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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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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 human 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 (100% complete)
Major Task 2: Establish baseline responses to toxicants in the SNCA triplication line and compare exposure phenotypes across other mutations (subtask 1, 100% complete; subtask 2 100% complete; subtask 3 25% 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 (subtask 1, 50% complete, subtask 2 pending)

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) (100% complete)
Major Task 6: Perform Screens on DA neurons (80% complete)
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 modification, and characterization of patient iPSC lines used for insertion of the tyrosine hydroxylase::tdtomato (THtd) reporter construct, toxicant sensitivity primary assays, optimization of secondary assays, screening assay development, and performing screens. Major activities with the PEG epidemiologic study included assessing *SNCA* and *GBA* gene-set gene-environment (GxE) interactions with the specific pesticides we identified as being associated with PD risk in a 'pesticideome' analysis.
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 and corrected lines, E46K mutant and corrected lines, and synuclein triplication line (part of Major Task 2).
    d. Assay development for toxicant and custom screens, build custom toxicant library (part of Major task 5)
    e. Perform compound screen and toxicant screen (Major Task 6, subtask 1 and 2)
    f. Establish calcium imaging assay for characterization of toxicant responses
    g. Assess for evidence of GxE interaction in the PEG study (n=1870), using pesticide profiles for specific chemicals based on quantitative measures from GIS models and using a SNP-set kernel association test to aggregate individual SNP test statistics for all markers in the *SNCA* and *GBA* genes. (Major Task 3 & 7)
    h. Assess pesticides associated with PD in PEG (FDR <0.05) for evidence of gene level GxE interaction (Major Task 3 & 7). Major task 7 (next research period) will involve identifying disease pathway-relevant SNPs for evaluation in the PEG cohort.

Significant results from the Rubin lab primarily relate to objective 2a-e. Production of the Synuclein knockdown and knockout lines was substantially more time consuming and challenging than the other THtdtomato lines generated for this project. Previous experience with the recombination plasmid template and guide RNA plasmids used for the reporter knock in resulted in about 40-50% of clones with a NHEJ error on the unmodified allele of tyrosine hydroxylase, typically with a length of 7-14 bases. The potential knock-in clones recovered from the synuclein knockdown and knockout lines showed substantially higher frequency of NHEJ errors (approximately 70-90% of clones) and the errors were larger than those previously encountered in other lines (**Figure 1**). This may be a function of the parental line itself or a function of a role for synuclein protein in DNA repair processes/NHEJ (Schaser et al Sci Reports 2019). Failure to recover viable clones that passed all the typical QC steps due to this increased frequency of NHEJ errors required an iterative process that has increased the time needed to recover clones and required picking and analyzing 4-5x the normal number of colonies for propagation and analysis. One THtdtomato clone from the synuclein knockout line has passed all QC steps and is being used for primary and secondary assays as a comparison to the synuclein triplication line. Due to budget and time constraints, attempts to recover a THtdtomato knockin clone for the 2-copy isogenic line have been suspended. We will perform the stated analysis on mixed cultures of the unmodified parental 2-copy clone using antibody staining for tyrosine hydroxylase to provide the necessary comparison data on toxicant sensitivity.

Substantial progress was made in Major task 2, subtask 1 and 3. Additional replicates were performed with the synuclein (SNCA) triplication line, the GBA mutant and corrected lines, and the E46K mutant line. The toxicant responses for each of these lines has provided insight into variation in sensitivity to the initial set of toxicants as a function of cell line and genotype (**Figure 2**). The aggregated and normalized results provided a few important insights: 1) the sensitivity to toxicants varied greatly based on the toxicant used, with ziram being the most potent toxicant in all cells lines tested and cyanazine being the least potent in all cell lines tested; 2) Differences in sensitivity to toxicants were more pronounced between cell lines than between isogenic pairs with the exception of paraquat where E46K mutant synuclein appeared to result in higher sensitivity than the corrected control; 3) Replicates with the A07-E46K isogenic pair (mutant and corrected) showed less sensitivity to ziram, compared to the other lines used in the analysis. The finding that mutations in synuclein or GBA do not confer increased sensitivity to most toxicants tested in this small subset is not surprising given that this set of toxicants was derived from a cohort of PD patients that did not take into account mutations or SNPs in SNCA or GBA. This limited set of toxicants did provide useful assay parameters for the design of our upcoming toxicant screen and has helped with selection of the upper and lower limits of dosing to be used for that screen.

Assay planning and development for the toxicant screen and compound screen were completed during the reporting period. The toxicant list provided by the Ritz lab was classified by solubility in DMSO, water, or ethanol in preparation for screening library construction. Acquisition, resuspension, and aliquoting of these toxicants was completed. Of the 64 toxicants, three have very high dermal toxicity in humans and mammals, making their use problematic from an occupational health and safety standpoint. These were omitted from testing. Eighteen were soluble in DMSO, twenty-three were soluble in water and seventeen were soluble in ethanol. Different template plates were generated for each solvent. Four concentrations for each toxicant were assayed in duplicate (water soluble toxicants) or triplicate (DMSO and ethanol soluble toxicants) in the toxicant screen. The concentrations were chosen based on solubility, previously published toxicant/pesticide-based screens and experience with other toxicants in Aim 1. Data from this custom toxicant library will simultaneously address objectives stated in Major Task 4, subtask 1 for the synuclein triplication line, and Major Task 6, subtask 1. Performing the assay in this manner is more cost effective because the SNP-associated PEG toxicants are contained in the custom screening library. During the reporting period, these tasks were completed for the synuclein triplication line. Subsequent experiments in the next reporting period with the GBA mutant line will complete the work for Major Task 4, subtask 1.

Results from the toxicant screen indicated a successful dynamic range between negative controls (DMSO, water, DMSO+Ethanol) and positive controls (rotenone, ziram). The Z prime score for DMSO as the negative control and rotenone positive control was 0.549. The following toxicants produced cell death >4 standard deviations below the mean at the 30uM screening concentration: propargite, copper sulfate (basic and pentahydrate), folpet, dicofol, naled, endothall, trifluralin, endosulfan, and diquat dibromide (**Figure 3**).

A few additional toxicants had cell death between 2-4 standard deviations below the mean or had large well to well variability in the screen. Secondary assays and biochemistry will be performed on a subset of the toxicants that clearly resulted in extensive cell death (>4 standard deviations). Four-concentration dose response curves designed into the screening library indicated that propargite is the most toxic, followed by diquat dibromide, folpet, and naled—all of which are significantly more toxic than DMSO control at 6uM in addition to 30uM (**Figure 4**). Our finding that propargite causes dopaminergic neuron cell death provides an independent validation of other studies which also identified this toxicant in screening assays (Zhou, T. Nat Communications 2018). Multiple toxicants identified in our custom screen of the PEG toxicants remain actively registered with the EPA and are used in agriculture. Evidence that they directly cause death of dopaminergic neurons in vitro could have implications for usage and policy decisions. Secondary assays performed by collaborators in the Khurana lab will provide additional mechanistic detail for a subset of these toxicants (see below).

As described in the project narrative, we also undertook a more traditional compound screen using a subset of compounds from a commercially available bioactive compound library (SelleckChem Bioactive library). The number of screened compounds was 776. This was a subset of the full library (>3,000 compounds) and was chosen to overrepresent compounds with targets involved in neuronal signaling pathways and inflammation. In the live imaging screen, surviving cells and neurite length were measured using a Molecular Devices IXM high content microscope. Image analysis using Columbus (Perkin Elmer) software was designed to select cells based on area, roundness, and THtdtomato signal intensity. Exported data was then processed using GeneData Screener software to perform Robust Z score normalization of the data. At a screening concentration of 3uM, 56 compounds resulted in cell death at >4 standard deviations greater than the DMSO negative control as measured by live imaging analysis of the THtdtomato dopamine neurons (**Figure 5**). Berzosertib, an ATR inhibitor identified in a previous toxicity screen in the Rubin lab (unpublished) on E46K-synuclein mutant dopamine neurons was used as the positive control. The compounds resulting in cell death >3 standard deviations greater than the DMSO control wells and their corresponding annotations provided by the commercial vendor are provided in **Table 1**. To provide an overview of which pathways are over-represented among the hit compounds, the percentage of various pathway annotations in the screened compounds were calculated and compared to the frequency of pathway annotations among the hit compounds. Pathways involving apoptosis, cytoskeletal signaling, microbiology, NFkB, transmembrane transporters and ubiquitin were overrepresented in the list of hit compounds compared to the input compound library. Multiple compounds affecting dopamine receptor activity are also among the toxic compounds. A subset of these compounds will be validated by dose response curves in an upcoming reporting period and the list of compounds will be evaluated as described in Major Task 7 by our collaborators in the Ritz lab.

