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Glia-Free Cortical Neuronal Feeding Schedule - Synapse Formation

In 1 collection

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ABSTRACT

Protocol for isolating rat cortical astrocytes and producing astrocyte-conditioned media for synaptogenesis assays.

MATERIALS

Penicillin-Streptomycin (10,000 U/mL) (Gibco, 15140148)

Sodium Pyruvate (GIBCO 11360)

Neurobasal plus (Gibco, A35029-01)

B27 plus (Gibco, A35828-01)

Forskolin (5 mM; 1000X)
Add 12ml DMSO to 50 mg of Forskolin (Sigma, F6886).
Make 20 ul aliquots; store -20˚C.

BDNF – (Peprotech, 450-02). Master stock should be prepared at 1mg/ml in 0.2%BSA DPBS and is stored at -80˚C as 100ul aliquots.
Preparation of 1000x Working Stocks
Thaw on ice one master stock aliquot (100ul) of BDNF. Cool down on ice 2ml of filtered 0.2% BSA (Sigma A-8806) in DPBS solution. Add BDNF master stock aliquot to the BSA solution. Mix well but gently to avoiding foaming. Aliquot in 0.5ml tubes

20ul each, concentration 50ug/ml (1000X). Flash freeze the aliquots in liquid nitrogen and store at -80C. Working concentration: 50ng/ml, final concentration on neurons 25ng/ml**.**

CNTF – (Peprotech, 450-13). Master stock should be prepared at 1mg/ml in 0.2%BSA DPBS and is stored at -80˚C as 50ul aliquots.
Preparation of 1000x Working Stocks
Thaw on ice one master stock aliquot (100ul) of CNTF. Cool down on ice 2.5ml of filtered 0.2% BSA (Sigma A-8806) in DPBS solution. Add CNTF master stock aliquot to the BSA solution. Mix well but gently to avoiding foaming. Aliquot in 0.5ml tubes 20ul each, concentration 20ug/ml (1000X). Flash freeze the aliquots in liquid nitrogen and store at -80C. Working concentration: 20ng/ml, final concentration on neurons 10ng/ml**.**

Antibody Blocking Buffer - 150 mM NaCl, 50 mM Tris-Base, 1% BSA, 100 mM L-lysine.
In a 1L baker add 4.383g NaCl, 3.025g Tris-Base (Fisher, Cat. No: BP152-5), 5g BSA (Sigma, Cat. No:A2153) and 9.125g L-Lysine monohydrochloride (Sigma, Cat. No: L-1137). Add 350-400ml of ddH2O and mix well. Once mixed, adjust pH with HCl to 7.5. Finally, add 5ml of Sodium Azide (NaN3). Add ddH2O to final volume 500ml. Filter through 0.22µm filter and store 4C.

Mounting media:
Final Composition: 20mM Tris pH8.0, 90% Glycerol, 0.5% N-propyl gallate.
In a 50ml conical tube add: 9ml Glycerol, 1ml 200mM Tris pH8.0 and 50mg N-propyl gallate. Mix on nutator in 37C incubator overnight. Store at 4C.

PFA 16% (Electron Microscopy Sciences, 15710)
Normal Goat Serum (NGS) - (ThermoFisher 01-6201)
Triton X-100 (Roche, 11332481001)

Anti-Basson antibody(Enzo/Assay Designs, SAP7F07/VAM-PS003F)

Anti-Gephyrin antibody (Synaptic System, 147 011)

Anti-Homer1 antibody (Synaptic System, 160 011)

Anti-VGAT antibody (Synaptic Systems, 131004)

Anti-Vglut1 antibody (Millipore, AB5905)

Alexa Fluor 488 goat anti-Mouse IgG2a (H+L) (Invitrogen, A-21131)

Alexa Fluor 568 goat anti-Mouse IgG1 (H+L) (Invitrogen, A-21124)

Alexa Fluor 647 goat anti-Guinea pig IgG (H+L) (Invitrogen, A21450)

DAPI (Invitrogen, D1306)

Neuronal growth media (NGM) for feeding

1 This recipe makes 20ml of neuronal media. Make fresh per use

2 1. To a 50ml tube, add the following media components:

Reagent	Volume
Neurobasal plus	19ml
Pen/strep (100x)	200µl
Sodium Pyruvate (100x)	200µl
B27 plus (50x)	400µl

3 Sterile filter these components through a syringe filter. Place media in the incubator with cap unscrewed. Allow the media to warm and equilibrate for at least 45 minutes. (Media will have an orange color and bubbles)

4 Add growth factors right before the time to use the media.

BDNF	20µl
CNTF	20µl
Forskolin	20µl

DIV 2 - AraC feeding

5

Note

Neurons are extremely sensitive to environmental changes, so only half of the media is replaced.

