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In this, the speed at which
in which

Many labs have adopted the
leading to the pharyngeal
Throughout the literature, the multiple

are important in

proportion is inherited (see, for a review, ref. 1) with the most
common genetic form being due to mutations in LRRK2 (Leucine-
Rich Repeat Kinase 2).

This gene is translated into a large, multi-domain protein, and
the pathogenic mutations include G2019S and I2020T,2 in which
the kinase activity is increased,3 and R1441C, a mutation in which
the GTPase activity is thought to be reduced.4

The excellent genetic toolkit provided by Drosophila led to the creation
of fly models of PD. These reflect many features of the
disease (loss of dopaminergic neurons, reduced movement,
motor abnormalities, oxidative stress, and visual defi-
cits).5,6 Many labs have adopted the fly negative geotaxis assay
(sometimes called the “startle response assay”) as their measure of
movement.7,8 In this, the speed at which flies walk up a glass
cylinder in response to a sharp tap is recorded. Although PD-
mimic flies have reduced movement, it is hard to specify exactly
where the changes are taking place (response to the startle
stimulus or gravity, or effects on the central pattern generator or
motor neurons or changes directly affecting the leg muscles
themselves). This assay also fails to discriminate between the
different possible movement defects (akinesia, hypokinesia, and
bradykinesia). We suggest the requirement for another, simpler
assay system.

This is reinforced by the difficulty of determining which of the
~125 dopaminergic neurons in the fly CNS10,11 are important in
the negative geotaxis response. Although dopaminergic innerva-
tion of the mushroom body by 15 “PAM” neurons plays a major
role in this negative geotaxis response,12 the subsequent neuronal
pathway is unclear. Further, manipulations of PD-related trans-
genics often lead to the loss of a relatively small proportion of
dopaminergic neurons, with many clusters remaining unaffected.
For example, with the LRRK2-G2019S mutation, the protocerebral
posterior medial (PPM) cluster dropped from 14 to 12 dopami-
ergic neurons but the protocerebral anterior lateral (PAL) cluster
remained unaffected.13 Throughout the literature, the multiple
processes involved in slowed negative geotaxis combined with
the observed small loss of dopaminergic neurons act to obscure
the functional relationship. To progress, we need to link a precise
measurement of movement with the physiology of a few specific
dopaminergic neurons.

An exciting solution to this problem is provided by the
discovery that a single dopaminergic neuron strongly modulates
the fly proboscis extension response (PER).14

As a fly walks into a solution containing sucrose, the Gr5a
chemosensory cells on its front legs are activated (Fig. 1, step 1).
Their axons project to the sub-esophageal zone of the CNS (SEZ;
the part signaling the taste response; Fig. 1, step 2). Within the
SEZ, the chemosensory inputs activate the interneuronal path-
way,15 leading to the pharyngeal Ed9 motoneurons, whose action
potentials elicit contraction of the M9 muscle (Fig. 1, step 3). This
well characterized neuronal pathway results in the reflex extrusion
of the proboscis towards the food (Fig. 1, step 4), allowing the fly
to ingest the solution. Although the sensory and motor steps in this pathway have been well defined (see for a review ref. 15 or ref. 13), the interneuronal steps mostly remain to be described.

One well-defined neuron that modulates the PER is TH-VUM, a single, unpaired neuron in the SEZ, which makes output synapses onto the sense cells and interneurons 14,18,19 (Fig. 1). Strong activity in the TH-VUM leads to contraction of the proboscis muscle, blocking the output of the TH-VUM reduces the probability of a sucrose-induced PER. The frequency of action potentials in the TH-VUM correlates with the length of starvation. 14 Interestingly, the TH-VUM fires steadily in a way reminiscent of mammalian substantia nigra dopaminergic neurons. 20

We have now found that expression of LRRK2-G2019S, the most common cause of genetic PD, in dopaminergic neurons results in a reduced PER, with bradykinesia, akinesia, and tremor, and that this is rescued by l-DOPA or by kinase inhibitors targeted at LRRK2.

RESULTS
Upregulation of LRRK2 kinase activity in dopaminergic neurons causes akinesia
In order to test the neuronal specificity of the PD-related mutation LRRK2-G2019S, we first expressed this in each of the components of the PER reflex pathway, recording the proportion of a population of starved flies that extended their proboscis in response to a moderate (100 mM) sugar stimulus (Fig. 2a, b).