In prior reports, the Khurana lab described optimization of assays in iPSC-derived dopaminergic neurons. This optimization has continued, as noted below, particularly to cope with the challenges of the long-term dopaminergic neuron cultures required to generate electro physiologically active neurons that are essential for measuring calcium fluxes. Beyond this optimization, in this last 6 months, important progress has been made using these optimized assays to study the effects of specific pesticide/toxicants identified by the Ritz group and screened by the Rubin group.

Significant results from the Khurana lab primarily relate to objective 2c and 2e. We have established the autophagy (lysosomal) and mitochondrial function assays which will allow us to evaluate the effect of toxicants in these organelles. The mitochondrial function assay is based on western blot assessment of the relative amounts of respiratory chain complexes, Complex I (assessed by NDUFB8), Complex II (assessed by SDHA and SDHB), Complex III (assessed by UQCRC2), Complex IV (assessed by COX II), Complex V (assessed by ATP5A), TOMM20 and actin. These assays have been optimized on the SNCA triplication cell line.
We evaluated several toxicants in the mitochondrial subunits assay using the SNCA triplication differentiated neurons at day 65, based on the results provided by Ritz/Rubin groups. For this evaluation, we used 8 wells in 24 well plate format, seeding 2 million cells per well and exposing them to toxicants at different concentrations for 6 hours and 24 hours. We used the EC50 concentration determined by the initial *in vitro* screening by the Rubin lab as a starting reference concentration to evaluate those toxicants in the mitochondrial subunit assay described above. We used mixed neuronal cultures in this biochemical assay. This necessitated a characterization of the nature of the cellular population exposed to the toxicants. To do this, we performed a quality control (QC) western blot targeting neuronal markers TUB III (TUJ1) and GAPDH as is described in **Figure 7**. This result clearly shows that all the mixed differentiated cultures were enriched in neuronal population.

Figure 6 depicts our classification scheme for the toolbox of assays we are currently establishing in this project for the evaluation of toxicants and the role they play in calcium homeostasis in mutant and corrected differentiated dopaminergic neurons. In addition to the biochemical assays, we have established the Agilent Seahorse XFCell Mito stress assay in our cells of interest. This functional assay provides real time assessment of the effect of a given toxicant on mitochondria respiration due to perturbations in the electron transport chain (ETC). Calcium imaging based on chemical indicators Fluo-4 and Fura-2 continue to be two of the pivotal functional assays in this study. In addition, we have begun reporter assays based on genetically encoded indicators (GEI)—including indicators to study calcium dynamics like GCaMP6s, mitochondrial dysfunction, and ER stability.

We made significant progress on the evaluation of toxicants in the mitochondria subunit assay. Initially, we tested the toxicants: Tribufos (30μM), Phorate (30μM), Ziram (75nM and 300nM), Trifluralin (10μM and 30μM) and Propargite (300nM) at or near their EC50 concentration in the mitochondrial subunits assay. In subsequent experiments, we narrowed down the set of toxicants to Ziram (300nM), Trifluralin (30μM) and Propargite (300nM and 1μM). We exposed the cells to each of these toxicants for 6- and 24-hours, as shown in **Figure 8**. A significant change in protein expression in the mitochondrial subunit assay is demonstrated by the quantification of complex I when differentiated SNCA triplication neurons are exposed to 30μM Trifluralin and 300nM Propargite (**Figure 8A)**. Quantification of this effect is shown in **Figure 8B** and **C** respectively. All the samples were normalized to TOM20. These results will be confirmed in additional experiments and the quantification of additional subunits will be performed.

We made substantial progress on the implementation of the Agilent Seahorse XFCell Mito stress assay. This functional assay allowed us to measure multiple physiologic parameters of differentiated neurons in real time that provide information about mitochondrial function and health. These include: the oxygen content in extracellular media; the oxygen consumption rate (OCR); the extracellular proton concentration (pH); and the extracellular acidification rate (ECAR). **Figure 9** shows the effect of Trifluralin on the OCR and ECAR of differentiated SNCA triplication neurons in a dose response manner. It is worth noticing that, even though trifluralin at 90 μM reduces the spare respiratory capacity to lower levels, the effect of 10μM trifluralin increases the spare respiratory capacity to levels higher than the DMSO control. More detailed assessment of the effect of trifluralin in OCR, ECAR and OCR vs ECAR is shown in **Figure 10**. **Figure 9B** shows the effect of Propargite on the OCR and ECAR of differentiated neurons in a dose response manner. Propargite reduces the spare respiratory capacity at its minimum when used at 1μM (**Figure 11)**.

We have implemented additional functionalities in the CaSiAn based image analysis pipeline. Now it is possible to classify and discriminate the TH-tdtomato positive neurons from the negative neurons. This functionality will allow us to specifically process and analyze the neuronal activity of dopaminergic neurons in the mixed cultures using the Fluo-4 based calcium imaging assay. This image analysis functionality utilizes the image acquired in the red channel (TH-tdtomato positive cells) of the fluorescence microscope. This image is then used to generate a mask that can be superimposed over the green channel (Fluo-4 positive cells), resulting in the ability to discriminate the differentiated neurons which are TH-tdtomato positive and spontaneously firing in the Fluo-4 calcium based assay. The assessment of those TH-td positive firing neurons is followed by the assignment of unique cell identifiers (numbering). We also implemented the functionality of generating violin plots for the statistical analysis portion of the pipeline. This serves to better represent the statistical significance of signal features from the fluorescent traces obtained in each dataset.
The mito stress assay required the seeding of 1x10^5 cells per PEI-laminin coated well in a Seahorse plate, which covers the entire culture area of the well. We observed that the clumping of THtdtomato positive cells is reduced under these higher density conditions. Therefore, we decided to increase the number of cells we seeded in the 96 well plates for subsequent calcium imaging assays—seeding between 5x10^4 and 1.8x10^5 cells per well. These high-density wells resulted in reduced formation of clumps and ensured the maintenance of a high number of cells in the culture during the differentiation and maturation process
We have successfully implemented a viral transfection protocol to use the genetically encoded calcium indicator (GECI) lenti vector pHAGE-RSV-GCaMP6s (Addgene plasmid 80146), in our differentiated dopaminergic neuron SNCA triplication line. This calcium indicator will allow us to take calcium imaging data from the same group of cells for longer periods of time, using a high content microscopy approach to determine the effect of toxicants in calcium dynamics in differentiated neurons.
Some preliminary measurements of the effect of 30μM trifluralin in differentiated neurons is reported in **Figure 12**. The implementation of this protocol has been performed in collaboration with the Wainger laboratory (Aaron Held and Brian Wainger) at Massachusetts General Hospital.

Significant results from the Ritz lab relate to Specific Aim 1 Major Task 3 and starting Specific Aim 2 Major Task 7, which will continue for the next reporting period. We have previously established pesticide exposure profiles and quantitative exposure measures, and generated a list of 33 pesticide toxicants that are both formally statistically significantly associated with PD (FDR<0.01) and for which we observed exposure in carriers and non-carriers of *SNCA* SNPs and *GBA* variants. This list of 33 pesticides is being used in the current phase of research to test for PD-related toxic mechanism in-vitro cell lines. This set of pesticides also provides the foundation for our GxE testing.

All of our analyses use the Parkinson's Environment and Genes (PEG) study from three agricultural counties in Central California. PEG is a population-based case-control study 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). In the previous period, we generated pesticide exposure profiles using a GIS-based model, which allows us to estimate ambient exposure to specific pesticides based on long-term residential and occupational proximity to agricultural pesticide applications. We used state-mandated pesticide use reports (CA-PUR database) and GRAPES, a GIS model we developed 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. We generated long-term exposure profiles to all pesticides applied in the study area (1974-interview/PD dx (with 10-year lag), nearly 700 pesticides applied in study area). In the previous reporting period, we identified 33 pesticides associated with PD in both the wave 1 discovery and wave 2 validation cohort at a meta-FDR<0.01, a total of 53 pesticides were associated with PD at a meta-FDR<0.05. More detail on this analysis has been provided in our previous report.

