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Original Research Article

Epidemiological Survey of Frontotemporal Lobar Degeneration in Tottori Prefecture, Japan

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Key Words
Prevalence · Frontotemporal dementia · Progressive nonfluent aphasia · Semantic dementia · Tau gene

Abstract
Background: The prevalence of frontotemporal lobar degeneration (FTLD) in Japan is unknown. An epidemiological survey study of FTLD was undertaken in Tottori Prefecture, a district in the western region of Japan. ***Methods:*** Hospitals in Tottori Prefecture were surveyed by a two-step questionnaire in 2010, and the prevalence of FTLD per 100,000 inhabitants was calculated using the actual number of patients and inhabitants in Tottori Prefecture on the prevalence day of October 1, 2010. ***Results:*** In this survey, 66 patients were diagnosed with FTLD. The subtypes of FTLD were as follows: 62 cases of frontotemporal dementia (FTD), 3 cases of progressive nonfluent aphasia, and 1 case of semantic dementia. Among the FTD cases, 5 cases were FTD with motor neuron disease and 1 case was FTD with parkinsonism linked to chromosome 17. The prevalence of FTD in the total population of Tottori Prefecture was 11.2 per 100,000 inhabitants. Based on these results, the prevalence of FTLD in Japan in 2008 was estimated to be 9.5 per 100,000 individuals. ***Conclusions:*** Our epidemiological survey results suggest that there are at least 12,000 FTLD patients in Japan, indicating that FTLD is not a rare disease.

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Introduction

Frontotemporal lobar degeneration (FTLD) is a neurodegenerative disorder predominantly affecting the frontal and temporal lobes. Two major clinical types are recognized in FTLD: behavioral variant frontotemporal dementia (FTD) and progressive aphasia. The latter is divided into progressive nonfluent aphasia (PNFA) and semantic dementia (SD) [1]. Few epidemiological surveys have been conducted concerning FTLD in Japan, and no epidemiological study focusing particularly on FTLD has been reported. In small community-based studies on the prevalence of dementia in individuals 65 years of age or older, a small percentage of the dementia cases were attributed to FTLD [2, 3]. In clinic-based survey studies, FTLD was the most third frequent cause of early-onset dementia in patients less than 65 years of age [4, 5]. The prevalence of FTLD in Japan is unknown. Here, we report a survey study of FTLD in Tottori Prefecture, the least populated district in Japan.

Methods

We used a questionnaire to perform a retrospective surveillance study of cases of FTLD in Tottori Prefecture. Tottori Prefecture is located in a rural area of western Japan (fig. 1); it had a population of 587,772 (280,602 males and 307,170 females) on the prevalence day of October 1, 2010.

In 2010, we sent inquiries with registration criteria for each category of FTLD to the departments of neurology and psychiatry in the 47 hospitals in Tottori Prefecture where patients with dementia were treated, asking if they had admitted or examined any cases of FTLD during the past year. We then sent a second questionnaire to the departments who responded affirmatively enquiring about the type of FTLD, sex and age of patients, age of onset, symptoms, neuroimaging results, and treatment. If permission was obtained, board-certificated neurologists (K.W.-I., S.I., S.N., and M.Y.) visited the hospitals to examine the patients.

The diagnosis of FTLD was based on the consensus criteria by Neary et al. [1] and the criteria of the International Behavioural Variant FTD Criteria Consortium [6]. Structural neuroimaging [cerebral computed tomography (CT) or magnetic resonance imaging (MRI)] was performed to support the clinical diagnosis. Functional imaging data [cerebral blood flow evaluated by single-photon emission computed tomography (SPECT)] were obtained, if available. Patients were diagnosed as having probable or possible amyotrophic lateral sclerosis using the El Escorial criteria [7, 8].

The prevalence of FTLD per 100,000 inhabitants and 95% confidence interval (CI) were calculated using the actual number of patients and inhabitants in Tottori Prefecture on the prevalence day, October 1, 2010.

This study was planned and conducted in accordance with the Declaration of Helsinki. The Ethics Committee of the Tottori University Faculty of Medicine approved the study prior to its implementation.

Results

Survey Results

Sixty-six patients were diagnosed with FTLD in Tottori Prefecture on the prevalence day (fig. 2). The subtypes of FTLD were as follows: 62 cases of FTD, 3 cases of PNFA, and 1 case of SD. Among the FTD cases, 5 were FTD with motor neuron disease, and 1 was FTD with parkinsonism linked to chromosome 17 (FTDP-17). Overall, the mean age of patients with

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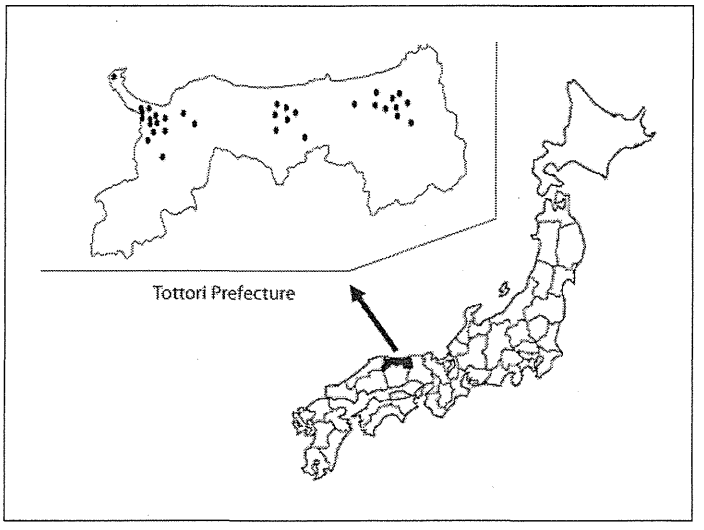

Fig. 1. Location of the surveyed hospitals in Tottori Prefecture. Tottori Prefecture is located in western Japan. The marked hospitals and clinics (circles) were included in our survey.

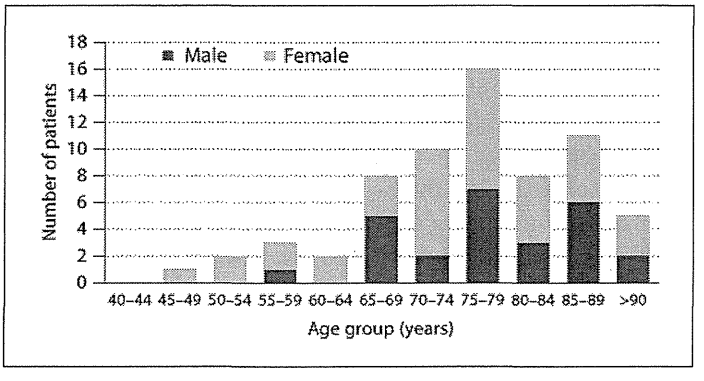

Fig. 2. Age-specific number of patients with FTLD.

FTD was 76.5 ± 11.0 years. The mean age of FTD patients with motor neuron disease was 65.8 ± 14.3 years, less than the mean age of FTD patients without motor neuron disease. The mean age of the 3 patients with PNFA was 71.7 ± 8.1 years, and the age of the patient with SD was 72 years. Three patients with FTLD had a family history of the disease. Genetic analysis revealed that the patient with FTDP-17 had an intronic mutation IVS10 C>T in the microtubule-associated protein tau (MAPT) gene.

Prevalence

Table 1 shows the age-specific prevalence of FTLD per 100,000 inhabitants of Tottori Prefecture in 2010. The survey results indicate that the overall prevalence of FTLD in Tottori Prefecture was at least 11.2 per 100,000 inhabitants. Based on the demographics of Japan in 2008, the data suggest an estimated prevalence of FTLD in Japan of 9.5 per 100,000 inhabitants, or an overall prevalence of at least 12,000 individuals.

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Table 1. Age-specific prevalence estimates of FTLD in Tottori Prefecture

	Patients, n	Prevalence (95% CI)[1]
Age group		
45–54 years	3	4.0 (–5.3 to 8.6)
55–64 years	5	5.6 (0.7–10.5)
65–74 years	18	25.8 (13.9–37.7)
75–84 years	24	40.5 (24.3–56.7)
>85 years	16	64.0 (32.6–95.0)
Population ≥45 years	66	20.7 (15.7–26.0)
Total Population	66	11.2 (8.5–14.0)

[1] Per 100,000 inhabitants.

Discussion

We conducted an epidemiological survey focusing on the prevalence of FTLD in Tottori Prefecture of Japan. Our previous epidemiological study in Ama-cho [3], a small island town, revealed that only 1 patient with FTLD was diagnosed among 943 subjects with dementia aged 65 years or older, suggesting that more subjects would be needed to examine the true prevalence of FTLD. Therefore, we decided to carry out an epidemiological survey in Tottori Prefecture, which has a total population of 587,772.

The prevalence of FTLD has also been reported in areas outside of Japan. In population-based studies, the prevalence of FTLD between 45 and 64 years of age has varied from 4.0 per 100,000 individuals in the Zuid-Holland district in the Netherlands to 22 per 100,000 individuals in Brescia, Italy [9–12]. A nationwide hospital-based clinicoepidemiological study in Germany showed a high estimated prevalence of FTLD of 43.1 per 100,000 individuals between 45 and 64 years of age [13]. In contrast, Ikejima et al. [14] reported the prevalence of restricted FTD patient in Ibaraki Prefecture, Japan, to be 2.0 per 100,000 individuals between 45 and 64 years of age. Taken together with our results, the prevalence of FTLD in individuals under 65 years of age in Japan might be less than that in Europe.

Although FTLD is generally considered to be a presenile dementia, a review of demographic characteristics of 353 FTLD patients by Johnson et al. [15] indicated that approximately one quarter of the patients diagnosed as having FTD and semantic dementia and half of PNFA patients had a disease onset after age 65 years. The prevalence of FTLD was 3.8 per 100,000 individuals between 70 and 79 years of age in the Zuid-Holland district in the Netherlands [9]. A nationwide study in Germany estimated the prevalence of FTLD to be 49.3 per 100,000 individuals between 70 and 79 years of age [11]. Gislason et al. [16] reported a much higher prevalence of FTD of 3% in a cohort of 85-year-old individuals in Gothenburg, Sweden. In the current study, the prevalence of FTLD was 37.6 per 100,000 inhabitants 65 years of age or older. These epidemiological data indicate that the prevalence of FTLD among elderly subjects might be higher than previously described. One challenging aspect of FTLD is that the clinicopathological features of FTLD in elderly patients may differ from those in patients with presenile-onset FTLD. Baborie et al. [17] proposed that FTLD in elderly patients might exist as a separate entity from presenile-onset FTLD in that the characteristics of clinically frequent memory loss and behavioral changes predominate over language and semantic dysfunction. It was suggested that FTLD in elderly patients is under-recognized, and FTLD should be considered in elderly subjects presenting with an 'atypical Alzheimer's disease' phenotype.

