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Estradiol Facilitates Functional Integration of iPSC-Derived Dopaminergic Neurons into Striatal Neuronal Circuits via Activation of Integrin α5β1

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SUMMARY

For cell transplantation therapy for Parkinson's disease (PD) to be realized, the grafted neurons should be integrated into the host neuronal circuit to restore the lost neuronal function. Here, using wheat-germ agglutinin-based transsynaptic tracing, we show that integrin α5 is selectively expressed in striatal neurons that are innervated by midbrain dopaminergic (DA) neurons. In addition, we found that integrin α5β1 was activated by the administration of estradiol-2-benzoate (E2B) in striatal neurons of adult female rats. Importantly, we observed that the systemic administration of E2B into hemi-parkinsonian rat models facilitates the functional integration of grafted DA neurons derived from human induced pluripotent stem cells into the host striatal neuronal circuit via the activation of integrin α5β1. Finally, methamphetamine-induced abnormal rotation was recovered earlier in E2B-administered rats than in rats that received other regimens. Our results suggest that the simultaneous administration of E2B with stem cell-derived DA progenitors can enhance the efficacy of cell transplantation therapy for PD.

INTRODUCTION

Cell transplantation therapies have great promise for treatment against neurodegenerative disorders such as Parkinson's disease (PD). Accordingly, several groups have reported methods to induce midbrain dopaminergic (DA) neurons from human pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (Kriks et al., 2011; Kirkeby et al., 2012; Denham et al., 2012; Sundberg et al., 2013), and a robust and efficient induction method of midbrain DA progenitors was established toward clinical application (Doi et al., 2014). These ESC/iPSC-derived DA neurons can improve the rotational behavior of 6-hydroxydopamine (6-OHDA)-lesioned hemi-parkinsonian rat models (Kriks et al., 2011; Kirkeby et al., 2012; Sundberg et al., 2013; Doi et al., 2014).

Histological and electrophysiological analyses have revealed that grafted DA neurons can functionally integrate into host neural circuits (Mahalik et al., 1985; Sortwell et al., 1998; Sørensen et al., 2005; Tønnesen and Kokaia, 2012). Importantly, the survival and maturation of grafted neural progenitor cells depend on conditional cues from the host brain environment (Nishino et al., 2000; Morizane et al., 2013; Nishimura et al., 2015). Moreover, it has been reported that the adult brain has endogenous potential to recruit neural stem/progenitor cells for neuronal function repair (Höglinger et al., 2004; Paez-Gonzalez et al., 2014). Evoking this potential may promote graft-to-host synaptic connection for effective cell transplantation therapy for PD. However, which neurons in the striatum optimally form functional synapses with the grafted DA neurons to achieve long-term recovery remains unknown.

To address this issue, we examined striatal neurons innervated by nigral DA neurons using the plant lectin wheat-germ agglutinin (WGA). WGA can be efficiently transferred between neurons via synapses and has been already used as a transsynaptic tracer (Fabian and Coulter, 1985; Broadwell and Balin, 1985). Classically, WGA was used to visualize specific neural pathways by microinjection into a target region (Peschanski and Ralston, 1985; Buttry and Goshgarian, 2014). A more recent technique for tracing induces WGA cDNA via adeno-associated virus-mediated gene transduction (Yoshihara et al., 1999; Ohashi et al., 2011). Using this technique and histological analysis, we show here that integrin α5 is selectively expressed in the striatal neurons innervated by nigral DA neurons. Furthermore, we show that activation of integrin α5β1 by systemic administration of estradiol-2-benzoate (E2B), an E2 derivative, can promote early improvement in the rotational behavior of hemi-parkinsonian rat models that received iPSC-derived DA neuron transplantation. Our findings provide a strategy that takes pharmacotherapeutic advantage of clinically approved drugs to promote efficacious cell transplantation therapy for PD.

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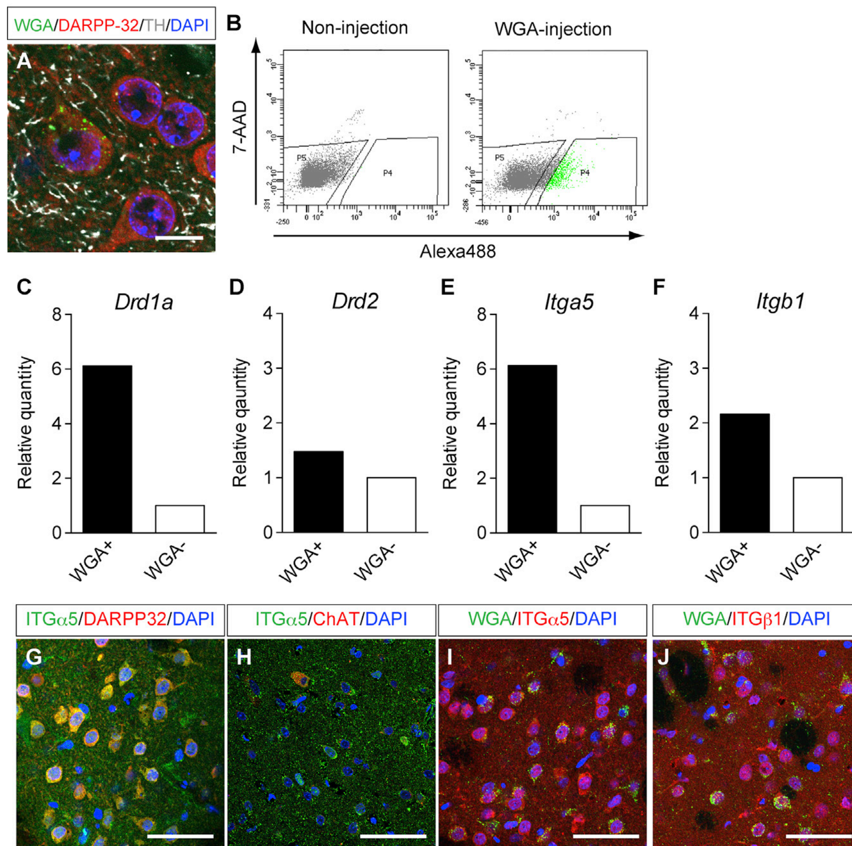

Figure 1. Characterization of Integrin α5 Highly Expressed in Striatum Neurons that Are Innervated from Midbrain DA Neurons in Mouse

(A) Typical image of striatal neurons that acquired WGA protein from midbrain DA neurons.

