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Using Homogeneous Primary Neuron Cultures to Study Fundamental Aspects of HSV-1 Latency and Reactivation

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Abstract

We describe a primary neuronal culture system suitable for molecular characterization of herpes simplex virus type 1 (HSV-1) infection, latency, and reactivation. While several alternative models are available, including infections of live animal and explanted ganglia, these are complicated by the presence of multiple cell types, including immune cells, and difficulties in manipulating the neuronal environment. The highly pure neuron culture system described here can be readily manipulated and is ideal for molecular studies that focus exclusively on the relationship between the virus and host neuron, the fundamental unit of latency. As such it allows for detailed investigations of both viral and neuronal factors involved in the establishment and maintenance of HSV-1 latency and in viral reactivation induced by defined stimuli.

Keywords

HSV-1; Latency; Reactivation; SCG neuron culture; In vitro system; Lentiviral delivery; RNA interference

1 Introduction

Despite years of investigation, our understanding of the molecular mechanisms underlying the establishment of herpes simplex virus type 1 (HSV-1) latency and reactivation remains incomplete [1, 2]. Animal models have proved invaluable for defining the roles of viral gene products and the host immune system in controlling latency, but continued progress is hampered by the involvement of host proteins essential for organismal viability, contributions from multiple cell types in addition to neurons, and the interconnected nature of physiological networks governing latency, cell homeostasis, and immune function. This complexity has made it extremely difficult to parse out the precise molecular signals that prevent or induce reactivation within the host neuron itself, the fundamental unit of the virus–host interaction. As revealed by a number of recent studies, neurons impose a significant degree of control over viral activity in the absence of other cell types [1, 3, 4]. To fully tease out these neuron-autonomous signals, we have refined a simple primary neuronal culture system [4] that is based on pioneering studies by Christine Wilcox, Eugene Johnson, and colleagues in the late 1980s [5, 6]. For the most part we use sympathetic neurons isolated from prenatal rat superior cervical ganglia (SCGs), but the general protocol

2. It is possible to reuse collagen to coat plates (maximum of five times), but generally coating with fresh collagen gives better adherence.

can be adapted to neurons isolated from other ganglia including those of sensory nerves [5, 7, 8]. Although sensory neurons are the most frequent site of latency in humans, HSV-1 latency is clearly documented in sympathetic neurons [9, 10], and the SCG is preferable for in vitro use because it yields extremely pure cultures of nerve growth factor (NGF)-responsive neurons capable of establishing latency with wild-type viruses at high efficiency. Access to cultures free of other cell types, especially CD8+ T cells, is of great benefit when focusing on the interplay between the virus and host neuron. All of the key hallmarks of HSV-1 latency as defined in live animal models are recapitulated with SCG cultures, most notably the absence of infectious virus particles, abundant expression of the latency-associated transcript (LAT), and ability of viruses to reactivate in response to defined stresses or disruption of important signaling pathways. From the experimental standpoint, the system is especially appealing because it can be easily manipulated using molecular genetic techniques such as RNA interference or gene delivery via adenoviral or lentiviral vectors as well as by treatment with a wide range of pharmacological reagents including compounds that are too toxic or difficult to administer for effective use in live animals. The purity and accessibility of the system allow for targeted studies of neuron-autonomous signaling as well as in-depth exploration of viral gene functions. Experiments using pure neuron cultures require a shorter turnaround time than with live animal models (as short as 15 days compared to 30 or more days) and can be monitored in real time using genetically modified HSV-1 expressing fluorescent proteins.

In this chapter, we describe how to isolate and culture primary SCG neurons, establish HSV-1 latency, induce viral reactivation, and, lastly, use lentiviral vectors to deliver functional RNAs or recombinant proteins into neurons that are already latently infected. In just the last few years, studies using this highly versatile system have provided valuable insight into the molecular basis of latency control and reactivation, and it is our hope that the system will be used and embellished by others to explore other equally important aspects of the HSV-1 life cycle such as the role of innate defense mechanisms, neuronal architecture, and participation of other cell types.

