Detection of NMDARs antibodies in encephalitis

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Summary/Abstract

There are a range of diseases of the brain that are associated with rapidly developing inflammation. These conditions are called encephalitis, and can be caused by infections, or autoimmune disorders when the subject's immune system reacts against host proteins. The most common form of autoimmune encephalitis is associated with antibodies against the NR1 subunit of the N-Methyl-D-Aspartate Receptor (anti-NMDAR-encephalitis). Antibody detection is crucial for diagnosis, and can be performed only with techniques that preserve the full tertiary structure (conformation) of the antigen. Here we describe the methods commonly used to detect NMDAR-antibodies with immunohistochemistry on rat brain slices and on live HEK cells transfected with components of the NMDAR.

Key Words: NMDAR, encephalitis, autoantibody

1 Introduction

N-Methyl-D-Aspartate receptors (NMDARs) are neuronal transmembrane ion channels implicated in several key functions of the central nervous system such as neuronal plasticity.[1] The structure of the channel on the surface of the neuron is hetero-tetrameric, composed of 2 GluN1 (also called NR1) subunits that express the binding site for glycine, and 2 GluN2 (or NR2) subunits that express the binding site for the excitatory neurotransmitter glutamate.

Antibodies directed against the NR1 subunit are the hallmark and probably the cause of “anti-NMDAR encephalitis”, a disease characterized by subacute onset (days to weeks) of psychiatric features, hypoventilation, movement disorders and seizures.[2] Techniques that rely on antibody linearization such as the enzyme-linked immunosorbent assay (ELISA) are inadequate for NMDAR-antibody detection, probably because the antibodies bind to the antigen only tertiary structure is preserved. To overcome this problem the most common technique employed is the cell-based assay (CBA). This commonly uses human embryonic kidney cancer (HEK 293) cells that are transiently transfected with plasmids encoding the NR1 and NR2B NMDAR subunits in order to obtain expression on the surface plasma membrane. In our laboratory, this test is usually performed on live cells, that allow antibodies to bind to extracellular regions of the fully-conformational membrane protein, and are not complicated by nonspecific binding to intracellular components.[3]. Other laboratories obtain largely similar results where cells are fixed prior to the staining procedure [4], and there is a commercial assay that pre-fixes the transfected cells for distribution. Unfortunately, no systematic multi-center studies of patients with clinically-defined NMDAR-antibody encephalitis has yet been performed and the true sensitivities and specificities of the different tests are unclear.

However, a useful and more generic approach that can indicate the presence of an NMDAR or related antibody is indirect immunohistochemistry (IHC) on thin sections of rodent (usually rat) brain. This procedure has been widely performed in the past to detect antibodies against intracellular neuronal components associated with neurological paraneoplastic syndromes.[5] For those antibodies, the heavy fixation often used markedly reduces the sensitivity of the assay for surface antigens such as

NMDAR. For this reason, a protocol for immunohistochemistry on rat brain slices optimized for surface antigens has been proposed.[4]

Here we provide details of the identification of NMDAR-antibodies combining these two complementary techniques [4].. IHC is considered the most sensitive method, especially when CSF is used [4], but since the antigenic repertoire of the rat brain is not limited to NMDAR, confirmation using an antigen-specific technique such as the CBA is essential to confirm the specific antigenic target.

2 Materials

2.1 Immunohistochemistry on rat brain slices

1. *Trizma buffered saline (TBS)*

   For 1 liter of TBS: Mix 12 g of Trizma base powder (Sigma-Aldrich, St. Louis, Missouri, U.S) and 9 g of sodium chloride, then add H2O to a final volume of 1 L. Correct pH to 7.5.

2. *4% PFA-TBS*

   Dissolve 4g of paraformaldehyde in 100 ml of TBS. Heat under constant stirring to 60°, then let cool down to room temperature and correct pH to 7.4. Store at 4° for several weeks.

3. *Hematoxylin solution*

   Dilute *hematoxylin solution (*Sigma-Aldrich, St. Louis, Missouri, U.S) 1:5 or 1:10 with H2O, according to the desired level of counter staining. Store protected from light at RT.

4. *Other reagents*

Goat anti-Human IgG biotinylated antibody. Avidin-Biotin kit (ABC). Vecta stain 3,3'-diamenobenzydine (DAB) kit (all from Vector labs, Burlingame, California, U.S.) (see note 1).