Strikingly, when we expressed LRRK2-G2019S in the dopaminergic neurons with TH-GAL4 (tyrosine hydroxylase GAL4), the proportion of young flies responding was about half that of control genotypes (no transgene expressed, $\chi^2$-post-hoc test, $p < 0.001$; from 76–35%; Fig. 2a). The same result was seen in a second sample, where the proportion of TH>hLRRK2 and found the same: only 36% of this set responded (step 1) and signal to the sub-esophageal zone of the CNS (SEZ, step 2). This leads to activation of the E49 motoneurons for the proboscis extension muscle (step 3), muscle contraction (step 4), and extension of the proboscis (step 5). In the sub-esophageal zone, the neuronal signal is modulated by a dopaminergic neuron, TH-VUM, and by other inputs from CNS neurons, possibly including other dopaminergic neurons. b Schematic neural circuit, showing the modulation of the sensory neuron–interneuron–motoneuron axis by TH-VUM. Other interneurons also modulate the proboscis extension response, as reviewed recently. 17 a Modified after refs. 52,53; b after refs. 14,16,19

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We conclude that drugs targeted at LRRK2 ameliorate the reduced PER response of TH>G2019S flies.

Fig. 1 The proboscis extension response (PER) of Drosophila. a The PER takes place when sugar-sensitive (Gr5a) neurons on the legs respond (step 1) and signal to the sub-esophageal zone of the CNS (SEZ, step 2). This leads to activation of the E49 motoneurons for the proboscis extension muscle (step 3), muscle contraction (step 4), and extension of the proboscis (step 5). In the sub-esophageal zone, the neuronal signal is modulated by a dopaminergic neuron, TH-VUM, and by other inputs from CNS neurons, possibly including other dopaminergic neurons. b Schematic neural circuit, showing the modulation of the sensory neuron–interneuron–motoneuron axis by TH-VUM. Other interneurons also modulate the proboscis extension response, as reviewed recently. 17 a Modified after refs. 52,53; b after refs. 14,16,19

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Dopaminergic expression of \( LRRK2-G2019S \) slows movement and increases tremor in the PER

For \( TH>G2019S \) flies showing a PER, recordings were made using a camera with high frame rate (200 per second), and digitized the distance from the eye to the end of the proboscis, to measure the movement during the PER. Individual traces showed that some \( TH>G2019S \) flies had very slow PER, taking three or even four times longer than the median wild-type control (Fig. 4a). A second mutant, \( I2020T \), also showed a slower PER (Fig. 4b). For both \( TH>G2019S \) and \( TH>I2020T \) flies, the speed of the PER is fully rescued to wild type by feeding 2.5 \( \mu \)M BMPPB-32.

Using the independently generated \( G2019S \) and \( hlRRK2 \) lines, we also found a slower PER with \( TH>G2019S \). In this case the duration of the PER increased from 0.30 ± 0.025 s (\( TH/+ \)) to 0.57 ± 0.11 s (\( TH>G2019S \)) (mean ± SE, Tukey-post-hoc test, \( p = 0.017 \)). The \( TH>hlRRK2 \) was the same as \( TH/+ \) (\( p = 0.81 \)).

Additionally, it appeared that the \( TH>G2019S \) traces were more irregular. To assess this quantitatively, each trace was fitted by a smooth curve (a piecewise cubic spline), and the deviation of the actual trace from smoothed determined (Fig. 4c). This showed that the \( TH>G2019S \) flies had a much longer "path", about twice that of the control flies. The proboscis does not move out in a smooth trajectory, but oscillates, showing tremor. No such changes were seen in the \( TH>hlRRK2 \) flies; they were the same as the no-transgene cross (Fig. 4c).

Thus dopaminergic expression of \( LRRK2-G2019S \) induces bradykinesia and tremor as well as akinesia.

The single dopamine neuron (\( TH-VUM \)) is mainly responsible for akinesia

Our next step was to test which dopaminergic neurons are responsible for akinesia in the PER response, focusing on the difference between flies with increased kinase activity (\( G2019S \) or \( I2020T \)) and the kinase dead (KD) line.

