1 **Abnormal visual gain control and excitotoxicity in early-onset Parkinson's**

2 **disease *Drosophila* models**

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12 MODELS

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Abstract

The excitotoxic theory of Parkinson's disease (PD) hypothesises that a pathophysiological degeneration of dopaminergic neurons stems from neural hyperactivity at early stages of disease, leading to mitochondrial stress and cell death. Recent research has harnessed the visual system of *Drosophila* PD models to probe this hypothesis. Here, we investigate whether abnormal visual sensitivity and excitotoxicity occur in early-onset PD (EOPD) *Drosophila* models $DJ\text{-}1\alpha^{\Delta 72}$, $DJ1\text{-}\beta^{\Delta 93}$, and $PINK1^5$. We used an electroretinogram to record steady state visually evoked potentials driven by temporal contrast stimuli. At 1 day of age, all EOPD mutants had a twofold increase in response amplitudes when compared to w^- controls. Further, we found that excitotoxicity occurs in older EOPD models after increased neural activity is triggered by visual stimulation. In an additional analysis, we used a linear discriminant analysis to test whether there were subtle variations in neural gain control that could be used to classify *Drosophila* into their correct age and genotype. The discriminant analysis was highly accurate, classifying *Drosophila* into their correct genotypic class at all age groups at 50-70% accuracy (20% chance baseline). Differences in cellular processes link to subtle alterations in neural network operation in young flies – all of which lead to the same pathogenic outcome. Our data are the first to quantify abnormal gain control and excitotoxicity in EOPD *Drosophila* mutants. We conclude that EOPD mutations may be linked to more sensitive neuronal signalling in prodromal animals that may cause the expression of PD symptomologies later in life.

New and Noteworthy: SSVEP response amplitudes to multivariate temporal contrast stimuli were recorded in early-onset PD *Drosophila* models. Our data

51   indicate that abnormal gain control and a subsequent visual loss occur in these PD

52   mutants, supporting a broader excitotoxicity hypothesis in genetic PD. Further, linear

53   discriminant analysis could accurately classify *Drosophila* into their correct genotype

54   at different ages throughout their lifespan. Our results suggest increased neural

55   signalling in prodromal PD patients.

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57   **Keywords**: Parkinson's disease, Gain Control, Excitotoxicity, SSVEPs, *Drosophila*,

58   Linear Discriminant Analysis

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Introduction

Parkinson's Disease (PD) is the second most common progressive neurodegenerative disorder, affecting ~0.2-3% of the population, with an increased prevalence in those aged over 50 (Clarke, 2007; de Rijk et al., 1997). PD is thought to stem from the pathophysiologic degeneration and subsequent loss of dopaminergic neurons within the pars compacta of the substantia nigra, a basal ganglia structure that plays a key role in movement (Clarke, 2007). It is hypothesised that neuronal death in PD is caused by an excitotoxic mechanism, in which neuronal hyperactivity leads to neurodegeneration. Neuronal hyperactivity causes an increase in demand for ATP from mitochondria, leading to oxidative stress and eventual neuronal death (Beal et al., 1993; Surmeier, Obeso, & Halliday, 2017). In both mammals and invertebrates, neuronal responses are regulated by a tightly-linked network of excitatory and inhibitory gain control mechanisms that, collectively, we refer to as 'normalization' (Carandini & Heeger, 1994; Carandini, Heeger, & Movshon, 1997; Carandini & Heeger, 2011; Single, Haag, & Borst, 1997). Normalization mechanisms can be measured across the animal kingdom using a range of methods, including steady state visually evoked potential (SSVEP) recordings, a sensitive technique commonly used to measure the amplitude of neural population responses to periodic flickering stimuli (Busse, Wade, & Carandini, 2009; Norcia, Appelbaum, Ales, Cottereau, & Rossion, 2015; Regan, 1966; Tyler, Apkarian, & Nakayama, 1978).

In *Drosophila*, SSVEP recordings are collected from the surface of the eye and can be made in both healthy and PD mutant *Drosophila* (Afsari et al., 2014; West, Elliott, & Wade, 2015a). Previously we have shown that young flies carrying

101 the late-onset gain-of-function PD mutation *LRRK2-G2019S* showed *increased*

102 visual contrast sensitivity to full field flicker stimuli, reflecting a failure in regulation of

103 neural activity (i.e. abnormal gain control or normalization) at one day of age (Afsari

104 et al., 2014). This regulatory failure is followed by a decline in visual function over

105 time, with physiological and anatomical degeneration in older *LRRK2-G2019S*

106 *Drosophila* (Hindle et al., 2013; Mortiboys et al., 2015).

107

108   Feeding *LRRK2-G2019S Drosophila* with BMPPB-32, a kinase inhibitor

109 specifically targeted at *LRRK2*, restored normal contrast sensitivity at both 1 and 14

110 days of age, indicating that both the early neuronal hypersensitivity and the

111 subsequent neurodegeneration are due to abnormal kinase domain activity (Afsari et

112 al., 2014). Vision loss was accelerated by increasing neural activity via photic

113 stimulation of the *Drosophila* visual system using flashing LED lights. Together,

114 these findings support an excitotoxicity theory of the *LRRK2-G2019S* form of PD.

115 This excitotoxicity theory of PD has also found support in rodent models of the

116 *G2019S* mutation (Longo, Russo, Shimshek, Greggio, & Morari, 2014; Matikainen-

117 Ankney et al., 2016; Ponzo et al., 2017; Sloan et al., 2016; Volta et al., 2017).

118

119   We have previously demonstrated that linear discriminant analysis (LDA) is a

120 useful tool in the analysis of SSVEP data obtained from *Drosophila* (West, Elliott, &

121 Wade, 2015b). Here, our findings indicated differences in SSVEP amplitude both

122 between and within wild type flies and EOPD mutants, in response to spatiotemporal

123 patterns. These differences had enough statistical regularity for LDA to accurately

124 discriminate between genotypes. When compared to wild-type controls, qualitative

125 observations indicated an elevation in SSVEP response in 1 day old EOPD flies.

126   Although LDA has diagnostic utility, it does not allow for the quantification of

127   directional differences in such responses. Having established this method, we now

128   seek to expand upon this and investigate abnormal gain control and excitotoxicity in

129   EOPD models.

130

131   Is excitotoxicity a general feature of all Drosophila PD mutants? If so, it would

132   suggest that rather than being an epiphenomenon of some metabolic dysfunction

133   that causes PD, the excitotoxicity itself is central to the disease. In the current paper,

134   we use SSVEP techniques combined with principal components analysis, general

135   linear modelling, and multivariate classification analysis, to investigate abnormal gain

136   control and excitotoxicity in EOPD *Drosophila* models. We hypothesised that

137   abnormal gain control would occur in young *Drosophila* carrying EOPD mutations

138   due to disease related changes in retinal dopaminergic neurons, reflected by

139   increased SSVEP amplitudes in 1 day old EOPD *Drosophila* mutants. We also

140   hypothesised that abnormal gain control would cause an excitotoxic cascade in older

141   EOPD *Drosophila.* Consequently, we expected to observe a decrease in SSVEP

142   amplitudes at later ages. Finally, we wondered if all mutations affected neuronal gain

143   control in the same manner or if there were subtle mechanistic variations that could

144   be used to differentiate the genotypes. To address this, we used linear discriminant

145   analysis based on SSVEP responses to a range of temporal modulation rates and

146   contrast levels to attempt to classify flies into their correct genotypic class at different

147   points throughout their lifespan. The greater the differences in the gain control

148   profiles across genotypes, the greater the accuracy we expected from this

149   classification.

