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Characterization of oligophrenin-1, a RhoGAP lost in patients affected with mental retardation. Lentiviral injection in organotypic brain slice cultures

Nael Nadif Kasri¹, Eve-Ellen Govek², and Linda Van Aelst¹

¹Cold Spring Harbor Laboratory 1 Bungtown Road Cold Spring Harbor New York, 11724, USA
Tel: + 516 367 8449 Fax: + 516 367 8815

²The Rockefeller University Laboratory of Developmental Neurobiology 1230 York Avenue
Rockefeller Research Building New York New York, 10021, USA Tel: + 212 327 7211 Fax: + 212 327 7145

Abstract

Mutations in regulators and effectors of the Rho GTPases underlie various forms of mental retardation (MR). Among them, *oligophrenin-1* (*OPHN1*), which encodes a Rho-GTPase activating protein (Rho-GAP), was one of the first Rho-linked MR gene identified. Upon characterization of *OPHN1* in hippocampal brain slices, we obtained evidence for the requirement of *OPHN1* in dendritic spine morphogenesis and synaptic function of CA1 pyramidal neurons. Organotypic hippocampal brain slice cultures are commonly used as a model system to investigate the morphology and synaptic function of neurons, mainly because they allow for the long-term examination of neurons in a preparation where the gross cellular architecture of the hippocampus is retained. In addition, the maintenance of the tri-synaptic circuitry in hippocampal slices enables the study of synaptic connections. Today, a multitude of gene transfer methods for postmitotic neurons in brain slices are available to easily manipulate and scrutinize the involvement of signaling molecules, such as Rho GTPases, in specific cellular processes in this system. This chapter covers techniques detailing the preparation and culturing of organotypic hippocampal brain slices, as well as the production and injection of lentivirus into brain slices.

Introduction: Rho GTPases, synaptic structure and function

Dendritic spines are highly specialized, actin rich structures that protrude from dendrites, and serve as the post-synaptic compartment for the majority of excitatory synapses (Hering and Sheng, 2001). Spine morphology is ultimately linked to synaptic function that underlies cognitive functions, such as learning and memory. In accordance with this idea, pathological studies have revealed dendritic spine abnormalities in patients with mental retardation (MR) (Fiala *et al.*, 2002). Recent progress in the field of MR suggests that defects in such crucial cellular processes as spine morphogenesis, synaptogenesis and synaptic plasticity contribute to cognitive impairment resulting from mutations in MR-related genes (Newey *et al.*, 2005; van Galen and Ramakers, 2005).

Small GTP-binding proteins of the Rho subfamily, and in particular Rac1, Cdc42 and RhoA GTPases, have emerged as key modulators of dendritic spine morphogenesis through their regulation of the actin cytoskeleton (Govek *et al.*, 2005). Hence, it is not surprising that mutations in genes encoding regulators and effectors of the Rho GTPases have been found to underlie human neurological diseases. To date, several Rho GTPase-linked genes associated with MR have been identified (Newey *et al.*, 2005), including *OPHN1* (Bienvenu *et al.*, 1997; Billuart *et al.*, 1998; Govek *et al.*, 2004), *PAK3* (Allen *et al.*, 1998; Bienvenu *et*

al., 2000; Boda *et al.*, 2004; Gedeon *et al.*, 2003; Meng *et al.*, 2005) and *ARHGEF6* (Kutsche *et al.*, 2000; Node-Langlois *et al.*, 2006). We embarked on the functional characterization of *OPHN1*. *OPHN1* was first found mutated in patients with nonsyndromic MR (Bienvenu, *et al.*, 1997; Billuart, *et al.*, 1998), but the presence of *OPHN1* mutations has been documented more recently in families with syndromic forms of MR (Bergmann *et al.*, 2003; des Portes *et al.*, 2004; Philip *et al.*, 2003; Zanni *et al.*, 2005). To better understand how mutations in *OPHN1* result in defects in neuronal signaling, we took advantage of the organotypic hippocampal slice culture system.