There are eight clusters of dopaminergic neurons in the fly CNS (Fig. 5b), and a range of GAL4 drivers have been developed to target various subsets of these. We started with a GAL4 driver \( DDC \), which has been widely used for studies of negative geotaxis and which gives generalized dopaminergic neuron expression, as well as expressing in serotonergic neurons. As with the \( TH-GAL4 \) (Fig. 1), fewer flies expressing \( G2019S \) or \( I2020T \) with \( DDC \) showed the PER compared with those flies expressing \( LRRK2-KD \) (Fig. 5). We next tested \( HL9 \) (ref. 24) which expresses in all the dopaminergic neurons except for the PAL cluster (Fig. 5b), though it may only label a proportion of each cluster. It may also...
We have not tested the feeding response and that weakly, but the three posterior neurons not being labeled at all. The presence of the three posterior neurons is confirmed by the anti-TH staining.

Consequently, we suggest that the effect of expressing LRRK2-G2019S in dopaminergic neurons is mostly mediated by the single TH-VUM neuron, because the two descending neurons do not show a direct link to feeding behavior. Only when G2019S is expressed in the TH-VUM do we see the reduction in PER, i.e. akinesia. However, we cannot rule out an effect of the PPL2 neurons, which also modulate the feeding system\textsuperscript{26} as they are also not targeted by the $D'$-GAL4.

**DISCUSSION**

Our principle finding is that expressing LRRK2 forms with increased kinase activity (G2019S, I2020T) in sets of dopaminergic neurons that include the TH-VUM is sufficient to induce akinesia, bradykinesia, and tremor in the fly PER. Although previous work with flies and rodents have identified movement disorders in PD models, our PER assay uniquely identifies the components of the response, in the context of changes mostly due to a single dopaminergic neuron (TH-VUM).

A key point is that our PER assay demonstrates dopaminergic-bradykinesia even at 3 days. In comparison, data from negative geotaxis (startle-induced climbing response) assays of G2019S, I2020T, or R1441C transgenics is more complex, depending on age and genotype. One report shows that while locomotion in DDC>l2020T flies is already compromised at 3 days,\textsuperscript{29} another study shows that TH>l2020T flies show little deficit at any age.\textsuperscript{31} This may be because DDC-GAL4 includes serotonergic, as well as dopaminergic neurons, and thus more cells compared with TH-GAL4. Using DDC-GAL4, to express LRRK2 in old (>5 weeks) flies, G2019S and R1441C movement is shown to be reduced,\textsuperscript{32,33} while younger flies show no deficit. In contrast, our data show movement deficits in young TH>g2019s and TH>l2020T flies, which are maintained over the first few weeks of adult life. Indeed, LRRK2 mutations already start to affect Drosophila larvae, indicating effects at an earlier timepoint.\textsuperscript{31,34,35}

One disadvantage of working with older flies is that, by 5 weeks, a proportion of flies will have died, potentially those most strongly influenced by the transgene, so that negative geotaxis assays may underestimate the real impact of LRRK2 mutations. Our assay has the advantage of working at 3 days, before flies have started to die, and potentially could be developed so that the same individual fly might be tested at different time points. This would permit comparison of the individual and population responses.

In our visual assays with TH>g2019s, we found that 3-day-old flies showed no detectable visual deficits, though younger flies (1-day-old) had overactive vision, and old flies (28-day-old, or visually stressed) had much reduced response.\textsuperscript{6,36,37} Overactivity has also been reported in young transgenic LRRK2 rats, followed later by loss of movement.\textsuperscript{38-40} We have not tested the feeding response of flies less than 3 days old, because these newly emerged flies rest and expand their cuticle, and are not feeding: this makes them unsuitable for PER assay.

However, the PER of our mildly starved 3-day-old TH>g2019s flies is already reduced, and remains well below wild-type levels for at least 18 days. A more pronounced PER deficit might arise in older flies (5 weeks), and/or those kept at 29°C to enhance transgene expression.

Further, the movement deficits in our PER assay on flies starved for 2–3 h are mainly a consequence of expressing G2019S in a single dopaminergic neuron, TH-VUM, rather than the mixed effect of a range of dopaminergic clusters.

In this respect the PER assay differs from both negative geotaxis and our visual assay (three different kinds of dopaminergic neuron are present in the retina). However, we note that longer term modulation of feeding appears to involve other dopaminergic clusters, interacting in the mushroom bodies.\textsuperscript{71} Additionally, our
PER deficit occurs in young flies in which the TH-VUM is still present, offering the potential to understand the processes by which LRRK2 leads to neuronal silencing in a single identified neuron.

