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1 **Glial Nrf2 signaling mediates the neuroprotection exerted by *Gastrodia***  
2 ***elata* Blume in Lrrk2-G2019S Parkinson's disease**

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17

18 **Abstract**

19       The most frequent missense mutations in familial Parkinson's disease (PD) occur in the highly  
20 conserved *LRRK2/PARK8* gene with G2019S mutation. We previously established a fly model of PD  
21 carrying the *LRRK2-G2019S* mutation that exhibited the parkinsonism-like phenotypes. An herbal  
22 medicine—*Gastrodia elata* Blume (GE), has been reported to have neuroprotective effects in toxin-  
23 induced PD models. However, the underpinning molecular mechanisms of GE beneficiary to G2019S-  
24 induced PD remain unclear. Here, we show that these G2019S flies treated with water extracts of GE  
25 (WGE) and its bioactive compounds, gastrodin and 4-HBA, displayed locomotion improvement and  
26 dopaminergic neuron protection. WGE suppressed the accumulation and hyperactivation of G2019S  
27 proteins in dopaminergic neurons, and activated the antioxidation and detoxification factor Nrf2 mostly in  
28 the astrocyte-like and ensheathing glia. Glial activation of Nrf2 antagonizes G2019S-induced Mad/Smad  
29 signaling. Moreover, we treated *LRRK2-G2019S* transgenic mice with WGE and found the locomotion  
30 declines, the loss of dopaminergic neurons, and the number of hyperactive microglia were restored. WGE  
31 also suppressed the hyperactivation of G2019S proteins and regulated the Smad2/3 pathways in the mice  
32 brains. We conclude that WGE prevents locomotion defects and the neuronal loss induced by G2019S  
33 mutation via glial Nrf2/Mad signaling, unveiling a potential therapeutic avenue for PD.

34

## 35 **Introduction**

36        Parkinson's disease (PD) is a highly prevalent neurodegenerative disorder characterized by the loss  
37 of dopaminergic neurons in the substantia nigra projecting to the striatum of the basal ganglion,  
38 representing a circuit involved in motor planning and coordination. As a consequence, PD is associated  
39 with motor abnormalities, bradykinesia, hypokinesia, rigidity and resting tremor. Currently, the most  
40 frequently applied pharmacological treatment, levodopa (L-DOPA), exerts limited motor improvement  
41 and elicits negative side-effects (Ray Chaudhuri et al., 2018). Hence, identifying and developing  
42 alternative or complementary treatments may assist in mitigating PD progression.

43        PD is a multi-causal disease with a complicated etiology, including familial inheritance. More than  
44 20 *PARK* genes have been genetically linked to PD, a number that is increasing (Houlden & Singleton,  
45 2012). Missense mutations in *PARK8*, or *Leucine-rich repeat kinase 2 (LRRK2)* induce characteristic PD  
46 symptoms and pathologies such as loss of dopaminergic neurons and the appearance of Lewy bodies  
47 (Martin et al., 2014). Notably, the most commonly observed mutation, dominant G2019S, among familial  
48 PD cases is located in the kinase domain of *Lrrk2*, which augments its kinase activity via auto- and hyper-  
49 activation (Sheng et al., 2012). The hyperactive G2019S mutant protein alters several cellular processes,  
50 including vesicle trafficking, microtubule dynamics, autophagy, mitochondrial function (Martin et al.,  
51 2014), and most commonly, increases susceptibility to oxidative stress that contributes to neuronal  
52 degeneration (Angeles et al., 2011; Nguyen et al., 2011). These indicate that regulation of the  
53 hyperactivation of G2019S mutant protein appears to be a disease-modifying strategy.

54        Glia provide structural and metabolic supports to neurons and regulate synaptic transmissions, so  
55 they are important for the function and survival of dopaminergic neurons (Lin et al., 1993; Sofroniew &  
56 Vinters, 2010). Dysfunction in two major glial types, astrocytes and microglia, contribute to the onset and  
57 progression of both sporadic and familial PD (Kam et al., 2020). Astrocytes and microglia of postmortem  
58 PD brains exhibit pathologic lesions and hyper-immunoactivity (Miklossy et al., 2006). A clinical trial  
59 involving down-regulation of microglial oxidative stress highlights the significance of glia to PD (Jucaite  
60 et al., 2015). Moreover, *Lrrk2* regulates the inflammatory response in microglia and the autophagy-

61 lysosome pathway in astrocytes, with the G2019S mutation altering the size and pH of lysosomes (Henry  
62 et al., 2015; Moehle et al., 2012). Expression of G2019S mutant protein in neurons was previously shown  
63 to induce the secretion of Glass bottom boat (Gbb)/bone morphogenetic protein (BMP) signal that, in  
64 turn, upregulates Mothers against decapentaplegic (Mad)/Smad signaling in glia, which prompts feedback  
65 signals to promote neuronal degeneration (Maksoud et al., 2019). These studies indicate that G2019S  
66 mutant protein alters the homeostasis and interaction between neurons and glia, contributing to PD  
67 pathogenesis. However, whether any dietary or pharmacological treatment blockading this neuron-glia  
68 interaction beneficiary to G2019S-induced PD is unclear.

69       Given that up to 70% of human *PARK* genes are conserved in the *Drosophila* genome, *Drosophila* is  
70 frequently used as a PD model for studying gene function, such as the *PARK1/SNCA* (Chen & Feany,  
71 2005) and *PARK8/LRRK2* (Liu et al., 2008). Genetic and molecular linkage between *PINK1/PARK6* and  
72 *Parkin/PARK2* was first established in *Drosophila* (Clark et al., 2006). When overexpressed in  
73 *Drosophila* dopaminergic neurons, *LRRK2* transgenes carrying G2019S or other dominant mutations  
74 induce dopaminergic neuron loss and locomotion impairment, two age-dependent symptoms of PD (Lin  
75 et al., 2010; Liu et al., 2008). The G2019S model has further been used to screen a collection of FDA-  
76 approved drugs to suppress these PD phenotypes. Thus, *Drosophila* represents an amenable model of PD  
77 for genetic, molecular and pharmacological study of potential therapeutic interventions. Strikingly,  
78 lovastatin was found to prevent dendrite degeneration, dopaminergic neuron loss, and impaired  
79 locomotion and, critically, a lovastatin-involved Nrf2 pathway proved neuroprotective (C. H. Lin et al.,  
80 2016). Nevertheless, whether the Nrf2-mediated neuroprotection is cell- or non-cell-autonomous,  
81 remaining elusive.

82       Traditional Chinese Medicine (TCM) is often used as an alternative or dietary treatment for human  
83 diseases, including PD (Kim et al., 2012; Li et al., 2017). Although the results were inconclusive, some  
84 TCM could display adjuvant effects when used in combination with L-DOPA, reducing the L-DOPA  
85 dosage required in long-term treatments and relieving non-motor symptoms (Kim et al., 2012). As a  
86 prominent component in TCM, *Gastrodia elata* Blume (GE; Orchidaceae) has been used to treat

87 neurological disorders for centuries (Chen & Sheen, 2011). GE has been shown to exert neuroprotective,  
88 anti-inflammatory and antioxidative effects in neurodegenerative disease models (Jang et al., 2015). The  
89 major bioactive compounds in GE include gastrodin and 4-hydroxybenzyl alcohol (4-HBA), both of  
90 which display pharmacological effects on neurobiological and psychological disorders (Chen et al., 2016;  
91 Kumar et al., 2013). Additionally, gastrodin and 4-HBA have been reported to activate the Nrf2 signaling  
92 in dopaminergic neurons and astrocytes, respectively (Jiang et al., 2014; Luo et al., 2017), highlighting a  
93 potential benefit of incorporating GE in PD treatments. However, the effects and mechanisms underlying  
94 how GE moderate Lrrk2-G2019S PD remain unclear.

95 In the present study, we treated G2019S animals with water extract of GE (WGE), and its bioactive  
96 compounds, gastrodin and 4-HBA. We have investigated the impact of WGE treatment on PD in restoring  
97 locomotion and protecting dopaminergic neurons in the *Drosophila* G2019S model. We identified two  
98 distinct pathways induced by WGE in the model, i.e., suppression of Lrrk2 protein accumulation and  
99 hyperphosphorylation in neurons, and activation of the Nrf2 pathway in glia, particularly in astrocyte-like  
100 and ensheathing glia. We show that WGE-induced Nrf2 activation antagonizes the Gbb-activated Mad  
101 signaling in glia, contributing to neuronal protection. WGE also suppressed the hyperactivation of  
102 G2019S proteins and antagonized Smad2/3 signaling in a *LRRK2-G2019S* mouse model, which restored  
103 locomotion, protected dopaminergic neurons and regulated the microglia hyperactivation. Conservation  
104 of the pathways impacted by WGE treatment in both the *Drosophila* and mouse G2019S models implies  
105 that the beneficial effects of GE and represent a reliable and effective complementary therapy for PD.

106

107 **Results**

108 **WGE treatment improves locomotion of *Ddc>G2019S* flies**

109 We employed the *GAL4-UAS* system to express the human G2019S mutant of Lrrk2 by the *Ddc*-  
110 *GAL4* driver (*Ddc>G2019S*) in dopaminergic neurons and then assessed the anti-geotactic climbing  
111 activity of adult flies. We observed that locomotion of *Ddc>G2019S* flies was affected significantly  
112 relative to control flies expressing human wild-type Lrrk2 (*Ddc>Lrrk2*) (Figure 1A and Figure 1-figure  
113 supplement 1A). At weeks 1 and 2, more than 80% of *Ddc>G2019S* flies could successfully climb above  
114 an 8-cm threshold, a proportion comparable to that of *Ddc>Lrrk2* flies. However, the success rate  
115 declined to ~40% at week 3, ~20% at week 4, and to less than 10% at weeks 5 and 6. These proportions  
116 are significantly lower than the ~80% at week 3, ~60% at week 4, and ~40% at weeks 5 and 6 displayed  
117 by *Ddc>Lrrk2* flies. Although both *Ddc>G2019S* and *Ddc>Lrrk2* flies failed to reach the 8-cm mark at  
118 weeks 7 and 8, *Ddc>Lrrk2* flies could still climb the wall, whereas almost all *Ddc>G2019S* flies could  
119 not (Figure 1-figure supplement 1A). We also tested the climbing activity of another control expressing  
120 GFP in dopaminergic neurons (*Ddc>mCD8-GFP*). Both *Ddc>Lrrk2* and *Ddc>mCD8-GFP* flies showed  
121 comparable climbing activities in the first six weeks, and a significant number of *Ddc>mCD8-GFP* flies  
122 were still able to climb above the 8-cm mark at week 7. Therefore, we used the *Ddc>Lrrk2* line as a  
123 control for *Ddc>G2019S* flies in subsequent experiments to dissect the specific mode of pathogenicity of  
124 the G2019S mutation.

125 Next, we examined the effect of feeding flies with water extracts of GE (WGE) as a dietary  
126 supplement at different concentrations (0.1, 0.5 or 1.0%, w/w). WGE treatment of *Ddc>G2019S* flies at  
127 all three doses elicited a significant improvement in their climbing ability (Figure 1A). Strikingly, the  
128 lowest concentration (0.1%) of WGE proved the most effective, with *Ddc>G2019S* flies still performing  
129 well at climbing (i.e., comparably to *Ddc>Lrrk2* control flies) in weeks 5 and 6. The higher doses of  
130 WGE (0.5 % and 1.0%) still exerted beneficial effects at weeks 3 and 4, albeit not as significantly as the  
131 0.1% dose, but had no beneficial effect in weeks 5 and 6. As 0.1% is the lowest among the three doses  
132 tested, we lowered the dose of WGE to 0.02% and found that 0.02% WGE was less effective than 0.1%

133 starting at week 3 till week 6, suggesting that 0.1% is the optimal dose in restoring the climbing activity  
134 of *Ddc>G2019S* flies (Figure 1-figure supplement 1B). We also tested the WGE effect on the  
135 *Ddc>G2019S* flies that were fed with regular food without WGE for 3 weeks. At week 4, these  
136 *Ddc>G2019S* flies also showed a significant improvement in their climbing ability, compared to the age-  
137 matched *Ddc>G2019S* flies fed continuously on regular food (Figure 1-figure supplement 2). The effect  
138 of improving climbing activity in the WGE-fed *Ddc>G2019S* flies was reduced at week 5 and diminished  
139 at week 6, suggesting that WGE feeding starting at earlier stages is important for long-term locomotion  
140 improvement.

141 Gastrodin and 4-HBA are two major phenolic compounds in GE displaying neuropharmacological  
142 effects (Zhan et al., 2016). Feeding *Ddc>G2019S* flies with food containing gastrodin (0.1 mM)  
143 equivalent to the amount in 0.1% WGE also restored locomotion of *Ddc>G2019S* flies in weeks 2-4,  
144 though its impact diminished to non-significant levels at weeks 5 and 6. However, increasing the  
145 gastrodin dose ten-fold (1.0 mM) resulted in improved climbing activity at weeks 5 and 6 (Figure 1B).  
146 Similarly, the equivalent 0.1 mM of 4-HBA, the aglyconic form of gastrodin and the bioactive form in the  
147 brain (Wu et al., 2017), was sufficient to restore the climbing ability of *Ddc>G2019S* flies, and a ten-fold  
148 dose at 1.0 mM had an even better effect (Figure 1C). These results indicate that both gastrodin and 4-  
149 HBA are primary bioactive compounds in GE that prevent locomotion decline in *Ddc>G2019S* flies, and  
150 higher doses are more beneficial to reach the effect as WGE did.

151 Success in the anti-gravity wall-climbing assay also requires an immediate response to startle  
152 knockdown. Accordingly, we performed a second assay, free-walking in an open arena, to assess  
153 improved locomotion. Consistently, free-walking by *Ddc>G2019S* flies was greatly impaired, with total  
154 walking distance reduced to less than 20% that displayed by control *Ddc>Lrrk2* flies (Figure 1D).  
155 Moreover, *Ddc>G2019S* flies displayed centrophobism, i.e., they avoided walking into the central open  
156 space. Strikingly, 0.1% WGE feeding greatly extended walking distance and suppressed the  
157 centrophobism of *Ddc>G2019S* flies. Together, these two assays strongly indicate that the defective  
158 locomotion exhibited by *Ddc>G2019S* flies is greatly improved by feeding them with 0.1% WGE.



159 Because the improvement on the locomotion was more effective in flies fed with 0.1% WGE than the  
160 pure compounds, we therefore fed the flies with 0.1% WGE in the following experiments.

161

### 162 **WGE treatment suppresses dopaminergic neuron loss in *Ddc>G2019S* brain**

163 Expression of G2019S mutant protein has been shown to induce a gradual loss of dopaminergic  
164 neurons in the adult fly brain, contributing to impaired locomotion (Lin et al., 2010; Liu et al., 2008).  
165 Several clusters of dopaminergic neurons have been identified in the adult brain of *Drosophila*. Here, we  
166 focused on the PPL1, PPL2, PPM1/2 and PPM3 clusters that have well-defined roles in modulating  
167 locomotion (Mao & Davis, 2009) to assess the effect of WGE treatment. We detected reduced numbers of  
168 dopaminergic neurons in the PPL1, PPL2, PPM1/2, and PPM3 clusters of *Ddc>G2019S* flies relative to  
169 *Ddc>Lrrk2* controls, which increased in severity from week 2 to 6 (Figure 2 and Figure 2-figure  
170 supplement 1A to C). Consistently, feeding *Ddc>G2019S* flies with 0.1% WGE restored numbers of  
171 dopaminergic neurons in these clusters to the levels observed in controls (Figure 2 and Figure 2-figure  
172 supplement 1A to C). Thus, concomitant rescue of locomotion and dopaminergic neuron populations in  
173 *Ddc>G2019S* flies indicates that WGE treatment likely promotes dopaminergic neuron survival to restore  
174 locomotion.

175

### 176 **WGE treatment suppresses G2019S-induced Lrrk2 hyperactivation**

177 The enhanced survival of dopaminergic neurons due to WGE treatment implies that WGE induces  
178 neuroprotective mechanisms in *Ddc>G2019S* flies. The G2019S mutation causes Lrrk2  
179 hyperphosphorylation, protein accumulation, and aberrant cellular signaling (Price et al., 2018).  
180 Therefore, we explored if WGE-induced neuroprotection is responsible for abrogating these processes in  
181 G2019S flies. We detected comparable levels of Lrrk2 proteins in 3-day-old adult brains pan-neuronally  
182 expressing wild-type Lrrk2 (*elav>Lrrk2*) or G2019S mutant protein (*elav>G2019S*) (Figure 3A).  
183 However, phosphorylation levels at the Ser<sup>1292</sup> autophosphorylation site was higher in the *elav>G2019S*  
184 flies compared to *elav>Lrrk2* (Figure 3A and B). This outcome was also observed at week 4 (Figure 3F

185 and G), consistent with the idea that Lrrk2 is hyperactivated upon G2019S mutation.  
186 Hyperphosphorylation in *elav>G2019S* flies led to Lrrk2 protein accumulation, as determined by total  
187 Lrrk2 levels at weeks 2 and 4 (Figure 3D and E). However, both hyperphosphorylation and protein  
188 accumulation were suppressed upon feeding *elav>G2019S* flies with 0.1% WGE (Figure 3D to G). In the  
189 *elav>Lrrk2* control, WGE feeding had no effect on levels of wild-type Lrrk2 or Ser<sup>1292</sup> phosphorylation  
190 (Figure 3-figure supplement 1A to C). We further examined the G2019S mutant-activated downstream  
191 effector Rab10, the phosphorylation status of which can serve as an indicator of Lrrk2 kinase activity  
192 (Karayel et al., 2020). We observed that levels of phosphorylated Rab10 were increased in *elav>G2019S*  
193 flies, but this phenotype was suppressed by WGE feeding (Figure 3F and H). Hence, feeding flies with  
194 WGE suppresses G2019S-induced Lrrk2 protein phosphorylation, accumulation, and signaling.

#### 195 196 **WGE treatment restores Akt/GSK3 $\beta$ /Nrf2 pathway activity**

197 The Akt/GSK3 $\beta$ /Nrf2 signaling axis has been shown to promote survival of dopaminergic neurons  
198 and ameliorate motor dysfunction in PD models (C. H. Lin et al., 2016). We assayed the phosphorylation  
199 status of Akt at Ser<sup>505</sup> in 3-day-old adult fly head extracts and found reduced levels of pAkt in  
200 *elav>G2019S* flies relative to *elav>Lrrk2* controls (Figure 3A and C). This pAkt reduction persisted at  
201 weeks 2 and 4, but was abrogated by feeding *elav>G2019S* flies with 0.1% WGE (Figure 4A and B). We  
202 also examined phosphorylation levels of Nrf2 at Ser<sup>40</sup> and GSK3 $\beta$  at Ser<sup>9</sup>, both of which were reduced  
203 upon expression of G2019S mutant protein and were equally offset by WGE feeding (Figure 4C and D).  
204 Induction of Akt/GSK3 $\beta$ /Nrf2 signaling activates expression of heme oxygenase 1 (HO-1), and we  
205 observed diminished levels of this latter protein in *elav>G2019S* flies, which could be rescued by WGE  
206 feeding (Figure 4C and D). Therefore, WGE feeding restores the Akt/GSK3 $\beta$ /Nrf2 signaling activity  
207 compromised by the G2019S mutation.

#### 208 209 **Glial Nrf2 mediates the beneficial effect of WGE on restored locomotion and neuronal protection in** 210 **G2019S flies**

211 Restoration of Akt/GSK3 $\beta$ /Nrf2 signaling activity by WGE treatment prompted us to test by genetic  
212 assays if that signaling pathway mediates the WGE mode of action. We focused on the downstream  
213 effector Nrf2 encoded by *cap-n-collar (cnc)* in *Drosophila*. Intriguingly, neither overexpression (*UAS-*  
214 *cncC-FL2*) nor RNAi knockdown (*UAS-cncTRiP*) of Nrf2 in dopaminergic neurons had an impact on the  
215 climbing ability of *Ddc>G2019S* flies (Figure 5A). Also, WGE feeding still rescued locomotion of  
216 *Ddc>G2019S* flies with Nrf2 knockdown (*Ddc>G2019S; cncTRiP*) to levels comparable to WGE-fed  
217 control flies (*Ddc>G2019S; mCD8-GFP*) without Nrf2 knockdown (Figure 5A). Thus, the Nrf2 pathway  
218 activation by WGE that appears to be effective in protecting neurons and restoring locomotion is likely  
219 exerted in cells other than neurons.

220 Nrf2 activation may be examined by assessing GFP signal from the *ARE-GFP* reporter that harbors  
221 Nrf2 binding sites and responds to Nrf2 activation (Chatterjee & Bohmann, 2012). *ARE-GFP* adult fly  
222 brains displayed low basal GFP signals (Figure 5-figure supplement 1A), but feeding with 0.1% WGE  
223 elicited many GFP-positive signals in Repo-positive glia (arrowheads in Figure 5-figure supplement 1B),  
224 evidencing that glia may be the primary cell type in which Nrf2 is activated.