Recently, we began assessing these PD-associated pesticides in conjunction with *SNCA* and *GBA* genetic data to explore possible GxE interactions. We have previously done GWAS genotyping (with 100k PD markers added for good coverage of PD genes) and exome sequencing (PD patients only) for both the SNCA and GBA genes. The PEG study has data on variations in 1,061 SNCA markers and 321 GBA markers (including a 100k bp buffer around the gene). The majority of markers in both the *SNCA* and *GBA* genes have a low or rare minor allele frequency (MAF), which is shown in **Figure 13**. Both rare and common variants are likely important contributors to PD etiology. We therefore assessed these genes as a whole using a SNP-set kernel association test (SKAT). SKAT is a SNP-set level test for association between a set of rare and/or common variants and dichotomous or quantitative phenotypes. SKAT aggregates individual score test statistics of SNPs in a SNP-set and computes SNP-set level p-values, e.g. a gene or a region level p-value, while adjusting for covariates, such as principal components to account for population stratification.

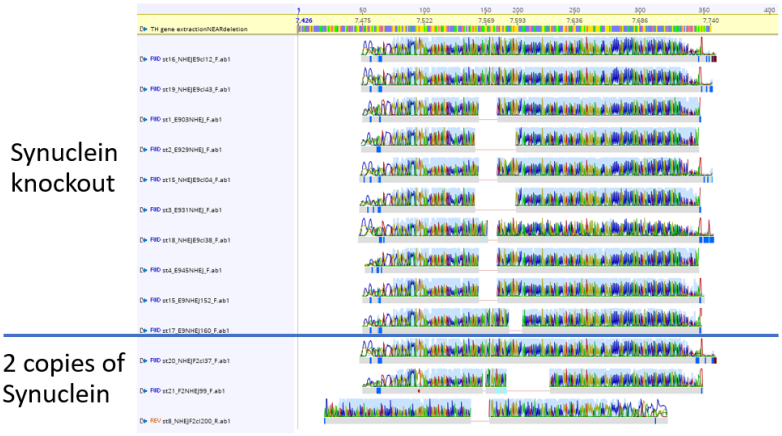
We first tested whether the *SNCA* and *GBA* SNP-sets were associated with PD - as expected based on large idiopathic PD genetic studies. Our results indicated as expected the genetic risk implied by variants in these genes in PEG, with mainly common variants in *SNCA* but rarer variants in GBA being associated with PD. Again, this is expected, as meta-GWAS associated GBA variants have MAF<0.01, while common *SNCA* variants have been identified as risk variants associated with PD. Our findings are detailed below:

			Employing weights allows for assumptions about rare variants			
			No variant weighting (linear) [a]	**Upweighted Rare Variant** (weights.beta =c(1,25))[b]	**Madsen and Browning weights – Common variants** (weights.beta= c(0.5,0.5))[c]	**Combined common and rare**[d]
	Sample Size	**Number of Variants**	**p-value**	**p-value**	**p-value**	**p-value**
SNCA	1501	1061	4.03E-05	0.22	1.54E-05	2.60E-04
GBA	1501	321	0.25	3.89E-03	0.17	0.12

a flat-weight (no weight), no variant weighting
b The weight is designed to incorporate an assumption that rare variants are more likely to be causal variants with larger effect sizes. For common variants, this weighting scheme does not work because it assigns almost zero weight to common variants (e.g., w = 0.0004 for a MAF of 0.30 but w = 7.28 for a MAF of 0.05).
c To incorporate more effects from common variants, the weight slowly decreases with increasing MAF. For example, for MAF = 0.05, $w = 1.46$, for MAF = 0.10, $w = 1.06$, for MAF = 0.30, $w = 0.69$, and for MAF = 0.5, w = 0.64.
d To test for both common and rare variants together, SKAT-CommonRare function uses a weighting scheme in which common and rare variants contribute to the test equally.

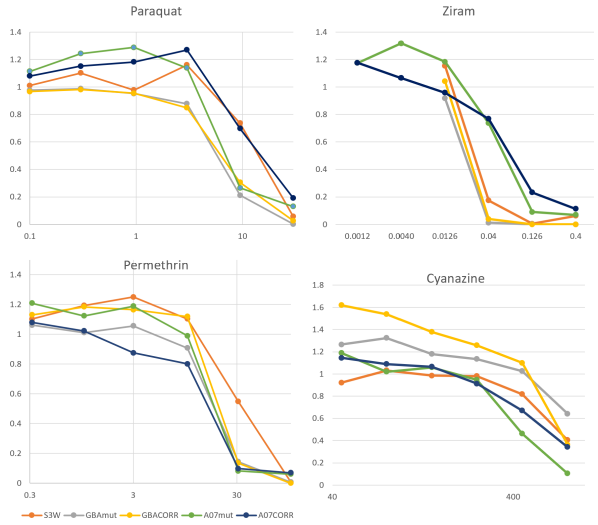
After generating the *SNCA* and *GBA* SNP-set summary results and confirming their contributions to PD risk, we next assessed whether there was evidence for GxE interactions. We selected a case-only design, as we have exome sequencing data only available for the patients and also to increase statistical power. In a case-only design, if genetic factors are not interacting with pesticide exposure to influence PD risk, we expect exposure to be similar across all risk genotypes among PD patients. Thus, to screen for interaction, we first tested whether the SNP-sets were associated with differences in exposure among the PD patients (n=728), controlling for genetic ancestry, sex, and age. We tested all pesticides we previously found to be associated with PD risk and employed two weighting schemes for handling rare and common variants (see footnotes b and c in table above). The results have been visualized in **Figure 14**, which displays the SNP-set p-values between each exposure and the *SNCA* or *GBA* SNP-sets. We found evidence of interaction with two related pesticides and the *SNCA* SNP-set, Dicamba (p=0.001) and Dicamba, other related salt (p=0.009). This indicates that PD patient carriers of more variants in the *SNCA* gene had also been exposed more to these two pesticides i.e. that the increased risk reflects both their genetic susceptibility and higher levels of exposure. The *GBA* SNP-set showed evidence of interaction with 14 pesticides, including MCPA (p=7.35e-04) and dinoseb (p=3.91e-05), with the burden of rare variants being more important than the common variants. We know that some of these pesticides are correlated, as they are often co-applied within the same or across different seasons on the same fields. We plan to handle this in two ways in the GxE analysis, both assessing orthogonal hierarchical exposure clusters, which we have already built, and using single pesticides and mixtures in the next phase of research in the in-vitro cell lines, to pinpoint whether toxicity is driven by a single pesticide driving the results or whether it is a mixture of pesticide exposures that is most harmful. In the next phase of this research, we will use validated hits from the lab-based screens to assess pathways relevant and also assess known functional SNPs in GxE analyses.

Figure 1



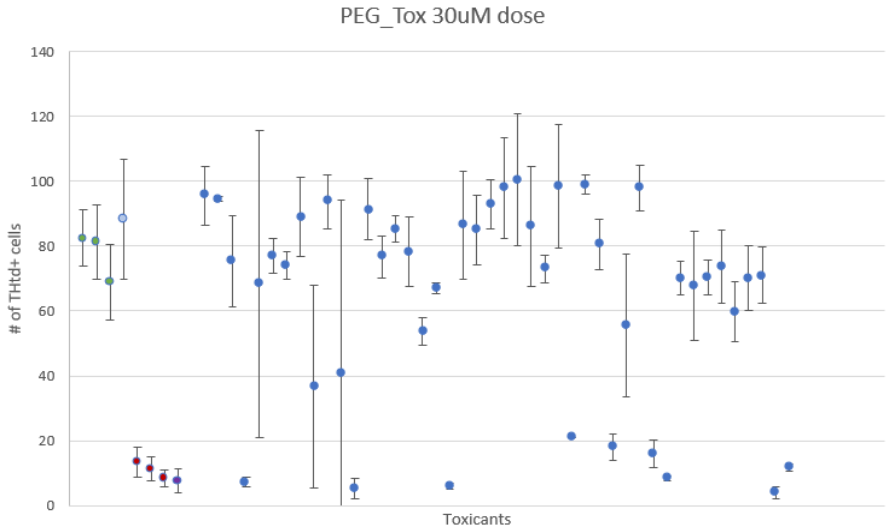
Sequence alignment of each clone at unmodified TH allele demonstrating substantial NHEJ error prevalence in synuclein knockout and knockdown lines. First two lines listed passed all PCR and differentiation quality control criteria. Clone 43 has a normal karyotype and is used for experiments going forward.

