Constitutive activity of a G protein-coupled receptor, DRD1, contributes to human cerebral organoid formation

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Abstract
The intricate balance of neural stem cell (NSC) amplification and neurogenesis is central to nervous system development. Dopamine D1 receptor (DRD1) is a typical G protein-coupled receptor (GPCR) mainly expressed in neurogenic area, with high constitutive activity. The receptor appears in the embryonic period before the formation of mature synaptic contacts, which indicates that dopamine receptor and its constitutive activity play crucial roles in the embryonic brain development. Here, we found that DRD1 was enriched in human NSCs. Inhibition of the receptor activity by its inverse agonists promoted human NSCs proliferation and impeded its differentiation. These results were also mimicked by genetic knockdown of DRD1, which also blocked the effects of inverse agonists, suggesting a receptor-dependent manner. More interestingly, knock-in A229T mutant with reduced DRD1 constitutive activity by CRISPR-Cas9 genome editing technology resulted into increased endogenous human NSCs proliferation. These results were well reproduced in human cerebral organoids, and inhibition of the DRD1 constitutive activity by its inverse agonists induced the expansion and folding of human cerebral organoids. The anatomic analysis uncovered that decreasing the constitutive activity of DRD1 by its inverse agonists promoted human NSCs proliferation and inhibited the DRD1 constitutive activity by its inverse agonists induced the expansion and folding of human cerebral organoids. The anatomic analysis uncovered that decreasing the constitutive activity of DRD1 by its inverse agonists led to the development of human nervous system. Further mechanistic studies revealed that the PKC-CBP pathway was involved in the regulation by DRD1. Thus, our findings indicate that the constitutive activity of DRD1 and possibly other GPCRs plays an important role in the development of human nervous system.

KEYWORDS
cerebral organoids, constitutive activity, DRD1, neural stem cell, neurogenesis

1 | INTRODUCTION

The intricate balance of neural stem cell (NSC) amplification and neurogenesis is central to nervous system development, and its deficits have been implicated in many brain disorders, such as megaloccephaly, microcephaly, autistic spectrum disorders, and schizophrenia.1-3 Radial glia stem cells persist as the principal progenitor type during development of the embryonic and postnatal CNS, function as NSCs, which is thought to be sequentially giving rise to neurons residing in different cortical layers in a highly coordinated process.4,5 In the nervous system, an ever-increasing number of factors that can change NSCs activity and fate are being uncovered. Signaling pathways mediated by these factors orchestrate the dynamics of mammalian cortical neurogenesis.

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In the recent decades, most G protein-coupled receptors (GPCRs) are revealed to show activity even in the absence of ligand binding.\textsuperscript{6-9} This pharmacological characteristic is called receptor constitutive activity, and the ligands to reduce this, named inverse agonists, have been developed for research or clinical use.\textsuperscript{10,11} The functions of GPCRs in the brain have been learnt by studying the effects of receptor endogenous agonists on the regulation of NSCs maintenance, proliferation, and differentiation.\textsuperscript{12} So far, little is known about the role of receptor constitutive activity in NSCs functions and brain development. Dopamine receptors, belonging to GPCRs, regulate emotional, reward system, locomotors activity, learning, and memory in the brain.\textsuperscript{13,14} Five different subtypes of dopamine receptors have been described so far: dopamine D1 receptor (DRD1), DRD2, DRD3, DRD4, and DRD5; in the human CNS, DRD1 is most abundantly expressed.\textsuperscript{15} DRD1 appears in the developing brain early in the embryonic period before the mature synaptic contacts emerge,\textsuperscript{16,17} whereas the receptor endogenous agonist dopamine presents interaction with synapse in the mature brain. This raises the possibility that the constitutive activity of DRD1 could play a development role in the embryonic brain. The constitutive activity of DRD1 has been established at Gα signaling in recombinant cells overexpressing wild-type or mutant receptors.\textsuperscript{18-21} However, the physiological significance of the receptor’s constitutive activity remains unclear.\textsuperscript{22}