2.2 Live cell CBA

2.2.1 Cells and cell culture reagents

1. *Poly-L-Lysine (PLL) 0.01%*

   Prepare a 1% PLL solution (Sigma-Aldrich, St. Louis, Missouri, U.S) with sterilised H2O, and store in 5 ml aliquots at -20° for later use. Defrost and dilute 5 ml aliquots in 500 ml of sterilised H2O. Filter sterilise before use. The unfrozen aliquots can be stored at 4° for several weeks.

2. *Phosphate Buffered Saline (PBS)*

   Dissolve 5 PBS tablets (Sigma-Aldrich, St. Louis, Missouri, U.S.) in 1 liter of sterile H2O

3. *Trypsin*

   Prepare aliquots of 1-5 ml of trypsin (Sigma-Aldrich, St. Louis, Missouri, U.S) and store at -20° for later use. When needed, defrost and prepare a 1:10 dilution of trypsin-PBS. This can be stored at 4° for several weeks.

4. *Complete Culture medium*

   Dulbecco's Modified Eagle's Medium with high glucose (DMEM containing 4500 mg/L of glucose, L-glutamine, sodium pyruvate and sodium carbonate) plus 10% foetal bovine serum and antibiotics/antimycotics solution 1:100 (penicillin, streptomycin and amphotericin B; Life technologies, Carlsbad, California, U.S.).

2.2.2 Transfection reagents

1. *Polyethylenimine (PEI)*

   Dissolve polyethylenimine (Polyscience, Warrington, Pennsylvania, U.S.) in sterile H2O heated to 80° (final concentration 1mg/ml). Stir until dissolved completely, let cool to room temperature and adjust pH to 7. Stock in 0.2-1 ml aliquots at -80° for later use.

2.2.3 Staining reagents

1. *4% paraformaldehyde (PFA)-PBS*

   Dissolve 4g of paraformaldehyde in 100 ml of sterilised PBS. Heat under constant stirring to 60°, then let cool down to room temperature and correct pH to 7.4. Store at 4° for several weeks.

2. 20 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) in DMEM

   Dissolve 2.38 g of HEPES in 500 ml of DMEM.

3. *Blocking medium*

   DMEM-HEPES plus 1% Bovine Serum Albumin (BSA).

4. *Other reagents*

   Goat anti-Human IgG (Fc specific, Sigma-Aldrich, St. Louis, Missouri, U.S). Fluorescent donkey anti-goat (Alexafluor, 568). Fluorescent mounting medium with 4',6-Diamidino-2-Phenylindole (DAPI).

3 Methods

3.1 Immunohistochemistry on rat brain slices

Freezing the brain

1. Sacrifice Sprague-Dawley rat with CO2, and extract the brain without perfusing the animal.

2. Chop the brain sagitally in half, and immerse in cold 4% PFA for 1 hour.
3. Transfer the brain to 40% Sucrose-TBS solution and store at 4° until the brain starts sinking (usually 48 hours).
4. Remove the brain from the sucrose and suck out the excess sucrose to prevent the formation of air bubbles when freezing.
5. Embed in OCT until the half brain is covered, and let rest for 15 minutes
6. Dip the embedded brain in chilled isopentane until frozen
7. Store at -80° for later use.

Obtaining rat brain slices

1. Prepare the cryostat chamber for slicing (usually temperature between -21° and -25° are ideal).
2. Remove the brain from the -80° freezer and put inside the cryostat. Wait for at least 30 minutes for the brain to reach the same temperature as the chamber.
3. Slice at 7 micron thickness (see note 2).
4. Let the slides dry overnight at room temperature or 2 hours at 37°.
5. Use slides immediately, or store at -20° (see note 3).

Staining (see note 4)

1. If slides are frozen, allow at least 20-30 minutes at room temperature for them to defrost.
2. Draw a line around each slice with a dako-pen.
3. Rehydrate slices with TBS for 1 minute.
4. Incubate for 15 minutes with cold 0.3% H2O2-TBS to quench residual peroxidase activity.
5. Wash 3 times with TBS.
6. Block for 1 hour with TBS-10% normal goat serum (NGS).

