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Targeted single-neuron infection with rabies virus for transneuronal multisynaptic tracing

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1. Introduction

Neurotropic viral tracers have been successfully used to reveal connectivity of neural circuits (Callaway, 2008). Among these, the rabies virus has become a major contributor (Callaway, 2008; Ugolini, 2010). While doing little or no damage to the host cell, the virus infects only neurons, spreads exclusively in the retrograde direction, and crosses neurons only at synapses (Kelly and Strick, 2000; Ugolini, 1995). For high-resolution analysis of transsynaptic transport, sensitive immunolabeling or imaging viruses that express fluorescent proteins has been performed.

For transsynaptic tracing, rabies virus is injected either intramuscularly or directly into the central nervous system where it is taken up by many neurons. Infection and labeling of higher-order, presynaptic neurons occurs at specific time points (Kelly and Strick, 2000), which is determined by the time it takes the virus to replicate and cross synapses. Recently, the use of a deletion-mutant rabies virus (Marshall et al., 2010; Rancz et al., 2011; Wickersham et al., 2007a; Wickersham et al., 2007b) restricted the spread of virus to monosynaptic connections specifically revealing exclusively first-order, presynaptic neurons.

Here, we report on a novel approach that used an unpseudotyped, fully replication-competent form of rabies to initially infect only a single neuron-of-choice, allowing one to dissect neuronal circuits on a single-cell level. Our method has a high success-rate and was

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relatively easy to implement. Labeled connectivity patterns monitored over a relatively long time are shown for both cultured cells and brain slices.

2. Materials and Methods

2.1. Rabies virus construction

A replication-competent form of rabies virus was genetically modified to express GFP. The cDNA encoding the rabies virus (RABV) vaccine strain SPBN (containing the glycoprotein of the strain CVS-N2c) has been described previously (Tan et al., 2007). The coding region of eGFP was PCR amplified with primers RP63-FP+(TTT *CGTACGATGGTGAGCAAG*, BsiWI italics) and RP64-GFP-(CCC *GCTAGCTTACTTGTACAGCTCGTCC*, BsiWI italics) and cloned into the *BsiWI* and *NheI* sites of cSPBN-N2c. The resulting plasmid was designated cSPBN-N2c-GFP and the recombinant RABV SPBN-N2c-GFP was recovered and grown as described previously (Gomme et al., 2011; Wirblich and Schnell, 2011).

2.2. Primary cell culture

Cortical cultures were prepared from embryonic day 16 Sprague – Dawley rats as previously described (Aizenman et al., 2000). Briefly, cortices were dissociated and plated onto poly-L-lysine-treated gridded coverslips (for easier identification of cells; Bellco Glass, USA) in a growth medium composed of 80 % Dulbecco's modified Eagle's medium, 10 % Ham's F12-nutrients, and 10 % bovine calf serum (heat-inactivated and iron-supplemented) with 25 mMHEPES, 24 U/ml penicillin, 24 mg/ml streptomycin, and 2 mM L-glutamine. Glial cell proliferation was inhibited after 2 weeks in culture with 1–2 mMcytosine arabinoside, at which time the culture medium was reduced to 2 % serum without F12-nutrients. The medium was partially replaced three times a week.

2.3. Organotypic brain slice culture

Cortical slices were obtained from C57BL/6 mice (postnatal day 2–8) and cultured according to a modified version of a published protocol (De Simoni and Yu, 2006). Briefly, each slice was laid on low-height cell culture insert (0.4 μ m-pore, PICMORG50, Millipore, MA, USA) that had been placed in a Petri dish filled with 1.0 ml culture medium. Culture medium consists of 50 % minimal essential medium with GlutamaxTM-I (42360-032, Gibco, CA, USA), 25 % horse serum (26050-070, Gibco, CA, USA), 24 % Earle's balanced salt solution (24010-068, Gibco, CA, USA), 1 % penicillin (5000 U/ml)–streptomycin (5000 μ g/ml) (15070-063, Gibco, CA, USA), and additional D-glucose (G5767, Sigma, MO, USA) to yield a final concentration of 40 mM. Because slices were incubated for only a relatively short time (one day), they did not have sufficient time to fully attach to the inserts and tended to float up when immersed in artificial cerebrospinal fluid (ACSF). This problem was alleviated by culturing slices on membrane filter paper with large pore-size (10 μ m) (K99CP81030, GE Osmonics, MN, USA), which had been previously attached onto the inserts with superglue.

