METHOD OF GENERATING MIDBRAIN DOPAMINE PROGENITORS AND NEURONS FROM STEM CELLS

FIELD OF THE INVENTION

[0001] The present invention generally relates to a method of neuron progenitors and neuron generation. In particular, the present invention relates to a method of generating midbrain dopamine neuron progenitors and midbrain dopamine neurons from human stem cells.

BACKGROUND

[0002] Parkinson's Disease (PD) results from degeneration of dopamine (DA) neurons in the midbrain. Current therapies, including the use of L-dopa, treat symptoms but do not stop disease progression. Hence, the efficacy of the treatments often wears off in several years. There is an unmet need to develop lasting and effective therapies for PD.

[0003] Replacement of the degenerated DA neurons, first explored by fetal tissue transplantation, is a potential option with long-term efficacy (Bjorklund and Lindvall, 2017). Such a therapy is becoming realistic with the development of human pluripotent stem cells (hPSCs), including embryonic stem cells (Thomson et al., 1998) and induced pluripotent stem cells (iPSCs, (Takahashi et al., 2007). In particular, technology has been developed to guide hPSCs to *bona fide* midbrain DA neurons (Kirkeby et al., 2012; Kriks et al., 2011; Tao and Zhang, 2016; Xi et al., 2012). Transplantation of these human DA neurons into the rodent and monkey brain (Chen et al., 2016; Hallett et al., 2015; Kikuchi et al., 2017; Piao et al., 2021; Steinbeck et al., 2015; Tao et al., 2021; Wang et al., 2018; Xiong et al., 2021) has led to the realization that the grafted DA neurons not only mature and secrete DA but also project axons in a long distance and find the appropriate targets as well as receive presynaptic inputs, thus repairing the dysfunctional circuit. These exciting findings have led to the clinical trials of stem cell therapy for PD (Barker et al., 2017; Parmar, 2018) with a first PD patient receiving autologous cell transplantation (Schweitzer et al., 2020).

[0004] A key to safe and effective cell therapy is the quality and purity of the donor cells, midbrain DA neuron progenitors (mDAP). These progenitors, especially produced at the good manufacturing practice (GMP) or Good Laboratory Practice (GLP) grade, generate 10~30% tyrosine hydroxylase $(TH)^+$ DA neurons *in vitro* or after transplantation into the mouse or rat brain (Piao et al., 2021; Song et al., 2020), suggesting that the mDAPs have a broader

differentiation potential than giving rise to DA neurons and/or that the donor population contains other cells besides mDAPs. Indeed, current methods for inducing mDAPs (actually floor plate progenitors in the literature) are done by using a single Wnt agonist, CHIR, which unavoidably results in the generation of cells of the forebrain and hindbrain regions besides those of the midbrain. Secondly, the midbrain progenitors (or floor plate progenitors) not only produce dopamine neurons, but also other types of neurons and even non-neural cells like vasculature cells (Tiklová et al., 2020). Furthermore, there are many different types of midbrain dopamine neurons, including those in the substantia nigra (relating to motor control) and those in the ventral tegmental area (VTA, relating to rewarding and psychiatric functions). Current methods do not distinguish these dopamine neuronal types. The contamination of cells in brain regions other than the midbrain and other cell types poses serious safety issues for cell therapy.

[0005] Midbrain dopamine neurons (mDANs) are specified from a small area in the midbrain called floor plate. During early embryo development, the anterior-posterior (A-P) axis is specified mainly by the gradient of Wnts. *In vitro*, CHIR99021 (CHIR), a small molecule activating the WNT signaling by inhibiting GSK3β-mediated β-catenin degradation, is commonly used for mimicking the role of Wnt ligands *in vivo*. There are currently two categories of approaches to use CHIR to pattern human stem cells, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) to floor plate progenitors and mDANs, the "low CHIR methods" that used CHIR below 1 μM from the starting day (D0) of differentiation and the "delayed and escalating CHIR method" that used CHIR at 1~3 μM from 2~4 days after differentiation followed by increasing its concentration to 7.5 μM. Both approaches successfully induced floor plate progenitors, indicated by expression of OTX2, FOXA2 and LMX1A, but generating different populations of mDANs (see Table 1 and Table 2).

[0006] **Table 1.** Low CHIR methods (concentration-dependent)

Reference	**CHIR**		**Efficiency**	
	Concentration	Period	Floor plate progenitor	mDAN
Xi et al., 2012	0.4 μM	D0-12	At D18, 92.6% of FOXA2, and more than 90% of OTX2, LMX1A and EN1+ cells.	At D35, 43.6% of TH+ cells.
Nolbrant et al., 2017	0.6 μM	D0-9	At D11, 80% of FOXA2 and LMX1A+ cells and	At D44, 10.7% of TH+ cells.

			95% of OTX2 and LMX1A+ cells.	
Kirkeby et al., 2017	0.7 μM	D0-9	At D11, 90.4% of FOXA2 and LMX1A+ cells and more than 80% of OTX2, FOXA2 and LMX1A+ cells.	Didn't show.
You et al., 2023	0.4 μM	D1-9	Didn't describe clearly.	After D45, around 20% of TH+ cells.

[0007] **Table 2.** Delayed and escalating CHIR methods (treatment timing-dependent)

Reference	**CHIR**		**Efficiency**	
	Concentration	Period	Floor plate progenitor	mDAN
Kriks et al, 2011	3 μM	D3-12	At D11, 60% of FOXA2 and LMX1A+ cells and 80% of OTX2 and FOXA2+ cells.	At D50, 75% of TH+ cells.
Fedele et al., 2017	3 μM	D2-12	At D11, more than 90% of FOXA2 and LMX1A+ cells.	At D20, 8.7% of TH+ cells.
Gantner et al., 2020	2~3 μM	D2-12	At D13, more than 80% of OTX2 and FOXA2+ cells.	At D20, 5% of TH+ cells. (D25, 25% of TH+) (D50, 50% of TH+)
Song et al., 2020	1 μM	D4-15	Didn't show	At D28, 15% of TH+ cells.
Piao et al., 2021	0.7 μM → 7.5 μM	D0-3(0.7) D4-9(7.5)	At D21, 90.1%–94.4% of FOXA2+	In vivo, after 3W of transplantation, 15.91% of TH+cells.

[0008] The fact that the nearly pure population of floor progenitors ends up with a much smaller proportion of dopamine neurons indicates that these progenitors are not restricted to midbrain dopamine neuron progenitors (mDAPs). This phenomenon can be attributed to at

least two possibilities. One is that the progenitors are not limited to the midbrain fate and the other is that the midbrain floor plate progenitors (mFPPs) are not limited to the mDAPs.

[0009] Therefore, there is a need for a novel method which is capable of generating pure and authentic mDAPs and then mDANs from human pluripotent stem cells (hPSCs).

SUMMARY

[0010] The present disclosure describes a new method that generates pure mDAPs and mDANs from human pluripotent stem cells (hPSCs).

[0011] In one aspect, the present disclosure refers to a method of generating midbrain dopamine progenitors (mDAPs) from human pluripotent stem cells (hPSCs), comprising:

(a) treating hPSCs with a Wnt agonist and a Wnt antagonist, to generate midbrain floor plate progenitors (mFPPs);

(b) treating the mFPPs with Activin A and a sonic hedgehog (SHH) agonist to restrict the mFPPs to medial mFPPs; and

(c) removing the SHH agonist and adding the Wnt agonist to differentiate medial mFPPs to mDAPs.

[0012] In another aspect, the present disclosure refers to a plurality of midbrain dopamine progenitors (mDAPs) generated by the method as disclosed herein, wherein at least 70%, at least 80%, at least 90%, preferably 95%, and more preferably 98% of the mDAPs have expression of at least one of FOXA2, LMX1A, MSX1 and OTX2, and substantially no expression of NKX6.1 and NKX2.1.