Efforts to study human brain development and complex human diseases have been hampered by the lack of access to the human brain. The human organoids generated from human induced pluripotent stem cells (hiPSCs) is an alternative in vitro system to investigate the human cortical formation.\textsuperscript{23-27} The induced cerebral organoids display human-specific features such as the ventricular zone (VZ) and outer subventricular zone (SVZ) and recapitulate early stages of cortical formation.\textsuperscript{26,28} Brain organoid contains multiple cell types and shows neuronal functionality,\textsuperscript{29} making it more representative of in vivo physiology than two-dimensional (2D) cell cultures. It also represents a novel method to investigate the molecular, cellular, and anatomical paradigms of early human cortical development.\textsuperscript{25,30,31} Here, we show that the decreased constitutive activity of DRD1 increased human NSCs proliferation and hindered it differentiation, thereby causing the excessive expansion and folding in human cerebral organoids. This indicates the potential role of the constitutive-mediated DRD1 activity for the regulation of neurogenesis in human brain development.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human iPSC/iPSC-derived NSCs were provided by IxCell Biotechnology, Ltd. The hiPSCs were cultured under feeder-free culture conditions on Matrigel (Corning)-coated dishes in mTeSR1 Medium (Stem cell technologies). Passaging was performed enzymatically using Accutase (Gibco) by splitting colonies in clumps every 6-7 days followed by replating on Matrigel-coated dishes. Medium were changed every day.

The human iPSC-derived NSC cells were maintained as adherent culture in 50% DMEM-F12 and 50% Neurobasal-A, containing 1× N2 supplement, 1× B27 supplement (Minus Vitamin A), 1× NEAA, 1× Glutamax, 10 ng/mL FGF-Basic (AA10-155) Recombinant Human Protein (basic fibroblast growth factor—bFGF, Gibco), 10 ng/mL LIF Recombinant Human Protein (hlif, Gibco), 3 μM CHIR99021 (Selleckchem), 5 μM SB431542 (Selleckchem), and 200 μM L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma). For neurospheres assays, cells were cultured in DMEM-F12 with 1× B27 supplement, 20 ng/mL Recombinant Human Protein (EGF, Gibco), 20 ng/mL bFGF, and 10 ng/mL hlif using low-attachment culture dishes (Corning).

SK-N-SH were purchased from ATCC and cultured in Modified Eagle’s Medium (MEM) with 10% (wt/vol) heat-inactivated fetal bovine serum. All cells were maintained at 37°C in humidified air with 5% CO₂.

2.2 | Compounds and reagents

SGC-CBP30, SKF83566, SCH23390, and SKF38393 were purchased from Tocris Bioscience; Y-27632 dihydrochloride, GF109203, and GO6983 were from Selleck Chemicals; heparin and DAPI were purchased from Sigma; GloSensor cAMP Reagent and CellTiter-Glo were from Promega.

2.3 | Plasmids and lentiviral constructs and Infection

For RNA interference experiments, shRNA targeting human DRD1 was cloned into a plKO.1 vector following the online protocol (Addgene, http://www.addgene.org/tools/protocols/plko/). All targeting sequences

Significance statement

In the current study, the authors first demonstrated a potential role of dopamine D1 receptor (DRD1) constitutive activity for the regulation of neurogenesis in human brain development. Inhibiting the constitutive activity of the DRD1 promoted human neural stem cell (NSC) amplification and thus induced expansion and folding of human cerebral organoids, thereby impeded the process of neurogenesis. The DRD1 mediates its action on NSCs partially through downregulating the genesis of neurons in a CBP/p300-dependent fashion by reducing the level of PKC phosphorylation. The study also provides another insight that there might be strategies to effectively control the time of neural precursor amplification or neuron transition by flexibly and precisely regulating the constitutive activity of DRD1 and modulating the multiple stages of adult NSC development in a single therapeutic strategy is possible to fight against age-related decline in hippocampal NSC.
are listed in Table S1. A pLKO.1-sh-SCRAM vector expressing a scrambled sequence not complementary to any human gene was used as a control. Lentivirus packing and infection were previously reported with minor modification. Briefly, human NSCs was seeded in 60-mm dishes before concentrated lentiviruses infection (minimum multiplicity of infection) in the presence of polybrene (Sigma, 4 μg/mL). After 8 hours, the medium was refreshed. The efficiency of the shRNA was determined by quantitative real-time polymerase chain reaction (qRT-PCR) or Western blot at 72 or 96 hours post-infection (h.p.i.).

### 2.4 ATP assay

Cell growth rate was measured by an ATP assay using CellTiter-Glo Luminescent kit according to the manufacturer's instructions. Human NSCs was plated in matrigel-coated 96-well plates at 2000 cells per well, in the reduced amount of bFGF (10 or 1 ng/mL). ATP assay was performed on day 1-3. Luminescence was recorded on a SpectraMax M5 Microplate Reader (MD).

### 2.5 Cell Counting Kit 8 assay

Human NSCs (5000 cells/well) were seeded into a 96-well plate and incubated with SKF83566 for 24 or 48 hours. Dimethylsulfoxide was used as a control. After treatment, Cell Counting Kit 8 (CCK8) solution (10 μL) (Genomeditech, Co., Ltd., Shanghai, China) was added to each well, and cells were cultured for 2 hours at 37°C. Absorption values at 405 nm were measured.

