Dendritic aggregation of the postsynaptic cell adhesion molecule NGL-3 by pre-clustered antibodies to induce postsynaptic protein clustering

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Method Article

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Abstract

Introduction

Direct aggregation of synaptogenic cell adhesion molecules on the plasma membrane of dendrites and axons can induce the secondary clustering of pre- and postsynaptic proteins at sites of aggregation. For instance, direct clustering of neurexin-1 on the surface of axons by pre-clustered antibodies induces the recruitment of presynaptic vesicle markers including synapsin I. In this experiment, cell adhesion molecules are usually epitope-tagged in the extracellular region to provide a site for antibody recognition. Alternatively, antibodies raised against the extracellular region can be used for target protein clustering. This assay is similar to direct aggregation of cell adhesion molecules by antibody coated-beads. However, this method appears to give final immunofluorescence signals with lower background at least in our experimental conditions. We employed this assay to demonstrate that direct aggregation of NGL-3, a postsynaptic cell adhesion molecule, on dendrites induces the clustering of excitatory postsynaptic proteins including PSD-95 and NMDA receptors.

Reagents

An expression construct carrying N-terminally EGFP-tagged NGL-3 \(EGFP-NGL-3). Cultured hippocampal neurons. EGFP antibodies. FITC-conjugated secondary antibodies directed against the Fc region of EGFP antibodies. Antibodies against postsynaptic marker proteins. Cy3-conjugated secondary antibodies.

Procedure

1. Transfect cultured hippocampal neurons at days _in vitro_ \(DIV 14) with EGFP-NGL-3, followed by incubation of the neurons for 2 more days. 2. For antibody pre-clustering, mix 1 µg of EGFP antibodies and 0.1 µg of FITC-conjugated secondary antibodies \(ratio of 10:1) in 500 µl complete neurobasal media \(CNB) and incubate the mixture for 2 hrs at 4 °C. 3. Transfer the transfected neurons \(DIV 16) to a 12-well plate containing ~400 µl of prewarmed CNB in each well, and add 100 µl of the preclustered antibody solution to make the final antibody concentration of 400 ng/ml. Save the conditioned culture media for step 5. 4. Incubate the neurons for 1 hr at 37 °C. 5. Wash the neurons three times with pre-warmed PBS, and transfer the coverslip with neurons back to the conditioned culture media. 6. After 24 hr incubation, fix and immunostain the neurons with primary antibodies against various postsynaptic proteins, followed by the incubation with Cy3-conjugated secondary antibodies. To boost the signal for EGFP-NGL-3 clusters, add FITC-conjugated secondary antibodies during the incubation of Cy3 secondary antibodies. 7. Capture Z-stacked images of the stained neurons by confocal microscopy. 8. For the analysis of the captured images, trace the boundaries of each NGL-3 clusters on dendrites. Quantify the percentage of NGL-3 clusters that are positive for postsynaptic marker proteins. In addition, quantify the average immunofluorescence intensities of coclustered postsynaptic proteins and normalize them to those in nearby dendrites.

References

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