(B) Dot plot of FACS analysis of striatal cells 2 days after intranigral injection of WGA.

(C–F) Gene-expression comparisons between WGA$^+$ and WGA$^-$ populations by qPCR. The gene-expression level of the WGA$^-$ population was normalized to 1.

(G and H) Immunofluorescence images of normal mouse striatum.

(I and J) Immunofluorescence images of the mouse striatum 2 days after intranigral injection of WGA.

Scale bars: 10 μm (A) and 50 μm (G–J). See also Figures S1–S3 and Tables S2 and S3.

RESULTS

The Main Target of DA Neurons in the Nigrostriatal Pathway Is Medium-Sized Spiny Neurons in the Striatum

To identify the striatal neurons that are innervated by DA neurons in the nigrostriatal pathway, we injected Alexa 488-conjugated WGA (WGA-Alexa488) into the substantia nigra pars compacta (SNpc) of mice. Two days later, we found that WGA was incorporated into DA neurons of the SNpc (Figure S1A). Interestingly, approximately half of striatal NeuN$^+$ cells (post-mitotic neurons) were also positive for WGA (Figures S1B and S1F). In contrast, striatal GFAP$^+$ cells (astrocyte), Iba-1$^+$ cells (microglia), and CNPase$^+$ cells (oligodendrocyte) were not (Figures S1C–S1F).

We next examined the subtypes of the WGA$^+$ striatal neurons. The striatum contains various types of neurons including γ-aminobutyric acid (GABA) neurons, cholinergic neurons, and peptidergic neurons (Kaneko et al., 2000; Tepper and Bolam, 2004). Dopamine-regulated and cyclic AMP-regulated neuronal phosphoprotein 32-kDa (DARPP32)$^+$ medium-sized spiny neurons (MSNs) are a major cell population that covers approximately 95% of striatal neurons in rodents (Kawaguchi et al., 1995; Fienberg et al., 1998). Most MSNs express *GAD1* mRNA and DARPP32 protein (Trifonov et al., 2012), indicating that they are GABAergic neurons. Immunofluorescence staining of the striatum revealed that both DARPP32$^+$ neurons and choline acetyltransferase (ChAT)$^+$ neurons contained WGA protein (Figures 1A, S1G, and S1H). DARPP32$^+$ neurons were smaller than ChAT$^+$ neurons, and these two markers never overlapped (Figure S1I). Approximately 83% and 6% of WGA-incorporated cells expressed DARPP32 and ChAT, respectively, indicating that the main target of DA neurons in the nigrostriatal pathway is MSNs (Figure S1J). The neurons innervated by nigral DA neurons are expected to express DA D_1 receptor (DRD1) and/or D_2 receptor (DRD2) (Gerfen and Surmeier, 2011). Therefore, we investigated the expression of these two receptors, finding that approximately 80% and 50% of WGA-incorporated cells expressed DRD1$^+$ and DRD2$^+$ neurons, respectively (Figures S1K–S1M).

Integrin α5 Plays an Important Role in the Innervation of Nigral DA Neurons to Striatal Target Neurons

Next, we tried to identify cell adhesion molecules that are highly expressed in the striatal neurons innervated by nigral DA neurons. Two days after the injection of WGA-Alexa488

Table 1. Gene Screening of Cell Adhesion Molecules that Are Highly Expressed in the WGA$^+$ Population

Gene Symbol	RefSeq	Microarray (Fold Change; n = 3) [WGA$^+$]/[WGA$^-$]	qPCR (Relative Quantity; n = 2) [WGA$^+$]/[WGA$^-$]
Integrin			
Itgam	NM_001082960	5.47	2.89
Itgb2	NM_008404	4.07	NA
Itga6	NM_008397	3.59	NA
Itgb1	NM_010578	3.02	2.16
Itgav	NM_008402	3.01	1.24
Itgb5	NM_001145884	2.93	1.98
Itga5	NM_010577	2.24	6.13
Itgb4	NM_001005608	2.13	1.35
Itgb8	NM_177290	2.07	0.82
Protocadherin			
Pcdh9	NM_001081377	3.31	1.21
Lectin			
Lgals9	NM_010708	4.01	1.93
Lgals1	NM_008495	2.47	1.34
Lgals3	NM_001145953	2.17	4.07
Semaphorin			
Sema4d	NM_013660	3.62	3.09
Sema4c	NM_001126047	2.31	2.28
Sema6d	NM_199241	2.20	2.70
Sema7a	NM_011352	2.08	13.0
Extracellular matrix			
Fn1	NM_010233	6.10	3.73
Ecm1	NM_007899	3.67	2.56

NA, not available.

into mouse SNpc, we quickly dissociated striatal tissue and separated WGA$^+$ and WGA$^-$ populations by fluorescence-activated cell sorting (FACS) (Figures 1A and 1B). qPCR analyses revealed that the gene-expression levels of *Drd1a* and *Drd2* in the WGA$^+$ population were higher than those in the WGA$^-$ population (Figures 1C and 1D), suggesting that we succeeded in separating neurons innervated by nigral DA neurons. Subsequently, we performed microarray analysis to compare the gene-expression profiles of the WGA$^+$ and WGA$^-$ populations. We focused on cell adhesion molecules, finding 19 candidates that had higher expression in the WGA$^+$ population (Table 1). We confirmed the expression of these candidate genes in striatal tissue by RT-PCR analysis (Figure S2A). Since the expression levels of *Itgb2* and *Itga6* were low in striatum tissue, these genes were excluded from the following experiments. We then examined the expression levels of the remaining candidates in several brain regions (Figure S2B). Intriguingly, integrin α5 was highly expressed in the striatum, and more abundantly so in the WGA$^+$ population (Figure 1E). It is known that integrin α5 forms heterodimers with integrin β1 for cell adhesion to fibronectin (FN) (Hynes, 1992). Integrin β1 also showed higher expression in the WGA$^+$ population according to microarray and qPCR analyses (Figure 1F and Table 1). Consistently, immunofluorescence studies showed that integrin α5 was expressed by DARPP32$^+$ neurons and ChAT$^+$ neurons in mouse striatum (Figures 1G and 1H). Furthermore, in mice that received intranigral injection of WGA, almost all WGA$^+$ cells expressed both integrins α5 and β1, suggesting that striatal neurons innervated by nigral DA neurons expressed integrin α5β1 (Figures 1I and 1J).

