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Human LRRK2 G2019S mutation represses post-synaptic protein PSD95 and causes cognitive impairment in transgenic mice

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Abstract

Background—LRRK2 G2019S mutation is associated with increased kinase activity and is the most common mutation associated with late-onset PD. However, the transgenic mouse model has not recapitulated cardinal PD-related motor phenotypes. Non-motor symptoms of PD including cognitive impairments are very common and may appear earlier than the motor symptoms. The objective of this study was to determine whether human LRRK2 with G2019S mutation causes hippocampus-dependent cognitive deficits in mice.

Results—Male (LRRK2-G2019S) LRRK2-Tg mice showed impairments in the early portion of the Two-day radial arm water maze acquisition trial as well as in the reversal learning on the third day. However, their performance was similar to Non-Tg controls in the probe trial. LRRK2-Tg mice also displayed impairments in the novel arm discrimination test but not in the spontaneous alternation test in Y-maze. Interestingly, there was no statistically significant locomotor impairment during any of these cognitive test, nor in the locomotor tests including open field, accelerating rotarod and pole tests. Expression of the postsynaptic protein PSD-95 but not the presynaptic protein synaptophysin was lower in hippocampal homogenates of LRRK2-Tg mice.

Conclusion—Consistent with previous reports in human LRRK2 G2019S carriers, the current data suggests that cognitive dysfunctions are present in LRRK2-Tg mice even in the absence of locomotor impairment. LRRK2 G2019S mutation represses the postsynaptic protein PSD-95 but not the presynaptic protein synaptophysin. This study also suggests that mild cognitive impairment may appear earlier than motor dysfunctions in LRRK2-G2019S mutation carriers.

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Competing interests

The authors declare that they have no competing interests.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.nlm.2017.05.001>.

Keywords

LRRK2; Hippocampus; Learning and memory; Parkinson's disease; Cognitive impairment

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disease characterized by a progressive deterioration of motor functions. Neuropathologic hallmark of PD primarily involves the loss of a specific population of midbrain dopaminergic neurons of the *substantia nigra*. Even though PD is primarily a movement disorder, studies have shown that certain domains of cognitive functions may be affected in PD as demonstrated in animal models and human studies (Chaudhuri & Odin, 2010; Magen et al., 2012; Mochizuki-Kawai, Mochizuki, & Kawamura, 2010; Paisan-Ruiz et al., 2004). The etiology of PD is not clearly known but certain genetic factors may predispose an individual to the disease (Melrose, 2008). Mutations in the Leucine-rich repeat kinase 2 gene (LRRK2) is the most important known genetic risk factors associated with PD (Paisan-Ruiz et al., 2004). The most prevalent LRRK2 mutation associated with late-onset PD is the G2019S mutation characterized by the main pathologic mechanism of increased kinase activity (Melrose, 2008). Although, various LRRK2 mice models have not accurately recapitulated the classic PD pathology and symptoms, some studies, including those employing *in vitro* techniques have demonstrated the role of LRRK2 in cellular and molecular mechanisms that may impact learning and memory. For example, LRRK2 has been shown to play important roles in synaptic functions, apoptosis and perhaps more importantly neurogenesis – a hippocampus process that is important for learning and memory (Iaccarino et al., 2007; Jessberger et al., 2009; Matta et al., 2012; Winner et al., 2011). The cognitive features of PD pose a significant challenge for clinical management of the disease because the symptoms are not as responsive to dopamine-based therapy as motor symptoms (Chaudhuri & Odin, 2010). Although, contrary reports exist, several studies have reported cognitive impairments which precede or accompany the appearance of motor symptoms in PD patients and even among healthy LRRK2 G2019S subjects (Mochizuki-Kawai et al., 2010; Saunders-Pullman et al., 2006; Thaler et al., 2012). The objective of this study is to determine whether human LRRK2 with the G2019S mutation causes hippocampus-dependent cognitive deficits in middle aged (9–10 month old) mice.

2. Methods

2.1. Mice

The previously described human LRRK2-Tg mice (with G2019S mutation) in FVB background were crossed with Non-Tg littermate of LRRK2-Tg mice (also with G2019S mutation) in C57BL/6J background. The resulting offsprings were genotyped by tail biopsy with the previously described primers (Melrose et al., 2010). The animals used throughout this study (in behavior and biochemical assays) are twelve (12) male mice consisting of six LRRK2 G2019S-Tg (henceforth referred to as LRRK2-Tg) and six Non-Tg littermates from the cross above, at 8.6–9.7 months of age (9.7 ± 0.9 month average $\pm$ standard deviation for both Tg and Non-Tg mice). All procedures were in compliance with University of

Mississippi Medical Center Institutional guidelines, approved by the Animal Care and Use Committees (protocol #1242A).

