Dopaminergic expression of the Parkinsonian gene LRRK2-G2019S leads to non-autonomous visual neurodegeneration, accelerated by increased neural demands for energy

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Received December 20, 2012; Revised and Accepted February 5, 2013

Parkinson’s disease (PD) is associated with loss of dopaminergic signalling, and affects not just movement, but also vision. As both mammalian and fly visual systems contain dopaminergic neurons, we investigated the effect of LRRK2 mutations (the most common cause of inherited PD) on Drosophila electroretinograms (ERGs). We reveal progressive loss of photoreceptor function in flies expressing LRRK2-G2019S in dopaminergic neurons. The photoreceptors showed elevated autophagy, apoptosis and mitochondrial disorganization. Head sections confirmed extensive neurodegeneration throughout the visual system, including regions not directly innervated by dopaminergic neurons. Other PD-related mutations did not affect photoreceptor function, and no loss of vision was seen with kinase-dead transgenics. Manipulations of the level of Drosophila dLRRK suggest G2019S is acting as a gain-of-function, rather than dominant negative mutation. Increasing activity of the visual system, or of just the dopaminergic neurons, accelerated the G2019S-induced deterioration of vision. The fly visual system provides an excellent, tractable model of a non-autonomous deficit reminiscent of that seen in PD, and suggests that increased energy demand may contribute to the mechanism by which LRRK2-G2019S causes neurodegeneration.

INTRODUCTION

Parkinson’s disease (PD), characterized by a loss of dopaminergic neurons, is generally described as a movement disorder. However, patients also experience a range of other symptoms, including cognitive changes (e.g. mood, dementia) and visual problems (from dry eyes and difficulties with reading, to hallucinations and perceptual disorders) (1). Some of these symptoms may be due to difficulties in the coordination and contraction of the eye muscles, but other symptoms may arise from deficits in signalling in the visual system as a result of dopaminergic neuron loss. Tyrosine-hydroxylase (TH) staining in the human retina identifies the A18 amacrine cells as dopaminergic (2). A18 neurons have major roles in the processing of edges and outlines (crucial to shape discrimination), in light adaptation and in diurnal rhythms (3,4).

These neurons have decreased levels of dopamine in PD patients (5), possibly explaining the physiological deficits in retinal signalling in these patients (6).

Like mammals, insects have dopaminergic neurons with axons branching in the visual system (7–9) (see also Fig. 7A). Other similarities between insects and mammals lie in the functions suggested for dopamine in the retina: adaptation to bright lights (10) and diurnal rhythms (11,12). Thus homology not only exists between fly and human in the PD-related fields of genetics and cell biology, but also in the organization of the visual system. Advantageously, the anatomy of the fly eye makes it straightforward to record the electroretinogram (ERG, Fig. 1A), while fly genetics provides the experimental selectivity of the GAL4-UAS system to analyse the effects of expressing PD-related genes carrying associated mutations. Here we focus on the dominant
RESULTS

Our key result is that expressing the LRRK2-G2019S mutation in just the dopaminergic neurons leads to a rapid decline in visual function. The response to a 500 ms pulse of blue light is recorded. A sample recording from a fly with G2019S expressed in the dopaminergic neurons using the tyrosine hydroxylase (TH) GAL4 driver (TH > G2019S, red trace), shows a normal ERG at 3 days, but a minimal response by 28 days. Flies expressing wild-type hLRRK2 and control outcrosses (green) show normal ERGs at both 3 and 28 days. The decline in visual function with TH > G2019S begins at 10 days and drops steeply thereafter, but flies expressing hLRRK2 or control outcross (TH+/+) maintain constant visual function. Analysis of 178 flies (8–13 flies of each genotype at each timepoint, see Supplementary Material, Table S1), kept dark in a 29°C incubator.

Figure 1. Expressing the G2019S mutation of LRRK2 in the dopaminergic neurons causes a rapid decline in visual function. (A) Visual function is assessed by electroretinograms (ERGs), in which an electrical recording is made from the surface of the eye, with a reference electrode in the mouthparts. The response to a 500 ms pulse of blue light is recorded. (B) A sample recording from a fly with G2019S expressed in the dopaminergic neurons using the tyrosine hydroxylase (TH) GAL4 driver (TH > G2019S, red trace), shows a normal ERG at 3 days, but a minimal response by 28 days. Flies expressing wild-type hLRRK2 and control outcrosses (green) show normal ERGs at both 3 and 28 days. (C) The decline in visual function with TH > G2019S begins at 10 days and drops steeply thereafter, but flies expressing hLRRK2 or control outcross (TH+/+) maintain constant visual function. Analysis of 178 flies (8–13 flies of each genotype at each timepoint, see Supplementary Material, Table S1), kept dark in a 29°C incubator.