Previous studies have demonstrated that the striatum shows a unique mosaic structure, the so-called striosome, in which DARPP32$^+$ neurons accumulate during developmental and neonatal stages (Gerfen, 1992; Antonopoulos et al., 2002). In addition, DA neuronal fibers heterogeneously form the high-density structure of the striosome (Graybiel, 1984). This unique structure disappears as the brain develops, and the distribution of DARPP32$^+$ neurons and DA neuronal fibers become uniform at the adult stage. Consistently, we observed that the soma of DARPP32$^+$ neurons accumulated in the striosome and that DA neuronal fibers were highly enriched in the striosome from postnatal day 0 (P0) to P7 (Figures S3A–S3C and S3A′–S3C′). These distributions became uniform from P16 (Figures S3D, S3E, S3D′, and S3E′). Interestingly, integrin α5 was also highly expressed in the striosome during the neonatal stage (Figures S3A″–S3E″). Taken together, these results suggest that integrin α5 plays a pivotal role in the innervation of nigral DA neurons to striatal target neurons.

Finally, we examined the gene-expression levels of integrins α5 and β1 in human brain. qPCR analyses using human tissue samples revealed that both integrins had higher expressions in the putamen than in the cortex (Figure 2A). We also examined their expression levels in postmortem putamen samples from healthy control and PD patients, but found no significant differences between the two conditions (Figure 2B and Table S1).

Estradiol-Induced Activation of Integrin α5β1 Promoted Attachment of Striatal Neurons to Fibronectin

Activation of integrin α5β1 enhances the connection with FN via the reelin pathway during cortical development

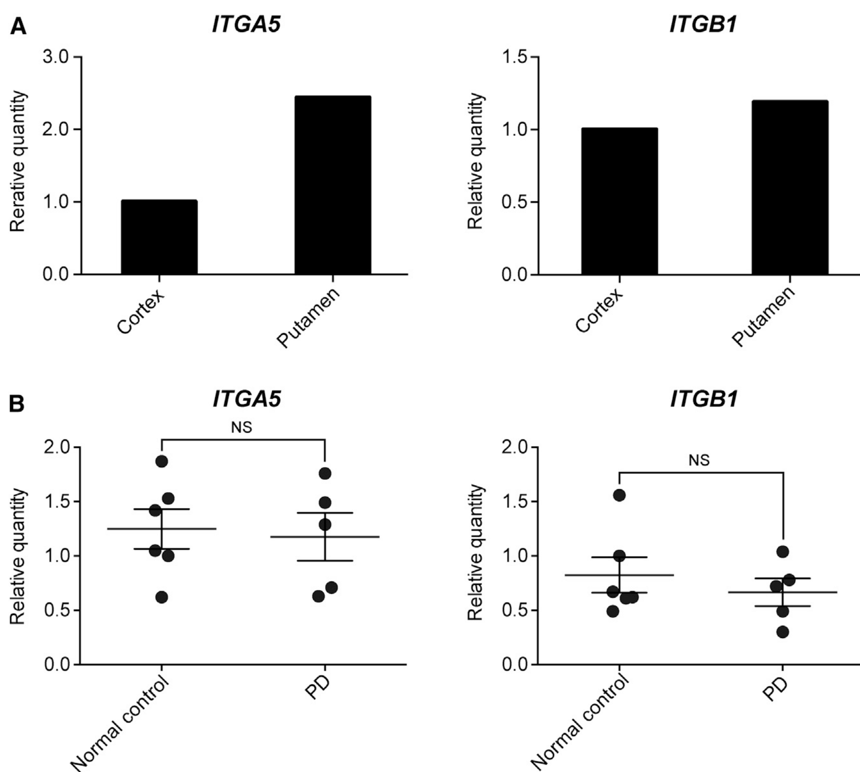

Figure 2. Comparison of Integrin α5 and Integrin β1 Gene Expressions in Human Samples

(A) qPCR-based relative comparison of integrin α5 and integrin β1 in the cortex and putamen of normal samples purchased from Clontech. Gene-expression levels of the cortex were normalized to 1.0.

(B) qPCR-based relative comparison of integrin α5 and integrin β1 in the postmortem putamen samples of normal control and PD patients. Gene-expression levels of control were normalized to 1.0. Quantitative data are represented as the mean ± SEM (n = 5–6 independent samples). Significance (Student's t test): NS, not significant.

See also Tables S1 and S2.

(Sekine et al., 2012). The reelin pathway can be activated by the effect of E2 in neonatal rat hippocampus (Bender et al., 2010). Based on these previous findings, we determined whether integrin α5 in striatal neurons could be activated by E2 administration in adult rats. First, we confirmed that estrogen receptor α (ERα) and ERβ, both E2 receptors, were expressed in striatal neurons (Figures 3A and 3B), and key molecules of the reelin pathway were also expressed in the striatum (Figures 3C–3E). Next, adult rats were treated with vehicle (sesame oil), 5 μg/kg E2B, 50 μg/kg E2B, or 50 μg/kg E2B plus 1 mg/kg ICI182.780 for 7 days, and the expression and activation of integrin α5β1 in the striatal tissue were examined by western blot analysis. The expressions of integrins α5 and β1 and the activation of integrin β1 were increased by the administration of 50 μg/kg E2B for 7 days (Figures 3F–3I).