7. Without washing, tap off blocking solution and incubate with patient's IgG in TBS-5% NGS (sera 1:200 dilution, CSF 1:2 dilution) overnight at 4°.
8. Wash 3 times in TBS.
9. Incubate for 2 hours with secondary biotinylated anti-human IgG 1:1000 in TBS-%NGS at room temperature.
10. Wash 3 times in TBS
11. Incubate for 1 hour with ABC complex (must be prepared at least 30 minutes before use).
12. Wash 3 times in TBS
13. Develop chromogenic reaction with Vectastain kit for 6-7 minutes (this step should be performed under the fume hood since DAB is carcinogenic).
14. (Optional) counterstain for 10-15 seconds with hematoxylin solution (see note 5).
15. Dehydrate the slides in increasing EtOH concentration and xylene (EtOH 50% for 5 minutes, EtOH 70% for 5 minutes, EtOH 100% for 5 minutes, EtOH 100% for 5 minutes, xylene I for 5 minutes, xylene II for 5 minutes) (This step should be perfomed under the fume hood since Xylene is carcinogenic).
16. Mount slides with DPX mounting medium (Sigma-Aldrich, St. Louis, Missouri, U.S), and air dry under a fume hood.
17. Evaluate under a light microscope. NMDAR antibodies should provide a specific staining of the neuropil of the hippocampus (see figure 1).

3.2 Live cell based Assay

All the procedures involving cells should be performed under a laminar flow hood in a tissue culture facility. It is not essential to perform the staining procedure under the hood.

To maintain sterility perform all steps under a laminar flow hood until the incubation with patient sera.

Coating coverslips

1. Put circular glass coverslips (13 mm) in the wells of a six well plate (3 or 4 coverslips/well can be handled easily).
2. Cover and submerge the coverslips with 3 ml of PLL solution per well (make sure no coverslips are floating on the surface of the liquid).
3. After one hour at room temperature aspirate the PLL solution.
4. Leave the 6-well plates open under sterile conditions until completely dry (usually about 1-2 hours). Then close and store at RTfor later use (PLL-plates can be stored for several weeks).

Cell culture preparation and seeding

1. Grow HEK293T cells in culture medium in a T175 flask until 90-100% confluent.
2. Detach cells from flask using trypsin. Count cells and seed approximately 450,000-550,000 cells into each well in complete culture medium (total volume 2 ml/well; seeding concentration may have to be corrected according to cell growth rate).
3. 4 hours after seeding, push down floating coverslips and move overlapping coverslips with a sterile pipette-tip.
4. After 36 hours, make sure that cells are at least 90-95% confluent (see note 6).

5. Before proceeding with the transfection procedure, replace complete culture medium in all wells.

Transfection

1. Prepare a mixture of the reagents necessary for transfection following this order:
    a. cDNA, total amount of 3 ug/well (NR1: 2.1 ug/well; NR2B: 0.7 ug/well; Enhanced Green Fluorescent Protein (EGFP): 0.2 ug/well)(see note 7).
    b. DMEM (50 uL/well)
    c. PEI (1.5 ug/well)
2. Mix well, and let rest for at least 10 minutes protected form light (both EGFP and PEI are photosensitive).
3. Add the mixture to the wells and incubate for 8-10 hours (see note 8)
4. Replace the complete culture medium from the wells with complete culture medium plus 500 uM ketamine, to prevent glutamate toxicity.
5. Let the cells incubate for 24 hours for optimal surface expression.
6.

Staining

1. Invert one of the coverslips with transfected cells face down onto a microscope slide onto a drop of fluorescent mounting medium. Evaluate under a fluorescence microscope the presence of an adequate number of cells and of green cells expressing EGFP.
2. Prepare samples (serum diluted 1:20 in blocking medium, CSF 1:2 or undiluted) directly in the wells of a 24-well plate (total volume 250 uL/well)