2.2. Materials

Rabbit anti-PSD-95 (Cat# Ab18258), Rabbit anti-synaptophysin (Cat# ab68851) and Mouse anti- β -Actin (Cat# Ab6276) were obtained from Abcam (Cambridge, MA). Pierce™ Fast Western Blot Kit, ECL substrate (Cat# 35050) was obtained from ThermoFisher Scientific, (Waltham, MA).

2.3. Learning and memory tasks

We used a Two-day *Radial-arm water maze* (RAWM) task followed by a one-day reversal learning component to study spatial memory and cognitive flexibility respectively. The tasks were conducted and analyzed as we have previously described (Adeosun et al., 2014; Alamed, Wilcock, Diamond, Gordon, & Morgan, 2006; Hou et al., 2015). Visual cues were included in the RAWM set up both near the tank and on the walls of the room.

Novel arm discrimination (NAD), a hippocampus-dependent spatial recognition memory task was carried out and analyzed as we have previously described (Adeosun et al., 2014; Hou et al., 2015; Wright & Conrad, 2005) with a shorter inter-trial interval (ITI) of 30 min. *Spontaneous alternation in Y-maze* (SAYM), which tests the mice working memory was also carried out and analyzed as previously described (Adeosun et al., 2014; Holcomb et al., 1999). The test lasted for 10-min and the first and last 5 min data were analyzed separately.

2.4. Locomotor tasks

Distance travelled in an *Open field task* was obtained by recording the mice activity in a 40 cm $\times$ 40 cm $\times$ 40 cm Plexiglas box for 10 min. The total distance travelled every 2 min was obtained by analyzing the captured overhead video offline with Noldus Ethovision XT software (Wageningen, The Netherlands).

An accelerating *Rotarod task* was also conducted using a 4-lane rotarod device as previously described with major modifications described below (Adeosun et al., 2012). Mice were tested in 2 batches of 4 animals per batch. The acceleration settings were 8, 20, 40 and 80 rev/min², starting from the lowest acceleration. Mice were settled on the rod facing away from the investigator after which the device, which preset to the right acceleration (starting with 8 rev/min²), was turned on. The time each mouse was able to ride on the accelerating rod before falling on the padded platform under the rod was recorded. The test was repeated at the same setting after all the mice had fallen off the rod. The second batch of mice were tested at the same setting while the first batch rests. Then the first batch was tested at the next setting (20 rev/min²) and so on until both batches have been tested at all the acceleration settings. Replicate data for each animal at each acceleration setting was combined as an average before the average for each genotype was calculated.

Although, ours was an accelerating rod, we used the overall rod performance (ORP) method, which is the area under the curve of a fixed speed rotarod, to calculate a performance score for each genotype (Rozas, Guerra, & Labandeira-Garcia, 1997). We also used a novel

method that consider performance in each acceleration setting individually and scores the animal such that the relative difficulty (*Coefficient of Challenge*) of each setting is factored into the total score. Thus, a 10 sec ride at 40 revs/min² contributes twice as much as a 10 sec ride at 20 revs/min² to the total score of the animal. We have termed this scoring method *Challenge-adjusted Rotarod Performance* (ChaRP). Thus, for the 8, 20, 40 and 80 revs/min² that we used in the current report, the ride time at the four settings were multiplied by coefficients of challenge 1, 2.5, 5 and 10 respectively before being summed to arrive at the ChaRP score.

Pole test was conducted using a 1 cm diameter, 50 cm long wooden pole with rough surface as previously described. The test was repeated four times (10 min inter-trial interval) and the scores were averaged for each mouse before the averages of the genotypes were calculated (Ogawa, Hirose, Ohara, Ono, & Watanabe, 1985).

2.5. Western blot

After all the behavior tests, the mice were sacrificed by isoflurane anaesthesia and cardiac perfusion. The left hemisphere of the brain was fixed in PFA for immunohistochemistry while the right hemisphere was reserved for protein extraction. The hippocampus was micro-dissected out of fresh right hemisphere brain samples and flash frozen in liquid nitrogen. Protein extraction from the hippocampal homogenates were done as previously described (Adeosun et al., 2014; Hou et al., 2015). The proteins were separated in polyacrylamide gel electrophoresis and transferred to PVDF membranes. PSD-95 (1:500) or Synaptophysin (1:1000) were used to probe the membranes. Membranes were also probed with β -Actin antibody (1:5000). Detection of the blots were completed using the Pierce™ Fast Western Blot Kit, ECL substrate. Optical densities were read in a Biorad Chemidoc imager (Hercules, CA).