Next, we determined whether the E2B-induced activation of integrin α5β1 promotes the attachment of striatal neurons to FN. Adult rats were treated with the same conditions as above for 7 days, and the striatal tissue was dissociated to single cells and seeded on an FN-coated dish. At first we confirmed that the neutralizing antibody against integrin α5 never prevented the attachment of striatal cells to other matrices, such as poly-L-lysine, laminin, and poly-L-ornithine (Figure 3J). The number of attached cells was significantly higher when the animal was treated with 50 μg/kg E2B, and this increase was significantly suppressed by treatment with a neutralizing antibody against integrin α5 (Figures 3K and 3L). As expected, E2 concentration levels in serum were elevated by the administration of E2B (Figure 3M). These results indicated that the E2B-induced activation of integrin α5β1 promoted the attachment of striatal neurons to FN.

Activation of Integrin α5β1 Contributes to Synapse Formation In Vitro

To determine whether integrin α5β1 is involved in synapse formation, we performed an in vitro co-culture assay of human iPSC-derived DA neurons and integrin α5β1-overexpressing HEK293 cells (Kim et al., 2011). HEK293 cells are not of a neural cell line and therefore do not express endogenous neural proteins. An assay using HEK293 cells, however, should reduce the complexity of the transsynaptic signal and neuronal modification (Biederer and Scheiffele, 2007). The human integrin α5β1-overexpressing HEK293 cells were designed to also express GFP by lentiviral transduction to distinguish them from iPSC-derived DA neurons. The expressions of integrin α5 and integrin β1 were confirmed by western blot analysis and immunocytochemistry (Figures 4A–4C). Next, DA progenitors were induced from human iPSCs (1039A1) according to a previously reported protocol (Doi et al., 2014). The DA progenitors (day 28) and mature DA neurons (day 42) expressed FN, which is a ligand of integrin α5β1 (Figures 4D and 4E).

DA progenitors were replated on ornithine/laminin (O/L)-coated dishes and cultured until day 49. As shown in Figure 4F, the cells extended TH$^+$ fibers that also expressed FN. Integrin α5β1-overexpressing HEK293 cells were seeded on the DA progenitors (day 49) and cultured for 2 days. Immunoreactivities against two presynaptic markers, synapsin and bassoon, were more abundantly detected around integrin α5β1-overexpressing HEK293 cells than around mock-transfected HEK293 cells (Figures 4G–4J). These results indicate that integrin α5β1 is involved in the synapse formation of human iPSC-derived DA neurons.

E2B-Induced Activation of Integrin α5β1 Accelerates Behavioral Recovery of 6-OHDA-Lesioned Rats

To evaluate transsynaptic transmission, we generated an iPSC line which stably expressed WGA by *piggybac* vector (Figures 5A–5C). We induced DA progenitors from the WGA-expressing iPSCs according to a previously reported protocol (Figure 5D; Doi et al., 2014). The differentiated cells expressed two midbrain markers, NURR1 and FOXA2, on day 28 (Figure 5E). When these cells were incubated in an attached culture until day 56 they became TH$^+$ mature DA neurons, which also expressed WGA (Figure 5F).

Next, we grafted the WGA-expressing DA progenitors (day 28) into the striatum of X-linked severe combined immunodeficiency (X-SCID) F344 rats (Samata et al., 2015). These rats were pre-treated with 6-OHDA and showed abnormal rotation in response to methamphetamine because of an imbalance between right and left extremities (Schwarting and Huston, 1996). Along with the cell transplantation, daily subcutaneous injection of vehicle, 50 μg/kg E2B, or 50 μg/kg E2B plus 1 mg/kg ICI182.780 was made until 1 day before the rats were euthanized. At the time of death, the average trough E2 concentration in serum was 36.7 ± 10.6 pg/ml, 277.4 ± 93.8 pg/ml, and 285.0 ± 172.4 pg/ml in the vehicle, E2B, and E2B + ICI groups, respectively (n = 5–7). As shown in Figure 6A, every group showed a gradual reduction of abnormal rotation. Intriguingly, the rats with E2B showed significant improvement at earlier time points compared with rats that received vehicle or E2B + ICI (Figure 6B). Immunofluorescence staining of the grafts at 16 weeks revealed that a large number of mature DA neurons (TH$^+$ FOXA2$^+$) survived in each group (Figures 6C–6E) and extended TH$^+$ neuronal fibers toward DARPP32$^+$ striatal neurons (Figure 6G). When we counted the number of mature DA neurons per graft volume at 12 and 16 weeks, we found no significant difference among the three groups (Figure 6F). The grafted cells and their fibers also expressed WGA and human synaptophysin (hSyn), a presynaptic protein (Figure 6H). WGA was transferred into DARPP32$^+$ host striatal neurons, which were co-localized with hSyn (Figure 6I), suggesting that the grafted DA neurons formed synapses with host striatal neurons. On the other hand, we never observed WGA staining in DARPP32$^+$ neurons in the contralateral striatum (Figures S4A and S4B). The percentage of WGA$^+$ cells per DARPP32$^+$ striatal neuron at 12 weeks was significantly higher in the rats with E2B treatment (13.75% ± 2.47%) compared with those that received vehicle (6.58% ± 3.34%) or E2B + ICI (6.55% ± 3.18%) (Figure 6J). In addition, TH$^+$ hSyn$^+$ neuronal terminals were detected on the surface of the host DARPP32$^+$ neurons (Figure S4C).

DISCUSSION

In this study, we demonstrated that integrin α5 is highly expressed in striatal neurons innervated by nigral DA neurons. In addition, systemic administration of E2B activated integrin α5β1 in the striatum and promoted behavioral recovery of 6-OHDA-lesioned rats by mediating synapse formation of grafted DA neurons with host striatal neurons. E2 is an important female sex steroid that is widely used to treat menopausal disorders. That we show its derivative, E2B, has effects on the brain environment suggests a pharmacotherapeutic strategy for cell transplantation therapy against PD.

