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Differentiation of human medium spiny neurons (MSNs) from induced pluripotent stem cells (iPSCs) V.1

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Quyen Do[1,2,3], Nora Bengoa-Vergniory[1,2,4,5,6,7], Richard Wade-Martins[1,2,3]

[1]Oxford Parkinson's Disease Centre and Department of Physiology, Anatomy and Genetics, University of Oxford, Dorothy Crowfoot Hodgkin Building, South Parks Road, Oxford, OX1 3QU, UK;
[2]Kavli Institute for Neuroscience Discovery, University of Oxford, Dorothy Crowfoot Hodgkin Building, South Park Road, Oxford OX1 3QU, United Kingdom;
[3]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, 20815, USA.;
[4]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, 20815, USA;
[5]Achucarro Basque Center for Neuroscience, Leioa, Spain;
[6]University of the Basque Country (UPV/EHU), Department of Neuroscience, Leioa, Spain;
[7]Ikerbasque - Basque Foundation for Science, Bilbao, Spain

Quyen Do

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Abstract

This protocol generates human medium spiny neurons (MSNs) from induced human pluripotent stem cells. Incorporating key findings from **Telzehkin et.al., 2016** and **Arber et.al., 2016**, this protocol produces MSNs following the *in vivo* developmental trajectory of MSN via the subpallium and subsequently, the lateral ganglionic eminence. These neurons expresses canonical markers of striatal projection neurons including glutamic acid decarboxylase as well as co-expression of the dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa (DARPP-32) and the Coup-TFI interacting protein 2 (CTIP2). They are also functionally active demonstrated by presence of intrinsic voltage-dependent sodium and potassium currents as well as the capacity to fire action potential upon current stimulation.

Materials

Reagents:

- Accutase (Stem Cell Tecnologies, CAT# 07920)
- Activin A (Sigma-Aldrich, SKU# SRP3003)
- AraC (Merck, CAT# C1768-100MG)
- B-27™ Supplement (50X), serum free (ThermoFisher Scientific, CAT# 17504044)
- B27 Plus (with vitamin A) (ThermoFisher Scientific, CAT# A3582801)
- β-Mercaptoethanol (ThermoFisher Scientific, CAT# 21985023)
- CaCl2 (sigma, SKU# C5670)
- cCAMP (Sigma-Aldrich, CAT# D0627. CAS# 16980-89-5)
- CHIR 99021 (Bio-Techne/Tocris/R&D, CAT# 4423)
- Cytosine β-D-arabinofuranoside (AraC) (Sigma-Aldrich, CAS# 147-94-4, SKU# C1768)
- DAPT, gamma-Secretase inhibitor (Abcam, CAT# ab120633, CAS# 208255-80-5)
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, CAS# 67-68-5)
- DMEM/F12 basal medium (ThermoFisher Scientific, CAT# 11320033)
- DMEM/F-12, GlutaMAX™ supplement (ThermoFisher Scientific, CAT# 10565018)
- GABA (Sigma-Aldrich, SKU# A2129)
- Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (ThermoFisher Scientific, CAT# A1413202)
- Laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane (Sigma-Aldrich, CAS# 114956-81-9, SKU# L2020-1MG)
- L-Ascorbic acid (Sigma-Aldrich, CAS# 50-81-7, SKU# A4544-25G)
- LDN193189 hydrochloride (Sigma-Aldrich, CAS# 1062368-24-4, SKU# SML0559-5MG)
- L-glutamine (CAT# 25030-024)
- LM22A4 (Tocris, CAT# 4607)
- Matrigel (CAT# 354277)
- MEM Non-Essential Amino Acids Solution (100X) (NEEA) (ThermoFisher Scientific, CAT# 11140050)
- N-2 Supplement (100X) (ThermoFisher Scientific, CAT# 17502048)
- Neurobasal (CAT# 21103049)
- Penicillin-Streptomycin (10,000 U/mL) (ThermoFisher Scientific, CAT# 15140122)
- PD0332991 (Selleck Chemicals, CAT# S1579)
- Poly-D-Lysine (ThermoFisher Scientific, CAT# A3890401)
- Poly-L-ornithine solution (Sigma-Aldrich, CAS# 27378-49-0, SKU# P4957-50ML)
- Phosphate-buffered saline, pH 7.4 (PBS) (Life Technologies, CAT# 10010056)
- Recombinant Human/Murine/Rat BDNF (BDNF) (PeproTech, CAT# 450-02)
- Recombinant Mouse FGF basic/FGF2/bFGF Protein (FGF) (biotechne | r&d systems, CAT# 3139-FB)
- ROCK inhibitor Y-27632 (ROCKi) (Bio-Techne, CAT# 1254)
- SB 431542 (Bio-Techne | tocris, CAT# 1614, CAS# 301836-41-9)
- Sodium pyruvate (CAT# 11360070)
- XAV 93939 (Tocris, CAT# TB3748-GMP)