6 Check neurons for normal morphology under a microscope.

7 Prepare neuronal media with Neurobasal plus + B27 plus according to recipe fresh + add AraC (1:10000). Let equilibrate for at least 45 min in the incubator with the cap unscrewed.

8 Remove 230µl of old neuronal media.

9 Add 250µl of fresh media.

10 Store in the incubator

DIV 3 - remove AraC from media

11 Check neurons for normal morphology under a microscope.

12 Prepare neuronal media with Neurobasal plus + B27 plus according to recipe fresh. Let equilibrate for at least 45 min in the incubator with the cap unscrewed.

13 Remove 230µl of old neuronal media.

14 Add 250µl of fresh media.

15 Store in the incubator.

DIV 6 - Media feeding

16 Check neurons for normal morphology under microscope.

17 Prepare neuronal media with Neurobasal plus + B27 according to recipe fresh. Let equilibrate for at least 45 min in the incubator with the cap unscrewed.

18 Remove 230µl of old neuronal media.

19 Add 250µl of fresh media.

20 Store in the incubator.

DIV 8 - ACM feeding

21 Check neurons for normal morphology under a microscope.

22 Prepare neuronal media with Neurobasal plus + B27 according to recipe fresh. Let equilibrate for at least 45 min in the incubator with the cap unscrewed.

23 Place the NGM on ice to cool down.

24 Thaw the ACM on ice.

25 Add AGM to the NGM for a final concentration of 50ug/ml for excitatory synapses and 100ug/ml for inhibitory synapses.

26 Equilibrate and warm up the NGM with ACM in the incubator (37C, 10% CO2) before adding it to the cells.

27 Remove 230µl of old neuronal media.

28 Add 250µl of fresh media.

29 Store in the incubator.

DIV 11 - ACM feeding

30 Check neurons for normal morphology under a microscope.

31 Prepare neuronal media with Neurobasal plus + B27 according to recipe fresh. Let equilibrate for at least 45 min in the incubator with the cap unscrewed.

32 Place the NGM on ice to cool down.

33 Thaw the ACM on ice.

34 Add AGM to the NGM for a final concentration of 50ug/ml for excitatory synapses and 100ug/ml for inhibitory synapses.

35 Equilibrate and warm up the NGM with ACM in the incubator (37C, 10% CO2) before adding it to the cells.

36 Remove 230µl of old neuronal media.

37 Add 250µl of fresh media.

38 Store in the incubator.

DIV 13 - Neuron culture fixation and staining

39 The following solutions should be prepared:
a) 4% PFA in PBS – In a 50ml conical tube add one 16% PFA ampule (10ml), 26ml of ddH2O and 4ml of 10X PBS. Mix and place at 37˚C to warm up. For storing keep away from the light at 4C.

b) Blocking and permeabilization solution (0.2% Triton) - This recipe makes 5ml of blocking solution, sufficient for 1 24-well plate.
• 2.4ml of antibody-blocking buffer
• 2.5ml of Normal Goat Serum (NGS)
• 100ul of 10% Triton.

c) Primary antibodies solution: This recipe makes for 5ml, sufficient for 1 24-well plate.
• 4500µl Antibody blocking buffer
• 500µl Normal Goat Serum (NGS)
• 10ul of anti-Bassoon antibody (presynaptic marker)
• 10ul of anti-Homer1 antibody (for excitatory synapses) or anti-Gephyrin antibody (for inhibitory synapses)
• 5ul of anti-Vglu1 antibody (for excitatory synapses) or anti-VGAT antibody (for inhibitory synapses)

40 In a chemical fume hood, aspirate media from cells and immediately add 500µl of warm 4% PFA to each well.

41 Fix the neurons for 7 minutes at room temperature.

42 Wash 3 times with PBS.

43 Add 200ul of blocking solution to each well and block for 30min at room temperature.

44 Remove the blocking solution and add 200µl of primary antibody solution to each well. Incubate at 4˚C overnight.

DIV 14 - Secondary antibody staining and mounting

45 a) Secondary antibodies solution: This recipe makes for 5ml, sufficient for 1 24-well plate.

- 4500µl Antibody blocking buffer
- 500µl Normal Goat Serum (NGS)
- 10ul of secondary antibody Alexa Fluor anti-Mouse IgG2a 488
- 10ul of secondary antibody Alexa Fluor anti-Rabbit IgG1 564
- 10ul of secondary antibody Alexa Fluor anti-Guinea Pig IgG 647
- 0.5ul DAPI (1/10000)

46 Remove the primary antibody and wash 3 times with PBS

47 Add 200µl of secondary antibody solution to each well and incubate at room temperature for 2h, keeping it protected from light.

48 Wash 3 times with PBS.

49 Mount coverslips onto slides using one small drop of mounting media for each coverslip.

50 Seal with nail polish and dry at room temp for at least 30min before storing at 4˚C.