225 Next, we investigated if WGE-induced Nrf2 activity in glia is effective in promoting locomotion in  
226 *G2019S* flies. To this end, we employed the *LexA-LexAop* system to overexpress wild-type *Lrrk2* or  
227 *G2019S* mutant protein in dopaminergic neurons (*Ddc-LexA>Lrrk2* and *Ddc-LexA>G2019S*,  
228 respectively), and used the *GAL4* driver to manipulate Nrf2/CncC activity in glia (*repo>cncC-FL2* for  
229 overexpression and *repo>cnc-RNAi* for knockdown). As a first step, we validated the age-dependent  
230 locomotion decline of *Ddc-LexA>G2019S* flies that was severer than *Ddc-LexA>Lrrk2* and could be  
231 rescued by 0.1% WGE feeding (Figure 5-figure supplement 2). We then assayed the climbing ability of  
232 *Ddc-LexA>G2019S* flies exhibiting *repo-GAL4*-driven Nrf2 overexpression in glia (*Ddc-LexA>G2019S;*  
233 *repo>cncC-FL2*). Significantly, the *Ddc-LexA>G2019S; repo>cncC-FL2* flies performed better in the  
234 climbing assay than *Ddc-LexA>G2019S; repo-GAL4* without Nrf2 overexpression (Figure 5B). We also  
235 performed Nrf2 knockdown in glia (*Ddc-LexA>G2019S; repo>cnc-RNAi*), which had little impact on the  
236 already declined climbing activity in *Ddc-LexA>G2019S; repo-GAL4* (Figure 5B). Importantly, WGE

237 feeding could not rescue locomotion deficits of glial Nrf2-knockdown flies (*Ddc-LexA>G2019S*;  
238 *repo>cnc-RNAi*) (Figure 5B, compare to *Ddc-LexA>G2019S*; *repo-GAL4* with WGE feeding). Thus,  
239 glial overexpression of Nrf2 partially restores locomotion of *Ddc-LexA>G2019S* flies, and glial depletion  
240 of Nrf2 abolishes the ability of WGE to rescue impaired locomotion.

241 We stained dopaminergic neurons of 6-week-old adult fly brains and confirmed that numbers of TH-  
242 positive dopaminergic neurons in the PPL1 cluster were reduced in the *Ddc-LexA>G2019S*; *repo-GAL4*  
243 flies compared to *Ddc-LexA>Lrrk2*; *repo-GAL4* controls (Figure 5C and D). Importantly, numbers of  
244 dopaminergic neurons in the PPL1 cluster were restored upon glial overexpression of Nrf2 in the *Ddc*-  
245 *LexA>G2019S*; *repo>cnc-FL2* flies. In contrast, glial Nrf2 knockdown had little impact on the already  
246 reduced dopaminergic neurons. Taken together, these results support that glial Nrf2 is compromised in  
247 *Ddc-LexA>G2019S* flies, and glial expression of Nrf2 protects the dopaminergic neurons.

248 Although WGE feeding suppressed hyperactivity of G2019S mutant protein (Figure 3D to G), it was  
249 not clear if WGE-mediated Nrf2 activation in glia could directly suppress mutant protein hyperactivity in  
250 dopaminergic neurons. To test this possibility, we compared the levels of total Lrrk2 protein and  
251 phosphorylated Lrrk2 protein in *Ddc-LexA>G2019S* with and without glial Nrf2 overexpression (*Ddc*-  
252 *LexA>G2019S*; *repo-GAL4* and *Ddc-LexA>G2019S*; *repo>cncC*). We observed comparable levels of  
253 Lrrk2 and pLrrk2 in the control and Nrf2 overexpression lines (Figure 6-figure supplement 1A to C).  
254 Thus, the neuroprotective effects of Nrf2 activity are unlikely to operate through modulation of Lrrk2  
255 levels or activity.

256

### 257 **Nrf2 in astrocyte-like and ensheathing glia are the major targets of WGE that alleviate *Lrrk2*-** 258 ***G2019S* locomotion deficits**

259 Five types of glia with different morphologies and functions have been identified in the fly brain  
260 (Freeman, 2015). We decided to identify specific subtypes of glia that may mediate the WGE-induced  
261 Nrf2 activity endowing neuronal protection, given that dysfunctional astrocytes and microglia have been  
262 linked to onset and progression of both sporadic and familial PD (Kam et al., 2020). In *Ddc*-

263 *LexA>G2019S* flies, *GAL4*-driven *cnc* knockdown in astrocyte-like (*Ddc-LexA>G2019S; alrm>cnc-*  
264 *RNAi*) or ensheathing glia (*Ddc-LexA>G2019S; R56F03>cnc-RNAi*) abolished the improved locomotion  
265 elicited by WGE treatment, recapitulating the effect of pan-glial Nrf2 knockdown (*Ddc-LexA>G2019S;*  
266 *repo>cnc-RNAi*) (Figure 6A). This outcome was not observed when we used *GAL4* drivers to knock  
267 down *cnc* in cortex (*np2222*), perineurial (*np6293*), or subperineurial (*moody*) glia. We confirmed the  
268 involvement of astrocyte-like and ensheathing glia by means of Nrf2 overexpression in astrocyte-like  
269 (*Ddc-LexA>G2019S; alrm>cncC-FL2*) or ensheathing (*Ddc-LexA>G2019S; R56F03>cncC-FL2*) glia,  
270 with both treatments improving the climbing activity of *Ddc-LexA>G2019S* flies not subjected to WGE  
271 feeding (Figure 6B). These analyses indicate that WGE feeding induces Nrf2 activity in astrocyte-like  
272 and ensheathing glia, which mitigates the reduced locomotion displayed by G2019S mutant-expressing  
273 flies.

274 Next, we assayed Nrf2-regulated *ARE-GFP* expression in astrocyte-like and ensheathing glia of flies  
275 expressing G2019S mutant protein and subjected to WGE treatment. We focused on the astrocyte-like  
276 and ensheathing cells located adjacent to dopaminergic neurons. In control flies expressing wild-type  
277 *Lrrk2*, we detected basal levels of GFP signal in mCherry-positive astrocyte-like (*alrm-GAL4*) or  
278 ensheathing glia (*R56F03*) (Figure 6C to F). We detected lower levels of GFP signal in these cells when  
279 G2019S mutant protein was expressed in dopaminergic neurons. However, upon feeding with 0.1%  
280 WGE, we observed higher levels of GFP signal in both types of glia. Changes in the intensities of GFP  
281 signals were also detected in TH-positive dopaminergic neurons, although the levels were lower than in  
282 glia (Figure 6-figure supplement 2A and B). Thus, overexpression of G2019S mutant protein in  
283 dopaminergic neurons elicited reduced Nrf2 signaling activity, but WGE feeding restored or further  
284 enhanced Nrf2 activities in these two glial subtypes.

285

## 286 **Nrf2 activation antagonizes BMP signaling in glia**

287 G2019S mutant protein in dopaminergic neurons has been shown previously to enhance the  
288 expression of the proprotein convertase Furin 1 (*Fur1*) that processes the bone morphogenetic protein

289 (BMP) signaling molecule Glass bottom boat (Gbb) for maturation and release, leading to activation of  
290 the BMP signaling pathway in glia (Maksoud et al., 2019). We confirmed that finding by removing one  
291 copy of *Mad* that encodes the pathway's downstream effector to restore locomotion in *Ddc>G2019S* flies  
292 (Figure 7-figure supplement 1). To address if WGE-induced Nrf2 activation could antagonize BMP  
293 signaling activity in glia, first we assessed expression of the phosphorylated Mad (pMad) activated by  
294 BMP signaling. In glia of *Ddc>G2019S* adult fly brains, pMad levels were higher than in *Ddc>Lrrk2*  
295 brains and they could be suppressed by WGE treatment (Figure 7A and B). Similar to the previous report  
296 (Maksoud et al., 2019), glial overexpression of Mad (*UAS-Mad*) or constitutively active type I receptor  
297 Tkv (*UAS-tkv<sup>Q253D</sup>*) was sufficient to impair locomotion, even without expressing G2019S mutant protein  
298 in neurons. However, impaired locomotion was rescued in both cases by 0.1% WGE feeding (Figure 7C  
299 and D). In addition, numbers of dopaminergic neurons in the PPL1 cluster were reduced upon glial  
300 overexpression of *Mad* or *tkv<sup>Q253D</sup>* and they were restored by 0.1% WGE treatment (Figure 7E to H).  
301 Together, these analyses indicate that activation of BMP signaling in glia recapitulates the phenotypes  
302 observed in flies overexpressing G2019S mutant protein in dopaminergic neurons and, furthermore, that  
303 these effects can be suppressed by WGE treatment.

304 Next, we explored if WGE-induced Nrf2 activation antagonizes BMP signaling in glia. Glial Nrf2  
305 overexpression in *Ddc-LexA>G2019S* flies (*Ddc-LexA>G2019S; repo>cncC-FL2*) partially rescued the  
306 locomotory impairment caused by G2019S mutation (Figure 5B), and removing one copy of *Mad* (*Ddc-*  
307 *LexA>G2019S, Mad<sup>+/-</sup>; repo>cncC-FL2*) further enhanced this effect (Figure 8A). Indeed, this outcome  
308 was equivalent to removing one copy of *Mad* but without Nrf2 overexpression (*Ddc-LexA>G2019S,*  
309 *Mad<sup>+/-</sup>; repo-GAL4*), suggesting that Nrf2 functions mainly to antagonize Mad activity (Figure 8A).  
310 Consistently, Nrf2 overexpression in glia or WGE treatment suppressed the up-regulation of pMad levels  
311 in glia caused by G2019S mutant protein overexpression in dopaminergic neurons (*Ddc-LexA>G2019S;*  
312 *repo-GAL4*) (Figure 8B and C). Depletion of Nrf2 from glia (*Ddc-LexA>G2019S; repo>cnc-RNAi*), even  
313 in the presence of WGE treatment, maintained high pMad levels in glia (Figure 8B and C). Thus,

314 modulation of Nrf2 activity, either by genetic manipulation or by WGE treatment, has an antagonistic  
315 effect on pMad levels in glia.

316 We also addressed if *Mad* modulates expression of the Nrf2 target *ARE-GFP*. We observed  
317 diminished GFP signal in PPL1-surrounding glia of *Ddc-LexA>G2019S* adult fly brains relative to the  
318 *Ddc-LexA>Lrrk2* control. Heterozygosity of *Mad* in *Ddc-LexA>G2019S* flies (*Ddc-LexA>G2019S*,  
319 *Mad*<sup>+/-</sup>) restored the level of GFP signal, implying that Mad modulates targeted gene expression induced  
320 by Nrf2 activity (Figure 8D and E).

321

### 322 **The effects of WGE treatment in a *LRRK2-G2019S* mouse model**

323 To further study the effect of WGE on G2019S mutation-induced neurodegeneration in a  
324 mammalian system, we fed *LRRK2-G2019S* transgenic mice with WGE starting at the age of 8.5 months,  
325 i.e., prior to onset of impairments in locomotion and dopaminergic neurons (Chou et al., 2014). We  
326 quantified three locomotor activities from video-tracking paths of an open-field test, i.e., accumulative  
327 moving distance, average velocity, and percentage of time moving (Figure 9A and B). At the age of 8.5  
328 months, three groups—non-transgenic (nTg), transgenic *LRRK2-G2019S*, and WGE-fed transgenic  
329 *LRRK2-G2019S* mice—presented comparable locomotor activities. At 9.5 months of age, the *LRRK2-*  
330 *G2019S* mice displayed clearly impaired locomotion, which was statistically significant at the age of 11.5  
331 months relative to nTg littermates (Figure 9B), consistent with a previous report (Chen et al., 2012).  
332 Importantly, the *LRRK2-G2019S* mice fed with WGE showed improved locomotion throughout the 3-  
333 month treatment period, an outcome that was statistically significant at 11.5 months (Figure 9A and B).  
334 WGE treatment also suppressed the centrophobism displayed by *LRRK2-G2019S* mice (Figure 9A). We  
335 also analyzed the gait of these three groups of mice (Figure 9C). Similar to our findings from the open  
336 field test, stride length of *LRRK2-G2019S* mice was significantly reduced at 11.5 months, but it was  
337 restored to the level of nTg mice by WGE feeding (Figure 9C and D). Collectively, these analyses show  
338 that WGE feeding is an effective means of restoring G2019S mutation-induced locomotory declines in  
339 this mouse model of PD.

340 Since the nigral-striatal system contributes to locomotor function, we counted the number of TH-  
341 positive dopaminergic neurons in the substantia nigra. In comparison to nTg littermates, the number of  
342 dopaminergic neurons in 11.5-month-old *LRRK2-G2019S* mice was significantly reduced, but WGE  
343 treatment for three months abrogated this loss of dopaminergic neurons (Figure 10A and B). In the  
344 *Ddc>G2019S Drosophila* model, glia mediate the protective effects of WGE on dopaminergic neurons.  
345 We found that activated microglia marked by ionized calcium-binding adapter molecule 1 (Iba-1) were  
346 increased in the substantia nigra of *LRRK2-G2019S* mice, but this increase was suppressed by WGE  
347 treatment (Figure 10C and D, and Figure 10-figure supplement 1). We further analyzed levels of activated  
348 LRRK2 (pLRRK2) in lysates isolated from the nigra-striatum. Levels of pLRRK2 normalized to LRRK2  
349 signal were higher in *LRRK2-G2019S* mice than in nTg littermates, but WGE treatment partially  
350 abrogated that outcome (Figure 10E and F). Since G2019S mutation enhanced Mad signaling activation  
351 in the fly brain (Figure 8B and C), we also tested this scenario in the mouse model. Immunoblots revealed  
352 that the ratio of phosphorylated Smad2 to total Smad2 (pSmad2/Smad2) was significantly elevated in  
353 *LRRK2-G2019S* mice, but this increase was suppressed by WGE treatment (Figure 10E and G).  
354 Moreover, although the level of pSmad3/Smad3 was not significantly enhanced by G2019S mutant  
355 protein expression, it was suppressed by WGE treatment (Figure 10E and G). Thus, WGE feeding  
356 suppresses G2019S mutation-induced microglia activation and Smad signaling in the substantia nigra of  
357 the *LRRK2-G2019S* mouse model of PD.

358 In summary, the potential mechanisms of WGE involved in the modulation of the neuron-glia  
359 interaction are proposed (Figure 11). WGE regulates the hyperactivation of G2019S mutant protein in  
360 dopaminergic neurons, and antagonizes the Mad signaling by activating the Nrf2 pathway in glia. Both  
361 actions provide neuroprotection.

362



363 **Discussion**

364 Motor dysfunction in *Drosophila* neurodegeneration models has been frequently evaluated by means  
365 of negative-geotaxis assay that measures the insect innate response. The assay begins with a sudden  
366 external stimulation, which initially inhibits spontaneous locomotion but is followed by climbing  
367 behavior. The entire response requires motor circuit coordination and muscle tone regulation, two  
368 processes that progressively decline with age and that are impacted by neuropathological insults  
369 (Grotewiel et al., 2005). In contrast, the open-arena walking assay allows free exploration without  
370 disturbance, representing an assay for locomotor activities that can reveal deficits like bradykinesia (Chen  
371 et al., 2014). Our G2019S flies exhibited reduced locomotor activity in both assays. Moreover, the  
372 G2019S flies also exhibited centrophobism-like behavior in the open-arena (Figure 1D). Centrophobism  
373 is indicative of emotional abnormalities, such as anxiety and depression, both of which are often  
374 associated with PD (Kulisevsky et al., 2008), and was also displayed by the *LRRK2-G2019S* mice (Figure  
375 9A). WGE treatment suppresses centrophobism in both fly and mouse PD models has important  
376 implications for tackling major symptoms of PD and even non-PD-related depression, as reported for  
377 rodent models (Lin et al., 2018; Y. E. Lin et al., 2016). Thus, WGE treatment exerts beneficial effects in  
378 both of our PD models.

379 We found that the 0.1% dosage of WGE is optimal in suppressing age-dependent locomotion decline  
380 in G2019S flies, with higher and lower doses being less effective (Figure 1A and Figure 1-figure  
381 supplement 1B). Although an inverted U-shaped drug response is common, a plausible explanation for the  
382 diminished effectiveness of higher doses is that WGE downregulates the day-time but not night-time  
383 locomotor activity of flies (Jo et al., 2017). In mice, higher WGE doses have sleep-promoting effects by  
384 activating adenosine A<sub>1</sub>/A<sub>2A</sub> receptors in the ventrolateral preoptic area (Zhang et al., 2012). Thus,  
385 dosage level is critical to the beneficial effects of GE in both PD models.

386 Gastrodin and 4-HBA are considered the principal active components in GE (Zhan et al., 2016).  
387 Although both compounds can cross the blood-brain barrier (Wu et al., 2017), the capability of gastrodin  
388 to do so is relatively poor compared to aglyconic 4-HBA due to the glucose moiety (Lin et al., 2007).

389 Gastrodin is quickly metabolized to 4-HBA and undetermined metabolites in the brain (Lin et al., 2008),  
390 perhaps explaining the lower effectiveness of gastrodin in G2019S flies. However, it is likely that the  
391 combination of gastrodin with other components in GE might exert optimal beneficial effects.

392 Expression of G2019S mutant protein impacts different clusters of dopaminergic neurons in the fly  
393 brain that are known for their connectivity and function. Activation of two specific mushroom body  
394 (MB)-projection dopaminergic neurons in the PPL1 cluster inhibits climbing performance (Sun et al.,  
395 2018). Mutations in the circadian gene *Clock* (*Clk*) cause PPL1 dopaminergic neuron degeneration,  
396 accelerating impaired age-associated climbing ability (Vaccaro et al., 2017). Dopaminergic neurons in the  
397 PPL2 cluster extend processes to the calyx of the MB, which has been linked to climbing activity (Sun et  
398 al., 2018). In contrast, PPM3 neurons project to the central complex, activation of which enhances  
399 locomotion (Kong et al., 2010). A significant reduction in dopaminergic neurons in the PPM1/2 cluster  
400 was only found at week 4 when impaired locomotion of G2019S flies was prominent. In a PD fly model  
401 involving *SNCA* overexpression and *aux* knockdown, dopaminergic neurons in the PPM1/2 cluster were  
402 selectively degenerated and this phenotype was accompanied by impaired locomotion in relatively young  
403 adult flies (Song et al., 2017). We postulate that the age-dependent impaired locomotion displayed by  
404 G2019S flies could be caused by gradual and differential loss of dopaminergic neurons in these clusters,  
405 thereby affecting different aspects of locomotion. However, further study is needed to test that hypothesis.

406 Expression of the G2019S mutant protein induces Lrrk2 auto- and hyper-phosphorylation, as well as  
407 protein accumulation, together enhancing cellular Lrrk2 activity and causing aberrant downstream  
408 signaling (Sheng et al., 2012). We have shown here that neuronal expression of Lrrk2-G2019S reduced  
409 Akt phosphorylation (Figure 4A and B). Consistently, hyperactivated G2019S mutant protein impaired  
410 interaction with and phosphorylation of Akt, resulting in compromised signaling and accelerated  
411 neurodegeneration (Ohta et al., 2011; Panagiotakopoulou et al., 2020). However, WGE feeding restored  
412 downstream Akt signaling by suppressing G2019S mutant protein hyperactivation. Rab10, one of the  
413 best-characterized substrates for Lrrk2, mediates several of Lrrk2's cellular functions (Karayel et al.,  
414 2020). In *Drosophila*, Rab10 and Lrrk2-G2019S synergistically affect the activity of dopaminergic

415 neurons, mediating deficits in movement (Fellgett et al., 2021; Petridi et al., 2020). We have shown that  
416 WGE treatment downregulated levels of Lrrk2 accumulation and phosphorylated Rab10 (Figure 3F to H),  
417 thus alleviating their synergistic toxicity. Several kinase inhibitors have been developed to block the  
418 kinase activity of Lrrk2, including of both wild-type Lrrk2 and the G2019S mutant, which could affect  
419 endogenous Lrrk2 activity (Sheng et al., 2012). Instead, WGE treatment modulates the phosphorylation  
420 status and protein level of the G2019S mutant but not those of wild-type Lrrk2. The new hydrogen bond  
421 created at the Ser<sup>2019</sup> autophosphorylation site may provide a docking site for some chemicals in WGE,  
422 representing a possible explanatory mechanism that warrants further study (Lang et al., 2015).

423 The antioxidation and detoxification factor Nrf2 is a target of Akt activation. Nrf2 phosphorylation and  
424 HO-1 expression levels revealed that Nrf2 is inactivated in G2019S flies, but it was activated by WGE  
425 treatment (Figure 4C and D). Intriguingly, our genetic data indicate that Nrf2 primarily functions in the  
426 glia of G2019S flies, with Nrf2 depletion from glia eliminating the beneficial effects of WGE and glial  
427 Nrf2 activation partially substituting for WGE feeding (Figure 5B). Cortical neurons express much lower  
428 levels of Nrf2 than astrocytes owing to hypo-acetylation and transcriptional repression of the *Nrf2*  
429 promoter (Bell et al., 2015). Moreover, neurons express greater amounts of Cullin 3, the scaffold  
430 component of the E3 ubiquitin ligase that targets Nrf2 for proteasomal degradation (Jimenez-Blasco et al.,  
431 2015). Both those mechanisms render neuronal Nrf2 inert to activation. Nrf2 activation in astrocytes  
432 maintains neuronal integrity and function against oxidative insults in response to stress by supplying  
433 antioxidants such as glutathione and HO-1 (Kraft et al., 2004; Vargas & Johnson, 2009). Previous study  
434 showed that 4-HBA triggers glia to secrete HO-1 via the Nrf2 pathway, protecting neurons from hydrogen  
435 peroxide in the primary culture (Luo et al., 2017). In PD models in which wild-type or mutant  $\alpha$ -  
436 synuclein is overexpressed, activation of neuronal Nrf2 (Barone et al., 2011; Skibinski et al., 2017) or  
437 astrocytic Nrf2 (Gan et al., 2012) proved neuroprotective. In a previous study, lovastatin treatment  
438 provides neuroprotection in the G2019S-induced PD model, also through the Akt/Nrf2 pathway (C. H.  
439 Lin et al., 2016). As activation of neuronal Nrf2 plays a non-conventional role in promoting  
440 developmental dendrite pruning (Chew et al., 2021), it remains interesting to further study the cell types

441 that mediate the action of lovastatin. By genetically manipulating the G2019S fly model, we have shown  
442 that WGE-induced Nrf2 activation in glia but not in neurons protects dopaminergic neurons from  
443 degeneration and ameliorates impaired locomotion.