150

151   We found that SSVEP response amplitudes to spatial stimuli are significantly

152   increased in EOPD mutants at 1 day of age – indicating that neuronal gain control is

153   abnormal in these animals. Generating additional neuronal stress by exposing flies

154   to randomly pulsating light for 7 days resulted in a profound loss of vision in all PD

155   mutants, supporting the excitotoxicity model of PD. Finally, there are robust

156   differences between the temporal contrast response profiles of the different PD

157   mutants which allow our multivariate classification algorithms to classify flies into

158   their respective genotypes at well above chance levels throughout their lifespan.

159

160   **Materials and Methods**

161   *Drosophila stocks and maintenance*

162   *Drosophila* were raised in a 12hr:12hr light:dark (LD) cycle at 25° on standard

163   food consisting of agar (1% w/v), cornmeal (3.9%), yeast (3.7%), and sucrose

164   (9.4%). All flies were outcrossed and stabilised where appropriate to remove any

165   naturally occurring mutations. Three EOPD mutations (*DJ-1α$^{\Delta 72}$, DJ1-β$^{\Delta 93}$*, and

166   *PINK1^5*), one knockout of the fly *LRRK2* homologue (*dLRRK^{ex1}*) and one wild-type

167   control genotype (*w^{1118}*, herein *w$^-$*) were deployed. *w$^-$* strains were gifted by Sean

168   Sweeney. *PINK1^5* and *dLRRK^{ex1}* strains were obtained from the Bloomington

169   *Drosophila* Stock Centre (Indiana, USA), whilst *DJ-1α$^{\Delta 72}$* and *DJ1-β$^{\Delta 93}$* strains were

170   kind gifts from Alex Whitworth. Male flies all had white eyes, and were tested at 1, 7,

171   14, 21, and 28 days post eclosion.

172

173   *Preparation of Drosophila for Testing*

174   Male flies were collected within 8 hours of eclosion and transferred to a new

175   vial of standard food that additionally contained nipagin (0.1% w/v). Flies were

176 maintained in these vials and transferred to fresh food weekly. Flies were kept in a

177 12hr:12hr LD cycle at 25°C until they had reached appropriate age for testing.

178

179 *Photic stress*

180   To explore as to whether an increase in neural demand resulted in a

181 decrease in SSVEP amplitudes, all *Drosophila* genotypes were exposed to a photic

182 stressor condition (Afsari et al., 2014; Hindle et al., 2013). Male flies were collected

183 within 8 hours of eclosion and transferred to a new vial of standard food containing

184 nipagin. These flies were maintained within a 29°C incubator with irregularly

185 pulsating LED lights at ~1.5s intervals to force the *Drosophila* visual system to adapt

186 to new light levels and increase photoreceptor response. Flies were maintained here

187 for 7 days, as this was the age at which *G2019S* mutants had previously shown

188 visual loss (Hindle et al., 2013). Ten flies of each genotype tested (except for *DJ-*

189 *$1\alpha^{\Delta72}$* where eight were tested) (N=48).

190

191 *Preparation for Electroretinogram*

192   On the day of testing, flies were collected using a pooter and aspirated into a

193 shortened pipette. Once the fly's head was protruding from the tip of the pipette, it

194 was restrained by placing a small layer of nail varnish on the back of the fly's neck.

195 Two pipettes at a time were mounted onto a customised *Drosophila*

196 electroretinogram (ERG) recording system, with both flies placed 22cm away from

197 the dual display monitors (West et al., 2015). ERG recordings were made through

198 hollow drawn-glass electrodes containing simple saline (130mM, NcCl, 4.7 mM KCl,

199 1.9mM $CaCl_2$) connected to a high-impedance amplifier (LF356 op-amp in the circuit

200 [Fig.7] of (Ogden, 1994)) via thin silver wires. The reference electrode was inserted

201   gently onto the *Drosophila* proboscis, and the recording electrode was placed on the

202   surface of the right eye. Ten unique flies of each genotype at each age were tested

203   (total N=250).

204

205   *Stimuli*

206   Stimuli were contrast-reversing achromatic sine wave gratings with a range of

207   Michelson contrasts (Michelson, 1927) and temporal frequencies. Spatial frequency

208   was held at 0.056 cycles per degree as this had previously been found to be the

209   optimal spatial frequency to measure SSVEP recordings from *Drosophila* (West et

210   al., 2015a). Stimuli were generated using the Psychophysics Toolbox on a Windows

211   7 PC and were displayed on dual 144Hz LCD monitors (XL240T, BenQ, Tiwam).

212   Stimuli swept through unique combinations of 8 levels of temporal frequency (1, 2, 4,

213   6, 8, 12, 18 and 36 Hz) and 8 levels of contrast (1, 4, 8, 16, 32, 64, 99%) to generate

214   64 different combinations of temporal contrast stimuli. Parameter combinations were

215   presented in a random order for an 11 second trial, with a 4 second inter-stimulus

216   interval. The first second of each trial was removed prior to analysis to remove onset

217   transients. Each parameter combination was presented 3 times per fly to create a

218   ~1-hour recording session.

219

220   *Analysis*

221   *Steady state visually evoked potentials*

222   The periodic modulation of a contrast reversing grating evokes steady-state,

223   visually evoked potentials (SSVEPs) with a phase-locked, periodic time course which

224   is analysed most conveniently in the frequency domain (see Figure 1A and C for

225   examples of SSVEP response from w^- and *PINK1* mutants). For a single contrast

reversing grating, the ERG records responses from both the photoreceptors and the

subsequent neuronal signalling pathways (Afsari et al., 2014). Individual

photoreceptors will track the luminance modulations of the grating bars at the input

frequency (F1) but because the signal elicited by a grating is a population average of

photoreceptors driven by different transition polarities (some dark->light, some light-

>dark) the overall photoreceptor contribution is largely self-cancelling. Residual

responses at F1 arise from asymmetries in photoreceptor sampling of the relatively

low spatial frequency grating. The majority of the signal is composed of the transient

responses arising from the visual neurons which are confined to even multiples of

the input frequency. Of these responses, the second harmonic is by far the largest

and we restrict our analyses to 2f for each input frequency. A coherently averaged

(phase-sensitive) Fourier amplitude was calculated for each temporal frequency and

contrast combination by averaging complex frequency-domain data obtained for

each condition over 3 runs (see Figure 1B and D for examples of Fourier amplitudes

from w^- and *PINK1* mutants). Due to the phase-locked nature of VEPs, coherent

averaging preserves the signal while phase-randomized noise sums to zero (Norcia

et al., 2015).This results in a high signal to noise ratio for SSVEP recordings.

FIGURE 1 HERE

Linear discriminant analysis

We assessed LDA as a tool to accurately assign flies into their correct

genotype based on multivariate visual response profiles. We used ERG

measurements recorded in response to 64 combinations of contrast and temporal

frequency, thus, providing a 64-dimensional dataset to input into the LDA. Each fly

was therefore located in a 64-dimensional space. Flies that showed similar

251 responses to these combinations of contrast and temporal frequency clustered

252 together in this space. Thus, if different classes showed different visual responses,

253 unique clusters for each class would form in this 64-dimensional space. The LDA

254 algorithm then attempted to identify a single linear boundary between these clusters

255 and classified each fly into a genotypic class by asking which side of this linear

256 boundary the fly was situated. The accuracy of the LDA algorithm depends on the

257 degree of separation between the genotypic clusters in the multidimensional feature

258 space. This is further expanded upon in Figure 2, where we illustrate the process of

259 raw data collection through to a range of possible classifications.