2.6. Immunohistochemistry

The left hemisphere of the brains were transferred from overnight PFA into 30% sucrose until they sank to the bottom. The brains were sectioned coronally at 40 μ m thickness and then subjected to immunohistochemical staining with specific antibodies for PSD-95 or Synaptophysin along with NeuN as previously described (Adeosun et al., 2014; Hou et al., 2015). Appropriate negative controls were done (i.e. excluding either the primary or respective secondary antibodies) to ascertain specificity of the antibody staining.

2.7. Statistics

In all cases where independent values were compared (Non-Tg vs. LRRK2-Tg), Independent sample *T*-test was used. When values were compared to chance levels like 50% in NAD or SAYM and 20% in RAWM tasks, One-sample *T*-test was used for each genotype (Non-Tg vs. LRRK2-Tg average vs. the Chance level). In all cases, significance level was set at 0.05. Repeated measure Two-way ANOVA was used as required, followed by Sidak's multiple comparison post hoc test.

3. Results

3.1. Spatial and reversal learning is impaired in LRRK2-Tg

We used a 2-day training in radial arm water maze (RAWM) to assess how the mice learned the location of a hidden target which is an escape platform in one of the alleys filled with water from which they are motivated to escape (Adeosun et al., 2014; Ahmad et al., 2005; Hou et al., 2015). For the latency (time) to find the target, Two-way ANOVA with Genotype and Day as factors shows significant effects for both Genotype and Day ($F(1,10) = 5.36$; $p = 0.043$ and $F(1,10) = 31.2$; $p = 0.0002$ respectively; Fig. 1A). Post-hoc analysis (Sidak's multiple comparisons test) shows that on the first day of training, it took LRRK2-Tg mice a significantly longer time to find the target (23.0 ± 2.8 sec vs. 14.4 ± 1.3 sec; $p = 0.020$). Both Non-Tg and LRRK2-Tg mice improved their performance significantly from day-1 to day-2 (to 7.3 ± 0.9 sec; $p = 0.039$; and to 10.0 ± 2.9 sec; $p = 0.001$ respectively). Consequently, on day-2, there was no more significant difference in the latency between Non-Tg and LRRK2-Tg mice ($p > 0.05$). Similarly, for errors made, there was a significant Genotype and Day effect ($F(1,10) = 5.00$; $p = 0.049$ and $F(1,10) = 42.43$; $p < 0.0001$ respectively; Fig. 1B). On day-1 LRRK2-Tg mice made significantly more errors than Non-Tg mice (3.0 ± 0.4 vs. 1.8 ± 0.1 errors; $p = 0.022$). Errors significantly decreased from day-1 to day-2 in both Non-Tg and LRRK2-Tg mice (to 0.6 ± 0.1 ; $p = 0.014$ and to 1.1 ± 0.5 ; $p < 0.001$ respectively). By day-2, the LRRK2-Tg mice have learned the task and their errors were not significantly different from those of the Non-Tg controls ($p > 0.05$).

To further confirm that the mice have learned the location of the escape platform, a probe trial was done 24 h after the last training trial on day-2. One sample *T*-test analysis shows that both Non-Tg and LRRK2-Tg mice have learned the location of the target as they showed preference (versus 20%; dotted line) platform location (already removed from the tank) in terms of time spent in the target (Fig. 1C; $41.2 \pm 4.8\%$ and $49.5 \pm 6.2\%$; $p = 0.007$ and 0.005 respectively). Percentage of frequency of entry further confirmed that the mice have learnt the location of the target (Fig. 1D; $34.3 \pm 3.4\%$ and $33.6 \pm 1.5\%$; $p = 0.009$ and 0.0003 respectively). Independent sample *T*-test shows no difference in the preference for the target between Non-Tg and LRRK2-Tg mice in terms of time and frequency in the target (Fig. 1C and D).

