Melatonin attenuates hLRRK2-induced sleep disturbances and synaptic dysfunction in a *Drosophila* model of Parkinson's disease

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Abstract. Sleep problems are the most common non-motor symptoms in Parkinson's disease (PD), and are more difficult to treat than the motor symptoms. In the current study, the role of human leucine-rich repeat kinase 2 (hLRRK2), the most common genetic cause of PD, was investigated with regards to sleep problems, and the therapeutic potential of melatonin in hLRRK2-associated sleep problems was explored in *Drosophila*. hLRRK2 was selectively expressed in the mushroom bodies (MBs) in *Drosophila* and sleep patterns were measured using the *Drosophila* Activity Monitoring System. MB expression of hLRRK2 resulted in sleep problems, presynaptic dysfunction as evidenced by reduced miniature excitatory postsynaptic current (mEPSC) and excitatory postsynaptic potential (EPSP) frequency, and excessive synaptic plasticity such as increased axon bouton density. Treatment with melatonin at 4 mM significantly attenuated the sleep problems and rescued the reduction in mEPSC and EPSP frequency in the hLRRK2 transgenic flies. The present study demonstrates that MB expression of hLRRK2 in flies recapitulates the clinical features of the sleep disturbances in PD, and that melatonin attenuates hLRRK2-induced sleep disorders and synaptic dysfunction, suggesting the therapeutic potential of melatonin in PD patients carrying LRRK2 mutations.

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Introduction

Parkinson's disease is the second most common neurodegenerative disease and affects nearly 1% of the worldwide population over 65 (1,2). Traditionally, PD is regarded as the most common movement disorder due to the fact that the majority of patients with PD present with predominantly motor symptoms including tremor, rigidity, slow movements and gait problems (3). However, non-motor symptoms are also very common in PD. For example, sleep problems, the most frequent non-motor symptoms, affect 65-95% of patients (4-7). Furthermore, the non-motor symptoms may equally or more adversely affect the quality life of patients with PD.

Although the etiology of PD is not clear, it is generally believed that both genetic susceptibility and environmental factors contribute to the pathogenesis of PD. Mutations in leucine-rich repeat kinase 2 (LRRK2) gene is the most common genetic cause of familial and sporadic PD (8-11). Similar to sporadic late-onset PD, sleep problems are the major complaints of patients with PD who have LRRK2 mutations. It has been reported that sleep disruptions are present in 60-98% of patients with LRRK2-associated PD (12). Thus, studying the role of LRRK2 in sleep disturbances may generate new insights into the pathophysiology of PD, providing therapeutic options for the management of sleep disorders in LRRK2-associated PD.

The fruit fly, *Drosophila melanogaster*, has been increasingly used to model neurologic diseases due to the similarity in the nervous system between fruit flies and humans (13-17). Several LRRK2 transgenic flies have been created to study the role of LRRK2 mutations (18,19). These LRRK2 transgenic flies recapitulate several key features of human PD including locomotor dysfunction and selective loss of dopaminergic neurons (20). However, little is known about sleep disorders

in these LRRK2 transgenic flies. Sleep in *Drosophila* is regulated by the MBs. MB output regulates the sleep through different downstream neurons, while ablation of MB output leads to sleep disturbances (21,22).

Clinically, sleep problems are more difficult to treat than motor symptoms in PD due to the multifactorial causes of sleep disturbances. Furthermore, current treatments for PD sleep disorders are often associated with undesirable adverse effects. Melatonin is an endogenous regulator of the sleep/wake cycle. Melatonin secretion patterns change in PD patients, and treatment with melatonin replenishes the inadequate melatonin levels, relieving the sleep symptoms (23). In addition to its sleep-promoting effects, melatonin is an effective antioxidant. The neuroprotective activity of melatonin has been well documented in a range of models of PD (24,25). However, these beneficial effects of melatonin have not been explored in LRRK2-associated PD. In the present study, transgenic flies were generated to express human (h)LRRK2 in the MBs. Transgenic flies expressing hLRRK2 were observed to exhibit sleep problems such as sleep fragmentation during the night. Additionally, hLRRK2 was observed to disrupt the presynaptic function in the Kenyon cells (KCs) in the MB and increase bouton density. Furthermore, administration of melatonin significantly attenuated the sleep problems and rescues the presynaptic dysfunction in the hLRRK2 transgenic flies.

Materials and methods

Generation of human LRRK2 transgenic flies. The transgenic flies were constructed to bear the different hLRRK2 constructs [wild type (WT) and G2019S] under the control of a yeast upstream activating sequence (UAS). A green fluorescent protein (GFP) *Xba*I-*Eco*RI fragment was first ligated into the pUAST vector to generate a UAS-GFP construct. Flag tagged hLRRK2 was then inserted between the *Kpn*I and *Bgl*II sites of the UAS-GFP vector. The constructs were injected into w^{1118} embryos (Bloomington Drosophila Stock Center, Bloomington, IN, USA; n=6 in each group) to obtain transformant lines. Two transgenic lines each were generated for UAS-GFP-hLRRK2 and UAS-GFP-hLRRK2-G2019S. The hLRRK2 expression levels were examined by western blot analysis using anti-Flag staining. OK107-GAL4 was used to selectively express UAS-GFP-hLRRK2 and UAS-GFP-hLRRK2-G2019S in the MBs. The phenotypic characterization was conducted on hLRRK2-WT and hLRRK2-G2019S lines. w^{1118} served as a negative control. *Drosophila* were grown on standard cornmeal medium at 25˚C under 12/12 h light/dark (L/D) conditions. For melatonin treatment, flies were transferred to regular fly food containing 4 mM melatonin.