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Although epidemiological studies in Europe have reported that a large percentage of FTLD patients have a family history (29% in the UK, 43% in the Netherlands), only 4.5% of FTLD patients in our current study had a family history. Only 1 patient (1.5%) of 66 FTLD patients in Tottori Prefecture had a mutation of MAPT, compared with 32 (13.8%) of 245 FTD patients in the study in the Zuid-Holland district in the Netherlands. These results suggest that genetic factors for the development of FTLD may have a less important role in the Japanese population.

There are several limitations in our estimates of the prevalence of FTLD in Tottori Prefecture of Japan. First, the diagnosis of FTLD was dependent on clinical symptoms only, due to the absence of a definitive biomarker. We could not confirm the diagnosis neuropathologically in any case in this survey. Careful clinical examinations are needed because of the similarities in symptoms between syndromes such as corticobasal degeneration, progressive supranuclear palsy, Alzheimer's disease, vascular dementia, and FTLD. In this study, board-certificated neurologists and psychiatrists reported the diagnosis of FTLD patients based on their clinical assessment and results of neuroimaging such as cerebral CT, MRI, or cerebral blood flow by SPECT. Further, board-certificated neurologists who have scientific interest in dementia or neurodegenerative disorders visited the clinic or hospital for assessment of the patients when required. For the diagnosis of FTD, we applied the criteria by Neary et al. [5] as well as the criteria of the International Behavioural Variant FTD Criteria Consortium [6]. The former criteria are thought to be relatively insensitive and difficult to apply in the early stage of FTD, whereas the sensitivity of the latter criteria is reported to be better [6].

Further, only patients diagnosed with FTLD who had a medical consultation with the department of neurology or psychiatry were included in the survey. A clinical survey in an academic hospital indicated that the prevalence of PNFA or SD was similar to that of FTD [4]. In the current survey, the proportion of patients with PNFA or SD was much less than that reported in the previous survey, suggesting that the prevalence of PNFA and SD may have been underestimated.

In conclusion, the results of this study suggest that there are as many as 12,000 patients with FTLD in Japan, indicating that FTLD is not a rare disease at all.

Acknowledgment

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Disclosure Statement

We certify that there is no conflict of interest.

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Cerebrospinal fluid amyloid β and tau in *LRRK2* mutation carriers

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ABSTRACT

Objective: The goal of the current investigation was to examine a cohort of symptomatic and asymptomatic *LRRK2* mutation carriers, in order to address whether the reported alterations in amyloid β (Aβ) and tau species in the CSF of patients with sporadic Parkinson disease (PD) are a part of PD pathogenesis, the aging process, or a comorbid disease in patients with PD, and to explore the possibility of Aβ and tau as markers of early or presymptomatic PD.

Methods: CSF Aβ42, total tau, and phosphorylated tau were measured with Luminex assays in 26 *LRRK2* mutation carriers, who were either asymptomatic (n = 18) or had a phenotype resembling sporadic PD (n = 8). All patients also underwent PET scans with ^{18}F-6-fluoro-L-dopa (FD), ^{11}C-(±)-α-dihydrotetrabenazine (DTBZ), and ^{11}C-d-*threo*-methylphenidate (MP) to measure dopaminergic function in the striatum. The levels of CSF markers were then compared to each PET measurement.

Results: Reduced CSF Aβ42 and tau levels correlated with lower striatal dopaminergic function as determined by all 3 PET tracers, with a significant association between Aβ42 and FD uptake. When cases were restricted to carriers of the G2019S mutation, the most common *LRRK2* variant in our cohort, significant correlations were also observed for tau.

Conclusions: The disposition of Aβ and tau is likely important in both *LRRK2*-related and sporadic PD, even during early phases of the disease. A better understanding of their production, aggregation, and degradation, including changes in their CSF levels, may provide insights into the pathogenesis of PD and the potential utility of these proteins as biomarkers. *Neurology*® 2012;78:55-61

GLOSSARY

Aβ = amyloid β; **AD** = Alzheimer disease; **DTBZ** = ^{11}C-(±)-α-dihydrotetrabenazine; **FD** = ^{18}F-6-fluoro-L-dopa; **MP** = ^{11}C-d-*threo*-methylphenidate; **p-tau** = phosphorylated tau; **PD** = Parkinson disease; **t-tau** = total tau.

Recent studies, particularly those with large cohorts of subjects, have reported altered CSF levels of amyloid β (Aβ) and tau species in patients with sporadic Parkinson disease (PD).[1–6] However, it is unclear whether these alterations are mechanistically important to PD pathogenesis, or represent a comorbidity or aging process. To address this question, Aβ and tau species need to be measured in patients with early (ideally preclinical) PD. Moreover, unique protein alterations in early/preclinical cases, alongside with other early (nonmotor) symptoms,[7] could assist in identifying PD at premotor stages.

Studying sporadic PD prior to the onset of clinical symptoms has been impractical, making early intervention exceedingly challenging. Autosomal dominant mutations in the leucine-rich repeat kinase (*LRRK2*) gene, the most common known genetic cause of parkinsonism, result in a

Supplemental data at www.neurology.org

Supplemental Data

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clinical phenotype similar to sporadic PD.[8,9] *LRRK2* mutation carriers have been extensively characterized by PET studies, demonstrating neurochemical changes similar to sporadic PD cases, as well as detectable dopaminergic dysfunction in asymptomatic carriers.[10,11] Thus, subjects with *LRRK2* mutations constitute an excellent cohort for studying preclinical and early PD.

In the present study, we examined CSF Aβ1–42 ($A\beta_{42}$) peptide, total tau (t-tau), and phosphorylated tau (p-tau) in symptomatic and asymptomatic *LRRK2* mutation carriers. CSF protein levels were correlated with dopamine denervation (assessed by PET measurements), to determine whether $A\beta_{42}$ and tau species are altered during early PD processes, and to explore the utility of these proteins as early or presymptomatic PD diagnostic or progression markers.

METHODS **Subjects.** This study focused on preclinical and asymptomatic *LRRK2* mutation carriers: 18 of the 26 carriers included in the study were asymptomatic at the time of evaluation. The remaining 8 subjects had clinically confirmed PD; most of them were at early disease stages that permitted long-range travel and limited confounding due to complicated CNS changes associated with advanced PD. Details on subject recruitment can be found in the e-Methods on the *Neurology*© Web site at www.neurology.org. Demographic and clinical information is listed in table 1 for all subjects.

Table 1 **Summary of demographics and PET values of subjects**

Subject	Source	Age, y	Sex	Mutation status	Clinical status	UPDRS III[a]	FD (k_{occ})[b]	DTBZ (BP_{ND})[b]	MP (BP_{ND})[b]
1,451	Norway	74	F	G2019S	PD	41	0.3831	0.1662	0.2051
1,454	Norway	69	F	G2019S	U	6	1.0824	0.8420	0.7905
1,457	Norway	60	M	G2019S	U	8	—	0.9504	0.9653
1,464	Norway	60	M	G2019S	U	7	0.9491	1.0489	0.9913
1,465	Norway	57	M	G2019S	U	4	0.9725	0.8173	0.7843
1,474	Norway	51	F	G2019S	U	5	0.8061	0.6640	0.6422
1,515	Norway	40	M	G2019S	U	7	1.0561	0.9743	0.7584
1,521	Norway	29	M	N1437H	U	2	1.0585	1.1693	0.8132
1,522	Norway	51	F	N1437H	PD	31	0.3854	0.2746	0.2264
1,523	Norway	50	F	N1437H	U	11	0.5273	0.3620	0.3035
1,526	Norway	26	M	N1437H	U	2	1.0094	0.9345	0.6876
1,529	Norway	30	M	N1437H	U	2	0.7339	1.0166	0.7537
1,539	US	80	M	G2019S[c]	PD	13	0.4759	0.4088	0.5153
1,540	US	77	M	G2019S[c]	PD	25	0.4113	0.2216	0.2442
1,541	US	56	F	R1441H	U	0	1.0138	1.0873	0.9621
1,542	US	55	M	R1441C	U	0	0.7791	0.8508	0.6589
1,543	US	61	F	R1441C	U	11	0.8858	0.8479	0.7901
1,544	US	57	M	R1441C	PD	7	0.9704	0.8878	0.9806
1,546	US	57	F	R1441C	U	0	0.8215	0.8584	0.7140
1,547	US	67	F	R1441C	PD	10	0.3771	0.3159	0.3188
2,241	Norway	55	F	G2019S	U	8	0.7326	0.6026	0.4629
2,246	Norway	47	F	G2019S	U	7	0.9997	1.0639	0.8189
2,250[d]	Japan	65	M	I2020T	PD	9	0.2328	0.2005	0.1914
2,251[d]	Japan	66	F	I2020T	PD	4	0.2579	0.1829	0.1536
2,252	Japan	62	M	I2020T	U	NA	0.9490	0.8032	0.6567
2,253[d]	Japan	59	M	I2020T	U	NA	0.9741	0.9950	0.8013

Abbreviations: DTBZ = ^{11}C-(+)-α-dihydrotetrabenazine; FD = ^{18}F-6-fluoro-L-dopa; MP = ^{11}C-d-*threo*-methylphenidate; PD = Parkinson disease; U = unaffected/asymptomatic; UPDRS = Unified Parkinson's Disease Rating Scale.

[a] Subject 1,523 had mainly mild left-sided bradykinesia and rigidity, as well as mild axial symptoms, but no tremor. She had no other signs or complaints consistent with Parkinson disease during the follow-up assessments. Subject 1,543's high UPDRS score might be due to a locomotion problem caused by other previous disease, rather than reflecting the parkinsonian phenotype. Some symptomatic subjects had low UPDRS scores, likely due to the medication (levodopa).

[b] Values shown are averages of left and right putamen expressed as a fraction of age-matched control values.