We find that both the akinesia and bradykinesia components of the TH>G2019S effect on PER are dependent on the kinase role of LRRK2. We observe no effect of expressing the KD (kinase-dead, G2019S-K1906M) form of LRRK2, although the expression level is stronger than G2019S. In this respect, it resembles the visual assay, where expressing G2019S, but not this KD construct led to retinal neurodegeneration. The TH>R1441C flies also showed no reduction in PER, or in visual degeneration, though it is possible that this is because R1441C is not so effectively expressed. The rescue by the specific LRRK2 inhibitor, BMPPB-32, argues that the reduction in LRRK2 is a consequence of phosphorylation of substrate(s) by LRRK2. We previously showed this inhibitor was effective in a visual assay, reverting TH>G2019S phenotypes in both young and old flies. Another specific inhibitor LDN-73794 prevents loss of DDC>G2019S induced locomotion in old flies. The assay has an advantage over the climbing assay (startle response), where degeneration is usually measured at 4–5 weeks, as our flies only need to be fed with the inhibitors for 3 days, reducing compound requirements.

GENETICALLY ACTIVATING OR SILENCING THE TH-VUM RESPECTIVELY INCREASE OR DECREASE THE PROBABILITY OF A PER. Thus, our data showing kinase active LRRK2 transgenes in the TH-VUM reduce PER could be explained by a reduction in dopamine release by this neuron. We hypothesize that expressing G2019S in the TH-VUM could lead to either (i) failure of TH-VUM neurites to grow, (ii) a reduction in its tonic firing, (iii) less dopamine synthesis, or (iv) lower probability of release of dopamine onto the reflex pathway. Cultured mammalian neurons, fly motoneurons, and sensory cells all have reduced neurites with G2019S. While it is possible that G2019S also reduces neuritic branching in the TH-VUM neuron, our data rather favor hypotheses (iii) or (iv) since we found that feeding flies L-DOPA rescued the TH>G2019S loss of PER. Reduced dopamine levels have been reported with DDC>G2019S, and with ubiquitous expression of an increased kinase form of the fly homolog dLRRK in both Drosophila and mammals, L-DOPA can cross the blood–brain barrier, but dopamine cannot. Thus we suggest that uptake of L-DOPA into the TH-VUM leads to increased dopamine levels and release, rescuing the effect of TH>G2019S. Increasing the amount of dopamine released onto the sugar-sensing Gr5a neurons would then rescue the proportion of flies that show the PER (akinesia), while release onto second order, local interneurons, might affect the motoneurons and thence speed (bradykinesia), and tremor of the proboscis extension. Such a dual output onto Gr5a neurons and onto local interneurons is suggested by the fact that 2.5 μM BMPPB-32 fully rescues bradykinesia, but only partially rescues akinesia. Although a number of interneurons in the SEZ with roles controlling proboscis extension, ingestion, and memory have recently been identified (e.g. see refs. 48–50), the link between Gr5a sense cells and the E49 motoneurons remains to be established.

METHODS

Flies, Drosophila melanogaster, were raised at 25 °C on.commeal-sugar-agar-yeast food. The following GAL4 lines were used: TH (tyrosine hydroxylase) GAL4,51 DDC-GAL4,25 HLG,26 or the C′ and D′-GAL4 stocks,27 the pan-neuronal nSyb-GAL4 (Stephen Goodwin), the sensory Gr5a-GAL4,30 and motoneuron E49-GAL4.18 The UAS lines were: wild-type hLRRK2 or LRRK2-G2019S,13 hLRRK2-I2020T and the kinase dead line LRRK2-G2019S-K1906M (hereafter, KD);31 hLRRK2-R1441C,31 elGFP (el4AAll:GFP, Andreas Prekop). In some confirmatory experiments, independent LRRK2-G2019S and hLRRK2 lines were used.21 The lab stocks of CS (Canton-S), w1118 (w), and w1118 (w) (Bloomington stock 148) provided "wild-type" outcross controls.