### 2.6 EdU detection

For EdU detection, human NSCs (5000 cells/well) were plated on Matrigel-coated 96 well plates and cultured for 24 hours. The culture medium was then replaced with the medium containing required chemical for up to 24 hours. EdU (2.5 μM, Sigma-Aldrich) was added, and cells were incubated for another 0.5 hours. During this period, all the cells entering S-phase incorporated the thymidine analog. Cells were fixed in 4% paraformaldehyde and stained with EdU (Sigma). Nuclei were labeled with DAPI. The numbers of EdU-positive cells were analyzed using HTS (Cellomics ArrayScan VTi 700).

### 2.7 cAMP assay

Intracellular cAMP was measured using a GloSensorTM cAMP assay kit following the manufacturer's instruction with minor modification. Cells were infected with F20-packaging lentivirus and seeded in white 96-well plates (Costar). The medium was then removed and replaced with fresh medium containing 2% (vol/vol) GloSensorTM cAMP reagent. After 90 minutes incubation at 37°C, cells were equilibrated at room temperature (RT) for 15 minutes and treated with the ligands at the indicated concentrations for another 15 minutes followed by the measurement of luciferase activity.

### 2.8 Immunostaining and imaging

For immunostaining, human NSCs or hiPSCs were seeded in 24-well culture dishes with a 1 × 1 cm diameter glass coverslip in each well. After infection or treatment, cells were fixed with 4% paraformaldehyde in PBS at 4°C for 10 minutes and permeabilized with 0.3% Triton X-100 in PBS for 15 minutes before being treated with 5% bovine serum albumin (BSA) for 1 hour at RT. Cells were incubated overnight at 4°C with various combinations of the following primary antibodies: rabbit antibody to Ki67 (Abcam, 1:500), rabbit antibody to Nestin (Millipore, 1:500), mouse antibody to TuJ1 (Covance, 1:500), goat antibody to Sox2 (R&D, 1:300), rabbit antibody to Oct4(Abcam, 1:500), or goat antibody to Sox1(R&D, 1:300). After washing with PBS/1% BSA three times, cells were incubated with Alexa Fluor 594-labelled donkey anti-goat IgG, Alexa Fluor 647-labelled donkey anti-rabbit IgG or Alexa Fluor 488-labelled donkey anti-mouse secondary antibodies in the dark for 1 hour. Followed by washing with PBS/1% BSA, cells were stained with DAPI (Sigma) and mounted on slides. Images were acquired under microscope (LAS SP8; Leica, FV10-ASW 4.2; Olympus, Zeiss Z1 and Zeiss Z.1 Lightsheet microscope; Zeiss). A Canny Edge Detection plugin of ImageJ was used to measure fold density in images of Hoechst-stained organoids by stereoscopic microscope.

### 2.9 Human cerebral organoid culture

Organoids were generated using a STEMdiff Cerebral Organoid Kit assay following the manufacturer's instruction. On day 0, human iPSCs at 90% confluence were dissociated into single cells using Accutase (5 minutes, 37°C). After centrifugation at 1000g for 5 minutes, iPSCs were resuspended in EB Formation Medium with 10 μM Rock Inhibitor Y27632 and diluted to the concentration of 9 × 10^5 cells per mL. Then, 100 μL of cell suspension was distributed into each well of a low-attachment 96-Well U-bottom plate (Coming) to form single EBs, medium was changed every two days. On days 5-6, half of the medium was replaced with induction medium. On day 7, organoids were harvested and embedded in Matrigel (Coming) and continued to grow in expansion medium in suspension culture in ultra-low attachment 6-well plates (Coming). After 3 days of maintenance, embedded organoids were cultured in maturation medium and the plates were transferred to a shaker for the continuous culturing, medium was changed every 3 days.

### 2.10 CRISPR-Cas9-mediated gene manipulation in human NSCs/iPSCs

To generate CRISPR-Cas9 plasmid for gene mutation, the sgRNAs were designed using the CRISPR tool (http://tko.ccbr.utoronto.ca/crispr_targets.pl), and their sequences as well as the target sequences...
are listed in Table S1. The sgRNAs of target genes were synthesized, annealed, and ligated into the pX330-mCherry plasmid that was digested with BbsI (New England Biolabs). Human NSCs were transfected with corresponding PX330-mCherry plasmids expressing sgRNA using Lipofectamine Stem Transfection Reagent (Thermofisher) in accordance with the manufacturer's instruction manual. After 24- to 48-hour transfection, the cells expressing mCherry were enriched with flow cytometry (FACS flux, BD Biosciences) in a 96-well plate. After 6-7 days plating, single colonies were picked and those carrying expected genotypes were selected.
for further analysis by DNA sequencing of PCR products amplified from targeted sites.