[c] Homozygote.

[d] Excluded from $A\beta_{42}$ analyses due to high blood contamination in the CSF sample.

Standard protocol approvals, registrations, and patient consents. This study was approved by the institutional review boards of all participating institutions. All individuals provided written informed consent to participate.

PET, CSF samples, and Luminex assays. Within 1 year of CSF sample collection, each subject was scanned with 3 tracers, ^{18}F-6-fluoro-L-dopa (FD, a marker for the uptake and decarboxylation of levodopa as well as the trapping of dopamine in synaptic vesicles), ^{11}C-(±)-α-dihydrotetrabenazine (DTBZ, a vesicular monoamine transporter 2 [VMAT2] ligand), and ^{11}C-d-*threo*-methylphenidate (MP, a dopamine transporter [DAT] ligand). CSF $A\beta_{42}$, t-tau, and p-tau levels of all cases were measured as previously described.[2] To minimize any age effects, PET data were normalized to age-matched control values. Details can be found in the e-Methods.

Statistical analysis. All analyses were performed with PASW Statistics 18.0 (SPSS Inc., Chicago, IL). Nonparametric correlation methods (Kendall rank correlation) were used to assess the relationship between the skewed PET measurements and the CSF biomarkers and to minimize the effects of outliers. See more details in the e-Methods.

RESULTS **PET measurements.** Subjects were scanned for putaminal tracer binding/uptake, and the average values of left and right putamen, expressed as a fraction of age-matched control values, are shown in table 1. Representative PET images from *LRRK2* PD patients, asymptomatic mutation carriers, and healthy controls are shown in figure e-1.

Except for subject 1,544, all *LRRK2* PD subjects had significantly reduced PET values for all 3 tracers (figure 1 and table 1). Among asymptomatic mutation carriers, 4 subjects had significantly reduced values for all 3 tracers; in subject 1,523, PET abnormalities demonstrated asymmetry, typical of sporadic PD. Subject 1,542 had significantly reduced MP (DAT) binding and FD uptake values only. In subject 1,526, DAT binding was significantly reduced; reduced DTBZ (VMAT2) binding was observed only in the right putamen. Subjects 1,454 and 2,246 showed borderline decrease in right putaminal FD uptake and DAT binding, respectively. All other asymptomatic mutation carriers demonstrated normal uptake/binding of all 3 tracers in the putamen. Subjects 1,465 and 1,543 showed abnormal VMAT2 and DAT binding in the caudate, but not the putamen. Subject 1,457 could not complete the FD scan.

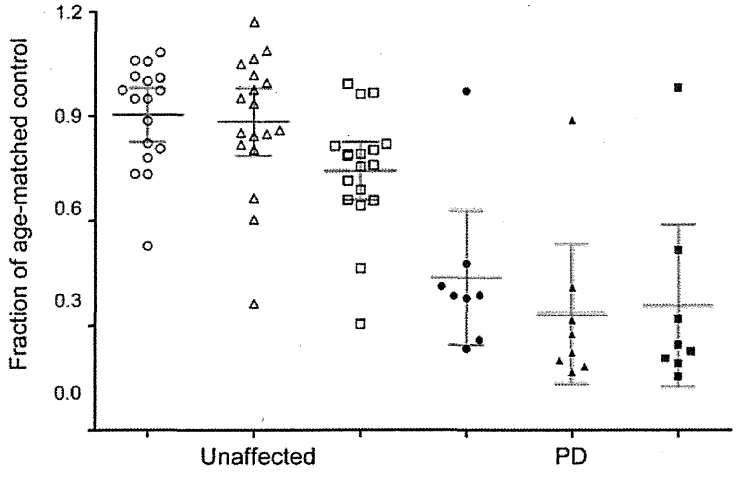
Correlation of CSF biomarkers with PET data. Blood contamination of CSF, which occasionally occurs during lumbar puncture collection, can significantly affect CSF levels of $A\beta_{42}$ (but not t-tau or p-tau).[2] To control for this variable, 3 subjects (1 *LRRK2* PD and 2 asymptomatic) whose CSF hemoglobin levels were >250 ng/mL were excluded from further analysis of $A\beta_{42}$.

Similar to our previous report in patients with sporadic PD,[2] CSF levels of all analytes were lower in symptomatic *LRRK2* mutation carriers compared to asymptomatic carriers (symptomatic [mean ± SD]: $A\beta_{42}$, 504.1 ± 128.5 pg/mL, t-tau, 24.4 ± 22.6 pg/mL, p-tau, 16.7 ± 3.8 pg/mL; asymptomatic: $A\beta_{42}$, 601.5 ± 234.1 pg/mL, t-tau, 36.4 ± 32.0 pg/mL, p-tau, 21.1 ± 9.0 pg/mL; individual values can be found in table e-1); but this trend did not reach significance, likely due to the limited number of subjects included. Additionally, because symptomatic subjects were older than asymptomatic carriers (mean 67.1 ± 9.8 vs 51.3 ± 12.3 years), the extent of decrease in CSF tau levels in *LRRK2* PD may have also been reduced by the known age-dependent increase in CSF tau levels ($A\beta_{42}$ is stable as a function of age in healthy controls).[2] Nonetheless, the correlations between CSF analyte levels and PET measurements are unlikely to be substantially affected by age because all the PET data were standardized to age-matched control values.

CSF levels of $A\beta_{42}$ in the entire *LRRK2* cohort decreased with decreasing uptake of FD (Kendall tau = 0.316, p = 0.040), indicating that decreasing protein levels correspond with progressive loss of dopamine function (figure 2A). Decreased levels of $A\beta_{42}$ also corresponded to lower values of the other PET tracers, as did decreased levels of t-tau or p-tau with all PET tracers, but these relationships did not reach significance (figure 2).

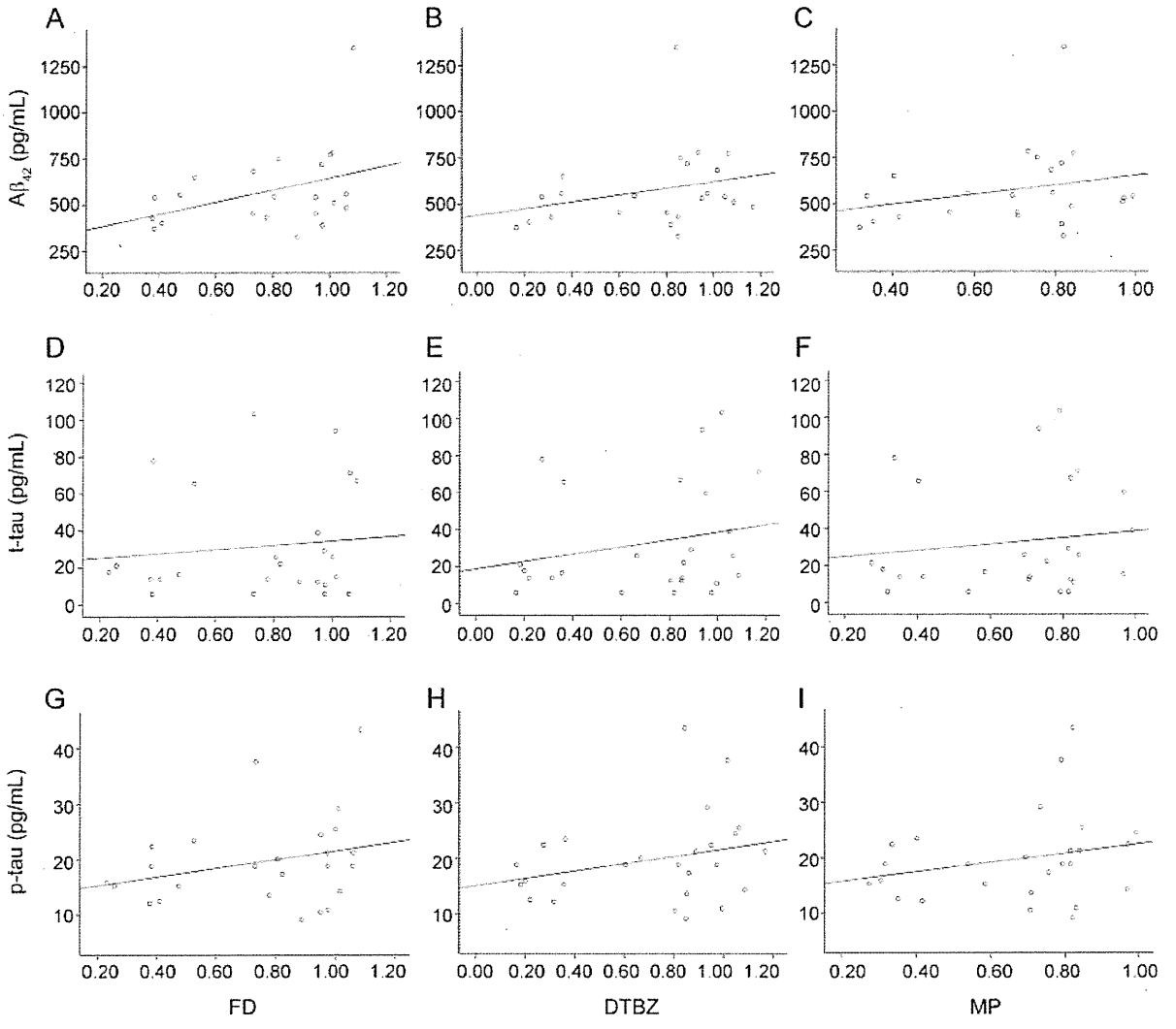
Because *LRRK2* mutations are heterogeneous, this analysis was repeated using the subset of patients

Figure 1 **Putaminal tracer binding/uptake in *LRRK2* mutation carriers**



The average left and right putaminal tracer binding/uptake values in *LRRK2* mutation carriers are given as a fraction of age-matched healthy control values. The means with 95% confidence intervals for asymptomatic (unaffected) subjects and subjects with clinically confirmed *LRRK2*–Parkinson disease (PD) are also shown. Circles indicate ^{18}F-fluoro-L-dopa (FD) uptake, triangles indicate ^{11}C-dihydrotetrabenazine (DTBZ) binding, and squares indicate ^{11}C-d-threo-methylphenidate (MP) binding.