G2019S mutation in LRRK2 kinase, as this mutation is the most common cause of inherited late-onset PD.

Even though hLRRK2 may be expressed at a slightly higher level (Supplementary Material, Fig. S2B) (14). Normal ERGs were also recorded when expression of the fly homologue dLRRK is increased (Fig. 2B, left-hand bar) or in control outcrosses (Fig. 2C, right-hand bars; Supplementary Material, Fig. S3).

In order to investigate the selectivity of the decline in visual function for G2019S, we expressed transgenes bearing seven further single amino-acid mutations in the dopaminergic neurons of the fly brain, all using the same driver (TH GAL4). These mutations are all at sites known to be pathogenic or to segregate with PD, and were encoded either in the human UAS-hLRRK2 sequence or the fly UAS-dLRRK sequence (Fig. 2A). None of the other transgenes showed a significant reduction in ERG amplitude by 28 days (Fig. 2B), showing this phenotype is highly sensitive to the G2019S mutation. Western blots suggested that expression of the other FLAG-tagged LRRK2 transgenes was at a comparable (or greater) level than G2019S (Supplementary Material, Fig. 2A and B).
Visual degeneration is specific for expression of the G2019S mutation in dopaminergic neurons. (A) Location of the LRRK2 mutations used in this study, on a scale diagram of the protein domains [(modified after Kumari and Tan (54)]. The position and pathological status of each mutation (orange/red/purple) is shown above the domain diagram. (B) Expression of these LRRK2 mutations with the TH GAL4 shows that only the G2019S mutation leads to a significant reduction in electroretinogram (ERG) amplitude at 28 days. No effect was seen with the expression of hLRRK2. This difference was seen in two independently generated lines [† (14), all the other data in this paper are derived from these lines; * (13)]. We found similar levels of expression of LRRK2 transgenics derived from different labs (Supplementary Material, Fig. S2), so that the differences in ERGs cannot be ascribed to differences in expression levels. No decline in visual response is seen with kinase dead mutations (dLRRK-3KD and G2019S-K1906M), or with those in the GTP-binding domain (e.g. R1441C). The Y1383C and I1915T mutations are expressed in the fly LRRK sequence. (C) The loss of visual function at 28 days is most severe when G2019S is driven with TH or HL9 GAL4 which only express in the dopaminergic neurons. Expression of G2019S in all neurons using either the elav or nSyb GAL4 drivers gives a significant reduction in the ERG amplitude, but it is not as severe as when G2019S is only expressed in the dopaminergic neurons. There is no decline in ERG amplitude when G2019S is expressed with the L1L2B GAL4, into the second order lamina neurons. Although the mean ERG response appears less when G2019S is expressed with the ubiquitous (Act5c) or with the photoreceptor-specific (LongGMR) GAL4, this was not significant (Bonferroni P = 1.0 and 0.11, respectively). In all these cases, expressing the normal hLRRK2 gave no significant loss of visual function over 28 days. Neither UAS-control showed any decline in visual function. Data from 295 flies for (B) and 360 for (C), at least eight flies of each genotype at a time point (see Supplementary Material, Table S1). Bonferroni post-hoc test, **P < 0.01; ***P < 0.001; all flies were kept in the dark incubator.
We took advantage of the range of highly specific GAL4 drivers to identify the susceptibility of fly tissues to the LRRK2-G2019S mutation (Fig. 2C). With both the TH GAL4 and a second dopaminergic neuron driver (HL9 GAL4), we observed a severe loss of visual function at 28 days with G2019S expression (down to 23% and 37%, respectively), but expressing hLRRK2 did not affect visual function by 28 days (ANOVA, Bonferroni comparison of G2019S and hLRRK2 expression: P < 0.001 for both GAL4 drivers). We then used two different GAL4 lines (elav and nSyb), both specific for broad neuronal expression. In both conditions, we found a ~50% loss of ERG amplitude at 28 days when G2019S was expressed, but no loss of response with hLRRK2 (ANOVA, Bonferroni P < 0.001 for both elav and nSyb). We next expressed these LRRK2 transgenes in the lamina neurons (second-order neurons which make synaptic contact with the photoreceptors) using the L1L2B GAL4 (15). Here we found no change over 28 days in the peak–peak amplitude of the ERG with either LRRK2 transgene. When LRRK2-G2019S is expressed in just the photoreceptors (LongGMR GAL4), or ubiquitously (Act5C GAL4) the ERG amplitude declines over 28 days, but there is no significant difference between expression of G2019S or hLRRK2. In control experiments (four different GAL4/wild-type outcrosses, one wild-type outcross, and with two UAS/ wild-type outcrosses), we found no significant change in ERG amplitude (Fig. 2C, Supplementary Material, Fig. S3).

