Trophoblast glycoprotein is a new candidate gene for Parkinson’s disease

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INTRODUCTION
Parkinson’s disease (PD) is a movement disorder caused by progressive degeneration of the midbrain dopaminergic (mDA) neurons in the substantia nigra pars compacta (SNc). Despite intense research efforts over the past decades, the etiology of PD remains largely unknown. Here, we discovered the involvement of trophoblast glycoprotein (Tpbg) in the development of PD-like phenotypes in mice. Tpbg expression was detected in the ventral midbrain during embryonic development and in mDA neurons in adulthood. Genetic ablation of Tpbg resulted in mild degeneration of mDA neurons in aged mice (12–14 months) with behavioral deficits reminiscent of PD symptoms. Through in silico analysis, we predicted potential Tpbg-interacting partners whose functions were relevant to PD pathogenesis; this result was substantiated by transcriptomic analysis of the SNc of aged Tpbg knockout mice. These findings suggest that Tpbg is a new candidate gene associated with PD and provide a new insight into PD pathogenesis.

Trophoblast glycoprotein (TPBG), also known as Wnt-activated inhibitory factor 1 (WAIF1), is a 72 kDa, heavily N-glycosylated, single-pass transmembrane protein10–12. It is highly expressed not only in trophoblast cells and carcinoma but also in normal adult tissues, including the ovary, bone, retina, and brain13,14. TPBG has been primarily studied in embryonic stem (ES) cell differentiation and cancer metastasis15–18. During embryonic development, TPBG acts as a feedback inhibitor of canonical Wnt signaling by interfering with the internalization of low-density lipoprotein receptor-related protein 6 (LRP6), a key component of the LRPS/LRP6/Frizzled co-receptor group, and enhances the activation of non-canonical Wnt signaling by stimulating the functions of Dickkopf-related protein 1 (DKK1)19. Additionally, TPBG modulates cell adhesion, cytoskeletal organization, and mobility by facilitating functional C-X-C chemokine receptor type 4 (CXCR4) expression, leading to C-X-C motif chemokine 12 (CXCL12)-mediated chemotaxis in differentiating ES cells, embryonic fibroblasts, and cancer cells16–18,20. More recently, analysis of a single-cell RNA sequencing (RNA-seq) dataset of the developing human ventral midbrain (VM) obtained from 6 to 11 week embryos revealed that Tpbg is significantly expressed in early neural progenitor cells of the floor plate and in a subset of dopaminergic neurons (referred to as “DA2” population) whose fate is determined to substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) 1 and 2 after birth21, implicating the role of Tpbg in the development of midbrain dopaminergic (mDA) neurons. Furthermore, we have recently discovered that Tpbg expression is specifically enriched in mDA precursors differentiated from human pluripotent stem cells (hPSCs) and proposed that it can be used as a marker for mDA precursor isolation from neural progenies of hPSCs22. Given its specific expression in the mDA population, TPBG has been considered as one of the PD-related genes23,24. Indeed, a study analyzing gene expression in postmortem tissues showed that TPBG was significantly downregulated in the SNc of patients with PD than in the SNc of age-matched healthy subjects25. Despite the evidence, detailed information including spatial and temporal expression of TPBG during embryonic development and its role in fate determination or maintenance of mDA neurons remain largely unknown; more importantly, the involvement of TPBG in PD etiology remains circumstantial.

In this study, we investigated the spatiotemporal expression of Tpbg in the VM of developing mouse embryos and adult mice. Our...
results demonstrate that Tpbg was specifically expressed in the VM region of developing embryos and in mDA neurons of the SNc and VTA in adults. In addition, we found that genetic ablation of Tpbg negatively affected the survival of mDA neurons in adult mice and resulted in motor impairments reminiscent of PD symptoms. This study provides evidence of the specific expression of Tpbg in developing and mature mDA neurons and its involvement in the maintenance of the mDA neuronal population, suggesting that Tpbg is a candidate gene associated with PD etiology.