Figure 2 **Correlation of CSF biomarkers with PET measurements in the entire *LRRK2* cohort**



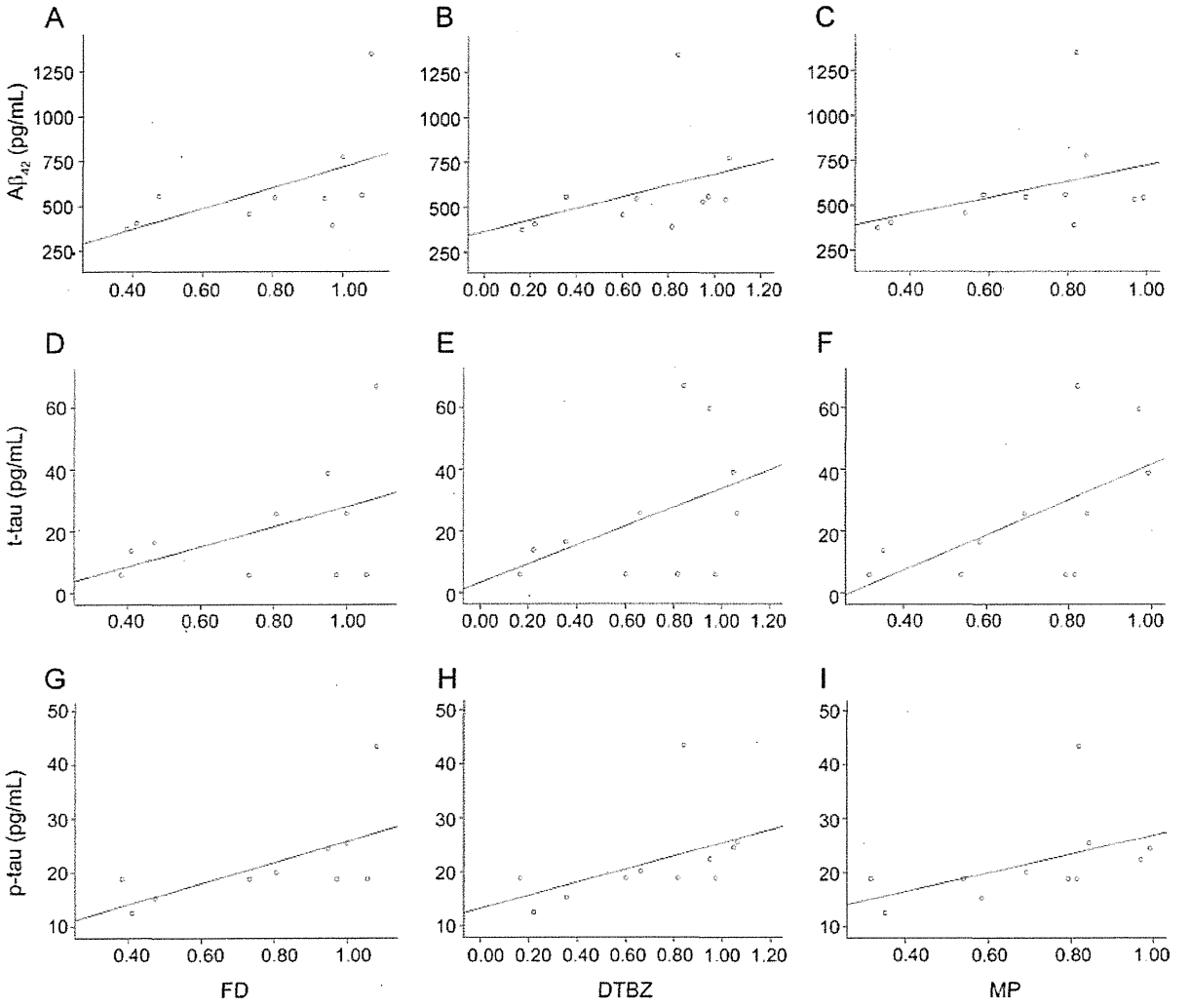
The correlations of protein levels in CSF with putaminal PET values expressed as fractions of age-matched healthy control values are shown. For $A\beta_{42}$ (A–C), FD: Kendall tau = 0.316, p = 0.040; DTBZ: Kendall tau = 0.223, p = 0.119; MP: Kendall tau = 0.217, p = 0.146. For total tau (t-tau) (D–F), FD: Kendall tau = 0.065, p = 0.656; DTBZ: Kendall tau = 0.182, p = 0.200; MP: Kendall tau = 0.119, p = 0.401. For phosphorylated tau (p-tau) (G–I), FD: Kendall tau = 0.226, p = 0.117; DTBZ: Kendall tau = 0.203, p = 0.151; MP: Kendall tau = 0.190, p = 0.178. $A\beta_{42}$ values restricted to 23 out of 26 cases due to high blood contamination in CSF, which does not influence t-tau or p-tau values. Note that regression lines were generated from linear regression for visualization only. DTBZ = ^{11}C-dihydrotetrabenazine; FD = ^{18}F-fluoro-L-dopa; MP = ^{11}C-d-threo-methylphenidate.

with the G2019S mutation (n = 11), the most common variant in our cohort. Remarkably, there was a clear reduction in the levels of all proteins with decreasing PET measurements in the striatum, regardless of the tracer used (figure 3). CSF levels of $A\beta_{42}$ and p-tau were both reduced with greater degrees of reduced FD uptake (i.e., diminished dopamine function; $A\beta_{42}$: Kendall tau = 0.556, p = 0.025; p-tau: Kendall tau = 0.597, p = 0.020). In addition, reduced CSF p-tau significantly correlated with reduced DTBZ binding (Kendall tau = 0.597, p = 0.013) and MP binding (Kendall tau = 0.559, p = 0.020), but t-tau only significantly correlated with MP binding (Kendall tau = 0.506, p = 0.037).

One symptomatic *LRRK2* PD subject (1,544) with normal PET measurements demonstrated relatively lowered t-tau and p-tau levels, i.e., behaved more like a patient with PD than a normal control, suggesting that CSF tau might be a more sensitive indicator than the PET measurements.

DISCUSSION Several independent investigations reported that CSF Aβ levels decreased (but not as substantially as in Alzheimer disease [AD]) in pa-

Figure 3 **Correlation of CSF biomarkers with PET measurements in *LRRK2* G2019S mutation carriers**



The correlations of protein levels in CSF with putaminal PET values expressed as fractions of age-matched healthy control values are shown ($\hat{n}$ = 11). For $A\beta_{42}$ (A–C), FD: Kendall tau = 0.556, p = 0.025; DTBZ: Kendall tau = 0.455, p = 0.052; MP: Kendall tau = 0.382, p = 0.102. For total tau (t-tau) (D–F), FD: Kendall tau = 0.339, p = 0.192; DTBZ: Kendall tau = 0.331, p = 0.199; MP: Kendall tau = 0.506, p = 0.037. For phosphorylated tau (p-tau) (G–I), FD: Kendall tau = 0.597, p = 0.020; DTBZ: Kendall tau = 0.597, p = 0.013; MP: Kendall tau = 0.559, p = 0.020. Note that the regression lines were generated from linear regression for visualization only. DTBZ = ^{11}C-dihydrotetrabenazine; FD = ^{18}F-fluoro-L-dopa; MP = ^{11}C-d-threo-methylphenidate.

tients with sporadic PD, particularly in those with cognitive impairment.[2,5,6,12,13] Additionally, studies with large cohorts (≥50 subjects in either sporadic PD or control group) suggest that, unlike what is typically described for AD, tau levels tend to decrease in PD compared to controls.[2,4,6] The obvious trend of decreasing CSF levels of $A\beta_{42}$ and tau in *LRRK2* PD and asymptomatic carriers (figure 2 and 3, table e-1) is in line with these observations. This raises the question of whether decreased concentrations of CSF tau and Aβ might be mechanistically involved in PD pathology. Tau pathology and amyloid plaques, though increasingly reported,[14,15] are not typically seen in the brains of patients with PD. Therefore, it is possible that the decrease in CSF tau and Aβ levels occurs as soluble tau/p-tau and Aβ oligomers, often considered more toxic,[16] deposit in the brains of patients with PD without formation of amyloid plaques and neurofibrillary (tau) tangles.

Regardless of the cause of decreased CSF $A\beta_{42}$, t-tau, and p-tau in asymptomatic and symptomatic mutation carriers, our data suggest that metabolism of Aβ and tau in brain is likely dysfunctional in *LRRK2*-related PD, even before motor symptoms appear. If the same is true of sporadic PD, then tau and amyloid pathology in patients with PD, rather than just indicating comorbidity or general aging pro-

cesses, might interact with synucleinopathy as a pathologic network that contributes to PD development and progression, even at premotor stages. In addition to experiments showing that both tau and Aβ might directly interact with α-synuclein, facilitating its aggregation,[17,18] this argument is further supported by recent independent genome-wide association studies indicating that the *SNCA* and *MAPT* genes/regions (encoding α-synuclein and tau, respectively) are consistently associated with an increased risk of developing PD.[19,20]

Our results, if applicable to sporadic PD, also suggest that Aβ_{42} and tau could potentially be used as biomarkers for early PD diagnosis. However, since they are not particularly strong predictors of the disease condition, it is likely that a combination of Aβ_{42} and tau with other markers will be necessary for effective preclinical diagnosis or monitoring disease progression. A major limitation in focusing on *LRRK2* mutation carriers is that this approach is logistically difficult in that it requires recruitment from a very limited pool of subjects, lengthy and potentially uncomfortable procedures (PET imaging and lumbar puncture), and in many instances, international travel. Thus, we were only able to study a modest number of participants, and because this was an exploratory study we did not perform statistical correction for multiple comparisons. Therefore, the data need to be interpreted with caution and the results should be replicated, ideally using a longitudinal design with serial PET scans and CSF measurements.

AUTHOR CONTRIBUTIONS

J.Z. conceived and supervised the project, and drafted the manuscript with M.S., J.O.A., T.S., and K.K.J., J.O.A., K.K.J., Z.K.W., R.J.U., K.H., T.Y., C.P.Z., H.M.K., and J.B.L. were responsible for patient characterization and sample collection. M.S. assisted in experimental design and execution, as well as in data interpretation and statistical analyses. V.S. and A.J.S. were responsible for PET analysis. C.G. worked on CSF sample handling and Luminex assays. J.A. worked on data management and statistical analyses. K.L.E. and K.W.S. were involved in statistical analyses. All authors critically reviewed the manuscript.