We next tested cognitive flexibility in the mice using a reversal learning paradigm (Burghardt, Park, Hen, & Fenton, 2012). This involved switching the location of the target to a different area of the tank as shown in Fig. 1E. Two-way repeated measure ANOVA latency to target shows significant trial effect ($F(3,30) = 27.21$; $p < 0.0001$) and matching effect ($F(10,30) = 3.51$; $p = 0.0037$) but no genotype effect ($p > 0.05$). The latency to target was significantly reduced (versus 1st trial) from 2nd through 4th trial sets in both Non-Tg and LRRK2-Tg mice ($p < 0.05$; Fig. 1F). Similarly, in the number of errors made, there was a significant trial effect ($F(3,30) = 11.99$; $p < 0.0001$) and matching effect ($F(10,30) = 5.13$; $p = 0.0002$) but no genotype effect ($p > 0.05$). However, while Non-Tg mice had a significant reduction (versus 1st trial set) in the number of errors right from 2nd trial set through 4th trial set ($p < 0.05$), this decrease in errors did not occur in LRRK2-Tg mice until the 3rd trial

set (Fig. 1G). Taken together, these data suggest that, though quite subtle, LRRK2-Tg mice have impairments in both spatial learning and cognitive flexibility.

3.2. Spatial recognition memory is impaired in LRRK2-Tg but working memory is intact

We used the novel arm discrimination task in Y-maze to study hippocampus-dependent spatial memory in the mice. This test is based on the rodents natural preference for novel environment over a familiar one (Wright & Conrad, 2005). Non-Tg littermate controls show preference for the novel arm in both parameters measured (dwell time and frequency of visit to the novel arm; Fig. 2A and B), achieving $75.4 \pm 4.3\%$ and $68.6 \pm 2.9\%$ for novel arm dwell and frequency respectively (Paired-sample *T*-test $p = 0.002$ and 0.001 respectively). In contrast, LRRK2-Tg mice did not show preference for the novel arm in either dwell time or frequency ($p > 0.05$ in both cases). More detailed analysis shows that this scenario was consistent through each minute of the retention trial (data not shown). Furthermore, an independent sample *T*-test also showed that Non-Tg mice had a significantly higher percentage novel-arm frequency than LRRK2-Tg mice ($68.6 \pm 2.6\%$ vs. $57.9 \pm 3.7\%$; $p = 0.046$, Fig. 2B) and a trend for a higher percentage novel arm dwell time ($75.4 \pm 4.3\%$ vs. $61.9 \pm 4.7\%$; $p = 0.06$, Fig. 2A). Data was also collected for the general locomotor measures during the test. Analysis shows that there was no significant difference in velocity, total time moving, latency to leave the start arm, frequency and duration in the triangular center. Also, the total dwell time and frequency in both novel and familiar arms, as well as the total distance travelled during the retention trial were not different between Non-Tg and LRRK2-Tg mice (data not shown; $p > 0.05$ in all cases).

To test another domain of memory in the Y-maze apparatus, we did a spontaneous alternation task on a different day. While this task also depends on the innate behavior of mice to prefer exploring a novel environment, the continuous nature of the setup targets it towards probing working memory (Holcomb et al., 1999; Wright & Conrad, 2005). Both Non-Tg and LRRK2-Tg mice performed above chance level (50%) during each half of the 10-min test (One-sample *T*-test vs 50%; $p < 0.05$ in both genotypes). There was also no significant difference in the percent alternation score between the Non-Tg and LRRK2-Tg mice during both halves of the task (Independent sample *T*-test $p > 0.05$; Fig. 2C). The general motor functions (total complete alternations and total number of arm entries) were also not significantly different between Non-Tg and LRRK2-Tg mice during the whole test. However, for both genotypes, complete alternations and total arms entered significantly decreased during the second half of the test by up to 48% for complete alternations and up to 52% for total arm entries (data not shown). Taken together, this suggests that while working memory is intact in LRRK2-Tg mice, spatial recognition memory in the novel arm discrimination task is impaired and this is not due to any confounds in locomotor function or exploratory behavior.

3.3. LRRK2-Tg mice show no locomotor impairment in open field rotarod and pole tests

There was no significant difference in the total distance travelled by Non-Tg and LRRK2-Tg mice during the 10-min exploration of the open field box (3484.4 ± 282.2 cm vs. 3836.0 ± 297.0 cm; $p = 0.41$). A more detailed analysis of the five 2-min blocks of the test also showed no difference within any block between Non-Tg and LRRK2-Tg mice (Fig. 3A).

Other behavioral/motor parameters including time and frequency at the center of the box, total time the animal was moving and average speed of the mice throughout the test also showed no significant difference between Non-Tg and LRRK2-Tg mice ($p > 0.05$; data not shown).