260

261   *FIGURE 2 HERE*

262

263 **Results**

264 *Early-onset PD temporal contrast profile amplitudes are larger than controls*

265   A series of exemplar raw SSVEP responses from both w^- and *PINK1* mutants

266 at different ages and stimulus contrasts are illustrated in Figure 3. Average Fourier

267 amplitudes at 2*f* for each temporal contrast combination for each genotype are

268 illustrated in Figure 4. Higher peak response amplitudes are represented by lighter

269 colours whilst lower amplitudes are represented by darker colours. Visual response

270 changes as a function of both contrast and temporal frequency, with responses in

271 both wild-type and EOPD models peaking at high contrast (99%) and an

272 intermediate temporal frequency (6-8Hz).

273

274   *FIGURE 3 HERE*

275   *FIGURE 4 HERE*

276

Principal Components Analysis

278   We computed a Principal Components Analysis (PCA) on the full dataset

279 (N=250) (See Figure 5). This allowed us to retain just those principal components

280 (PCs) that explain significant amounts of the overall variance, simplifying our 64-

281 dimensional data significantly (Jolliffe & Cadima, 2016; West et al., 2015a). Our first

282 PC explained 89.9% of total variance within the dataset and the univariate analysis

283 that follows is based on the amplitude of this component while the multivariate

284 analysis later in the paper is performed on the full dataset.

285

286   *FIGURE 5 HERE*

287

Main effects

289   A 5x5 between groups ANOVA was performed on the first principal

290 component score (representing SSVEP amplitude) to assess if there was a

291 difference in SSVEP amplitudes between *Drosophila* genotypes or ages. The

292 analysis found a significant main effect of genotype, $F(4,225) = 21.428$, $p < .001$,

293 indicating a difference in response amplitude between the five genotypes, when

294 collapsed over age. The analysis also found a significant main effect of age $F(4,225)$

295 $= 5,558$, $p < .001$, indicating a difference in response amplitude between the 5 ages,

296 when collapsed over genotype. Finally, there was a significant interaction effect

297 $F(16,225) = 2.984$, $p < .001$, indicating that response amplitude differed between

298 genotype depending on age. A simple effects analysis was performed to tease out

299 differences in our conditions and explore our interaction effect.

300

301   *Simple effects analysis comparing between genotypes within each age group*

302   A simple effects analysis was undertaken to explore differences in the SSVEP

303   amplitudes of *Drosophila* genotypes within each age group, with Sidak corrections

304   applied to all possible comparisons. The SSVEP amplitudes of each genotype as a

305   function of age are illustrated in Figure 6, whilst all corresponding *p* values are

306   presented in Statistical Supplements Tables 1-10. Analysis revealed that at 1 day

307   of age, all EOPD mutations (i.e. excluding *dLRRK^{ex1}*) had significantly higher SSVEP

308   amplitudes when compared to *w⁻* control flies, ($p < .01$)**.** When comparing between 1

309   day old PD mutants, *PINK1^5* produced significantly higher SSVEP amplitudes when

310   compared to both *DJ-1α^{Δ72}* ($p < .05$) and *dLRRK^{ex1}* mutants ($p < .01$). There were no

311   other significant differences in the SSVEP amplitudes of PD mutants. The larger

312   amplitudes of EOPD mutants did not hold over later ages as wild type response

313   increased at 7 days of age (see Figure 6). However, differences between the SSVEP

314   amplitudes of PD mutants was found at these later ages. At 7 days of age *PINK1^5*

315   mutants produced significantly higher amplitudes when compared to *dLRRK^{ex1}* ($p <$

316     $.005$), whilst at 14 days of age *DJ1-β^{Δ93}* had significantly higher amplitudes when

317   compared to *DJ-1α^{Δ72}* ($p < .001$) and *dLRRK^{ex1}* ($p < .001$) mutants. This trend

318   continued at 21 days of age, with *DJ1-β^{Δ93}* continuing to show higher SSVEP

319   amplitudes when compared to *DJ-1α^{Δ72}* ($p < .01$) and *dLRRK^{ex1}* ($p < .05$). At 28

320   days of age, *DJ1-β^{Δ93}* ($p < .01$) and *PINK1^5* ($p = .01$) produced significantly higher

321   SSVEP amplitudes when compared to *DJ-1α^{Δ72}*.

322

323   *FIGURE 6 HERE*

324

325   *Simple effects analysis comparing between age group within each genotype*

13

326   A simple main effects analysis was undertaken to explore differences in the

327   SSVEP amplitudes within each *Drosophila* genotype over its lifespan, with Sidak

328   corrections applied to all possible comparisons. The *p* values for all simple effects

329   are presented in Supplements. Analysis revealed that w^- response amplitudes

330   increased between 1 and 7 days of age ($p = .001$), however there was no significant

331   difference when comparing between further consecutive ages within this genotype,

332   thus, visual response held stable between 7 to 28 days of age. There was a

333   significant increase in *DJ1-$\beta^{\Delta93}$* response amplitudes between 7 and 14 days of

334   age ($p < .001$), which then held steady from 14 to 28 days of age. There was no

335   significant difference in response amplitudes within *DJ-1$\alpha^{\Delta72}$*, *PINK1^5* or *dLRRK^{ex1}*

336   at any consecutive ages between 1 and 28 days.

337

338   *Increased demand for energy in the visual system leads to loss of visual response in*

339   *old PD flies*

340   While we demonstrated that abnormal gain control occurs in 1 day old EOPD

341   mutants, at later ages, responses were comparable to those of wild-type flies (w^-).

342   This represents a difference between EOPD mutant flies and flies mimicking the late-

343   onset *LRRK2-G2019S* mutation, where responses fall to zero at later ages (Hindle et

344   al., 2013). We hypothesized that maintaining our *Drosophila* stocks at 25° and a

345   12:12 LD cycle did not produce enough neuronal demand on the visual system to

346   see any effect. To test this hypothesis, we increase the demand for energy by

347   exposing *Drosophila* to irregular ~1.5s flashes of light of at random periodic intervals

348   over seven days. Here, we hypothesise that the abnormal gain we have observed in

349   young EOPD flies will interact with a visually induced increase in neural demand to

350   cause an excitotoxic cascade.

14

351   Observation of temporal contrast response profiles (see Figure 7) indicated a

352 profound reduction in SSVEP amplitudes across temporal frequency and contrast

353 combinations for PD mutants (but not wild-type flies) after seven days exposure to

354 photic stress.

355

356   *FIGURE 7 HERE*

357

358   A one way between groups ANOVA was performed on the first principal

359 component score (representing SSVEP amplitude) extracted via the PCA analysis to

360 assess if there was a significant difference in visual response between five

361 *Drosophila* genotypes after they had been exposed to seven days of photic stress.

362 The analysis found a significant main effect of genotype, $F(1,43) = 5.965$, $p = .001$,

363 $\eta^2 = .357$, indicating a difference in response amplitude between the five genotypes.

364 Pairwise comparisons revealed that all PD mutants produced significantly lower

365 SSVEP amplitudes when compared to w^- control flies ($p < .05$), indicating an

366 interaction between visual stimulation and *Drosophila* genotype on visual response

367 amplitudes (see Figure 8). There was no significant difference between the PD

368 mutants' SSVEP responses.

369

370   *FIGURE 8 HERE*

371

372 *Linear discriminant analysis classifies flies into their correct genotypic class*

373   Thus, all EOPD mutants show both an early increased visual response and a

374 loss of vision after 7 days of visual stimulation, compared to w^- control flies.