Western blotting. The heads of adult flies (3-7 days post eclosion) were collected and homogenized in radioimmunoprecipitation assay lysis buffer (EMD Millipore, Billerica, MA, USA) containing a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Following centrifugation at 8,000 x g for 15 min at 4˚C, the supernatants were subjected to Bradford protein assays (Beyotime Institute of Biotechnology, Haimen, China) to ensure equal protein loading (50 μg) and resolved on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, and then transferred onto polyvinylidene difluoride membranes (0.45 mm; EMD Millipore). The membranes were blocked in Tris-buffered saline-0.1% Tween 20 containing 5% nonfat milk for 1 h at room temperature and then probed with primary antibodies overnight at 4˚C and secondary antibodies for 1 h at room temperature. The primary antibodies used were as follows: a mouse monoclonal anti-Flag-tag antibody (1:2,000; Sigma-Aldrich, St. Louis, MO, USA; cat. no. F3165) and mouse monoclonal anti-β-actin (1:3,000; ProteinTech Group, Inc., Chicago, IL, USA; cat. no. 60008-01). The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse antibody (1:6,000; EarthOx Life Sciences, Millbrae, CA, USA; cat. no. E030110-01). Protein was detected using chemiluminescence reagents (Beyotime Institute of Biotechnology).

Sleep assays. Individual female virgin flies (3-7 days post eclosion; n=32 in each group) were transferred into monitor tubes (5x65 mm) containing 5% sucrose and 2% agar media at one end, enabling the continuous recording of behavior over a number of days. Locomotor activity was recorded by the *Drosophila* Activity Monitoring System (DAMS; TriKinetics, Inc., Waltham, MA, USA). Sleep was defined as periods of 5 min without recorded activity. Data were collected for 3 days. Experiments were performed in an incubator at a temperature of 25±1˚C and a relative humidity of 60±5%. Lights were turned on at zeitgeber time (ZT)0 (circadian time 06:00) and off at ZT12 (circadian time 18:00).

Electrophysiological recordings from KCs in isolated whole brains. All brains were obtained from flies 1-3 days in age. The entire brain including the optic lobes was removed from the head. The dissected brains were then mounted in an RC-26 perfusion chamber (Warner Instruments, LLC, Hamden, CT, USA) containing the recording solution bubbled with 95% O_2 and 5% CO_2 (2 ml/min) throughout the experiments with the ventral surface of the brain facing up (26). The standard external solution contained (in mM): 101 NaCl, 1 $CaCl_2$, 4 $MgCl_2$, 3 KCl, 5 glucose, 1.25 NaH_2PO4, and 20.7 $NaHCO_3$, at pH 7.2, osmolarity 250 Osm. Recording pipettes were fabricated from capillary glass using a four stage micropipette puller, and had tip resistances of 15-20 MΩ, when filled with the intracellular solution of the following composition (in mM): 102 K-gluconate, 17 KCl, 0.94 ethylene glycol tetraacetic acid, 8.5 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1.7 $MgCl_2$, 17 NaCl, at pH 7.2, osmolarity 235 Osm. Pipettes were targeted to GFP-labeled KCs in the MBs. Current-clamp (n=6 in each group) and voltage-clamp (n=6 in each group) recordings were performed using patch-clamp electrodes. Giga-ohm seals were achieved prior to recording in an on-cell configuration, followed by whole-cell configuration while in voltage-clamp mode. Recordings were made at room temperature, and only a single KC neuron was examined in each brain. Excitatory postsynaptic potential (EPSP) frequency and amplitude were recorded using whole-cell current-clamp. For measurements of cholinergic miniature excitatory postsynaptic current (mEPSC), neurons were held at -75 mV followed by whole-cell configuration in voltage clamp, tetrodotoxin (1 μM) was added to the external solution to block voltage-gated sodium currents and γ-aminobutyric acid-ergic synaptic currents were blocked by picrotoxin

Table I. Sleep behavior of flies expressing hLRRK2-WT and hLRRK2-G2019S (with and without melatonin treatment), driven by the OK107-GAL4-mediated expression of upstream activating sequence transgenes and w^{1118}.

	Light phase			Dark phase		
Group	Sleep bout (number)	Mean bout length (min)	Sleep (min)	Sleep bout (number)	Mean bout length (min)	Sleep (min)
Control	8.281±0.743	141.997±19.541[b]	579.484±9.063[b]	11.500±1.067[b]	41.449±1.170[c]	470.687±11.698[b]
WT	9.938±0.853[d]	85.648±9.493[e]	542.078±9.282[d]	13.859±1.778	39.627±4.256	387.625±12.625
G2019S	14.875±1.162[f]	74.109±10.287[f]	523.797±11.293[e]	17.906±0.867[f]	22.975±1.463[f]	348.125±10.718[e]
G2019S (M)	9.094±0.714[c]	121.475±16.085[b]	552.313±9.083[a]	15.359±0.892[a]	26.100±2.219	310.016±13.212

Data are presented as the mean ± standard error of the mean (n=32, 3 independent experiments performed). Differences between means were determined by one-way analysis of variance followed by the Newman-Keuls multiple comparison post hoc test. [a]P<0.05, [b]P<0.01, [c]P<0.001 vs. the hLRRK2- G2019S mutant without melatonin and [d]P<0.05, [e]P<0.01, [f]P<0.001 vs. the control (w^{1118}) group. hLRRK2, human leucine-rich repeat kinase 2; WT, wild type; G2019S, glycine 2019 serine.