2 Materials

2.1 Dissecting SCGs from E21 Rat Embryos

1. Timed pregnant rats at embryonic day 21.
2. CO_2 supply and CO_2 chamber.
3. 15-cm petri dish.
4. Hemostats/forceps.
5. Dissection scissors, surgical grade.
6. Laminar flow hood.
7. Dissection microscope.
8. Light source.
9. Dissection tray: A styrofoam block covered with aluminum foil.

10. 23 G (1) syringe needles.
11. Straight-edge dissection forceps.
12. 70 % ethanol for sterilization.
13. Kimwipes.
14. 15-mL conical tube.
15. L-15 medium: 500 mL of Leibovitz's L-15 medium with L-glutamine supplemented with 5 mL of 40 % D-glucose. Filter sterilize. Store at 4 °C.

2.2 Dissociating, Seeding, and Culturing SCG Neurons onto 96- or 24-Well Plates

1. 96- or 24-well culture plates.
2. Rat tail collagen type I: To prepare a working stock solution, dilute in sterile H_2O to a final concentration of 0.66 mg/mL. Store at 4 °C.
3. Laminin from Engelbreth–Holm–Swarm murine sarcoma basement membrane: For a working stock solution, dilute in sterile H_2O to a final concentration of 2 μg/mL.
4. 2.5 % Trypsin (10×) without EDTA or phenol red.
5. C-medium: Into 500 mL of minimum essential medium (MEM) with Earle's salt and L-glutamine, add 5.5 mL of 40 % D-glucose, 50 mL of FBS, and 5.5 mL of 200 mM L-glutamine. Filter to sterilize, and store at 4 °C.
6. NGF: Prepare a 50 μg/mL stock solution by dissolving 1 mg NGF in 20 mL sterile 1× PBS. Store at −80 °C.
7. 5 mL syringes.
8. 21 G (1½) syringe needle.
9. 23 G (1) syringe needle.
10. 70 μm nylon filter cell strainer.
11. Hemocytometer.
12. Trypan blue solution.
13. NBM: Into 500 mL neural basal medium, add 5.2 mL of 40 % D-glucose, 5.2 mL of L-glutamine, and 10 mL of B-27 supplement. Filter sterilize, and store at 4 °C.
14. 5-fluorouracil (FU): To prepare a 20 mM stock solution, dissolve 24.4 mg uridine and 24.6 mg 5-fluoridine in 10 mL MEM. Store at −20 °C.
15. Aphidicolin: To prepare a 10 mM stock solution, dissolve 1 mg aphidicolin in 300 μL DMSO. Store at −20 °C.

2.3 Establishment and Reactivation of HSV-1 in SCG Neurons

1. Acyclovir: For a 31 mM stock solution, dissolve 25 mg acyclovir in 3.58 mL DMSO. Store at −20 °C.

2. HSV-1 virus stock: Experiments described below used HSV-1 GFP-Us11 (Patton strain).
3. NBM, *see* Subheading 2.2.
4. NGF, *see* Subheading 2.2.
5. LY294002: Resuspend 5 mg LY294002 in 1.62 mL DMSO for a 10 mM stock solution and store at −20 °C in the dark.
6. For RT-qPCR: RNeasy Mini Kit, DNase I, SuperScript III Reverse Transcriptase, FastStart Universal SYBR Green Master (Rox). Primer sets optimized for the detection of HSV-1 ICP27, UL5, and UL36 mRNAs in the context of the rat neuronal transcriptome [4, 11] are as follows:

   ICP27 FW 5′-TTTCTCCAGTGCTACCTGAAGG-3′.

   ICP27 RV 5′-TCAACTCGCAGACACGACTCG-3′.

   UL5 FW 5′-ACGTCGAGCTGTTGTTCGTCCA-3′.

   UL5 RV 5′-GGCGAGCGTGCGTTTGATTT-3′.

   UL36 FW 5′-CGCTGCACGAATAGCATGGAATC-3′.