Our data—that the decline in visual response is specific for the G2019S mutation—is in accordance with the hypothesis that the key function of LRRK2 (at least in the visual system) is as a kinase. We tested this hypothesis by expressing the LRRK2-G2019S-K1906M transgene, in which kinase activity has been abolished by the K1906M amino-acid substitution in the (presumed) ATP-binding site. We found no loss of ERG response in the TH > LRRK2-G2019S-K1906M flies, as would be expected if the kinase is the key function of LRRK2 in the visual system. Western blots suggest that the G2019S and G2019S-K1906M proteins are expressed at similar levels (Fig. 2A), so the difference does not seem to be due to a failure of protein production.

It has been suggested (16,17) that G2019S may be a simple gain-of-function (GOF) mutation, increasing the rate of phosphorylation of LRRK2 substrates, or it might act as a dominant negative (DN). If G2019S were a DN mutation, we would expect the effect of G2019S expression to be stronger when the amount of the flies’ own dLRRK is reduced. We directly tested this by putting TH > G2019S in a dLRRK heterozygote background. Our data do not support the DN hypothesis: G2019S was much less effective at reducing the amplitude of the ERG in the dLRRK heterozygote background (Fig. 3). Our data rather accords with the hypothesis that the G2019S is a simple GOF mutation.

We next asked if the functional decline in vision is accompanied by anatomical evidence of neurodegeneration. We found that, after 28 days, the TH > G2019S flies showed strong neurodegeneration throughout the internal structure of the retina, which became disorganized, while the visual lobes (lamina and medulla) showed frequent vacuoles (Fig. 4A). No such features were seen in TH > hLRRK2 flies or in wild-type controls. This was accompanied by evidence of increased autophagy and apoptosis around the microvilli of the photoreceptors in 21–23-day-old TH > G2019S flies (Fig. 5). Additionally, the photoreceptor mitochondria become swollen (70% increase in area), and the cristae wider (80% wider) and become more broken, fragmented and rounded (Fig. 6). Although the internal structure of the TH > G2019S retina is degenerating, the exterior surface of the eyes of all 28-day-old flies was normal in all our experiments. We observed none of the developmental abnormalities (irregular ommatidia or black pigmentation) reported in flies raised at 29°C (18). This included our TH > G2019S and TH > hLRRK2 crosses (Fig. 4B) and the flies expressing G2019S or hLRRK2 with the Act5C, LongGMR and elav GAL4 drivers (Supplementary Material, Fig. S4).

A second anatomical question is whether the loss of visual response and degeneration of the photoreceptor layer is preceded by (or correlated with) a loss of dopaminergic innervation of the visual lobes. As dopaminergic transgenics and antibodies have been reported to highlight different groups of neurons (19), we used three GFP reporters under the control of two different dopaminergic GAL4 drivers and compared this with staining by tyrosine hydroxylase antibody (α-TH). In young control flies, we found three kinds of innervation of the visual lobes. First, the PPL neurons, which branch densely in the innermost visual neuropil (the lobula), which are α-TH positive and which express all the GFPs tested with TH GAL4 (Fig. 7B and C). We did not see the PPL neurons whenever the HL9 GAL4 was used. Secondly, the abundant MC neurons (Fig. 7B and C), with their cell
bodies at the edge of the medulla and axons projecting radially in towards the core of the medulla, stained reliably with α-TH. As expected (8), only a proportion of the MC neurons are reported by any GFP. Thirdly, we observed a cluster of approximately four neurons projecting from the lateral edge of the protocerebrum, across the anterior face of the medulla to the lamina. As these neurons have not previously been reported, we have labelled them as LA neurons as they branched extensively and gave rise to blebs in the lamina, suggesting synaptic release sites. These neurons were reliably seen both with TH and H9-GFP (Fig. 7D) and hLRRK2 in the dopaminergic neurons (TH > G2019S, TH > hLRRK2, respectively), and of a control (TH+/-) fly shown at 28 days. In all panels, anterior is to the top. In the eye surface images, dorsal is to the right. All flies were kept dark in the incubator.