444 Astrocyte-like and ensheathing glia are two major types of glia in the *Drosophila* nervous system,  
445 surrounding and also extending long processes into neuropils of the brain. These astrocyte-like glia  
446 exhibit a morphology and function similar to those of mammalian astrocytes, including reuptake of  
447 neurotransmitters and phagocytosis of neuronal debris (Freeman, 2015; Tasdemir-Yilmaz & Freeman,  
448 2014). Ensheathing glia of varying morphologies encase axonal tracts and neuropils, regulating neuronal  
449 excitability and participating in phagocytosis and injury-induced inflammation (Doherty et al., 2009; Otto  
450 et al., 2018). Thus, given their proximity to neurons and similar functions, it is not surprising that both  
451 types of glia collectively mediate the protective effects of WGE.

452 Communication between neurons and glia maintains homeostasis, yet also confers the disease state  
453 during neurodegeneration. In the *Drosophila* G2019S model, upregulation of the BMP ligand Gbb in  
454 dopaminergic neurons activates Mad/Smad signaling in glia, which promotes neuronal degeneration via a  
455 feedback mechanism (Maksoud et al., 2019). Surprisingly, although the number of dopaminergic neurons  
456 in the fly brain is relatively small, the upregulated pMad signal spreads throughout the brain (Figure 7B),  
457 suggesting that BMP can be disseminated over long distances. In PD patients, higher levels of TGF- $\beta$ 1  
458 have been detected in the striatum and ventricular cerebrospinal fluid (Vawter et al., 1996). Thus,  
459 members of the TGF- $\beta$ 1 superfamily such as TGF- $\beta$ 1 and BMP signaling molecules may represent  
460 indicators of neuronal degeneration. Accordingly, disrupting the glia-to-neuron feedback mechanism may  
461 sustain neuronal survival. In glia, we found that WGE treatment downregulated the pMad levels that had  
462 been increased in the G2019S flies (Figure 7A and B). Nrf2 activation in glia also suppressed the  
463 enhanced levels of pMad in G2019S flies (Figure 8B and C). Indeed, our genetic assays indicate that the  
464 Nrf2 and Mad pathways interact in the glia of G2019S flies. Thus, WGE exerts its beneficial effects by  
465 activating Nrf2 to antagonize the Mad activity that would otherwise contribute to the degeneration of  
466 dopaminergic neurons. As a transcriptional activator, Nrf2 induces expression of the inhibitory

467 component Smad7 to form inactive Smad complexes (Song et al., 2019) and the phosphatase subunit  
468 PPM1A to alter Smad2/3/4 phosphorylation and DNA binding (Lin et al., 2006). Given that these  
469 components are conserved in *Drosophila*, Nrf2 may employ similar pathways to block Mad signaling in  
470 glia.

471 That glial Nrf2 activation protects neurons is evidenced by our observations of enhanced HO-1  
472 expression (Figure 4C) and increased numbers of dopaminergic neurons (Figure 5C and D). These results  
473 support that the role of Nrf2 in glia is to induce expression of antioxidation building blocks, such as  
474 phase-II detoxification enzymes, and to enhance inflammatory processes (Hirrlinger & Dringen, 2010;  
475 Rojo et al., 2010). In a model of fibrosis, TGF- $\beta$ /Smad2/3 suppressed expression of the *ARE*-luciferase  
476 reporter and glutathione (Ryoo et al., 2014). Moreover, Nrf2 knockdown was shown to reduce expression  
477 of the antioxidative enzyme NAD(P)H quinone dehydrogenase 1 (NQO1), thereby elevating cellular  
478 oxidative stress and upregulating TGF- $\beta$ /Smad targeted gene expression (Prestigiacomo & Suter-Dick,  
479 2018). Hence, we propose that WGE promotes Nrf2 activation to antagonize the Smad signaling in glia  
480 that is induced by dopaminergic neuron-secreted BMP/Gbb signal during degeneration.

481 In our study, the *LRRK2-G2019S* mice show locomotor defects and dopaminergic loss at the age of  
482 11.5 months. A previous study shows only earlier signs of defects, the reduction of the dopamine level  
483 and release at the age of 12 months in the *LRRK2-G2019S* mice (Li et al, 2012), which could be  
484 contributed by the genetic background (FVB/NJ v.s. C57BL/6J). Nevertheless, we have demonstrated that  
485 feeding these mice with WGE rescues their locomotor coordination, suppresses their centrophobism, and  
486 recovers their numbers of dopaminergic neurons and hyperactivated microglia (Figure 9, and Figure 10A  
487 to D). Significantly, we found that activity of the TGF- $\beta$ /Smad2/3 pathway was elevated in nigrostriatal  
488 brain lysates, and this activity was also suppressed by WGE treatment (Figure 10E and G). Collectively,  
489 these results from fly and mouse PD models indicate that the effectiveness of WGE is likely mediated  
490 through conserved Nrf2/Mad pathways (Figure 11). Our findings contribute to our mechanistic  
491 understanding of PD and provide potential therapeutic strategies that incorporate the traditional herbal  
492 medicine GE.

## 493 **Materials and Methods**

### 494 ***Drosophila* stocks and maintenance**

495 All fly stocks were maintained on standard cornmeal-based food medium at 25°C. *Drosophila* stocks  
496 sourced from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, USA) were:  
497 *UAS-mCD8-GFP* (#5137), *elav-GAL4* (#8760), *repo-GAL4* (#7215), *Ddc-LexA* (#54218), *UAS-cncTRiP*  
498 (#25984), *Tub-GAL80<sup>ts</sup>* (#7108), *alrm-GAL4* (#67032), *R56F03-GAL4* (#39157), *UAS-tkv<sup>Q253D</sup>* (#36536),  
499 and *Mad<sup>K00237</sup>* (#10474). *NP2222-GAL4* (#112830) was from the *Drosophila* Genomics Resource Center  
500 and *NP6293-GAL4* (#105188) was from the Kyoto Stock Center. Other stocks include *UAS-Flag-LRRK2-*  
501 *WT* (Lin et al., 2010), *UAS-Flag-LRRK2-G2019S* (Lin et al., 2010), *Ddc-GAL4* (Sang et al., 2007), *ARE-*  
502 *GFP* (Sykiotis & Bohmann, 2008), *UAS-cncC-FL2* (Sykiotis & Bohmann, 2008), *moody-GAL4* (Bainton  
503 et al., 2005), and *UAS-Mad* (Takaesu et al., 2006). The two *LexAop* fly lines—*LexAop-LRRK2-WT* and  
504 *LexAop-LRRK2-G2019S*—were generated in this study. In brief, the cDNAs for *LRRK2-WT* and *LRRK2-*  
505 *G2019S* were isolated from the pDEST53-LRRK2-WT and pDEST53-LRRK2-G2019S plasmids  
506 (Addgene, Massachusetts, USA) for subcloning into *LexAop* plasmids (Addgene, Massachusetts, USA),  
507 which were for microinjection (Fly facility, University of Cambridge, UK). The transgenes were site  
508 landed at an attP site on the 2nd chromosome (25C6). For temperature-shift assay of *GAL80<sup>ts</sup>* flies,  
509 parental flies were maintained at 19°C and allowed to mate, before collecting the F1 adults and shifting  
510 them to 29°C to inactivate GAL80.

511

### 512 **Preparation of WGE and related chemical compounds**

513 Authentication of GE and preparation of WGE were as described previously (Lin et al., 2018; Y. E.  
514 Lin et al., 2016). WGE (KO DA Pharmaceutical Co. Ltd., Taoyuan, Taiwan), gastrodin (Wuhan YC Fine  
515 Chemical Co., Wuhan, China), and 4-HBA (Sigma-Aldrich, Darmstadt, Germany) were added to freshly-  
516 prepared cornmeal-based fly food at indicated final concentrations (w/w). For experiments, one- to three-  
517 day-old post-eclosion flies were collected and transferred to fresh food medium twice per week.

518

## 519 **Fly locomotion assay**

520 A negative geotaxis climbing assay was performed to assess locomotor activity, and it was  
521 conducted according to a previous study with minor modification (Madabattula et al., 2015). Cohorts of  
522 35 flies from each genotype were assayed weekly for six consecutive weeks. Success rates were  
523 calculated as the percentage of flies that could climb above the 8-cm mark of a 20-cm cylinder within 10  
524 s.

525 The free-walking assay protocol was conducted based on a previous report with minor modification  
526 (Chen et al., 2014). Cohorts of eight flies were habituated on a 10-cm agar-filled dish for 30 min. The  
527 dishes were gently tapped to encourage the flies to walk, which was video-taped for 5 min. Movement  
528 tracks were processed in ImageJ and quantified using the Caltech multiple fly tracker (Ctrax).

## 529 **Immunostaining and immunoblotting of adult fly brains**

531 The protocol for immunostaining whole-mount adult brains was essentially as described previously  
532 (Lin et al., 2010; Maksoud et al., 2019). Adult fly brains for each genotype were dissected at the indicated  
533 time-points for immunostaining with the following primary antibodies: mouse anti-TH (Immunostar,  
534 22941, 1:1000); mouse anti-repo (Hybridoma Bank DSHB, 8D12, 1:500); chicken anti-GFP (Abcam,  
535 ab13970, 1:10000); and rabbit anti-phospho-Smad3 (Ser423/425) (Abcam, ab52903, 1:250) (Smith et al.,  
536 2012). Fluorophore-conjugated secondary antibodies were: FITC-conjugated goat anti-mouse IgG  
537 (Jackson ImmunoResearch, AB\_2338589, 1:500); Alexa Fluor 488-conjugated goat anti-mouse IgG  
538 (Invitrogen, A28175, 1:500); Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch,  
539 AB\_2338680, 1:500); Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, A27034, 1:500); and  
540 Cy5-conjugated goat anti-rat IgG (Invitrogen, A10525, 1:500). Phalloidin-TRITC (Sigma-Aldrich,  
541 P1951, 1:5000) that binds F-actin was also used for counterstaining. Immunofluorescence signals were  
542 acquired under confocal microscopy (ZEISS LSM 710, Germany).

543 Adult brain extracts were prepared according to a previously described protocol (Lin et al., 2010). In  
544 brief, ~80 fly heads for each genotype were isolated for extract preparation. Equivalent amounts of

545 samples (30 µg/20 µL/well) were resolved by SDS-PAGE for immunoblotting. The following primary  
546 antibodies were used: rabbit anti-human LRRK2 (Abcam, ab133474, 1:1000); rabbit anti-phospho-  
547 LRRK2 (Ser<sup>1292</sup>) (Abcam, ab203181, 1:500); rabbit anti-Akt (Cell Signaling, #4691, 1:1000); rabbit anti-  
548 *Drosophila* phospho-Akt Ser<sup>505</sup> (Cell Signaling, #4054, 1:500); rabbit anti-Nrf2 (Thermo Fisher  
549 Scientific, 710574, 1:1000); rabbit anti-phospho-Nrf2 (Ser<sup>40</sup>) (Thermo Fisher Scientific, PA5-67520,  
550 1:1000); mouse anti-HO-1-1 (Thermo Fisher Scientific, MA1-112, 1:1000); rabbit anti-GAPDH  
551 (GeneTex, GTX100118, 1:5000); and rabbit anti-alpha tubulin (Cell Signaling, #2144, 1:10000),  
552 followed by blotting with secondary antibodies peroxidase-conjugated goat anti-rabbit IgG (Jackson  
553 ImmunoResearch, AB\_2307391, 1:7500) and peroxidase-conjugated goat anti-mouse IgG (Jackson  
554 ImmunoResearch, AB\_10015289, 1:7500).

555

#### 556 ***ARE-GFP* reporter assay**

557 The antioxidant response element (*ARE*)-*GFP* reporter assay was a modification of the protocol  
558 from a previous study (Sykiotis & Bohmann, 2008). *ARE-GFP* flies of one week old (for [Figure 5-figure](#)  
559 [supplement 1](#)) or 6 weeks old ([Figure 6](#)) were fed with regular food or food containing 0.1% WGE prior  
560 to brain dissection and GFP immunostaining. To quantify *ARE-GFP* in the 6-week-old adult fly brain,  
561 confocal images were processed in ImageJ. Mean GFP fluorescence intensities in *mCherry*-labeled glial  
562 cells and TH-positive dopaminergic neurons of the PPL1 cluster were quantified and normalized as  
563 GFP/mCherry.

564

#### 565 **Animal care and treatments**

566 Transgenic *LRRK2-G2019S* mice were purchased from the Jackson Laboratory (JAX stock #009609,  
567 Bar Harbor, ME, USA) and they were maintained at the animal center of the National Taiwan University  
568 Hospital (NTUH). Non-transgenic (nTg) and heterozygous transgenic *LRRK2-G2019S* mice were  
569 obtained by crossing heterozygous *LRRK2-G2019S* mice with wild-type FVB/NJ mice (JAX stock  
570 #001800). Mice at 8.5 months-old were assigned to one of three groups (5 to 6 mice per group): nTg,



571 *LRRK2-G2019S*, and *LRRK2-G2019S* fed with WGE (0.5 g/kg body weight per day) (Lin et al., 2018) for  
572 three months.

573

#### 574 **Behavioral assays**

575 We employed two behavioral tests to assay mouse motor function, i.e., an open field assay to assess  
576 spontaneous locomotor activity and CatWalk XT gait analysis to assay coordination. Behavioral  
577 experiments were conducted blind to genotype, as described previously (Lin et al., 2020).

578

#### 579 **Immunohistochemical staining**

580 After three months of WGE treatment, mice were sacrificed at the age of 11.5 months. The  
581 substantia nigra and striatum were dissected out. The substantia nigra was subjected to immunostaining,  
582 as described previously (Lin et al., 2020). Anti-tyrosine hydroxylase (TH) (Millipore, AB152, 1:200) and  
583 anti-ionized calcium-binding adapter molecule 1 (Iba-1) (GeneTex, GTX100042, 1:200) were used as  
584 primary antibodies for 24 h at 4°C. Secondary antibodies were DyLight 488 goat anti-rabbit 1:300 and  
585 Alexa Fluor 546 goat anti-rabbit at 1:200 (25°C for 1 h). Mounting medium with DAPI (GeneTex,  
586 GTX30920) was used as a counterstain.

587

#### 588 **Immunoblotting**

589 Frozen nigrostriatal brain tissues were homogenized and mixed with lysis buffer to determine  
590 protein content and for immunoblotting, as described previously (Lin et al., 2020). The membrane was  
591 incubated overnight at 4°C with the following primary antibodies: anti-LRRK2 (Abcam, ab133474,  
592 1:5000); anti-phospho-LRRK2 (Ser<sup>1292</sup>, Abcam, ab203181, 1:1000); anti-Smad2 (Cell Signaling, #5339,  
593 1:1000); anti-phospho-Smad2 (Ser465/467, Cell Signaling, #3108, 1:1000); anti-Smad3 (Cell Signaling,  
594 #9523, 1:1000); anti-phospho-Smad3 (Ser423/425, Abcam, ab52903, 1:1000); and anti-beta actin  
595 (Sigma-Aldrich, A5441, 1:5000). After washing, peroxidase-conjugated goat anti-rabbit IgG (GeneTex,

596 GTX213110-01, 1:5000) or peroxidase-conjugated goat anti-mouse IgG (GeneTex, GTX213111-01,  
597 1:5000) were used as secondary antibodies.

598

### 599 **Statistical Analysis**

600 All statistical analyses were carried out in GraphPad PRISM 6 (La Jolla, CA, USA). Data are  
601 presented as mean  $\pm$  standard error (SEM). Statistical analysis was performed using either student *t*-test or  
602 one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. *P* values less than  
603 0.05 ( $p < 0.05$ ) were considered indicative of significance. For exact *n* numbers, *p* values, *F* values, *t*-  
604 values, and degrees of freedom of each statistical test, please see the statistical information in the  
605 Additional file-Supporting file 1.

606

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612

613 **Additional information**

614 **Ethics approval**

615 All animal procedures were approved by the local ethics committee and the Institutional Animal  
616 Care and Use Committee (IACUC) of the National Taiwan University (IACUC approval no. 20180103).

617

618 **Data availability**

619 All data generated or analyzed during this study are included in the manuscript and supporting files.

620

621 **Competing interests**

622 The authors declare that they have no competing interests.

623

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635 **Additional files**

636 **Supporting file 1**

637 [Source data 1-Statistical Information.xlsx](#)

638 The file includes all data and the statistical analyses in this article.

639

640 **Supporting file 2**

641 [Source data 2-Data-WB.pdf](#)

642 The file includes the uncropped images of the western blotting in this article.

643

644 **Appendix 1**

645 [Appendix-Key Resources Table-73753.docx](#)

646 The file includes the Key Resources Table in this article.

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922

923

924 **Figure legends**

925 **Figure 1. WGE treatment rescues the diminished locomotion of *Ddc>G2019S* flies.**

926 (A to C) Climbing activities of *Ddc>G2019S* flies fed on food supplemented with 0.1%, 0.5% or 1.0%  
927 WGE (A), 0.1 or 1.0 mM gastrodin (B), and 0.1 or 1.0 mM 4-HBA (C). Controls are *Ddc>Lrrk2* and  
928 *Ddc>G2019S* flies fed regular food. Bar graphs show the percentages of flies (mean  $\pm$  SEM, N = 6) that  
929 climbed above 8 cm within 10 sec. (D) Five-minute walking tracks pooled from eight flies each of the  
930 *Ddc>Lrrk2*, *Ddc>G2019S*, and *Ddc>G2019S* + 0.1% WGE groups at week 4. Bar graph at right  
931 summarizes their walking distances (mean  $\pm$  SEM, N = 8). One-way analysis of variance (ANOVA) with  
932 Tukey's post-hoc multiple comparison test: \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ , ns, not significant.

933

934 **Figure 1-figure supplement 1. Climbing activity assay of *Ddc>mCD8-GFP* and *Ddc>Lrrk2* flies.**

935 (A) Comparable climbing activities were detected for *Ddc>mCD8-GFP* and *Ddc>Lrrk2* flies. Both  
936 exhibited better climbing activities than *Ddc>G2019S* flies in the climbing assay from week 1 to 6. Bar  
937 graph shows percentage (mean  $\pm$  SEM, N = 5) of flies that successfully climbed above 8 cm within 10  
938 sec. One-way analysis of variance (ANOVA) and Tukey's post-hoc multiple comparison test: \*  $p < 0.05$ ,  
939 \*\*\*  $p < 0.001$ , ns, not significant. (B) *Ddc>G2019S* flies fed with 0.02% or 0.1% WGE exhibited  
940 improved climbing activity (mean  $\pm$  SEM, N = 6). One-way ANOVA and Tukey's post-hoc multiple  
941 comparison test: \*  $p < 0.05$ , \*\*\*  $p < 0.001$  (relative to *Ddc>G2019S*); and ##  $p < 0.01$ , ###  $p < 0.001$   
942 (comparing different doses of WGE), ns, not significant.

943

944 **Figure 1-figure supplement 2. Locomotion improvement of *Ddc>G2019S* flies starting WGE feeding**  
945 **at week 4.**

946 The climbing activities of *Ddc>Lrrk2*, *Ddc>G2019S*, and *Ddc>G2019S* with 0.1% WGE feeding at week  
947 4 were assessed at weeks 3, 4, 5 and 6. Bar graphs show success rates (mean  $\pm$  SEM, N = 6) of flies  
948 climbing over 8 cm height in 10 sec. One-way ANOVA and Tukey's post-hoc multiple comparison test

949 were performed and statistical significance was shown as \*\*\* for  $p < 0.001$ , \* for  $p < 0.05$  and ns for no  
950 significance.

951

952 **Figure 2. WGE prevents loss of dopaminergic neurons in *Ddc>G2019S* flies.**

953 (A) Representative adult brain images showing TH-positive dopaminergic neurons in the PPL1 cluster of  
954 2-, 4-, and 6-week-old flies of the *Ddc>Lrrk2*, *Ddc>G2019S*, and 0.1% WGE-fed *Ddc>G2019S* groups.  
955 Scale bar: 10  $\mu\text{m}$ . (B) Average numbers (mean  $\pm$  SEM, N = 5) of TH-positive dopaminergic neurons in  
956 the PPL1, PPM1/2, PPL2, and PPL3 clusters per brain hemisphere. One-way ANOVA with Tukey's post-  
957 hoc multiple comparison test: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , ns, not significant.