(10 μM). mEPSC amplitudes <20 pA were detected. All electrophysiological recordings were conducted using a BX51WI upright microscope (Olympus Corporation, Tokyo, Japan). Data were acquired by MultiClamp 700B amplifier and an Axon Digidata1440A (Molecular Devices, LLC, Sunnyvale, CA, USA), and were filtered at 5 kHz using a built-in filter and digitized at 5 kHz. Data were analyzed offline using Clampfit 10.2 (Molecular Devices, LLC).

Biocytin staining and two-photon laser scanning fluorescence microscopy. To examine the morphology of the recorded single neurons, post hoc staining with biocytin was utilized. In these cells (n=7 in each group), 0.4% biocytin was added to the internal pipette solution. Following electrophysiological recordings, the brain was fixed in phosphate buffered 4% formaldehyde for 3 h on ice. The brains were then washed 5 times with phosphate-buffered saline-Tween 20 on the orbital shaker for 10 min at room temperature and then blocked in blocking buffer (0.1 M Tris-HCl, 0.1% Triton X-100, 10% goat serum) for 3 h on ice. Brains were incubated in Cy3 (1:100 dilution) for 24 h at 4˚C.

A Leica TCS SP5 microscope (Leica Microsystems GmbH, Wetzlar, Germany) with a 40x objective was used to acquire optical slices through the MBs. The position of the soma was determined by both the position of electrode tip and the intense biocytin staining. An argon laser provided the excitation line at 546 nm with the gate for the multiplier opened between 500 and 600 nm. Slices of the brain (~120) were generated at a thickness of 1 μm per step (from the top of the soma to bottom of the dendrite in the z-axis through the tissue), at a 1024x2048 Hz scanning speed. Two photon images were saved as lif, the preferred file format for image processing using Imarisx64 software, version 7.2.1 (Bitplane AG, Zurich, Switzerland), which were used to create 3D optical volumes of the neuronal dendrites, from which the synaptic boutons can be detected.

Data analysis. All statistical analysis was conducted using SPSS 19.0 software (IBM SPSS, Armonk, NY, USA). The Shapiro-Wilks test was used to determine the normality of data. The normally distributed data were analyzed by 2-tailed, unpaired one-way analysis of variance followed by the Newman-Keuls multiple comparison post hoc test. Data are presented as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

MB expression of hLRRK2 did not result in gross morphological alterations. hLRRK2 was selectively expressed in the MBs using the OK107 promoter to target GAL4 expression in all lobes of the MBs. In these transgenic flies, hLRRK2 was expressed in all MB lobes as indicated by the GFP fluorescence (Fig. 1A). Structurally, MB lobes remained intact in flies expressing either hLRRK2-WT or hLRRK2-G2019S. The size and thickness of the MB lobes were similar between control and hLRRK2 flies (Fig. 1A). Confocal microscopy did not reveal any cell loss and axonal degeneration of KCs in all the examined LRRK2 files compared with the control. Western blotting demonstrated that the protein expression was stable and at similar levels between the hLRRK2-WT and hLRRK2-G2019S flies (Fig. 1B). Taken together, these results indicate that expression of hLRRK2 in the MBs does not result in any significant morphological alterations.

MB expression of hLRRK2 disrupted normal sleep patterns. To explore whether MB expression of hLRRK2 results in sleep problems, sleep patterns were investigated in hLRRK2 flies. Compared with the control flies, the bout number of hLRRK2-WT flies were increased (P=0.035; Fig. 2A) and the mean length of each sleep episode in the light phase were significantly reduced (Fig. 2B, P=0.002), however, not in the dark phase (Fig. 2D and E). By contrast, the expression of hLRRK2-G2019S markedly increased bout number (counts; Fig. 2A, P<0.001; Fig. 2D, P<0.001) and reduced bout length (min) in both the light and dark phases (Fig. 2B, P<0.001; Fig. 2E, P<0.001). Furthermore, the total sleep (min) of the hLRRK2-G2019S mutant flies was significantly reduced (Fig. 2C, P=0.007; Fig. 2F, P=0.004), however, not in

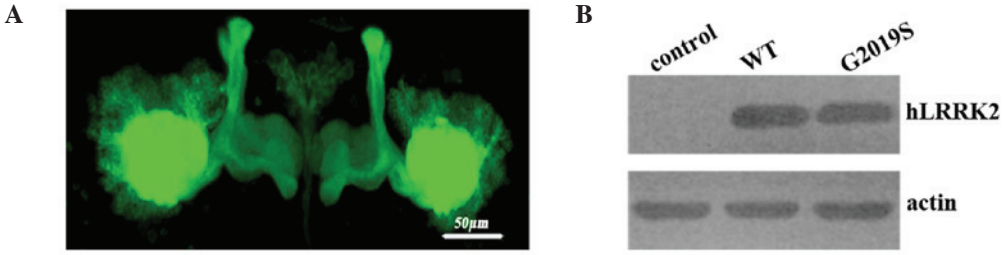

Figure 1. hLRRK2 was expressed in the MBs in flies. (A) Image showing MB expression of hLRRK2 and green fluorescent protein driven by GAL4-OK107 (Scale bar, 50 μm). (B) Image of western blot showing the expression of hLRRK2 in the MBs. hLRRK2, human leucine-rich repeat kinase 2; MBs; mushroom bodies; WT, wild type; G2019S, glycine 2019 serine.