In search of a mechanism for loss of visual response, we tested if physiological and neurodegenerative decline is driven by activity (or energy) demand. We addressed this in two experiments, first by keeping flies in irregularly pulsating light, so that the visual system continually has to adapt to new light levels, and second by increasing the activity of the dopaminergic neurons genetically. If these treatments were to accelerate visual neurodegeneration, we might expect to see a loss of ERG amplitude sooner than normal; therefore we tested these flies at 10 days, a time before any loss of visual response is normally seen in the TH > G2019S flies (Fig. 1C).

In our first experiment, we kept flies in constant dark, constant light, and in vials where the light was pulsed on and off randomly at ~1.5 s intervals. When we tested 10-day-old flies, we first found that those kept in constant light were less sensitive to our test stimulus than flies kept in the dark (Fig. 9A). This is as expected: flies have long-term adaptations to constant light. Importantly, there was no difference between TH > hLRRK2 and G2019S lines in either constant dark or constant light. However, the G2019S flies kept in pulsating light showed reduced ERG response to the test stimulus compared with the hLRRK2 flies, or with their counterparts in constant light. No such decrement is seen in the hLRRK2 flies.

In our second experiment, we manipulated the electrical activity of the dopaminergic neurons. We took advantage of the...
electrical-knock-in (EKI) transgene, a combination of shaker (sh) and ether a go-go (eag) dominant negative constructs (20,21), which reduces potassium-channel function and so makes neurons (in which it is expressed) more active. At 3 days, all genotypes had normal vision (Fig. 9B). At 10 days, the TH > G2019S animals, TH > EKI animals and control out-cross animals all had the expected normal visual response. However, flies expressing both the G2019S and EKI constructs in the dopaminergic neurons had already started to lose their visual function: they had significantly smaller ERGs (71%) than those expressing G2019S or EKI alone. Thus knockout of the shaker and ether a go-go channels in just the dopaminergic neurons increases their sensitivity to the G2019S form of LRRK2.

DISCUSSION

We have demonstrated functional and anatomical loss of visual response as a consequence of expressing the most common Parkinson’s disease-related mutation (LRRK2-G2019S) selectively in the dopaminergic neurons. This is caused by progressive neuronal dysfunction rather than a developmental defect because young flies (≤10 days old) have normal vision, the external structure of the eye is not compromised and the loss of visual response and anatomical disorganization occurs gradually. The loss of visual function is highly specific for the combination of the dopaminergic neurons with LRRK2-G2019S mutation. Of the mutations which we tested in vivo, in previous biochemical assays, only G2019S more than doubles the LRRK2 kinase activity with all the substrates tested (22). Some of the kinase assays suggested that the G2019S mutation may be acting in a dominant negative manner (16,17), but our data indicate a simpler, GOF effect of the G2019S mutation.

Physiologically, our light stimuli excite the photoreceptors, which in turn excite second-order lamina or medulla neurons. The loss of the maintained component of the ERG in TH > G2019S flies indicates failure of these primary photoreceptors
to respond. The loss-of-function is accompanied by extensive anatomical changes, with both autophagy and apoptosis occurring around the photoreceptors. The photoreceptors also show evidence of mitochondrial failure. Anatomical evidence of degeneration of fly photoreceptors has been seen following expression of PD-related genes (α-synuclein, parkin and several LRRK2 transgenes) in the eye using the GMR GAL4 (14,18,23,24), though this was not seen by all studies (25,26). Similarly, mitochondrial degeneration is seen in the fly flight muscle when LRRK2-G2019S is expressed using a muscle-specific driver (27). Degeneration and mitochondrial failure are key features of LRRK2-G2019S PD (28). While cell models indicate a loss of neuronal growth with LRRK2-G2019S (29), this is not the key finding of this report. However, we cannot exclude a moderate loss of the dopaminergic neurons, especially of the numerous MC cells. In all these previous papers, the degeneration is occurring in the tissue in which the G2019S transgene is expressed. Our data are different: we report a non-autonomous dysfunction and anatomical degeneration.