In the accelerating rotarod task, there was no difference in the performance of Non-Tg vs. LRRK2-Tg mice in any of the acceleration settings (Fig. 3B). Consequently, the ChaRP scores between the genotypes were not significantly different (340.5 ± 35.4 vs. 319.6 ± 22.9 ; $p = 0.63$). There was a strong and significant correlation between ChaRP and ORP scores ($R = 0.988$; $p = 0.0002$); thus ORP scores were also not significantly different between Non-Tg vs. LRRK2-Tg mice (1600.2 ± 159.9 vs. 1490.2 ± 117.8 ; $p > 0.05$).

In the pole test which is a more challenging test that require fine motor co-ordination, although it took LRRK2-Tg mice 55% and 23% longer to turn and descend respectively, there was no statistically significant difference in either the *Turn* time (15.5 ± 5.0 sec vs. 24.1 ± 6.1 sec; $p = 0.31$) nor the *Descent* time (24.7 ± 5.0 sec vs. 30.5 ± 5.4 sec; $p = 0.45$) between Non-Tg and LRRK2-Tg mice. Given the relatively high, though non-significant percentage difference in the performance of LRRK2-Tg mice in the pole test, we wondered whether this might be related to their cognitive performance. Therefore, we ran Spearman correlation between each of Turn time or Descent time versus data from the cognitive tests (as in Fig. 1A and B; RAWM Day1 Latency and Errors and Fig. 2A and B; NAD Percent time in Novel arm and Percent entry into Novel arm) where LRRK2-Tg mice showed clear impairment. There was no significant correlation in any of the eight pairs ($p > 0.05$; Table 1). Thus, taken together with the locomotor data from the cognitive tasks, male LRRK2-Tg mice at this 10-month age show no statistically significant locomotor deficits.

3.4. LRRK2-Tg mice have reduced expression of PSD-95 in their hippocampus

Since these animals show impairments in hippocampus dependent tasks and synaptic protein expression in the hippocampus plays important role in the pathophysiology of learning and memory impairment in various animal models (Migaud et al., 1998; Schmitt, Tanimoto, Seeliger, Schaeffel, & Leube, 2009) as well as in Alzheimer's disease subjects (Counts, Nadeem, Lad, Wu, & Mufson, 2006; Proctor, Coulson, & Dodd, 2010; Sze et al., 1997), we decided to assess the level of synaptic protein expression in their hippocampus. LRRK2-Tg mice showed a modest but significant 20% reduction in the postsynaptic protein PSD-95 (Fig. 4A; 1.00 ± 0.06 vs 0.80 ± 0.04 for Non-Tg and LRRK2-Tg respectively; $p = 0.021$). We also measured the expression of synaptophysin in the same tissue homogenate. Interestingly, unlike PSD-95, there was no significant difference in expression of synaptophysin (Fig. 4B; 1.00 ± 0.04 vs 1.07 ± 0.04 for Non-Tg and LRRK2-Tg respectively; $p > 0.05$). To further confirm the reduction in PSD-95 expression in the hippocampus, we assessed the distribution of PSD-95 immunoreactivity by fluorescence staining. PSD-95 immunoreactivity appeared as punctate signals which were very few within the cell bodies but abundant within the intercellular spaces of the granular cell layer (GCL) neurons (Fig. 4C iii and vii). This distribution is consistent with what has been described in rat brains (Cho, Hunt, & Kennedy, 1992; Hunt, Schenker, & Kennedy, 1996). On close observation, while the PSD-95 immuno-signals in the hilus were not different, there is a clear reduction

in the punctate signals within the GCL of the LRRK2-Tg mice versus the Non-Tg. Hence, the lower expression of PSD-95 in LRRK2-Tg mice determined by western blot (Fig. 4A) is probably due to the region-specific reduction of the protein in the GCL of the hippocampus.

4. Discussion

Several LRRK2 models with different mutations have been generated but background genetic issues, especially the *Pde6br^{d1}* mutation in FVB mice which causes retinal degeneration and blindness had been an impediment in trying to assay cognitive functions in those mice (Counts et al., 2006; Melrose et al., 2010; Proctor et al., 2010; Sze et al., 1997; Winner et al., 2011). We have used LRRK2 mice with a mixed FVB-C57BL/6J background to overcome this problem. In general, our data shows that LRRK2-Tg mice have cognitive dysfunction, which is dependent on the presence of the transgene as we have used Non-Tg littermates as controls. Also, our data suggest that the cognitive impairment in LRRK2-Tg mice are quite subtle and perhaps also mild at least at the age analyzed, as only very sensitive and more difficult aspects of the tasks were able to uncover the deficits. Apart from using the Non-Tg littermates as controls which attempts to address the potential question about poor vision, the improvement of LRRK2-Tg mice in RAWM training, normal performance in RAWM probe trial and in the working memory (Y-maze) tasks supports the likelihood of normal visual acuity of the mice. However, even though, we also included both proximal and distal visual cues in the RAWM tasks, it is worth noting that we did not directly test visual acuity in these mice. The mixed performance of the LRRK2-Tg mice in these battery of cognitive tests thus supports the idea that specific domains of learning and memory, for example, cognitive flexibility, is impaired in this middle-aged LRRK2-mice, just as it has been reported in human PD subjects (Mochizuki-Kawai et al., 2010).