2.11 Reverse transcription and qRT-PCR

RNA extraction and reverse transcription were performed with Trizol reagent (Sigma) and PrimeScriptTMRT Master Mix (Takara, #RR036A) following the manufacturer's instructions. All gene transcripts were quantified by qRT-PCR performed with a 2× HotStart SYBR Green qPCR Master Mix (ExCell Bio) on a Stratagene Mx3000P (Agilent Technologies). The primers used for the detection of mRNA levels of human genes are listed in Table S1. All the primers were synthesized and purified by Shanghai Sunny Biotechnology Co., Ltd.

2.12 Statistical analysis

All experiments were repeated at least three times. Data are representative or mean ± SEM. All data were analyzed by Prism 6.0 (GraphPad Software Inc, San Diego, California). Concentration-response curves were analyzed using a three-parameter nonlinear regression analysis. Unpaired Student's t-test was applied for the comparisons of two data sets. One-way or two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was used where more than two data sets or groups were compared.

3 RESULTS

3.1 Inhibition of the constitutive activity of DRD1 promotes human NSC proliferation

In order to determine the role of dopamine receptors on human NSCs, first, we tested the expression pattern of each subtype by qRT-PCR. DRD1 showed relatively high gene expression in human NSCs compared with other subtypes (Figure S1A). While differentiation to neurons, the expression of DRD1 continued to grow (Figure S1B), suggesting its possible physiological involvement in normal neural development. The human NSCs were induced from pluripotent stem cells and characterized by staining with specific NSCs markers. More than 80% of the human NSCs expressed Sox1, Sox2, and Nestin. Additionally, proliferation marker ki67 was also detected (Figures 1A and S1C). In 2D adherent culture system, cells were cultured in either the presence or absence of respective ligands for 48 hours, followed by observation under a phase contrast microscope and subsequent determination of ATP levels by CellTiter-Glo assay as an index of cell proliferation. We found that SKF83566, an inverse agonist that inhibits the constitutive activity of the DRD1 (Figure S1D), induced a significantly higher growth rate of human NSC in the culture medium containing low bFGF (Figure 1B). The SKF83566 dose-dependently increased the growth of human NSCs, reaching the peak at 10 μM (Figure 1C). The response was also increased with time (Figure 1D). The promotion of cell proliferation by SKF83566 was further confirmed by CCK8 assay and EdU incorporation (Figure 1E,F). And another DRD1 inverse agonist SCH23390 showed the similar effect on human NSCs proliferation (Figure S1E). Additionally, the effect of SKF83566 was not observed in a neuroblastoma cell line SK-N-SH which is also enriched of DRD1, showing the cell-specific effect (Figure S1F, G). For the measurement of self-renewal, neurospheres that had been grown with SKF83566 were dissociated and replated in mitogens without the SKF83566. The secondary neurospheres treated with SKF83566 were formed in growth media at a higher number than those without SKF83566 (Figure 1G). These data indicate that SKF83566 promotes self-renewing symmetric divisions and multipotent maintenance of human NSCs.

To examine the receptor-specific effect of SKF83566 on cell growth, the shRNA technique was applied to knock-down DRD1 level. The knock-down of DRD1 also promoted increased cell proliferation compared with the negative control (Figure 1H), indicating its DRD1-dependent effect. The reduction of DRD1 mRNA by gene-specific shRNA was confirmed by qRT-PCR (Figure 1I). We further used genetic methods to confirm it is the constitutive activity of the DRD1, dopamine D1 receptor; iPSC, induced pluripotent stem cells; NSC, neural stem cell

![Inhibition of the constitutive activity of DRD1 promotes human NSC proliferation](image)