Thus, a crucial point here is that we are expressing the transgene in the dopaminergic neurons, but the physiological and anatomical loss we see is occurring elsewhere, in the photoreceptors and other regions of the visual system. What possible connections are there between the dopaminergic neurons in which G2019S is produced and the cells that die? Three kinds of dopaminergic neurons innervate the visual system: the PPL neurons in the lobula, the MC neurons in the medulla and the LA neurons which project from protocerebrum to the lamina. We may initially discount the PPL neurons because (i) they are not marked by the HL9 GFP reporter (and HL9. G2019S is as effective as TH. G2019S in causing loss of visual function) and (ii) these neurons only project to the lobula, well away from the photoreceptor terminals. We have no evidence that the MC neurons connect to the photoreceptors or laminal layers: the closest they approach is in the outer part of the medulla (7). While the axons of the ultraviolet sensitive R7 and R8 photoreceptors do terminate in the medulla (9), electron microscopy indicates there are no direct synapses between the MC dendrites and photoreceptor axons (30). The most likely type of dopaminergic neurons to interact with the photoreceptors is therefore the LA neurons: they are GFP-positive with both the TH and HL9 GAL4 drivers, and their blebbed endings in the lamina allow us to speculate that they might release neurotransmitter near the photoreceptor terminals.

Nonetheless, whether there is synaptic contact or not, we are expressing G2019S in one class of cells, and recording functional and anatomical loss in a second kind of cell. Both

Figure 6. The photoreceptor mitochondria are dilated and have aberrant cristae in 28-day-old TH > G2019S flies. (A) Electron micrographs at the outer level of the microvilli from TH > G2019S, TH > hLRRK2 and control (CS/+ ) flies. (Ai) Images centred on the ommatidium; the mitochondria are distributed around the edge of the ommatidia. (Aii) Higher magnification images, showing the enlarged mitochondria with dilated and disorganized cristae. (B) Quantification of the mitochondrial area. (C) Quantification of the width of the cristae. Summary data from 85 mitochondria in nine preparations. Bonferroni post-hoc tests, **P < 0.01; ***P < 0.001.
physiological and anatomical criteria therefore indicate a spreading problem, with the loss of neuronal integrity occurring non-autonomously. Possible explanations of this include misregulation of dopamine (31) (or some co-transmitter), changes in the secretion of growth factors or diffusion of reactive oxygen species, or cell–cell transmission of mis-folded proteins (32).

Our data also show that increasing the demands on the visual system to adapt, or increasing the activity of just the dopaminergic neurons, accelerates the decline in visual function due to G2019S expression. After each action potential, the dopaminergic neurons are required to pump cations back across the plasma membrane. Such pumping requires energy from the mitochondria in the form of ATP. Ionic pumping already accounts for ~40% of the energy requirements of the CNS (33); the neurons of the fly visual system also have high demands for ATP (34). Thus, increase in neuronal activity is likely to couple harmfully with mitochondrial dysfunction, now accepted as a key feature of many forms of inherited PD. This could also lead to elevated or mis-regulated calcium flux, further exacerbating the situation. Increases in cation flux in fly photoreceptors or manipulations of mitochondrial ATP production in the fly retina cause rapid neurodegeneration (35–37). We recently reported a failure to maintain the normal resting potential at the Drosophila neuromuscular junction in the parkin knockout (38), while in mouse models of PINK1, HtrA2/Omi and DJ-1, hyperexcitability has also been reported (39,40). Our current data take this approach a step further, by showing that the progression of neurodegeneration (as monitored by loss of ERG response) can be affected by manipulation of the demand for energy.