375

376 In the presentation of our data so far, we utilized PCA to reduce the

377 dimensionality in our data to a single variable, thereby removing any nuanced

378 differences between full *Drosophila* temporal contrast profiles. We now explore how

379 linear discriminant analysis can use the additional small, but significant sources of

380 variation in our SSVEP data to classify *Drosophila* into their correct genotypic class

381 and age group.

382

383 Linear Discriminant Analysis (LDA) is a statistical method that aims to answer

384 both binary and multi-class classification problems by seeking linear combinations of

385 variables that best explain the variance within the data, working under the

386 assumption that unique classes generate unique Gaussian distributions (Izenman,

387 2008). We assess the accuracy of our LDA in two ways. First, we use a standard

388 linear classifier (Fisher, 1936) as implemented in MATLAB's (2017a, Mathworks,

389 MA) 'classify' function to conduct a leave one out (LOO) analysis, where the

390 classifier receives training data from all flies to be assessed except one, then we

391 measure the classifiers accuracy in classifying the excluded fly. This fly is

392 resubstituted and the classification is repeated for every fly in the dataset to return a

393 generalized LOO accuracy. Second, use MATLAB's classification function 'fitcdiscr'

394 to fit an LDA model to our raw 64-dimensional data. We then use Monte Carlo

395 resampling methods to produce 3 estimates of accuracy – an overall model

396 accuracy, an N-way classification accuracy (the accuracy of correctly classifying a fly

397 into one of the 5 genotypes at each age group or 5 age groups for each genotype)

398 and a pair-wise classification accuracy (the accuracy of correctly classifying a fly into

399 one of two correct genotypes at each age group). For detailed description of the

400   methods we used to apply LDA to multivariate *Drosophila* data, please see West et

401   al. (2015).

402   Here, we hypothesise that *Drosophila* will be classified into their correct

403   genotypic class at above-chance levels based on temporal contrast profiles, in line

404   with previous findings using spatiotemporal profiles (West et al., 2015a).

405

406   *Overall Model Discrimination Accuracy*

407   We first ran our full dataset of 25 classes through the LDA to assess how well

408   it could classify *Drosophila* when considering both their genotype and age. In this

409   case, baseline (chance) performance was 4% (1/25). Next, to assess how well we

410   could discriminate between *Drosophila* genotypes within each age group, our data

411   were partitioned into 5 genotypes and LDA was applied with a 20% chance baseline

412   (1/5). Finally, to assess how well we could classify between *Drosophila* at different

413   ages within each genotype, our data were divided into 5 age groups within each

414   genotype and analysed using LDA, again with a 20% chance baseline (1/5).

415

416   The full overall classification accuracies for both LOO analysis and Monte

417   Carlo resampling analysis for all 3 sets of data are presented in Table 11. The

418   overall accuracy of our model in classifying *Drosophila* into their correct genotypic

419   class differed depending on the age of the genotypes included in the model. The

420   highest classifications occurred at 1 and 28 days of age. Although there was a slight

421   decrease in accuracies when classifying *Drosophila* into their correct age within a

422   genotype, the algorithm still performed above 20% chance baseline for all

423   genotypes.

424

Class	LOO Classification	Monte Carlo Resampling
All 25 classes	24.8%	29.6%
1 day post eclosion	58%	68%
7 days post eclosion	52%	64%
14 days post eclosion	46%	54%
21 days post eclosion	48%	50%
28 days post eclosion	64%	70%
w^-	54%	54%
$DJ\text{-}1\alpha^{\Delta 72}$	38%	38%
$DJ1\text{-}\beta^{\Delta 93}$	52%	52%
$PINK1^5$	34%	50%
$dLRRK^{ex1}$	26%	34%

425

426   Table 11: Classification accuracy differs when flies are grouped by age and

427   classified into genotype, and when they are grouped by genotype and classified into

428   age. Generally, both LOO and Monte Carlo resampling methods provide similar

429   classification accuracies. N=50 for per class (chance baseline 20%), except 'All 25

430   classes' N=250 (chance baseline 4%).

431

432   *N-Way Classification Accuracy*

433   The confusion matrix was used to establish the accuracy of our LDA model to

434   classify *Drosophila* into their correct genotypic class. Again, we investigated the

435   precision of our model when all 25 classes were included in the model, with a 4%

436   chance baseline (1/25). All classifications were reported above chance, bar $PINK1^5$

437   at 21 days of age. The highest accuracy was for w^- *at* 1 day of age, where the

438   model performed with 34.49% accuracy, whilst most other conditions were classified

439   with ~25% accuracy. A profile of classification accuracies when all 25 classes are

440   considered is presented in Figure 9.

441

442   *FIGURE 9 HERE*

443

444   Next, we assessed the ability of the classifier to accurately genotype

445   *Drosophila* within each age group, thus, five genotypes at each age were included in

446   the model, with a 20% chance baseline (1/5). Our classification accuracy is deduced

447   by normalizing our confusion matrix by dividing by the number of flies in each

448   condition (n=10). As illustrated in Figure 10, at 1 day of age our model could classify

449   w^- control flies into their correct genotypic class with 78.8% accuracy, whilst we

450   could classify *DJ-1$\alpha^{\Delta 72}$* at 45.5% accuracy, *DJ1-$\beta^{\Delta 93}$* at 52.9% accuracy, *PINK1^5* at

451   73.6% accuracy and *dLRRK^{ex1}* at 60.0% accuracy.

452

453   *FIGURE 10 HERE*

454

455   These accuracies shifted at seven days of age, with our model classifying w^-

456   with 29.8% accuracy, *DJ-1$\alpha^{\Delta 72}$* with 50.0% accuracy, *DJ1-$\beta^{\Delta 93}$* with 64.7%

457   accuracy, *PINK1^5* with 62.2% accuracy and *dLRRK^{ex1}* at 46.9% accuracy. At 14

458   days of age our model could accuracy classify w^- at 50.0% accuracy, *DJ-1$\alpha^{\Delta 72}$* at

459   68.1% accuracy, *DJ1-$\beta^{\Delta 93}$* at 50.3% accuracy, *PINK1^5* at 36.4% accuracy and

460   *dLRRK^{ex1}* at 29.1% accuracy. At 21 days of age with our model classified w^- at

461   58.35% accuracy, *DJ-1$\alpha^{\Delta 72}$* at 50.5% accuracy, *DJ1-$\beta^{\Delta 93}$* at 50.2% accuracy,

462   *PINK1^5* at 25.7% accuracy and *dLRRK^{ex1}* 53.8% accuracy**.** At 28 days of age our

463 model classified w^- with 53.7% accuracy, $DJ\text{-}1\alpha^{\Delta72}$ with 71.5% accuracy, $DJ1\text{-}\beta^{\Delta93}$

464 with 62.6% accuracy, $PINK1^5$ with 55.1% accuracy and $dLRRK^{ex1}$ at 46.35%

465 accuracy.

466

467 *N-Way Classification Accuracy: Age*

468 Here, our LDA model was used to classify *Drosophila* mutants into their

469 correct age within a single genotype, with a 20% chance baseline (1/5).

470 Comparatively, the model was generally weaker in accurately classifying into age

471 when compared to classifying into genotype, although all classifications exceeded

472 chance baseline. Age N-Way classification accuracies for each genotype are

473 presented in Table 12.