**FIGURE 1** Inhibition of the constitutive activity of DRD1 promotes human NSC proliferation. A. Representative images showing the validation of human NSC differentiation from human iPSC. Shown is a sample confocal image. Scale bars, 200 μm. N = 3. B. The effect of DRD1 agonist/antagonist (SKF8393/SKF83566), DRD2 agonist/antagonist (Sumanitrole/L-741,626) or DRD4 agonist/antagonist (PD168707/L741,742) on human NSC growth in the presence of low bFGF concentration determined by ATP assay in 2D adherent culture after 48 hours. Data are mean ± SEM. **P < .001, unpaired Student's t-test. N = 4. C, Dosage-dependent response of cell growth after treatment with SKF83566 in the presence of low bFGF concentration determined by ATP assay in 2D adherent culture. Data are mean ± SEM. ***P < .001, one-way ANOVA, followed by Tukey's multiple comparisons test. N = 4. D, Time lapses of cell growth in response to SKF83566. Data are mean ± SEM. ***P < .001, one-way ANOVA, followed by Tukey's multiple comparisons test. N = 4. E, The effect of SKF83566 on human NSC growth in the presence of low bFGF concentration determined by CCK8 assay in 2D adherent culture. Data are mean ± SEM. ***P < .001, unpaired Student's t-test. N = 4. F, The percentage of EdU-positive cells to the control after treatment with SKF83566. Data are mean ± SEM. **P < .001, one-way ANOVA, followed by Tukey's multiple comparisons test. N = 4. G, The number of secondary neurospheres formed from human NSCs treated with SKF83566 and its representative images. Data are + SEM. *P < .05, unpaired Student's t-test. Scale bars, 200 μm. N = 4. H, The effect of knockdown of DRD1 on human NSCs proliferation. Data are mean ± SEM. ***P < .001, unpaired Student's t-test. N = 3. I, Quantitative real-time PCR analyzing the mRNA level of DRD1 in the cells with or without receptor knockdown. Data are mean ± SEM. *P < .05, unpaired Student's t-test. N = 3. J. CRISPR/Cas9-mediated targeting of the human DRD1 locus and the sequences of the knock-in DRD1 human NSC clone. K, The cell growth of human NSCs without or with A229T/L286A mutant knock-in by CRISPR-Cas9. Data are mean ± SEM. **P < .001, *P < .05, one-way ANOVA, followed by Tukey's multiple comparisons test. N = 4. ANOVA, analysis of variance; bFGF, basic fibroblast growth factor; DRD1, dopamine D1 receptor; iPSC, induced pluripotent stem cells; NSC, neural stem cell
receptor that regulates the function of human NSCs. A CRISPR-Cas9 genome editing technology was performed to knock-in A229T or L286A mutant (Figure 1J), which was reported to reduce or increase DRD1 constitutive activity, respectively. We verified the presence of receptor mutants changed the basal level of cAMP (Figure S1G) but did not affect both the total and surface expression of receptor by monitoring the level and localization of GFP-tagged receptors (data not shown). The A229R mutation was reported to reduce the constitutive activity of receptor, and our experiment proved that the mutant could increase the proliferation of NSCs. The L286A mutation could increase the constitutive activity of receptor and reduce the proliferation of NSCs (Figure 1K). All these imply the constitutive activity of DRD1 plays the key role in human NSCs function.

3.2 | The decreased constitutive activity of DRD1 maintains human NSC stemness

We next analyzed whether SKF83566-induced proliferation is coupled to maintenance of the stem cell state. The number of kinase-positive cells...
was largely increased by the treatment with SKF83566 on human NSCs in the low factor defined media for 3 days (Figure 2A, B). In addition, compared with the control, SKF83566-expanded human NSCs were more primitive progeny, expressing the NSC markers Sox2 and Nestin with only a few immature, neuron-like cells positive for neuron marker DCX (Figure 2C-F). We further examined the expression of Sox2 and DCX which associated with stemness or neural commitment at the molecular level. As expected, we found increased Nestin mRNA and decreased DCX mRNA in human NSCs cultured with SKF83566 relative to the control (Figure 2G). Hence, our data indicate that SKF83566 contributes to NSCs proliferation, as manifested by increased number of cells with proliferation capacity and molecular changes underlying a stem cell state. But, SKF83566-expanded human NSCs still could be differentiated into neurons after SKF83566 depletion (Figure S1I).

**FIGURE 3** Legend on next page.
3.3 | Inhibition of the constitutive activity of DRD1 induced expansion and folding of human cerebral organoids