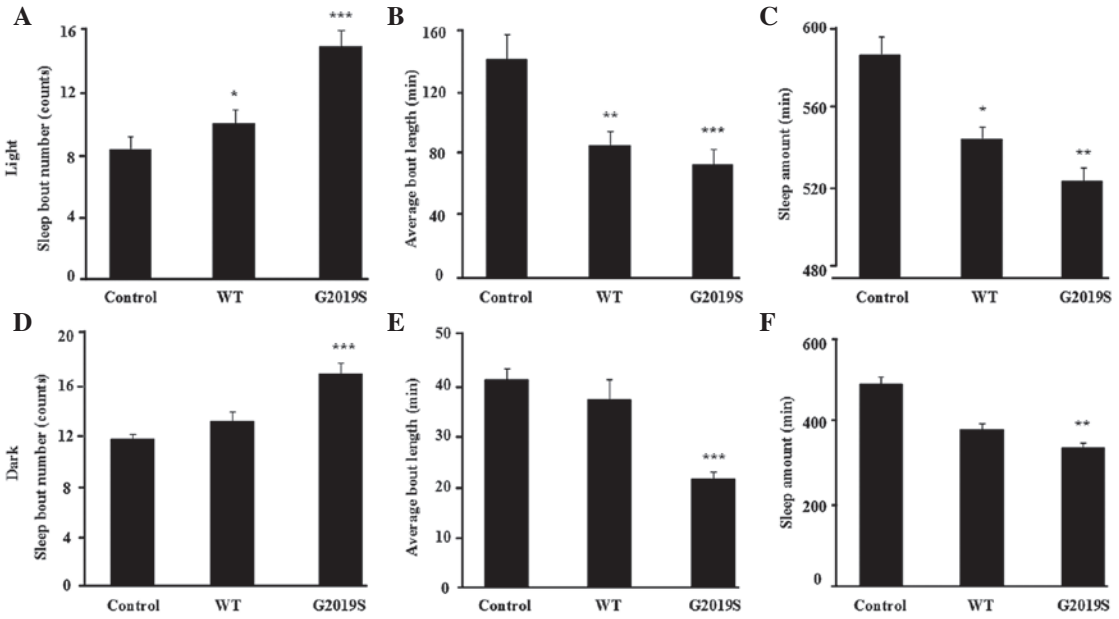

Figure 2. Sleep behavior of flies expressing hLRRK2-WT and hLRRK2-G2019S, driven by the OK107-GAL4-mediated expression of UAS transgenes and w^{1118}. Expression of the hLRRK2-G2019S mutation in young flies (3 days after eclosion) affected (A and D) the average number of sleep episodes (sleep bout number), (B and E) the average length of sleep episodes (bout length) and (C and F) the sleep amount (total sleep) to a greater extent than w^{1118} and hLRRK2-WT expression during the (A-C) light and (D-F) dark phase. Data are presented as the mean ± standard error of the mean from 32 animals that were individually recorded in each experiment, from a minimum of 3 independent experiments. Differences between means were determined by one-way analysis of variance followed by the Newman-Keuls multiple comparison post hoc test. *P<0.05, **P<0.01, **P<0.001 vs. the control (w^{1118}) group. hLRRK2, human leucine-rich repeat kinase 2; WT, wild type; G2019S, glycine 2019 serine.

the hLRRK2-WT flies compared with the control group (Fig. 2C and F). The sleep behavior data is presented in Table I. Collectively, the data suggest that MB expression of hLRRK2 disrupted normal sleep patterns in flies, predominantly by increasing arousal during sleep and reducing total sleep time.

MB expression of hLRRK2 resulted in presynaptic dysfunction. The synaptic functions of KCs, such as membrane excitability, are thought to be critical for sleep regulation. To investigate whether hLRRK2 modulates the synaptic function of KCs, whole-cell current-clamp recording (Fig. 3A) and voltage-clamp recordings in MB KCs was performed (Fig. 3D). During the recordings, the frequency and amplitude of the EPSP and mEPSC were detected, with the amplitude and frequency of the mEPSC indicating the postsynaptic function and presynaptic function, respectively. When the holding potential was -75 mV in voltage-clamp mode, and the mean resting potential was -60±1.25 mV in current-clamp mode, none of the examined KCs exhibited spontaneous firing activities, however, some exhibited subthreshold spontaneous activity in normal control flies (Fig. 3A and D). The EPSP amplitude in WT (1.624±0.1759 mV; P=0.572) and G2019S (0.954±0.169 mV, P=0.994) were not significantly different from the control flies (1.091±0.219 mV; Fig. 3B). By contrast, the EPSP frequency in WT (12.300±1.174 Hz, P=0.008) and G2019S (6.850±1.053 Hz, P=0.004) were significantly lower compared with the control flies (25.525±3.957 Hz; Fig. 3C). Similarly, the mEPSCs frequency of WT (0.388±0.0557 Hz; P<0.05) and G2019S (0.3101±0.462 Hz; P<0.01; Fig. 3F) were significantly lower than the control w^{1118} (0.778±0.078; Fig. 3F), however, the mEPSCs amplitude of WT and G2019S indicated no significant difference compared with the control w^{1118} (Fig. 3E). However, the amplitude but not the frequency of either mEPSCs or EPSPs were similar between the hLRRK2 flies and control flies (Fig. 3B and E), indicating that postsynaptic but not presynaptic function remained intact in hLRRK2 flies. Taken together, these results indicate that hLRRK2