474

N-Way Classification Accuracy

Genotype	1 day	7 days	14 days	21 days	28 days
w^-	81.3%	29.5%	32%	53.5%	53.5%
$DJ\text{-}1\alpha^{\Delta72}$	26.6%	34.1%	50.0%	29.7%	48.4%
$DJ1\text{-}\beta^{\Delta93}$	55.3%	59.5%	51.0%	45.0%	57.3%
$PINK1^5$	39.7%	49.1%	35.0%	27.2%	49.3%
$dLRRK^{ex1}$	37.6%	23.7%	22.7%	30.2%	43.7%

475 Chance baseline: 20% (1/5)

476 Table 12: N-Way classification of flies into their correct age differs between

477 genotypes. All classes can be classified above 20% chance baseline, with the

478 highest accuracy sitting at 81.3% for 1 day old w^- classifications. n=10

479

480

Pairwise Classification Accuracy

482   To assess the accuracy of our model in classifying *Drosophila* between pairs

483   of genotypes within each age group we bootstrapped our data through 1000

484   iterations of a two-way classification analysis. Here, we assess the accuracy of the

485   algorithm estimation in classifying a fly from a pair of genotypes into its correct class.

486   Classification is significantly above chance when fewer than 5% of the bootstrapped

487   2-way classification probabilities are .5 or greater.

488

489   As presented in Table 13, the algorithm classified one-day old *Drosophila*

490   genotypes with accuracy between 73.7% - 94.1% (p<.05). Notably, all PD mutants

491   could be accurately distinguished from w^- control flies.

492

	w^-	DJ1-$\beta^{\Delta 93}$	DJ-1$\alpha^{\Delta 72}$	dLRRK^{ex1}
PINK1^5	94.1%*	84.7%*	78.8%*	88.9%*
w^-	-	86.3%*	75.8%*	77.6%*
DJ1-$\beta^{\Delta 93}$	-	-	57.9%	73.7%*
DJ-1$\alpha^{\Delta 72}$	-	-	-	65.3%

493   ** = p < .05*

494   Table 13: LDA can accurately compute pairwise classifications between PD and

495   control genotypes at 1 day of age (n=10).

496

497   As presented in Table 14, at 7 days of age the model had a reduction in the

498   amount of significant comparisons, performing between 74.5% - 85.6% accuracy. At

499   this age, the LDA could not accurately discriminate between any of the PD mutants

500   and control flies.

	w^-	$DJ1\text{-}\beta^{\Delta93}$	$DJ\text{-}1\alpha^{\Delta72}$	$dLRRK^{ex1}$
$PINK1^5$	69.9%	74.7%*	76.1%*	85.6%*
w^-	-	60.8%	60.5%	63.3%
$DJ1\text{-}\beta^{\Delta93}$	-	-	67.7%	76.3%*
$DJ\text{-}1\alpha^{\Delta72}$	-	-	-	66.9%

501   * = $p < .05$

502   Table 14: LDA had a reduction in total significant comparisons at 7 days of age, and

503   cannot accurately discriminate between any of the PD mutants when compared

504   against control flies (n=10).

505

506   At 14 days of age there appeared to be an overall improvement in pairwise

507   classifications with significant pairwise classifications between 78.0% - 81.3%

508   accuracy, as illustrated in Table 15.

509

	w^-	$DJ1\text{-}\beta^{\Delta93}$	$DJ\text{-}1\alpha^{\Delta72}$	$dLRRK^{ex1}$
$PINK1^5$	61.7%	57.8%	78.6%*	79.2%*
w^-	-	78.4%*	78.0%*	79.9%*
$DJ1\text{-}\beta^{\Delta93}$	-	-	89.6%*	91.3%*
$DJ\text{-}1\alpha^{\Delta72}$	-	-	-	52.1%

510   * = $p < .05$

511   Table 15. LDA can accurately compute pairwise classifications between PD and

512   control genotypes at 14 days of age (n=10). There are differences in accuracy when

513   compared to 7 and 1 day old classifications.

514

515   This held at 21 days of age, where our pairwise classification accuracy

516   reached between 75.2% - 85.1% for significant comparisons, as illustrated in Table

517   16, however there was a reduction in significant comparisons at this age.

	w^-	$DJ1\text{-}\beta^{\Delta 93}$	$DJ\text{-}1\alpha^{\Delta 72}$	$dLRRK^{ex1}$
$PINK1^5$	63.3%	65.2%	75.2%*	52.9%
w^-	-	78.4%*	77.4%*	69.4%
$DJ1\text{-}\beta^{\Delta 93}$	-	-	85.1%*	77.7%*
$DJ\text{-}1\alpha^{\Delta 72}$	-	-	-	60.6%

518   *$= p < .05$

519 Table 16. LDA can accurately compute pairwise classifications between PD and

520 control genotypes at 21 day of age (n=10), however there are less significant

521 comparisons compared to earlier ages.

522

523   In line with our peak in overall model accuracy, our model was most accurate

524 in classifying between flies at 28 days of age, with all possible comparisons

525 statistically significant and sitting between 72.7% and 86.2% accuracy (Table 17).

526 Similar to one day old comparisons, all PD mutants could be accurately

527 distinguished from w^- control flies at 28 days of age. We note that these statistics

528 differ from the comparisons on the PCA simple effects analysis data, as will be

529 addressed in our discussion.

530

531

532

533

	w^-	DJ1-$\beta^{\Delta 93}$	DJ-1$\alpha^{\Delta 72}$	dLRRK^{ex1}
PINK1^5	78.9%*	78.7%*	79.7%*	73.7%*
w^-	-	86.2%*	81.0%*	75.6%*
DJ1-$\beta^{\Delta 93}$	-	-	88.4%*	83.6%*
DJ-1$\alpha^{\Delta 72}$	-	-	-	72.7%*

534   *= $p < .05$

535   Table 17. LDA accurately computes pairwise classifications between all genotypes at

536   28 days of age (n=10). All comparisons are significant and above 72.7% accuracy.

537

538   **Discussion**

539   *Abnormal gain control in early-onset PD Drosophila models*

540   We have demonstrated that abnormal gain control occurs in young EOPD

541   mutants; *DJ-1$\alpha^{\Delta 72}$*, *DJ1-$\beta^{\Delta 93}$*, and *PINK1^5*. *Drosophila* with these mutations have

542   significantly higher SSVEP response amplitudes when compared to *w^-* controls at

543   day 1. Notably, there appears to be no difference between response amplitudes of 1

544   day old *w^-* controls and knockout of the fly *LRRK2* homologue *dLRRK^{ex1}*. These

545   results are consistent with previous studies, and point to a common phenotype of

546   abnormal gain control occurring in the current studied EOPD mutants and the

547   *LRRK2-G2019S* late-onset mutant (Afsari et al., 2014; West et al., 2015a).

548

549   What common biological mechanism might explain these findings?

550   Dopaminergic terminals are found in the *Drosophila* ommatidium, lamina, and

551   medulla, where dopamine is thought to regulate contrast sensitivity, light adaptation,

552   and circadian rhythms (Afsari et al., 2014; Chyb et al., 1999; Hirsh et al., 2010;

553   Jackson et al., 2012; Nassel & Elekes, 1992). Thus, dopamine acts as a

neuromodulator within the *Drosophila* visual system, effectively regulating neural response to visual excitation. PD-model flies may have less dopamine content, and/or fewer dopaminergic neurons, or disrupted dopamine signalling, though the reduction may depend on the environmental conditions (Navarro et al., 2014; Ng et al., 2012; Park et al., 2006; Wang et al., 2006). Any reduction in dopamine release will cause photoreceptors to respond faster and with greater amplitude (Chyb et al., 1999). This hyperactivity causes increased SSVEP amplitudes, manifesting as abnormal gain control. Humans, like flies, have retinal dopamine within the amacrine cells and inner border of the nuclear layer, where it is thought to be responsible for light adaptation, contour perception, and contrast sensitivity (Crooks & Kolb, 1992; Dowling, 1979; Witkovsky, 2004). Human patients also show a reduction in retinal dopamine and report a range of low-level visual deficits, including poor contrast sensitivity and reduced light sensitivity (Archibald, Clarke, Mosimann, & Burn, 2011; Beitz, 2014; Chaudhuri & Schapira, 2009; Weil et al., 2016). These homologies in retinal structure, function, and disease pathology point to the possibility that prodromal gain control abnormalities occur in human PD patients.