The hippocampus is a structure central to learning and memory. Previous studies have demonstrated experience- and learning-dependent structural changes in dendrites and spines of hippocampal neurons in mammalian brain (Yuste and Bonhoeffer, 2001). The highly organized and laminar arrangement of synaptic pathways, from the entorhinal cortex to the dentate gyrus (DG), DG to CA3, and CA3 to CA1, makes the hippocampus a convenient model for studying synaptic connections. Identifiable neurons (e.g. CA1 pyramidal cells) in hippocampal slices are amenable to specific genetic manipulations, including RNAi and overexpression, and neighboring, untransfected neurons with comparable connectivity, developmental history, and exposure to culture conditions in the same slice can serve as ideal control neurons.

Preparation of organotypic brain slice cultures

Several methods have been developed for the preparation of organotypic brain slice cultures. Here we describe the interface culturing technique introduced originally by Stoppini *et al.* (Stoppini *et al.*, 1991), in which the slices are placed on Millicell® inserts so that the top of the slice is exposed to the incubator atmosphere (35 °C and 5 % CO₂), while the bottom of the slice contacts the culture medium.

Preparation of media

1. Place an insert (Millicell-CM 0.4 µm culture plate insert, 30 mm diameter, Millipore, Cat# PICM03050) in a culture dish (Corning, 35 mm diameter, Cat# 430165).
2. Add 750 µl of filter sterilized (using 0.22 µm disposable filter, Millipore Cat# SCGPU02RE) long-term slice culture medium (MEM hanks medium, 1 mM L-glutamine, 1 mM CaCl₂, 2 mM MgSO₄, 1 mg/l insulin, 1 mM NaHCO₃, 20 % heat inactivated horse serum, 0.5 mM L-ascorbate, 30 mM Hepes) under the insert. Make sure that the insert is completely wet on the bottom, avoiding any air bubbles.
3. Place 2 ml of long-term slice medium in a second cell culture dish. This will be used for the transfer of the sliced hippocampi.
4. Place both cell culture dishes in a cell culture incubator at 35 °C/ 5 % CO₂ until the hippocampi are ready.

Preparation for dissection and slicing

1. Prepare the tissue chopper (Stoelting Cat# 51425). Tape a square piece of Teflon to the chopping platform. Insert a clean razor blade and align it with the chopping platform. Clean the chopping platform and blade with 70 % ethanol.
2. Clean the dissection tools, including large scissors, small 3 1/2" straight micro-dissecting scissors (such as Biomedical Research Instruments, Cat# 11-2000), straight forceps, a straight, tapered spatula for removing the brain from the skull, a

flat, spoon-shaped spatula for handling the whole brain, curved spatulae (such as Fine Science Tools, Cat# 10092-12), scalpels or dissecting chisels (such as Fine Science Tools, Cat# 10095-12), and wide-bore pipettes.

3. Prepare dissection buffer (low Na^+ Artificial Cerebral Spinal Fluid (ACSF): 1 mM CaCl_2 , 5 mM MgCl_2 , 4 mM KCl , 26 mM NaHCO_3 , 8 % sucrose, 0.5 % phenol red). Filter sterilize and oxygenate with 95 % O_2 / 5 % CO_2 gas mixture in a beaker chilled on salted ice. To make salted ice, simply add a large scoop of NaCl to a bucket of ice and mix the salt into the ice. The buffer will be ready when the color changes from purple to orange.
4. Prepare a cell culture dish (Corning, 60 mm diameter well, Cat#. 430166) with a Whatman filter (Whatman 42.5 mm Cat# 1003-42) in the lid, and cover it with cold dissection buffer. Keep it on ice until ready to use.