   UL36 RV 5′-CCAGCTCCCCGGAACACATTTA-3′.
7. Plaque assay overlay medium: 2× MEM, 1 % agarose in H_2O.
8. For indirect immunofluorescence microscopy: Poly-D-lysine, 1× PBS, 4 % paraformaldehyde (PFA) in 20 % sucrose/1× PBS, 100 mM ammonium chloride, Triton-X 100, 1 % BSA in 1× PBS, 1 μg/mL 4′,6-diamino-2-phenylindole (DAPI nuclear stain) in 1 % BSA.

2.4 Transduction of Latent SCG Neurons with Lentivirus

1. 293LTV cell line (Cell BioLabs, Inc., LTV-100).
2. 293LTV growth medium: 1× DMEM supplemented with 10 % FBS, 2 mM L-glutamine, and 0.1 mM MEM nonessential amino acids (NEAA).
3. Lipofectamine 2000 transfection reagent.
4. 1× OptiMEM.
5. Lentiviral packaging and vector plasmids.
6. Millex-HV syringe filter unit with 0.45 μm pore PVDF membrane.
7. Syringes.
8. Acyclovir, *see* Subheading 2.3.
9. NGF, *see* Subheading 2.2.

3 Methods

3.1 Dissecting SCGs

1. Prior to use, coat 96- and/or 24-well plates by filling each well with a 0.66 mg/mL solution of rat-tail collagen, and then remove the collagen solution immediately. Leave the plates open in a running laminar flow hood to allow the wells to dry completely. Drying will take approximately 10 min for smaller wells but closer to 45 min for larger wells. After the wells are completely dry, wash once with sterile H_2O, and then fill each well with a 2 μg/mL solution of laminin. Store the laminin-filled plates at 37 °C until the SCG neurons are ready to be seeded (*see* Notes 1–3).

2. Euthanize the pregnant female rat(s) on embryonic day 21 by CO_2 asphyxiation. Spray the animal(s) with 70 % ethanol, make a U-shaped incision, and fold back the skin away from the abdominal cavity. Carefully cut through the abdominal musculature to reveal the uterus and unborn pups. Remove the entire uterus, being careful to not injure the pups, and place in a 15-cm dish. Remove each pup from the uterus and embryonic sac, cut the umbilical cord, and clean using 70 % ethanol and Kimwipes.

3. Sacrifice one pup at a time by decapitation using a pair of dissecting scissors to cut just above the shoulders at the base of the neck. Mount the severed head on the dissection tray. Pin the exposed spinal cord with a 23 G needle. Pull the anterior skin over the nose away from the carotid arteries, and hold the skin down using a second 23 G needle. Push the esophagus and trachea away from the carotid arteries, and place a third 23 G needle through the esophagus and trachea (Fig. 1).

4. The two SCGs can be found by first locating the two carotid arteries (Fig. 1). Gently pulling up one artery at a time with a pair of straight-edge dissection forceps will reveal a bifurcation in the blood vessel. The SCG sits at the branching point of the artery and is a light, almond-shaped tissue that is less yellow than the surrounding adipose tissue. Remove each SCG by gently pulling it away from the artery, and place it in a 15-mL conical tube filled with 12 mL of L-15 medium.

5. Repeat **steps 3** and **4** for each of the pups.

3.2 Dissociating, Plating, and Culturing SCG Neurons

1. Centrifuge the SCGs for 1 min at 133×*g*, and remove the medium (*see* Note 4).

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1. Allow collagen to dry completely on plates before rinsing with H_2O. Since the collagen is H_2O soluble, rinsing the plates with H_2O before drying may cause the collagen to wash off and SCG neurons to detach from the plates later on.
3. Remove the laminin solution used to coat plates just before the neurons are to be plated. Doing this too early may cause the laminin to dry and precipitate.
4. Do not centrifuge the SCGs faster than the recommended speed of 133 × *g*, because the tissues can be damaged, reducing the number of viable neurons.