[0013] In another aspect, the present disclosure refers to a plurality of midbrain dopamine neurons (mDANs) generated by the method as disclosed herein, wherein at least 70%, at least 80%, at least 90%, preferably 95%, and more preferably 98% of the mDANs have expression of at least one of Tyrosine Hydroxylase (TH), dihydroxyphenylalanine (DOPA) decarboxylase (DDC), FOXA2, OTX2, LMX1A, EN1, and NURR1, and substantially no expression of NKX6.1 and NKX2.1.

[0014] In another aspect, the present disclosure refers to a pharmaceutical composition comprising the plurality of mDAPs as disclosed herein, and a pharmaceutically-acceptable diluent, carrier or excipient.

[0015] In another aspect, the present disclosure refers to a method of treating a neurological disease of a subject, comprising administering the plurality of mDAPs as disclosed herein or

the pharmaceutical composition as disclosed herein into a neurological disease site of the subject.

[0016] In another aspect, the present disclosure refers to use of the plurality of mDAPs as disclosed herein or the pharmaceutical composition as disclosed herein in the manufacture of a medicament for treating a neurological disease of a subject, wherein the plurality of mDAPs or the pharmaceutical composition is to be administered into a neurological disease site of the subject.

[0017] Advantageously, the present disclosure discloses a technology that overcomes the major issues of existing protocols in the differentiation of mDAPs and mDANs from human stem cells, especially the contamination of non-target cells and lower yield. In addition, the present protocol is simple and generates mDAPs from stem cells within 3 weeks.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[0019] **Figure 1 illustrates midbrain regional specification using different approaches of WNT regulation.** (A) Concentration-dependent WNT regulation using the low concentration of CHIR99021 (0.4 μM) during the differentiation. (B) Time-dependent WNT regulation using the high concentration of CHIR99021 (3 μM), but treated from D3 of differentiation. (C) Agonist + antagonist combination used Wnt agonist (CHIR99021) with Wnt antagonist (IWR1) for the efficient and precise specification of the midbrain identity.

[0020] **Figure 2 illustrates patterning of OTX2 and FOXA2 expression by combining Wnt agonist and antagonist.** (A and B) Wnt agonist (CHIR99021) affected OTX2 and FOXA2-positive population according to the concentration of CHIR99021. Above 1.2 μM of CHIR99021 reduced OTX2-positive population. (C and D) Treatment of Wnt antagonist (IWR1) increased the range of CHIR99021 concentration that maintained OTX2 and FOXA2-positive population. With IWR1, 1.2 μM to 2.1 μM of CHIR99021 maintained OTX2-positive population efficiently. (E and F) XAV-939 (XAV) treatment showed the similar results with IWR1 treatment, however, WNT-C59 (C59) didn't maintain OTX2 expression above 1.5 μM of CHIR99021 treatment.

[0021] **Figure 3 illustrates the medial floor plate specification.** Early ventral midbrain floor plate contains NKX6.1-positive population. During development, the medial site of the ventral midbrain begins to express LMX1A and reduce NKX6.1 and there are LMX1A and NKX6.1-double positive intermediate progenitors in the middle of the ventral midbrain. When the specification completes, medial floor plate region only expresses LMX1A but not NKX6.1 which expression sustains in the lateral regions of the ventral midbrain.

[0022] **Figure 4 illustrates that sustained NKX6.1 expression occurred by high WNT-mediated neurogenesis of floor plate progenitors.** (A) NKX6.1 and LMX1A-double positive cells observed in high WNT (3 μM of CHIR99021) condition. (B) TH-positive neurons derived from this condition expressed NKX6.1 in their nucleus. (C) NURR1 expression was not detected in the nucleus of TH-positive neurons in this condition.

[0023] **Figure 5 illustrates that Activin A and SHH induced the medial floor plate identity.** Activin A and SHH agonist (A+S) resulted in FOXA2 and LMX1A-positive, but NKX6.1-negative population. However, BMP4 and SHH agonist (B+S) showed cytoplasmic LMX1A expression different from A+S condition

[0024] **Figure 6 illustrates differentiation potential of mDAPs derived from the medial FPPs.** (A) Highly enriched LMX1A and MSX1-positive cells observed during differentiation. (B) mDAP-derived neurons expressed mDAN markers including FOXA2, LMX1A, TH, NURR1 and TUBB3.

[0025] **Figure 7 illustrates functional analysis of differentiated mDANs.** (A) mDANs showed the rapid axon elongation during neuronal maturation. TH expression observed in most neurons. (B) Most cells expressed TH (above 80%) which was functional enzyme for generating dopamine from mDANs. (C) Neuronal maturation was confirmed by MAP2 (mature dendrite marker) and SMI312 (mature axon marker). (D) Patch clamp analysis showed the electrophysiological capacity of mDANs.

[0026] **Figure 8** illustrates that during brain development, the anterior-posterior (A-P) axis is precisely patterned by opposing morphogens like Wnts and their antagonists.

[0027] **Figure 9** is a schematic diagram of the method of generating midbrain dopamine progenitors (mDAPs) and midbrain dopamine neurons (mDANs) from human pluripotent stem cells (hPSCs) as disclosed herein.

DETAILED DESCRIPTION

[0028] The present disclosure describes a method of generating pure mDAPs and mDANs from human stem cells. The ability to generate such authentic human mDAPs and mDANs allows modeling disease processes that affect mDANs, testing drugs for these diseases, and developing cell therapy for the diseases through cell (mDAPs) transplantation. Diseases affecting mDANs include movement disorders like Parkinson's disease and numerous psychiatric disorders. In particular, the present method to generate pure mDAPs and mDANs without the contamination of non-target cells enables safe and effective cell therapy for Parkinson's disease.

[0029] In one aspect, the present disclosure refers to a method of generating midbrain dopamine progenitors (mDAPs) from human pluripotent stem cells (hPSCs), comprising:

(a) treating hPSCs with a Wnt agonist and a Wnt antagonist, to generate midbrain floor plate progenitors (mFPPs);

(b) treating the mFPPs with Activin A and a sonic hedgehog (SHH) agonist to restrict the mFPPs to medial mFPPs; and

(c) removing the SHH agonist and adding the Wnt agonist to differentiate medial mFPPs to mDAPs.

[0030] A schematic diagram of the method as disclosed herein is illustrated in Fig. 9. In one example, mDAPs are generated from human pluripotent stem cells (hPSCs) which are embryonic stem cells (ESCs). In another example, mDAPs are generated from hPSCs which are induced pluripotent stem cells (iPSCs). iPSCs are derived from adult cells, most often from fibroblasts or blood cells, and programmed into an embryonic-like pluripotent state.

[0031] The method as disclosed herein comprises at least three steps:

[0032] ***Step (a) - Induction of mFPPs by Wnt agonist and antagonist***

In step (a) of the method as disclosed herein, hPSCs are treated with a Wnt agonist and a Wnt antagonist, to generate midbrain floor plate progenitors (mFPPs). As used herein, the term "treat" refers to incubating hPSCs in an *in vitro* culture medium containing the Wnt agonist and the Wnt antagonist as disclosed herein.

[0033] In one example, the hPSCs are treated with a Wnt agonist and a Wnt antagonist from day 0 to day 4-6. In another example, the hPSCs are treated with a Wnt agonist and a Wnt antagonist from day 0 to day 4. In another example, the hPSCs are treated with a Wnt agonist

and a Wnt antagonist from day 0 to day 5. In another example, the hPSCs are treated with a Wnt agonist and a Wnt antagonist from day 0 to day 6.