3. Transfer the coverslips with transfected cells into the 24 well-plates (with the cells facing up) and incubate for 1 hour at room temperature protected from light.
4. Aspirate primary antibody and wash 3 times with DMEM-HEPES.
5. Incubate the coverslips with 4% PFA in PBS for 5 minutes at RT.
6. Wash 3 times with DMEM-HEPES
7. Incubate with goat anti-human IgG (Fc specific) 1:750 in blocking medium for 1 hour at room temperature.
8. Wash 3 times with DMEM-HEPES
9. Incubate for 45 minutes with donkey anti-goat fluorescent secondary antibody 1:750 in blocking medium.
10. Wash 3 times with DMEM-HEPES and once with PBS.
11. Invert the coverslips with cells face down onto one drop of anti-fade mounting medium per coverslip on microscope slides with (maximum 6 coverslips per slide).
12. Protect from the light and air-dry for 15-20 minutes (to avoid damaging the microscope lenses with wet mounting medium).
13. Evaluate under a florescent microscope. The presence of NMDAR antibodies will be revealed by a thin rim of fluorescent staining on the surface of the cells (see figure 2).

4 Notes

1. The reagents indicated are used in our laboratory for reproducibility. Any chromogenic method using DAB is suitable, if performed adequately.

2. Since the blade has to cut through materials with different consistency, the slicing can be particularly challenging. Often, the slices can show several artifacts such as folds and bubbles when they are picked up. The following tips could help in the procedure, but it must be considered that slicing with the cryostat requires experience and optimal settings must be set in each laboratory:
    a. Start cutting at 20 micron thickness, and progressively reduce the thickness as you become more experienced
    b. Reducing the specimen temperature helps in keeping the slices flat, but a specimen that is too cold can present cutting artifacts
    c. Using warm slides (37°) can help to pick up the rat brain slices quickly, and avoid air bubbles.
3. Best results are always obtained with freshly prepared tissue, but for practical reason long term storage is often needed. A reduction in staining intensity after several weeks of storage may occur.
4. In every step of the staining slices must be fully covered by buffer. Usually 120-150 uL/slice are sufficient to cover the whole brain without overfilling.
5. Hematoxylin counterstaining is optional. Conditions must be optimized in each laboratory, but our suggestion is to perform a very light counterstaining for optimal evaluation.
6. Since NR1/NR2B transfection is harmful to the cells because binding of glutamate to the NMDAR leads to excessive influx of calcium that can be toxic and some cells will die after transfection. Healthy and confluent cells at this stage are crucial to perform a reliable staining.

7. Even though pathogenic antibodies target the NR1 subunit, in the assay optimized in our laboratory a combination of NR1 and NR2B was necessary to obtain good surface expression of the channel. The cells are also transfected with EGFP to check the efficiency of the transfection before the staining procedure.
8. We usually perform this procedure overnight, even though shorter time is probably sufficient for an efficient transfection. Medium has to be replaced in any case before 12-16 hours to avoid excessive glutamate toxicity.

5 References

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Figure Legend

Figure 1: Immunohistochemistry on rat brain slices

Indirect immunohistochemistry using peroxidase labelled goat anti-human IgG as the secondary antibody on sagittal rat brain slices with hematoxylin counterstaining. Hippocampal staining is visible with patient serum NMDAR antibody (A, black arrow) but not with serum from a healthy individual (B). Detail of the hippocampus (C,D and E). In the Cornus ammonis the staining pattern involves the oriens layer and the radiatum layer, sparing the pyramidal layer; a thin rim of stronger staining is seen adjacent to the stratum laconosum molecularis (D, black arrows). In the dentate gyrus, the staining is visible in the molecular layer with sparing of the granular and polymorph layer and the hilus; a thin rim of stronger staining is seen adjacent to the pyramidal layer (E, black arrows). The serum was diluted 1:100 but CSF can be used undiluted if sufficient is available and gives a very clean and specific result.

Figure2: Live cell based assay

The figure shows the serum of a patient with anti-NMDAR-encephalitis (A-B) and the serum from a healthy individual (C-D) tested on the live cell based assay for NMDAR antibodies. The rim of red staining around the cell represents the binding of the NMDAR antibodies on the cell surface revealed with a fluorescent-labelled secondary antibody (Alexa fluor 568, A and B, white arrows). The red staining is absent with the negative sample (C-D). EGFP has been co-transfected with the NR1 and NR2B subunit plasmids to demonstrate good transfection, but does not

necessarily co-localize with the NMDAR antibodies. Sera were diluted 1:20, but CSF undiluted or 1:1 can be used. Magnification 40X.