Removing and dissecting the hippocampus

1. Wipe the head of a postnatal day (P)6-P8 rat pup with 70 % ethanol.
2. Using a large pair of scissors, collect the head of the animal.
3. Cut the skin down the midline, from the neck up to and between the eyes. Insert the scissors into the foramen magnum and cut the skull along the midline up to and between the eyes. Make four small incisions perpendicular to the main cut at the ends of the main incision. Peel the skull back with straight forceps, remove the brain with a straight, tapered spatula, and place it immediately in a small beaker with chilled dissection buffer.
4. Place the brain in the culture dish lid containing the chilled dissection medium with a Whatman filter (from step 4 under *Preparation for dissection and slicing*). Keep the brain covered with chilled dissection medium during the rest of the dissection.
5. Using a scalpel, remove the cerebellum by making a coronal cut just behind the inferior colliculi. Make a second cut sagittally down the midline, completely through the brain. Separate the hemispheres. Turn the hemibrain so that the medial surface is facing up. Separate the neocortex with the underlying hippocampus from the midbrain and brainstem. The hippocampus will now be exposed. Using a curved spatula, disrupt the connection of the hippocampus on the ventral side (fimbria). Rotate the hippocampus gently with the curved spatula around the longitudinal axis of the hippocampus to separate it from the neocortex.
6. Repeat this procedure to isolate the second hippocampus.
7. Remove the hippocampi from the dish with a wide-bore pipette and place them on the chopping block.
8. Position the hippocampi, smooth side up, using a pipette filled with dissection medium to push the hippocampi into the correct position. Place them so that the long axis of each hippocampus is parallel to the direction of stage movement, but allow the septal end to curve away from the parallel axis.
9. Remove the excess of liquid. It is important to achieve the right amount of wetness. If the tissue is too wet, the hippocampi tend to move each time the blade is lifted, whereas if the tissue is too dry, the hippocampi tend to stick to the blade.
10. Position the stage of the tissue chopper to the edge of the hippocampi. To make 400 μm slices, raise the blade, adjust the micrometer by 400 μm , and drop the blade. Continue to raise the blade and adjust the micrometer by 400 μm before each drop of the blade until you are done cutting.

11. Transfer the slices from the chopping platform into a tissue culture plate with pre-warmed medium (from step 3 under *Preparation of media*), using a wide-bore pipette.
12. Separate the slices under a dissecting microscope, either by gently agitating the dish or using a curved spatula and forceps. Be careful not to poke or tear the slices.
13. Select slices with an intact structure that display distinct CA1, CA3 and DG cell layers. Typically, you will obtain 12 to 15 slices for each preparation.
14. Place 3 to 4 slices per Millicell-CM insert in a well with pre-warmed long-term slice medium (from steps 1 and 2 under *Preparation of media*). Keep slices distant from each other, but relatively centered in the middle of the membrane. Gently remove excess medium from the top of the insert, as medium on top of the insert will prevent oxygen exchange.
15. Place the cell culture dishes in a cell culture incubator (35 °C and 5 % CO₂).

When medium is changed every 3 days, the slices will continue to survive for several weeks in culture, forming layer-specific connections. It is important to assess viability of the organotypic slices before proceeding with the experiment. Healthy slices should look transparent and the DG should be visible by eye. Viable slices are most easily obtained from young animals. We usually use P6 to P8 rat pups.

The quality of organotypic brain slice cultures will strongly depend on the isolation and dissection procedure described above. Time is an important parameter. Typically, the actual dissection should not take longer than 10 min for obtaining healthy slices. Although time is an important parameter, it is more important to do the dissection well than fast. During the procedure, it is essential to treat the slices gently, and avoid touching them with any sharp objects. Slices that are treated too harshly tend to become epileptic and will die prematurely.

Lentivirus preparation and injection

A multitude of gene transfer methods for postmitotic neurons are now available, including different viral vectors (Ehrenguber *et al.*, 2001) to make adenovirus, adeno-associated virus, Sindbis virus (Ehrenguber *et al.*, 1999), Semliki Forest virus, measles virus and lentivirus (Zufferey *et al.*, 1997). Each viral vector has its own specificity and advantages (Ehrenguber *et al.*, 2001). Here we describe the use of lentiviral vectors to transduce genes into CA1 pyramidal cells in hippocampal brain slices, primarily for long-term expression studies.