[0034] In one example, the Wnt agonist is a Wnt ligand. Wnt ligands bind to Wnt receptors and activate WNT signaling pathway. In another example, the Wnt agonist is a GSK3 inhibitor. GSK3 inhibitors inhibit Destruction complex and result in the activation of WNT signaling pathway via the accumulation of WNT down-stream protein, β-catenin.

[0035] In one example, the Wnt ligand is selected from the group consisting of Wnt1, Wnt2, Wnt2b, Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, Wnt8a, Wnt8b, Wnt9a, Wnt9b, Wnt10a, Wnt10b, Wnt11, and Wnt 16. In another example, the GSK3 inhibitor is selected from the group consisting of the CHIR99021(Laduviglusib), SB415286, LY2090314, AZD1080, AR-A014418, BIO, Elraglusib, BRD0705, KY19382, SB216763, TWS119, CHIR-98014, Tideglusib, TDZD-8, 1-Azakenpaullone, AZD2858, IM-12, Bikinin, CP21R7, 5-Bromoindole, (E/Z)-GSK-3β inhibitor 1, PF-04802367, and WAY-119064. In one particular example, the Wnt agonist is CHIR99021.

[0036] In one example, the Wnt antagonist is a Tankyrase inhibitor. Tankyrase inhibitors stabilize Axin protein which is a part of Destruction complex and promote the degradation of WNT down-stream protein, β-catenin. In another example, the Wnt antagonist is not a Porcupine inhibitor selected from the group consisting of Wnt-C59, IWP-1, LGK-974, IWP-L6, GNF-6231, and ETC-159. Porcupine Inhibitors inhibit Porcupine-mediated secretion of Wnt ligands from cells and result in the low level of Wnt ligands. The Wnt antagonist as disclosed in the present disclosure being a Tankyrase inhibitor but not a Porcupine inhibitor suggests that the present disclosure is closely related to the downstream of canonical Wnt signaling, but not derived from the endogenous Wnt secretion mechanism.

[0037] In one example, the Tankyrase inhibitor is selected from the group consisting of IWR1, XAV-939, G007-LK, WIKI4, NVP-TNKS656, RK-287107, JW55, M2912, and Nesuparib. In one particular example, the Wnt antagonist is IWR-1. In another particular example, the Wnt antagonist is XAV-939. XAV-939 and IWR-1 are tankyrase inhibitors, acting as reversible Wnt pathway inhibitors. IWR-1 exerts its effect via interaction with Axin, while XAV-939 binds TNKS directly.

[0038] In one example, the Wnt agonist used in step (a) of the method as disclosed herein is CHIR99021, and the concentration of CHIR99021 is 1.2 to 2.1 μM. In another example, the concentration of the Wnt agonist CHIR99021 is 1.2-1.5 μM. In another example, the

concentration of the Wnt agonist CHIR99021 is 1.3-1.6 μM. In another example, the concentration of the Wnt agonist CHIR99021 is 1.4-1.7 μM. In another example, the concentration of the Wnt agonist CHIR99021 is 1.5-1.8 μM. In another example, the concentration of the Wnt agonist CHIR99021 is 1.6-1.9 μM. In another example, the concentration of the Wnt agonist CHIR99021 is 1.7-2.0 μM. In another example, the concentration of the Wnt agonist CHIR99021 is 1.8-2.1 μM. In another example, the concentration of the Wnt agonist CHIR99021 is about 1.2 μM. In another example, the concentration of the Wnt agonist CHIR99021 is about 1.3 μM. In another example, the concentration of the Wnt agonist CHIR99021 is about 1.4 μM. In another example, the concentration of the Wnt agonist CHIR99021 is about 1.5 μM. In another example, the concentration of the Wnt agonist CHIR99021 is about 1.6 μM. In another example, the concentration of the Wnt agonist CHIR99021 is about 1.7 μM. In another example, the concentration of the Wnt agonist CHIR99021 is about 1.8 μM. In another example, the concentration of the Wnt agonist CHIR99021 is about 1.9 μM. In another example, the concentration of the Wnt agonist CHIR99021 is about 2.0 μM. In another example, the concentration of the Wnt agonist CHIR99021 is about 2.1 μM.

[0039] In one example, the Wnt antagonist is IWR1 or XAV-939, and the concentration of IWR1 or XAV-939 is about 0.5 μM.

[0040] Using both the "low-CHIR method" and the "delayed CHIR method" available in the literature (Table 1 and 2), contamination of cells of the anterior and posterior to the midbrain are observed by examining genes characteristic of fore-, mid-, and hind-brain regions. Thus, a single morphogen results in the differentiation of hPSCs with a spectrum of regional identities. Contrary to these methods in the literature, by using the method as disclosed herein, after step (a), the mFPPs generated are restricted to only midbrain identity, and do not contain forebrain or hindbrain floor plate progenitors. In one example, after step (a), the mFPPs generated contain 0% NKX2.1+ forebrain floor plate progenitors or GBX2+ hindbrain floor plate progenitors. In another example, after step (a), the mFPPs generated contain less than 0.5% NKX2.1+ forebrain floor plate progenitors or GBX2+ hindbrain floor plate progenitors. In another example, after step (a), the mFPPs generated contain less than 1% NKX2.1+ forebrain floor plate progenitors or GBX2+ hindbrain floor plate progenitors. In another example, after step (a), the mFPPs generated contain less than 3% NKX2.1+ forebrain floor plate progenitors or GBX2+ hindbrain floor plate progenitors. In another example, after step

(a), the mFPPs generated contain less than 5% NKX2.1+ forebrain floor plate progenitors or GBX2+ hindbrain floor plate progenitors. In another example, after step (a), the mFPPs generated contain less than 10% NKX2.1+ forebrain floor plate progenitors or GBX2+ hindbrain floor plate progenitors.

[0041] In step (a), the hPSCs are incubated in a basal medium supplemented with (i) Dual-Smad inhibitors (Kriks et al., 2011; Xi et al., 2012) comprising a BMP inhibitor selected from the group consisting of DMH1, Noggin and LDN-193189 and a TGF-β inhibitor selected from the group consisting of SB431542 and A-83-01, and (ii) an SHH agonist selected from the group consisting of SHH, SAG and Purmorphamine. In one example, the concentration of DMH1 is about 2 μM. In another example, the concentration of Noggin is 200-500 ng/mL. In another example, the concentration of LDN-193189 is about 0.1 μM. In another example, the concentration of SB431542 is 2-10 μM. In another example, the concentration of A-83-01 is about 2 μM. In another example, the concentration of SHH is 100-500 ng/mL. In another example, the concentration of SAG is 0.1-1 μM. In another example, the concentration of Purmorphamine is 1-3 μM. The two inhibitors disclosed above, the BMP inhibitor and the TGF-β inhibitor are termed as "dual SMAD inhibitors" since they are regulating the activity of SMAD proteins which are the downstream of BMP and TGF-β signaling.

[0042] The basal medium for step (a) is 1:1 mixture of DMEM/F12 and Neurobasal medium with 1X of N2 supplement.

[0043] After step (a), the mFPPs generated are OTX2+/FOXA2+. Co-expression of OTX2 and FOXA2 indicates the ventral midbrain cell population called the midbrain floor plate progenitors (mFPPs).

[0044] ***Step (b) - Restriction of mFPPs to the medial mFPPs by Activin A and SHH activation***

In step (b) of the method as disclosed herein, mFPPs generated from step (a) are treated with Activin A and a sonic hedgehog (SHH) agonist to restrict the mFPPs to medial mFPPs. As used herein, the term "treat" refers to incubating mFPPs in an *in vitro* culture medium containing Activin A and the SHH agonist as disclosed herein.

[0045] In one example, the mFPPs are treated with Activin A and the SHH agonist for about 5 days, from day 4-6 up to day 9-11. In another example, the mFPPs are treated with Activin A and the SHH agonist from day 4 up to day 9. In another example, the mFPPs are treated with

Activin A and the SHH agonist from day 5 up to day 10. In another example, the mFPPs are treated with Activin A and the SHH agonist from day 6 up to day 11.