2.4. Fluorescence imaging

Images were taken with a 12-bit digital camera (Quantix, Photometrics, AZ, USA) mounted on top of the microscope (BX51WI, Olympus, PA, USA). For imaging GFP-fluorescence, samples were exposed with excitation light at 488-nm produced by a monochromator (Polychrome V, TILL Photonics, Germany) for 5–10 s, and GFP-emission light was filtered out using a standard dichroic filter. Both image acquisition and control of monochromator was done by computer software (Imaging Workbench 6.0, INDEC BioSystems, CA USA).

2.5. Visualization of virus solution

The ability to visualize the rabies virus solution inside a patch pipette was important for estimating virus location and fluid movement. Virus pipette solution consisted of ACSF (composition [in mM]: 140 NaCl, 24 D-glucose, 10 HEPES, 5 KCl, 1 MgCl₂, and 1 CaCl₂; pH=7.2) containing 10 % by volume viral stock (titer ~10⁹ plaque-forming units (pfu)/ml) of a rabies virus strain (SPBN-N2c-GFP) genetically modified to express the green fluorescent protein (GFP; see Section 2.1). The end concentration of virus in the pipette was 10⁸ pfu/ml. At 40x magnification, particles of varying sizes (< 1 μm) were observed undergoing Brownian motion inside the pipette (Fig. 1A). Because the bullet-shaped virus (0.1 μm in diameter and 0.2 μm in length) was below the optical resolution, these particles most likely consisted of aggregates of virions and/or large proteins.

Using still and time-elapsd video images, we counted particles within a specified volume and estimated the particle density to be ~10⁻⁴ particles/μm³, corresponding to a concentration of 0.2 pM, consistent with a value obtained using the viral titer. By tracking the displacements of a few particles over time, we estimated a diffusion constant of 0.7 ± 0.1 μm²/s, signifying that, in 70 s, a particle will have a root-mean-square displacement of 10 μm. Finally, by applying a small voltage to the pipette electrode, these particles were found to be electrophoretic possessing a negative charge in water at pH 7.2, which is consistent with the fact that pH 7.0 is the known value for the isoelectric point of the rabies virus (Atanasiu et al., 1979; Dietzschold et al., 1978).

2.6. Single-cell inoculation procedure

Prior to inoculation, it was necessary to perform a pipette “flush” procedure in a separate Petri dish. This flush served three purposes. First, because capillary action initially pushed the virus solution 500–1000 μm from the pipette tip, it was necessary to push the virus solution closer to the tip by increasing the pipette pressure (1–2 Torr), which was controlled by a perfusion pump (Model 2PK+, ALA Scientific Instruments, USA). Second, to keep virus solution near the tip, the pressure was set to a value which gives zero or slight inward fluid flow. Finally, any debris at the pipette tip was ejected by administering short pulses of high pressure (> 500 Torr in amplitude, ~ 100 ms in duration) generated by a second perfusion pump (Model PV820, World Precision Instruments, USA) connected in parallel to the first pump.

Once the flush procedure was complete, the pipette (1.0–1.5 MΩ corresponding to ~ 4 μm diameter tips) was brought into a dish containing the target neuron to be infected. A second pipette (“suction pipette”, tip diameter (~ 25 μm, negative pressure of 1–2 Torr), was placed 10–20 μm above the target cell and opposite to the virus pipette (Fig. 1B). The suction pipette served to remove any viral solution that had leaked from the virus pipette. The virus pipette was pushed slowly onto the cell until the seal resistance was about 1.5–2.0 times the pipette resistance. To monitor fluid movement, a particle in the virus solution close to the tip was chosen for observation. The pressure in the pipette was increased slowly until this particle began to drift at a speed of ~5 μm/s while keeping the seal resistance 10–20 % above the pipette resistance. The pipette was kept in this “loose-seal” cell-attachment mode for 30 to 50 min with periodic adjustments to the pressure and/or pipette position in order to keep the flow rate constant (as indicated by monitoring particle movement). Following inoculation, pipettes were withdrawn and cells were transferred back into the incubator.