Another problem associated with evaluating cognitive deficits in PD mouse models is that behavioral assays depend on motor functions of the animal, for example, swimming in water maze, exploration in Y-maze, etc. Thus, since the critical phenotype of PD is motor impairment, interpretation of cognitive impairments from the motor readouts may not be accurate as the poor performance of the animal may be due to motor impairment rather than its cognitive capacity (Lindgren & Dunnett, 2012). Interestingly, all motor parameters analyzed during the cognitive tests were normal in the LRRK2-Tg mice, suggesting that the impairment in cognitive functions of LRRK2-Tg mice may not be due to motor impairments. To specifically test locomotor functions in the mice, we used a battery of widely used locomotor tasks including the open field, rotarod and pole tests. The locomotor test results further confirm that LRRK2-Tg mice has no impairment in any of the locomotor function tested (Fig. 3A and B). It is however worth noting that LRRK2 mice spent considerably longer time to turn and descend in the pole test (55% and 23% respectively), but this did not reach statistical significance. A significant difference might be detectable in a larger sample size at this age or in an older population of these mice. The lack of any overt motor phenotypes in these mice is not surprising as similar results have been reported in young and up to 12-month old LRRK2 G2019S or R1441G mice despite reduced extracellular dopamine levels (Li et al., 2010; Melrose et al., 2010; Volta et al., 2015).

The current results suggest that LRRK2 may play a bigger role in the cognitive phenotype of PD than previously thought and perhaps precede the principal motor phenotype. This is supported by the fact that LRRK2 is expressed at a very high level in the hippocampus – even higher than in the midbrain by several folds (Melrose et al., 2010; Winner et al., 2011). Secondly, In addition to the regulation of synaptogenesis (Parisiadou et al., 2014), LRRK2 play an important role in neurogenesis, which is a hippocampus process that is central to the process of learning and memory (Jessberger et al., 2009; Winner et al., 2011). Thirdly, increased abnormal phosphorylation of tau, which is one of the hallmarks of Alzheimer's disease has also been reported in LRRK2 mice with G2019S or R1441G mutations. It has been suggested that LRRK2, whose kinase activity is increased by G2019S mutation, may directly or indirectly phosphorylate tau (Bailey et al., 2013; Li et al., 2009; Lin, Tsai, Wu, & Chien, 2010; Melrose et al., 2010). Fourthly, apart from neurogenesis, the LRRK2 G2019S mutation is associated with impairment is several cellular and molecular mechanisms of learning and memory including dendritic arborization as well as the formation of spines and synaptic connections within the hippocampus (Winner et al., 2011). Synaptic protein loss correlates with, and may mark or precede the earliest stages of memory impairment (Counts et al., 2006; Proctor et al., 2010; Scheff, Ansari, & Mufson, 2016; Sze et al., 1997; Yuki et al., 2014). We specifically addressed this point in this study by assessing synaptic protein expression in the hippocampus of the mice where LRRK2 is most highly expressed in the brain (Melrose et al., 2010; Winner et al., 2011). Consistent with a recent report on the role of post-but not presynaptic function in G2019S LRRK2 effect on hippocampus-dependent behavior (Sweet, Saunier-Rebori, Yue, & Blitzler, 2015), we found that only the postsynaptic protein PSD-95 (Fig. 4A) but not presynaptic synaptophysin (Fig. 4B), might play a role in the cognitive effect of G2019S LRRK2 reported in the current study. Sweet et al. (2015) further showed that the postsynaptic-specific effect of G2019S LRRK2 depends on the kinase activity of the mutant LRRK2.