[0046] In one example, the SHH agonist is a SHH ligand. SHH ligands bind to Patched receptors and activated SHH signaling pathway. In another example, the SHH agonist is a Smo activator. Smo activators bind SHH down-stream protein, Smo, and increase its activity.

[0047] In one example, the SHH ligand is SHH. In another example, the Smo activator is selected from the group consisting of SAG and Purmorphamine.

[0048] Activin A repressed NKX6.1 while maintaining the floor plate markers FOXA2 and LMX1A expression when combining with SHH activation. In one example, after step (b), the medial mFPPs generated do not contain NKX6.1+ lateral floor plate progenitors. In another example, after step (b), the medial mFPPs generated contain 0% NKX6.1+ lateral floor plate progenitors. In another example, after step (b), the medial mFPPs generated contain less than 0.5% NKX6.1+ lateral floor plate progenitors. In another example, after step (b), the medial mFPPs generated contain less than 1% NKX6.1+ lateral floor plate progenitors. In another example, after step (b), the medial mFPPs generated contain less than 3% NKX6.1+ lateral floor plate progenitors. In another example, after step (b), the medial mFPPs generated contain less than 5% NKX6.1+ lateral floor plate progenitor. In another example, after step (b), the medial mFPPs generated contain less than 10% NKX6.1+ lateral floor plate progenitors.

[0049] During development, mFPPs are divided into the medial and the lateral mFPPs. The mFPPs in the medial part generate midbrain dopaminergic neurons (mDANs) whereas those in the lateral floor plate give rise to glutamatergic neurons of the Red nucleus or Oculomotor nucleus. The use of Activin A and a SHH agonist to restrict mFPPs to the medial mFPPs was never reported before. The resulting medial mFPPs generated from step (b) maintain the expression of the floor plate markers FOXA2 and LMX1A, but have repressed expression of NKX6.1.

[0050] The basal medium for step (b) is 1:1 mixture of DMEM/F12 and Neurobasal medium with 1X of N2 supplement.

[0051] ***Step (c) – Generation of mDA precursors (mDAPs) from medial mFPPs***

[0052] In step (c) of the method as disclosed herein, medial mFPPs generated from step (b) are allowed to differentiate to midbrain dopamine progenitors (mDAPs) by removing the SHH agonist from the culture medium and adding a Wnt agonist to the culture medium. Removal of the SHH agonist and adding of the Wnt agonist is crucial to allow the medial mFPPs to undergo

further differentiation. In one example, the Wnt agonist is a Wnt ligand. Wnt ligands bind to WNT receptors and activate WNT signaling pathway. In another example, the Wnt agonist is a GSK3 inhibitor. GSK3 inhibitors inhibit Destruction complex and result in the activation of WNT signaling pathway via the accumulation of WNT down-stream protein, β-catenin. In one example, the Wnt ligand is selected from the group consisting of Wnt1, Wnt2, Wnt2b, Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, Wnt8a, Wnt8b, Wnt9a, Wnt9b, Wnt10a, Wnt10b, Wnt11, and Wnt 16. In another example, the GSK3 inhibitor is selected from the group consisting of the CHIR99021(Laduviglusib), SB415286, LY2090314, AZD1080, AR-A014418, BIO, Elraglusib, BRD0705, KY19382, SB216763, TWS119, CHIR-98014, Tideglusib, TDZD-8, 1-Azakenpaullone, AZD2858, IM-12, Bikinin, CP21R7, 5-Bromoindole, (E/Z)-GSK-3β inhibitor 1, PF-04802367, and WAY-119064. In one particular example, the Wnt agonist is CHIR99021. In another example, the concentration of CHIR99021 used in step (c) of the method as disclosed herein is 3.0 to 5.0 μM. In another example, the concentration of the Wnt agonist CHIR99021 used in step (c) of the method as disclosed herein is selected from the group consisting of 3.0-3.5 μM, 3.5-4.0 μM, 4.0-4.5 μM, 4.5-5.0 μM, 3.0-4.0 μM, 3.5-4.5 μM, 4.0-5.0 μM, about 3 μM, about 3.1 μM, about 3.2 μM, about 3.3 μM, about 3.4 μM, about 3.5 μM, about 3.6 μM, about 3.7 μM, about 3.8 μM, about 3.9 μM, about 4.0 μM, about 4.1 μM, about 4.2 μM, about 4.3 μM, about 4.4 μM, about 4.5 μM, about 4.6 μM, about 4.7 μM, about 4.8 μM, about 4.9 μM, and about 5.0 μM.

[0053] In one example, the medial mFPPs are incubated for about 5 days, from day 9-11 to day 16-18 after the removal of the SHH agonist and adding the Wnt agonist. In another example, the medial mFPPs are incubated from day 9 to day 16 after the removal of the SHH agonist and adding the Wnt agonist. In another example, the medial mFPPs are incubated from day 10 to day 17 after the removal of the SHH agonist and adding the Wnt agonist. In another example, the medial mFPPs are incubated from day 11 to day 18 after the removal of the SHH agonist and adding the Wnt agonist.

[0054] After step (c), the mDAPs generated do not contain other type of cells. In one example, after step (c), the mDAPs generated are at least 70%, at least 80%, or at least 90% pure. In another example, example, after step (c), the mDAPs generated are at least 95% pure. In another example, example, after step (c), the mDAPs generated are at least 98% pure. In another example, after step (c), the mDAPs generated are 100% pure.

[0055] The resulting midbrain dopamine progenitors (mDAPs) generated from step (c) express FOXA2, LMX1A, MSX1 and OTX2. In another example, at least 70%, at least 80%, at least 90%, preferably 95%, and more preferably 98% of the mDAPs generated have expression of at least one of FOXA2, LMX1A, MSX1 and OTX2, and substantially no expression of NKX6.1 and NKX2.1. As used herein, "substantially no expression of NKX6.1 and NKX2.1" refers to that 0% or 1-10% of the produced mDAPs express NKX6.1, and 0% or 1-10% of the produced mDAPs express NKX2.1.

[0056] The population of LMX1A and MSX1-expressing cells after step (c) indicates that these cells become committed to the dopaminergic fate, and they are hereby termed committed dopamine neurons progenitors (mDAPs).

[0057] The basal medium for step (c) is 1:1 mixture of DMEM/F12 and Neurobasal medium with 1X of N2 supplement.

[0058] ***Step (d) – Differentiation of mDAPs to authentic mDANs***

In another example, in step (d) of the method as disclosed herein, the mDAPs are treated with a notch inhibitor in a conventional neuron maturation medium for 1-3 days, and the notch inhibitor is removed after the mDAPs are differentiated to midbrain dopamine neurons (mDANs). As used herein, the term "treat" refers to incubating mDAPs in an *in vitro* culture medium containing the notch inhibitor as disclosed herein. In one example, the conventional neuron maturation medium is Neurobasal medium, which comprises B27 supplement (1x), vitamin C (200 μM), GlutaMAX supplement (1x), BDNF (brain-derived neurotrophic factor; 10 ng/ml), GDNF (glia-derived neurotrophic factor; 10 ng/ml), db-cAMP (1 μM, or Forskolin (10 μM)), and TGFβ3 (1 ng/ml).

[0059] In one example, the notch inhibitor is DAPT. In another example, the notch inhibitor is compound E. In another example, the concentration of DAPT is about 10 μM. In another example, the concentration of compound E is 0.1-1 μM.

