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

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ABSTRACT

The transynaptic and retrograde spread of rabies virus make it an efficient and robust transneuronal tracer, capable of revealing connectivity patterns of multisynaptic, neuronal circuits with great detail. Current techniques begin by infecting many neurons simultaneously, from which higher-order neurons are then labeled sequentially in time. Here we report on a method that can initially infect a single neuron-of-choice, allowing for greater precision and specificity of labeled circuits.

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). To achieve high-resolution analysis of transsynaptic transport, sensitive immunolabeling or tracing with 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 (Marshel et al., 2010; Rancz et al., 2011; Wickersham et al., 2007a,b) 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 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 the green fluorescent protein (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*CGTACG*ATGGTGAGCAAG, BsiWI italics) and RP64-GFP-(CCC*GCTAGC*TTACTTGTACAGCTCGTCC, BsiWI italics) and cloned into the *Bsi*WI and *Nhe*I sites of cSPBN-N2c. The resulting plasmid was designated cSPBN-N2c-GFP and the

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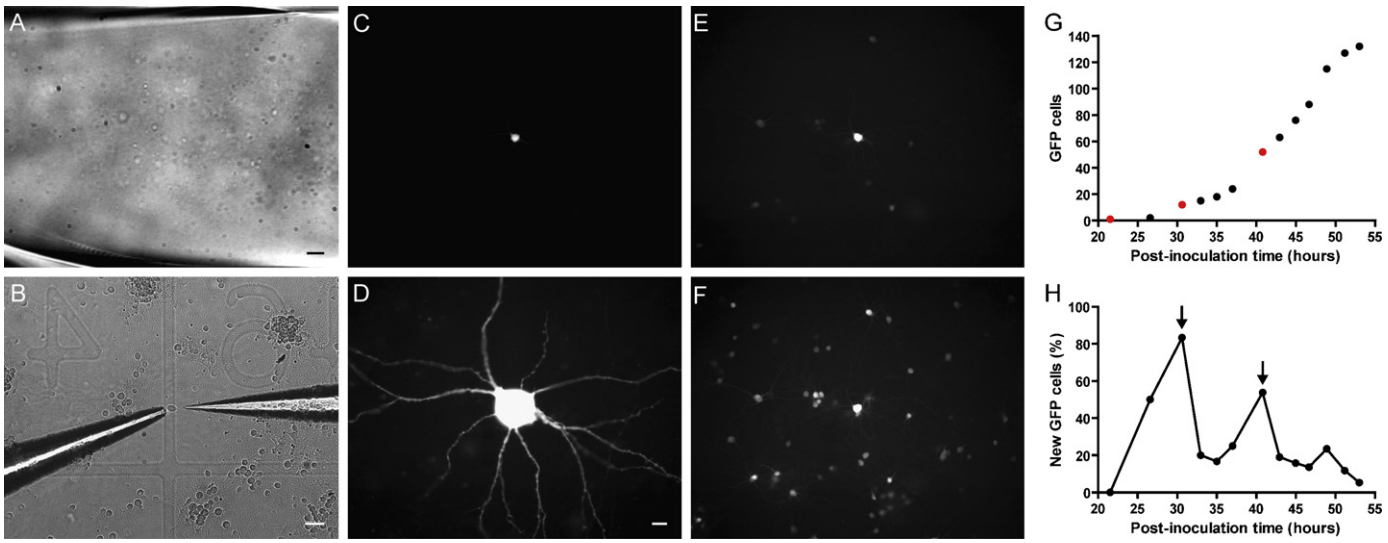

Fig. 1. Targeted-infection of a single neuron in cortical cultures. (A) Bright-field image of rabies virus solution inside pipette. Observed particles likely consisting of aggregates of virus and large proteins, were used to visualize fluid flow and to estimate location of rabies virus. (B) Bright-field 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 and D) 22 h, (E) 31 h, and (F) 41 h 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 h, 31 h, and 41 h 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 and D) and 50 μm (B, C, E and F).

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 mM HEPES, 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 mM cytosine 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 μm-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 Glutamax™-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 μg/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 μm) (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 $MgCl_2$, and 1 $CaCl_2$; pH = 7.2) containing 10% by volume viral stock (titer $\sim 10^9$ 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 10^8 pfu/ml. At 40× 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-elapsed video images, we counted particles within a specified volume and estimated the particle density to be $\sim 10^{-4}$ particles/μm^3, 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 $\mu m^2/s$, 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