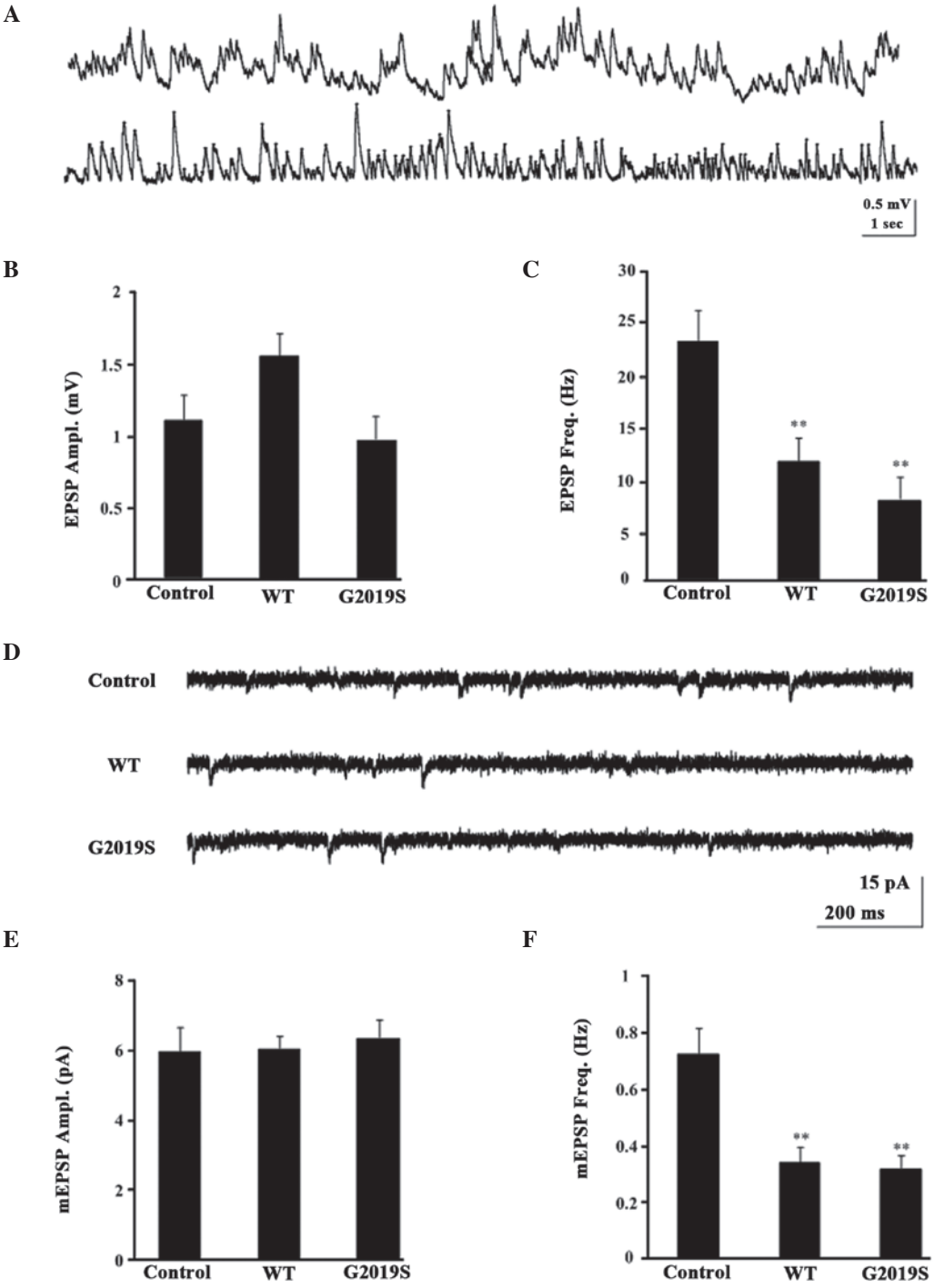

Figure 3. mEPSC and EPSP are affected in hLRRK2 transgenic flies. (A) Representative waves of the EPSP. (B) The amplitude of the EPSP was not affected in hLRRK2 transgenic flies. (C) The frequency of the EPSP was reduced in hLRRK2 transgenic flies. (D) Representative waves of the mEPSC. (E) The amplitude of the mEPSC was not affected in hLRRK2 transgenic flies. (F) mEPSC frequency was reduced in hLRRK2 transgenic flies. **P<0.01 vs. the control (w^{1118}) group. Data are presented as the mean ± standard error of the mean, n=6. mEPSC, miniature excitatory postsynaptic current; EPSP, excitatory postsynaptic potential; hLRRK2, human leucine-rich repeat kinase 2; WT, wild type; G2019S, glycine 2019 serine.

expression results in presynaptic dysfunction, as evidenced by the reduction in the cellular excitability of KCs.