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The Nitric Oxide-Cyclic GMP Pathway Regulates FoxO and Alters Dopaminergic Neuron Survival in *Drosophila*

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Abstract

Activation of the forkhead box transcription factor FoxO is suggested to be involved in dopaminergic (DA) neurodegeneration in a *Drosophila* model of Parkinson's disease (PD), in which a PD gene product LRRK2 activates FoxO through phosphorylation. In the current study that combines *Drosophila* genetics and biochemical analysis, we show that cyclic guanosine monophosphate (cGMP)-dependent kinase II (cGKII) also phosphorylates FoxO at the same residue as LRRK2, and *Drosophila* orthologues of cGKII and LRRK2, DG2/For and dLRRK, respectively, enhance the neurotoxic activity of FoxO in an additive manner. Biochemical assays using mammalian cGKII and FoxO1 reveal that cGKII enhances the transcriptional activity of FoxO1 through phosphorylation of the FoxO1 S319 site in the same manner as LRRK2. A *Drosophila* FoxO mutant resistant to phosphorylation by DG2 and dLRRK (dFoxO S259A corresponding to human FoxO1 S319A) suppressed the neurotoxicity and improved motor dysfunction caused by co-expression of FoxO and DG2. Nitric oxide synthase (NOS) and soluble guanylyl cyclase (sGC) also increased FoxO's activity, whereas the administration of a NOS inhibitor L-NAME suppressed the loss of DA neurons in aged flies co-expressing FoxO and DG2. These results strongly suggest that the NO-FoxO axis contributes to DA neurodegeneration in *LRRK2*-linked PD.

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Introduction

PD, one of the most common movement disorders, is characterized by age-dependent impairments of several nervous systems including the midbrain DA system. The degeneration of DA neurons in the substantia nigra produces the prominent motor symptoms of PD. Postmortem inspections and studies with neurotoxin-based PD models suggest a multifactorial etiology involving inflammation, mitochondrial dysfunction, iron accumulation and oxidative stress. NO, a free gaseous signaling molecule, has also been implicated in PD [1,2]. The signaling function of NO is dependent on the dynamic regulation of its synthase, NOS. There are three types of NOS, neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), in humans whereas the *Drosophila* genome has only a single orthologue, dNOS. High levels of nNOS and iNOS have been reported in the substantia nigra of PD patients [3,4] and animal models of PD [5,6]. Overproduction of NO is suggested to cause DNA damage, protein modifications and cell toxicity mainly mediated by the reactive species peroxynitrite, which may be generated with dopamine metabolism in DA neurons. In the etiology of PD, overproduction of NO could be caused either by upregulation of iNOS in activated glia cells [3,5] or by an increase in intracellular calcium, for example, after glutamate excitotoxicity [7].

The discovery of genes linked to rare familial forms of PD has provided vital clues to understanding the cellular and molecular pathogenesis of the disease. Missense mutations in the *Leucine-rich repeat kinase 2 (LRRK2)/Dardarin* gene cause autosomal dominant late onset familial PD as well as sporadic PD [8,9,10]. The clinical symptoms and pathology caused by *LRRK2* mutations closely resemble those of the sporadic form of PD, suggesting that the LRRK2 pathogenic pathway may underlie the general PD etiology. The *LRRK2* gene encodes a large protein with multiple domains including a GTPase domain and a kinase domain [8,9]. Several amino acid substitutions are identified as pathogenic mutations linked to PD [11]. Mutations in the kinase domain of human LRRK2 such as G2019S and I2020T have been reported to produce enhanced kinase activity *in vitro*, suggesting that gain-of-function mutations of LRRK2 cause neurodegeneration [12,13,14]. However, how these mutations present in the LRRK2 gene lead to the progressive loss of DA neurons and other associated pathologies is still unknown.

Because various key signaling pathways are conserved between humans and *Drosophila*, genetic and functional studies using *Drosophila* models for familial PD have revealed crucial signal transductions that affect the pathogenesis of PD [15]. We have previously reported that a *Drosophila* LRRK2 orthologue, dLRRK phosphorylates *Drosophila* FoxO (dFoxO) at Ser259, which stimulates the expression of a pro-apoptotic dFoxO target, *hid*, and leads to neurodegeneration in *Drosophila* [16]. The event was further enhanced by transgenic expression of pathogenic dLRRK proteins such as dLRRK I1915T (corresponding to I2020T in humans). However, a kinase-dead form of dLRRK (dLRRK 3KD) did not completely suppress a synergic effect caused by the co-expression of dFoxO with dLRRK, suggesting that some other factor(s) modulates this pathway. Here, we report that cGKII also phosphorylates FoxO and activates FoxO-transcriptional activity in the same manner as LRRK2/dLRRK by using biochemical studies of mammalian cGKII and FoxO1. Moreover, by using *Drosophila* models, our data suggest that NO signaling and its downstream effector cGKII/DG2 contribute to DA neurodegeneration.

Results

cGK genetically interacts with FoxO and activates FoxO activity

We previously reported a genetic interaction between FoxO and LRRK2/dLRRK in *Drosophila* [16]. To identify components of the LRRK2-FoxO signaling pathway, we screened for modifiers (Fig. 1 and Fig. S1A). Kinases reported to affect the activity of FoxO were expressed with dFoxO in the *Drosophila* eye. As reported, transgenic expression of AKT suppressed FoxO-mediated developmental defects in the eye. The expression of MST/Hippo resulted in extensive degeneration, which did not appear to be dependent on FoxO (Fig. 1). Expression of one of the *Drosophila* cGMP-dependent kinases (cGKs), DG2, leads to strong optic degeneration in conjunction with dFoxO (Fig. 1 and Fig. S2A), while the other kinases had little effect on the developmental defects caused by FoxO (Fig. 1). Removal of one copy of the *dg2* gene improved the defects, suggesting that endogenous DG2 activity contribute to the dFoxO-mediated neurodegeneration (Fig. 2H compared with B).

Next we examined whether DG2 is an upstream kinase of dLRRK, or whether DG2 acts independently of dLRRK by means of a combination of genetic interaction tests, reporter assays for FoxO and *in vitro* kinase assay. Co-expression of dLRRK harboring a PD-related mutant I1915T together with DG2 dramatically enhanced the toxicity of dFoxO (Fig. 2D compared with C). However, expression of dLRRK 3KD or removal of the dLRRK gene did not suppress the eye phenotype caused by dFoxO-DG2 at all (Fig. 2E and J compared with C). Co-expression of DG2 and dLRRK I1915T produced a normal eye, suggesting that the phenotype is dependent on the level of dFoxO protein (Fig. 2I compared with D).

Co-expression of dFoxO with DG2, but not GFP or DG1, in *Drosophila* eyes caused appearance of a slower migrated dFoxO protein in western blot analysis (Fig. 3A), which indicates phosphorylation of dFoxO [16]. Consistent with the result, knockdown of DG2 decreased a phosphorylated form of endogenous dFoxO in *Drosophila* brain tissue (Fig. 3B). In *Drosophila* S2 cells, transient expression of DG2 together with 8-bromoguanosine-3′, 5′-cyclic monophosphate (8-Br-cGMP), a membrane permeable analogue for cGMP, also stimulates phosphorylation of endogenous dFoxO (Fig. 3C, lane 3).

Two groups of cGKs, the soluble type I (cGKI α and β) and the membrane-bound type II (cGKII), have been reported in vertebrates. In *Drosophila*, there are two genes encoding cGK, namely *dg1* and *dg2* [17]. As reported [18], the gene products DG1 and DG2 are located in the cytoplasm and at the cytoplasmic membrane, respectively (Fig. 3E and F). Interestingly, expression of DG1 had little effect on the degeneration of the eye mediated by dFoxO, suggesting that DG1 and DG2 have different roles *in vivo* (Fig. S1B, S2B and S2C). Although predictions of amino acid sequence indicate that DG2 is more similar as a cGKI α/β homologue [19], their subcellular distribution suggests that DG2 is functionally more similar to cGKII (Fig. 3G–J) [18,20,21]. Consistent with the idea, transgenic expression of human cGKII exacerbated eye degeneration by dFoxO (Fig. 2G compared with B) whereas expression of cGKII alone did not affect the eye development (Fig. 2F). Interestingly, cGKII appeared to recruit FoxO1 to the cytoplasmic membrane of human cultured cells (Fig. 3K–M) while there was no evidence that cGKI associates with cGKII *in vivo* (Fig. S3). In addition, we observed that cGKII is