Figure 2



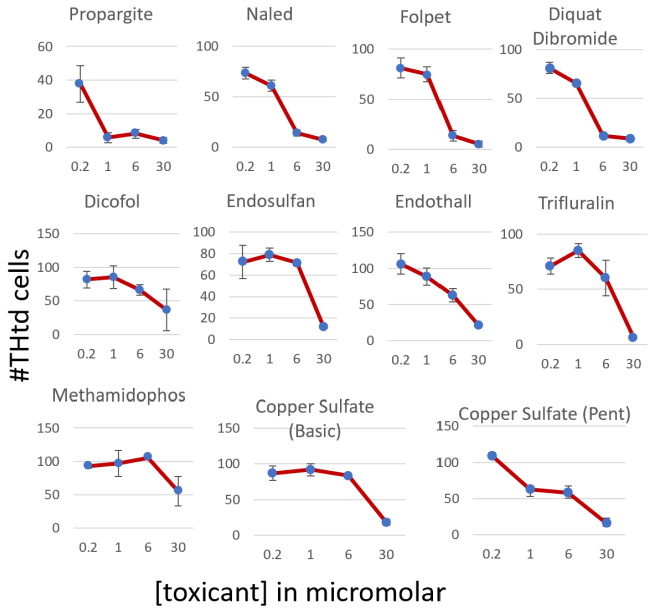
FACS purified THtd+ neurons from the SNCA-tripl (“S3W”), GBA mutant (“GBAmut”), GBA corrected (“GBACORR”), E46K mutant (“A07mut”) and E46K corrected (“A07CORR”) cell lines were treated with multiple doses of toxicants (x-axis in micromolar) linked to PD risk. Cell numbers measured by high content imaging of live cultures 11 days after first treatment and normalized to DMSO control for each line (y axis).

Figure 3



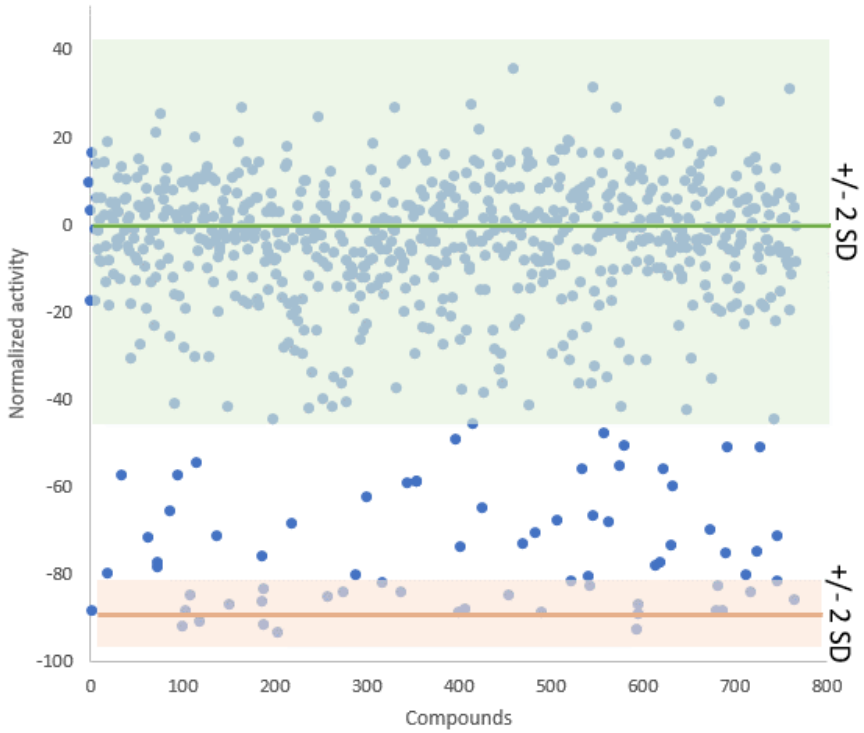
Scatter plot of PEG_tox screen on synuclein triplication line. Y-axis represents number of cells counted at the end of the survival assay. Negative and positive controls are on the left: green dots are DMSO, light blue dots are water, red dots are rotenone, purple dot is ziram, blue dots are screened toxicants. Large standard deviation from three toxicants each have a single outlier of the three technical replicates.

Figure 4



Four concentration dose curves of PEG toxicants producing death in synuclein triplication THtdtomato sorted neurons. Cell numbers measured by high content imaging of live cultures 11 days after first treatment. Error bars = SD

Figure 5



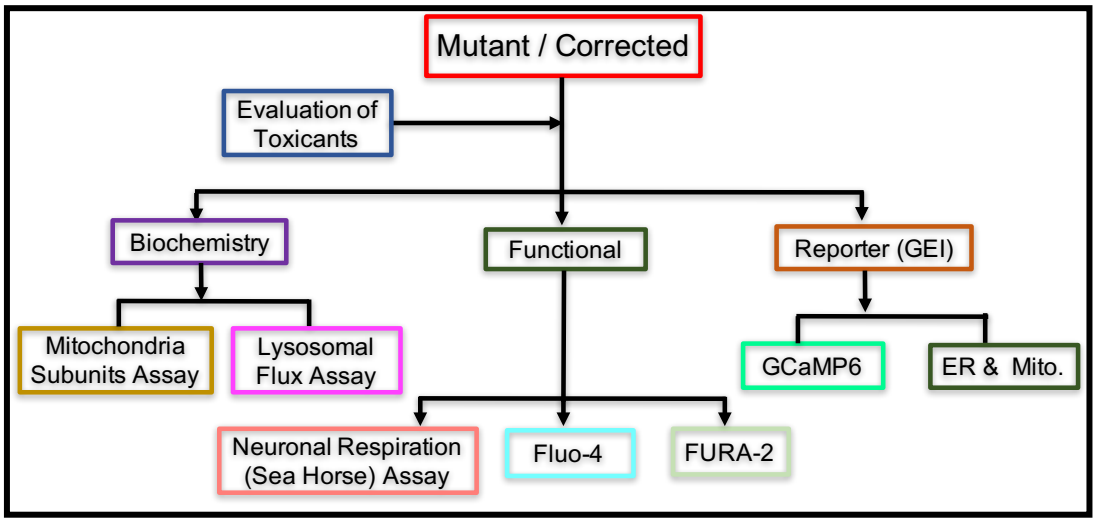
Scatter plot of normalized compound screen result. Green shaded area is two standard deviations above and below the DMSO control. Orange shaded area is two standard deviations above and below the positive control, Berzosertib, which had an activity of -89.6. Standard deviation of DMSO control is 22.3. Seventy compounds had activity <-44.9 (2SD below control) and fifty-two compounds had activity <-66.9 (3SD below control).