2. Resuspend the ganglia in 1 mL of L-15 medium containing 0.25 % trypsin and incubate at 37 °C for 20–30 min, agitating approximately every 10 min (*see* Note 5).

3. Add 10 mL of C-medium, and centrifuge for 1 min at 133×*g*.

4. Remove the medium, and resuspend cells in 1 mL of C-medium. To dissociate the cells, pass the tissue through a 21 G needle using a 5 mL syringe until clumps are not seen, usually about 8–10 times. Then repeat three times using a smaller bore 23 G needle (*see* Note 6).

5. Filter the dissociated cells through a 70 μm nylon filter to remove any remaining clumps.

6. Remove 10 μL of cell suspension and mix with trypan blue. Count the live cells that are able to exclude the dye, and calculate the concentration of the cell suspension.

7. Remove the laminin solution from the wells, and, without allowing the plate to dry, immediately add cells in C-medium supplemented with 50 ng/mL of NGF to the wells (*see* Notes 3 and 7).

    a. 96-well plate: Add 5–6 × 10^3 cells/well in a final volume of 50–100 μL/well.

    b. 24-well plate: Add 4–5 × 10^4 cells/well in a final volume of 500 μL/well.

8. The next day, referred to as *day* in vitro (DIV) 1, remove the C-medium and replace with NBM supplemented with 50 ng/mL of NGF, 5 μM aphidicolin, and 20 μM 5-FU. The NGF will sustain the post-mitotic neurons, and the other compounds will kill any dividing cells (*see* Notes 8 and 9).

3.3 Establishment of Latency and Reactivation of HSV-1 in SCG Neurons

1. On DIV 6, the day before HSV-1 infection, refeed the neuron cultures with media containing acyclovir at a final concentration of 100 μM (*see* Note 10). Acyclovir is a chain terminator that blocks any low level of HSV-1 lytic replication in the culture, allowing infected neurons to establish latency.

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5. Incubate SCGs in trypsin for approximately 20–30 min but no longer than 1 h. Excessive incubation will unduly stress the neurons, reducing survival.
6. Pass the SCGs gently through the syringes since neurons may be stressed and not survive.
7. Seed SCG neurons at the recommended density. Plating a fewer number of the neurons results in unhealthy cultures.
8. While changing the medium, it is advised to be extremely careful and gentle. Do not use an aspirator. Discard and replenish one well by one well for 24-well plates using a pipette and one row by one row for 96-well plates using a multichannel pipette. SCG neurons may come off from the plates otherwise.
9. By DIV 2 or 3, newly plated SCG neurons should have extended viable axons.
10. Avoid adding an acyclovir stock solution directly to SCG neurons because this can overly stress the neurons. Instead dilute the stock solution in NBM before adding to the well. Neurons should be treated with acyclovir for at least 6 h before HSV-1 infection. Overnight treatment is recommended.

a. 96-well plate: Make a solution of NBM supplemented with 50 ng/mL NGF and 300 μM acyclovir. Add 25 μL to each well to a final volume of 75 μL.

b. 24-well plate: Make a solution of NBM supplemented with 50 ng/mL NGF and 500 μM acyclovir. Carefully remove 100 μL of medium from each well and replenish with 100 μL of the 5× acyclovir solution to a final volume of 500 μL.

2. The next day (DIV 7), infect the neurons with HSV-1 at a multiplicity of infection (m.o.i.) corresponding to 1–2 plaqueforming units (pfu) per neuron (*see* Note 11), by adding an appropriate amount of virus diluted in NBM. This point in the protocol is referred to as 0 days post-infection (0 d.p.i.). Mock and/or an acute infection (omitting acyclovir) should be included as control(s).

3. After incubating the neurons with virus for 2–3 h at 37 °C, gently remove and replace the media with NBM supplemented with 50 ng/mL NGF and 100 μM acyclovir (*see* Note 12).