Robust and reproducible protocols for the induction of midbrain DA neurons from human ESCs/iPSCs have been reported by several groups (Kriks et al., 2011; Denham et al., 2012; Kirkeby et al., 2012; Sundberg et al., 2013; Doi et al., 2014). To understand the therapeutic mechanisms of grafted DA neurons, it is necessary to investigate how the grafted cells integrate into the host neuronal circuit. Recently, several technologies such as diphtheria toxin-based neuronal ablation, rabies virus-mediated monosynaptic tracing, and optogenetics have been used for this purpose (Abematsu et al., 2010; Grealish et al., 2015; Steinbeck et al., 2015). In the present study we describe another experimental method, WGA-based transsynaptic tracing, to observe transsynaptic connection. Using this technique, we demonstrated that integrin α5β1 is highly expressed in DARPP32$^+$ striatal neurons innervated by nigral DA neurons. The integrin superfamily consists of cell adhesion molecules that bind to the extracellular matrix and form αβ heterodimers, and includes at least 18 α and 8 β subunits in humans (Takada et al., 2007). Integrin α5 is broadly distributed in the body, but at low levels in the brain (Pinkstaff et al., 1999). According to experiments on cortical and hippocampal neurons, integrin α5β1 interacts with FN and regulates cellular migration, synaptogenesis, and the maintenance of synaptic plasticity (Chan et al., 2003; Webb et al., 2007; Gardiner et al., 2007; Hu and Strittmatter, 2008; Sekine et al., 2012). These findings are consistent with our in vitro data showing that human

Figure 3. Activation of Integrin $\alpha 5\beta 1$ in the Striatal Tissue of E2B-Treated Adult Female Rats

(A–E) Immunofluorescence images (A, B, D, and E) and western blot (C) of striatal neurons in adult female F344 rat. Arrows indicate $ER\alpha^{+}$ and $DARPP32^{+}$ neurons (A) and $ER\beta^{+}$ and $DARPP32^{+}$ neurons (B). VLDLR, very-low-density lipoprotein receptor. (D) Scale bars represent 50 μm.

(F) Western blot analysis of the striatal tissue in E2B-treated adult female rats.

(G–I) Semi-quantitative analysis of integrin α5 (G), integrin β1 (H), and activated integrin β1 (I) in E2B-treated adult female F344 rats. Data represent the mean ± SEM (n = 3–4 independent samples). Significance (one-way ANOVA with Tukey's multiple comparisons test): $*p < 0.05$; $**p < 0.01$.

(legend continued on next page)

iPSC-derived DA neurons efficiently form synapses with integrin α5β1-overexpressing HEK293 cells (Figure 4).

Systemic administration of E2B induced activation of integrin α5β1 in rat striatum (Figure 3). Since the activation of integrin α5β1 could facilitate attachment to FN, it is possible that the affinity between graft-derived neurites and target neurons is enhanced. Accordingly, the transmission of WGA from the grafted DA neurons to DARPP32$^+$ striatal neurons was promoted by the administration of E2B (Figure 6). E2B did not directly promote the expression of several midbrain DA markers (*NURR1*, *FOXA2*, *TH*, *SLC18A2*, and *SLC6A3)*, synaptic markers (*SYNAPSIN* and *DREBRIN*), or estradiol receptors (*ESR1* and *ESR2*) by iPSC-derived DA progenitors (day 28), whereas a combination of glial cell-derived neurotrophic factor (GDNF), ascorbic acid (AA), brain-derived neurotrophic factor (BDNF), and dibutyryl cyclic AMP (dbcAMP) promoted the expression of *NURR1*, *TH*, *SLC18A2*, and *SYNAPSIN* in vitro (Figures S4D–S4N). These findings suggested that the enhanced synapse formation and function of the grafted DA neurons was mediated by E2B-induced activation of integrin α5β1 of host striatal neurons.

Neuroanatomical studies indicate that DARPP32$^+$ neurons could be divided into two populations: one that constructs a direct pathway and another that constructs an indirect pathway. DARPP32$^+$ neurons in the direct pathway express DRD1 and are located in the striosome. On the other hand, those in the indirect pathway express DRD2 and are located in the matrix (Gerfen et al., 1990; Crittenden and Graybiel, 2011). A recent neurotracing study indicated that DARPP32$^+$ neurons in both the striosome and matrix are innervated by nigral DA neurons (Matsuda et al., 2009). Our study revealed that DRD1$^+$ neurons are more frequently innervated by nigral DA neurons than are DRD2 $^+$ neurons. Interestingly, integrin α5 was dominantly expressed in the striosome, where the soma of DARPP32$^+$ neurons and TH$^+$ neuronal fibers had accumulated during the neonatal stage (Figure S3). These results suggested that the process of synapse formation between the grafted DA neurons and striatal neurons recapitulates the DA innervation process during development.

In conclusion, we show that E2B could be used to activate integrin α5β1 in adult rodent striatum. Importantly, a qPCR analysis of postmortem human brains indicated that the expression levels of integrins α5 and β1 were still maintained in the putamen of PD patients (Figure 2). These results suggest that E2B could be used to modify the host brain environment in a way that improves the outcome of cell transplantation therapy for PD.

EXPERIMENTAL PROCEDURES

Culture of Undifferentiated iPSCs

Human iPSC line 1039A1 (XY, passages 15–30) (Nakagawa et al., 2014; Samata et al., 2015) was maintained on an LN511-E8-coated dish with StemFit AK03 medium (Ajinomoto) (Nakagawa et al., 2014). These cells were replated at a density of 1.5×10^4 cells per six-well plate for each passaging.

Establishment of WGA-Expressing Human iPSC Line

Piggybac vector PB-EF1-MCS-IRES-Neo was purchased from System Bioscience. The coding region of WGA cDNA (a generous gift from Dr. Yoshihara of the RIKEN Brain Science Institute) was PCR cloned into the PB-EF1-MCS-IRES-Neo vector. The PB-WGA-IRES-Neo vector and transposase plasmid were then transfected into iPSCs (1039A1) with FUGENE HD (Roche) 5 days after seeding on an LN511-E8-coated dish. Three days after transfection, 500 mg/ml G418 selection was started to obtain WGA-expressing iPSCs. iPSCs were reseeded on an LN511-E8-coated dish 3 days after G418 selection to obtain a single clone of WGA-expressing iPSCs. We then picked 24 single colonies that seemed to express WGA 7 days after G418 selection and chose the clone that most stably expressed the WGA protein.