958

959 **Figure 2-figure supplement 1. WGE treatment prevents dopaminergic neuron loss in *Ddc>G2019S***  
960 **flies.**

961 (A to C) Representative adult whole-brain images for TH staining to reveal dopaminergic neurons in the  
962 PPL1, PPL2, PPM1/2 and PPM3 clusters of 2-, 4-, and 6-week-old flies. Scale bar: 40  $\mu\text{m}$ . The images  
963 for the PPL1 cluster are shown as enhanced views of the dashed boxes in Fig. 2.

964

965 **Figure 3. WGE modulates *Lrrk2* accumulation and hyperactivation in *elav>G2019S* flies.**

966 (A) Representative immunoblots of 3-day-old adult brain lysates showing expression levels of *Lrrk2*,  
967 p*Lrrk2* (phosphorylated at Ser<sup>1292</sup>), Akt, and pAkt (phosphorylated at Ser<sup>505</sup>) in *elav>Lrrk2* and  
968 *elav>G2019S* flies. (B and C) Quantifications (mean  $\pm$  SEM, N = 3) of *Lrrk2* and p*Lrrk2*/*Lrrk2* (B), and  
969 Akt and pAkt/Akt (C). (D) Representative immunoblots of 2- and 4-week-old adult brain lysates showing  
970 *Lrrk2* levels in the fly groups *elav>Lrrk2*, *elav>G2019S*, and *elav>G2019S* fed with 0.1% WGE. (E)  
971 Quantification of *Lrrk2* levels in 2- and 4-week-old adult brains (mean  $\pm$  SEM, N = 3). (F) Representative  
972 immunoblots of 4-week-old adult brain lysates showing expression levels of *Lrrk2*, p*Lrrk2*, Rab10 and  
973 pRab10 (phosphorylated at Thr<sup>73</sup>). (G and H) Quantification (mean  $\pm$  SEM, N = 3) of levels of *Lrrk2* and

974 pLrrk2/Lrrk2 (G), and of Rab10 and pRab10/Rab10 (H). One-way ANOVA with Tukey's post-hoc  
975 multiple comparison test: \*  $p < 0.05$ , \*\*  $p < 0.01$ , ns, not significant.

976

977 **Figure 3-figure supplement 1. WGE specifically modulates Lrrk2 accumulation and**  
978 **hyperactivation in *elav>G2019S* but not *elav>Lrrk2* flies.**

979 (A) Representative immunoblots of 2-week-old adult brain lysates showing levels of Lrrk2 and pLrrk2  
980 (Ser<sup>1292</sup>) in *elav>Lrrk2*, WGE-fed *elav>Lrrk2*, *elav>G2019S* and WGE-fed *elav>G2019S* flies. (B and  
981 C) Quantification (mean  $\pm$  SEM, N = 3) of Lrrk2 (B) and pLrrk2/Lrrk2 (C) levels. One-way ANOVA and  
982 Tukey's post-hoc multiple comparison test: \*  $p < 0.05$ , \*\*\*  $p < 0.001$ , ns, not significant.

983

984 **Figure 4. WGE activates the Akt-Nrf2 pathway in *elav>G2019S* flies.**

985 (A) Representative immunoblots of 2- and 4-week-old adult brain lysates showing levels of Akt and pAkt  
986 in brain extracts of *elav>Lrrk2*, *elav>G2019S*, and WGE-fed *elav>G2019S* flies. (B) Quantification  
987 (mean  $\pm$  SEM, N = 3) of pAkt/Akt levels in 2- and 4-week-old adult brains. (C) Representative  
988 immunoblots of 4-week-old adult brain lysates showing levels of Nrf2, pNrf2 (phosphorylated at Ser<sup>40</sup>),  
989 GSK3 $\beta$ , pGSK3 $\beta$  (phosphorylated at Ser<sup>9</sup>), and HO-1 in *elav>Lrrk2*, *elav>G2019S*, and WGE-fed  
990 *elav>G2019S* flies. GADPH acted as a loading control in (A) and (C). (D) Quantification (mean  $\pm$  SEM,  
991 N = 3) of relative protein levels to respective Nrf2, GSK3 $\beta$ , and HO-1. One-way ANOVA with Tukey's  
992 post-hoc multiple comparison test (relative to *elav>G2019S*): \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , ns,  
993 not significant.

994

995 **Figure 5. Activation of Nrf2 in glia rescues locomotion defects in *Ddc>G2019S* flies.**

996 (A and B) Requirement of Nrf2 in glia but not neurons for WGE-improved *Ddc>G2019S* climbing  
997 activity. (A) Climbing success rates of flies in which *Ddc-GAL4* drives co-expression of *Lrrk2-G2019S*  
998 and *mCD8-GFP*, *cncC-FL2* or *cnc-RNAi*. As a control line, *Ddc-GAL4* drives co-expression of *Lrrk2* and  
999 *mCD8-GFP*. (B) Climbing success rates of flies in which *Ddc-LexA* drives wild-type *Lrrk2* or *Lrrk2-*



1000 *G2019S* expression and *repo-GAL4* drives *cncC-FL2* or *cnc-RNAi* expression (mean  $\pm$  SEM, N = 6 for  
1001 (A) and (B)). WGE was added to food at a concentration of 0.1% (w/w). (C) Adult brain images showing  
1002 TH-positive dopaminergic neurons in the PPL1 clusters of 6-week-old *Ddc-LexA>Lrrk2* or *Ddc-*  
1003 *LexA>G2019S* flies with *repo-GAL4* control or *repo-GAL4*-driven *cncC-FL2* or *cnc-RNAi* expression.  
1004 Scale bar: 10  $\mu$ m. (D) Average numbers (mean  $\pm$  SEM, N = 5) of TH-positive dopaminergic neurons in  
1005 PPL1 clusters per brain hemisphere are shown. One-way ANOVA with Tukey's post-hoc multiple  
1006 comparison test (relative to *Ddc>G2019S* (A) or *Ddc-LexA>G2019S; repo-GAL4* (B and D)): \*\*  $p <$   
1007 0.01, \*\*\*  $p <$  0.001, ns, not significant.

1008

1009 **Figure 5-figure supplement 1. WGE treatment specifically activates glial Nrf2 signals in the ARE-**  
1010 **GFP reporter flies.**

1011 (A and B) WGE treatment activates glial Nrf2 signals in one-week-old *ARE-GFP* reporter flies. ARE-  
1012 GFP (green) and glial Repo (red) in whole-mount adult brains without (A) or with 0.1% WGE treatment  
1013 (B) for 5 days. Phalloidin (in blue) reveals brain structures. White arrowheads indicate GFP signals. Scale  
1014 bar: 50  $\mu$ m. Dotted boxes are enhanced views and shown as separate channels at right.

1015

1016 **Figure 5-figure supplement 2. WGE rescues the locomotion defect displayed by *Ddc-LexA>G2019S***  
1017 **flies.**

1018 WGE treatment improves the climbing ability of *Ddc-LexA>G2019S* flies. Bar graph shows percentage  
1019 (mean  $\pm$  SEM, N = 6) of flies that successfully climbed above 8 cm within 10 sec. One-way ANOVA and  
1020 Tukey's post-hoc multiple comparison test (relative to *Ddc-LexA>G2019S*): \*  $p <$  0.05, \*\*  $p <$  0.01, \*\*\*  
1021  $p <$  0.001.

1022

1023

1024 **Figure 6. Nrf2 in astrocyte-like and ensheathing glia mediates the effect of WGE treatment in**  
1025 ***Ddc>G2019S* flies.**

1026 (A) Nrf2 knockdown in astrocyte-like and ensheathing glia abolishes the improved locomotion elicited by  
1027 WGE treatment in *Ddc>G2019S* flies. Composite bar graph shows climbing success rates for 6-week-old  
1028 *Ddc-LexA>G2019S* flies with *cnc-RNAi* driven by *repo-GAL4* in all glia, *alrm-GAL4* in astrocyte-like  
1029 glia, *np2222* in cortex glia, *np6293* in perineurial glia, *R56F03* in ensheathing glia, and *moody-GAL4* in  
1030 subperineurial glia. (B) Composite bar graph shows climbing success rates (mean  $\pm$  SEM, N = 6) for 6-  
1031 week-old *Ddc-LexA>G2019S* flies with overexpression of Nrf2 in astrocyte-like glia (*alrm>cncC-FL2*)  
1032 or ensheathing glia (*R56F03>cncC-FL2*). One-way ANOVA and Tukey's post-hoc multiple comparison  
1033 test (relative to *Ddc-LexA>G2019S; GAL4>cnc-RNAi* (A) or *Ddc-LexA>G2019S; GAL4* (B)): \*  $p < 0.05$ ,  
1034 \*\*\*  $p < 0.001$ , ns, not significant. (C and E) Representative images showing expression of *ARE-GFP* in  
1035 astrocyte-like glia (*alrm>mCherry*) (C) and ensheathing glia (*R56F03>mCherry*) (E), together with TH-  
1036 positive dopaminergic neurons in the PPL1 clusters of 6-week-old *Ddc-LexA>Lrrk2*, *Ddc-LexA>G2019S*,  
1037 or WGE (0.1% w/w)-fed *Ddc-LexA>G2019S* flies. Bar: 5  $\mu$ m. GFP channels in the dashed boxes are  
1038 shown as enhanced views in the lower panel, with glial signals labeled by dashed lines. (D and F)  
1039 Quantifications (mean  $\pm$  SEM, n > 25 for each genotype) of GFP intensities in astrocyte-like glia (D) or  
1040 ensheathing glia (F). GFP intensities in glia have been outlined manually using the mCherry-positive  
1041 signals. One-way ANOVA and Tukey's post-hoc multiple comparison test (relative to *Ddc-*  
1042 *LexA>G2019S; alrm>mCherry* (D) or *Ddc-LexA>G2019S; R56F03>mCherry* (F)): \*  $p < 0.05$ , \*\*\*  $p <$   
1043 0.001, ns, not significant.

1044

1045 **Figure 6-figure supplement 1. Lrrk2 and pLrrk2 levels are maintained upon glial Nrf2**  
1046 **overexpression.**

1047 (A) Representative immunoblots of 2-week-old adult brain lysates showing protein expression levels of  
1048 Lrrk2 and pLrrk2 (Ser<sup>1292</sup>) in *Ddc-LexA>G2019S; repo-GAL4* and *Ddc-LexA>G2019S; repo>cncC-FL2*  
1049 flies. (B and C) Quantification of Lrrk2 (B) and pLrrk2/Lrrk2 (C) (mean  $\pm$  SEM, N = 3). Student *t*-test:  
1050 ns, not significant.

1051

1052 **Figure 6-figure supplement 2. WGE treatment induces a mild Nrf2 activation in dopaminergic**  
1053 **neurons in *Ddc>G2019S* flies.**

1054 (A and B) Quantifications (mean  $\pm$  SEM,  $n > 20$  for each genotype) of GFP intensities in TH-positive  
1055 dopaminergic neurons nearby the astrocyte-like glia (A) or ensheathing glia (B). GFP intensities were  
1056 measured within dopaminergic neurons outlined by the TH-positive signals and were normalized to  
1057 mCherry intensities. One-way ANOVA and Tukey's post-hoc multiple comparison test with reference to  
1058 *Ddc-LexA>G2019S; alrm>mCherry* (A) or *Ddc-LexA>G2019S; R56F03>mCherry* (B) were performed  
1059 and shown as \*\* for  $p < 0.01$ , \*\*\* for  $p < 0.001$ , and ns for no significance.

1060

1061 **Figure 7. WGE down-regulates G2019S-induced BMP/Mad signaling.**

1062 (A) Representative images of glial pMad staining in the adult PPL1 clusters of *Ddc>Lrrk2*,  
1063 *Ddc>G2019S*, and WGE-fed *Ddc>G2019S* flies. White arrows indicate pMad signals co-localized with  
1064 Repo, with single channels for pMad signals shown as insets. Bar: 10  $\mu$ m. (B) Quantification (mean  $\pm$   
1065 SEM,  $n > 60$  for each genotype) of pMad signals normalized to Repo levels in glia of the indicated  
1066 genotypes. One-way ANOVA and Tukey's post-hoc multiple comparison test (relative to *Ddc>G2019S*):  
1067 \*\*\*  $p < 0.001$ . (C and D) Climbing success rates (mean  $\pm$  SEM,  $N = 10$ ) at weeks 1-4 demonstrating that  
1068 WGE treatment rescues locomotion deficits induced by glial overexpression of *Mad* (C) or *tkv<sup>Q253D</sup>* (D) in  
1069 *Tub-GAL80<sup>ts</sup>; repo-GAL4* flies. One-way ANOVA and Tukey's post-hoc multiple comparison test  
1070 (relative to *repo>Mad* or *repo>tkv<sup>Q253D</sup>*): \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . (E and G) Representative images of  
1071 4-week-old adult brain showing TH staining of the PPL1 clusters of *Tub-GAL80<sup>ts</sup>; repo-GAL4*, *Tub-*  
1072 *GAL80<sup>ts</sup>; repo>Mad*, and WGE-fed *Tub-GAL80<sup>ts</sup>; repo>Mad* flies (E), and *Tub-GAL80<sup>ts</sup>; repo-GAL4*,  
1073 *Tub-GAL80<sup>ts</sup>; repo>tkv<sup>Q253D</sup>*, and WGE-fed *Tub-GAL80<sup>ts</sup>; repo>tkv<sup>Q253D</sup>* flies (G). Bars: 12.5  $\mu$ m. (F and  
1074 H) Bar graphs show mean  $\pm$  SEM ( $N = 5$ ) of TH-positive dopaminergic neurons in the PPL1 clusters of  
1075 4-week-old flies. One-way ANOVA and Tukey's post-hoc multiple comparison test (relative to *Tub-*  
1076 *GAL80<sup>ts</sup>; repo>Mad* (F) or *Tub-GAL80<sup>ts</sup>; repo>tkv<sup>Q253D</sup>* (H)): \*\*\*  $p < 0.001$ .

1077

1078 **Figure 7-figure supplement 1. *Mad* heterozygosity rescues the impaired locomotion of *Ddc>G2019S***  
1079 **flies.**

1080 Removing one copy of *Mad* improves *Ddc>G2019S* climbing activity. Bar graphs show climbing success  
1081 rates (mean  $\pm$  SEM, N = 6) of 6-week-old *Ddc>Lrrk2*, *Ddc>G2019S*, and *Ddc>G2019S, Mad<sup>+/-</sup>* flies.  
1082 One-way ANOVA and Tukey's post-hoc multiple comparison test (relative to *Ddc>G2019S*): \*\*\*  $p <$   
1083 0.001.

1084  
1085 **Figure 8. Nrf2 antagonizes BMP/Mad signaling in glia.**

1086 (A) Heterozygosity of *Mad* suppresses G2019S mutation-induced locomotion impairment in a negative  
1087 geotaxis assay. Bar graph shows percentages (mean  $\pm$  SEM, N = 6) of 6-week-old flies that climbed  
1088 above 8 cm within 10 sec. One-way ANOVA and Tukey's post-hoc multiple comparison test: \*  $p <$  0.05,  
1089 \*\*  $p <$  0.01, \*\*\*  $p <$  0.001, ns, not significant. (B) Representative images of pMad staining in the adult  
1090 brains of *Ddc-LexA>Lrrk2* or *Ddc-LexA>G2019S* flies in which *repo-GAL4* drives expression of *cncC-*  
1091 *FL2* or *cnc-RNAi*. Impact of WGE treatment is shown in the rightmost panels. Arrows indicate pMad and  
1092 Repo dual-positive cells. Insets are enlarged images of the dashed boxes, and dashed lines encompass  
1093 Repo-positive cells. Bar: 20  $\mu$ m. (C) Quantification of the ratio of pMad to Repo (mean  $\pm$  SEM, N > 40).  
1094 One-way ANOVA and Tukey's post-hoc multiple comparison test: \*\*\*  $p <$  0.001, ns, not significant. (D)  
1095 Images show pan-glial *ARE-GFP* expression (*repo>mCherry*) in *Ddc-LexA>Lrrk2*, *Ddc-LexA>G2019S*,  
1096 and *Ddc-LexA>G2019S, Mad<sup>+/-</sup>* fly brains. GFP channels within the dashed boxes are shown as enhanced  
1097 views in the inset, with glial signals outlined by dashed lines. Bar: 20  $\mu$ m. (E) Quantification for GFP  
1098 expression levels (mean  $\pm$  SEM, n > 13). One-way ANOVA and Tukey's post-hoc multiple comparison  
1099 test: \*  $p <$  0.05, \*\*\*  $p <$  0.001.

1100  
1101 **Figure 9. WGE treatment rescues impaired locomotion of *LRRK2-G2019S* mice.**

1102 (A) Video-tracked paths for nTg, *LRRK2-G2019S* and WGE-fed *LRRK2-G2019S* mice (8.5 and 11.5  
1103 months old) during the open-field test. (B) Quantification of total distance (meters, m), average velocity

1104 (centimeters per sec, cm/s), and percentage moving time for 8.5, 9.5, 10.5 and 11.5 months old mice. (C)  
1105 Captured and converted images of single stance for each paw of 11.5-month-old nTg, *LRRK2-G2019S*  
1106 and WGE-fed *LRRK2-G2019S* mice in a catwalk analysis. (D) Quantification of stride length for each  
1107 paw of 8.5- and 11.5-month-old mice. Data in (B and D) are presented as mean  $\pm$  SEM (nTg; N = 5,  
1108 *G2019S*; N = 6, WGE-fed *G2019S*; N = 6). One-way ANOVA and Tukey's post-hoc multiple comparison  
1109 test: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , ns, not significant. RF – right front; RH – right hind; LF – left  
1110 front; LH – left hind.

1111

1112 **Figure 10. WGE prevents dopaminergic neuron loss, microglial activation, and phosphorylation of**  
1113 **LRRK2, Smad2, and Smad3.**

1114 (A and C) Representative images showing TH-positive dopaminergic neurons (A) or Iba-1-positive  
1115 microglia (C) in the substantia nigra of 11.5-month-old nTg, *LRRK2-G2019S* and WGE-fed *LRRK2-*  
1116 *G2019S* mice. Bars: 100  $\mu$ m in (A) and 50  $\mu$ m in (C). (B and D) Quantification of numbers of TH-  
1117 positive (B) or Iba-1-positive cells (D) relative to DAPI cells. (E) Representative immunoblots of  
1118 nigrostriatal lysates prepared from 11.5-month-old nTg, *LRRK2-G2019S* and WGE-fed *LRRK2-G2019S*  
1119 mice reveal expression levels of LRRK2, pLRRK2 (Ser<sup>1292</sup>), Smad2, pSmad2 (Ser<sup>465</sup> and Ser<sup>467</sup>), Smad3,  
1120 and pSmad3 (Ser<sup>423</sup> and Ser<sup>425</sup>). Actin acted as a loading control. (F and G) Quantifications of LRRK2  
1121 and pLRRK2/LRRK2 (F), as well as pSmad2/Smad2 and pSmad3/Smad3 (G). Data in (B, D, F, G) are  
1122 presented as mean  $\pm$  SEM (N = 3). One-way ANOVA and Tukey's post-hoc multiple comparison test: \*  
1123  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , ns, not significant.

1124

1125 **Figure 10-figure supplement 1. WGE suppresses microglia activation in *LRRK2-G2019S* mice.**

1126 Single channel images of Figure 10C show Iba-1-positive microglia in the substantia nigra of 11.5-  
1127 month-old nTg, *LRRK2-G2019S* and WGE-fed *LRRK2-G2019S* mice. Bars: 50  $\mu$ m.

1128

1129 **Figure 11. The proposed model of WGE in the *G2019S*-induced neurodegeneration.**

1130 Accumulation of the hyperactivated G2019S mutant protein enhances the BMP ligand (Gbb) maturation  
1131 via up-regulation of Furin 1 translation in dopaminergic neurons. Secreted Gbb binds to the BMP  
1132 receptor, Tkv, and turns on Mad signaling in glia. The G2019S mutation also decreases the Nrf2 activity  
1133 in the brain, particularly in glia. Both up-regulated Mad and down-regulated Nrf2 pathways contribute to  
1134 neurodegeneration. WGE feeding suppresses G2019S hyperactivation in neurons and restores Nrf2  
1135 activity mostly in the astrocyte-like and ensheathing glia. WGE-elevated Nrf2 activity in glia antagonizes  
1136 the BMP/Mad signaling and initiates Nrf2/HO-1 axis in glia, attenuating the stress signals from glia and  
1137 promoting neuroprotection. Red and green solid arrows (→) indicate the observed effects exerted by  
1138 G2019S overexpression and WGE feeding, respectively, in the present study. Red and green dashed  
1139 arrows (-->) indicate the proposed actions triggered by Mad signaling and WGE feeding, respectively. Red  
1140 and green blunt-ended lines (---|) indicate the proposed inhibitions by Mad and Nrf2 overexpression,  
1141 respectively. (P) indicates phosphorylation. The Furin 1-mediated Gbb pathway labeled in gray is  
1142 modified from previous findings (Maksoud et al., 2019).