4. Allow the virus to establish latency by incubating the neurons at 37 °C for the next 7 days. Occasionally, monitor the health of the cultures using light microscopy (Fig. 2). If a virus expressing a fluorescent protein as a lytic gene is being used (e.g., EGFP-Us11), cultures can be checked for fluorescence indicative of unwanted reactivation or a failure to establish latency.

5. On DIV 14, induce reactivation by very carefully replacing the media with NBM containing 50 ng/mL NGF and 10–20 μM LY294002, a PI3-kinase inhibitor that serves as a potent inducer of reactivation (Fig. 2). DMSO, the vehicle, can be used as a negative control.

6. Incubate LY294002-treated cultures for 20 h at 37 °C, and then gently remove the media and replace with NBM supplemented with 50 ng/mL NGF (*see* Note 13).

7. Measure viral reactivation using one of the followings assays:

a. RT-qPCR: Detect and quantitate viral productive cycle transcripts such as the ICP27 (immediate–early gene), UL5 (early gene), and/or UL36 (true-late gene) mRNAs. Extract RNA from approximately 10^5 neurons per sample (RNeasy Mini Kit). Treat the RNA with DNase I to eliminate contaminating DNA, and then synthesize cDNA using Superscript III Reverse Transcriptase following the manufacturer's guidelines. Perform RT-qPCR with FastStart Universal SYBR Green

11. The highest m.o.i. to be used may vary slightly between different HSV-1 preparations, and this needs to be determined empirically using neurons. Stocks at a titer of 10^7 pfu per mL (as determined in Vero cells) or greater is recommended, but a titer of 10^6 may be sufficient.

12. Sometimes the neurons at the outer edges of a well will detach but will usually reattach if left undisturbed.

13. Each reactivation inducer must be tested to determine the optimal amount of time of exposure to the cells. Some inducers may be left on the cells throughout the experiment, but others may interfere with important cellular functions such as gene expression or metabolic homeostasis. This may be detrimental to cell viability or replication of reactivated virus; however, toxic compounds can still be used as inducers if they are added and then removed within a suitable time frame. This should be addressed empirically.

Master (Rox) or equivalent. Sequences of primer sets to detect ICP27, UL5, and UL36 are shown in Subheading 2.3.

b. Monitor fluorescent protein expression: Using a fluorescent microscope, count the number of wells with one or more neurons expressing the fluorescent protein expressed by the virus. Calculate the efficiency of reactivation by dividing this number by the total number of wells per condition.

c. Plaque assay: Collect the neurons in NBM medium 2–7 days after inducing reactivation. Dissociate the virus from cell debris by freezing/thawing and sonicating, make serial dilutions, and perform plaque assays (using plaque assay overlay medium) on monolayers of Vero cells or another suitable cell line.

d. Western blot analysis: Collect the neurons in 1× SDS sample buffer, heat denature, and resolve on a SDS-PAGE gel prior to immunoblotting for viral antigens using appropriate primary and secondary antibody combinations. Approximately 10^5 cells are required per sample. For details on how to prepare and probe immunoblots *see* refs. 12, 13.

e. Indirect immunofluorescence microscopy: In situ detection of viral proteins may be a useful measure of reactivation efficiency. Neurons should be plated onto glass cover slips or glass-bottomed culture chambers. Prior to use, cover slips should be cleaned with 1N HCl, rinsed with H_2O, and sterilized. Coat the cover slips with poly-D-lysine, collagen, and laminin. After inducing reactivation, fix neurons with 4 % PFA and 20 % sucrose in 1× PBS and then quench in 100 mM ammonium chloride. After thorough washing and blocking, stain neurons with primary antibodies against viral proteins and appropriate secondary antibodies. Counterstain the nucleus with DAPI. Include a specificity control by omitting the primary antibody.

Neurons are notoriously difficult to transfect, and conventional methods are likely to trigger reactivation of latent HSV-1 (our unpublished observations). Fortunately, neurons are amenable to lentiviral transduction allowing for the efficient delivery of a variety of bioactive molecules. It is possible to deplete host or viral proteins using appropriate short-hairpin RNAs (shRNAs) or to overexpress wild-type or mutant proteins. Introduction of the lentivirus can occur either before or after infection of HSV-1, enabling the researcher to selectively study the impact on latency establishment, maintenance, and reactivation. Methodology to prepare and infect SCG neurons with lentiviral based vectors is described below.