PSD-95 is one of the most abundant components of the synapse which in turn is the most abundant and most distinguishing structural feature of the brain (Grant, 2012). PSD-95 functions as a scaffolding protein in dendrites and regulate AMPAR and NMDA receptor functions –both of which are important for synaptic plasticity vis-à-vis learning and memory (Rao & Finkbeiner, 2007). Furthermore, in a mouse model of fragile $\times$ syndrome, characterized by dendritic spine abnormalities, training-induced up-regulation of PSD-95 was attenuated in the mutant mice and maze errors correlated negatively with PSD-95 protein expression (Gandhi, Kogan, Messier, & Macleod, 2014). In addition, donepezil, a clinically used therapy for dementia improved learning and memory in aged C57BL/6J mice with concomitant increase in PSD-95 but not Synaptophysin protein levels in the hippocampus CA1 and dentate gyrus (Jiang et al., 2015). Thus, the lower expression of PSD-95 in LRRK-2 mice may be central to the observed cognitive impairment given the central role of PSD-95 in the hippocampus-dependent learning and memory functions.

The immunofluorescence data (Fig. 4C) suggests that the reduction in PSD-95 expression in LRRK2-Tg mice may be limited to the GCL and not the hilus of the hippocampus dentate gyrus. Synaptophysin which unlike PSD-95 did not show reduction in western blot, is distributed in a similar manner in Non-Tg and LRRK2-Tg mice within both the GCL and hilus (Supplementary material Fig. S1). PSD-95 signals in the GCL are postsynaptic targets

of the perforant pathway, that is, entorhinal cortex neurons synapsing on the dendrites of GCL neurons. These signals may also include the synapses of the projections of hilar mossy cells and interneurons which send excitatory and inhibitory signals respectively to the GCL neurons. The GCL neurons then project to the CA3 pyramidal neurons through their mossy fibers (Deng, Aimone, & Gage, 2010). Although, the dendrites of matured GCL neurons are mostly located within the molecular layer, we also found abundant punctate PSD-95 signals within the GCL. The PSD-95 signals within the GCL may be from the dendrites of newborn neurons that are just being added to the deeper layers of GCL from the GCL-hilus junction (Adeosun et al., 2014). Interestingly, Winner et al. previously reported significant reduction in adult neurogenesis and neurite outgrowth in LRRK2 G2019S mice (Winner et al., 2011). Thus, reduced neurogenesis and neurite outgrowth in LRRK2-Tg mice may contribute to the observed reduction in PSD-95 expression. This in turn may lead to disruption of the tri-synaptic neural network of the hippocampus and the resultant functional impairment in learning and memory performance of LRRK2-Tg mice.

Lastly, the previously reported, most consistent and most PD-relevant phenotype in LRRK2-Tg mice –reduced dopamine neurotransmission in the nigrostriatal pathway, may also contribute to the cognitive deficits observed in the current study. This is because a bilateral striatal 6-OHDA PD mouse model also exhibit cognitive deficits in domains similar to the ones in the current report (De Leonibus et al., 2007; Melrose et al., 2010).

It is also worth noting that, in human wildtype LRRK2 (hLRRK2-WT) transgenic mice, the transgene may be up to 6-folds higher in the hippocampus (versus mouse LRRK2 in Non-Tg mice), this might have also contributed to the impairment observed in these mice. Previous reports show that LRRK2 G2019S Tg mice showed no impairment in learning and memory tasks at age 3 months but did at 12 months (Volta et al., 2015). Furthermore, LRRK2-WT transgenic mice showed no impairment in short-term object recognition task but displayed an impairment in the long term variant of the task (24 h I-T-I) (Beccano-Kelly et al., 2015). Volta et al. (2015) posited that there is a temporal distinction in the effects of overexpression of wildtype versus G2019S LRRK2 (Volta et al., 2015). In the same vein and taken together with previous reports, our data suggests that the G2019S effect accelerates the detrimental effect LRRK2 overexpression. The current results show that the G2019S effect occurs earlier than previously shown since the mice in the current study are in between the ages previously studied (6 and 12 months old).

5. Conclusion

In conclusion, the current data suggests that cognitive dysfunctions are present in 9–10 month old LRRK2-Tg mice even in the absence of locomotor impairment. This study also suggests that mild cognitive impairment may appear earlier than motor dysfunctions in LRRK2-G2019S mutation carriers. The dysfunction of the post-synaptic transmission in hippocampus may be the underpinning mechanism for the early cognitive impairment induced by LRRK2-G2019S mutation.

Supplementary Material

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Abbreviations

ChaRP	challenge-adjusted rod performance
ITI	inter-trial interval
LRRK2	leucine-rich repeat kinase 2
NAD	novel arm discrimination
ORP	overall rod performance
PD	Parkinson's disease
PSD-95	post synaptic density protein
RAWM	radial arm water maze
SAYM	spontaneous alternation in Y-maze
Tg	transgenic

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Fig. 1.