1143

<b>Key Resources Table</b>				
<b>Reagent type (species) or resource</b>	<b>Designation</b>	<b>Source or reference</b>	<b>Identifiers</b>	<b>Additional information</b>
Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>UAS-mCD8-GFP</i>	Bloomington <i>Drosophila</i> Stock Center	BDSC Cat# 5137; RRID:BDSC_5137	
Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>UAS-cncTRiP</i>	Bloomington <i>Drosophila</i> Stock Center	BDSC Cat# 25984; RRID:BDSC_25984	
Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>UAS-tkv<sup>Q253D</sup></i>	Bloomington <i>Drosophila</i> Stock Center	BDSC Cat# 36536; RRID:BDSC_36536	
Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>UAS-Flag-LRRK2-WT</i>	Lin et al., 2010	N/A	
Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>UAS-Flag-LRRK2-G2019S</i>	Lin et al., 2010	N/A	
Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>UAS-cncC-FL2</i>	Sykiotis & Bohmann, 2008	N/A	
Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>UAS-Mad</i>	Takaesu et al., 2006	N/A	

Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>elav-GAL4</i>	Bloomington <i>Drosophila</i> Stock Center	BDSC Cat# 8760; RRID:BDSC_8760	
Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>repo-GAL4</i>	Bloomington <i>Drosophila</i> Stock Center	BDSC Cat# 7215; RRID:BDSC_7215	
Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>alrm-GAL4</i>	Bloomington <i>Drosophila</i> Stock Center	BDSC Cat# 67032; RRID:BDSC_67032	
Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>R56F03-GAL4</i>	Bloomington <i>Drosophila</i> Stock Center	BDSC Cat# 39157; RRID:BDSC_39157	
Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>NP2222-GAL4</i>	Bloomington <i>Drosophila</i> Stock Center	BDSC Cat# 112830; RRID:BDSC_112830	
Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>NP6293-GAL4</i>	Kyoto Stock Center	DGRC Cat# 105188; RRID:Kyoto Stock Center_105188	
Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>Ddc-GAL4</i>	Sang et al., 2007	N/A	
Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>moody-GAL4</i>	Bainton et al., 2005	N/A	



Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>Tub-GAL80<sup>ts</sup></i>	Bloomington <i>Drosophila</i> Stock Center	BDSC Cat# 7108; RRID:BDSC_7108	
Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>Ddc-LexA</i>	Bloomington <i>Drosophila</i> Stock Center	BDSC Cat# 54218; RRID:BDSC_54218	
Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>Mad</i> <sup>K00237</sup>	Bloomington <i>Drosophila</i> Stock Center	BDSC Cat# 10474; RRID:BDSC_10474	
Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>ARE-GFP</i>	Sykiotis & Bohmann, 2008	N/A	
Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>LexAop-LRRK2-WT</i>	This paper	N/A	See Materials and Methods, Section 1
Genetic reagent ( <i>Drosophila melanogaster</i> )	<i>LexAop-LRRK2-G2019S</i>	This paper	N/A	See Materials and Methods, Section 1
Genetic reagent ( <i>Mus musculus</i> )	FVB/NJ	The Jackson Laboratory	JAX stock #001800	
Genetic reagent ( <i>Mus musculus</i> )	FVB/N-Tg (LRRK2*G2019S)1Cjli/J	The Jackson Laboratory	JAX stock #009609	
Antibody	anti-TH (Mouse monoclonal)	Immunostar	Cat# 22941; RRID:AB_572268	IF (1:1000)

Antibody	anti-TH (Rabbit polyclonal)	Millipore	Cat# AB152 RRID:AB_390204	mouse-IHC (1:200)
Antibody	anti-Repo (Mouse monoclonal)	Hybridoma Bank DSHB	Cat# 8D12; RRID:AB_528448	IF (1:500)
Antibody	anti-GFP (Chicken polyclonal)	Abcam	Cat# ab13970; RRID:AB_300798	IF (1:10000)
Antibody	anti-LRRK2 (Rabbit monoclonal)	Abcam	Cat# ab133474 RRID:AB_2713963	fly-WB (1:1000) mouse-WB (1:5000)
Antibody	anti-LRRK2 (phospho Ser1292) (Rabbit monoclonal)	Abcam	Cat# ab203181	fly-WB (1:500) mouse-WB (1:1000)
Antibody	anti-Akt (Rabbit monoclonal)	Cell Signaling	Cat# 4691 RRID:AB_915783	WB (1:1000)
Antibody	anti-Phospho- <i>Drosophila</i> Akt (Ser505) (Rabbit polyclonal)	Cell Signaling	Cat# 4054 RRID:AB_331414	WB (1:500)
Antibody	anti-Nrf2 (Rabbit polyclonal)	Thermo Fisher Scientific	Cat# 710574 RRID:AB_2532742	WB (1:1000)
Antibody	anti-Phospho-Nrf2 (Ser40) (Rabbit polyclonal)	Thermo Fisher Scientific	Cat# PA5-67520 RRID:AB_2691678	WB (1:1000)
Antibody	anti-HO-1-1 (Mouse monoclonal)	Thermo Fisher Scientific	Cat# MA1-112 RRID:AB_2536823	WB (1:1000)

Antibody	anti-GAPDH (Rabbit polyclonal)	GeneTex	Cat# GTX100118 RRID:AB_1080976	WB (1:5000)
Antibody	anti-alpha Tubulin (Rabbit polyclonal)	Cell Signaling	Cat# 2144 RRID:AB_2210548	WB (1:10000)
Antibody	anti-Smad2 (Rabbit monoclonal)	Cell Signaling	Cat# 5339 RRID:AB_10626777	mouse-WB (1:1000)
Antibody	anti-Phospho-Smad2 (Ser465/467) (Rabbit monoclonal)	Cell Signaling	Cat# 3108 RRID:AB_490941	mouse-WB (1:1000)
Antibody	anti-Smad3 (Rabbit monoclonal)	Cell Signaling	Cat# 9523 RRID:AB_2193182	mouse-WB (1:1000)
Antibody	anti-Smad3 (phospho S423 + S425) (Rabbit monoclonal)	Abcam	Cat# ab52903; RRID:AB_882596	IF (1:250) mouse-WB (1:1000)
Antibody	anti-beta Actin (Mouse monoclonal)	Sigma-Aldrich	Cat# A5441 RRID:AB_476744	mouse-WB (1:5000)
Antibody	anti-Iba-1 (Rabbit polyclonal)	GeneTex	Cat# GTX100042 RRID:AB_1240434	mouse-IHC (1:200)
Antibody	anti-Mouse Alexa 488 (Goat polyclonal)	Invitrogen	Cat# A28175 RRID:AB_2536161	IF (1:500)
Antibody	anti-Rabbit Alexa 488 (Goat polyclonal)	Invitrogen	Cat# A27034 RRID:AB_2536097	IF (1:500)

Antibody	anti-Rabbit DyLight 488 (Goat polyclonal)	Thermo Fisher Scientific	Cat# 35552 RRID:AB_844398	mouse-IHC (1:300)
Antibody	anti-Rabbit Alexa 546 (Goat polyclonal)	Thermo Fisher Scientific	Cat# A-11035 RRID:AB_2534093	mouse-IHC (1:200)
Antibody	anti-Mouse FITC (Goat polyclonal)	Jackson ImmunoResearch	Cat#115-095-003 RRID:AB_2338589	IF (1:500)
Antibody	anti-Mouse Cy3 (Goat polyclonal)	Jackson ImmunoResearch	Cat# 115-165-003 RRID:AB_2338680	IF (1:500)
Antibody	anti-Rat IgG Cy5 (Goat polyclonal)	Invitrogen	Cat# A10525 RRID:AB_2534034	IF (1:500)
Antibody	anti-Rabbit peroxidase (Goat polyclonal)	Jackson ImmunoResearch	Cat# 111-035-144 RRID:AB_2307391	WB (1:7500)
Antibody	anti-Mouse peroxidase (Goat polyclonal)	Jackson ImmunoResearch	Cat# 115-035-003 RRID:AB_10015289	WB (1:7500)
Antibody	anti-Mouse peroxidase (Goat polyclonal)	GeneTex	Cat# GTX213111-01 RRID:AB_10618076	mouse-WB (1:5000)
Antibody	anti-Rabbit peroxidase (Goat polyclonal)	GeneTex	Cat# GTX213110-01 RRID:AB_10618573	mouse-WB (1:5000)
Chemical compound, drug	Phalloidin-TRITC	Sigma-Aldrich	Cat# P1951 RRID:AB_2315148	IF (1:5000)

Chemical compound, drug	WGE	Lin et al., 2016; Lin et al., 2018	N/A	KO DA Pharmaceutical Co. Ltd.
Chemical compound, drug	gastrodin	Lin et al., 2016	N/A	Wuhan YC Fine Chemical Co.
Chemical compound, drug	4-HBA	Sigma-Aldrich	Cat# H20806-10G	
Recombinant DNA reagent ( <i>Homo sapiens</i> )	pDEST53-LRRK2-WT (plasmid)	Addgene	Addgene plasmid # 25044; RRID:Addgene_25044	
Recombinant DNA reagent ( <i>Homo sapiens</i> )	pDEST53-LRRK2-G2019S (plasmid)	Addgene	Addgene plasmid # 25045; RRID:Addgene_25045	
Recombinant DNA reagent ( <i>Drosophila melanogaster</i> )	pJFRC19-13XLexAop2-IVS-myr::GFP (plasmid)	Addgene	Addgene plasmid # 26224; RRID:Addgene_26224	
Software, algorithm	ImageJ	PMID: 22930834	<a href="https://imagej.nih.gov/ij/">https://imagej.nih.gov/ij/</a> ; RRID:SCR_003070	
Software, algorithm	Ctrax	PMID: 19412169	N/A	
Software, algorithm	CatWalk XT	Lin et al., 2020	N/A	Noldus Information Technology
Software, algorithm	Prism 6	Graphpad	RRID:SCR_002798	

Other	DAPI	GeneTex	Cat# GTX30920	
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1

**Fig. 1**

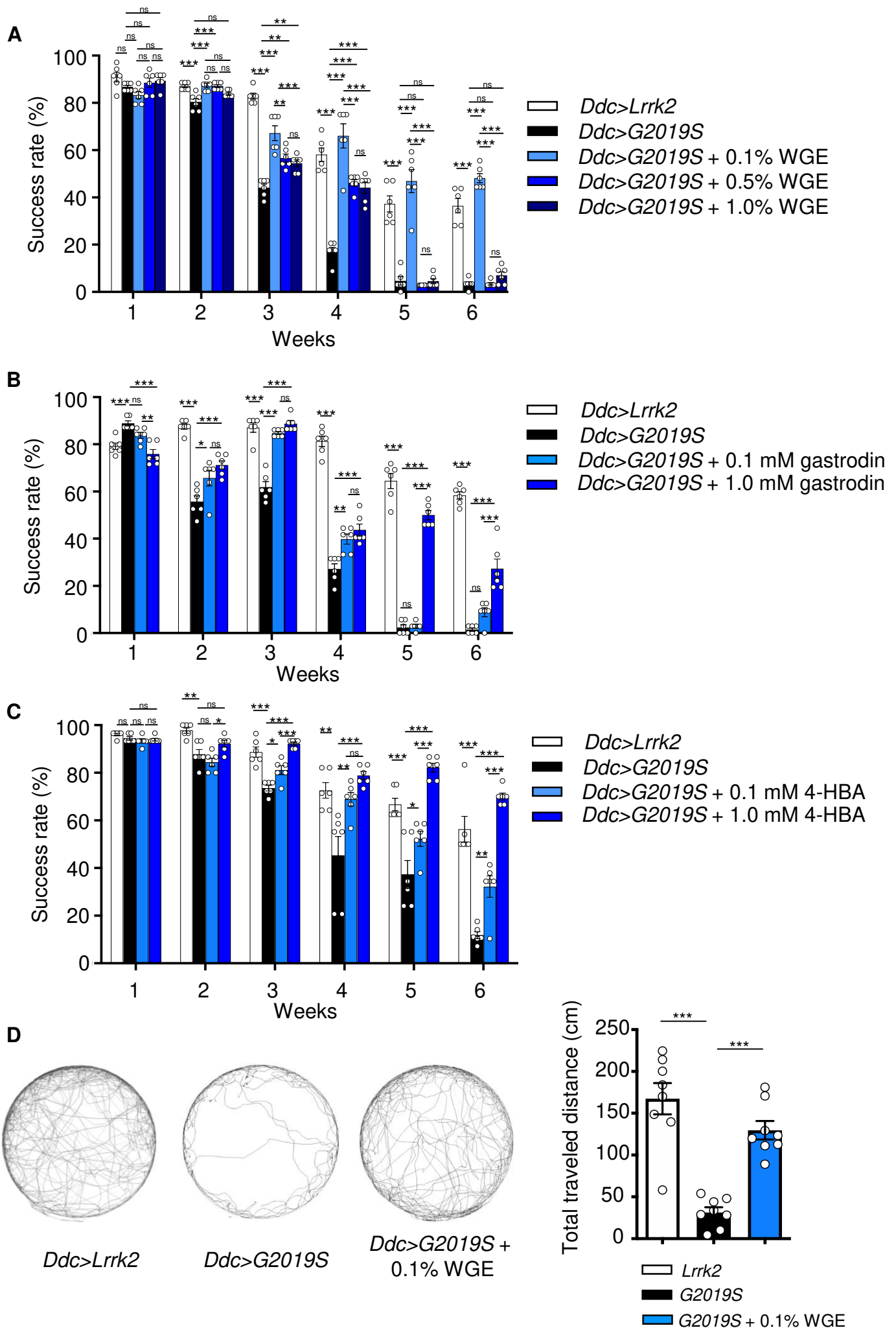


Figure 1-figure supplement 1

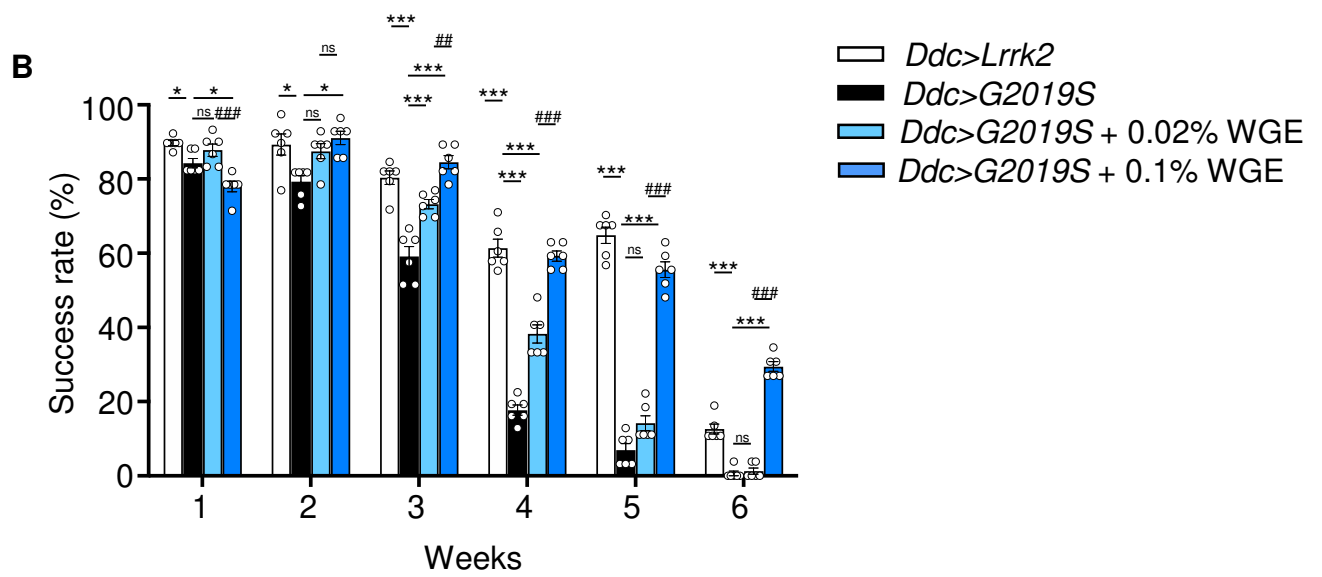
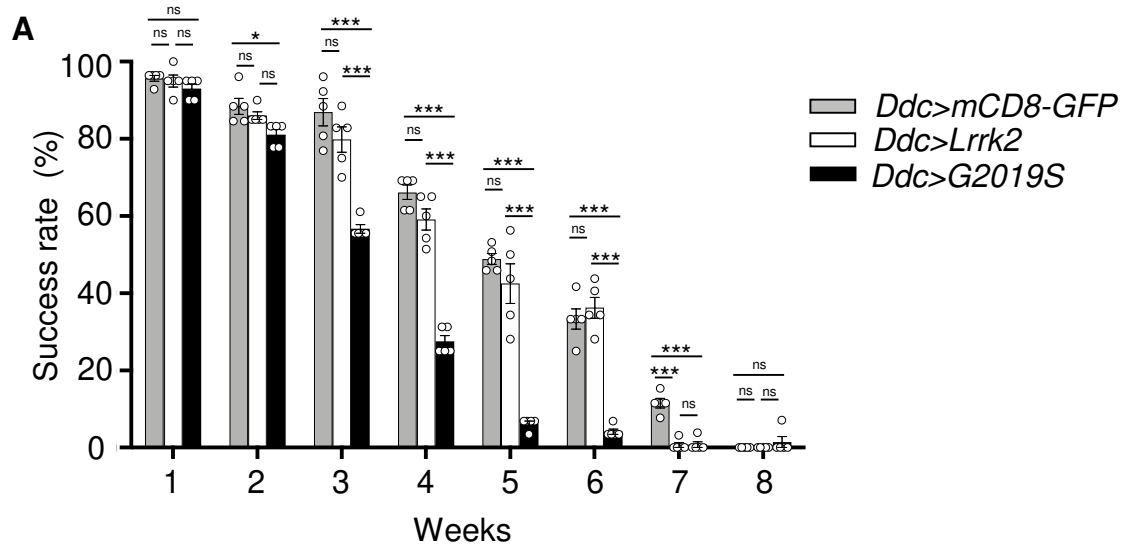
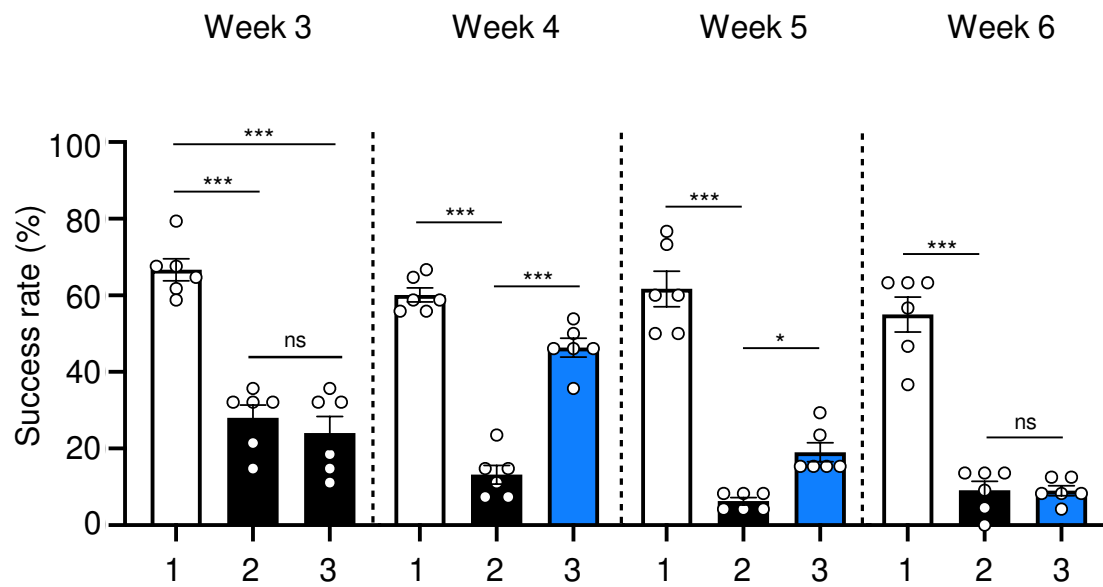




Figure 1-figure supplement 2



1. *Ddc>Lrrk2* at week 3, 4, 5 and 6
2. *Ddc>G2019S* at week 3, 4, 5 and 6
3. *Ddc>G2019S* at week 3 and starting 0.1% WGE feeding at week 4