MB expression of hLRRK2-G2019S increases the density of synaptic boutons. To investigate whether hLRRK2-G2019S affects synaptic plasticity, the synaptic bouton density (synaptic terminals) was investigated. Biocytin staining was used to label the synaptic boutons of the KCs in the MBs (Fig. 4Aa) and Fig. 4Ab presents a single KC neuron. To identify the density of neurons accurately and efficiency, an imaging method of the KCs was tested, based on two-photon laser scanning fluorescence microscopy, which enabled the visualization of the density of KCs synaptic boutons. The density of boutons was significantly higher in hLRRK2-G2019S mutant flies (0.2010±0.0238 μm; P=0.025) while the density of boutons remained unaltered in hLRRK2-WT flies (0.1145±0.0465 μm; P=0.89) compared with the w^{1118} control flies (0.1246±0.0145 μm; Fig. 4B). Thus, these results demon-

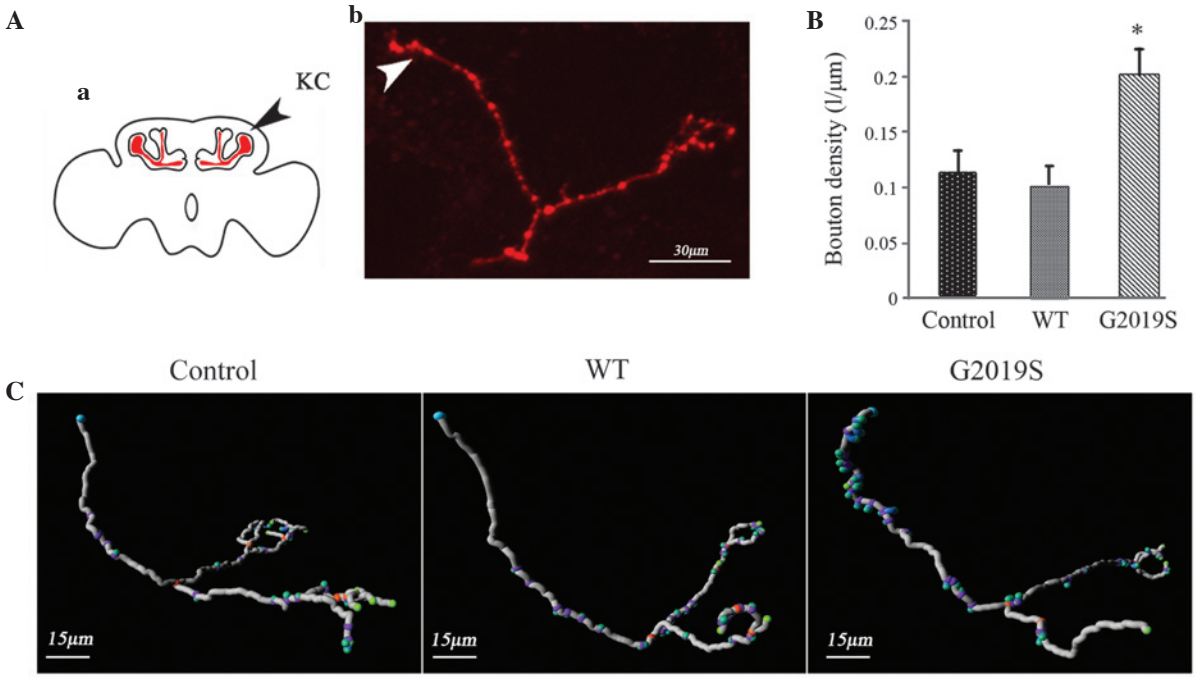

Figure 4. Axon bouton density is altered in hLRRK2-G2019S mutant flies. (A) Representative images of KC axons. (a) Dissected *Drosophila* whole brain with the mushroom bodies indicated in red. (b) A single KC indicated with biocytin. (B) The density of the axon boutons was increased in hLRRK2-G2019S mutant flies. (C) Representative images of the 3D reconstruction of the axonal boutons. Scale bar, 15 μm, *P<0.05 vs. the control w^{1118} group, n=7. Data are presented as the mean ± standard error of the mean. hLRRK2-G2019S, human leucine-rich repeat kinase 2-glycine 2019 serine; KC, Kenyon cell; WT, wild type.