The proliferation of NSCs has been reported to induce expansion and folding of the cerebral organoids. To investigate the effect of DRD1 constitutive activity on human brain function, we adopted cerebral organoids culture system to differentiate human iPSCs into 3D neural tissues. Consistent with previous reports, we obtained organoids exhibiting brain-like architectures (Figure S2A). Immunostaining revealed that organoids contained complex heterogeneous tissues, displaying regions reminiscent of the VZ expressing NSCs marker Sox2 and neuronal marker such as TuJ1 at the basal side away from the lumen and cortical tissue patterning markers (eg, TBR2, BRN2) (Figure S2B). Consistent with the results in NSCs, in the organoids, DRD1 gene expression was relatively low at the early stage of induction and then increased as differentiation and maturation, further suggesting possible physiological involvement of DRD1 during normal human neural development (Figure S3C). To compare the organoid formation between SKF83566-treated and vehicle-treated conditions, equal numbers of dissociated single cells from human iPSCs were seeded to form embryoid bodies. SKF83566 was added in organoids on day 7 before the stem cell expansion. After 7 days treatment, the SKF83566-treated organoids exhibited more ventricle-like structures and displayed a markedly increased outgrowth of neuroepithelial tissue surrounding ventricle-like structures compared with vehicle-treated organoids that were relatively spherical and smooth. The neuroepithelial overgrowth was even more evident with time in SKF83566-treated organoids leading to a drastic increase in size and an overall folded surface (Figure 3A). The phenotype seems to be more obvious from 4 days post-treatment when NSCs just emerged (Figure 3B). The organoids were then imaged on a light-sheet fluorescence microscope to get a more stereoscopic outline of the organoids at 2 weeks (Figure 3C). SKF83566-treated organoids displayed a markedly increased surface area, overall volume, and reduced sphericity compared with vehicle-treated (Figure 3D). Because the increased surface area was often organized in continuous folds, we further demonstrated a significant increase in fold density in SKF83566-treated organoids (Figure 3E-G). The knock-out of DRD1 also induced human organoids expansion and weakens the effect of SKF83566 (Figure 3H). The absence of DRD1 mRNA was confirmed by qRT-PCR (Figure 3I). The dopaminergic neurons in human organoids could produce and secrete dopamine which would bind to and activate dopamine receptors and the effect of SKF83566 on cell proliferation observed here could simply due to the competition of SKF83566 with dopamine. To exclude this possibility, we subsequently suppressed the activity of DOPA decarboxylase (DDC), the rate-limiting enzyme in dopamine synthesis, in human organoids using carbidopa, a selective inhibitor known to drastically reduce dopamine levels. The dopamine content was indeed reduced by carbidopa pretreatment (Figure S2D). Under this circumstance, the effect of SKF83566 on human organoids was consistently observed. The surface folding was also not affected (Figure 3J-L). Furthermore, SKF83566-promoted human organoids expansion and folding were not changed by DDC knockout (Figure 3N). The absence of DDC mRNA was confirmed by qRT-PCR (Figure 3M). The results eliminate the influence of endogenous dopamine and suggest a putative role of constitutive activity of DRD1 as a growth or maintenance power in human NSCs.

3.4 | The constitutive activity of DRD1 is required for the neurogenesis in human cerebral organoids

To investigate whether the expansion and folding of SKF83566-treated organoids was due to disruption of the intricate balance of NSC amplification and differentiation transition, we performed molecular anatomical staining on the cerebral organoids to determine the cellular organizations. At 2 weeks, neuronal differentiation was initiated in control organoids, and TuJ1+ neurons began to emerge; in contrast, SKF83566-treated organoids contained predominantly Sox2+ NSCs (Figure 4A, B). At 3 weeks, more neurons appeared in the control group, but human cerebral organoids treated with SKF83566 mainly contained Sox2+ NSCs, with a small number of neurons (Figure 4C, D). The organoids with A229T [Image 77x743 to 95x761] DDC, DOPA decarboxylase; DRD1, dopamine D1 receptor; PCR, polymerase chain reaction.