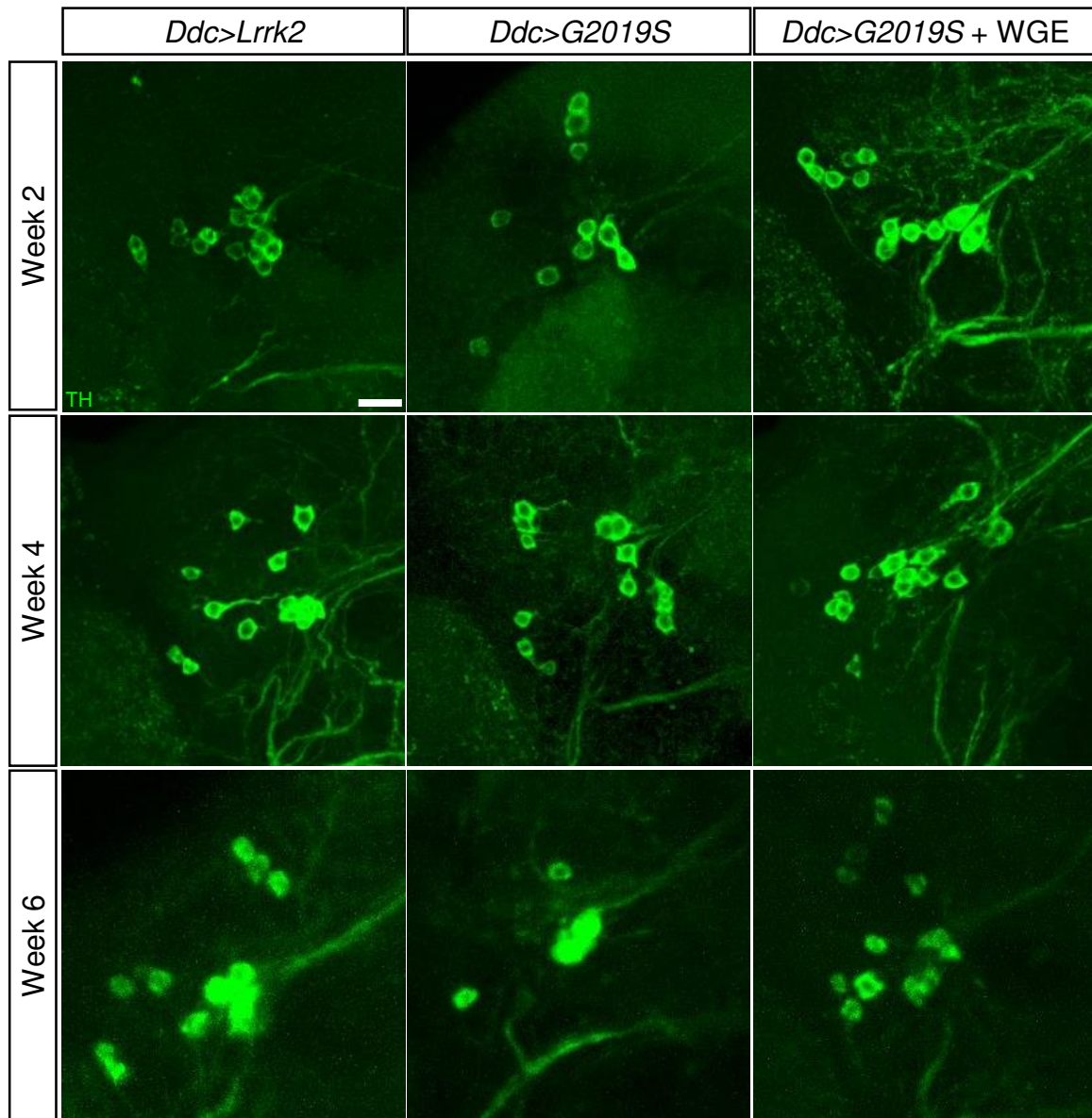
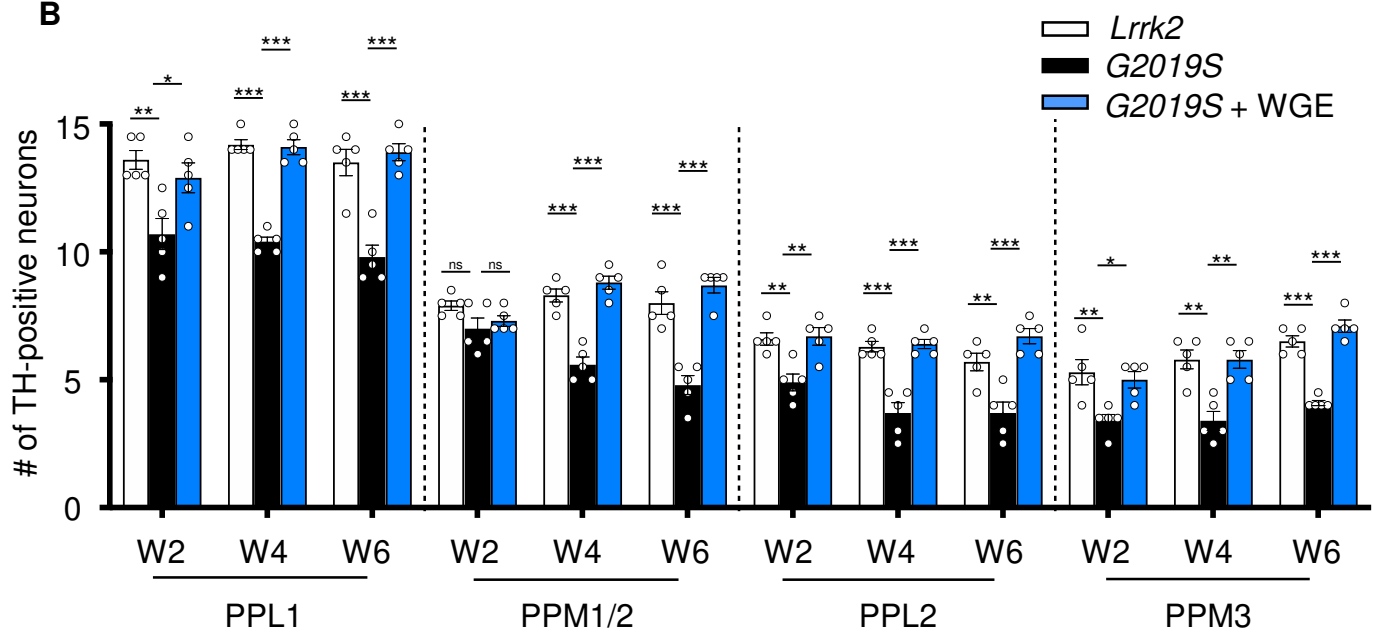
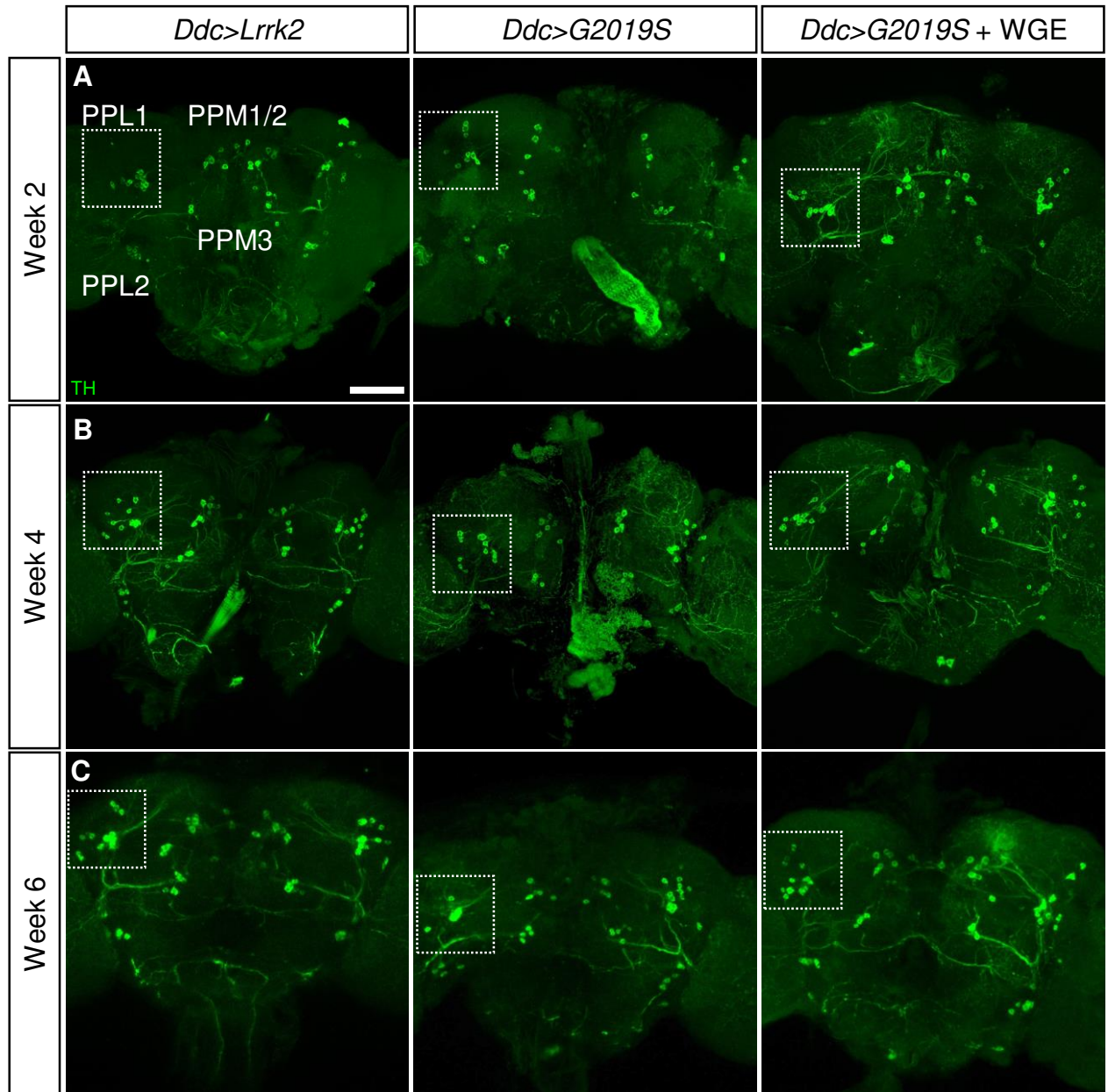
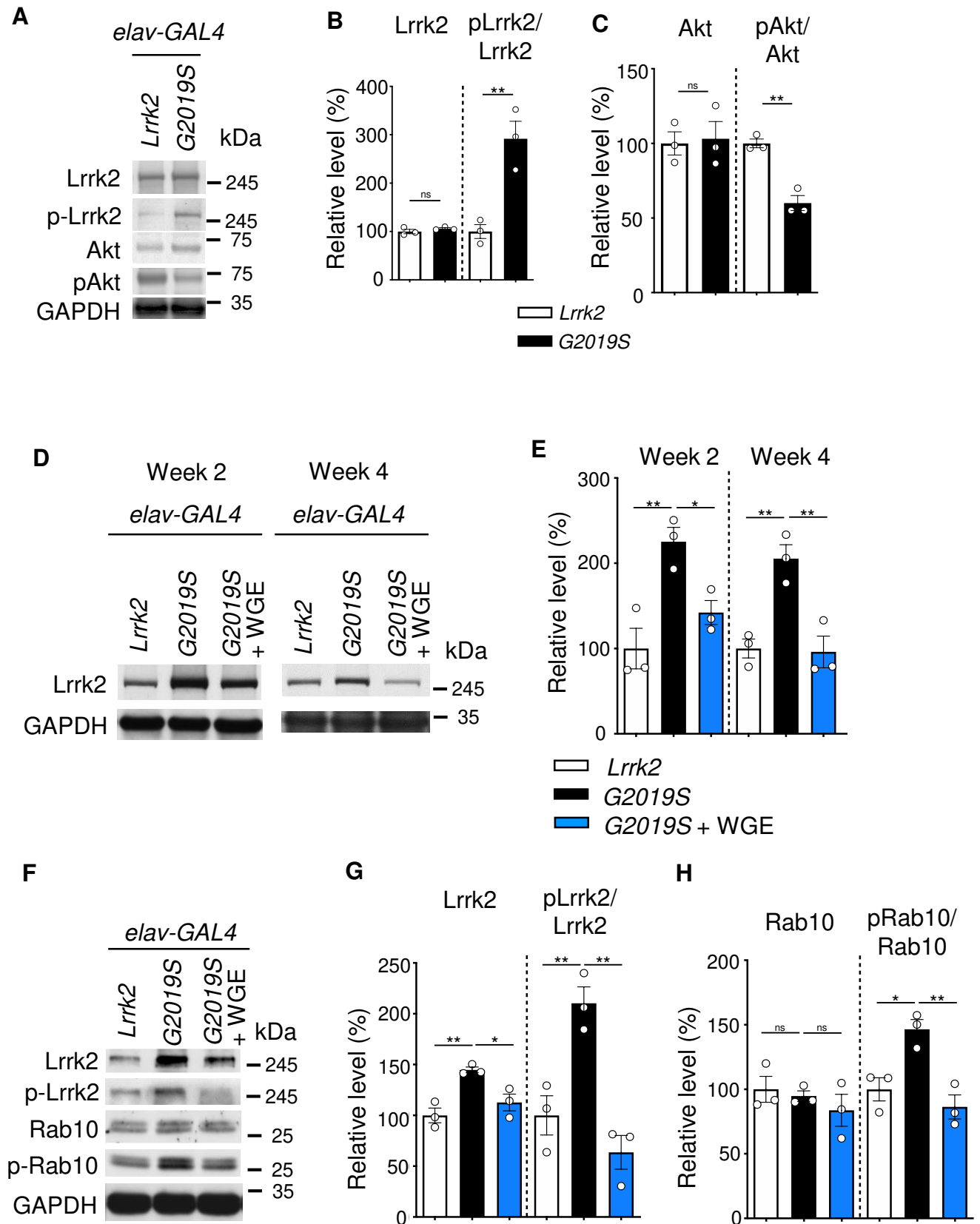
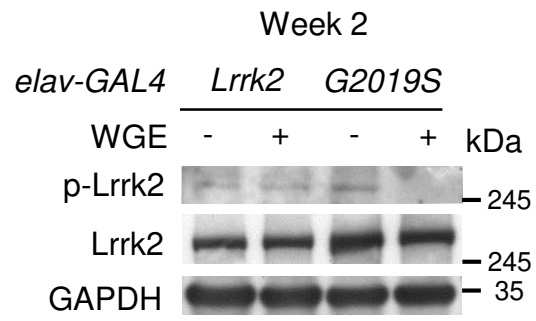
**Fig. 2****A****B**

Figure 2-figure supplement 1

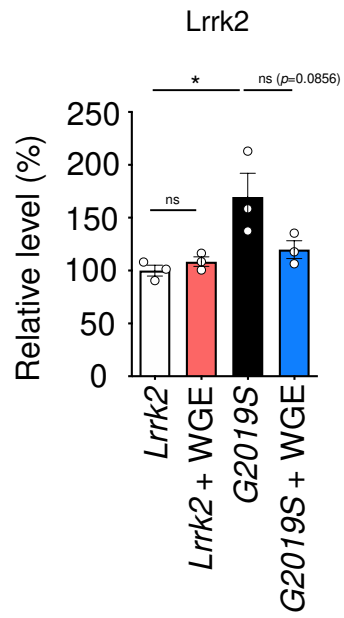


**Fig. 3**

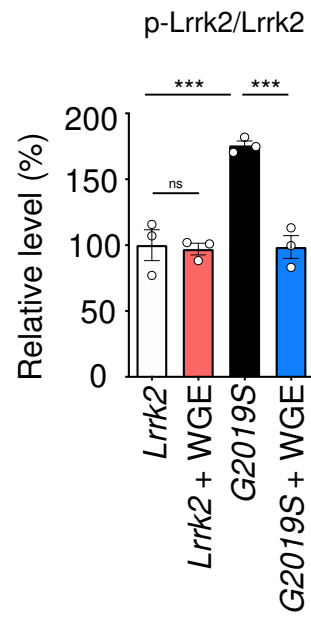
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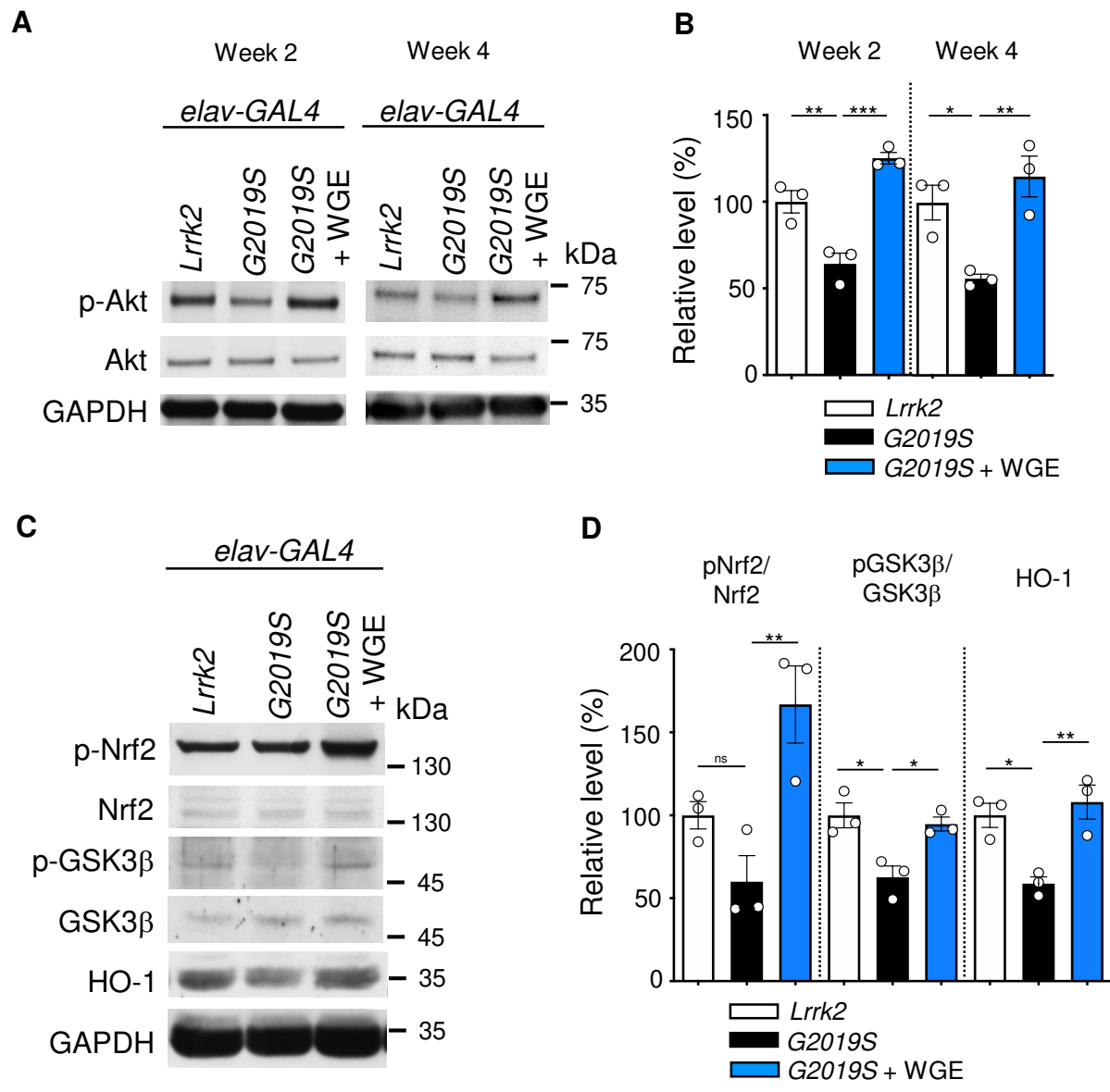
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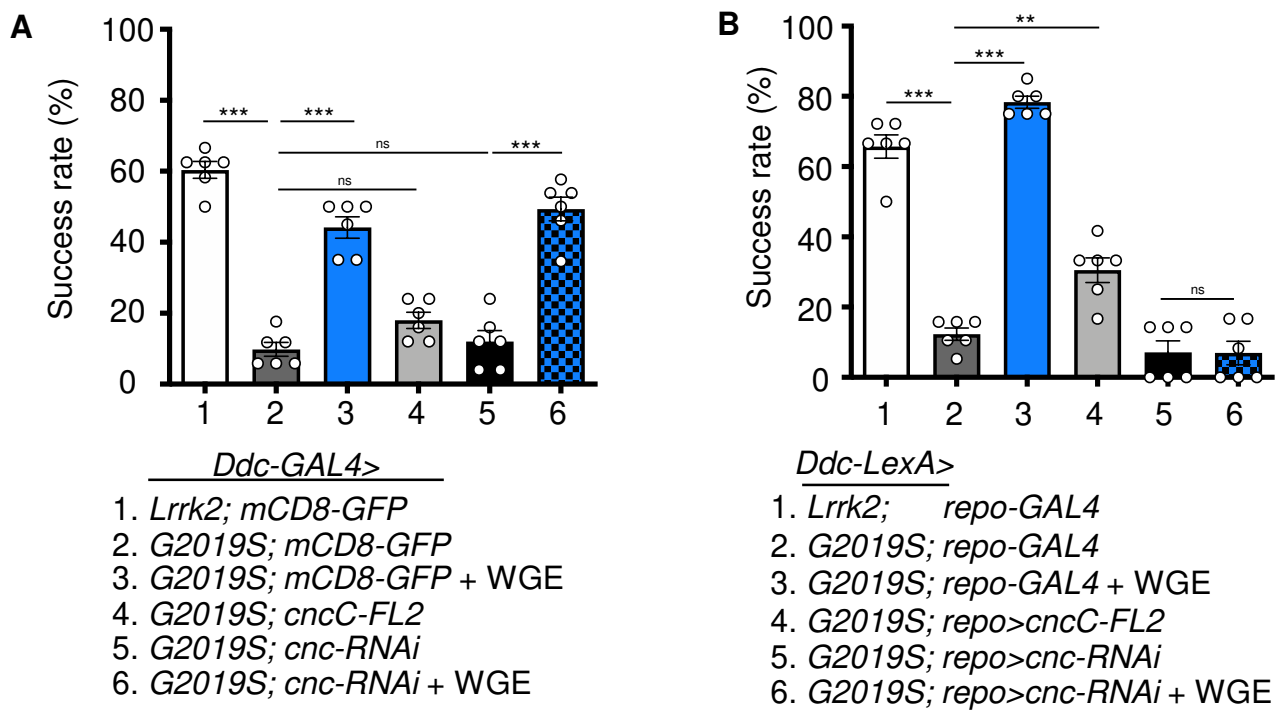
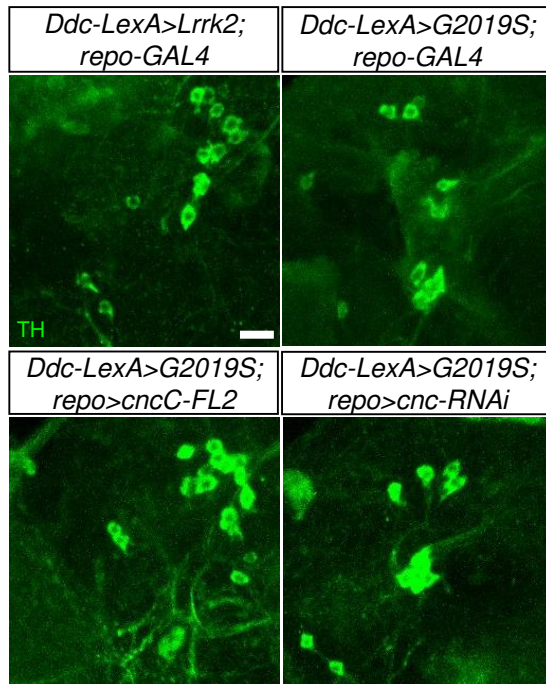
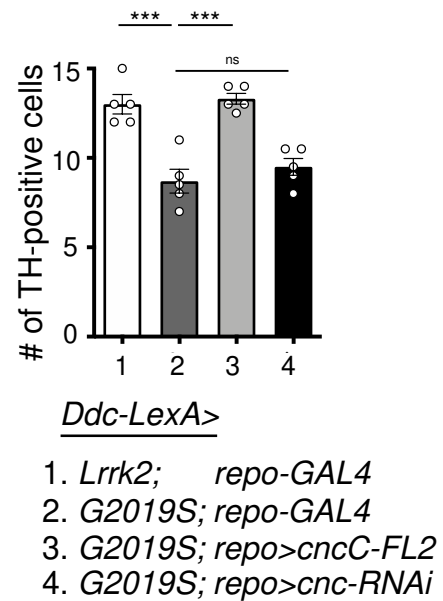