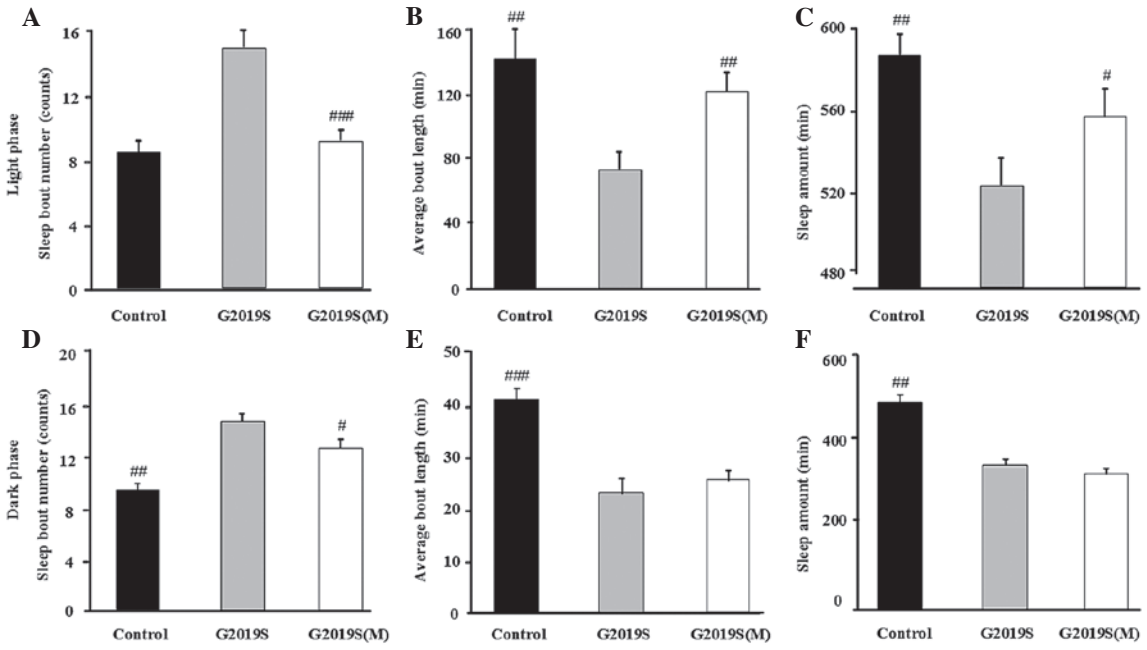

Figure 5. Melatonin attenuates sleep disorders in hLRRK2-G2019S mutant flies. (A) Melatonin reduced the number of light phase of sleep in hLRRK2-G2019S mutant flies. (B) Melatonin increased the light phase length of bouts of sleep in hLRRK2-G2019S mutant flies. (C) Melatonin increased the total amount of light phase sleep in hLRRK2-G2019S mutant flies. (D) Melatonin reduced the number of dark phase bouts of sleep in hLRRK2-G2019S mutant flies. (E) Melatonin did not affect the length of dark phase bouts of sleep in hLRRK2-G2019S mutant flies. (F) Melatonin did not affect the dark phase sleep amount in hLRRK2-G2019S mutant. Data are presented as the mean ± standard error of the mean (n=32). $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. the hLRRK2-G2019S mutant without melatonin treatment. hLRRK2-G2019S, human leucine-rich repeat kinase 2-glycine 2019 serine; M, melatonin.

strate that hLRRK2-G2019S induces excessive synaptic plasticity (Fig. 4C).

Melatonin attenuates sleep disturbances and rescues presynaptic dysfunction in hLRRK2 flies. Whether melatonin attenuates hLRRK2-associated sleep problems and the relationship between sleep disorder and synaptic dysfunction was investigated. It was observed that melatonin significantly attenuated the hLRRK2-induced increase in light and dark phase sleep bouts (Fig. 5A and D), and melatonin attenuated the

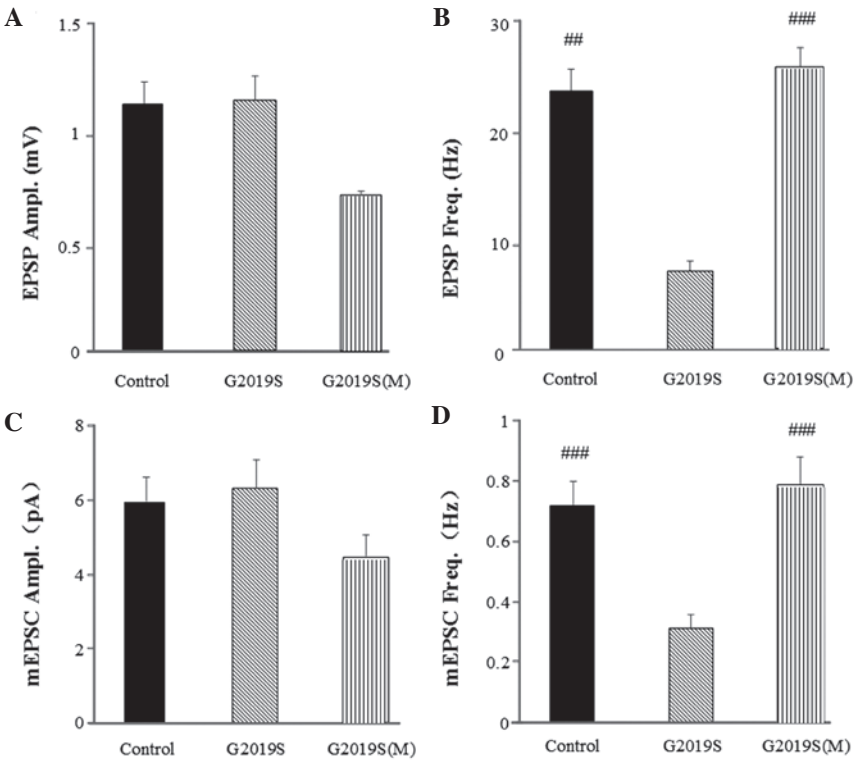

Figure 6. Melatonin increases mEPSC and EPSP frequency in hLRRK2-G2019S mutant flies. (A) Melatonin did not affect EPSP amplitude. (B) Melatonin increased EPSP frequency. (C) Melatonin did not affect mEPSC amplitude. (D) Melatonin increased mEPSC frequency. Data are presented as the mean ± standard error of the mean (n=6). $^{\#}$P<0.05; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. the hLRRK2-G2019S mutant without melatonin treatment. mEPSC, miniature excitatory postsynaptic current; EPSP, excitatory postsynaptic potential; hLRRK2-G2019S, human leucine-rich repeat kinase 2-glycine 2019 serine; M, melatonin.

hLRRK2-induced reduction in the mean bout length and the amount of sleep in the light phase compared with the G2019S flies without melatonin (Fig. 5B and C). However, the mean bout length and the amount of sleep in the light phase were not altered (Fig. 5E and F). All sleep behavior data is presented in Table I. Furthermore, the EPSP and mEPSC frequencies in the hLRRK2 flies were restored to the levels of the control group following melatonin treatment compared with the flies without melatonin treatment (Fig. 6). The EPSP frequency of G2019S flies treated with melatonin was restored to 26.022±6.534 Hz (P<0.001; Fig. 6B), which was significantly different from the EPSP frequency of the G2019S flies without melatonin (6.850±1.953 Hz). The mEPSC frequency in the G2019S flies treated with melatonin was restored to 0.785±0.104 Hz (P<0.001; Fig. 6D), which was significantly different from the mEPSC frequency of the G2019S flies without melatonin (0.310±0.046 Hz). Amplitude differences in mEPSCs suggest changes to postsynaptic function and frequency differences suggest changes to presynaptic function. The results from the present study indicated neither the EPSP (Fig. 6A) or mEPSC (Fig. 6C) amplitudes were altered in the hLRRK2 flies, with or without melatonin treatment. However, the frequency of EPSP (Fig. 6B) and mEPSC (Fig. 6D was altered with melatonin, thus, suggesting that melatonin improves sleep problems and presynaptic dysfunction in hLRRK2 flies.