Induction of DA Progenitors

The induction of DA progenitors was performed according to a previous report (Doi et al., 2014). Undifferentiated iPSCs were replated at a density of 6×10^6 cells per 6-well LN511-E8-coated plate. Cells were differentiated in Glasgow minimum essential medium (Invitrogen) supplemented with 8% knockout serum replacement (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), sodium pyruvate (Sigma), and 0.1 mM 2-mercaptoethanol (Wako). 100 nM LDN193189 (Stemgent) and 500 nM A83-01 (Wako) were supplemented from day 0, 2 μM purmorphamine and 100 ng/ml

(J) Effects of neutralizing antibody against integrin α5 in the cell adhesion assay to several matrices using the striatal cells of adult female rats. Quantitative data are represented as the mean ± SEM (n = 4 independent experiments) and normalized to non-Ab. Significance (one-way ANOVA with Tukey's multiple comparisons test): ***p < 0.001. NS, not significant.
(K) Cell adhesion assay to FN using the striatal cells of E2B-treated adult female rats. Quantitative data are represented as the mean ± SEM (n = 6 independent samples). Significance (one-way ANOVA with Tukey's multiple comparisons test): *p < 0.05; **p < 0.01; ***p < 0.001.
(L) Analysis of neutralizing antibody against integrin α5 in the cell adhesion assay to FN using the striatal cells of E2B-treated adult female rats. Quantitative data are represented as the mean ± SEM (n = 4 independent experiments). Significance (one-way ANOVA with Tukey's multiple comparisons test): *p < 0.05; **p < 0.01; ***p < 0.001.
(M) E2 concentration in serum of E2B-treated adult female rats for 7 days. Quantitative data are represented as the mean ± SD (n = 3–4 independent animals). Significance (one-way ANOVA with Tukey's multiple comparisons test): **p < 0.01; ***p < 0.001.
See also Table S3.

Figure 4. Co-culture Assay for Synaptic Formation between iPSC-Derived DA Neurons and Integrin α5β1-Overexpressing HEK293 Cells

(A) Western blot analysis of human integrin α5β1-overexpressing HEK293 cells.

(B–C′) Immunofluorescence of integrin α5β1-overexpressing HEK293 cells (B and C) and mock-transfected HEK293 cells (B′ and C′). Inset boxes indicate immunofluorescence of integrin α5 (B and B′) and integrin β1 (C and C′).

(D) Western blot analysis of FN in iPSC-derived DA progenitors (day 28) and DA neurons (day 42).

(E and F) Immunofluorescence of iPSC-derived DA progenitors (day 28, E) and DA neurons (day 49, F).

(G–I′) Immunofluorescence of presynaptic markers synapsin (G and G′) and bassoon (I and I′) 2 days after co-culture of hiPSC-derived DA neurons with integrin α5β1-overexpressing HEK293 cells (G and I) or mock-transfected HEK293 cells (G′ and I′).

(H) Relative intensity of synapsin immunoreactivity. Quantitative data are represented as the mean ± SEM (n = 3 independent experiments). Significance (Student's t test): $^{*}p < 0.05$.

(J) Relative intensity of bassoon immunoreactivity. Quantitative data are represented as the mean ± SEM (n = 5 independent experiments). Significance (Student's t test): $^{***}p < 0.001$.

Scale bars: 25 μm (B, C, and F), 100 μm (E), and 10 μm (G and I). See also Table S3.

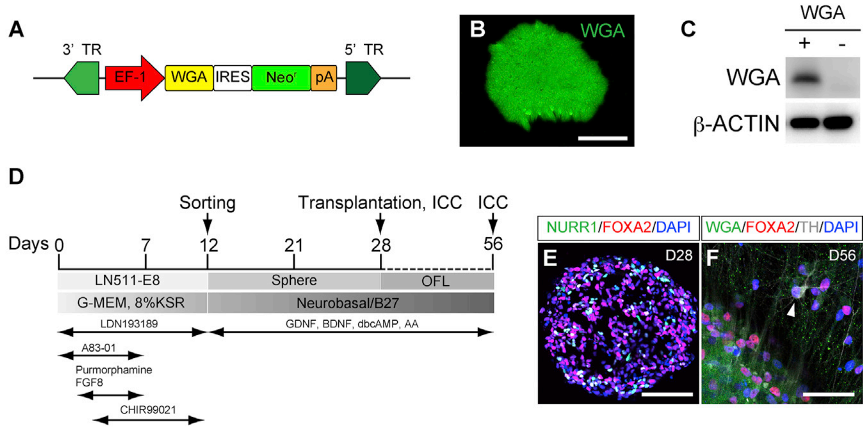

Figure 5. Establishment of a WGA-Expressing iPSC Line

(A) Vector construct of WGA-expressing *piggybac* vector.

(B) Typical image of WGA-expressing undifferentiated iPSCs.

(C) Western blot analysis of WGA expression in undifferentiated iPSCs.

(D) Protocol for induction of DA progenitors from iPSCs.

(E and F) Immunofluorescence images of iPSC-derived DA progenitor on day 28 (E) and DA neurons on day 56 (F). Arrowhead indicates WGA$^+$ FOXA2$^+$ TH$^+$ neurons.

Scale bars: 200 μm (B), 100 μm (E), and 25 μm (F). See also Table S3.

fibroblast growth factor 8 (FGF8; Wako) were supplemented from day 1, and 3 μM CHIR99021 (Wako) was supplemented from day 3. A83-01, purmorphamine, and FGF8 were removed from culture medium on day 7. CORIN$^+$ cells were then sorted using FACS AriaIII and quickly aggregated on low-cell-adhesion 96-well plates at a density of 2 × 10^4 cells/well. Neuronal differentiation was induced in Neurobasal medium (Invitrogen) supplemented with B27 supplement (Invitrogen), 2 mM L-glutamine (Invitrogen), 10 ng/ml GDNF (Wako), 200 mM AA (Wako), 20 ng/ml BDNF (Wako), and 400 mM dbcAMP (Sigma). iPSC-derived DA progenitors on day 28 were used for transplantation experiments. Additional differentiation was extended on an ornithine/fibronectin/laminin-coated dish until day 56 for in vitro analysis.