**C**



**Fig. 4**



**Fig. 5****C****D**

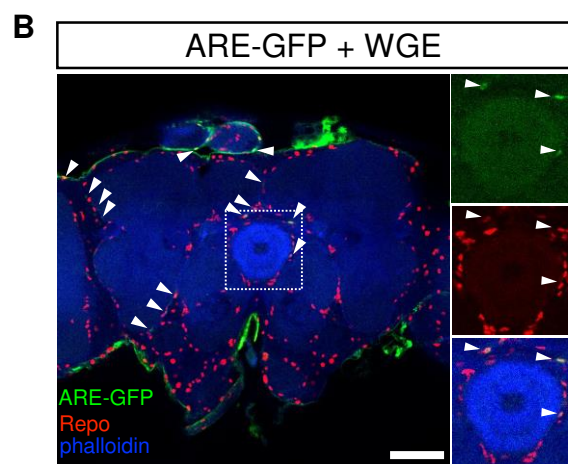
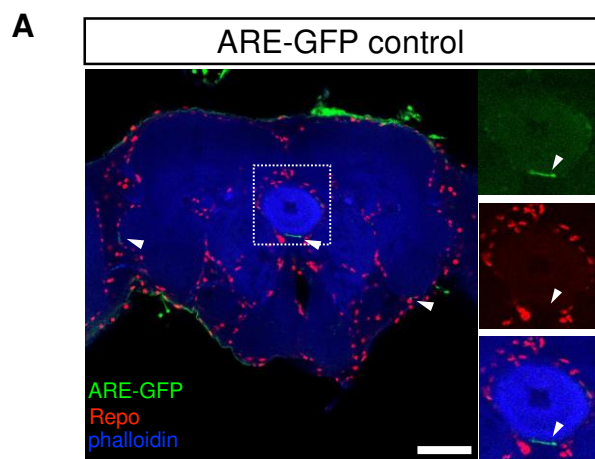
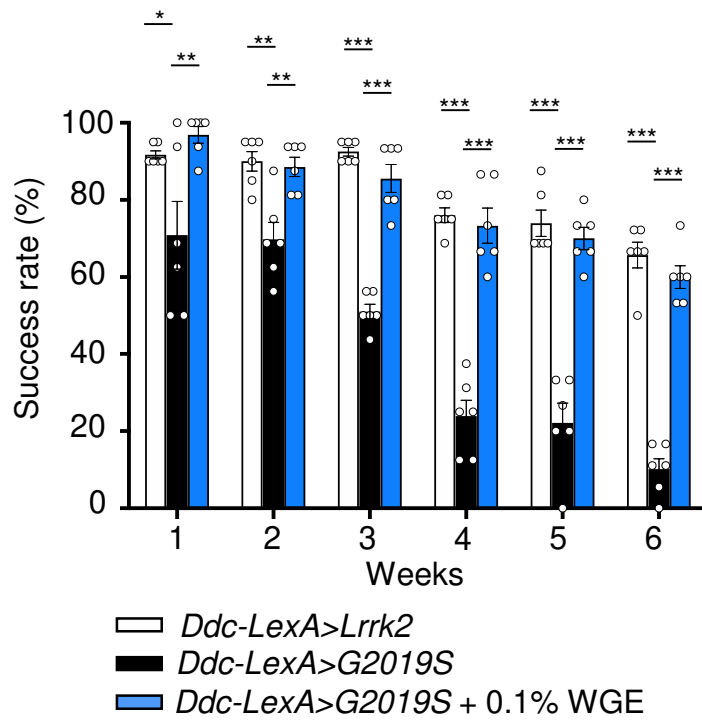
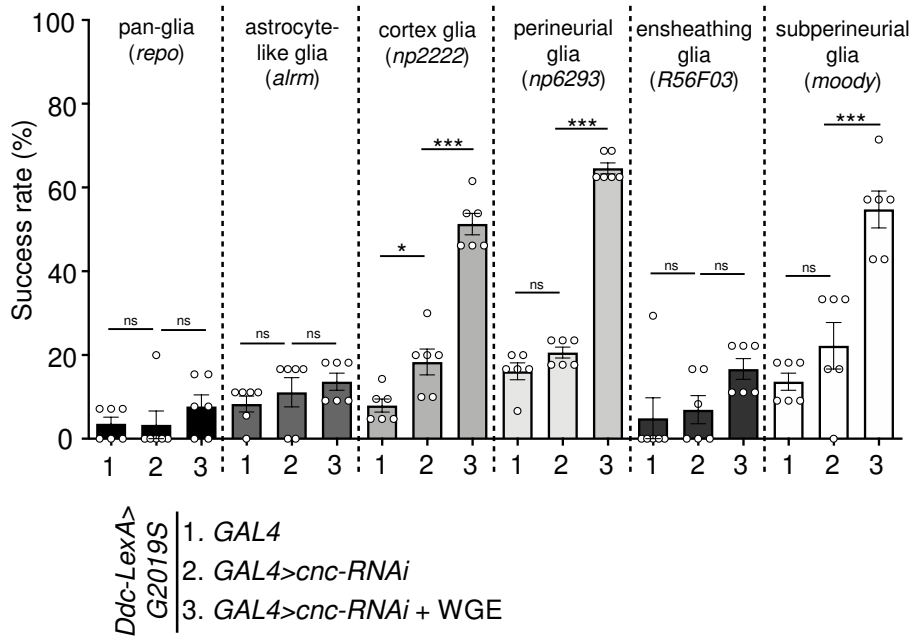
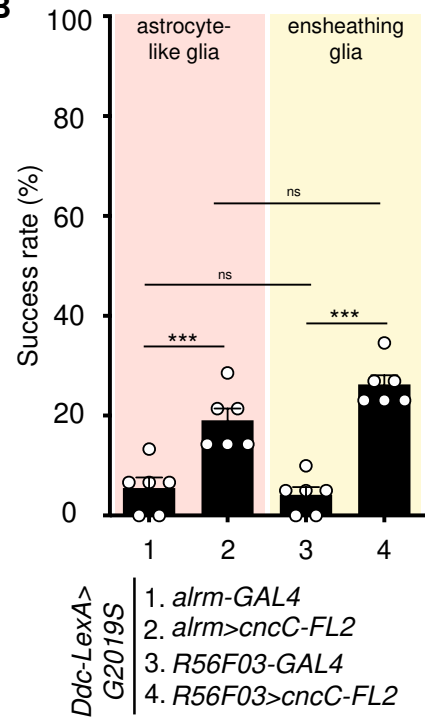
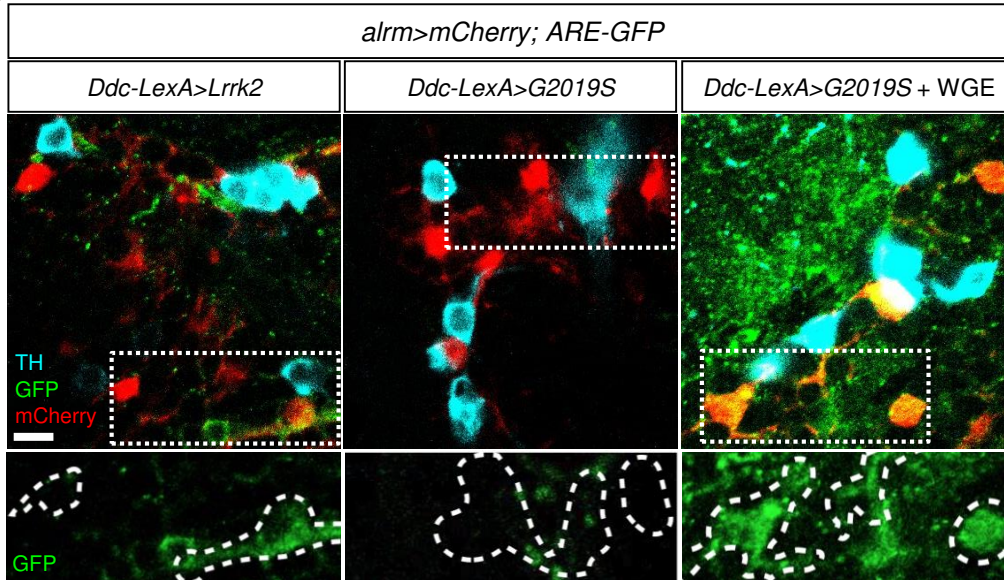
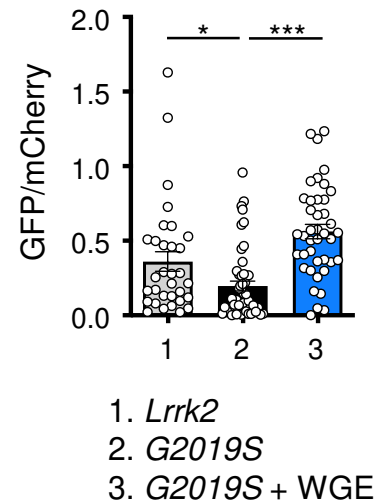
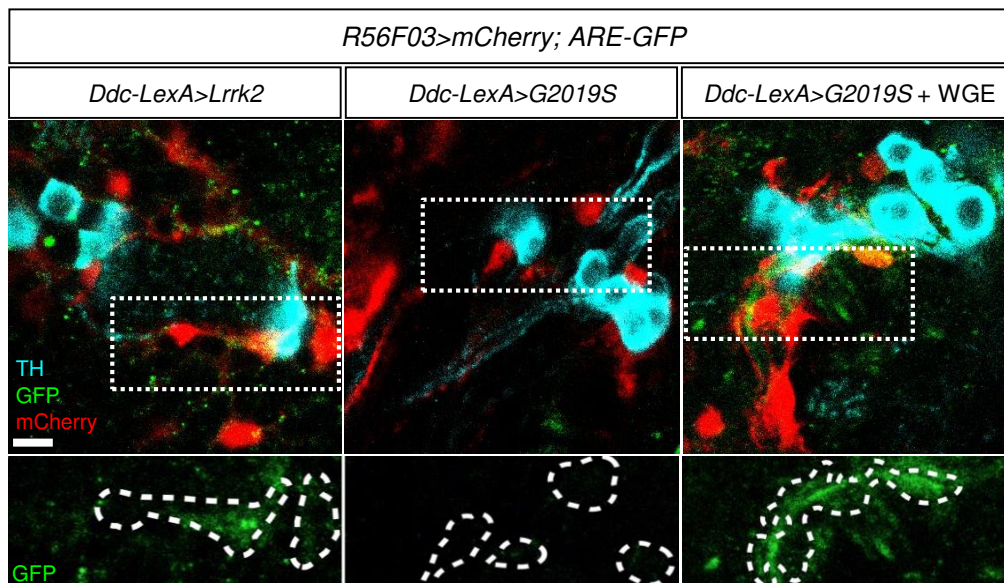
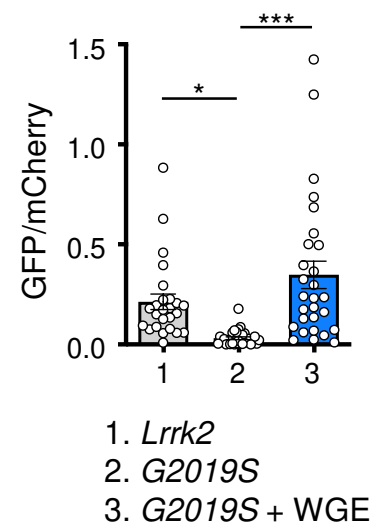




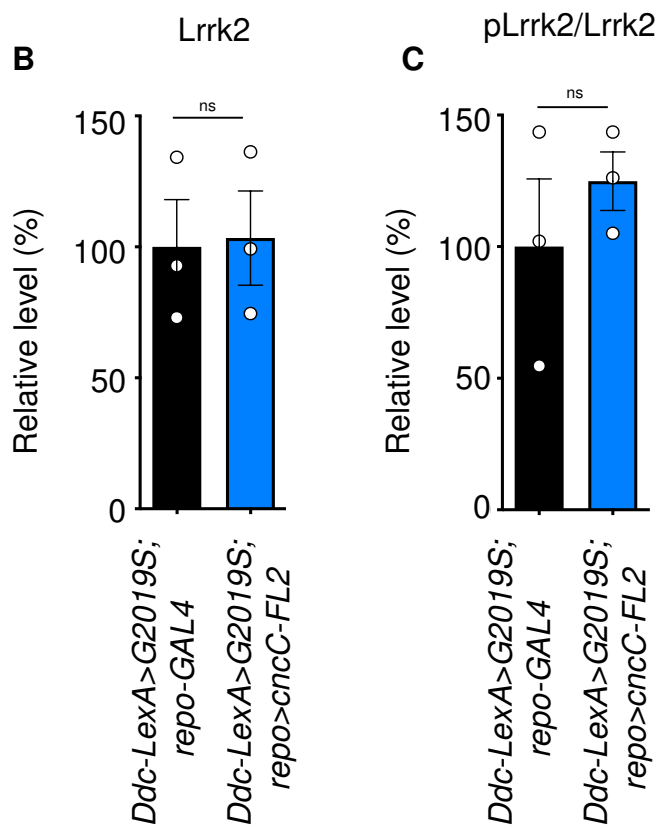
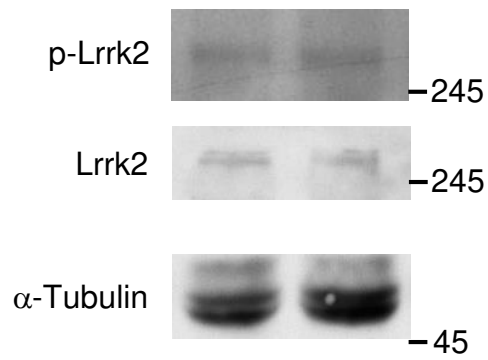
Figure 5-figure supplement 2



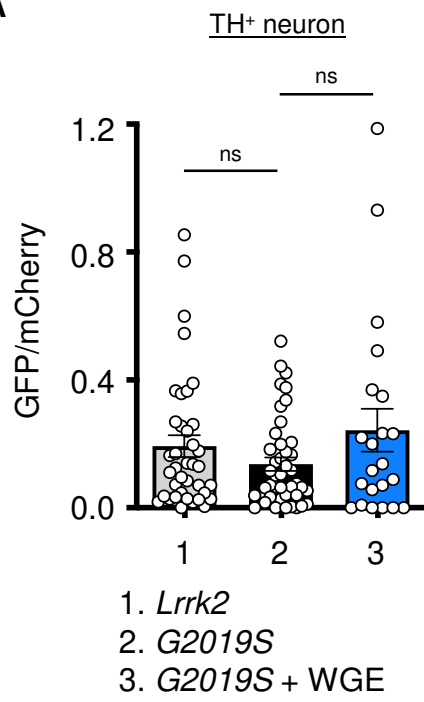
**Fig. 6****A****B****C****D****E****F**