RESULTS

Tpbg is expressed in the developing mouse ventral midbrain
The spatiotemporal expression of Tpbg in the developing VM was investigated using immunohistochemical analysis of mouse brain tissue at different embryonic stages (embryonic day [E]9.5–E15.5) (Fig. 1). To pinpoint the location of Tpbg expression, a transgenic mouse strain in which enhanced green fluorescent protein (EGFP) is expressed under the Tpbg promoter was used. Immunohistochemical staining for Tpbg showed that most EGFP+ cells co-expressed Tpbg, indicating that EGFP reflected endogenous Tpbg expression in Tpbg-EGFP mice. Further examination revealed that EGFP signal and immunoreactivity of Tpbg were detected at in the floor plate (FP) of the midbrain from E9.5 (Fig. 1a). The expression was also detected in the dorsal side of the diencephalic region, and it eventually became localized at the cortical hem (at E12.5, Fig. 1a). However, we focused on its expression in the VM region to investigate its potential involvement in the differentiation of mDA neurons. Strong expression was maintained until E12.5, at which mDA neurogenesis reaches at peak. To identify the cell types that express Tpbg in the FP, we labeled the EGFP-expressing tissue with antibodies against several mDA markers. At E11.5, the sagittal sections of the developing mouse brain highlighted EGFP expression throughout the rostro-caudal axis of the VM. This expression pattern overlapped with immunoreactivity to LMX1A and FOXA2, specific markers for mDA precursors and FP, respectively, suggesting that Tpbg-expressing cells are a subset of mDA neuron progenitors (Fig. 1b). Further examination of sagittal sections at E12.5 showed that a subset of EGFP+ cells co-expressed NURR1 and/or TH, conventional markers for mDA neurons (Fig. 1b). Most of the rostral population of EGFP+ cells exhibited co-labeling with mDA markers; however, few cells overlapped with these markers in the caudal midbrain (Fig. 1b). In the coronal sections, EGFP+ cells were initially concentrated at the medial part of the ventral midline, and they overlapped with FOXA2- and LMX1A-expressing mDA domains through the length of the VM at E11.5 and E12.5 (Fig. 1c). During this period, the neuroepithelium is divided into three layers, according to the developmental stage of the mDA lineage cells that compose each domain: the ventricular zone (VZ), intermediate zone (IZ), and marginal zone (MZ) (Supplementary Fig. 1a). EGFP expression was detected in SOX2+ progenitor cells of the VZ, NURR1+ mDA neuroblasts of the IZ, and TH+ postmitotic mDA neurons of the MZ (Supplementary Fig. 1b), suggesting that Tpbg is expressed in mDA lineage cells throughout the developmental stages of the rostral VM. Between E12.5 and E15.5, which is the migration phase of mDA progenitors, EGFP+ cells exhibited radially and tangentially oriented bipolar morphology in the mDA domain (i.e., IZ and MZ) (Fig. 1d). In addition, most EGFP+ cells in that domain overlapped with the NURR1+ mDA neuroblasts, while a fraction of them were also positive for TH (Supplementary Fig. 1c). These results indicate that Tpbg is initially expressed in the mDA progenitor population, and its expression is sustained in postmitotic mDA neurons. Notably, such characteristics of EGFP+ cells, including cellular composition, distribution, and morphology, were evident in the rostral part but not as much as in the caudal part of the VM region (Fig. 1). These features during a critical period of mDA neurogenesis were similar to those of the developing mDA cell population destined for the SNc.25,26

Tpbg remains expressed in the dopaminergic subsets of adult mouse ventral midbrain
We examined whether Tpbg expression was maintained in the mDA domains of the adult VM. In situ hybridization data from the Allen Institute database (https://mouse.brain-map.org/) showed that Tpbg transcripts were detected in the same region (SNc expressing Girk2 and in the VTA region marked by Calbindin1 (Calb) on postnatal day 56 (Supplementary Fig. 2). Consistent with the mRNA expression pattern, our immunohistochemical analysis showed that EGFP was detected in most TH-positive neurons in the SNc (Girk2+) and in a small portion of the VTA (CALB+) (Fig. 2a, b). Collectively, these data indicate that Tpbg was expressed in the mDA lineage cells of the mouse VM region during embryonic development, and its expression was maintained in mDA neurons in the adult mouse brain.

Genetic ablation of Tpbg induces loss of midbrain dopaminergic neurons, accompanied by axonal defects in aged mice
Histological analysis of Tpbg-EGFP mice revealed that Tpbg was expressed in the mDA domain in the developing embryonic brain, as well as in the adult brain. These results prompted us to hypothesize that Tpbg may not only be involved in the development of mDA neurons but also be required for the maintenance of mDA systems later in life. To answer this question, we explored the neuroanatomical and neurochemical changes in mDA systems using Tpbg knockout (KO) mice.

The ablation of the Tpbg gene in the Tpbg KO brain was confirmed by PCR-based genotyping and western blotting (Supplementary Fig. 3). Although previous studies using the same mouse line as ours (i.e., Tpbg<sup>km1<sub>l1 Ley</sub></sup>) did not report abnormal brain structure in their colony of Tpbg KO mice, we observed that a few subjects exhibited hydrocephalic phenotypes (e.g., unusually large head size and a dome-shaped head) in specific generations at F3 and their progenies (F4–), consistent with other studies using different mouse lines (Tpbg<sup>km1<sub>l1 Ley</sub></sup> or Tpbg<sup>km1<sub>l1 Ley</sub></sup>). We attributed the random occurrence of the hydrocephalic phenotype in our experimental difference in the genetic background of the mice (C57Bl/6 for the previous study demonstrating the hydrocephalus vs. C57Bl/6 × 129/Sv in this study). To focus on the phenotype in the midbrain, we excluded the colony exhibiting the hydrocephalic phenotype in their genealogy according to histological evaluation. Histological examination revealed that the overall structure of Tpbg KO mouse brains was comparable to that of wild-type (WT) counterparts except for structural abnormalities in the hippocampus of Tpbg KO mice (Supplementary Fig. 4).