[0060] After step (d), the mDANs generated do not contain other type of cells. In one example, after step (d), at least 70% of generated cells are TH+ mDANs. In another example, after step (d), at least 80% of generated cells are TH+ mDANs. In another example, after step (d), at least 90% of generated cells are TH+ mDANs. In another example, after step (d), at least 95% of generated cells are TH+ mDANs. In another example, after step (d), at least 98% of generated cells are TH+ mDANs. In another example, after step (d), 100 % of generated cells are TH+ mDANs.

[0061] Since the mDAPs have the limited potential, they are simply differentiated to mDANs in step (d) of the method as disclosed herein, by growing the mDAPs in the conventional neuron maturation medium (consisting of neural basal medium with B27, BDNF, GDNF, cAMP agonist, ascorbic acid, and TGFβ3) together with a notch inhibitor DAPT or compound E. By about day 20, the differentiated cells are nearly uniformly expressing Tyrosine Hydroxylase (TH). The mDANs generated exhibited long axons with extensive arborizations, and exhibited characteristic electrophysiological properties such rhythmic firing and sag potentials.

[0062] In one example, the method as disclosed herein further comprise detecting expression of at least one of Tyrosine Hydroxylase (TH), dihydroxyphenylalanine (DOPA) decarboxylase (DDC), FOXA2, OTX2, LMX1A, EN1, and NURR1, and substantially no expression of NKX6.1 and NKX2.1.

[0063] In another example, at least 70%, at least 80%, at least 90%, preferably 95%, and more preferably 98% of the mDANs generated have expression of at least one of Tyrosine Hydroxylase (TH), dihydroxyphenylalanine (DOPA) decarboxylase (DDC), FOXA2, OTX2, LMX1A, EN1, and NURR1, and substantially no expression of NKX6.1 and NKX2.1. As used herein, "substantially no expression of NKX6.1 and NKX2.1" refers to that 0% or 1-10% of the produced mDANs express NKX6.1, and 0% or 1-10% of the produced mDANs express NKX2.1.

[0064] In summary, the present disclosure discloses a method of generating mDAPs and mDANs by (a) inducing mFPPs without contamination of forebrain and hindbrain cells, by combination of both Wnt agonists and antagonists; (b) inducing mFPPs to the medial floor plate fate, restricting the mFPPs to the dopaminergic (mDAPs) but not glutaminergic fate, by using Activin A as a novel regulator and a combinatorial approach (Activin A + SHH activation); (c) producing enriched mDAPs that are suitable for transplantation, which express FOXA2, LMX1A, MSX1 and OTX2, by removing the SHH agonist from the cell culture and adding the Wnt agonist to the cell culture; and (d) producing enriched and authentic midbrain dopaminergic neurons (mDANs) from mDAPs in the presence of a notch inhibitor, the mDANs being highly enriched without the contamination of glutamate neurons and non-neural cells. The mDANs exhibit authentic features of those in the brain.

[0065] Advantageously, the present technology overcomes the major issues of existing protocols in the differentiation of mDAPs and mDANs from human stem cells, especially the

contamination of non-target cells and lower yield. In addition, the present protocol is simpler and generates mDAPs from stem cells within 3 weeks.

[0066] In another aspect, the present disclosure refers to a plurality of midbrain dopamine progenitors (mDAPs) generated by the method as disclosed herein, wherein at least 70%, at least 80%, at least 90%, preferably 95%, or more preferably 98% of the mDAPs have expression of at least one of FOXA2, LMX1A, MSX1 and OTX2, and substantially no expression of NKX6.1 and NKX2.1. As used herein, "substantially no expression of NKX6.1 and NKX2.1" refers to that 0% or 1-10% of the mDAPs express NKX6.1, and 0% or 1-10% of the mDAPs express NKX2.1.

[0067] In another aspect, the present disclosure refers to a plurality of midbrain dopamine neurons (mDANs) generated by the method as disclosed herein, wherein at least 70%, at least 80%, at least 90%, preferably 95%, and more preferably 98% of the mDANs have expression of at least one of Tyrosine Hydroxylase (TH), dihydroxyphenylalanine (DOPA) decarboxylase (DDC), FOXA2, OTX2, LMX1A, EN1, and NURR1, and substantially no expression of NKX6.1 and NKX2.1. As used herein, "substantially no expression of NKX6.1 and NKX2.1" refers to that 0% or 1-10% of the mDANs express NKX6.1, and 0% or 1-10% of the mDANs express NKX2.1.

[0068] In one example, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% of the mDAPs express genes selected from the group consisting of FOXA2, LMX1A, MSX1 and OTX2, and the mDAPs have substantially no expression of NKX6.1 and NKX2.1. In another example, less than 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 4%, less than 3%, less than 2%, less than 1%, or 0% of the mDAPs express NKX6.1, and less than 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 4%, less than 3%, less than 2%, less than 1%, or 0% of the mDAPs express NKX2.1.

[0069] In one example, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% of the differentiated mDANs express TH. In another example, the mDANs generated using the method as disclosed herein exhibited long axons with extensive arborizations, and characteristic electrophysiological properties such rhythmic firing and sag potentials. In another example, the mDANs express genes selected from the group consisting of TH, dihydroxyphenylalanine (DOPA) decarboxylase (DDC), FOXA2, OTX2, LMX1A, EN1, and NURR1, and the mDANs have substantially no expression of NKX6.1 and NKX2.1. In another example, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least

99%, or 100% of the mDANs express genes selected from the group consisting of TH, dihydroxyphenylalanine (DOPA) decarboxylase (DDC), FOXA2, OTX2, LMX1A, EN1, and NURR1, and the mDANs have substantially no expression of NKX6.1 and NKX2.1. In another example, less than 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 4%, less than 3%, less than 2%, less than 1%, or 0% of the mDANs express NKX6.1, and less than 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 4%, less than 3%, less than 2%, less than 1%, or 0% of the mDANs express NKX2.1.

[0070] In another aspect, the present disclosure refers to a pharmaceutical composition comprising the plurality of mDAPs as disclosed herein, and a pharmaceutically-acceptable diluent, carrier or excipient.

[0071] The carriers, diluents and adjuvants must be pharmaceutically "acceptable" in terms of being compatible with the other ingredients of the composition, and not deleterious to the recipient thereof.

[0072] Examples of pharmaceutically acceptable carriers or diluents are demineralized or distilled water; saline solution; vegetable based oils such as peanut oil, safflower oil, olive oil, cottonseed oil, maize oil, sesame oils such as peanut oil, safflower oil, olive oil, cottonseed oil, maize oil, sesame oil, arachis oil or coconut oil; silicone oils, including polysiloxanes, such as methyl polysiloxane, phenyl polysiloxane and methylphenyl polysolpoxane; volatile silicones; mineral oils such as liquid paraffin, soft paraffin or squalane; cellulose derivatives such as methyl cellulose, ethyl cellulose, carboxymethylcellulose, sodium carboxymethylcellulose or hydroxypropylmethylcellulose; lower alkanols, for example ethanol or iso-propanol; lower aralkanols; lower polyalkylene glycols or lower alkylene glycols, for example polyethylene glycol, polypropylene glycol, ethylene glycol, propylene glycol, 1,3-butylene glycol or glycerin; fatty acid esters such as isopropyl palmitate, isopropyl myristate or ethyl oleate; polyvinylpyrolidone; agar; gum tragacanth or gum acacia, and petroleum jelly. Typically, the carrier or carriers will form from 10% to 99.9% by weight of the compositions.

[0073] In another aspect, the present disclosure refers to a method of treating a neurological disease of a subject, comprising administering the plurality of mDAPs as disclosed herein or the pharmaceutical composition as disclosed herein into a neurological disease site of the subject.

[0074] In another aspect, the present disclosure refers to use of the plurality of mDAPs as disclosed herein or the pharmaceutical composition as disclosed herein in the manufacture of

a medicament for treating a neurological disease of a subject, wherein the plurality of mDAPs or the pharmaceutical composition is to be administered into a neurological disease site of the subject.