Table 1

Compound ID	Activity17	Target	Information	Pathway
Ciclopirox ethanolamine	-93.732758	ATPase	Ciclopirox ethanolamine (Ciclopirox olamine, HOE 296) is a broad-spectrur	Transmembrane Transporters
Pyrrolidinedithiocarbamate ammonium	-92.694618	NF-κB	Ammonium pyrrolidine dithiocarbamate (PDTC) is a potent inhibitor of nuc	NF-κB
b-AP15	-92.313362	DUB	b-AP15 is a deubiquitinases inhibitor for 19S proteasomes activity of Ub-Al	Ubiquitin
Chlorhexidine 2HCl	-91.716652	Antifection	Chlorhexidine hydrochloride is an antiseptic effective against a wide varie	Others
Berbamine (dihydrochloride)	-91.011749	Bcr-Abl	Berbamine (BBM) is a natural bisbenzylisoquinoline product isolated from	Angiogenesis
Quinacrine 2HCl	-89.505966	Phospholipase (e.g. PLA)	Quinacrine 2HCl is a lipophilic cationic drug with multiple actions that is cc	Others
IKK-16 (IKK Inhibitor VII)	-89.079231	IκB/IKK	IKK-16 (IKK Inhibitor VII) is a selective IκB kinase (IKK) inhibitor for IKK-2, IKK	NF-κB
Moxidectin	-88.812286	Antifection	Moxidectin is a potent, broad-spectrum endectocide with activity against	Microbiology
Thimerosal	-88.595421	Antifection	Thimerosal is a well-established antiseptic and antifungal agent and usua	Microbiology
Beclomethasone dipropionate	-88.526276	Glucocorticoid Receptor	Beclomethasone dipropionate is a potent glucocorticoid steroid used for t	Others
(+)-Fangchinoline	-88.484871	Others	Fangchinoline is a phytochemical that has been shown to elicit anti-cance	Others
Tenovin-6	-88.416237	p53,Sirtuin	Tenovin-6 is a small molecule activator of p53 transcriptional activity.	Apoptosis
Isavuconazole	-87.907898	Antifection	Isavuconazole is a new extended-spectrum triazole with activity against ye	Microbiology
QNZ (EVP4593)	-87.422554	NF-κB,TNF-alpha	QNZ (EVP4593) shows potent inhibitory activity toward both NF-κB activati	NF-κB
Cabozantinib malate (XL184)	-87.135986	TAM Receptor,VEGFR	Cabozantinib malate (XL184) is the malate of Cabozantinib, a potent VEGFI	Protein Tyrosine Kinase
Cepharanthine	-86.52002	TNF-alpha	Cepharanthine is a biscoclaurine alkaloid inhibiting tumor necrosis factor	Others
Zinc Pyrithione	-86.000175	Proton Pump,Antifection	Zinc pyrithione is an antifungal and antibacterial agent disrupting membra	Transmembrane Transporters
DHBP dibromide	-85.667145	Calcium Channel	DHBP, a viologen for electrochromic memory display agent, inhibits the ca	Transmembrane Transporters
Mefloquine HCl	-85.170624	Antifection	Mefloquine HCl is a blood schizonticide by inhibiting hemozoin formation,	Others
Benzethonium Chloride	-85.134384	AChR	Benzethonium chloride is a potent inhibitor of nAChRs, it inhibits α4β2 nAC	Others
Fangchinoline	-84.395454	Reverse Transcriptase	Fangchinoline, a bisbenzylisoquinoline alkaloid, is a novel HIV-1 inhibitor v	Microbiology
Triptolide (PG490)	-84.267509	NF-κB	Triptolide is a diterpene triepoxide, immunosuppresive agent extracted fr	Others
Digoxin	-84.113976	Sodium Channel	Digoxin is a classical Na,K-ATPase inhibitor, with selectivity for the α2β3 is	Transmembrane Transporters
Cetylpyridinium Chloride	-83.422546	Antifection	Cetylpyridinium chloride is a cationic quaternary ammonium compound us	Others
Terfenadine	-83.129807	Others	Terfenadine is an antihistamine, generally completely metabolizes to the	Others
Pentamidine isethionate	-82.797729	phosphatase	Pentamidine is an inhibitor of PRL Phosphatases and also inhibits synthes	Others
Ethacridine lactate monohydrate	-82.073158	Antifection	Ethacridine lactate monohydrate is an aromatic organic compound based	Others
Vinorelbine Tartrate	-81.855698	Microtubule Associated	Vinorelbine Tartrate is a semi-synthetic vinca alkaloid, and inhibits mitosi	Cytoskeletal Signaling
Ouabain	-81.740135	Sodium Channel	Ouabain is a selective Na+/K+, -ATPase inhibitor, binds to α2 /α3 subunit w	Transmembrane Transporters
Penfluridol	-80.726555	Dopamine Receptor	Penfluridol is a highly potent, first generation diphenylbutylpiperidine ant	Others
Trifluoperazine 2HCl	-80.619713	Dopamine Receptor	Trifluoperazine is a dopamine D2 receptor inhibitor with IC50 of 1.1 nM.	Ubiquitin
Domiphen Bromide	-80.397568	Others	Domiphen bromide is a quaternary ammonium antiseptic with actions as a	Others
2-Methoxy-1,4-naphthoquinone	-79.938271	PKC	2-Methoxy-1,4-naphthoquinone, isolated from the leaves of Impatiens gla	Cytoskeletal Signaling
Arctiin	-78.722435	Opioid Receptor	Arctiin acts on an agonists of the adiponectin receptor 1 with anti-cancer e	Others
Ropinirole HCl	-78.178589	Dopamine Receptor	Ropinirole a selective dopamine D2 receptors agonist with Ki of 29 nM.	Others
Salmeterol Xinafoate	-77.520355	Adrenergic Receptor	Salmeterol Xinafoate is a long-acting β2-adrenergic receptor agonist with a	GPCR & G Protein
Apoptosis Activator 2	-77.438316	Caspase	Apoptosis Activator 2 strongly induces caspase-3 activation, PARP cleavag	Apoptosis
Cetrimonium Bromide (CTAB)	-76.246231	Antifection	Cetrimonium Bromide is a known component of the broad-spectrum antise	Others
Thioridazine HCl	-75.186798	Others	Thioridazine is a trifluoro-methyl phenothiazine derivative, which blocks p	Others
Tulathromycin A	-74.885002	Others	Tulathromycin A is a novel long-acting semi-synthetic tribasic macyocyclic	Others
IMD 0354	-74.119423	IκB/IKK	IMD-0354 is an IKKβ inhibitor and blocks IκBα phosphorylation in NF-κB pat	NF-κB
Sertaconazole nitrate	-73.522911	Antifection	Sertaconazole nitrate is a topical broad-spectrum antifungal that is develc	Others
Methylene Blue	-73.399696	Others	Methylene Blue is used as a dye in chromoendoscopy. It Inhibits tau filame	Others
Amodiaquine dihydrochloride dihydrate	-72.147591	Transferase,Histone Methyltra	Amodiaquine is a potent, non-competitive inhibitor of histamine N-methyl	Others
Vinblastine sulfate	-71.654404	Microtubule Associated,AChR	Vinblastine sulfate inhibits microtubule formation and suppresses nAChR	Cytoskeletal Signaling
Brexpiprazole	-71.382271	5-HT Receptor,Dopamine Rece	Brexpiprazole is a novel D2 dopamine and serotonin 1A partial agonist, cal	Neuronal Signaling
MLN0905	-70.533165	PLK	MLN0905 is a potent inhibitor of PLK1 with IC50 of 2 nM.	Cell Cycle
Tazobactam	-69.708168	Antifection	Tazobactam is a β-lactamases Inhibitor with antibacterial activity. It also i	Others
Clofoctol	-68.595963	Others	0	Others
Pimozide	-68.191704	Others	Pimozide is an antipsychotic drug of the diphenylbutylpiperidine class, wh	Others
Nifuroxazide	-67.539307	STAT	Nifuroxazide is a cell-permeable and orally available nitrofuran-based ant	Others
Perphenazine	-67.078995	Dopamine Receptor	Perphenazine is a phenothiazine derivative and a dopamine antagonist wi	Neuronal Signaling