FIGURE 3 | Inactivation of the constitutive activity of DRD1 induces expansion and folding of human cerebral organoids. A, Representative bright-field images of control and SKF83566-treated human cerebral organoids (n = 8 organoids per condition). Scale bars, 200 μm. B, Time course of SKF83566-mediated human organoids formation (n = 6 organoids per condition). Scale bars, 200 μm. N = 3. C and D, Light sheet images and reconstructed models of control and SKF83566-treated organoids and quantification of volume, surface area, and sphericity at 2 weeks (n = 4 organoids per condition). Data are mean ± SEM. *P < .05, unpaired Student’s t-test. Scale bars, 300 μm. N = 4. E, Representative images of surface fold density in Hoechst-stained control and SKF83566-treated organoids (n = 8 organoids per condition). Scale bars, 300 μm. N = 4. F and G, Higher magnification view of the top of control and SKF83566-treated organoid using the stereological method and quantification of surface folds via canny edge detection (n = 6 organoids per condition). Results are mean ± SEM. **P < .005, N = 5. H, Representative bright-field images of control and SKF83566-treated cerebral organoids after knock-out of DRD1 at 2 weeks (n = 6 organoids per condition). Scale bars, 200 μm. N = 3. I, Quantitative real-time PCR analyzing the mRNA level of DRD1 in the cells with or without receptor knockout. Data are mean ± SEM. ***P < .001, unpaired Student’s t-test. N = 4. J, Representative bright-field images of control and SKF83566-treated cerebral organoids in the presence of carbidopa at 2 weeks (n = 6 organoids per condition). Scale bars, 200 μm. N = 3. K and L, Higher magnification view of the top of control and SKF83566-treated organoid with carbidopa pretreatment using the stereological method and quantification of surface folds (n = 6 organoids per condition). Results are mean ± SEM. **P < .005. N = 4. M, Representative bright-field images of control and SKF83566-treated cerebral organoids after DDC knock-out at 2 weeks (n = 6 organoids per condition). Scale bars, 200 μm. N = 4. N, Quantitative real-time PCR analyzing the mRNA level of DDC in the cells with or without receptor knock-down. Data are mean ± SEM. ***P < .001, unpaired Student’s t-test. N = 4. DDC, DOPA decarboxylase; DRD1, dopamine D1 receptor; PCR, polymerase chain reaction.
FIGURE 4  The constitutive activity of DRD1 is required for the neurogenesis in human cerebral organoids. A and B, Immunostaining of the Sox2+ and Tuj1+ cells in control and SKF83566-treated cerebral organoids at 2-3 weeks and the corresponding quantification (n = 4 organoids per condition). Scale bars, 250 μm. N = 3. E and F, Immunostaining of the Nestin+ and Sox2+ cells in control and SKF83566-treated cerebral organoids and the corresponding quantification (n = 4 organoids per condition). Scale bars, 250 μm. N = 3. G and H, Immunostaining of ki67+ cells in control and SKF83566-treated cerebral organoids and the corresponding quantification (n = 4 organoids per condition). Scale bars, 250 μm. N = 3. I, Quantitative real-time PCR analysis of Nestin and DCX mRNA levels in control and SKF83566-treated organoids at 3 weeks (n = 4 organoids per condition). Data were normalized to control. Data are mean ± SEM. * P < .05, *** P < .001, unpaired Student’s t-test. N = 4. DRD1, dopamine D1 receptor; PCR, polymerase chain reaction.
The PKC-CBP pathway mediates the effects of DRD1 constitutive activity in human NSCs and human cerebral organoids

To identify the molecular mechanism by which DRD1 inactivation enhanced human NSCs proliferation, we performed a small screening of kinase inhibitor library in our laboratory. First, we used PKA inhibitor (H89) to pretreat the cells, and we found that blocked the PKA activity did not abolish the enhancement of human NSCs.
proliferation, which suggests that Gαs-mediated signaling or the canonical cAMP pathway is not involved in the effect of DRD1 on human NSC proliferation (Figure S3A). Pretreatment with AKT inhibitor MK2206 also had no effect (Figure S3B). Unexpectedly, we found that PKC inhibitor could block the effect of SKF83566. Since the involvement of PKC-CBP pathway in mediating stem cell differentiation was previously reported,38 we hypothesized that the constitutive activity mediated by the PKC-CBP/P300 signaling may contribute to differentiation of human NSCs in culture/cerebral organoids. Inhibition of the constitutive activity of DRD1 by SKF83566 reduced the level of p-PKC. To investigate whether the reduction of p-PKC was responsible for the phenotypes, we treated human NSCs or developing human organoids with GF109203X, a PKC inhibitor, and found it could block the proliferation of human NSCs and the formation of expanded and folded organoids, accompanied by normalized neural marker gene expression (Figure S5A,B,E). The result was confirmed by another PKC inhibitor GO6983 (Figure S3C). Additionally, SKF83566 treatment led to significantly decreased level of p-PKC in human NSCs (Figure S5C) and human organoids (Figure S5D). Furthermore, the inhibition of CREBBP/EP300 by SGC-CBP30 blocked the effect of SKF83566 (Figure S5F, G), which was further confirmed by another CREBBP/EP300 inhibitor PF-CBP1 (Figure S3D). These suggest that SKF83566 mediates the human NSCs proliferation to induce expansion and folding of human cerebral organoids through reducing the PKC-CBP pathway activation. This transient and cell type-specific reduction of PKC-CBP signaling in NSCs echoed the pattern of enhanced proliferation in SKF83566-treated human organoids and strongly indicated that decreased PKC signaling drove the expansion of the NSCs pool and, consequently, the expansion and folding of cerebral organoids.