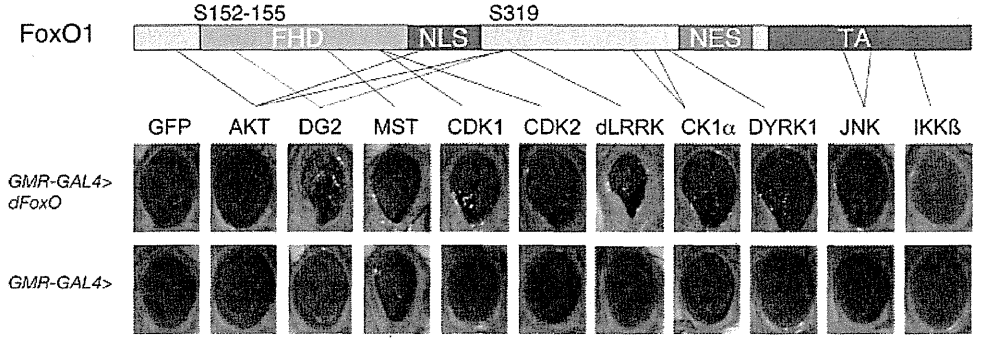

Figure 1. Screening of kinases that affect the eye phenotypes caused by dFoxO. *Drosophila* orthologues of reported FoxO kinases were expressed with (upper row) or without (lower) dFoxO in *Drosophila* eyes using the *GMR-GAL4* driver. GFP served as a control. The *Drosophila* DG2 is presumably functionally equivalent to the vertebrate cGKII. Reported phosphorylation sites and newly identified sites that are phosphorylated by cGKII (S152–155 and S319) in human FoxO1 are indicated by black and red lines, respectively. FHD, forkhead domain; NLS, nuclear localization signal; NES, nuclear export signal; TA, transactivation domain. Overexpressing lines used for crosses are: *UAS-GFP* (GFP), *UAS-AKT1* (AKT), *UAS-DG2* (DG2), *UAS-hippo* (MST), *UAS-CDK1-Myc* (CDK1), *UAS-CDK2-Myc* (CDK2), *UAS-dLRRK* (dLRRK), $Cdk\alpha^{EP1555}$ (CK1α), $mnb^{EY14320}$ (DYRK1), *UAS-bsk* (JNK), *UAS-dIKKß* (IKKβ).
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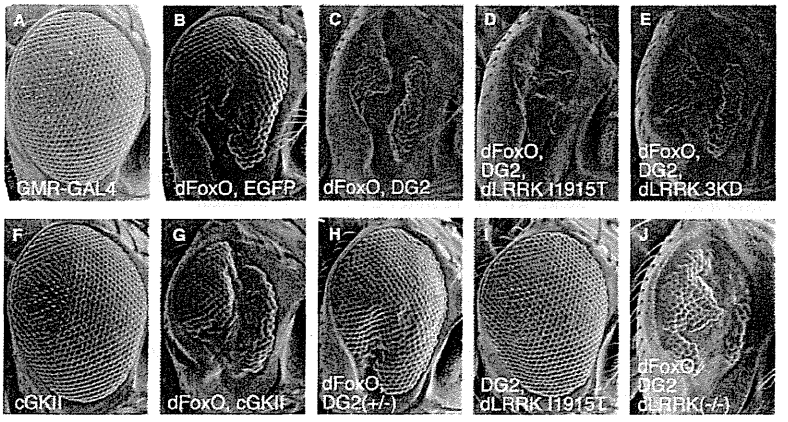

Figure 2. DG2 as well as dLRRK additively enhances FoxO-mediated developmental defects in the *Drosophila* eye. (**A–J**) SEM images of the eye of flies expressing the indicated genes. The genotypes are: *GMR-Gal4* (**A**), *GMR-Gal4/UAS-EGFP* (**B**), *UAS-DG2; GMR-Gal4, UAS-dFoxO* (**C**), *UAS-DG2; GMR-Gal4, UAS-dFoxO; UAS-dLRRK I1915T* (**D**), *UAS-DG2; GMR-Gal4, UAS-dFoxO; UAS-dLRRK 3KD* (**E**), *GMR-Gal4; UAS-cGKII* (**F**), *GMR-Gal4, UAS-dFoxO; UAS-cGKII* (**G**), *GMR-Gal4, UAS-dFoxO/DG2^{k04703}* (**H**), *UAS-DG2; GMR-Gal4; UAS-dLRRK I1915T* (**I**), *UAS-DG2; GMR-Gal4, UAS-dFoxO; e03680/e03680* (**J**).
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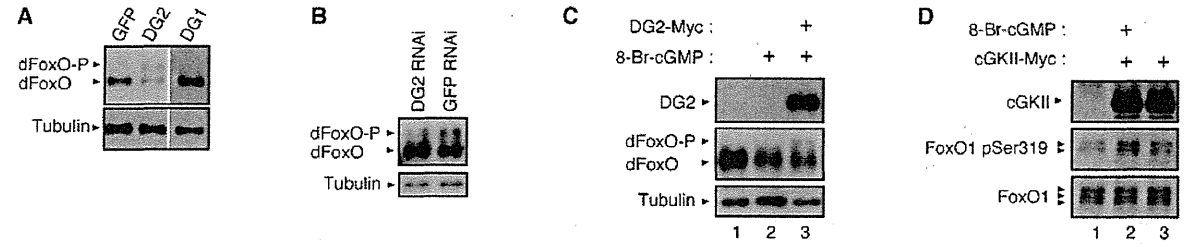

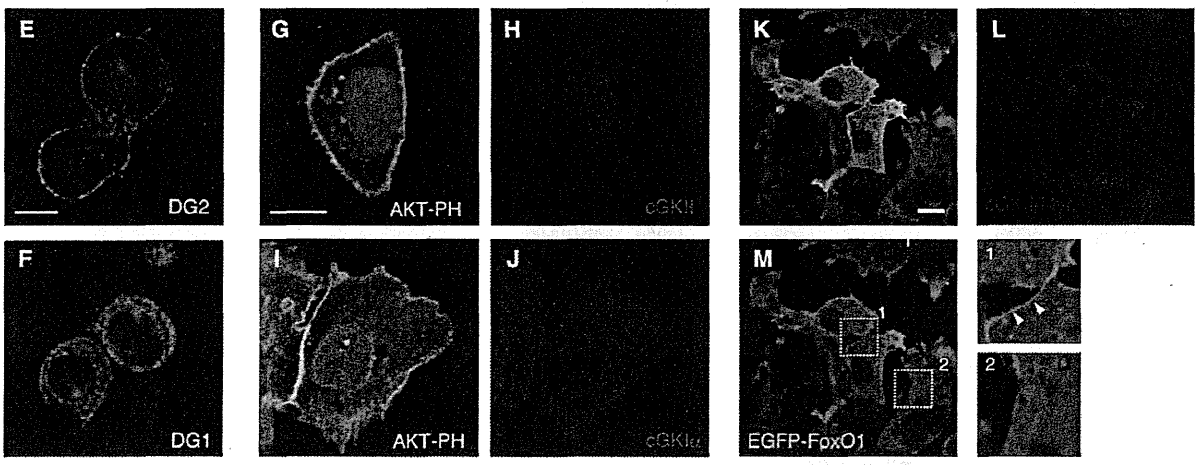

Figure 3. DG2 modulates FoxO *in vivo*. (**A**) dFoxO and the indicated transgenes were expressed in the *Drosophila* eyes using the *GMR-GAL4* driver. Extracts from brain tissues were subjected to western blot analysis. dFoxO-P; a phosphorylated form of dFoxO. (**B**) DG2 RNAi or GFP RNAi constructs were expressed in the *Drosophila* brain using the *elav-GAL4* driver. Western blot analysis for endogenous dFoxO was carried out as in (**A**). (**C**) *Drosophila* S2 cells were transfected with or without C-terminally Myc-tagged DG2 (DG2-Myc). Thirty-six hrs post transfection, cells were treated with or without 10 μM 8-Br-cGMP for 30 min. Cell lysate were then subjected to western blot analysis. (**D**) Human 293T cells were transfected with or without cGKII-Myc, and were treated with 8-Br-cGMP as in (**C**). Phosphorylation of the S319 site in endogenous FoxO1 was detected with phospho-specific antibody. (**E, F**) S2 cells expressing DG2-Myc (**E**) or DG1-Myc (**F**) were visualized with anti-Myc antibody (green), by counterstaining with DAPI (blue color). (**G–J**) HeLa cells expressing AKT-PH-GFP (green) along with cGKII-Myc (**G, H**) or cGKI-Myc (**I, J**) were visualized with anti-Myc antibody (red), by counterstaining with DAPI (blue color). AKT-PH-GFP was used for a marker protein of the plasma membrane [62]. (**K–M**) Flp-In T-REx-293 cells harboring EGFP-FoxO1 gene were transiently transfected with cGKII-Myc, and EGFP-FoxO1 was induced with doxycycline. Enlarged views of the plasma membrane regions in cGKII-positive (Box1) and negative (Box2) cells are also shown in (**M**). Accumulation of FoxO1 along with cGKII in the plasma membrane is indicated by arrowheads. Scale bars = 5 μm for (**E, F**), 25 μm for (**G–J**) and 10 μm for (**K–M**).
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abundantly expressed in DA neurons in the substantia nigra of mice (Fig. S4). We then focused on mammalian cGKII as a cGK that might be associated with the pathology of PD. Reporter assays for FoxO transcriptional activity revealed that cGKII stimulated FoxO activity in cultured mammalian cells and that co-expression of hLRRK2 with cGKII caused a 3-fold increase in FoxO activity (Fig. 4A). A kinase-dead form of hLRRK2 (hLRRK2 3KD) did not suppress the activation of FoxO by cGKII to the control level. Similarly, a kinase-dead form of cGKII (cGKII KD) failed to suppress FoxO's activation by LRRK2 (Fig. 4B). The results of the genetic interaction tests and the reporter assays suggested that cGKII and LRRK2 have additive effects on the regulation of FoxO activity.

cGK directly phosphorylates FoxO in vitro

Previously, we have demonstrated that LRRK2 phosphorylates, and enhances the neurotoxic activity of, FoxO. Using *in vitro* kinase assays, we tested whether cGKII stimulates the kinase activity of LRRK2 through phosphorylation, or whether cGKII directly activates FoxO as shown in a study on LRRK2 [16]. We transfected HEK293 cells with a FLAG-tagged cGKII or FLAG-cGKII KD plasmid and affinity-purified these proteins using anti-FLAG columns (Fig. 5B). We observed that cGKII WT but not KD specifically phosphorylated GST-FoxO1 in the presence of cGMP (Fig. 5C), and that cGKII targeted at least two sites of FoxO1, which were in FoxO-N and FoxO-C (Fig. 5A and D). A previous report has shown that cGKIα phosphorylates the human FoxO1 forkhead domain mainly at S152–155 and S184, by which the DNA-binding activity of FoxO1 is abolished [22]. We found that cGKII also phosphorylates FoxO1 at S152–155 and that these residues are major sites of phosphorylation in FoxO-N (Fig. S5A and B). However, the replacement of serine with alanine at S152–155 had little effect on the FoxO-transcriptional stimulation by cGKII and the binding to 14-3-3ε protein, which regulates the cytosolic localization of FoxO, in this context (Fig. S5C and D). Next, we determined phosphorylation sites in FoxO-C. Experiments with several truncated FoxO1 mutants narrowed down the phosphorylation sites in FoxO-C and identified S319 as a major phospho-residue targeted by cGKII (Fig. 5E and F). We also confirmed that overexpression of cGKII in the presence of 8-Br-cGMP stimulates the phosphorylation of the FoxO1 S319 site in human cultured cells (Fig. 3D, lane 2). Although cGKIα also phosphorylated GST-tagged full-length FoxO1 *in vitro*, the S319 site did not appear to be a major phosphorylation site (Fig. S6). The S319 site was also targeted by LRRK2 as shown previously (Fig. 5F) and co-incubation of cGKII and LRRK2 enhanced phosphorylation of the FoxO-C fragment in *in vitro* kinase assays (lane 5 compared with lane 1 in Fig. 5G). In contrast to the phosphorylation of FoxO at S152 155, the replacement of serine with alanine at S319 suppressed FoxO-transcriptional activity and abolished cGKII-mediated stimulation of FoxO, suggesting that phosphorylation at S319 has a major effect on the activity mediated by cGKII as well as LRRK2 (Fig. 4C) [16].