Loss of function by RNAi and overexpression are two convenient methods to study the involvement of a Rho GTPase, or a related molecule, in a specific cellular process. For the production of shRNAs in hippocampal neurons in organotypic slices, we have successfully used the TRIPΔU3-EF1α-EGFP (pTRIP) vector, a replication-defective self-inactivating lentiviral vector (Gimeno *et al.*, 2004; Stove *et al.*, 2005; Zennou *et al.*, 2000). Use of this vector resulted in a very efficient knockdown of OPHN1. Details concerning the pTRIP vector and how to clone the shRNA expression cassette are described in Janas *et al.* (Janas *et al.*, 2006). To ectopically express OPHN1, we successfully used a lentiviral backbone, based on a self-inactivating FUGW vector (described previously) (Lois *et al.*, 2002). The FUGW vector was modified by introducing an internal ribosomal entry site (IRES), followed by the green fluorescent protein (GFP) cDNA, to create the vector FUIGW that contains an IRES-GFP cassette downstream of the ubiquitin-C promoter. The full-length cDNA of *OPHN1* was then cloned into the BamHI and EcoRI sites. This lentiviral vector allowed us to ectopically express full-length OPHN1, driven by an ubiquitin-C promoter, while co-expressing GFP in order to visualize the infected neurons (Fig. 1).

Lentivirus production and concentration

The pTRIP and FUIGW vectors were pseudotyped with VSV-G encoded by pMD.G (Dull *et al.*, 1998). Gag, Pol, and Tat were expressed from the packaging construct pCMVΔR8.91 (Zufferey *et al.*, 1997).

1. Plate 2 to 3 X 10⁶ HEK 293T cells in a 10 cm tissue culture dish with 10 ml of medium 24 h before transfection. Cells should be approximately 60–80% confluent at the time of transfection.
2. Prepare 1 ml of calcium phosphate–DNA precipitate for the transfection of one 10 cm plate of HEK 293T cells as follows:
 - a. Combine 7 µg of pTRIP or FUIGW vector, 10 µg of pCMVΔR8.91, and 6 µg of pMD.G (VSVG) in a sterile microcentrifuge tube. Note: For optimal results, the relative amounts of plasmids used for cotransfection should be determined empirically.
 - b. Adjust the volume to 250 µl with 0.1x TE (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA).
 - c. Add 250 µl of 2x CaCl₂ solution (0.5 M CaCl₂, 1 mM Tris-Base, 0.1 mM EDTA) and mix by vortexing.
 - d. Place 500 µl of 2x HEPES buffered saline (HBS) (0.05 M HEPES-NaOH, pH 7.12, 0.28 M NaCl, 1.5 mM sodium phosphate buffer, pH 7.0) in a sterile tube (e.g., 2065 Falcon tube).
 - e. Add the Ca²⁺-DNA solution drop-wise to 500 µl of 2x HBS while vortexing slowly. Let the suspension stand for 10 min to allow precipitate formation.

Note: 2x HBS should be stored at 4 °C and can be kept for up to 2 weeks. Aliquots of 2x CaCl₂ solution and 0.5 M HEPES-NaOH stock should be stored frozen at –20 °C. All solutions should be thawed at room temperature before use.

3. Distribute the precipitate evenly over the cells in the culture dish. Mix gently by rocking the dish back and forth, and return the plates to the incubator.
4. Replace medium 5–8 h after transfection with 10 ml of fresh culture medium.
5. Collect the medium containing viral particles 48–60 h after transfection.
6. Centrifuge freshly collected supernatant at 2000 g to remove cell debris.
7. Filter the supernatant through a 0.45 µm filtration unit. The filter should be prerinsed with cell culture medium before use.
8. Aliquot and store at –80 °C or proceed to the concentration step.

For infecting neurons in brain slices, further concentration of the supernatant is required.

Concentration

1. Transfer the filtered supernatant containing the virus in an Ultra-Clear tube for an SW28 Rotor (Beckman Cat# 344058).
2. Centrifuge at 120,000 g for 3 h at 4 °C.
3. Remove the supernatant and resuspend the viral pellet in an appropriate volume of Hanks balanced salt solution without calcium (Ca²⁺) and magnesium (Mg²⁺) (HBSS) (Gibco/Invitrogen Cat# 14185-052) or suitable culture medium.

4. Store the tube containing the concentrated viral suspension at 4 °C for 12 h to ensure complete pellet resuspension. Aliquot and store at –80 °C.

Injection

Infection with lentivirus is performed as soon as possible after the preparation of the brain slices, since the efficiency of infection will decrease with time.