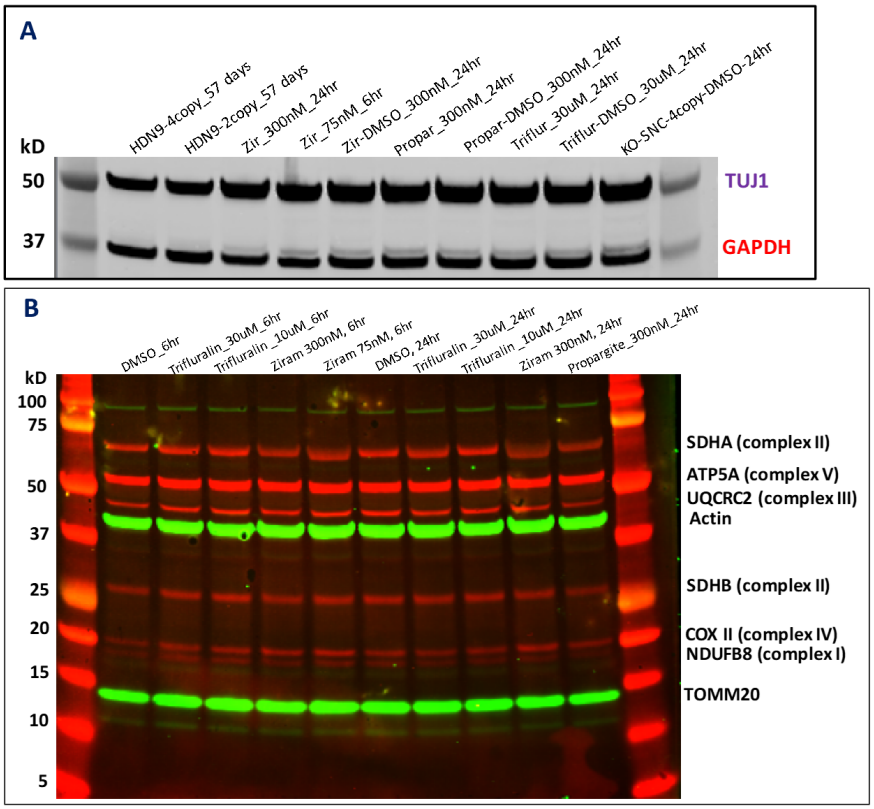
Screen results from synuclein triplication toxicity screen. The compounds resulting in cell death >3 standard deviations greater than the DMSO control wells and their corresponding annotations provided by the commercial vendor are listed. More negative “Activity” values indicate conditions with less surviving THtdtomato + neurons

Figure 6



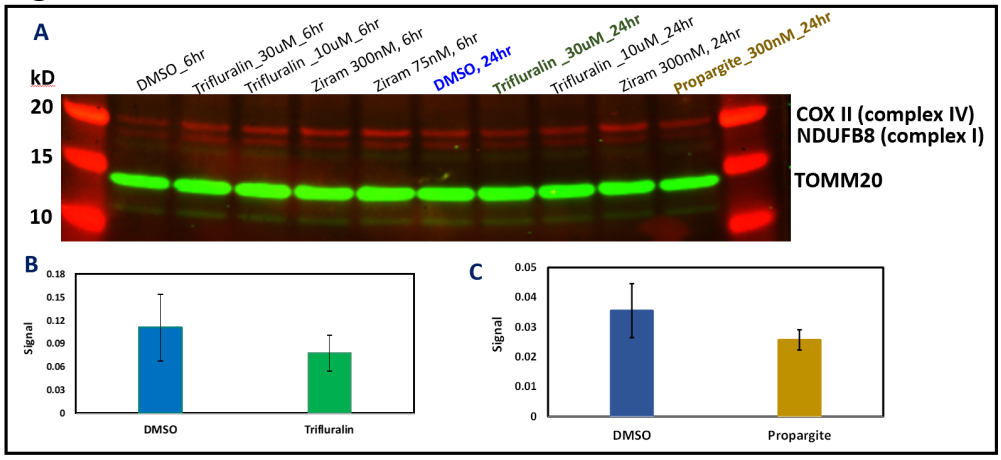
Distribution of assays to assess the effect of toxicants in differentiated dopaminergic neurons

Figure 7



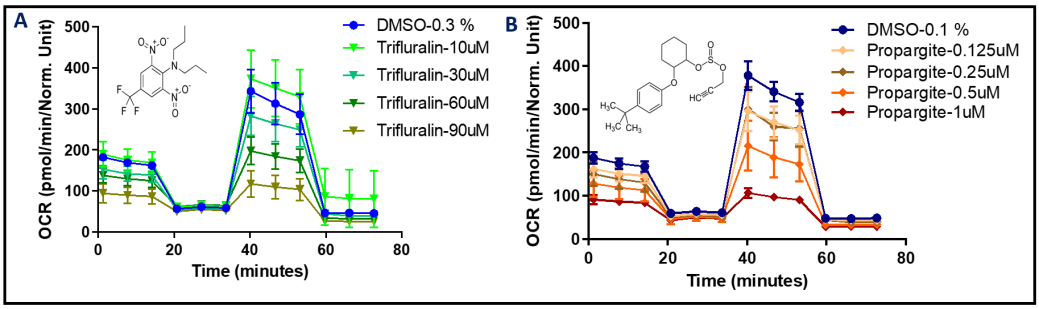
Western blot for (A) quality control of neuronal markers for Beta Tubulin- 3 and GAPDH. (B) Mitochondria subunits for SNC-triplication differentiated neurons exposed to selected toxicants for 6hr and 24hr

Figure 8



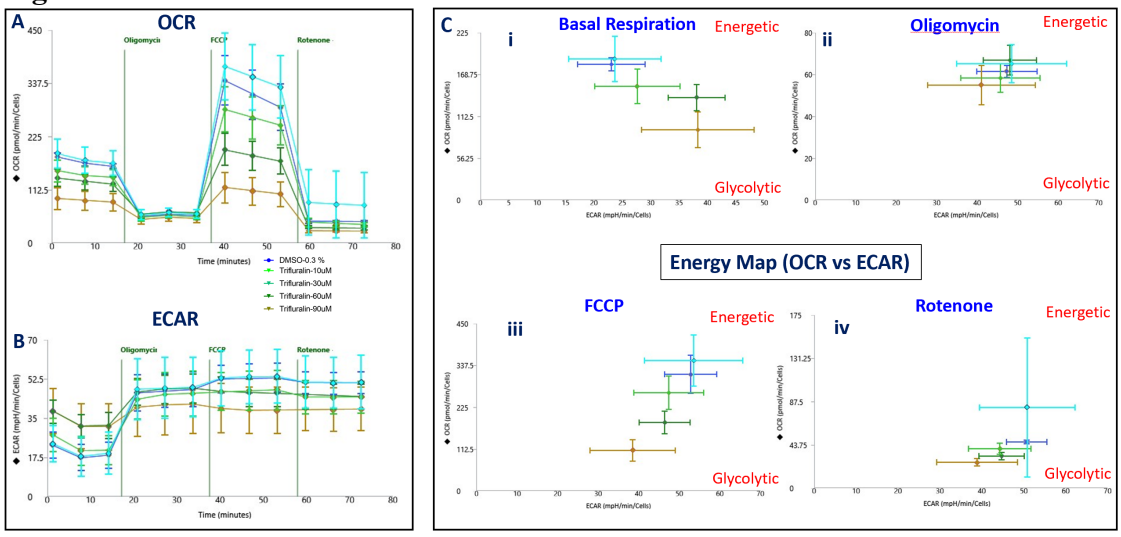
Western blot for (A) Complex IV and complex I Mitochondria subunits for differentiated SNC-triplication neurons exposed to DMSO, Ziram, Trifluralin and Propargite for 6hr and 24hr (B) Quantification of the effect of DMSO, Trifluralin and Propargite on differentiated SNCA-triplication neurons after 24hr exposure