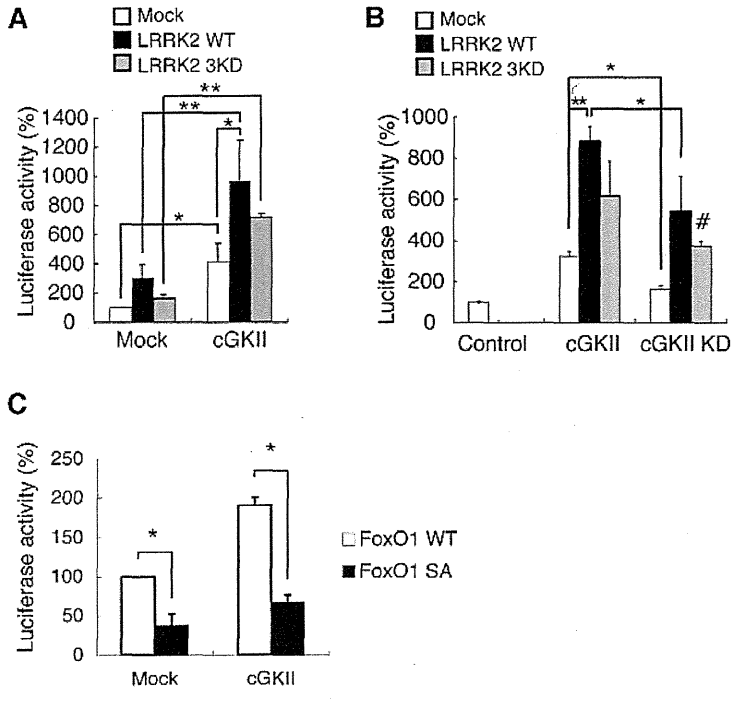

Figure 4. cGKII stimulates FoxO-transcriptional activity. **(A, B)** cGKII and LRRK2 additively stimulate FoxO-transcriptional activity. FoxO-transcriptional activity was measured in extracts prepared from 293T cells transfected with the indicated plasmids and a plasmid for FoxO1, a FoxO reporter plasmid containing *Firefly* luciferase, and a plasmid for *Renilla* luciferase to monitor the transfection efficiency. The relative FoxO-transcriptional activity (*Firefly* luciferase activity) normalized to *Renilla* luciferase activity is presented. Data are presented as the mean ± SE for three independent experiments. β-galactosidase (Mock) served as a transfection control. **(C)** Introduction of the S319A (SA) mutation in FoxO1 reduced FoxO activity. Data are presented as the mean ± SE for three independent experiments. *, $p<0.05$; **, $p<0.01$. Co-transfection of kinase-dead forms of cGKII and LRRK2 also sitmulated FoxO (#, $p<0.05$ *vs.* Control in **B**).
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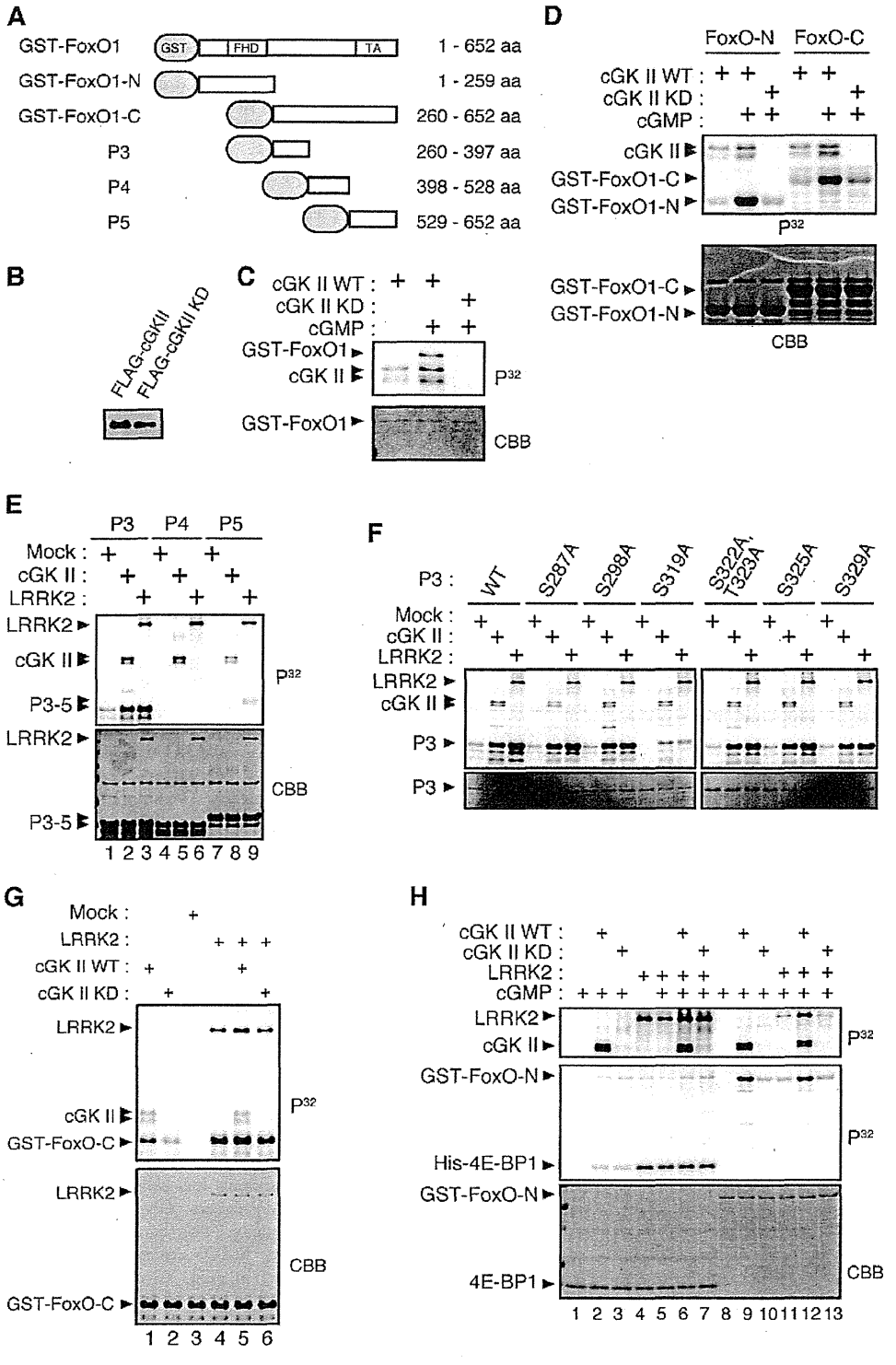

Figure 5. cGKII phosphorylates FoxO1 *in vitro*. (**A**) The recombinant FoxO1 proteins used as substrates. GST, GST-tag. Numbers indicate corresponding amino acid residues of FoxO1. (**B**) FLAG-tagged cGKII and FLAG-cGKII KD were immunoprecipitated from FLAG-tagged cGKII or FLAG-cGKII KD-transfected 293T cells as kinase sources. Western blotting confirmed that the amounts of the two proteins obtained were similar. (**C, D**) *In vitro* kinase assays of cGKII using recombinant GST-FoxO1 as a substrate. In the presence of cGMP, cGKII WT but not cGKII KD phosphorylated GST-FoxO, GST-FoxO-N, and GST-FoxO-C. Autoradiography (P^{32}) and Coomassie brilliant blue (CBB) staining of the gels are shown. Note cGKII proteins by

CBB staining were difficult to detect in spite of the presence of autophosphorylation signals of cGKII (cGKII in P^{32}) **(E)** cGKII and LRRK2 phosphorylated the P3 but not P4 or P5 protein. Autophosphorylation signals of cGKII and LRRK2 are also shown (cGKII and LRRK2 in P^{32}). The mock immunoprecipitate (Mock) served as a control. **(F)** *In vitro* kinase assay using P3 and a series of P3 mutants where the candidate phosphorylation residues are replaced with alanine (refer to [16] for information on the mutated residues). The phosphorylation by cGKII or LRRK2 was decreased in the P3 S319A mutant. **(G)** Co-incubation of cGKII and LRRK2 enhanced GST-FoxO-C phosphorylation. **(H)** cGKII failed to stimulate LRRK2 kinase activity and LRRK2 failed to stimulate cGKII kinase activity. His-tagged 4E-BP1 and GST-FoxO-N served as LRRK2-specific and cGKII-specific substrates, respectively.
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cGK phosphorylates LRRK2, but does not affect the kinase activity of LRRK2 in vitro

To examine the possibility that cGKII activates the kinase activity of LRRK2, or that LRRK2 activated cGKII, we further performed *in vitro* kinase assays using 4E-BP1 and FoxO-N as substrates (Fig. 5H). As reported [14], LRRK2 specifically phosphorylated 4E-BP1, which is not dependent on cGMP, while cGKII failed to do so (lanes 4 and 5 compared with lane 2 in Fig. 5H). cGKII and cGKII KD had little effect on the kinase activity of LRRK2 toward 4E-BP1 (lanes 6 and 7 *vs.* lanes 4 and 5 in Fig. 5H). cGKII but not cGKII KD or LRRK2 effectively phosphorylated FoxO-N (lane 9 compared with lanes 10 and 11 in Fig. 5H). Again LRRK2 had little effect on the kinase activity of cGKII toward FoxO-N (lane 12 compared with lanes 9 and 13 in Fig. 5H). However, cGKII also appeared to phosphorylate LRRK2 without modifying the kinase activity of LRRK2 (lane 6 *vs.* lanes 5, and lane 12 *vs.* lane 11 in Fig. 5H and Fig. S7). The *in vitro* observation that cGKII and LRRK2 act independently was consistent with the results of the genetic test (Fig. 2) and the reporter assay (Fig. 4).