The response profile of wild-type *w⁻ Drosophila* changes as a function of age. This genotype initially presented with comparatively low response amplitudes when compared to EOPD mutants. *w⁻* response then increased between 1 and 7 days of age. This reflects the anatomical plasticity of the young Drosophila visual system. Young *w⁻* flies are born with reduced visual sensitivity which then adapts to functional requirements, with visual maturity occurring between 4 - 7 days of age (Kral & Meinertzhagen, 1989). It is important to note that all *Drosophila* included in our study are white eyed, thus share the *w⁻* mutation. The increased sensitivity to

579   visual stimuli we observe in EOPD mutants, and mutants' unique developmental

580   profiles, is due solely to the PD mutation.

581

582   *Excitotoxicity as a pathological phenotype in Parkinson's disease*

583   Initially we saw no evidence of excitotoxic damage in the visual system of

584   older PD flies. However, *Drosophila* in the lab experience a relatively stable visual

585   environment: light levels are many orders of magnitude lower than those in the

586   outside world and they are modulated according to a strict 12hr:12hr LD cycle. We

587   theorised that purposeful visual stimulation of the PD *Drosophila* visual system may

588   be necessary to induce excitotoxicity in the lab. To increase neural demand for

589   energy we exposed flies to a rich visual environment which contained irregular bursts

590   of high intensity luminance modulations. This environment requires the

591   photoreceptors both to change their firing rates and their mean sensitivity over

592   relatively short time periods. Our hypothesis was that the abnormal gain control we

593   observed in young EOPD flies would interact with an increase in neural activity to

594   cause an excitotoxic cascade. Our data are consistent with this hypothesis – PD, but

595   not w^- flies, showed reduced visual functionality after prolonged exposure to these

596   visually demanding environments.

597

598   Our results provide evidence for an excitotoxic cascade in PD *Drosophila*

599   mutants, with *DJ-1α^{Δ72}*, *DJ1-β^{Δ93}*, and *PINK1^5* all showing a significant decrease in

600   SSVEP amplitudes after seven days of visual stimulation, with a minimum of 50%

601   reduction in response. Surprisingly, the response amplitudes of *dLRRK^{ex1}* mutants

602   were also reduced, even though we did not observe abnormal gain control in this

603   strain at one day of age.

26

604

605   We draw upon the previously established theory of excitotoxicity in PD explain

606   the biological processes underlying our observed visual loss. Here, abnormal gain

607   control interacts with a visually induced increase in neural demand. This causes an

608   increase in ionic flux across the cell membrane which in turn results in extra demand

609   for ATP from the ion exchange pumps. When mitochondria cannot meet this

610   increased demand for ATP, they release reactive oxygen species (e.g. superoxide,

611   hydrogen peroxide), so generating oxidative stress, which leads to autophagy,

612   apoptosis and other forms of cell damage. This is then followed visual decline and

613   eventual cell death (Hindle et al., 2013).

614

615   Mitochondrial dysfunction and oxidative stress appear to play a central role in

616   PD pathogenesis (Bogaerts, Theuns, & Van Broeckhoven, 2008; Büeler, 2009;

617   Henchcliffe & Beal, 2008; Schapira, 2008). The current paper has investigated

618   *Drosophila* PD mutations in genes whose human homologues are associated with

619   EOPD. In both humans and flies, *DJ-1* encodes a small protein that is thought to

620   protect against oxidative stress and assist in mitochondrial regulation by acting as a

621   sensor for Reactive Oxidative Species (ROS) (Oswald et al., 2016). Subsequently,

622   loss-of-function mutations in *DJ-1* appear to increase cell death in response to

623   oxidative stress. Further, animal studies have observed perturbations in dopamine

624   release in DJ-1 deficient animal models, although there is no physiological loss of

625   dopamine neurons (Goldberg et al., 2005; Martella et al., 2011; Menzies, Yenisetti, &

626   Min, 2005; Meulener et al., 2005; Pisani et al., 2006; Yang, Chen, Ding, Zhuang, &

627   Kang, 2007). *PINK1* is a protein kinase with a mitochondrial targeting sequence and

628   acts to maintain mitochondrial homeostasis in dopaminergic neurons (Park et al.,

629   2006). Likewise, studies in *PINK1* animal models have found evidence for abnormal

630   mitochondrial morphology and impaired dopamine release (Clark et al., 2006; Kitada

631   et al., 2007; Park et al., 2006)**.** Thus, the protein products of both *DJ-1* and *PINK1*

632   both play roles in the regulation of cellular energy production. However, loss-of-

633   function mutations on these genes negatively impact mitochondria in different ways.

634   Our data provide additional support for the hypothesis that mitochondrial impairment

635   plays a role in the pathogenesis of genetic PD.

636

637   *Classification of Drosophila PD genotype*

638   Previously, we demonstrated that discriminant analysis is a useful tool that

639   can accurately classify PD *Drosophila* into their correct genotypic class at 1 day of

640   age (West et al., 2015a). Here, we build upon this observation, establishing that

641   variability within temporal contrast response profiles obtained from *Drosophila* can

642   be used in a LDA to accurately classify *Drosophila* into their correct genotypic class

643   at various ages with above chance accuracy. When all 25 classes were included in

644   our model, our LOO classification accuracy sat at 24.8%, whilst our bootstrapped

645   classification accuracy was 29.6% (chance baseline of 4%). Our LDA model also

646   performed well when classifying five genotypes within a single age group. Highest

647   classifications occurred at one day (Monte Carlo sampling accuracy of 68% and LLO

648   accuracy of 58%) and 28 days of age (Monte Carlo sampling accuracy of 70% and

649   LOO accuracy of 64%) with a baseline of 20%. This indicates that there are

650   substantial differences between *Drosophila* genotypes at both one and 28 days of

651   age.

652

When all 25 classes were included in our model, all classifications (except *PINK1[5]*) perform above a 4% chance baseline, with most classifications occurring with ~25% accuracy. There is substantial variation between PD *Drosophila* visual response throughout their lifespan, indicating that EOPD mutations have unique effects on *Drosophila* visual pathways at not only one day of age, but throughout the *Drosophila* lifespan. After our data were partitioned into five genotypes for each age group, we could classify *Drosophila* into their correct genotypic class with 29.8% - 78.8% accuracy over all possible age groups, with no classifications falling under the statistical chance baseline of 20%. Our results illustrate that mutants can be accurately classified into their correct genotypic class beyond one day of age, indicating there are subtle differences in how EOPD mutations affect *Drosophila* neural gain control, as will be discussed.