[0075] The mDAPs may be administered, grafted or transplanted into a neurological disease site, which will then survive and differentiate to functional mDANs, to replace the injured or lost mDANs at the neurological disease site. As used herein, the term "neurological disease site" refers to a site resulted from a neurological disease selected from the group consisting of Parkinson's disease and a psychiatric disorder. In Parkinson's disease, nerve cells in the basal ganglia, an area of the brain that controls movement, become impaired and/or die. Classic clinical symptoms include bradykinesia, resting tremor, postural instability, and shuffling gait. Parkinson's disease is a result of neurodegeneration of the dopaminergic neurons. The substantia nigra, due to degeneration, loses its grossly visible dark pigmentation, a concomitant sign of dopamine biosynthesis dysfunction. This loss of dopamine depresses the nigrostriatal pathway. With decreased dopaminergic input the striatum exerts less positive motor activity and more negative motor inhibition. This gives the characteristic hypokinetic dysfunction found in these patients. In particular, the present technology generates pure mDAPs without the contamination of non-target cells, which enables safe and effective cell therapy for neurological diseases such as Parkinson's disease.

[0076] In one example, the psychiatric disorder is selected from the group consisting of depression, anxiety, addiction, and schizophrenia.

[0077] In one example, the neurological disease site is in the brain. In another example, the neurological disease site is the basal ganglia. In another example, the neurological disease site is selected from the group consisting of caudate, putamen, substantia nigra, nigra, and striatum. The basal ganglia or basal nuclei are a group of subcortical nuclei found in the brains of vertebrates. Positioned at the base of the forebrain and the top of the midbrain, they have strong connections with the cerebral cortex, thalamus, brainstem and other brain areas. Basal ganglia include the following structures: caudate nucleus, globus pallidus, putamen, substantia nigra, subthalamic nucleus and ventral pallidum. These structures intricately synapse onto one another to promote or antagonize movement.

[0078] As used herein, the term "treating" and grammatical variations of that term, refer to administration of the population of mDAPs or the pharmaceutical composition as disclosed herein to a subject as described herein by any appropriate means as described herein. Such

treatment includes any and all uses which remedy a disease state or symptoms, prevent the establishment of disease, or otherwise prevent, hinder, retard, or reverse the progression of disease or other undesirable symptoms in any way whatsoever.

[0079] The terms "subject", "host", and "patient" are used interchangeably. As used herein, a subject is preferably a mammal, such as a non-primate (e.g., cows, pigs, horses, cats, dogs, rats, etc.) or a primate (e.g., monkeys and humans), most preferably a human.

[0080] In one example, the number of mDAPs to be administered into a neurological disease site is $5x10^4$ to 20 million. In another example, the number of mDAPs to be administered into a neurological disease site is $5x10^4$-$5x10^5$. In another example, the number of mDAPs to be administered into a neurological disease site is $5x10^5$-5 million. In another example, the number of mDAPs to be administered into a neurological disease site is 5-10 million. In another example, the number of mDAPs to be administered into a neurological disease site is 10-20 million. In another example, the number of mDAPs to be administered into a neurological disease site is about $5x10^4$. In another example, the number of mDAPs to be administered into a neurological disease site is about $5x10^5$. In another example, the number of mDAPs to be administered into a neurological disease site is about 5 million. In another example, the number of mDAPs to be administered into a neurological disease site is about 10 million. In another example, the number of mDAPs to be administered into a neurological disease site is about 15 million. In another example, the number of mDAPs to be administered into a neurological disease site is about 20 million.

[0081] As used herein, the term "administration" and grammatical variations of that term, refer to transplanting the mDAPs or the pharmaceutical composition as disclosed herein to a neurological disease site of the subject as disclosed herein. In one example, the mDAPs or the pharmaceutical composition as disclosed herein is administered by stereotaxic injection, which allows injecting the mDAPs directly into the neurological disease site such as the basal ganglia as disclosed herein.

[0082] In one example, the plurality of mDAPs or the pharmaceutical composition is administered once.

[0083] Besides the therapeutic applications of the mDAPs generated using the method as disclosed herein, the pure populations of authentic mDANs which are generated after step (d) of the method as disclosed herein can be used in other applications such as modeling disease

processes that affect mDANs, testing drugs for these diseases, and developing cell therapy for the diseases through cell transplantation.

[0084] As used in this application, the singular form "a," "an," and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a primer" includes a plurality of primers, including mixtures and combinations thereof.

[0085] As used herein, the term "comprising" means "including." Variations of the word "comprising", such as "comprise" and "comprises," have correspondingly varied meanings. Thus, for example, a composition "comprising" X may consist exclusively of X or may include one or more additional unrecited components.

[0086] As used herein, the term "about" in the context of concentration of a substance, size of a substance, length of time, or other stated values means +/- 5% of the stated value, or +/- 4% of the stated value, or +/- 3% of the stated value, or +/- 2% of the stated value, or +/- 1% of the stated value, or +/- 0.5% of the stated value.

[0087] Throughout this disclosure, certain embodiments may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

[0088] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied herein disclosed may be resorted to by those skilled in

the art, and that such modifications and variations are considered to be within the scope of this invention.

[0089] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0090] Unless otherwise indicated, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.

[0091] Other embodiments are within the following claims and non-limiting examples.

EXAMPLES

[0092] Non-limiting examples of the disclosure will be further described in greater detail by reference to specific Examples, which should not be construed as in any way limiting the scope of the disclosure.

[0093] A new procedure that enables differentiating hPSCs to pure authentic mDAPs and mDANs was developed. First, midbrain floor plate progenitors (mFPPs) were induced without contamination of forebrain and hindbrain cells by both Wnt activators and inhibitors. Then, the mFPPs were restricted to the medial mFPPs by Activin A and SHH activation. Then, the medial mFPPs were further differentiated to midbrain dopamine neuron progenitors (mDAPs) in the presence of Activin A and the Wnt agonist but with removal of SHH. This stepwise fate restriction procedure enabled generation of committed mDAPs and finally authentic functional midbrain dopamine neurons (mDANs).

[0094] **Example 1 – Step 1: Induction of mFPPs by Wnt agonist and antagonist**

[0095] The fact that the highly-enriched floor plate progenitor populations produce different percentages of Tyrosine hydroxylase (TH, a marker for mDAN)-expressing cells (5%~75%) suggests the contamination of non-dopaminergic cells. Most of the published work did not account the presence of other cells. Two recent reports, by applying single-cell RNA sequencing (scRNAseq) analysis, revealed the contamination of cells from other brain regions. Kee et al demonstrated the presence of subthalamic (forebrain) cells that express BARHL1

when using a low concentration of CHIR (Kee et al., 2017). Even the low CHIR (0.4 μM) induced cells of the hindbrain and midbrain-hindbrain boundary (MHB) when it was combined with FGF8b treatment (You et al., 2023).

[0096] The contamination of cells of the anterior and posterior to the midbrain was confirmed by examining genes characteristic of fore-, mid-, and hind-brain regions using both the "low-CHIR method" and the "delayed CHIR method" (Table 1 and 2). When the "low-CHIR method" was used, there was contamination of anterior cells (forebrain, PAX6, NKX2.1). When the "delayed-CHIR method" was employed, the contamination of forebrain cells was largely suppressed but posterior cells (hindbrain, EN1) were present (**Fig. 1**). Hence, a single morphogen results in the differentiation of cells with a spectrum of regional identities. Therefore, a way to restrict the FPP to only the midbrain identity was explored.