Preparing Striatal neuronal induction base medium (sNIM):

- DMEM/F12 basal medium
- 1% MEM Non-Essential Amino Acids (NEAA)
- 1% Glutamax
- 1x B27 without vitamin A
- 1% penicillin/Streptomycin (P/S)
- 0.05% β-mercaptoethanol

Preparing Day 4 (D4) media:

1. Add to sNIM base media:

- 200 nM LDN (1:5000)
- 10 µM SB (1:1000)
- 4 µM XAV (1:5000)
- 10 µM ROCKi (1:1000)

Preparing Day 8 (D8) media:

1. Add to sNIM base media:

- 100 nM LDN (1:10000)
- 4 µM XAV (1:5000)
- 10 µM ROCKi (1:1000)

Preparing Day 16 (D16) media:

1. Add to sMM1 base media:

- 100 ng/ml BDNF (1:10000)
- 200 µM Ascorbic acid (1:1000)
- 10 µM DAPT (1:10000)
- 2 µM PD0332991 (1:500)
- 0.6 mM $CaCl_2$ (1:1000)
- 1 µM LM22A4 (1:1000)
- 200 nM cAMP (1:500)
- 3 µM CHIR (1:3333)
- 300 µM GABA (1:1000)
- 25 ng/ml Activin A (1:1000)
- 10 µM ROCKi (1:1000)

Preparing Striatal maturation base medium 1 (sMM1):

- DMEM/F12 basal medium
- 1% MEM Non-Essential Amino Acids (NEAA)
- 1% L-Glutamine
- 1x B27 without vitamin A
- 1% penicillin/Streptomycin (P/S)
- 0.05% β-mercaptoethanol

Preparing Striatal maturation base medium 2 (sMM2):

- 50% v/v DMEM/F12
- 50% v/v Neurobasal
- 1% MEM Non-Essential Amino Acids (NEAA)
- 1% L-Glutamine
- 1x B27 plus with vitamin A
- 1% penicillin/Streptomycin (P/S)
- 0.05% β-mercaptoethanol

Troubleshooting

Before start

Sterile working techniques are an absolute must to ensure cell viability and vitality. This includes, but not limited to, filtering of all media to be used with 0.22 µm filter, sterilisation of gloves, stripettes, falcons, or any materials to be in contact with cells or cell media.

All growth factors should be added fresh on the day of intended use, or within 48 hours. Prior to use media must be warmed preferentially to 37ºC, or room temperature at the very least, as these cells are temperature-sensitive.

Cells should be regularly checked under brightfield microscope for monitoring of normal growth and identification of potential contamination.

Frozen supplement in large quantity (e.g. B27, penicillin/Streptomycin) should be ideally thawed overnight in the fridge before use.

Differentiation of iPSCs into Neuronal Progenitor Cells (NPCs)

1 **Day -2: Preparing plates for replating**

Two days before intending on starting the differentiation (Day -2), add 1 mL/well in a 6-well plate of Geltrex one day prior to replating the iPSCs to begin the differentiation.

Note

Geltrex should be prepared in DMEM/F12 basal medium based on manufacturer's dilution instructions and should be kept cold at all times.

Cells are typically replated the day before beginning the differentiation.

2 **Day -1: Replating iPSCs for differentiation**

Replating iPSCs for differentiation is identical to described in **Protocol: Expansion and maintenance of human induced pluripotent stem cells (iPSCs)**, however, includes a cell counting step.

2.1 ***Prepare for splitting***

Follow steps described in **steps 6 and 7** of **Protocol: Expansion and maintenance of human induced pluripotent stem cells (iPSCs)**.

2.2 ***Prepare for cell counting***

Prepare 99 µl of Phosphate Buffered Saline (PBS) into one Eppendorf per cell line for cell dilution.

2.3 ***Replate iPSCs***

As described in **step 7** of **Protocol: Expansion and maintenance of human induced pluripotent stem cells (iPSCs)**, pausing when cell pellet is suspended in 1 mL of mTesR media (i.e. mTesR plus their accompanying Supplement and 1% Penicilline/Streptomycin) + ROCKi (1:1000) to count cells.

2.4 ***Count cells (manually using a haemocytometer)***

2.4.1. Dilute cells by adding 1 µL of cell suspension to 99 µL of previously prepared PBS in an Eppendorf.

2.4.2. Mix thoroughly.

2.4.3. Take 10 µL of diluted cell mixture and add to a haemocytometer.

2.4.4. Using a microscope, focus on the grid lines of the hemocytometer with a 10X objective.

2.4.5. Manually count cells from all 4 all 4 sets of 16 corners of the haemocytometer using a tally counter.

2.4.6. Average cell count from each of the sets of 16 corner squares and multiply by 10,000 (10^4).

2.4.7. Multiply by 100 to correct for the dilution in **step 2.4.1.**
2.4.8. Calculate and plate cells based on the following optimal density for Day -1 plating: 1.2 millions cells per 6 well (125 000/$cm^{2)}$.