Figure 9



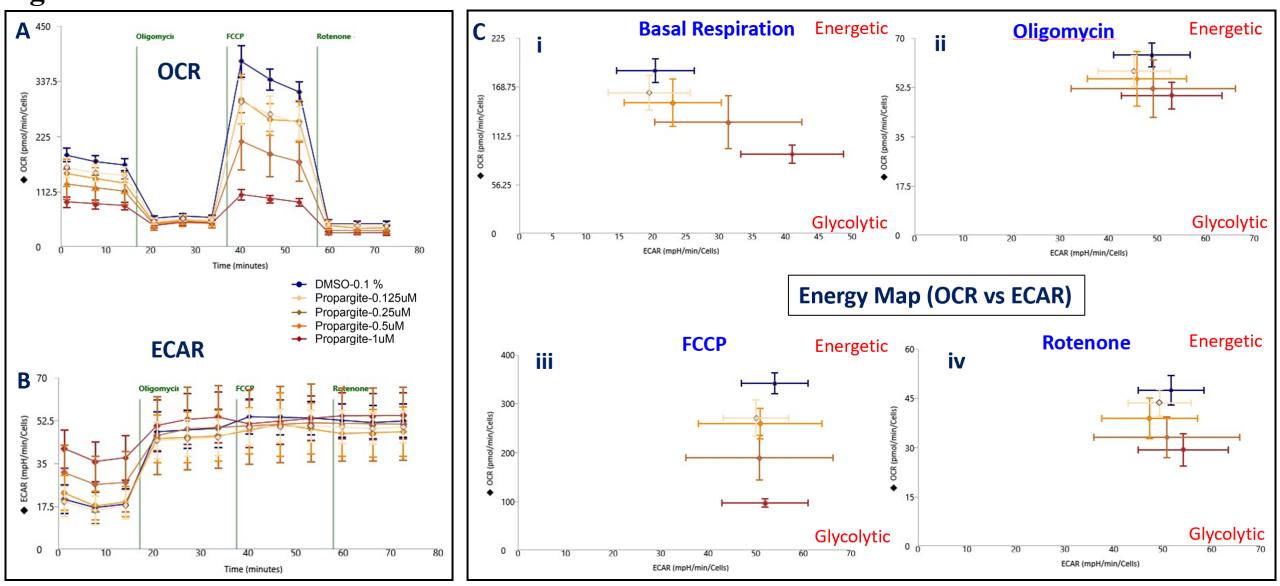
Measurement of Oxygen Consumption Rate (OCR) on Agilent Seahorse XFCell Mito stress assay for the dose response effect of (A) DMSO and Trifluralin on SNC-triplication differentiated neurons after 6hr exposure (B) DMSO and Propargite on SNC-triplication differentiated neurons after 6hr exposure.

Figure 10



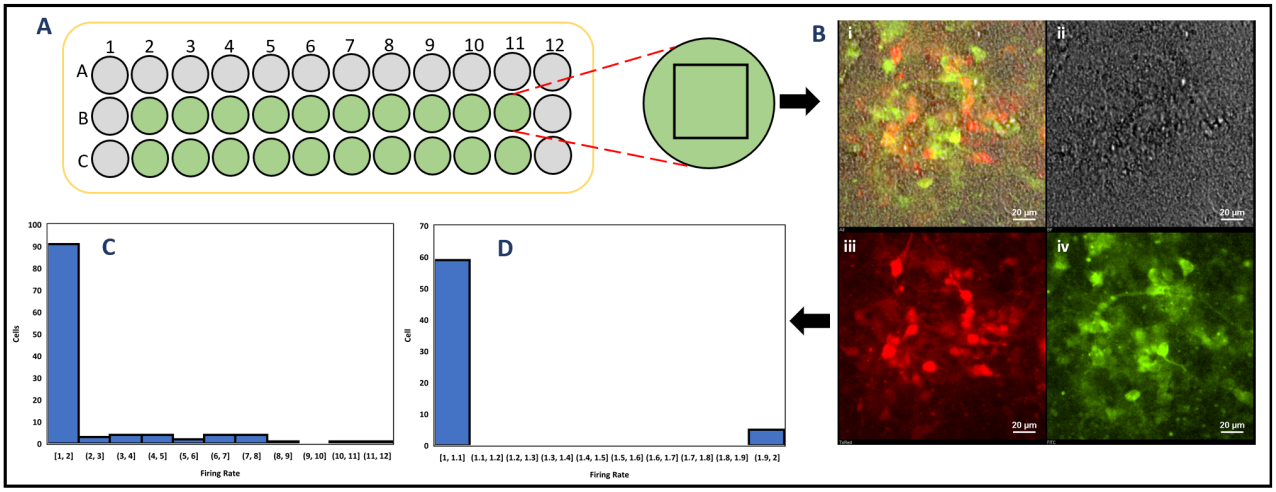
Agilent Seahorse XFCell Mito stress assay measurement of energy source on SNCA-triplication differentiated neurons after 6hr exposure to dose response of DMSO (0.3%) and Trifluralin (10, 30, 60, and 90 µM). (A) Oxygen Consumption Rate (OCR), (B) Extracellular Acidification Rate (ECAR) and (C) OCR vs ECAR

Figure 11



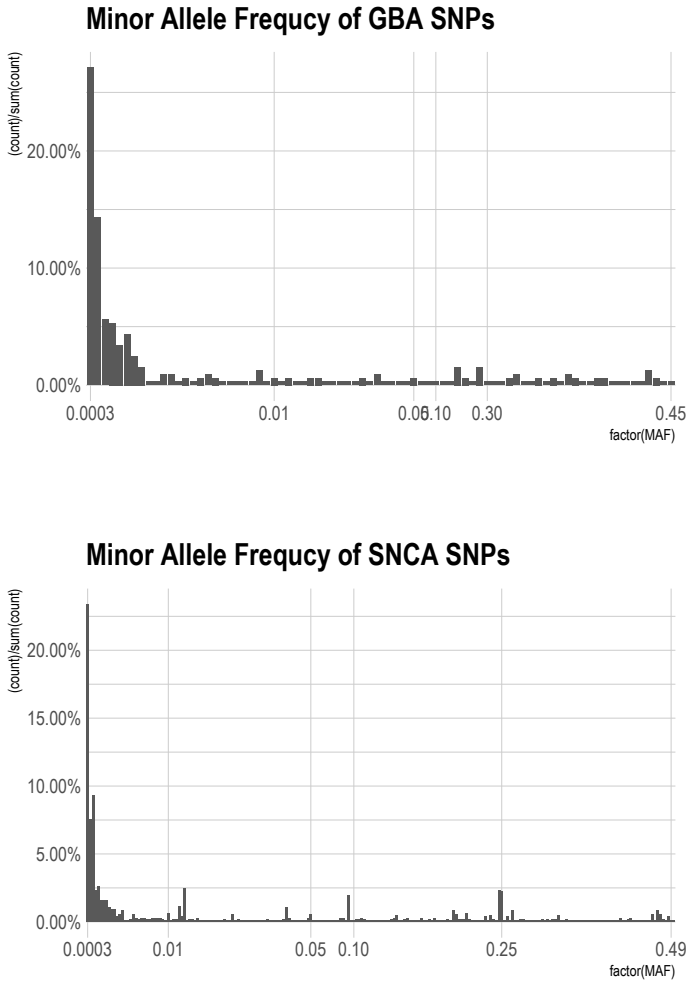
Agilent Seahorse XFCell Mito stress assay measurement of energy source on SNC-triplication differentiated neurons after 6hr exposure to dose response of DMSO (0.1%) and Propargite (0.125, 0.250, 0.5, and 1 µM). (A) Oxygen Consumption Rate (OCR), (B) Extracellular Acidification Rate (ECAR) and (C) OCR vs ECAR.

Figure 12



Workflow for High Content GCaMP6s Based Calcium Imaging. (A) schematic representation of a portion of a 96 well plate with an insert of a well highlighting the acquisition area at 4x (square inside the circle). (B) sample picture of GCaMP6s calcium imaging of synuclein triplication THtd differentiated neurons ((i) merged, (ii) bright field, (iii) Texas Red and (iv) FITC channels). (C) Histograms of firing rate before trifluralin treatment. (D) Histograms of firing rate after 30 µM trifluralin treatment.

Figure 13



Minor allele frequency (MAF) for all variants with data in *GBA* and *SNCA*, in the PEG study (n=1870).

Figure 14.

Results from the case-only GxE analysis in the PEG study, using a SNP-set kernel association test (SKAT) to test whether a higher burden of variants in the *SNCA* or *GBA* genes is associated with higher PD risk under pesticide exposure, using all PD-related pesticides. We show results for two different weighting schemes for handling rare variants, a weight designed to incorporate an assumption that rare variants are more likely to be causal variants with larger effect sizes, and a weight to incorporate more effects from common variants.