There was no significant difference in the number and distribution of mDA neurons between WT and Tpbg KO mice at E18.5 (data not shown), indicating that the loss of Tpbg did not alter the formation of mDA subsets during embryonic development. To investigate whether genetic ablation of Tpbg affects the maintenance of mDA neurons later in life, the number of TH+ mDA neurons was quantified in the SNc and VTA of adult Tpbg KO and WT mice at young and old ages (Fig. 3a–c). The number of TH+ mDA neurons in the midbrain of young mice showed no differences between the two genotypes (Fig. 3b, c). At old age, however, the total number of TH+ mDA neurons in the Tpbg KO mice was lower than that in WT counterparts, and the reduction was significantly greater in the SNc than in the VTA (Fig. 3b, c). Tpbg hemizygous (Hem) mice did not show a significant decrease in the number of TH+ cells in either the SNc or VTA (data not shown). Consistent with this result, we detected an increased number of TH+ cells positive for cleaved CASPASE-3 (CC-3) in the
SNc (Fig. 3d, e), indicating that the decrease in the number of TH$^+$ cells in aged Tpbg KO mice might be a result of apoptosis. The selective vulnerability of mDA neurons to Tpbg ablation correlates with the enriched expression of Tpbg in the SNc (Supplementary Fig. 2 and Fig. 2b) and the previous result showing differential Tpbg expression between SNc and VTA$^{29,30}$. Because the TH$^+$ neurons in the SNc were diminished in number, we explored whether the functional connectivity of the mDA neurons to the axonal projection target is affected by Tpbg ablation. In particular, neurochemical changes in the striatum were assessed. TH$^+$ fiber density in the striatum was not significantly different between Tpbg KO mice and WT controls (Supplementary Fig. 5). However, we observed spheroidal dystrophic terminals in the TH$^+$ nerve fibers in the striatum of aged Tpbg KO mice (Fig. 3f, g).

S. Park et al.  [Published in partnership with the Parkinson’s Foundation] npj Parkinson’s Disease (2021) 110
In accordance with the abnormal mDA nerve terminals, striatal dopamine contents in aged Tpbg KO mice were significantly reduced by 30% compared to that in the age-matched WT control (128.24 ± 15.6 pmol/mg in WT vs. 89.21 ± 20.23 pmol/mg in Tpbg KO) (Fig. 3h). Scattered TH⁺ cell bodies were also observed in the striatum of Tpbg KO mice (Supplementary Fig. 6), a likely compensatory mechanism for the dystrophic nerve terminal, as demonstrated by previous studies of the striata from PD brains⁵¹,⁵². Collectively, these data indicate that Tpbg ablation led to the loss of mDA neurons in the SNc with age, accompanied by impairment of dopaminergic innervation to the striatum in the mouse brain.

**Pathophysiological features of Parkinson’s disease were observed in the ventral midbrain of Tpbg knockout mice at old age**

Reduction in the number of TH⁺ cells in the SNc and striatal dopamine levels prompted us to further investigate whether genetic ablation of Tpbg induces the pathophysiological features of PD in mice. One of the pathological hallmarks of PD is the accumulation of abnormal α-SYN, which may contribute to Lewy-like inclusion formation and lead to neuroinflammation. To address this, we examined the presence of α-SYN and S129-phosphorylated α-SYN (P-α-SYN) in the SNc of aged Tpbg KO mice using western blotting. The results revealed that the band intensity for α-SYN and P-α-SYN significantly increased in the SNc of aged Tpbg KO mice compared with that in the SNc of age-matched WT mice (Fig. 4a–c). Furthermore, immunohistochemical analysis revealed that TH⁺ cells were more frequently labeled with antibodies for both α-SYN and P-α-SYN in aged Tpbg KO mice than in WT counterpart (Fig. 4d, e). However, the number of Lewy-like aggregates in the SNc was not significantly different between the WT and Tpbg KO mice (Supplementary Fig. 7). Next, to investigate whether the accumulation of α-SYN is accompanied by the sign of neuroinflammation, we performed immunohistochemical analysis with an antibody targeting microglia (anti-IBA1) (Fig. 5a). Total IBA1⁺ cell density (the number of IBA1⁺ microglia per unit area of 1 mm²) showed no significant difference between the two genotypes (Fig. 5b). However, upon analyzing the results on the basis of their morphological characteristics (Fig. 5c), we found that the proportion of IBA1⁺ cells with enlarged cell bodies and fewer short and thick branches (type C), which are the morphological features of activated microglia⁵³,⁵⁴, were significantly higher than that of the simply ramiﬁed IBA1⁺ cells (type A and B) in the SNc of the aged Tpbg KO mice (Fig. 5d). This result suggests that the SNc of aged Tpbg KO mice may present higher levels of neuroinflammation than that of age-matched WT counterpart.

Collectively, our results show that genetic ablation of Tpbg results in pathological changes in the VM of aged mice, which may create an unfavorable environment for the survival of mDA neurons.