Note

Cells could be plated at 1.5 million cells / well of 6 well plate for IPSC cell lines that usually proliferate at a slower rate than others by observation. This allows similar cell confluence for synchronise date of differentiation commencement.

2.4.9. Transfer 1.2 -1.5 millions cells / well to a 6-well plate after aspirating the Matrigel, and top up to have 2 mL media total.

Differentiation of iPSCs into Medium Spiny Neurons (MSNs)

3 Before starting, check the confluency. The iPSCs should be at least >75% confluent to start, otherwise feed the cells and wait another day. If in doubt, more confluent is better.

Thaw growth factors at room temperature and make every media fresh daily and filter immediately before use.

3.1 ***Day -1: Prepare differentiation media***
3.1.1. Thaw supplements (B27, N2, L-glutamin and penicillin/streptomycin) (ideally in fridge overnight).
3.1.2. Prepare Striatal neuronal induction base medium (sNIM)(see **Materials**).

3.2 ***Day 0 - Day 3***
1. Add to sNIM base media:
200 nM LDN (1:5000)
10 µM SB (1:1000)
4 µM XAV (1:5000)

Media is changed daily, 3 mL / well of a 6-well plate.

3.3 ***Day 3: Prepare for splitting***
Add 1 mL of Geltrex to each well of a 6-well plate and leave at 4°C overnight.

Note

Throughout this protocol, Geltrex and Matrigel can be used interchangeably, whichever available.

3.4 ***Day 4: Prepare for splitting***

3.4.1. Pre-warm spinning falcons containing 9 mL of KO DMEM basal medium.

3.4.2. Prepare Day 4 (D4) media (see **Materials**).

3.4.3. Thaw 1 mL per well of a 6-well plate and allow it to reach room temperature.

3.5 ***Day 4: 1:2 splitting of Neuronal Progenitor Cells (NPCs)***

3.5.1. Add 3 µL of 10 µM ROCKi directly into each well of the 6-well plate containing cells and incubate at 37ºC for 1 hour**.**

3.5.2. Aspirate media and wash each well with 1 mL of PBS**.**

3.5.3. Immediately aspirate PBS and add 1 mL of Accutase**.**

3.5.4. Incubate at 37ºC for 5 minutes**.**

3.5.5. Gently collect cells using a 1000 µL pipette and place in pre-warmed spinning falcon**.**

3.5.6. Spin cells for 5 minutes at 350g**.**

3.5.7. While cells are spinning, aspirate Geltrex and replace with 2 mL of pre-warmed D4 media + 10 µM ROCKi (1:1000)**.**

3.5.8. Aspirate media from pelleted cells, re-suspend pellet in 1 mL of D4 media + 10 µM ROCKi (1:1000) and add dropwise to each well of 6-well plate.

3.5.9. Gently swirl to distribute cells evenly around dish.

3.6 ***Day 5 - Day 8: Daily full media change***

1. Add to sNIM base media:
100 nM LDN (1:10000)
10 µM SB (1:1000)
4 µM XAV (1:5000)

Note

Full media changes are 3 mL / well of a 6 well plate.
Half media changes are 1.5 mL / well of a 6 well plate.

3.7 ***Day 8: 1:2 splitting of NPCs***

3.7.1. Prepare Day 8 (D8) media (see **Materials**).

3.7.2. Split cells by single-cell passaging as described in **step 7** of **Protocol: Expansion and maintenance of human induced pluripotent stem cells (iPSCs)**, but use D8 media instead of iMM media.

Note

Cultures might appear stickier at this stage and hence, incubation with Accutase for longer than 5 mins might be required. Periodically check the cells to avoid unnecessary long incubation with Accutase.

3.8 ***Day 9 - Day 11: Daily full media change***

1. Add to sNIM base media:
100 nM LDN (1:10000)
4 µM XAV (1:5000)

3.9 ***Day 12 - Day 15: Daily full media change***

1. Add to sNIM base media:
100 nM LDN (1:10000)
4 µM XAV (1:5000)
25 ng/ml Activin A (1:1000)

3.10 ***Day 16: Freezing Day 16 MSN precursors***

3.10.1. Passage cells as described in **step 3.5.3 to 3.5.6**

3.10.2. Resuspend pellet in freezing media Cryostore 10.

3.10.3. Store in cryovial in liquid nitrogen.

Note

Wells of cells can be frozen and thawed at a ratio of 1 well: 1 ml of freezing medium, or less, depending on plans of future use. Freezing at the density of less than 2 millions cells / ml of freezing media is not recommended due to low viability upon thawing.