1. Prepare a glass pipette pulled to an outer diameter of 5–15 µm (Premium standard wall borosilicate capillaries with filament, 2.00 mm OD, Warner Instruments, cat. no. 64-0796).
2. Add 0.3 µl of a 1 % fast green solution (Sigma, Cat# F7252) to 2.5 µl of concentrated virus for visualization during the injection.
3. Fill the glass pipette with the viral solution.
4. Position the glass pipette through a microelectrode holder (WPI Cat# MPH420) on a micromanipulator (World Precision Instruments, Cat# M3301R).
5. Connect the microelectrode holder to a controlled air pressure system, a Picospritzer (General Valve, Fairfield, NJ).
6. Under a stereomicroscope (Zeiss, Stemi 2000), lower the glass pipette into the desired cell layer (e.g. CA1 pyramidal cells), just under the surface membrane. The longitudinal axis of the glass pipette should be almost parallel with the dendritic projections of the pyramidal cells.
7. Inject virus using the Picospritzer. For each position, one or multiple injections of short duration (20 ms) can be performed, using controlled air pressure (10–20 psi). By varying the pulse duration and pressure on the Picospritzer, the multiplicity of infection can be adjusted from very few to 10–50 infected cells. Alternatively, a sharpened PCR micropipette with plunger (Drummond Cat# 5-00-1001-X10) can be used to manually inject the virus into the slice.

Typically, 2–3 injection sites are selected per slice, and a total of 0.5–1 µl of concentrated virus solution is applied. When injecting the virus, a rapid diffusion of the fast green dye should be observed between the cells.

8. A few hours (5–6h) after the injection, change the culture medium to prevent toxicity from the fast green dye.

Using the lentiviral based system described above, one can expect a very high efficiency of infection that is restricted to the area of injection (Fig. 2). Typically, transgene expression increases slowly with lentivirus. We observed the initial onset of GFP expression 4 days after infection. At this time point, the fluorescence intensity is still low, but rapidly increases at 6–8 days post-infection and last for many days. Lentiviral based infection is therefore ideal for long-term gene expression.

Note that the amount of infection (dependent on injection duration, pressure and number of injection sites) mainly depends on the purpose of the experiment. For electrophysiological experiments, 1 or 2 injection sites per slice are sufficient. In contrast, for biochemistry experiments, slices should receive multiple viral injections, spaced 100 to 150 µm apart, spanning the area of interest (e.g. CA1 area). These multiple injections and spacing ensure high infection efficiency. The infected regions can then be dissected under a fluorescence magnifying scope.

Conclusions

Lentiviral injection can be used reliably to infect postmitotic neurons in intact brain tissue. Once the specific parameters of the infection (titer, volume, pulse duration) are determined, this method is efficient, reproducible, and does not require advanced molecular biological facilities for its application. Significantly, the use of lentiviral vectors to express hairpins or recombinant proteins allows for gene manipulation in specific neurons (e.g. CA1 pyramidal cells) in an environment of normal background tissue at a particular time point during development, thus circumventing many of the problems of interpreting data from transgenic and knockout animals. The preserved tissue architecture in slices also facilitates the analysis of defined hippocampal cells, synapses and connections. Applications are numerous and include electrophysiology, morphology, biochemistry, pharmacology and development.

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Figure 1. Schematic representation of the FUIGW vector

The FUIGW vector was adapted from the previously described FUGW vector (Lois, et al., 2002). The woodchuck hepatitis virus posttranscriptional regulatory element (WRE) serves to increase the level of transcription and the human immunodeficiency virus-1 (HIV-1) flap element, between the 5' long terminal repeat (LTR) and the human ubiquitin-C promoter, serves to increase the titer of the virus. For visualization of the infected cells, an IRES-GFP cassette was introduced downstream of the ubiquitin-C promoter. Full-length cDNA of OPHN1 was inserted between the ubiquitin-C promoter and the IRES-GFP cassette, into the unique restriction sites BamHI and EcoRI.

Figure 2. Lentiviral mediated expression of GFP using the pTRIP lentiviral vector

A) Organotypic brains slice prepared from a postnatal day 6 rat was infected in the CA1 region with a GFP expressing lentivirus (pTRIP). The image was taken 7 days post infection. **B)** Higher magnification two-photon microscope image of CA1 pyramidal cells shows a clear fluorescent signal in the cell body and apical dendrites. Abbreviations used: CA1-CA3, Cornu Ammonis; DG, Dentate Gyrus.