**Aged Tpbg knockout mice display motor deﬁcits in nigrostriatal pathway-sensitive behavioral tests**

Since Tpbg KO mice show pathological features reminiscent of PD, we questioned whether the ablation of Tpbg results in the development of PD-like motor symptoms. To answer this question, we evaluated motor function using a battery of behavioral tests sensitive to alterations in the nigrostriatal dopaminergic system. When young and old Tpbg KO and WT mice were weighed before behavioral testing, no significant difference was observed with
respect to their genotypes and ages (Supplementary Fig. 8), confirming that the body weight could be excluded from factors affecting behavioral phenotype. Gait analysis was performed using the footprint method to quantify the potential difference in walking patterns. The results revealed that there was no significant difference in all parameters of gait analysis, including stride length, stride width, and intra-step distance among different genotypes (Fig. 6a, b and Supplementary Figs. 9, 10a, b).

A challenging beam travel test was conducted to further examine motor performance and coordination. Again, no significant difference was observed in the time to traverse and the number of steps taken while traversing the beam among different genotypes (Fig. 6c, d and Supplementary Fig. 10c, d). However, Tpbg KO mice made significantly more errors and errors per step while traversing the beam than old WT mice with the number of erroneous steps increasing with age (Fig. 6e, f). Consistent with
histological evidence, young Tpbg KO and old Tpbg Hem mice did not show significant motor deficits (Figs. 3b, 6c–f and Supplementary Fig. 10c–f). These results suggest that Tpbg KO mice exhibit deficits in motor performance and coordination only at old age. Lastly, to assess motor response to sensory stimuli, we measured the time to detect a stimulus and the time taken to remove it using the forepaws. In young mice, there were no significant differences in the ‘time to respond’ and ‘time to make a contact with sensory stimuli’ between the genotypes (Fig. 6g–i). On the contrary, the time taken to make contact with the sensory stimulus and the time taken to remove it after contact increased in aged Tpbg KO mice, although the latter was not statistically significant compared with age-matched WT and Tpbg Hem mice (Fig. 6g, h and Supplementary Fig. 10g, h). More importantly,
the total time elapsed between making contact with the stimulant and removing it was significantly longer for aged Tpbg KO mice than for either young Tpbg KO (age controls) or aged WT mice (genotype controls) (Fig. 6i). These results indicated that aged Tpbg KO mice showed mild impairment in both paw and snout sensitivity and dexterity; however, the cumulative effect of both produced a significant defect in sensorimotor function. Together with biochemical and histological evidence, impaired motor performance and sensorimotor dysfunction observed in aged Tpbg KO mice strongly suggests that Tpbg is a candidate gene associated with the development of PD-like phenotypes in mice.

**In silico prediction identified potential partners interacting with TPBG that are relevant to Parkinson’s disease pathogenesis**

To explore the mechanism underlying the PD-like phenotypes of Tpbg KO mice, we searched for proteins that may interact with TPBG using STRING, a biological database for visualizing protein–protein interaction (PPI) networks. Initially, we obtained 36 proteins from STRING that are expected to interact with TPBG with a cutoff value of 0.4 (medium interaction confidence)\(^3\). A network analysis of the proteins interacting with TPBG, using a clustering algorithm (MCODE)\(^3\) identified three major clusters involved in the biological functions of splicing, immune response, and neuronal development (Fig. 7a). Furthermore, GeneOntology (GO) enrichment analysis using the 36 proteins revealed the same three functional groups enlisted at the top of the list (Fig. 7b)\(^3\). Surprisingly, many of the TPBG-interacting partners are involved in splicing-related functions. The malfunction of splicing has been implicated in neurodegenerative diseases, including PD\(^3\). In particular, WD40 repeat-containing protein SMU1 (SMU1), serine/arginine-rich splicing factor 11 (SRSF11), ubiquitin-specific peptidase 39 (USP39), protein BUD31 homolog (BUD31), splicing factor 3B subunit 3 (SF3B3), and pre-mRNA-processing factor 6 (PRPF6) play a role in pre-mRNA splicing as components of the splicing machinery (spliceosome), and their aberrant functioning is highly implicated in PD\(^3\).

The enrichment of genes related to immune response, such as CD276 antigen (CD276), interleukin-2 (IL2), tumor necrosis factor ligand superfamily member 11 (TNFSF11), purine nucleoside phosphorylase (PNP), and mucin-1 (MUC1), was also intriguing. Although previous studies have implicated the innate and adaptive immune system in PD pathobiology and disease severity\(^4\), there is limited evidence linking TPBG to the immune response. Thus, our data cast an interesting possibility that the absence of functional TPBG may cause PD pathogenesis through the modulation of multiple genes related to immune responses.

Neuron development was the third on the list of biological functions of TPBG interactors from network analysis and GO enrichment analysis. Several genes enriched in this cluster have been involved in synaptic transmission and connectivity of neurons. For example, PPP1CA and PPP2R2C, subunits of protein phosphatase 1 and 2 (PP1 and PP2), are important for the regulation of dopamine release; they act on the SNARE complex and synaptic plasticity in the dopaminergic synapse\(^5\). Interaction with these factors was likely lost in Tpbg KO mice, which might be responsible for reduced dopamine content in the striatum of Tpbg KO mice (Fig. 3h).