3. Results

In our first set of experiments, we used primary cortical cell cultures (see Section 2.2) plated on gridded coverslips for easier identification of cells. GFP-fluorescence was detectable at about 16–25 hours following inoculation (Fig. 1C). In 19 out of 23 coverslips, GFP-

fluorescence appeared first in the target cell while in the remaining 4 coverslips no GFP-fluorescence was detected up to three days post-inoculation. Thus, the overall success-rate of this method was 83 % (n=23) with a 100 % selectivity for the targeted neuron.

To monitor the transsynaptic spread of infection originating from single neurons, coverslips were imaged every 2–4 hours (Fig. 1C-F). We considered a neuron as GFP-positive when its mean fluorescence was greater than 2.5 standard deviations of the background intensity. The number of GFP-positive cells increased in a stepwise manner (Fig. 1G), which was interpreted to mean that transneuronal spread of the virus to higher order-connected neurons had occurred between each step. These steps showed up as peaks in a plot of the percentage of GFP-positive cells that were newly-appearing versus time (Fig. 1H). The separation in time between 1st and 2nd order neurons could readily be made and, for these cultured neurons, the time separating these peaks was measured to be 7.6 ± 1.8 hours (mean $\pm$ SD; n=4) which was similar to the transneuronal transport times reported in in vivo tracing studies (Kelly and Strick, 2000).

In order to test our single-cell infection method in a more biologically intact system, we inoculated neurons in cortical slices that had been cultured for one day (see section 2.3). Using the same approach, we were able to successfully infect a single neuron in layer 2/3 and observed transsynaptic labeling in 60 % of slices (n=15) (see for example Fig. 2), while in the rest of the slices no GFP-fluorescence was observed even at 3 days after inoculation. Due to the high density of neurons in slices, it was not possible to unequivocally determine whether the first-infected cell was the target cell or a neighboring cell although the first-infected cell was usually within 10–20 μ m from the tip of the virus containing pipette. After 1.5 days post-inoculation, neurons labeled by transsynaptic, retrograde virus infection gave rise to a column with a diameter of approximately 100 μ m and a radial extent of 200 μ m. Both pyramidal-like (presumably excitatory) and non-pyramidal-like (presumably inhibitory) cells were labeled in this region (Fig. 2C-D), which indicated that the rabies virus crossed excitatory as well as inhibitory synapses. In addition, a small number of neurons in deep cortical layers were weakly labeled. This labeling pattern was consistent with what was observed in monosynaptic tracing using a deletion-mutant rabies virus (Marshall et al., 2010; Wickersham et al., 2007b).

4. Discussion

The method described here allowed for the initial infection of a single neuron-of-choice with an unsequenced, replication-competent form of rabies virus which was capable of multisynaptic neuronal tracing. Because infection starts from a single neuron, this method makes it possible to dissect neuronal microcircuits with a hitherto unachieved specificity. We have shown that this method is relatively easy to implement, has a high success-rate, and, by following the time-course of infection, can be used to identify higher-order connections.

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Fig. 1.

Targeted-infection of a single neuron in cortical cultures. (A) Bright-field image of rabies virus solution inside pipette. Observed particles, likely consisting of aggregates of virus and large proteins, were used to visualize fluid flow and to estimate location of rabies virus. (B) Bright-field image of cultured cortical neurons showing virus containing pipette (right) and suction pipette (left). Target neuron is shown between the pipettes after detachment of virus pipette from loose-seal cell-attachment. GFP-fluorescent images taken of a different coverslip (C, D) 22 hours, (E) 31 hours, and (F) 41 hours post-inoculation. (G) Number of GFP-positive cells as a function of time after inoculation. Red circles (left to right, respectively) indicate values at 22 hours, 31 hours, and 41 hours post-inoculation. (H) Percentage of newly appearing GFP-positive cells. Arrows (left to right, respectively) indicate bursts of new cells infected, suggesting 1st and 2nd order infections. Scale bars, 10 μm (A, D) and 50 μm (B, C, E, F).

Fig. 2. Infection of GFP-encoded rabies virus in cortical slice cultured from mouse (P4) brain. (A) Bright-field image showing pipette position at inoculation site. GFP-fluorescence of cortical slice at (B) 23 hours, (C) 35 hours, and (D) 41 hours after inoculation. Scale bars, 100 μ m. Insets: higher magnification image (scale bars, 50 μ m) of cells indicated by corresponding arrow. Orientation: pia is at the top (slice boundary marked by long dashes) and white matter is at the bottom (below short dashes).