Human Brain Tissue

Postmortem human putamen samples (n = 11; six normal controls, five PD patients) were provided from the Brain Bank at the Tokyo Metropolitan Institute of Gerontology. This research project was approved by ethics committees at Kyoto University and Tokyo Metropolitan Institute of Gerontology. We used the same samples from our previous work (Nishimura et al., 2015).

Animals

Male 8-week-old C57BL/6NCrSlc mice and female 10-week-old Fisher 344 (F344) rats were purchased from SLC. Female F344-Il2rg^{em2Kyo} X-SCID rats were maintained at Kyoto University (NBRP-Rat No. 0586, Kyoto University; http://www.anim.med.kyoto-u.ac.jp) (Mashimo et al., 2010). Animals were housed in a standard laboratory cage. All animal experiments were performed according to the Guidelines for Animal Experiments of Kyoto University and the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources.

Anterograde Transsynaptic Tracing of the Nigrostriatal Pathway by WGA and FACS

One percent WGA Alexa Fluor 488 solution (0.5 μl) was stereotactically injected into the bilateral SNpc (from the bregma: A −3.0, L ±1.2, V −4.2, and TB 0). Two days after injection, a striatum sample was dissected from the mouse brain and dissociated into a single-cell population by Accumax (Innovated Cell Technologies). To separate a two-cell population, we sorted Alexa Fluor 488$^+$ cells and Alexa Fluor 488$^-$ cells using a FACSAria III cell sorter. Dead cells were identified and eliminated by 7-amino-actinomycin D (7-AAD) staining.

Hemi-Parkinsonian Rat Model and Rotational Behavior

Female 10-week-old X-SCID rats were used for the 6-OHDA-lesioned hemi-parkinsonian model. A total of 15 μg of 6-OHDA hydrochloride (Sigma) in 3 μl of saline with 0.02% AA was stereotactically injected into the medial forebrain bundle in the left side of the rat brain (from the bregma: A −5.3, L +1.2, V −7.0, and TB +1.5). The methamphetamine-induced rotation assay was performed 2 or 4 weeks before transplantation and 4, 8, 10, 12, 14, or 16 weeks after transplantation. Rotational behavior was automatically recorded for 90 min after intraperitoneal administration of 2.5 mg/kg methamphetamine hydrochloride (Dainippon Sumitomo Pharma).

Cell Transplantation into Hemi-Parkinsonian Rat Models

iPSC-derived DA progenitors were stereotactically transplanted through a 22-gauge needle into the left side of the striatum (from the bregma: A +1.0, L +3.0, V −5.0 and −4.0, and TB 0). Each rat received approximately 4 × 10^5 cells in 2 μl (2 × 10^5 cells/μl for one point; 1 μl/10 s). Immunosuppressant was not used because X-SCID rats were used for the xenotransplantation.

E2B Administration to Rats

Female adult rats were subcutaneously administered vehicle (sesame oil; Nacalai Tesque) or E2B (5 or 50 μg/kg; Sigma) with or without 1 mg/kg ICI182.780 (AdooQ Bioscience) for 7 days, once daily. For xenotransplantation, X-SCID rats were administered vehicle (sesame oil) or 50 μg/kg of E2B with or without 1 mg/kg ICI182.780 once daily from 7 days before transplantation until 1 day before they were euthanized.

RT-PCR

Total RNA was extracted from the cells using an RNeasy Plus Micro kit (Qiagen), after which 1 μg of total RNA was used for reverse

Figure 6. Rotational Behavior and Histological Evaluation of Hemi-Parkinsonian Rat Models that Are Grafted iPSC-Derived DA Progenitors and Received E2B Treatment

(A and B) Methamphetamine-induced rotational behavior (A) and improvement ratio of rotational behavior (B) in 6-OHDA-lesioned rats after cell grafting. Quantitative data are represented as the mean ± SD (n = 9–12 independent animals, pre to 12 weeks; n = 5–7 independent animals, 14 and 16 weeks). Significance (two-way ANOVA with Tukey's multiple comparisons test): *$p < 0.05$, ***$p < 0.001$ versus pre of vehicle; $^{\dagger}p < 0.05$, $^{\dagger\dagger\dagger}p < 0.001$ versus pre of E2B; $^{\S}p < 0.05$, $^{\S\S\S}p < 0.001$ versus pre of E2B + ICI.

(legend continued on next page)

transcription by a Super Script III First-Strand Synthesis System with Oligo(dT)$_{20}$ primer (Invitrogen). Amplification was performed with TaKaRa Ex Taq (Takara Bio). The primer sequences and product sizes are shown in Table S2. Normal human samples were purchased from Clontech Laboratories.

qRT-PCR

qPCR was performed on a StepOne detection system (Applied Biosystems). Data analysis is based on the ΔC_T method with normalization of the raw data to *Gapdh* genes. All PCRs were performed in triplicate. Primer sequences are shown in Table S2.

Cell Adhesion Assay

A 12-well plate was coated with 20 μg/ml FN, 20 μg/ml poly-L-lysine, and 20 μg/ml poly-L-ornithine or 20 μg/ml laminin at 37°C for 10 min. Adult rat striatum was dissociated mechanically by pipetting, and the acquired cells were filtered through cell-strainer caps (35 μm mesh) (BD Biosciences). The cells were plated onto the coated wells (7×10^4 cells per well) for 10 min at 37°C. Attached cells were fixed in 4% paraformaldehyde (PFA) for 15 min at 4°C, stained with DAPI (Invitrogen) and automatically counted by BioRevo (Keyence). A neutralizing antibody against integrin α5 (MFR5; Abcam; 20 μg/ml) or isotype-matched control (rat IgG2a; MBL; 20 μg/ml) was added to the medium.