Several putative TPBG-interaction partners have also drawn our attention because they are involved in other cellular processes.
related to PD pathology. First, both tripartite motif-containing 33 (TRIM33), an E3 ubiquitin-protein ligase, and USP39, a deubiquitinating protein, play roles in the ubiquitin proteasome system (UPS). The UPS is an intracellular protein degradation system responsible for the majority of protein turnover within the cell\textsuperscript{51,52}. Dysfunction of the UPS has been strongly implicated in PD pathogenesis\textsuperscript{53–55} and \(\alpha\)-SYN clearance\textsuperscript{56,57}. Therefore, it is plausible to speculate that the dysfunction of USP39 due to loss of TPBG function might be linked to the accumulation of \(\alpha\)-SYN (Fig. 4). Second, the three isoforms of latrophilin (LPHN1, LPHN2, and LPHN3) have been identified as interaction partners of TPBG. Previous studies have implicated LPHNs in the dopamine metabolic system and dopamine neurotransmission\textsuperscript{58,59} thus, the reduced dopamine content in the striatum of \(Tpbg\) KO mice might be caused by the misregulation of LPHNs.

For the experimental validation of our hypothesis suggested from in silico analysis, we conducted RNA-seq analysis of the SNc of aged \(Tpbg\) KO mice and WT counterpart (Supplementary Fig. 11). Since the analysis was to confirm our proposed hypothesis, we investigated whether the genes involved in splicing and neuronal development were captured in the RNA-seq analysis. For this analysis, we performed gene set enrichment analysis (GSEA) using the KEGG and GO databases. As shown in Supplementary Fig. 11b–d, the expression patterns of the genes involved in spliceosome and neuronal development were significantly different between the \(Tpbg\) KO mice and WT counterpart, and those of the genes involved in PD were also significantly different. In the enrichment analysis, we were unable to capture specific immune responses because of the large number of child terms of immune response in GO and a large number of genes involved in immune response-related terms. In general, protein-network analysis is useful for identifying local and specific cellular processes for in-depth study, while enrichment analysis is useful for obtaining a broad insight into global phenomena. Therefore, further studies on specific immune responses are required for elucidating the association between immune response and \(Tpbg\) ablation. Despite of the missing of immune response in GSEA analysis results, the additional analysis results represent that \(Tpbg\) is implicated in PD via splicing and neuronal development processes. In addition, the similarities in histology and behavior

Fig. 6  Assessment of sensorimotor tests that are sensitive to alterations in the nigrostriatal dopaminergic system. Behavioral examination of WT and \(Tpbg\) KO mice at young (WT, \(n = 9\); KO, \(n = 5\)) and old age (WT, \(n = 9\); KO, \(n = 9\)) was performed as follows: a–b Gait patterns were measured as forelimb (a) and hindlimb (b) stride lengths. c–f Motor performance and coordination were assessed using the challenging beam travel test, as measured by the time to traverse (c), the number of steps (d), the number of errors (e), and errors per step (f). g–i Sensorimotor function was assessed by measuring the time to contact (g), contact to removal time (h), and time to removal (i) in the adhesive-removal test. Data is represented as the mean ± SEM (* \(P < 0.05\), ** \(P < 0.01\), *** \(P < 0.001\), **** \(P < 0.0001\); two-way ANOVA with Sidak's multiple comparison test).
between PD and Tpbg KO mice provide further evidence that Tpbg is a candidate gene associated with PD.

DISCUSSION
This study demonstrates that Tpbg is specifically expressed in the VM of the developing embryonic brain and adult midbrain in mice. Tpbg-expressing cells initially arise at the medial FP (FOXA2⁺LMX1A⁺) and subsequently migrate out to the MZ along a course similar to that of the differentiating mDA progenitors (NURR1⁺TH⁺). Eventually, they become restricted to the rostro-lateral domain of the VM and remain at the same position (both SNc and VTA) for the lifetime. These developmental features of Tpbg-expressing cells suggest that Tpbg may be intimately involved in the development and function of mDA neurons.

Given the previous reports demonstrating that TPBG regulates Wnt and CXCR4 chemokine signaling, it was surprising that neither late embryos of Tpbg KO mice nor young Tpbg KO mice showed any alteration in the distribution or number of mDA neurons.
neurons. The essential roles of Wnt signaling have been well characterized in the differentiation of mDA neurons. Thus, the absence of developmental defects suggests that TPBG may not be actively engaged with the Wnt signaling pathway in the development of mDA neurons. Additionally, CXCR4/CXCL12-mediated chemokine signaling was found to be required for the radial migration of mDA lineage cells. However, TPBG may not exert a critical influence on the CXCR4/CXCL12 signal in mDA neuronal migration. Alternatively, the migratory defect of mDA lineage cells in the absence of a functional CXCR4/CXCL12 signal is transient and limited to the initial radial migratory phase. Furthermore, numerous chemokine signals are involved in the migration and axon guidance of mDA lineage cells. Therefore, it is speculated that multiple cellular signals might complement each other to rescue the migration defect caused by the absence of TPBG. Lastly, the phenotype of a specific gene deficiency has often been differentially presented in mice with different genetic backgrounds; thus, we cannot exclude the possibility that the lack of developmental defects is only restricted in the mouse strain used in this study.