Impaired spatial and reversal learning in G2019S LRRK2-TG mice. (A) Average latency, or Time-to-target (escape platform) in seconds and (B) average number of errors made during the two-day radial-arm water maze (RAWM) training. In a probe trial (test without the platform) conducted 24 h after the last training trial of day 2, both Non-Tg and (G2019S) LRRK2-Tg mice showed preference for the target location which was significantly higher than chance level (dotted line; 20%) in both (C) Percent time spent in the target location and (D) Percent number of entry into the target location in the probe trial done 24 h after the last training trial. (E) On day 3, the location of the target was changed as shown i.e., target

previously at D was moved to the opposite side of the tank to either A or B; or target previously at C moved to A or E, etc. (F) Time to the new target and (G) errors made before finding the new target in the course of training the mice after changing the target location (each trial set vs. 1st trial set). Data expressed as Mean $\pm$ SEM. N = 6 for both groups. Sidak's post hoc test, One sample or Independent *T*-test p-values **p* < 0.05; ***p* < 0.01; ****p* < 0.005; Sidak's post hoc test Day 1 vs. Day 2 within each genotype p-value #*p* < 0.05; ##*p* < 0.01; ###*p* < 0.005.

Fig. 2.

Impaired spatial recognition but intact working memory in G2019S LRRK2-Tg mice. (A) Percentage of time spent in, and (B) percentage of number of entry (frequency) into both familiar and novel arms during the 3 min retention trial. (C) Percent alternation scores in Y-maze during two 5-min periods of the test. Dotted line represent chance level (at 50%). Paired and One-sample *T*-test *p*-values **p* < 0.05; ***p* < 0.01; ****p* < 0.005; Independent sample *T*-test (Non-Tg vs. (G2019S) LRRK2-Tg) #*p* < 0.05.

Fig. 3.

No evidence of motor dysfunctions in G2019S LRRK2-Tg mice in open field and Rotarod tasks (A) Total distance travelled during each of the five 2-min blocks of monitoring in an open field box. (B) Percentage of time spent in, and (B) Ridding time of Non-Tg and (G2019S) LRRK2-Tg mice on the rotarod at different acceleration settings. Data point (each acceleration setting) shows an average of the two consecutive trials at the same setting.

Fig. 4. Reduced expression of postsynaptic-but not presynaptic protein in G2019S LRRK2-Tg mice hippocampus. Immunoblots and relative optical density (ROD) quantifications of (A) PSD-95 and (B) Synaptophysin expression in hippocampal homogenates of (G2019S) LRRK2-Tg mice. Protein expression levels were determined by western blot using PSD-95 and Synaptophysin specific antibodies respectively. Protein expression levels expressed as ROD relative to β -Actin which was the loading control. Data expressed as Mean $\pm$ SEM. N = 6 for both groups. Independent *T*-test *p*-values **p* < 0.05; ns = not significant. (C) Representative immunofluorescence images of hippocampal dentate gyri of Non-Tg (i–iv) and LRRK2-Tg (v–viii) mice. The dotted lines in (C) iii, iv, vii and viii represent the boundaries of the hilus bordered by the granular cell layers (GCL) on either side. Note the punctate staining of PSD-95 in iii and vii which are absent within the nuclei and very rare in

the cell bodies of the granular cell layer (NeuN-positive) neurons (ii and vi). Also note the fairly even distribution of the PSD-95 punctate staining throughout the dentate gyrus (Hilus and around the cell bodies in the GCL) in Non-Tg mice sections (iii). This is not so in the LRRK2-Tg sections where the PSD-95 punctate staining is fewer especially in the GCL and the adjacent molecular layer. Scale bar 50 mm. DAPI in Blue, NeuN in Green, PSD-95 in Red.

Table 1

Relationship between motor performance and cognitive performance.

	Turn time	Descent time
Day 1 latency	0.36 (<i>0.25</i>)	0.33 (<i>0.29</i>)
Day 1 errors	0.41 (<i>0.19</i>)	0.41 (<i>0.18</i>)
Percent time in novel arm	−0.30 (<i>0.34</i>)	−0.26 (<i>0.42</i>)
Percent frequency of entry into novel arm	−0.34 (<i>0.29</i>)	−0.30 (<i>0.34</i>)

Spearman correlation co-efficient and p-value (*italics in brackets*) between performance measures in cognitive tasks (rows) and the pole test (columns).