4 | DISCUSSION

Increased NSC proliferation and failure to differentiate can cause developmental diseases.39 Human NSCs derived from iPSCs strongly resemble embryonic NSCs, which give rise to all cells in the developing brain. Here we found that the impairment of the constitutive activity of DRD1 caused stem cell defects, leading to imbalanced proliferation and differentiation in human NSCs. Our findings suggest that the constitutive activity of DRD1 may promote the activation of neurogenic genes in proliferating and differentiating human NSCs and reveal the developmental requirement of the DRD1 constitutive activity in human neurogenesis and cortical development. More molecular and developmental studies of the constitutive activity will likely improve the understanding of diseases associated with the receptor. Furthermore, there could be more GPCRs that harbor constitutive activity regulating NSC functions, and we suspect that all those GPCRs coordinate regulation of NSC, which could be essential for maintaining the physiological functions of neuron system. The present study also provides the other insight mechanism by which DRD1 and its inverse agonist regulates NSC proliferation, suggesting that inactivation of the receptor could be a strategy to amplify human NSCs pool and manipulating the receptor could improve age-related decline in hippocampal NSC and associated cognitive and affective defects.

Dopamine signaling is involved in neurogenesis at different time in intricate way, interweaving with synergy and antagonism. For example, activating DRD3 receptors could transit-amplify progenitor cells in the SVZ through AKT and ERK1/2 pathways and antagonizing the DRD2 or DRD4 could impair stem cells growth through influencing the autophagy-lysosome pathway.40-42 Although previous studies in mouse models have shown that genetic deletion of DRD1 can perturb normal brain development and produce lasting changes in brain structure and function, ultimately changing adult behavior,43-46 there is no complete and unified explanation, due to the limitations of the original experimental technology and equipment so far. One potential mechanism for these changes may be that receptors influence subsequent neurogenesis by affecting neural progenitor cells.47,48 But it is also difficult to explain the possible for the early appearance of the receptor in the neurogenic area before development of dopaminergic innervation. Additionally, little is known about its function in the human brain development. Because of the huge difference between humans and animal models, we wonder if the DRD1 has the same function in human brain development. Here we first showed that DRD1 displayed a high level of constitutive activity at the Gαs-mediated and cAMP-dependent signal pathway and several lines of evidence supported a role for DRD1 constitutive activity in human NSCs multipotent maintenance: (a) DRD1 inverse agonists increased the number of human NSCs; (b) DRD1 mutant with reduced constitutive activity showed promotion of human NSCs proliferation; (c) knockdown of DRD1 mimicked the result of inverse agonist. We further reported that inactivation of DRD1 induced human organoids developed more expanded VZ, increased overall size, and a larger surface area organized into continuous cortical folds. This expansion in vitro is triggered by increased proliferation, transiently arrested neuronal differentiation. It implies the potential role of the constitutive-mediated DRD1 activity for the regulation of neurogenesis in human brain development. The dopamine content in our culture organoids is barely detected by HPLC, indicating that our hybrid cerebral organoids culture system induce relatively small proportion of dopaminergic neuronal progenitors. There are mounting literatures reported the specific midbrain organoids culture system,29,50,51 which may generate a large number of dopaminergic neuronal progenitors. It would be interesting and meaningful to explore the function of constitutive activity of DRD1 on the development of midbrain.

Maintenance of an NSC pool depends on the combined action of different gene products, and it may therefore involve the activity of large regulatory complexes, including transcriptional factors and co-repressors/activators. The PKC-mediated CBP phosphorylation, as a signal sensor, regulates the neuronal differentiation of adult NP by modulating the activity of CBP histone acetyltransferase or chromatin binding to respond to changes in the microenvironment.38,52-54 We found blockage of the PKC-CBP pathway weaken the action of DRD1 inverse agonist. The constitutive activity of DRD1 may influence PKC-CBP-mediated H3K27me3, and to a greater extent H3K27ac, and is required to balance proliferation and differentiation of human NSCs. Identification of upstream modulator of the PKC-CBP pathway and isoform of PKC will be the subject of our future work.
5  | CONCLUSION

Altogether, here we showed the DRD1 was highly enriched in human NSCs. We used an inverse agonist SKF83566 to selectively inhibit the constitutive activity of DRD1 and found that inactivation of DRD1 promoted human NSCs proliferation and arrested neuronal differentiation, thereby causing the excessive expansion and folding in an in vitro model of human cerebral organoids. DRD1 partially mediates its action on NSCs by downregulating the genesis of neurons in a CBP/p300-dependent fashion through reducing PKC phosphorylation. These results possibly underscore a tonic role of DRD1’s constitutive activity in neurogenesis during brain development.

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CONFLICT OF INTEREST
The authors indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS
Q.W.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; X.D., T.H.: collection and/or assembly of data, data analysis and interpretation; J.L.: manuscript writing, final approval of manuscript; G.P.: conception and design, financial support, manuscript writing, final approval of manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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