Although there was no noticeable phenotype at the late embryonic stage and young age of Tpbg KO mice, we observed several PD-related pathological signs at old age. Aged Tpbg KO mice exhibited a significant reduction in the number of TH+ cells in the SNc, likely due to apoptosis, along with α-SYN accumulation, increased microglial activation, and a decline in striatal dopamine content. Interestingly, the loss of TH+ cells was more pronounced in the SNc than in the VTA. The molecular basis behind this selective loss is unclear; however, considering that TPBG was preferentially expressed in the SNc than in the VTA, ablation of Tpbg seems to have a more negative impact on the survival of TH+ cells in the SNc than in the VTA. Consistent with this idea, previous reports have demonstrated that differential gene expression between the SNc and VTA impacts the selective vulnerability of mDA neurons in PD.

Indeed, these cellular phenotypes were later accompanied by their PD-like behavioral phenotypes, albeit somewhat mild, and we observed impairments in motor performance and coordination and somatosensory function in aged Tpbg KO mice in the challenging beam travel test and adhesive removal test but no alteration in gait performance. However, motor dysfunction correlated well with the extent of dopaminergic loss: aged Tpbg KO mice showed 18% less mDA neurons in the SNc and 30% lower dopamine content in the striatum compared with WT counterparts. Since previous clinical studies have estimated that at least 30% of mDA neuronal cell bodies were lost in the SNc and that dopamine contents were decreased by 68–82% in the striatum at the onset of motor symptoms in PD patients, such mild defects in motor performance and coordination appear reasonable.

Our in silico analysis predicted that TPBG interacts with various protein partners involved in splicing, immune response, neuronal development, protein homeostasis, and several other minor cellular functions, including UPS and dopamine metabolism. Furthermore, our unbiased transcriptomic analysis substantiated the in silico prediction by demonstrating alterations in gene expression related to similar biological processes. Alteration in the expression of certain genes appeared to critically contribute to PD-like phenotypes, as a plethora of evidence has implicated aberrant splicing and unbalanced protein homeostasis in the pathophysiology of PD. Most importantly, the enrichment of PD-related genes strongly supports the pathological phenotypes that we observed in the histological and behavioral analyses. Nonetheless, it is still obscure whether Tpbg deficiency directly causes such transcriptomic alteration, because this result might also have been an indirect consequence of the pathological changes manifested in the SNc of aged Tpbg KO mice; that is, up-regulation of genes involved in PD may reflect the occurrence of mDA degeneration in aged Tpbg KO mice. A more detailed molecular investigation will be required to determine the causal relationship between ablation of TPBG and the altered expression of the genes involved in PD pathogenesis.

On the basis of these findings, we propose a hypothetical model in which TPBG plays a role in maintaining the homeostasis of mDA neurons while interacting with protein factors involved in RNA processing, protein quality control, and dopamine metabolism (Fig. 8). Given such multifaceted involvement of TPBG in various cellular mechanisms and its putative existence on the plasma membrane of mDA neurons, the in silico analysis, and transcriptomic analysis, it is pragmatic to suggest a hypothetical role of TPBG in PD pathogenesis.
TPBG may function as a “moderator” that modulates the proper functioning of diverse cellular mechanisms while monitoring both extracellular and intracellular environments. For example, as a potential regulator of Wnt signaling, TPBG may assist Wnt signaling in playing an important role in synaptic maintenance and function in the adult nervous system or may affect the interaction of LRP6 with leucin-rich repeat kinase 2 (LRKK2), a factor responsible for familial PD. The ability to interact with CXCR4/CXCL12 may also influence synaptic function and neuronal survival in the adult brain, as the alteration of CXCR4/CXCL12 has been involved in the impairment of synaptic transmission and neuro-regeneration in mice. In such a scenario, failure in the ability of TPBG to function would not be immediately disastrous; however, the interconnection of the cellular mechanisms moderated by TPBG could eventually disintegrate and become pathological. Subtle cellular and behavioral phenotypes of TPBG KO mice, which are observed only in old age, suggest that TPBG mutation is unlikely to be a causative factor of PD; instead, it may act as a risk factor that increases the odds of disease onset triggered by other critical factors such as aging.

To our knowledge, this is the first study to investigate the potential correlation between TPBG and PD pathogenesis. Although further functional studies should be conducted to elucidate the detailed molecular mechanisms of how TPBG interacts with its partners and how the interaction contributes to the homeostasis of the mDA system, our study suggests that TPBG is a potential candidate gene associated with PD and provides a new insight into the molecular mechanism underlying the pathophysiology of PD. Lastly, previous genome-wide association studies have never identified TPBG as a candidate gene for PD; thus, the involvement of TPBG in human disease needs to be verified through further genetic studies.

