Stimulation with EphB-Fc induces spine maturation in cultured rat hippocampal neurons

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Method Article

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Abstract

Introduction

Spine maturation is reflected by decreased spine length and increased size of spine heads leading to a mushroom-like appearance. The following protocol is used to study the involvement of ephrinB reverse signaling in this process. Hippocampal neurons from embryonic rat \(E18-19) were isolated and cultured for 14 days. In order to visualize the protrusion the neurons were transfected with Yellow Fluorescent Protein \(YFP) 2 days prior to stimulation \(12DIV) using Calcium Phosphate method. Activation of ephrinB ligands by soluble EphB receptor ectodomains fused to Fc portions of human IgG \(EphB2-Fc) promoted spine maturation in these neurons \(14DIV). Spine maturation was analyzed by comparing the average spine length and spine head area of stimulated neurons to that of Fc-stimulated control neurons.

Reagents

Coverslips \(CVS) \(Ø 13 mm): Poly-D-lysine-hydrobromide \(Sigma Aldrich) Borate-Buffer: 3.1mgml-1 Boric acid; 4.75 mg/ml Borax in 1l H_2O, pH 8.5 Laminin \(natural mouse laminin; Invitrogen #23017-015) Sterile PBS Sterile H_2O Isolation: Dissection Medium: HBSS 500ml \(Invitrogen #24020-091) ; 5 ml Penecillin/Streptomycin \(PAA #P11-026); 5 ml L-Glutamine \(PAA #M11-004); 5 ml 1M $MgCl_2$; 3.5 ml Hepes \[pH 7.2] \(Invitrogen) Trypsin: 0.25% Trypsin/1M EDTA 100 ml \(Invitrogen #25200-056); 0.7 ml Hepes \[pH 7.2] Serum Medium \(SM): D-MEM \(Invitrogen #61965-026); 10% Horse serum Cell culture: NB medium: Neurobasal Medium \(Gibco, Invirtogen #21103-049); 1/50 B27 supplement \(Gibco, Invitrogen); 0.5 mM L-Glutamine \(PAA #M11-004) Calcium Phosphate Transfection: 1M $CaCl_2$ 2x BES saline \(2x BBS) \[pH 7.26] HBSS \(Invitrogen #14025-050) Stimulation: Human Fc Fragment \(2.2 µg/µl) \(Dianova) Goat anti-human Fc \(1.8 µg/µl) \(Dianova) EphB2-Fc \(0.2 µg/µl) \(recombinant mouse Eph receptor/Fc chimera; R&D) Ephrinb2-Fc \(0.2 µg/µl) \(recombinatnt mouse ephrinb2/Fc chimera; R&D) PFA 4% \(4% sucrose) 50mM NH_4Cl chilled Gel/Mount anti-fading medium \(Biomeda)

Equipment

Dissection tools CO_2 Incubator \(37 ^oC; 5.0% CO_2) Epifluorescence microscope Metamorph Culture dishes \(24-well plates) Vortex

Procedure

A) CVS Treatment: CVS are treated with nitric acid o/n, briefly washed with H_2O \(5x + 4x 30 min) and spread separately on Whatmann paper to dry. Dry CVS are baked in the oven \(165 ^oC; o/n) **B) Coating:** 1. Place CVS into 24-well plates \(sterile) 2. Coat CVS with 400µl/well poly-D-lysine in Borate-Buffer \(1mg/ml; sterile filtered) for > 5 hrs into the incubator. 3. wash CVS 3x with H_2O and let them dry in the hood 4. coat with 5µg/ml laminin in PBS for > 2 hrs into the incubator \(400µl/well) 5. wash 3x

with PBS, replace it with NB medium \(400µl/well) and put it into the incubator **C) Isolation of Hippocampal Neurons \(adapted from Jenny Lauterbach - ref 1):** 1. put 4ml of SM and 4ml of NB medium each in a dish to pre-warm it in the incubator 2. pre-warm trypsin at 37 ^{o}C in the water bath 3. take E18-19 embryos from rat, cut off the heads and collect them in a dish with chilled dissection medium \(DM) 4. carefully open the skull, remove the brains and transfer them into a new dish with chilled DM 5. separate the 2 cortices from the rest of the brain, remove their meninges and cut out each hippocampus; collect hippocampi in a 15ml tube filled with 10ml DM on ice 6. remove DM, replace it with 1-2ml warmed trypsin and incubate 20 min at 37 ^{o}C in the water bath 7. flame Pasteur pipette to decrease the diameter of the tip, take off the trypsin and wash hippocampi 2x with 1ml warm SM 8. add 1.5ml SM and homogenize tissue with the fire-polished Pasteur pipette by pipetting up and down 20-30x 9. centrifuge 5 min at 80g \(650rpm) 10. remove supernatant, resuspend neurons in 1-3ml pre-warmed NB medium using the fire-polished Pasteur pipette 11. count cells, and plate them at designated cell density \(e.g. high density 25,000 cells/cm^2) on prepared CVS **D) Calcium Phosphate Transfection \(general protocol)** Mix for 4 wells \(24-well plate): 900µl fresh NB medium 100µl Transfection Mix containing: \(37.5 µl H_2O – DNA \(4-7µg) + 12.5 µl 1M $CaCl_2$ + 50 µl BBS) 1. mix H_2O with $CaCl_2$ 2. add DNA, mix by slowly pipetting 3. add BBS to DNA-H_2O-$CaCl_2$-mix 4. mix with 900 µl warm NB medium by vortexing 5. take away the old medium from the cells and quickly apply 250µl of the transfection-medium mix to each of the 4 wells. 6. incubate for 1.5-2.5 hrs 7. warm up HBSS for washing 8. wash cells with pre-warmed HBSS until most of the precipitate is removed 9. add 300 µl of NB medium per well to the cells 10. stimulate 2 days later **E) Stimulation/Fixation:** 1. cluster EphB- Fc, ephrinb-Fc or Fc one hour prior stimulation at RT: Mix EphB/ephrin 4 µg/ml + 1/20 anti-hFc \(0.2 µg/ml) e.g.: 300µl medium/well: take 6 µl EphB2/ephrinB2 + 0.325 µl anti-hFc \(1/10) 2. stimulate the cells with clustered EphB2, ephrinB2 or Fc by adding the appropriate amount \(4µg/ml) into the wells for 8 hrs in the incubator 3. put cells on ice to stop reaction 4. remove the medium and fix the cells by adding 200 µl/well 4% PFA \(4% sucrose) for 12 min on ice 5. wash 2x with cold PBS 6. remove PBS and add 200µl/well cold NH4Cl for 10 min on ice 7. wash 2x with cold PBS 8. wash 1x with RT PBS for 5 min 9. wash with H_2O 10. drop of H_2O and mount CVS using Gel/Mount anti-fading medium \(Biomeda) onto a glass slide 11. Take pictures using an epifluorescence microscope 12. Analyze changes in spine length and spine head size using MetaMorph software

Timing

Isolation of hippocampal neurons \(2-3 hrs) Cultivation time 14 days Ca-PO4-transfection: 40 min + 2 hrs incubation Stimulation: 1hour clustering + 8 hrs incubation

References

1. Lauterbach, J. & Klein, R. Release of full-length EphB2 receptors from hippocampal neurons to cocultured glial cells. _J Neuroscience_ **26**:11575-81 \(2006)

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