To plan accordingly for experiments, 1 well of 6 well plate will give roughly 5-10 million cells/ well, but can vary greatly for each line and differentiation.

In the event of Cryostore 10 unavailability, cells can be frozen in the self-made freezing media containing 90% Day 16 media and 10% DMSO.

Importantly, resuspension of cell pellets in the freezing media (be it Cryostore 10 or self-made) should be minimal to enhance cell viability upon thawing.

Maturation of MSN precursors into functionally active post-mitotic MSNs

4 **Thawing of Day 16 MSN precursors**

Note

This is the final replating step and hence, cells are deposited in the final plate format relevant for immediate downstream applications.

4.1 ***Prepare plates for replating***

4.1.1. Add 10 or 100 µg/mL of Poly-D-lysine onto plastic wells and glass coverslips, respectively and incubate at 37ºC overnight.

Note

These glass coverslips should have been sterilised in 70% ethanol for at least 1 hours and air dried completely in a tissue culture hood.

4.1.2. Wash plenty with PBS, at least 3 times.

4.1.3. Add Matrigel and incubate at 37ºC for at least 1 hour.

4.2 ***Prepare media for thawing and replating***

4.2.1. Pre-warm spinning falcons containing 9 mL of Neurobasal.

4.2.2. Prepare D16 media (see **Materials**) + ROCKi (1:1000) and allow it to reach room temperature.

4.3 ***Thawing and replating of Day 16 MSN precursors***

4.3.1. Thaw cryovial containing Day 16 MSN precursors in water bath until only a small component remains frozen.

4.3.2. Carefully transfer contents of cryovial to pre-warmed spinning tubes.

4.3.3. Centrifuge at 350g for 5 min.

4.3.4. While spinning, aspirate Matrigel and replace with D16 media + 10 µM ROCKi.

4.3.5. Aspirate media from cell pellet in spinning falcon and replace with D16 media + 10 µM ROCKi, slowly and gently resuspending the pellet.

4.3.6. Transfer an appropriate amount of cell suspension into previously prepared wells and swirl plate gently in a figure 8 motion.

5 **Further differentiation and maturation of MSN precursors**

Maturation media from this stage onwards is adopted and modified from the Synaptojuice™ recipe reported in **Telezhkin *et. al.*, 2016**.

5.1 ***Day 16: Full media change***

Add 3 mL of D16 media + 10 µM ROCKi (1:1000) to each well.

5.2 ***Day 18: Half media change***

1. Add to sMM1 base media:
100 ng/ml BDNF (1:10000)
200 µM Ascorbic acid (1:1000)
10 µM DAPT (1:10000)
2 µM PD0332991 (1:500)
0.6 mM $CaCl_2$ (1:1000)
1 µM LM22A4 (1:1000)
200 nM cAMP (1:500)
3 µM CHIR (1:3333)
300 µM GABA (1:1000)
25 ng/ml Activin A (1:1000)
200 nM AraC (1:10000)

5.3 ***Day 20: Half media change***

1. Add to sMM1 base media:
100 ng/ml BDNF (1:10000)
200 µM Ascorbic acid (1:1000)
10 µM DAPT (1:10000)
2 µM PD0332991 (1:500)
0.6 mM $CaCl_2$ (1:1000)
1 µM LM22A4 (1:1000)
200 nM cAMP (1:500)
3 µM CHIR (1:3333)
300 µM GABA (1:1000)
25 ng/ml Activin A (1:1000)

5.4 ***Day 22 : Half media change***

1. Add to sMM1 base media:
100 ng/ml BDNF (1:10000)
200 µM Ascorbic acid (1:1000)
10 µM DAPT (1:10000)
2 µM PD0332991 (1:500)
0.6 mM $CaCl_2$ (1:1000)
1 µM LM22A4 (1:1000)
200 nM cAMP (1:500)
3 µM CHIR (1:3333)
300 µM GABA (1:1000)
25 ng/ml Activin A (1:1000)

5.5 ***Day 24: Full media change***

1. Add to sMM2 base media:
100 ng/ml BDNF (1:10000)
200 µM Ascorbic acid (1:1000)
2 µM PD0332991 (1:500)

0.3 mM $CaCl_2$ (1:2000)
1 µM LM22A4 (1:1000)
3 µM CHIR (1:3333)

5.6 ***Day 26: Half media change***

1. Add to sMM2 base media:
100 ng/ml BDNF (1:10000)
200 µM Ascorbic acid (1:1000)
2 µM PD0332991 (1:500)
0.3 mM $CaCl_2$ (1:2000)
1 µM LM22A4 (1:1000)
3 µM CHIR (1:3333)

From now on media change is spaced out 3 times a week at half feed till relevant experimental timepoints.