METHODS

Animals

Tpbg-EGFP reporter line. Tpbg-EGFP mice on a mixed FVB/N and CrlCD1 (ICR) background (strain Tg(TPBG-EGFP)JN116 Gsat69, MMRCR, archived at the generation NF2) were purchased as cryo-preserved spermatozoa and recovered on a C57BL/6N (Orient Bio, Seongnam, South Korea) background at the Yonsei Biomedical Research Institute. F1/F2 heterozygous mice were backcrossed with C57BL/6N mice, and F3/F4 WT and heterozygous mice were sib-mated to generate F5 heterozygous mice. In all experiments, female heterozygous mice were used, which were obtained by intercross breeding of WT and heterozygous mice of the following age and number of mice: 9 embryos between E9.5 and E15.5 (E9.5, n = 1; E10.5, n = 2; E11.5, n = 2; E12.5, n = 2; E13.5, n = 1; E15.5, n = 1) and two adult mice (9 months old).

Tpbg knockout line. Tpbg KO mice on a mixed C57BL/6 and 129/SV/EvdBrd background (strain Tpbgtm1Lex5, MMRCR, archived at the sib-mated generation F4) were purchased as cryopreserved spermatozoa and recovered on C57BL/6N mice. Tpbg +/− (Tpbg Hem) progeny were intercrossed each generation thereafter to generate F5 Tpbg +/− (WT), Tpbg Hem, and Tpbg −/− (Tpbg KO) mice. In all experiments, female WT, Tpbg Hem, and Tpbg KO mice were used, which were obtained by intercross breeding of Tpbg KO mice as follows: 47 adult mice (young age, 3–4 months old; WT, n = 9; Tpbg KO, n = 5; old age, 12–14 months old; WT, n = 14; Tpbg KO, n = 13; Tpbg Hem, n = 6). All experimental procedures for this study were approved by the Institutional Animal Care and Use Committee of the Yonsei University Health System. Mice were maintained in a specific pathogen-free barrier facility with a 12 h light/dark cycle. The mouse genotypes were characterized using a PCR-based strategy. For genotyping, tails of pre- or postnatal mice were cut at 1 mm from the end, and genomic DNA was obtained using Direct PCR Lysis Reagent (Viagen Biotech, Inc., LA, CA, USA) according to the manufacturer’s instructions. Polymerase chain reaction (PCR) was performed using a PCR thermal cycler (Applied Biosystems Geneamp 2720, Emerald Amp’ GT PCR Master Mix (TAKARA Bio Inc., Shiga, Japan), and primers listed in Supplementary Table 1.
slices (from −2.92 to −3.64 mm, relative to the bregma) per mouse. Final counts were calculated and presented as the number of Lewy-like aggregates per 1 mm² area.

**Mouse brain striatum and SNC dissection**

For dissection of the mouse brain, the mice were anesthetized with isoflurane, and the whole brain was rapidly removed from the calvarium. The brain was placed on a mouse brain matrix (Zivic, Pittsburgh, PA, USA); 4 mm thick slices of the forebrain and 1 mm thick slices of the midbrain were obtained for the dissection of the striatum and SNC, respectively, under cool and sterile conditions. Using anatomical markers, the striatum and SNC were rapidly dissected out from these slices under a stereomicroscope, frozen in dry ice, and stored at −80 °C until analysis.

**Dopamine measurement**

For the analysis of dopamine content in the striatum, dissected striatal tissues were homogenized in 0.1 N HCl containing 1 mM EDTA and 4 mM sodium metabisulfite and centrifuged at 13,000 rpm for 20 min. The concentration of dopamine in the supernatant was determined using a dopamine ELISA Kit (Abnova, Walnut, CA, USA), according to the manufacturer’s instructions. Briefly, dopamine in the dialysate was extracted using a cis-diol-specific gel, acylated, and derivatized enzymatically. The optical density was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). Dopamine levels were normalized to the weight of the wet tissue.

**Western blot**

The dissected SNC tissues from the right hemisphere were homogenized in RIPA buffer (Thermo Scientific) with 1× proteinase inhibitor and phosphatase inhibitor (Thermo Scientific) and centrifuged at 16,000 rpm at 4 °C for 30 min. The protein concentration of the supernatants was determined using a BCA assay (Thermo Scientific). The proteins (10–15 µg) were denatured at 95 °C for 5 min, separated by SDS-PAGE on a 4–20% polyacrylamide bis-tris gel (iNtRON Biotechnology, Seongnam, South Korea), and transferred to a PVDF membrane (Millipore). The membranes were blocked in 5% skim milk in TBS with 0.1% Tween 20 (TBS-T) and incubated overnight at 4 °C with primary antibodies in blocking solution. The membranes were washed with TBS-T and incubated for 1 h at 25 °C with horseradish peroxidase (HRP)-conjugated secondary antibodies (Supplementary Table 2). The blots were visualized using an ECL Kit (Thermo Scientific), and chemiluminescence images were obtained using a LAS-4000 lumino-image analyzer system (Fujifilm, Tokyo, Japan). The intensity of each band was quantified using ImageJ (v1.53c, NIH) and normalized to β-actin levels.