Synapse Formation Assay in Co-culture System

HEK293 cells that constitutively expressed GFP were transfected with pCMV-human integrin α5-IRES-human integrin β1 plasmids using FuGENE6 DNA Transfection Reagent (Promega). Human integrin α5β1-overexpressing HEK293 cells were selected by 500 μg/ml G418 from 2 days after transfection. iPSC-derived DA progenitors were attached on an O/L-coated eight-well chamber slide on day 28 and cultured until day 49. Integrin α5β1-overexpressing HEK293 cells or mock-transfected HEK293 cells (5,000 cells/well) were co-cultured with iPSC-derived DA neurons (day 49) for 2 days. Cells were fixed in 4% PFA 2 days after co-culture.

Microarray

Total RNA was extracted using an RNeasy Plus Mini kit. 50 ng of total RNA was processed by an Ambion WT Expression Kit and Affymetrix GeneChip Whole-Transcript Expression Arrays (Ambion). Samples were hybridized to GeneChip Human Gene 1.0 ST Arrays (Affymetrix) according to the manufacturer's protocol. WGA$^+$ and WGA$^-$ populations were sorted by FACS and analyzed using GeneSpring GX13.0 software (Agilent Technologies). The expression signals of the probe sets were calculated using PRIER16.

In Vitro Assay in iPSC-Derived DA Progenitors with E2B

Differentiated iPSC-derived DA progenitors (day 28) were treated with 1, 10, or 100 nM E2B or with 10 nM E2B with 2 μM ICI182.780 for 7 days. 0.1% DMSO was used as the vehicle. Neurobasal (without phenol red) with B27 and L-glutamine was used for the culture medium during the E2B treatment. A combination of GDNF, AA, BDNF, and dbcAMP was used as positive control.

Western Blot Analysis

Cell and brain tissue were homogenized in the sample buffer, and 5–10 μg of protein was analyzed by SDS-PAGE using TGX Precast Gels (Bio-Rad). The immunoblotting was then carried out using antibodies (Table S3). Signal detection was visualized using ECL Plus and detected with ImageQuant LAS 4000 (GE Healthcare).

Immunofluorescence

Animals were perfused with 100 mM PBS and then 4% PFA in PBS under deep anesthesia with sodium pentobarbital (100 mg/kg intraperitoneally). Brain was post-fixed for 2 days with 4% PFA and then transferred to 30% sucrose solution at 4°C. The brain pieces were sectioned at 40-μm thickness using a cryostat microtome. The free-floating sections were washed in PBS containing 0.1% Triton X-100 (PBST). Sections then were incubated at 4°C overnight with primary antibodies (Table S3). Samples were incubated with fluorescent-dye-conjugated secondary antibody. In addition, the samples were incubated with DAPI. Finally, the sections were observed using a confocal fluorescence microscope (Olympus FV1000; Tokyo, Japan).

Immunocytochemistry

For in vitro multiple immunofluorescence, cells were fixed with 4% PFA in PBS for 30 min. Slides were pre-incubated with PBST

(C–E) Immunofluorescence images of grafted-iPSC-derived DA neurons 16 weeks after transplantation in rats that were administered vehicle (C), E2B (D), or E2B with ICI182.780 (E).
(F) Quantitative analysis of FOXA2$^+$ TH$^+$ neurons in the graft 12 weeks and 16 weeks after transplantation. Quantitative data are represented as the mean ± SD (n = 4–5 independent samples, 12W; n = 5–6 independent samples, 16W). Significance (one-way ANOVA with Tukey's multiple comparisons test): NS, not significant.
(G and H) Typical images of the elongation of TH$^+$ neuronal fibers into host striatum (G) and WGA-immunoreactivity in grafted neurons (H) 16 weeks after transplantation in E2B-administered rat. Arrows indicates the graft-derived TH$^+$ neuronal fibers (G).
(I–I‴) Representative image of WGA$^+$ DARPP32$^+$ neurons 12 weeks after transplantation in E2B-administered rat. Arrows indicate WGA$^+$ signal in DARPP32$^+$ neurons.
(J) Quantitative analysis of the percentage of WGA$^+$ per DARPP32$^+$ neuron in the graft 12 weeks after transplantation. Quantitative data are represented as the mean ± SD (n = 4–5 independent samples). Significance (one-way ANOVA with Tukey's multiple comparisons test): $^*p < 0.05$.
Scale bars: 200 μm (C, D, E, G, and H) and 10 μm (I). See also Figure S4 and Tables S2 and S3.

and 2% skimmed milk for 60 min and then incubated at 4°C overnight with primary antibodies.

3,3′-Diaminobenzidine Staining

Free-floating sections were treated with 0.3% hydrogen peroxide in PBST. Sections were then incubated with primary antibodies. The sections were incubated with biotinylated antibodies against mouse, rabbit, and rat immunoglobulin G (diluted 1:2,000; Vector Laboratories) and then incubated with avidin peroxidase (diluted 1:4,000; Vectastain ABC Elite kit, Vector Laboratories). Signal detection was done by 3,3′-diaminobenzidine (Dojindo Laboratories) with nickel ammonium.

Histological Evaluation

Immunopositive cells were manually counted for at least three independent samples to calculate the number and percentage of positive cells for each marker. The graft volume and the number of immunoreactive cells were determined by the $hSyn^+$ area in every sixth 40-μm-thick section using BZ-II Analyzer software (Keyence) and totaling the volumes of whole-tall cylinders according to Cavalieri's principle.

Statistical Analysis

Results are given as means ± SD or SEM. The significance of differences was determined by Student's t test for single comparisons and by one-way ANOVA or two-way ANOVA for multiple comparisons. Further statistical analysis for post hoc comparisons was performed using Tukey's test (Prism 6; GraphPad).

ACCESSION NUMBERS

The Gene Expression Omnibus accession number for the microarray data reported in this paper is GEO: GSE77274.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.02.008.

AUTHOR CONTRIBUTIONS

K.N. designed the project, performed all experiments, and wrote the manuscript. D.D. provided technical support for the iPSC culture and DA neuronal differentiation. B.S. provided technical support for the cell sorting and generation of hemi-parkinsonian rat models. S.M. collected postmortem brain samples and performed the neuropathological diagnosis. T.T. and H.O. provided plasmid and scientific discussions. J.T. supervised the whole project, cowrote the manuscript with K.N., and made final approval of the manuscript.

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