Although the N-Way classification accuracy decreased when the algorithm was required to classify *Drosophila* into their correct age within a single genotype, our model still performed above chance baseline. This is surprising considering the results of our first experiment, where, for the most part, within genotype responses did not significantly differ over time. Our analysis was run on a reduced number of genotypes and flies (n=10 and five genotypes, rather than n=20 and 10 genotypes as per West et al. (2015)), yet our model produced a consistently high classification accuracy, even with all 25 classes were included in the model. In West et al., (2015), we varied temporal and spatial frequency but kept contrast fixed. We observed relatively little dependence on spatial frequency up to a hard cut-off that was associated with spatial sampling limits. Our use of contrast rather than spatial frequency in the experiments described here allows us to measure the full contrast

678 sensitivity profile of each genotype and age, increasing the sensitivity of this

679 multivariate visual biomarker for EOPD genes in *Drosophila*. Further, our assay,

680 when combined with LDA, is sensitive enough to detect small differences in the

681 effect of EOPD mutations on *Drosophila* neural gain control. Our initial analysis

682 found a substantial difference between w^- and EOPD mutants at 1 day of age,

683 however our LDA results indicate that these mutations have their own subtle effects

684 on neural gain control across *Drosophila* lifespan. Our findings carry an important

685 implication. As noted, *DJ-1* acts as a ROS sensor, whilst *PINK1* acts to maintain

686 mitochondrial homeostasis in dopaminergic neurons (Lavara-Culebras, Muñoz-

687 Soriano, Gómez-Pastor, Matallana, & Paricio, 2010; Oswald et al., 2016; Park et al.,

688 2006). The ability of our LDA to accurately distinguish between mutations on these

689 genes indicates each mutation uniquely impacts the underlying cellular processes

690 thereby causing a subtle, dissimilar neural responses across *Drosophila* lifespan,

691 that then results in a common pathogenic outcome of visual loss and cell death.

692

693 A key benefit of using *Drosophila* as disease model is their convenience for

694 early-stage drug testing due to their fecundity and fast generation time. It is

695 advantageous to have phenotypic expression of PD mutations at early stages of

696 *Drosophila* lifespan as this supports their utility as an initial model for the rapid

697 testing of neuroactive drugs that have the potential to treat human disease. Like

698 *Drosophila*, perturbations in contrast sensitivity occur in human PD patients due to

699 reduced dopamine levels within the retina (Harnois & Di Paolo, 1990). Our current

700 findings may correspond to the changes seen in human PD patients, although there

701 is obvious difficulty in assessing whether a prodromal abnormal gain control occurs

702 in the early stages of pre-genotyped PD patients. We believe that it may be possible

703   for LDA to classify human PD patients genotype based on multivariate SSVEP

704   response profiles as measured by electroencephalogram (EEG). This would have

705   the potential to assist in early PD diagnosis, genotypic classification, and disease

706   expression. Our next step is to investigate *Drosophila* response to additional low

707   level visual parameters such as chromatic contrast and orientation, and deduce

708   whether a similar biomarker can be established in human PD patients.

709

710   Together, our experiments have uncovered abnormal gain control and an

711   excitotoxic cascade as a common pathological phenotype in three EOPD mutations,

712   *DJ-1α$^{\Delta 72}$*, *DJ1-β$^{\Delta 93}$*, and *PINK1^5*. In addition to furthering the link between abnormal

713   gain control and excitotoxicity in genetic forms of PD, our findings have built upon

714   the utility of LDA in genotyping *Drosophila* based on multivariate response profiles.

715   Further, we have illustrated that there are variations in how these EOPD mutations

716   affect neural gain control across *Drosophila* lifespan, indicating that these mutations

717   have unique effects upon underlying cellular processes that lead to a common

718   outcome – visual loss and cell death. Overall, it appears that these PD related

719   mutations are heterochronic: in young flies, mutations lead to stronger neural

720   signalling (increased sensory response may be beneficial in escaping behaviour) but

721   are detrimental in older flies (a loss of vision would hinder escape behaviour)

722   (Himmelberg, West, Wade, & Elliott, 2017). Should these findings in fly models prove

723   applicable to the human situation, it would suggest that prodromal PD may be linked

724   to changes in central nervous system processing that could, potentially, confer

725   advantages in early life at the cost of degenerative disease in old age.

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Figure Captions

978 **Figure 1.** Time-domain SSVEP with a stimulus input frequency of 8Hz contains 16

979 'reversals' / second and can be decomposed into a SSVEP response spectrum with

980 peaks at multiples of the input frequency. In A) we present an averaged time-domain

981 SSVEP response from a w^- fly to 99% contrast reversing sine grating over 1000ms,

982 modulating at 8Hz, whilst B) shows Fourier amplitudes decomposed from Fourier

983 transform the 8Hz waveform in A, with peaks occurring at multiples of our input

984 frequency (8Hz, 16Hz, 24Hz, 32Hz, 40Hz). The same is shown in C) and D) for a

985 *PINK1*[5] PD-mutant fly.

986

987 **Figure 2.** Analysis path for Linear Discriminant Analysis (LDA). The raw ERG

988 (electroretinogram) response to 64 different stimuli is collected – here from a control

989 (wild-type) w^- fly and an EOPD (*PINK1)* fly (A). For each stimulus, Fourier analysis

990 is used to measure the response of the fly at the second harmonic ($2f$) (B). Each fly

991 is exposed to 64 stimuli – each with a known contrast and temporal frequency. The

992 heat map (C) represents the amplitude of the second harmonic at each stimulus

993 condition. In this simple case, with just 2 genotypes at one time point, the LDA is

994 applied to the data from both genotypes, and determines the equation that best

995 separates the data into two classes based on the 64 responses. Three outcomes

996 could be envisaged – an optimal separation of the data. Di) a clear line separates the

997 data, or a partial separation (Dii), or no difference (Diii), all the data are mixed). In

998 this portrayal, the graph plots 'X' and 'Y' which will be calculated from the 64 Fourier

999 results by the LDA algorithm. In the more complex dataset explored below, 5

1000 genotypes and 5 ages were sampled, leading to a multi-dimensional 'cloud' of data

1001 which can still be separated by a (more complex) set of linear equations.

1002

1003 **Figure 3.** We use the ERG to obtain accurate SSVEP measurements from both wild-

1004 type and PD *Drosophila* mutants at different contrasts and ages. In A-F we present

1005 exemplar ERG responses at 8Hz obtained from *w⁻* and *PINK1* PD mutants at 1 and

1006 28 days of age, and at 64% and 99% contrast. SSVEP waveform peak amplitude

1007 increases with increasing contrast.

1008

1009 **Figure 4**: EOPD mutants show steeper response amplitudes at 1 day of age. A-E)

1010 Mean response amplitudes from all *Drosophila* genotypes (n=10 for each genotype).

1011 *Drosophila* exhibit visual tuning to temporal frequency and contrast, with peak

1012 sensitivity at 6-8Hz temporal frequency and 99% contrast. Further, the maps appear

1013 to show subtle differences outside of peak regions between 12-36Hz at 1-8%

1014 contrast. Profiles indicate that EOPD mutants have larger response amplitudes at

1015 'peak sensitivity' regions. F) Boxplot of the 2*f* peak response at 99% contrast and

1016 8Hz for each genotype.

1017

1018 **Figure 5**. High contrast (99%) and intermediate temporal frequency combinations (6-

1019 18Hz) conditions exhibit the strongest loading onto the first principal component. The

1020 entire dataset (N=250) is run through the PCA simultaneously to ensure that it is

1021 scaled by the same eigenvalue. Brighter colours represented a higher loading onto

1022 the first PC, whilst darker colours represent a lower loading.

1023

1024 **Figure 6**. One day old EOPD flies show increased SSVEP response amplitudes

1025 when compared to control flies (*w⁻*). Mean PC Score (representing response

1026   amplitude) as a function of age for five *Drosophila* genotypes (n=10 for each

1027   genotype/age group). Error bars show ±1SE.