Discussion

Mutations in LRRK2 are the most common genetic cause for familial and sporadic PD (27). Thus, studying the role of hLRRK2 in PD is important to further understand the pathophysiology of PD. The present study reports that the expression of hLRRK2 in the MBs induced sleep disorders in flies. hLRRK2-induced sleep problems were associated with reduced frequency of EPSPs and increased synaptic bouton density, indicating the important role of hLRRK2-induced synaptic dysfunction in sleep disorders. Furthermore, melatonin significantly attenuated hLRRK2-induced sleep problems and rescued hLRRK2-induced synaptic dysfunction, suggesting a potential clinical application of melatonin in patients with PD carrying LRRK2 mutations. Sleep problems are a major contributor to impairment in PD, with 80-90% of patients with PD experiencing disturbances of sleep patterns (28,29). Although the manifestations of sleep problems vary, sleep fragmentation is one of the most common sleep complaints of patients with PD (30-32). Consistently, MB expression of hLRRK2 in flies resulted in sleep fragmentation as indicated by the increase in arousal during sleep. Thus, the transgenic flies expressing hLRRK2 in the MBs are able to recapitulate the sleep disturbances observed in clinical PD. The underlying causes of sleep problems in PD are complex. Previous studies have demonstrated that a large proportion of sleep problems are associated with the involvement of non-dopaminergic brain regions with multiple neurotransmitter deficiencies such as cholinergic system degeneration (33-36). The MBs serve a central role in sleep regulation in flies (22). The MBs are a paired brain structure, and are composed of small neurons known as KCs. The synaptic functions of the KCs are critical for sleep regulation (21). In the present study, expression of hLRRK2

in the MBs did not result in any gross morphological damage however, reduced cholinergic synaptic mEPSC and EPSP frequency was observed, suggesting a negative modulation of hLRRK2 on presynaptic properties. Notably, in addition to sleep problems, the synaptic bouton density was increased in hLRRK2-G2019S flies. Synaptic boutons are the presynaptic terminals that contain neurotransmitters stored in synaptic vesicles. The increase in the number of synaptic boutons means greater levels of neurotransmitters present, which in turn increases the probability of the neurotransmitter release. Thus, the increase in synaptic bouton density seems contradictory to the negative modulation of hLRRK2 on synapses. This may be explained by the inhibitory effect of hLRRK2 on synaptic efficacy. Synaptic efficacy is the capacity of a presynaptic input to influence postsynaptic output. Previously, hLRRK2 has been reported to disrupt synaptic vesicle trafficking and distribution within the bouton (37). By doing so, hLRRK2 may prevent the release of neurotransmitters from the vesicles in the synaptic boutons and reduce synaptic efficacy. Furthermore, the increase in the number of synaptic boutons may lead to synaptic stress due to the increase in synaptic boutons occupying greater space and demanding increased cellular supply to synapses (38). As a result, low synaptic efficacy and synaptic stress may further disrupt synaptic homeostasis, leading to an exaggeration of hLRRK2-induced sleep problems.

The primary function of sleep is to restore brain energy metabolism, with sleep problems increasing energy consumption and exaggerating the metabolic disturbances in PD. In this regard, treatment of sleep disturbances with appropriate drugs may help to not only to solve sleep problems, but also to prevent the progression of PD. In the present study, melatonin significantly attenuated hLRRK2-induced sleep problems and rescued the reduced cholinergic synaptic mEPSC and EPSP frequencies, however, had no effect on the increase in synaptic bouton density. These results suggest that the beneficial effects of melatonin may be associated with the promotion of synaptic transmission. The direct action of melatonin on synaptic transmission remains inconclusive due to inconsistent results from different studies (39). Alternatively, the promotion of synaptic transmission may be explained by the antioxidant role of melatonin. Reactive oxygen species (ROS) have been reported to be involved in vesicular neurotransmitter release (40). At presynaptic sites, certain essential proteins responsible for the exocytosis of neurotransmitters such as synaptosomal-associated protein, 25kDa are vulnerable to ROS attack due to their chemical structures (41). The oxidation of these key proteins impairs the neurotransmitter release machinery. Indeed, ROS scavengers have been reported to prevent ROS attack and enhance synaptic transmission (41). Thus, melatonin may reduce ROS and attenuate hLRRK2-induced synaptic dysfunction. As a result, melatonin may break the cycle involving sleep disturbances and metabolic stress, and prevent the progression of LRRK2-associated PD. However, the precise mechanisms underlying the beneficial effects of melatonin require further study. Nevertheless, the present study highlights the potential of melatonin as a candidate neuroprotective agent with sleep-promoting properties in future clinical trials for patients with PD carrying hLRRK2 mutations.

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