[0097] During brain development, the anterior-posterior (A-P) axis is precisely patterned by opposing morphogens like Wnts and their antagonists. In other words, the cell identity of a brain region is restricted or "squeezed" by molecules with opposing effects from both sides (**Fig. 8**). Hence, a "converging modulation" method was developed by using CHIR together with a Wnt antagonist. Co-expression of OTX2 and FOXA2 indicates the ventral midbrain cell population called the midbrain floor plate progenitors (mFPPs). Recent reports, including those prepared for clinical trials, also used it as a standard for confirming the purity of the cell source for transplantation (Kirkeby et al., 2023 and Park et al., 2024). By examining the expression of OTX2 and FOXA2, it was found that the population of OTX2+/FOXA2+ cells rapidly decreased with increasing amount of CHIR at above 1.2 μM in the traditional CHIR (Wnt agonist)-only group (**Fig. 2A, B**). In the presence of a Wnt antagonist (IWR1), the population of OTX2+/FOXA2+ cells was maintained with increasing amount of CHIR up to 2.1 μM (**Fig. 2C, D**). Another Wnt antagonist, XAV939, had a similar effect (**Fig. 2E**). However, another type of Wnt antagonist, WNT-C59, didn't show the similar effect in the same condition (**Fig. 2F**). These results suggest that this effect is likely related to the activity of Destruction complex (IWR1 and XAV939) instead of the secretion of endogenous Wnt ligand (WNT-C59).

[0098] Together, an optimal combination of Wnt agonist (e.g., CHIR) and Wnt antagonist (e.g., IWR1, XAV939 but not C59) was developed to restrict the floor plate progenitors to the midbrain fate without contamination of forebrain or hindbrain cells.

[0099] **Example 2 – Step 2: Restriction of mFPPs to the medial mFPPs by Activin A and SHH activation**

[00100] During development, mFPPs are divided into the medial and the lateral mFPPs. The mFPPs in the medial part generate midbrain dopaminergic neurons (mDANs) whereas those in the lateral floor plate give rise to glutamatergic neurons of the Red nucleus or Oculomotor nucleus (**Fig. 3**). The separation of the medial floor plate from the lateral one is regulated at least partially by the dorsal-ventral patterning signals, such as Wnt or Bmp signaling, since the midbrain is expressing the common roof plate (dorsal) markers LMX1A and MSX1 in the floor plate. Therefore, the rational way and the presently common practice to induce medialization (or ventralization) and trigger neurogenesis is to activate the Wnt signaling pathway using high concentration of CHIR99021 treatment following the mFPP formation (Kim et al., 2021 and Nishimura et al., 2023).

[00101] It was found that the mFPPs expressed LMX1A but also NKX6.1, suggesting that the mFPPs at this stage are equivalent to the floor plate cells during early development. When Wnt signaling was activated by treating the mFPPs with CHIR99021, a common practice in mDA neuron differentiation, a sustained strong expression of NKX6.1 was found (**Fig. 4A, B**), suggesting that activation of Wnt signaling is not sufficient to restrict the mFPPs to the medial floor plate cells, at least in human cells. This strong level of NKX6.1 by WNT signaling is similar to the phenomenon observed in transgenic mice that continued expression of the active form of β-catenin in the floor plate region resulted in the mixed populations of neurons in the floor plate during development (Nouri et al., 2015). Indeed, the TH-expressing neurons generated still expressed NKX6.1 (most DA neurons do not express NKX6.1) but not NURR1 in their nuclei, which should have (**Fig. 4B, C**). These results indicated that WNT activation indeed triggered neurogenesis but the generated neurons were not authentic DA neurons. In other words, the presently common method to use WNT to trigger neurogenesis confuses the differentiation program, producing artificial cell types.

[00102] Another usual suspect is BMP activation. Indeed, NKX6.1 was effectively suppressed when the mFPPs were treated by various BMPs (BMP4, 7) at various concentrations. However, BMPs down regulated FOXA2 and LMX1A, the key floor plate markers and consequently inhibited mDAN differentiation, suggesting that dorsalization by BMPs has a negative effect on mDAN differentiation.

[00103] Then the search was extended to additional morphogen families like TGFβ family, which is used for DA neuron maturation but not for patterning. Similar to the effect of BMPs, Activin A (member of the TGFβ family) effectively down-regulated FOXA2 as well as

NKX6.1. Different from the effect of BMPs, however, Activin A retained the LMX1A expression, suggesting Activin A directly regulates LMX1A expression.

[00104] The above series of experiments indicates that any one of these morphogens is not effective in restricting the mFPPs to the medial floor plate progenitors. Considering mFPPs are induced by sonic hedgehog and express FOXA2, SHH or its agonist SAG were combined with the dorsal-patterning molecules BMP or Activin A. It was found that combination of SAG with BMP4 or Activin A successfully down-regulated NKX6.1 while maintaining FOXA2 expression. Nevertheless, the expression of LMX1A was localized to the cytoplasm instead of the nucleus in the BMP treated cells (**Fig. 5**). Thus, only Activin A, but not BMP4, repressed NKX6.1 while maintaining the floor plate markers FOXA2 and LMX1A when combining with SHH activation.

[00105] The use of Activin A and SAG (SHH activation) from day 7 to 9 to restrict mFPPs to the medial mFPPs was never reported before.

[00105] **Example 3 – Step 3: Generation of mDA precursors from medial mFPPs**

[00106] For clinical transplantation therapy, the donor cells should have a fixed fate potential (predictability) and limited proliferation capacity (safety). In mDAN development, FOXA2, OTX2 and LMX1A are expressed from early progenitors located to the ventricular zone (VZ) of the floor plate that is considered as the proliferating stem cell zone, suggesting that these markers are not sufficient to identify the appropriate cells for transplantation. Instead, there are markers in the late stage of the midbrain floor plate before progenitors reach the post-mitotic stage. MSX1 is expressed from LMX1A-expressing VZ in the late stage of the midbrain floor plate and is known as the intrinsic determinant factor for the neurogenic mDA precursor cells (Andersson et al., 2006). Once MSX1 expression appears, the progenitors at the midbrain floor plate begin to generate immature neuronal cells expressing NURR1 and are located in the intermediate zone (IZ) of the floor plate before immature neuronal cells reach the marginal zone (MZ) where terminal differentiation is completed. Furthermore, NURR1 is known to regulate many functional effectors of mDANs including TH, AADC, DAT, and VMAT2. Additionally, it has a role in protecting mDANs from inflammation-induced death (Le et al., 1999, Jankovic et al., 2005, and Saijo et al., 2009). However, NURR1-expressing immature neurons are post-mitotic cells, suggesting that this stage is too late for transplantation studies.

[00107] Following the induction of medial mFPPs, SHH or SAG was then removed and Wnt agonist was added to the culture at D11 to allow the progenitors to undergo further

differentiation. Under this condition, mFPPs retained the expression of FOXA2, indicating that SHH signaling is no longer required for FOXA2. By D18, highly enriched population of LMX1A and MSX1-expressing cells was observed (**Fig. 6A**), indicating that these cells become committed to the dopaminergic fate. Indeed, further differentiation of these LMX1A and MSX1-expressing cells resulted in the generation of TH-expressing DANs (**Fig. 6B**). These LMX1A and MSX1-expressing (but not TH) cells were called committed midbrain dopamine neurons precursors (mDAPs).

[00108] **Example 4 – Step 4: Generation of mDA precursors from medial mFPPs**

[00109] Since the mDAPs have the limited potential, they were simply differentiated to mDANs by growing the cells in the conventional neuronal differentiation medium (consisting of neural basal medium with B27, BDNF, GDNF, cAMP agonist, ascorbic acid, and TGFβ3) together with a notch inhibitor DAPT or compound E. By day 20, the differentiated cells were nearly uniformly expressing TH. Like those in the brain, the mDANs generated in the new method exhibited long axons with extensive arborizations, indicated by SMI312. They also exhibited characteristic electrophysiological properties such rhythmic firing and sag potentials (**Fig. 7**).