1028

1029   **Figure 7**. All EOPD mutants show perturbations in response amplitudes after

1030   exposure to pulsating light, indicating a decrease in temporal contrast sensitivity

1031   (n=10 per genotype). A-E) Mean response amplitudes from all *Drosophila* genotypes

1032   after 7 days of visual stimulation (each genotype n=10, except *DJ-1α$^{\Delta 72}$* n=8). Same

1033   scale as Figure 3. F) Boxplot of the 2*f* peak response at 99% contrast and 8Hz.

1034

1035   **Figure 8**. Visual loss occurs in all PD mutants after 7 days of exposure to pulsating

1036   light. Mean PC Score of 5 *Drosophila* genotypes after 7 days exposure (each

1037   genotype n=10, except *DJ-1α$^{\Delta 72}$* n=8).

1038

1039   **Figure 9.** LDA can accurately discriminate between all 25 classes when they are

1040   included in the model. All classifications sit above 4% chance baseline, except for

1041   *PINK1[5]* at 21 days of age.

1042

1043   **Figure 10**. Classification of young flies by genotypic class using data from temporal

1044   contrast response profiles. Mean classification accuracies for N-way LDA of 5

1045   genotypes at 1 day of age (n=10 per genotype). The chance baseline is set at 20%,

1046   with mean classification accuracies between 45.5% and 78.8%.

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Statistical Supplements

	w^-	$DJ1\text{-}\beta^{\Delta 93}$	$DJ\text{-}1\alpha^{\Delta 72}$	$dLRRK^{ex1}$
$PINK1^5$	$p <. 001^*$	$p = .724$	$p = .048^*$	$p < .001^*$
w^-	-	$p < .001^*$	$p = .006^*$	$p = .302$
$DJ1\text{-}\beta^{\Delta 93}$	-	-	$p = .892$	$p = .083$
$DJ\text{-}1\alpha^{\Delta 72}$	-	-	-	$p = .852$

1053

1054   Table 1. Simple Effects Analysis: *p*-values at 1 day of age

	w^-	$DJ1\text{-}\beta^{\Delta 93}$	$DJ\text{-}1\alpha^{\Delta 72}$	$dLRRK^{ex1}$
$PINK1^5$	$p = .208$	$p = .158$	$p = .185$	$p = .004^*$
w^-	-	$p =.208$	$p = 1.000$	$p = 1.000$
$DJ1\text{-}\beta^{\Delta 93}$	-	-	$p = 1.000$	$p = .940$
$DJ\text{-}1\alpha^{\Delta 72}$	-	-	-	$p = .917$

1055

1056   Table 2. Simple Effects Analysis: *p*-values at 7 days of age

	w^-	$DJ1\text{-}\beta^{\Delta 93}$	$DJ\text{-}1\alpha^{\Delta 72}$	$dLRRK^{ex1}$
$PINK1^5$	$p = 1.000$	$p = .221$	$p = .042^*$	$p = .019^*$
w^-	-	$p = .064$	$p = .156$	$p =.080$
$DJ1\text{-}\beta^{\Delta 93}$	-	-	$p < . 001^*$	$p < .001^*$
$DJ\text{-}1\alpha^{\Delta 72}$	-	-	-	$p = 1.000$

1057

1058   Table 3. Simple Effects Analysis: *p*-values at 14 days of age

1059

	w^-	$DJ1\text{-}\beta^{\Delta 93}$	$DJ\text{-}1\alpha^{\Delta 72}$	$dLRRK^{ex1}$
$PINK1^5$	$p = .897$	$p = .737$	$p = .440$	$p = .862$
w^-	-	$p = .052$	$p = .999$	$p = 1.0$
$DJ1\text{-}\beta^{\Delta 93}$	-	-	$p = .006^*$	$p = .042^*$
$DJ\text{-}1\alpha^{\Delta 72}$	-	-	-	$p = 1.000$

1062

1063   Table 4. Simple Effects Analysis: *p*-values at 21 days of age

	w^-	$DJ1\text{-}\beta^{\Delta 93}$	$DJ\text{-}1\alpha^{\Delta 72}$	$dLRRK^{ex1}$
$PINK1^5$	$p = .515$	$p = 1.000$	$p = .010^*$	$p = .275$
w^-	-	$p = .440$	$p = .753$	$p = 1.000$
$DJ1\text{-}\beta^{\Delta 93}$	-	-	$p = .007^*$	$p = .222$
$DJ\text{-}1\alpha^{\Delta 72}$	-	-	-	$p = .937$

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1065   Table 5. Simple Effects Analysis: *p*-values at 28 days of age

	7 days	14 days	21 days	28 days
1 day	$p = .001^*$	$p < .001^*$	$p = .05^*$	$p < .001^*$
7 days	-	$p = .811$	$p = 1.000$	$p = 1.000$
14 days	-	-	$p = .372$	$p = .991$
21 days	-	-	-	$p = .951$

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1067   Table 6. Simple Effects Analysis: *p*-values for *w⁻ Drosophila*

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	7 days	14 days	21 days	28 days
1 day	$p = 1.000$	$p = 1.000$	$p = 1.000$	$p = 1.000$
7 days	-	$p = .988$	$p = .938$	$p = .988$
14 days	-	-	$p = 1.000$	$p = 1.000$
21 days	-	-	-	$p = 1.000$

1071

1072 Table 7. Simple Effects Analysis: *p*-values for *DJ-1$\alpha^{\Delta 72}$ Drosophila*

	7 days	14 days	21 days	28 days
1 day	$p = .988$	$p = .005^*$	$p = .691$	$p = .507$
7 days	-	$p < .001^*$	$p = .178$	$p = .099$
14 days	-	-	$p = .427$	$p = .609$
21 days	-	-	-	$p = 1.000$

1073

1074 Table 8. Simple Effects Analysis: *p*-values for *DJ1-$\beta^{\Delta 93}$ Drosophila*

	7 days	14 days	21 days	28 days
1 day	$p = 1.000$	$p = 1.000$	$p = .768$	$p = 1.000$
7 days	-	$p = 1.000$	$p = .698$	$p = 1.000$
14 days	-	-	$p = .923$	$p = 1.000$
21 days	-	-	-	$p = .634$

1075

1076 Table 9. Simple Effects Analysis: *p*-values for *PINK1^5 Drosophila*

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	7 days	14 days	21 days	28 days
1 day	*p* = .998	*p* = .997	*p* = .852	*p* = .242
7 days	-	*p* = 1.000	*p* = .999	*p* = .733
14 days	-	-	*p* = .1.000	*p* = .806
21 days	-	-	-	*p* = .993

1080

1081   Table 10. Simple Effects Analysis: *p*-values for *dLRRK^{ex1} Drosophila*

1082

A)

B)

1 day old w^-

C)

D)

1 day old *PINK1*

Ai)

Aii)

Bi)

1 day *w⁻* F2 amplitude at 99% contrast, 8Hz

Bii)

1 day *PINK1* F2 amplitude at 99% contrast, 8Hz

Ci)

1 day *w⁻* F2 amplitude full profile

Cii)

1 day *PINK1* F2 amplitude full profile

Di)

High classification accuracy

Dii)

Medium classification accuracy

Diii)

Low classification accuracy

× *w⁻* flies
○ *PINK1* flies

A)

B)

C)

1 day old *w⁻* 64% contrast

28 day old *w⁻* 99% contrast

28 day old *w⁻* 64% contrast

D)

E)

F)

1 day old *PINK1* 64% contrast

28 day old *PINK1* 99% contrast

28 day old *PINK1* 64% contrast