**A** *Ddc-LexA>G2019S; repo-GAL4*

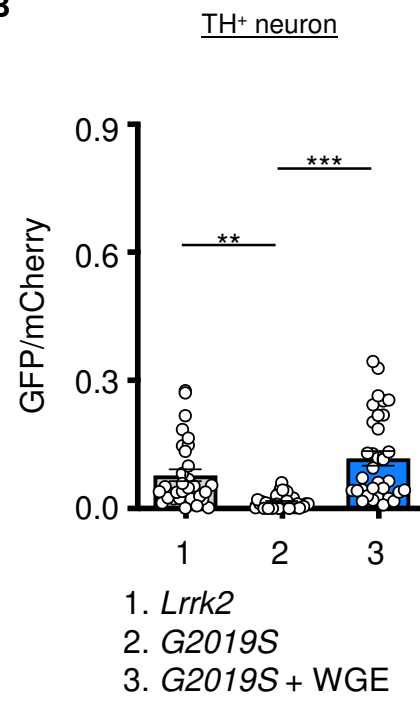
- *cncC-FL2*



**A**



**B**



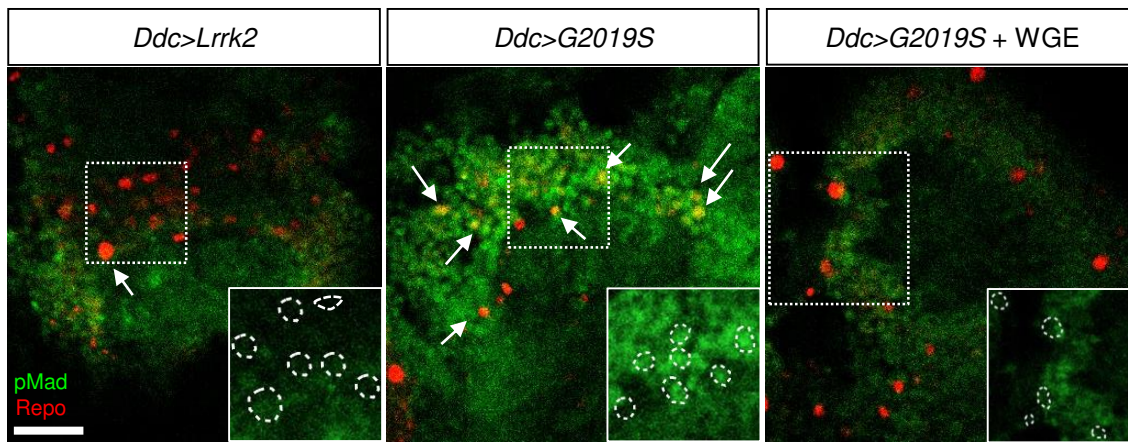
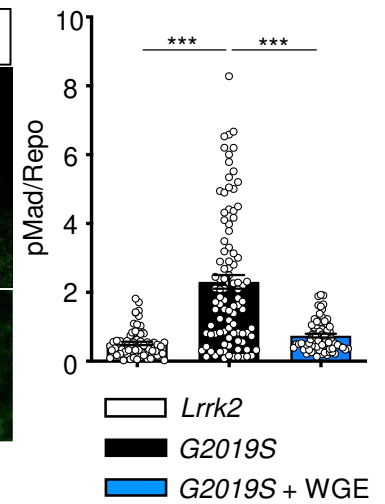
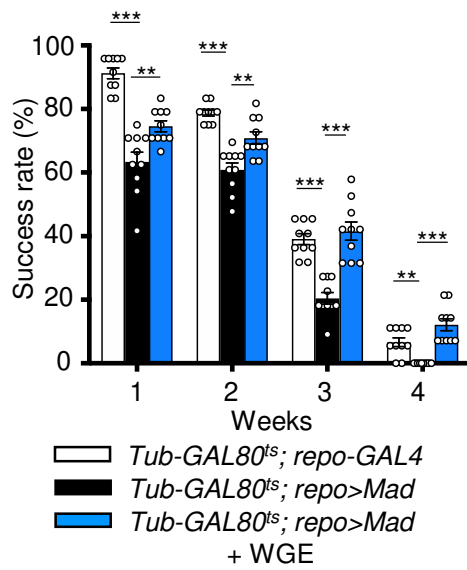
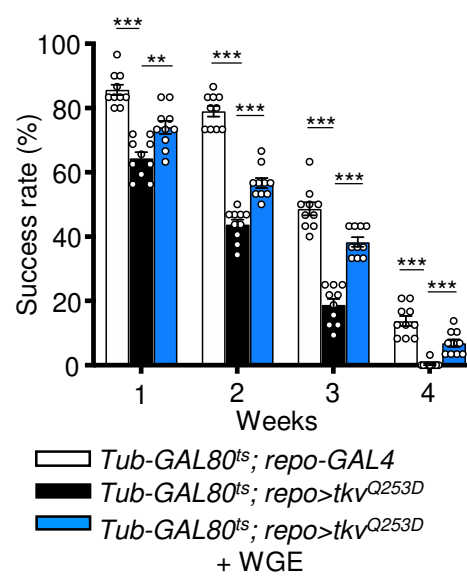
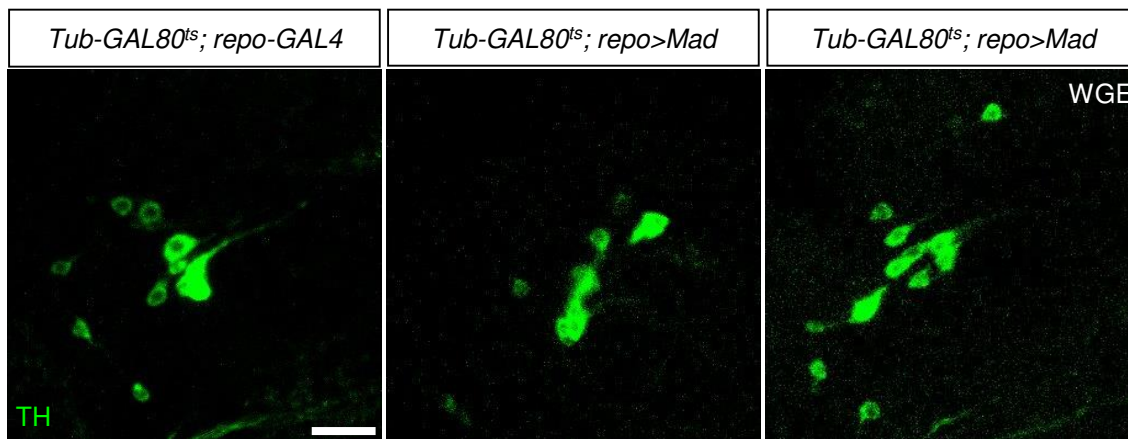
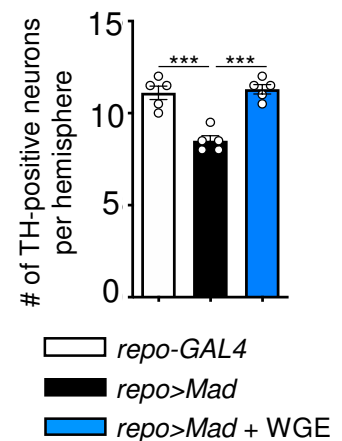
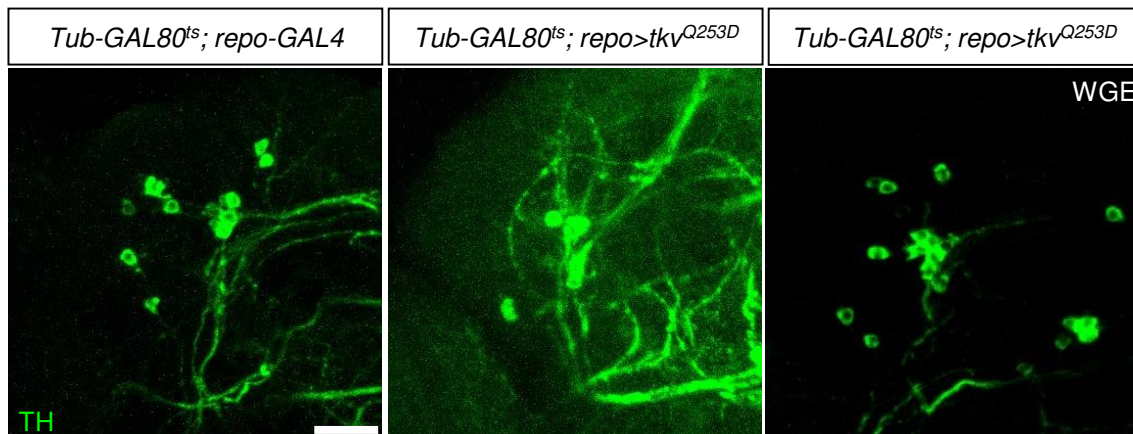
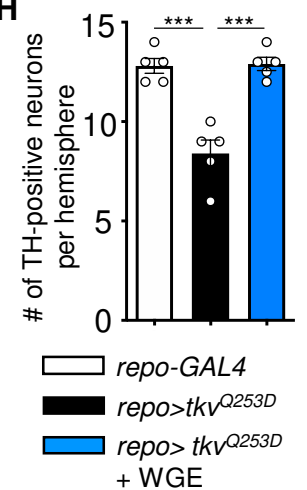
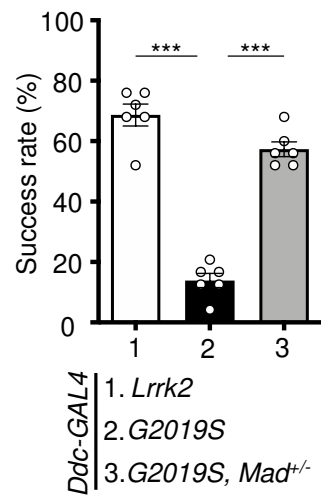
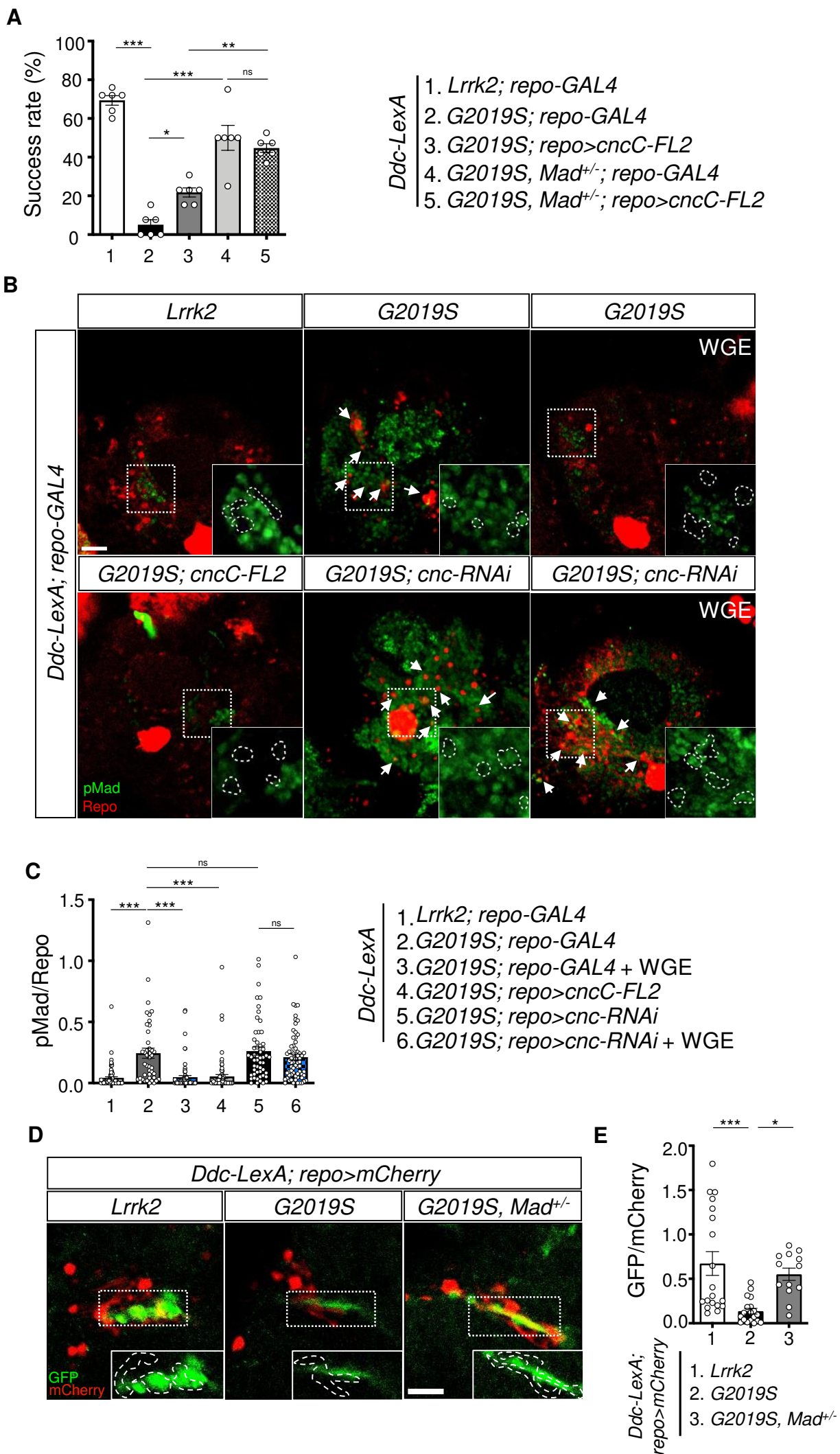
**Fig. 7****A****B****C****D****E****F****G****H**

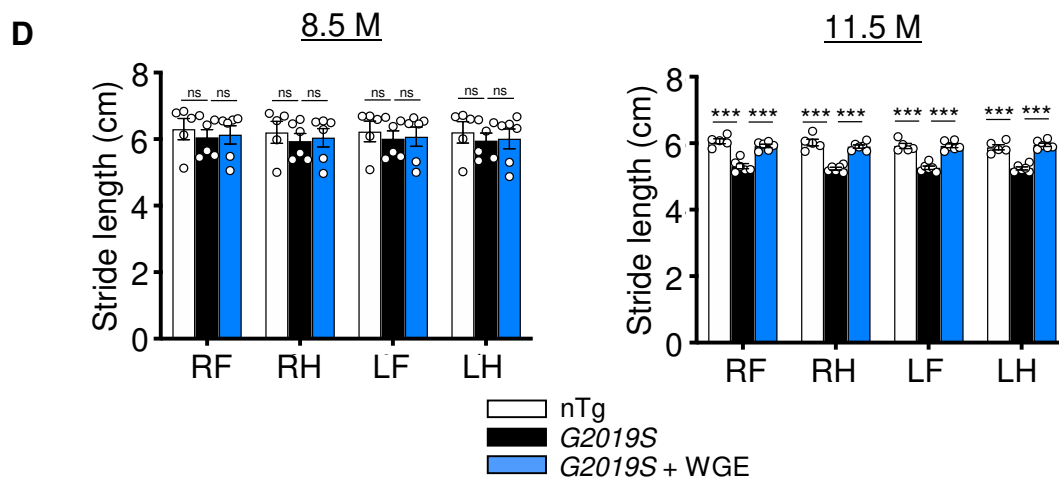
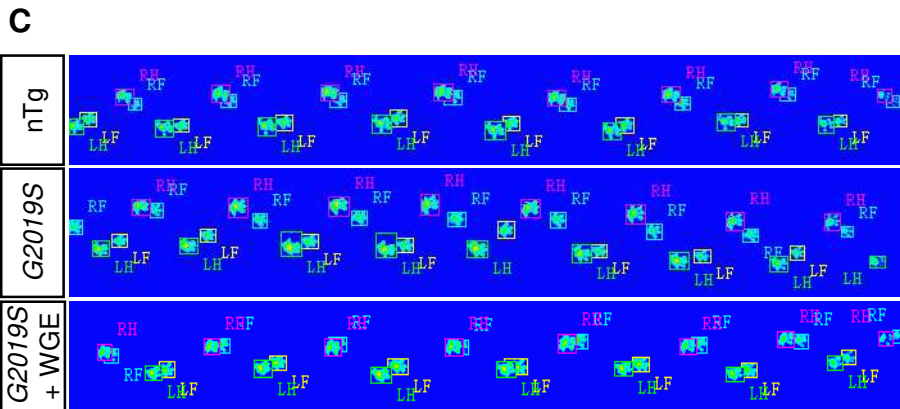
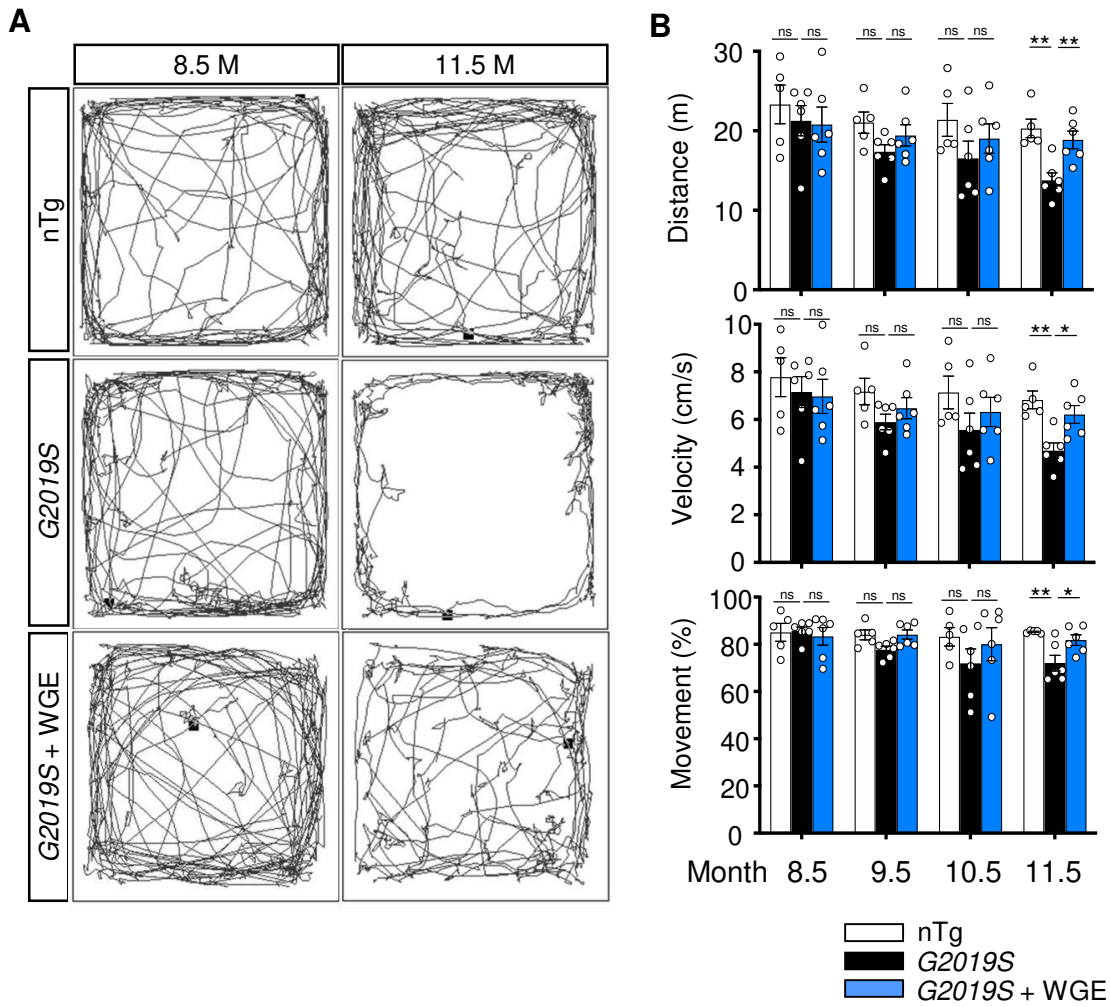
Figure 7-figure supplement 1



**Fig. 8**



**Fig. 9**





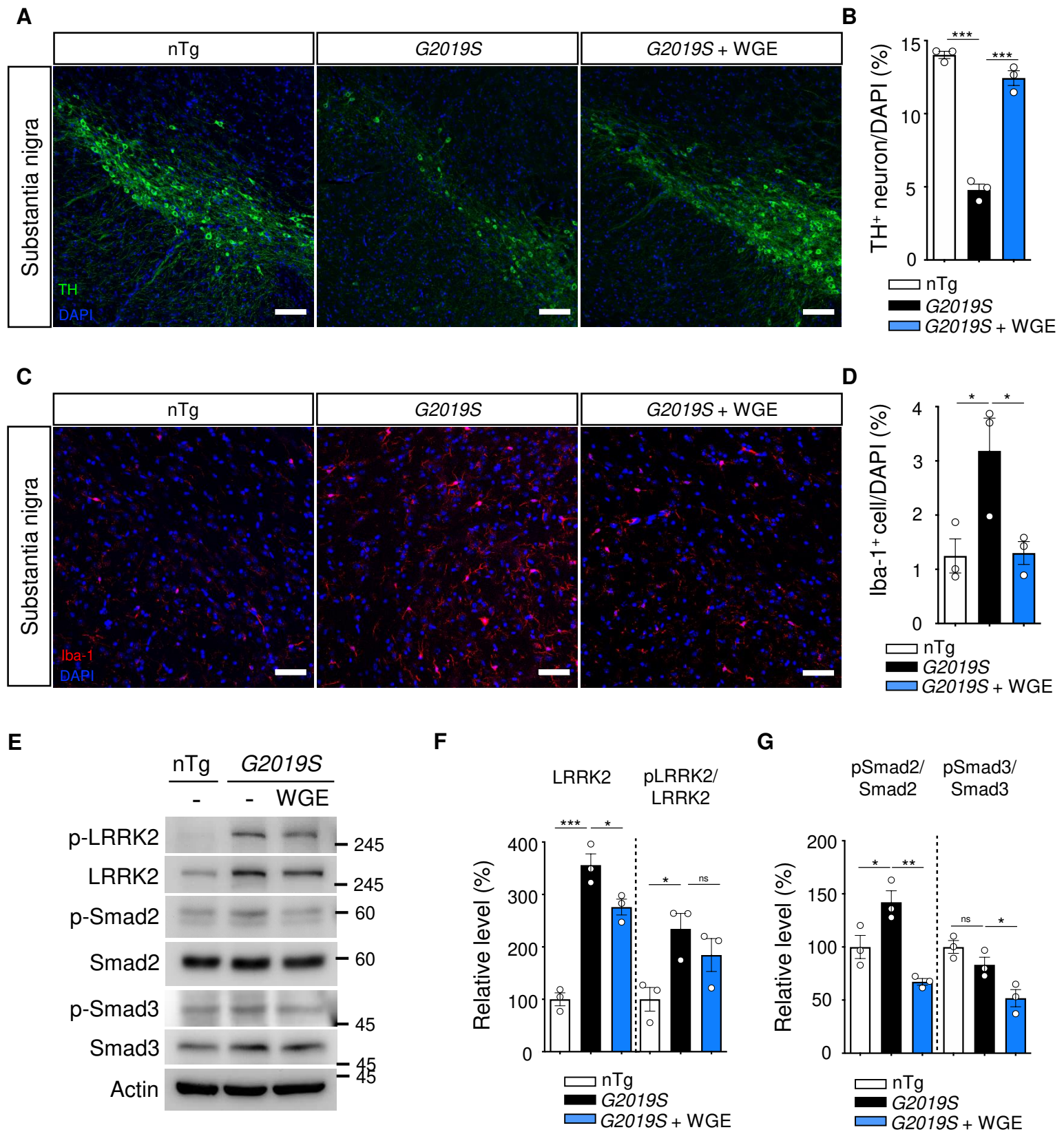
**Fig. 10**

Figure 10-figure supplement 1

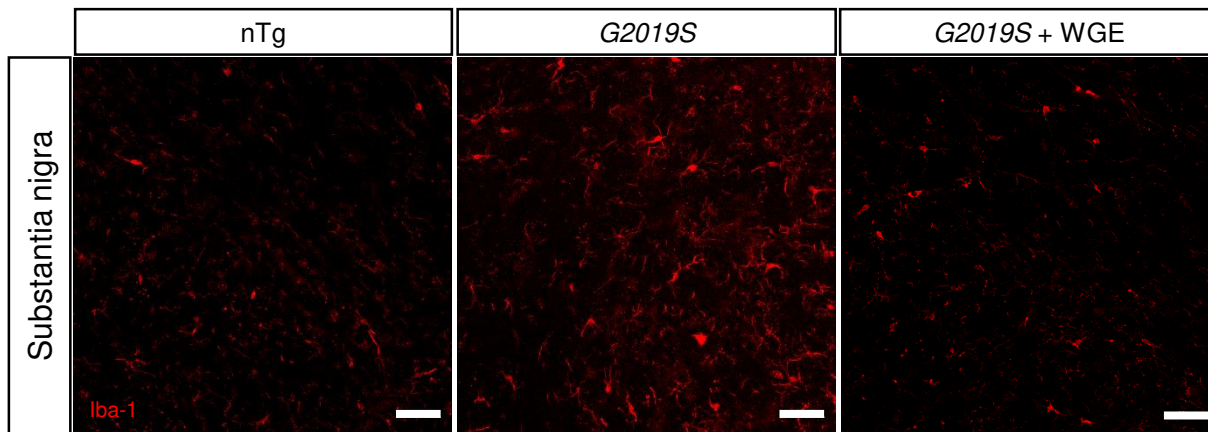


Fig. 11