PER was performed (A.C.C.) by collecting male flies of known age at the start of the working day, under CO2 anesthesia, and sticking them ventral side up to card with rubber cement (Fino Gum). Flies were left to recover for 2–3 h at 25 °C. They were presented with a droplet of 100 mM sucrose solution to the legs, and the immediate PER/no PER scored (response in <2 s). Experiments were designed so that each graph plotted here comes from flies scored over three adjacent days, with the genotypes mixed each day, to allow for the small variations in food and environmental conditions.
Fig. 5  The presence of dopaminergic TH-VUM neuron is essential for the G2019S/I2020T-mediated reduction in PER. a Proportion of flies responding when LRRK2 transgenes are expressed in different subsets of the dopaminergic neurons, using the DDC, HL9, C" or D" GAL4 drivers. There is no difference between the increased kinase mutants (G2019S/I2020T) and the kinase-dead construct (KD, G2019S-K1906M) with the D" GAL4, which does not express in the TH-VUM neurons. All the other GAL4 lines tested express in the TH-VUM neurons and show a smaller response in G2019S/I2020T than in KD. Exact genotypes: + is wildtype. b Summary maps of the expression patterns of the GAL4 drivers used in a. Figures redrawn after Mao and Davis (2009). c The lack of TH-VUM in the D" line is confirmed anatomically. Each panel shows the projection of a confocal stack through the sub-esophageal zone (as marked in the first panel by the dotted box). Neurons marked by expression of eIfGFP under the control of the relevant GAL4 and stained by anti-TH antibody. The SEZ contains a single anterior cell ("a") and a group of three posterior cells ("p"), marked with anti-TH antibody. With DDC, HL9, and C" GAL4 drivers, all four SEZ neurons were GFP positive. With D", the nucleus of the anterior neuron "a" has a weak GFP signal, but the three posterior neurons marked with anti-TH antibody do not fluoresce green (note the cytoplasm of the two left posterior cells is merged in this projection of the z-stack, but their empty nuclei are still visible). Scalebar 20 μm

Power calculations indicate that a "medium" effect size, with a sample of 500 flies, and 16 df, would be detected at the 1 % level >98% of the time. Drugs were fed to adult flies from eclosion until testing. L-DOPA (Sigma) was added to food (final concentration 50 μM). BMPPB-32 and LRRK2-IN-1 (Lundbeck) were dissolved to give a final concentration in the food at 2.5 μM.

PER was filmed using a Mikrotron MC-1362 camera mounted on a Zeiss Stemi microscope. Videos were acquired at 200 frames/s; sample movies for a wild type and TH-G2019S flies are presented in Movies S1 and S2. Only the first PER of each fly was analyzed. In Matlab, the eye and tip of the proboscis were marked and their separation was determined for each frame individually. The analyses of Movies S1 and S2 are shown in Movies S3 and S4, respectively.

Western blots were performed as described using the heads of 3-day-old female flies, raised at 29 °C using anti-LRRK2 (Neuromab, clone N241A/34, 1:1000) and anti-β-actin (Proteintech, 1:180,000, loading control). The data are representative of three blots.

Immunocytochemistry was as described recently, using mouse anti-TH (Immunostar, 1:1000) and driving eGFP using the required GAL4 line. All data are from male flies, aged 3–5 days. No anti-GFP was used in the data chosen for illustration. The brightness and contrast of the images was adjusted in ImageJ so that the cells could be seen in both color channels, as each GAL4 drove GFP with a different intensity in the VUM neurons. Original images available on request to cje2@york.ac.uk, Representative data from at least three preparations are shown.

Statistics
For analysis of the proportion of flies showing a PER, statistical significance was determined using the χ² post-hoc test in the "Fifer" package of R. Confidence limits were determined using the Binomial test in R. Measurements of the speed of the PER were analyzed by ANOVA and Tukey post-hoc tests. For a "medium" effect size, with 26 flies in each of three samples, and the probability of 0.05, the power is 63%. N values for each genotype/treatment are included in Supplementary Table 1.

Data availability statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Image 92x640 to 517x705

Image 97x419 to 528x510
AUTHOR CONTRIBUTIONS
A.C.C., designed and performed experiments; N.S. analyzed video; S.P. did western blots; C.A.M. was responsible for the immunocytochemistry; L.W. organized the video and its analysis; C.J.H.E. designed experiments, analyzed the results, and wrote the manuscript; all contributors revised the manuscript.

ADDITIONAL INFORMATION
Supplementary information accompanies the paper on the npj Parkinson’s Disease website (https://doi.org/10.1038/s41531-017-0036-y).

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