Although fly models of inherited PD have generally reported a more consistent phenotype than mouse models, there are discrepancies in the fly data, e.g. in counts of
dopaminergic neurons, retinal surface abnormalities and neurodegeneration (reviewed in 41). This has been ascribed to differences in microscopical technique (19,41) or food (42,43). Our data suggest another explanation: the level of neuronal demands for energy is important in the PD models. This leads to the idea that increasing the activity of the visual system in mouse models of PD may provide a more consistent phenotype. Finally, we suggest that the selective death of some

Figure 9. G2019S-induced degeneration is enhanced by neuronal activity. (A) Flies were kept for 10 days in constant dark, constant blue illumination or with the blue light turned on–off at randomized intervals (mean ~1.5 s). Comparison of TH > G2019S and TH > hLRRK2 only shows a difference in pulsed light, when the TH > G2019S showed reduced electroretinogram (ERG) amplitude (Bonferroni post-hoc test, \( P = 0.001 \)). The pulsed light G2019S flies also had reduced ERGs compared with those in constant light \( (P = 0.006) \), but there is no significant difference between hLRRK2 flies in constant and pulsed illumination. (B) Reducing the activity of potassium channels in the dopaminergic neurons accelerates the loss of visual function seen with G2019S. When TH GAL4 was used to express both the LRRK2-G2019S mutation and the EKI transgene (a combination of dominant negative forms of the shaker (sh) and ether a go-go (eag) potassium channels), a loss of visual function is already seen at 10 days \( (P = 0.001) \), whereas all the other genotypes shown have a normal visual response (comparison of the TH > G2019S; EKI with any other sample, \( P = <0.001 \)). Using TH to express the EKI transgene alone had no impact on visual function. Data in (A) from 60 flies kept at 29°C, in (B) from 122 flies kept in the dark in the 29°C incubator; for details of the number of flies see Supplementary Material, Table S1.
dopaminergic neurons and survival of others, both in flies (26,44) and mammals (45,46), could be due to differences in neuronal activity.

MATERIALS AND METHODS

Flies

Stock vials of Drosophila melanogaster were raised on yeast–sucrose–agar fly food. Directed expression of LRRK2-related transgenes was achieved using the GAL4/UAS system, with lines described as follows: UAS-hLRRK2 and UAS-G2019S (14), UAS-dLRRK and UAS-dLRRK-3KD (dLRRK-K1781M-D1882A-D1912A) (47), UAS-dLRRK I1915T and UAS-dLRRK Y1383C (25), UAS-J2020T and UAS-I1122V (18), UAS-G2019S-K1906M, UAS-G2385R and UAS-R1441C (13), HL9 GAL4 (48), LIL2B GAL4 (15), dLRRK (e03680) (49); and using lines from Matthias Landgraf (UAS-myr-GFP), Stephen Goodwin (TH and nSyb GAL4) and Manolis Fanto (UAS-ATG5 and UAS-ATG5-RNAi); from Bloomington (UAS-elfA43-GFP) or from laboratory stocks (Actin5c, LongGMR and elav3el GAL4 lines and the w1118 (+) and Canton-S (CS) wild-types). The EKI transgene is a combined UAS-shakerDN ether a go-goDN construct (20,21). In Figure 2, we also tested the UAS-hLRRK2 and UAS-G2019S lines from Lin et al. (13) (marked with *) and found their ERGs resembled those of Liu et al. (14) (marked with ?).

Experimental crosses were raised on maize-meal fly food at 25°C. On the day of emergence, females were transferred to vials of yeast–sucrose–agar fly food in a dark incubator at 29°C. Flies were moved to fresh vials every 3 or 4 days, and so were exposed to light occasionally. In pulsating light experiments, vials were placed in an opaque plastic tube, stoppered with cotton wool in the 29°C incubator. The side wall of the tube contained high-intensity tricolour light-emitting diodes (LEDs, Kingbright, KAF-5060PBSEEVGC, maximum blue emission wavelength 465 nm). These were either off, on or randomly toggled on/off under computer control. Only the blue component was ever turned on.

ERGs were by aspirating (unaesthetized) females into shortened pipette tips and restraining them using nail varnish (Creative Nail Design). Recordings were made between blunt glass pipette electrodes, filled with simple Drosophila saline (130 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl2) (50). The recording electrode was placed in the centre of the eye with a reference electrode in the mouthparts. Three to five stimuli (10 s apart, 0.5 s long) were presented after 2 min adaptation in the dark laboratory, from the blue component of LEDs, (Kingbright, KAF-5060PBSEEVGC, maximum emission wavelength 465 nm) placed ~6 cm in front of the fly. Stimuli were monitored with a BPX65 photodiode (Centronics) placed next to the pipette tip. The photodiode current was 0.5 nA in the darkened laboratory and 400 nA during the stimuli. Each genotype/timepoint sample is the average (± SE) from at least seven flies.