**Morphological characterization of IBA1-positive microglia**

The morphology of IBA1+ cells was analyzed and scored in five matching coronal slices of the midbrain (from −2.92 to −3.64 mm, relative to the bregma) per mouse, according to a previous method. On the basis of their morphological characteristics, IBA1+ cells were classified as resting microglia (type A, visible thin cytoplasm with long and thin processes), activated microglia (type B, dense and enlarged cell body with thick, short processes), and phagocytic microglia (type C, pseudo-amoeboid shape, large, dark cell body merging with processes).

**Behavioral assessments**

For behavioral tests, WT, Tpbg KO, and Tpbg KO mice were obtained through intercross breeding of Tpbg KO mice. Adult female mice at a young age (WT mice, n = 9; Tpbg KO mice, n = 5) and old age (WT mice, n = 9; Tpbg KO mice, n = 6; Tpbg KO mice, n = 9) were examined. All behavioral experiments were performed during the light phase of the mouse, with the mice habituated to the testing room for 1 h before the tests. All behavioral tests were conducted and analyzed in a genotype-blinded manner. For gait analysis, a 45 cm runway was placed between a bright light source and the home cage in a darkened room. The mice were trained over two consecutive days to walk across a 45 cm wide runway to their home cage. On the day of testing, the mice were placed on absorbent paper placed on the runway. The ink footprints were analyzed to measure stride length, stride width, and intra-step distance. For the challenging beam travel test, an acrylic beam was used consisting of four segments (25 cm each, 1 m total length) of varying widths (3.5, 2.5, 1.5, and 0.5 cm) and a wire mesh (1 cm width) of the corresponding width on each beam surface. The mice were trained to traverse the entire length of the beam without the mesh grid to their home cage for two consecutive days. On the day of the test, the mice were videotaped while traversing the grid-surfaced beam for a total of five trials. The time to traverse, number of steps, and number of errors were counted by viewing the videotapes in slow motion. For the adhesive removal test, a small adhesive stimulus was placed on the snout of the mouse using a tweezer. The mouse was placed back in its home cage and timed until it made contact with the stimulus (time to contact) and removed (time to removal) the stimulus. Each mouse underwent three trials with a cutoff of 1 min and an inter-trial interval of 2 min.

**Analysis of TPBG-interacting proteins**

To investigate the impact of alteration of Tpbg expression on the pathophysiology of PD, we identified TPBG-interacting proteins from the STRING database with a cutoff value of 0.4 (moderate confidence); the study yielded 36 proteins. Cytoscape and MCODE were used to identify dense clusters in the network of TPBG-interacting proteins. For functional analysis, enrichment analysis using DAVID was performed.

**Acquisition and analysis of RNA-seq data**

For RNA-seq analysis, total RNA was extracted from the dissected SNC tissues (Supplementary Fig. 11a) using the Easy- Spin Total RNA Extraction kit (iNTRON Biotechnology, Seongnam, South Korea) for paired-end sequencing, with the aim of generating over 60 million reads. Adapter sequences were trimmed using BBduk, and transcript quantification was performed using Salmon, using the reference transcript GENCODEvM23. To identify differentially expressed genes, transcript abundance estimates were imported into DESeq2 using tximport for further analysis. A correlation plot was generated using ggplot2 to visualize gene expression patterns. A heatmap was generated using heatmap (v1.0.12). GSEA pre-ranked analysis was performed using fgage (v1.16.0).

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism v9.0.2. For neurohistological and neurochemical analysis, data are presented as the mean ± standard deviation (SD) or standard error of the mean (SEM) of at least three individual subjects. Statistical significance was determined using the Mann–Whitney test for the comparison of genotypes (WT vs. Tpbg KO) or two-way ANOVA with Sidak’s multiple comparison test for the comparison of ages (young vs. old) and genotypes (WT vs. Tpbg KO). For behavior measurements, data are presented as the mean ± SEM. Differences between genotypes (young vs. old) and genotypes (WT vs. Tpbg KO) were analyzed using two-way ANOVA with Sidak’s multiple comparison test. Differences between WT, Tpbg KO, and Tpbg KO mice at old age were analyzed using the Kruskal–Wallis test with Dunn’s multiple comparison test. Significant differences were described in the graph when the p-value was less than 0.05 and assumed at ∗p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.001.

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**DATA AVAILABILITY**

RNA-seq data have been deposited in the Gene Expression Omnibus (GEO) database under accession codes GSE178400. All other relevant data supporting the key findings of this study are available within the article and its Supplementary Information file.
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AUTHOR CONTRIBUTIONS

S.P., D.-S.K., and D.-W.K. designed the experiments; S.P., J.-E.Y., J.S.L., and S.K.C. performed the experiments; S.P., J.-E.Y., G.-B.Y., J.H.K., Y.-G.H., C.W.P., M.S.C., J.K., D.N., and H.W.K. analyzed the data; and S.P., D.N., H.W.K, D.-S.K, and D.-W.K. wrote the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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