[00110] **Industrial Applicability**

[00111] It will be apparent that various other modifications and adaptations of the invention will be apparent to the person skilled in the art after reading the foregoing disclosure without departing from the spirit and scope of the invention and it is intended that all such modifications and adaptations come within the scope of the appended claims.

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Claims

1. A method of generating midbrain dopamine progenitors (mDAPs) from human pluripotent stem cells (hPSCs), comprising:
(a) treating hPSCs with a Wnt agonist and a Wnt antagonist, to generate midbrain floor plate progenitors (mFPPs);
(b) treating the mFPPs with Activin A and a sonic hedgehog (SHH) agonist to restrict the mFPPs to medial mFPPs; and
(c) removing the SHH agonist and adding the Wnt agonist to differentiate medial mFPPs to mDAPs.
2. The method of claim 1, further comprising: (d) treating the mDAPs with a notch inhibitor to generate midbrain dopamine neurons (mDANs).
3. The method of claim 1, wherein the hPSCs comprise embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs).
4. The method of claim 1, wherein step (a) comprises incubating the hPSCs in a medium supplemented with (i) Dual-Smad inhibitors comprising a BMP inhibitor selected from the group consisting of DMH1, Noggin and LDN-193189 and a TGF-β inhibitor selected from the group consisting of SB431542 and A-83-01, and (ii) an SHH agonist selected from the group consisting of SHH, SAG and Purmorphamine.
5. The method of claim 4, wherein the concentration of DMH1 is about 2 μM, the concentration of Noggin is 200-500 ng/mL, the concentration of LDN-193189 is about 0.1 μM, the concentration of SB431542 is 2-10 μM, the concentration of A-83-01 is about 2 μM, the concentration of SHH is 100-500 ng/mL, the concentration of SAG is 0.1-1 μM, and the concentration of Purmorphamine is 1-3 μM.
6. The method of claim 1, wherein in step (a), the hPSCs are treated with the Wnt agonist and the Wnt antagonist from day 0 to day 4-6.
7. The method of claim 6, wherein the Wnt agonist is a Wnt ligand, or a GSK3 inhibitor.
8. The method of claim 7, wherein the Wnt ligand is selected from the group consisting of Wnt1, Wnt2, Wnt2b, Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, Wnt8a, Wnt8b, Wnt9a, Wnt9b, Wnt10a, Wnt10b, Wnt11, and Wnt 16.
9. The method of claim 7, wherein the GSK3 inhibitor is selected from the group consisting of the CHIR99021(Laduviglusib), SB415286, LY2090314, AZD1080, AR-A014418, BIO,

Elraglusib, BRD0705, KY19382, SB216763, TWS119, CHIR-98014, Tideglusib, TDZD-8, 1-Azakenpaullone, AZD2858, IM-12, Bikinin, CP21R7, 5-Bromoindole, (E/Z)-GSK-3β inhibitor 1, PF-04802367, and WAY-119064.

10. The method of claim 6, wherein the Wnt antagonist is a Tankyrase inhibitor.
11. The method of claim 10, wherein the Tankyrase inhibitor is selected from the group consisting of IWR1, XAV-939, G007-LK, WIKI4, NVP-TNKS656, RK-287107, JW55, M2912, and Nesuparib.
12. The method of claim 6, wherein the Wnt antagonist is not a Porcupine inhibitor selected from the group consisting of Wnt-C59, IWP-1, LGK-974, IWP-L6, GNF-6231, and ETC-159.
13. The method of claim 9, wherein the concentration of CHIR99021 is 1.2 to 2.1 μM.
14. The method of claim 11, wherein the concentration of IWR1 or XAV-939 is about 0.5 μM.
15. The method of claim 1, wherein after step (a) the mFPPs do not contain or contain less than 10%, less than 5%, less than 3% or less than 1% of NKX2.1+ forebrain floor plate progenitors or GBX2+ hindbrain floor plate progenitors.
16. The method of claim 1, wherein in step (b), the mFPPs are treated with the Activin A and the SHH agonist for about 5 days, from day 4-6 to day 9-11.
17. The method of claim 16, wherein the SHH agonist is a SHH ligand or a Smo activator.
18. The method of claim 17, wherein the SHH ligand is SHH.
19. The method of claim 17, wherein the Smo activator is selected from the group consisting of SAG and Purmorphamine.
20. The method of claim 1, wherein after step (b) the medial mFPPs do not contain or contain less than 10%, less than 5%, less than 3% or less than 1% of NKX6.1+ lateral floor plate progenitors.
21. The method of claim 1, wherein in step (c), the medial mFPPs are incubated for about 5 days, from day 9-11 to day 16-18 after the removal of the SHH agonist and adding the Wnt agonist.
22. The method of claim 2, wherein in step (d), the mDAPs are treated with a notch inhibitor for 1-3 days and the notch inhibitor is removed after the mDAPs are differentiated to midbrain dopamine neurons (mDANs).
23. The method of claim 22, wherein the notch inhibitor is DAPT or compound E.

24. The method of claim 23, wherein the concentration of DAPT is about 10 μM, and the concentration of compound E is 0.1-1 μM.

25. The method of claim 24, wherein after step (d) at least 70%, at least 80%, at least 90%, preferably 95%, and more preferably 98% of generated cells are TH+ mDANs.

26. The method of any one of claims 1-25, wherein the method further comprises detecting expression of at least one of Tyrosine Hydroxylase (TH), dihydroxyphenylalanine (DOPA) decarboxylase (DDC), FOXA2, OTX2, LMX1A, EN1, and NURR1, and substantially no expression of NKX6.1 and NKX2.1.

27. A plurality of midbrain dopamine progenitors (mDAPs) generated by the method of any one of claims 1 and 3-26, wherein at least 70%, at least 80%, at least 90%, preferably 95%, and more preferably 98% of the mDAPs have expression of at least one of FOXA2, LMX1A, MSX1 and OTX2, and substantially no expression of NKX6.1 and NKX2.1.

28. A plurality of midbrain dopamine neurons (mDANs) generated by the method of any one of claims 2-26, wherein at least 70%, at least 80%, at least 90%, preferably 95%, and more preferably 98% of the mDANs have expression of at least one of Tyrosine Hydroxylase (TH), dihydroxyphenylalanine (DOPA) decarboxylase (DDC), FOXA2, OTX2, LMX1A, EN1, and NURR1, and substantially no expression of NKX6.1 and NKX2.1.

29. A pharmaceutical composition comprising the plurality of the mDAPs of claim 27 and a pharmaceutically-acceptable diluent, carrier or excipient.

30. A method of treating a neurological disease of a subject, comprising administering the plurality of mDAPs of claim 27 or the pharmaceutical composition of claim 29 into a neurological disease site of the subject.

31. Use of the plurality of mDAPs of claim 27 or the pharmaceutical composition of claim 29 in the manufacture of a medicament for treating a neurological disease of a subject, wherein the plurality of mDAPs or the pharmaceutical composition is to be administered into a neurological disease site of the subject.

32. The method of claim 30 or the use of claim 31, wherein the neurological disease is selected from the group consisting of Parkinson's disease and a psychiatric disorder.

33. The method or use of claim 32, wherein the psychiatric disorder is selected from the group consisting of depression, anxiety, addiction, and schizophrenia.

34. The method of claim 30 or the use of claim 31, wherein the neurological disease site is in the brain.

35. The method or use of claim 34, wherein the neurological disease site is selected from the group consisting of the basal ganglia, caudate, putamen, substantia nigra, nigra, and striatum.

36. The method or use of claim 35, wherein the plurality of mDAPs or the pharmaceutical composition is administered once.

37. The method of any of claims 30-36 or the use of any one of claims 31-36, wherein the subject is human.