Anatomy

Head sections were made as described recently (51). Heads were fixed (4% paraformaldehyde, 1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4), followed by 1% osmium tetroxide, dehydrated through an acetone series, followed by infiltration and embedding in Spurr’s resin. Semithick serial sections (1.0 μm; Leica Ultracut UCT) were stained with 0.6% toluidine blue in 0.3% sodium carbonate and imaged with a Zeiss Axiovert 200 microscope and Zeiss AxioCam HRm camera. Thin sections were cut and stained with uranyl acetate and Reynolds lead citrate. Images were captured with an Olympus-SIS Megaview III camera using analySIS software on a Tecnai 12 BioTWIN G2 (software version 2.1.8) operating at 120 kV. Tangential sections taken at the level of the outer part of the microvilli were analysed. The area of the mitochondria was measured from high-power images (samples in Fig. 6Aii) using ImageJ. The width of the cristae was estimated by counting the number of cristae which crossed a line drawn on the long axis of the mitochondria.

Confocal images

These were acquired from brains (including the optic lobes), dissected from TH > GFP expressing flies, fixed in 4% paraformaldehyde for 30 min. This and all subsequent incubations were performed at room temperature. After permeabilization in 0.5% Triton X-100 for 30 min, the brains were incubated in mouse anti-TH (Immunostar, 1:1000) together with rabbit anti-GFP (Abcam, 1:1000) for 3 h. After washing, the brains were transferred to a solution containing Cy3 conjugated goat anti-mouse IgG (Abcam, 1:250) and FITC conjugated goat anti-rabbit IgG (Abcam, 1:250) for a further 3 h. After further washing, the brains were mounted in Vectashield (Vector Laboratories). Eyes were dissected and fixed in 3.7% formaldehyde, and stained with ATG5 (Anti-Rabbit ATG5, Novus Biological 1:300 dilution) or cleaved caspase 3 antibody (anti-Rabbit Cleaved caspase-3 (Asp175), Cell Signaling Technology, 1:1600 dilution), which recognizes the effector caspases DRICE and DCP-1 in Drosophila (52,53). Confocal images were obtained using a Zeiss 780 microscope. The external surface of the eyes of etherized females was photographed using a Zeiss Stemi microscope and ERc 5 s camera.

Detection of protein expression in Drosophila lysates

FLAG-LRRK2

For each genotype, 30 fly heads were dissected and immediately homogenized on ice in 30 μl 2X SDS sample buffer (Sigma), boiled for 10 min and then separated by SDS–PAGE on a 7.5% polyacrylamide gel. The proteins were transferred to a PVDF membrane by electrophoresis and probed with mouse anti-FLAG (1:1000; M2 clone, Sigma) and a goat anti-mouse-HRP secondary antibody (1:5000; Sigma), followed by visualization with enhanced chemiluminescence (Millipore). To control for equal protein loading, the membrane was re-probed with mouse anti-β-actin (1:10 000; Proteintech) or rabbit anti-Synaptotagmin antibody, used at 1:1000.

Cleaved caspase 3

Eyes (40) were dissected on dry ice, lysed in sample buffer (10 μg/ml) and boiled for 5 min, before resolving on 12%
SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We are grateful to those who sent gifts of flies: Wanli Smith, Bingwei Lu, Yuzuru Imai, Cheng-Ting Chien, Katerina Venderova, Zhuhoua Zhang, Matthias Landgraf, Subhabrata Sanayal, Stephen Goodwin, Manolis Fanto, Gero Miesenboeck, Charlotte Foerster and Jens Rister. We would like to thank Ryan West, Shaun Calvert and Rebecca Furmston for assistance with data collection. John Sparrow and Alex Wade kindly commented on the manuscript.

Conflict of Interest statement. None declared.

FUNDING

We were supported by Parkinson’s UK (grant number K-1007), the Wellcome Trust (grant number 097829/Z/11/A) and Biotechnology and Biological Sciences Research Council. Funding to pay the Open Access publication charges for this article was provided by The Wellcome Trust.

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