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 exposing the PEG toxicants to the remaining patient-derived reporter lines (GBA-01THtd, E46KTHtd, SNCAtriplication knockdown) in survival and neurite outgrowth assays as described Major Task 4. Because we were unsuccessful in producing the reporter line from the SNCA triplication knockdown cells, traditional endpoint fixed cell antibody staining and nuclear labelling will be used to determine cell death and neurite length following toxicant exposure to mixed cultures. The compound screen will be repeated on NGN2-induced cortical neurons generated from a modified version of the synuclein triplication line allowing for robust production of cortical neurons. This will provide data on which compounds identified as hits are specifically toxic to dopamine neurons or generally toxic to dopamine neurons and cortical neurons. Additional follow up on the compound screen will involve performing dose curves on a subset of hits to determine the potency and EC50. This subset of compounds will then be assessed for toxicity in the GBA mutant and E46K mutant lines to determine whether these mutations produce additional sensitivity or protection from cell death. Pathways that are over-represented by multiple compounds in the initial list of toxic compounds will be explored with initial target validation experiments (i.e. NFkB).

The Khurana lab will continue evaluating the toxicants reported by the Rubin lab using the set of biochemical, functional and reporter assays already established in this study. In addition to the evaluation in the synuclein triplication line, we plan to expand those experiments to the remaining patient-derived reporter lines (GBA-01THtd, E46KTHtd, SNCA triplication knockdown). With respect to the degree of information about the functionality of mitochondria, we will test the Agilent Seahorse XFCell Mito stress assay on those additional cell lines. Taking advantage of the versatility of the repetitive measurements of calcium dynamics possible with cells expressing the GECI GCaMP6, we will transfect the additional cell lines with this calcium indicator and measure the effect of different toxicants in this system. Furthermore, in the Khurana lab. we are working intensively on generating the genetically encoded mitochondria and ER sensors. We expect to implement this organelle sensors to maximize the understanding of calcium homeostasis in the present of toxicants, for the next reporting period.

During the next reporting period, the Ritz lab will focus efforts on the final major task, 7. This includes continuing to assess validated pesticide hits from the experimental screens in GxE analysis with pathway relevant SNPs. We will work to accomplish major task 7, subtask 1, which is pathway analysis of hits, data/literature mining for SNPs in relevant pathway components. For this subtask, to assess biologic pathways for the pesticides hits through data mining, we will use the Comparative Toxicogenomics Database (CTD). The CTD is a publicly available database that builds networks of chemical, genetic, and disease-related information from manual curation of vast amounts of peer-reviewed literature. Through this data integration and computational toxicology, we will build biologic networks for each pesticide hit, including all links (established via peer reviewed literature) for pesticide-gene (or gene product) and specifically pesticide-PD genes. From this we will be able to assess relevant pathway components and enriched components in the pesticide-gene network. For instance, methomyl, which over the previous reporting period was linked to PD through GBA GxE (Figure 14), also has also been linked to PD through pathway analysis and the HSPA1A gene using the CTD. The HSPA1A gene protein product stabilizes existing proteins against aggregation and mediates the folding of newly translated proteins. Thus, making this a relevant pathway component, with methomyl linked to GBA in the PEG cohort (Figure 14), and both genes linked to autophagy pathways. We will systematically assess pathways for all validated pathway hits in the coming period, to identify pathway-relevant SNPs for evaluation in the PEG cohort, as the final aim of this project.

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. The findings will also prompt more in-depth mechanistic investigation of the implicated pesticides.

The results described by the Khurana lab provide the validation of gateway assays and conditions that can be used to characterize the effect of toxicants on differentiated dopaminergic neurons. This allows us to classify the toxicants according to their effect on the function of organelles (e.g. mitochondria), providing some clues into their mechanism of action beyond just information about the survival of cultured dopaminergic neurons. Trifluralin and Propargite are clear examples of a set of toxicants for which we quantified their effect on mitochondria at the biochemical level using the mitochondria subunit assay and at a functional level using the Agilent Seahorse XFCell Mito stress assay. Ideally, testing for metabolites in the culture conditions after exposure to toxicants would provide more information about the metabolic output of such effect on health and function of differentiated neurons, but at the moment we don't have the capabilities to generate these types of results.

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. Furthermore, our work now integrating relevant genetics will inform further research in the lab, both as part of this project and beyond, related to genetic susceptibility related to pesticide exposures and PD.

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?

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. The in vitro data showing death of dopaminergic neurons when exposed to certain toxicants combined with PEG pesticide exposure data indicating an association with PD strongly argues for additional studies on these toxicants and consideration of additional regulation of these pesticides and toxicants.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

The experiments performed by the Rubin lab shifted more focus onto 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

Although we have made excellent progress since resumption of normal activities following the reduction of lab capacity during the initial COVID-19 outbreak, 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. The overall experimental progress remains only a few months behind the proposed benchmarks in the original statement of work due to COVID-19. Plasticware and reagent shortages and backorders have necessitated the substitution of some plasticware and reagents that slowed production of dopamine neurons in the last 2-3 months. We have adapted our workflow and protocols as best as possible, and dopamine neuron production for experiments is nearly back to the levels prior to shortages. For example, iPSC had to be adapted to mTESRplus media from StemFlex media and cells were transitioned into Geltrex instead of Matrigel due to shortages and backorders of these reagents.

In the Khurana lab, we have encountered several challenges related to long term culture and continued differentiation of the neurons in adherent conditions required for maturation and calcium activity. The THtdtomato neurons often require 60 days of differentiation to demonstrate firing activity by calcium imaging. These differentiated neurons start forming clumps before this maturation benchmark (as early as 10 days after seeding in the desired 96 well plate format). The aggregates are mainly composed of our cells of interest, the TH-tdtomato positive cells, which interferes with and complicates the ability to obtain calcium imaging at the single cell resolution required to precisely characterize the firing pattern of differentiated neurons. In order to overcome this problem, we evaluated different culture conditions that may prevent the formation of clumps by the TH-td tomato positive cells. Of the conditions we tested, polyethylenimine-Laminin (PEI-Lam) offers a good alternative to prevent early clumping of cells plated in 96 wells plate format. We also found out that increasing the cell seeding density of dissociated neurons also reduces the formation of clumps under these culture conditions. In addition, we have tested different co-culture conditions using commercial human iPSC derived astrocytes (Science Cell Astrocytes Cat # 1800) with EBs derived dissociated neurons, to recreate a more brain-like environment for the THtd differentiated dopaminergic neurons. A ScienceCell astrocyte based co-culture protocol has been tested and we will continue implementing this co-culture protocol in future experiments.
A careful evaluation of the conditions in which the calcium imaging assay based on Fluo-4 was performed also led us to evaluate the use of BrainPhys imaging optimized neuronal medium from Stem Cell Technologies (Bardy et al PNAS 2015; Zobolocki et al. Nature Communications, 2020) as an alternative neuronal medium for functional imaging to measure the activity of differentiated neurons.

The Ritz lab has been able to continue research as planned, and does not anticipate problems or delays in the final year.

Changes that had a significant impact on expenditures

More resources were needed for FACS of neurons than originally proposed. The overall effect on budget was minimal because the use of THtdtomato sorted neurons reduced reliance on antibody staining to quantify the tyrosine hydroxylase expressing dopamine neurons and reduced the number of biological replicates needed due to more reproducible assays.

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: Vikram Khurana, MD, PhD
Project Role: PI
Nearest Person Month worked: 1
Contribution to Project: Analysis planning, results interpretation, coordination with co-PIs, supervision of Edinson Lucumi

Name: Edinson Lucumi, PhD
Project Role: Postdoctoral Scholar
Nearest Person Month worked: 6
Contribution to Project: Image analysis, biochemical assay development, dopamine neuron experiments, data analysis

Name: Lee Rubin, PhD
Project Role: PI
Researcher Identifier: 0000-0002-8658-841X
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: Richard Krolewski, MD, PhD
Project Role: Scientist
Research Identifier: N/A
Nearest person month worked: 4
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: 3
Contribution to project: Dopamine neuron experiments, generation of transgenic lines, data analysis.

Name: Beate Ritz, MD, PhD
Project Role: PI
Nearest Person Month worked: 1
Contribution to Project: Analysis planning, results interpretation, coordination with co-PIs, supervision of Kimberly Paul

Name: Kimberly Paul, PhD
Project Role: Postdoctoral Scholar (until Dec 31, 2020)/Asst Prof of Neurology (Jan 1, 2021-current)
Nearest Person Month worked: 4
Contribution to Project: All epidemiologic analysis, including analysis planning, data analysis, results presentation, and interpretation

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: "Khurana- Changes to Other Support 10.14.2021"