Phosphorylation of FoxO by DG2 as well as dLRRK causes DA neurodegeneration

We next examined the pathological consequence of the phosphorylation of FoxO by DG2 and dLRRK in *Drosophila*. Ubiquitous or pan-neuronal expression of DG2 or dFoxO using GAL4 drivers for constitutive expression caused death. We then employed the mifepristone-inducible GAL4 system (GeneSwitch-GAL4) that drives the tissue-specific expression of upstream activating sequence (UAS)-constructs in post-mitotic cells. Pan-neuronal co-expression of dFoxO with DG2, but not the expression of either dFoxO or DG2 alone, caused significant neuronal loss in the PPM1/2 cluster Tyrosine hydroxylase (TH)-positive neurons of the adult brain (Fig. 6A). Expression of dLRRK I1915T exacerbated the neurotoxicity mediated by dFoxO and DG2 co-expression (Fig. 6A). In this context, the introduction of the S259A mutation, which corresponds to S319A in human FoxO1, attenuated the toxic interaction of dFoxO with DG2 (Fig. 6B). Consistent with the viability of TH-positive neurons, the motor activity of the flies expressing dFoxO and DG2 was impaired (Fig. 6C). Co-expression of dLRRK I1915T further worsened the motor dysfunction (Fig. 6C). Treatment with 1 mM L-3,4-dihydroxyphenylalanine (L-DOPA) significantly improved the locomotor activity of dFoxO and DG2-coexpressing flies (Fig. 6D), suggesting that the reduction in motor activity reflects DA degeneration. The expression of only DG2 mildly affected lifespan (Fig. 6E), whereas the co-expression of DG2 and dFoxO significantly shortened lifespan (Fig. 6E). However, the dFoxO S259A mutation failed to suppress the decrease in lifespan caused by the co-expression of dFoxO and DG2, suggesting that the toxic interaction of DG2 with dFoxO that affects lifespan is produced by a different mechanism rather than phosphorylation at S259 by DG2 (Fig. 6F). We then examined whether endogenous dFoxO contributes to DG2-mediated toxicity in *Drosophila* (Fig. 7A and B). Pan-neuronal expression of DG2 alone by the GeneSwitch-GAL4 driver caused mild motor defect (Fig. 7A). Removal of one copy of functional FoxO allele had little effect on the motor function (Fig. 7A) and lifespan (Fig. 7B) whereas it partly suppressed DG2-mediated motor dysfunction (Fig. 7A) and reduction in lifespan (Fig. 7B). These results suggested that endogenous dFoxO is also involved in neurodegeneration by DG2.

NO signal leads to DA neurodegeneration through DG2-FoxO

The activation of cGK requires cGMP. cGMP is generated by the NO-mediated activation of sGCs as well as ligands-mediated activation of receptor GCs [23,24,25]. However, as NO generated by NOS has been implicated in PD, the role of NOS-sGC was investigated via functional assays in *Drosophila*. We tested whether the *Drosophila* NO signal components dNOS and sGC are indeed involved in FoxO and DG2-mediated DA neurodegeneration in *Drosophila* (Fig. 8). Genetic interaction tests showed that co-expression of dNOS enhances the FoxO-mediated degeneration in the eye (Fig. 8B). In contrast, knockdown of sGC α or β subunits partially improved the phenotype of dFoxO expression (Fig. 8C and D). Moreover, knockdown of sGCα or removal of one copy of the DG2 genes improved the eye degeneration caused by co-expression of dFoxO with dNOS (Fig. 8E and F compared with B). In the context of pan-neuronal expression of FoxO and DG2 in *Drosophila*, treatment with a NOS inhibitor, Nω-Nitro-L-Arginine-Methyl-Ester (L-NAME), but not the inactive D-enantiomer D-NAME, significantly suppressed loss of the PPM1/2 and PPL1 cluster DA neurons (Fig. 9A E). In this setting, L-NAME treatment specifically reduced phosphorylation of dFoxO (Fig. 9F). The endogenous function of dNOS-DG2 signaling in DA neurodegeneration was estimated by survival assays of DG2 or dNOS mutant flies administrated with a PD-related toxin, paraquat, where both mutant lines showed significant resistances to paraquat (Fig. 9G). These results suggested that DG2/cGKII activated by NO signal could affect the survival of DA neurons through FoxO.

Discussion

We have previously demonstrated that dLRRK/LRRK2 phosphorylates and stimulates FoxO, which confers neurotoxic activity to FoxO, activating the expression of pro-apoptotic proteins such as Bim/Hid [16]. Searching for LRRK2-FoxO signaling components, we found that *Drosophila* cGK DG2 also exacerbates FoxO-mediated neurotoxicity. The current study suggests that cGKII/DG2 activates FoxO similar to, but independently of, LRRK2. However, in spite of the similar activation mechanism, the genetic results suggested that the Hid-DIAP-Dronc pathway is not a major cause of the optic degeneration by DG2-FoxO (Fig. S8A–D). Supporting this result, a quantitative RT-PCR analysis showed that DG2 or DG2/dFoxO does not effectively stimulate FoxO-mediated transactivation of *hid* as well as *4E-BP* (Fig. S8E and F). We attempted to determine downstream effector(s) of DG2-dFoxO using a combination of microarrays, real-time PCR and *Drosophila* genetic screening, but could not identify any candidate genes, suggesting

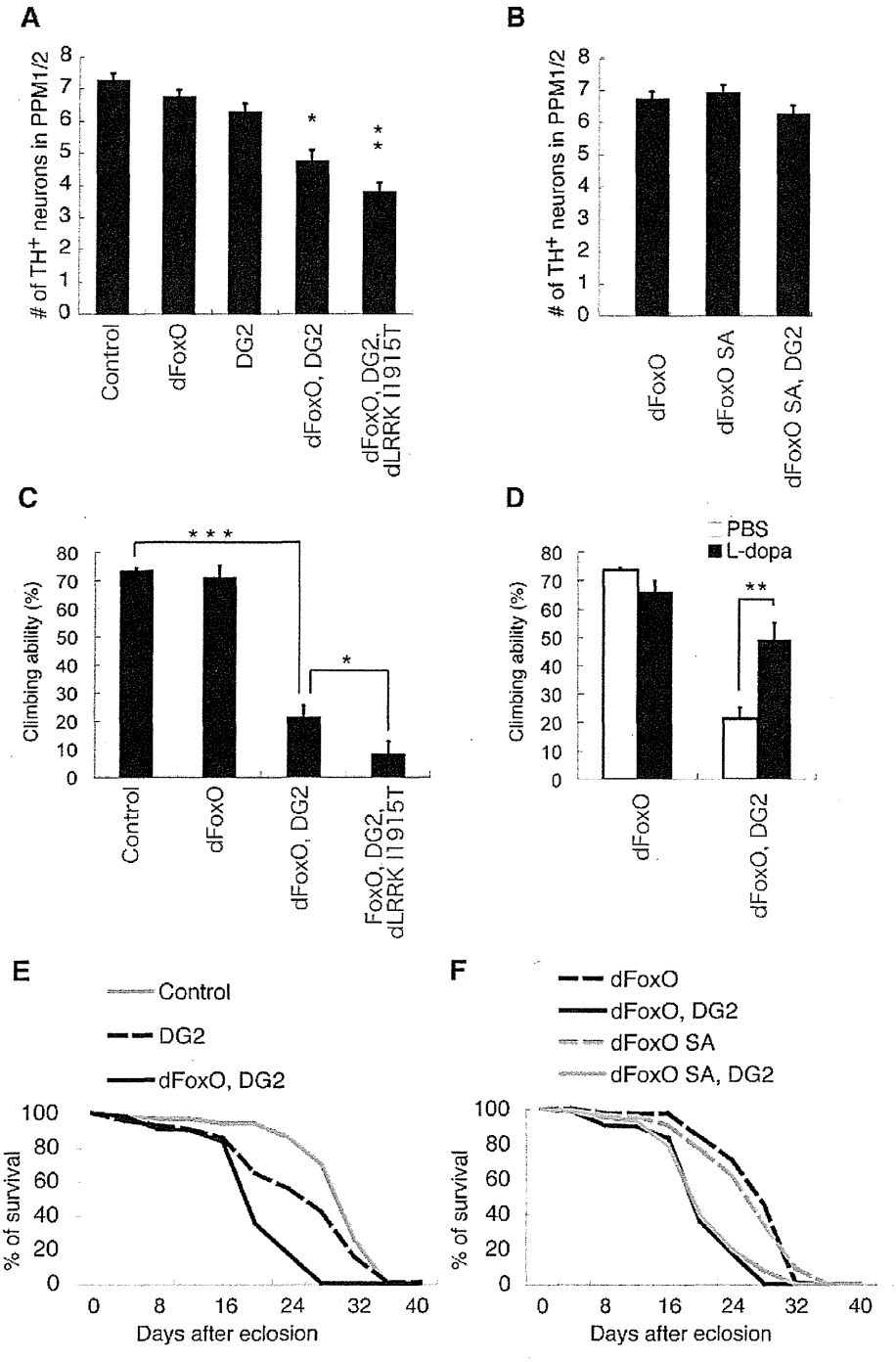

Figure 6. Neuronal activation of dFoxO by DG2 affects the maintenance of DA neurons in *Drosophila*. **(A)** The number of protocerebral posterior medial (PPM) 1/2 clusters of Tyrosine hydroxylase (TH)-positive DA neurons in 24-day-old adult flies. Neuron-specific expression of dFoxO, dLRRK I1915T and/or DG2 was induced following the administration of the activator RU486 (25 μg/mL) in the *elav-GeneSwitch-GAL4* (*elav-GS*) crosses. *elav-GS*/+ served as a control. Data are presented as the mean ± SE for three repeated experiments (*, $P<0.05$; **, $p<0.01$). **(B)** Co-expression of the dFoxO S259A (SA) mutant with DG2 suppressed the loss of PPM 1/2 TH-positive neurons. Flies were treated as in **(A)**. **(C)** Adult aged flies expressing dFoxO and DG2 under the control of *elav-GS* showed motor defects, while the expression of dFoxO alone had little effect. The values represent means ± SE for 20 trials in six independent experiments (*, $p<0.05$; ***, $p<0.001$). **(D)** Treatment with 1 mM L-DOPA in phosphate-buffered saline (PBS), but not with PBS alone, for 4 days rescued the loss of climbing ability in dFoxO and DG2-expressing flies. dFoxO served as a control. The values represent means ± SE for 20 trials in six independent experiments (**, $p<0.01$). **(E)** Flies from each genotype were subjected to survival assays