3.4 Preparation of Lentivirus Stocks

1. Transfect one 10-cm plate of 90 % confluent 293LTV producer cells with a total of 24 μg of an equimolar mix of the packaging and vector (engineered to express appropriate shRNAs or overexpress wild-type or mutant proteins) plasmids using Lipofectamine 2000 reagent (day 0) according to the manufacturer's guidelines.

Incubate the culture overnight at 37 °C. The packaging plasmids should provide the HIV *gag*, *pol*, and *rev* proteins (e.g., pCMV-dR8.91) and the vesicular stomatitis Indiana virus (VSIV) envelope glycoprotein (VSVG).

2. The next day (day 1), gently remove the medium and add 7 mL fresh 293LTV growth medium.
3. 2 days post-transfection (day 2), carefully collect the lentivirus-containing medium into a 15 mL conical tube and store at 4 °C. Refeed the cells with 7 mL 293LTV growth media, and then incubate the cells overnight at 37 °C.
4. 3 days post-transfection (day 3), gently remove the medium and pool with the lentivirus harvest from day 2.
5. Centrifuge for 10 min at 1,200×*g*, 4 °C, to pellet cell debris, and carefully filter the lentivirus preps through a 0.45 μm PVDF filter. Discard the pellet.
6. Aliquot the filtrate into 1.5 mL cryostorage tubes and store at −80 °C.

3.5 Transduction of Latently Infected SCG Neurons with Lentivirus

1. On DIV 12, or 5 days after infection with HSV-1, infect the neurons secondarily with lentivirus diluted into NBM media containing 50 ng/mL NGF and 100 μM acyclovir. The volume of a particular lentivirus stock that is added to the neurons must be tested empirically (*see* Note 14). This can be done on neurons without HSV-1 infection. Below are suggested ranges for lentivirus made using the above protocol.
    a. 96-well plate: Add 0.5–25 μL lentivirus/well diluted in a final volume of 50 μL/well.
    b. 24-well plate: Add 2.5–125 μL lentivirus/well in a final volume of 500 μL/well.
2. Incubate infected cultures overnight at 37 °C, and then gently remove the medium and replace with NBM supplemented with 50 ng/mL NGF and 100 μM acyclovir. Infection efficiency can be readily assessed using lentiviral vectors that constitutively express a fluorescent marker such as mCherry (*see* Fig. 3); the reporter gene should be chosen so as not to interfere with subsequent analyses.
3. Maintain the SCG neurons at 37 °C for 2–5 days. The exact time will depend on the degree of expression or knockdown efficiency or experimental design.
4. The SCG neurons may be tested for lentivirus-induced reactivation (Fig. 3). Carefully remove the medium and replace with NBM supplemented with 50 ng/mL NGF, omitting the acyclovir. Incubate at 37 °C and analyze by one of the methods described in Subheading 3.3.

14. The amount of lentivirus may need to be adjusted, either to optimize knockdown or maximize recombinant protein expression. Lentivirus exposure should be balanced against the impact on the health of the culture. Toxicity due to the lentivirus infection or presence of contaminants in the lentivirus stock can manifest as changes in neuronal morphology (often most evident as changes in the cell body or soma) or reduced attachment to the substrate.

To investigate the role of a neuronal or a viral factor on the establishment of latency, the lentivirus can be provided before infection with HSV-1 (*see* Note 15).

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15. Infections with HSV-1 and lentiviruses do not seem to interfere with each other, and thus the order of addition can be arranged to selectively investigate the role of viral or neuronal proteins in the establishment of latency rather than maintenance/reactivation. To precondition the neuronal environment prior to HSV-1 infection, add the lentivirus at DIV 6 at the same time as the acyclovir and perform the HSV-1 infection at DIV 7.

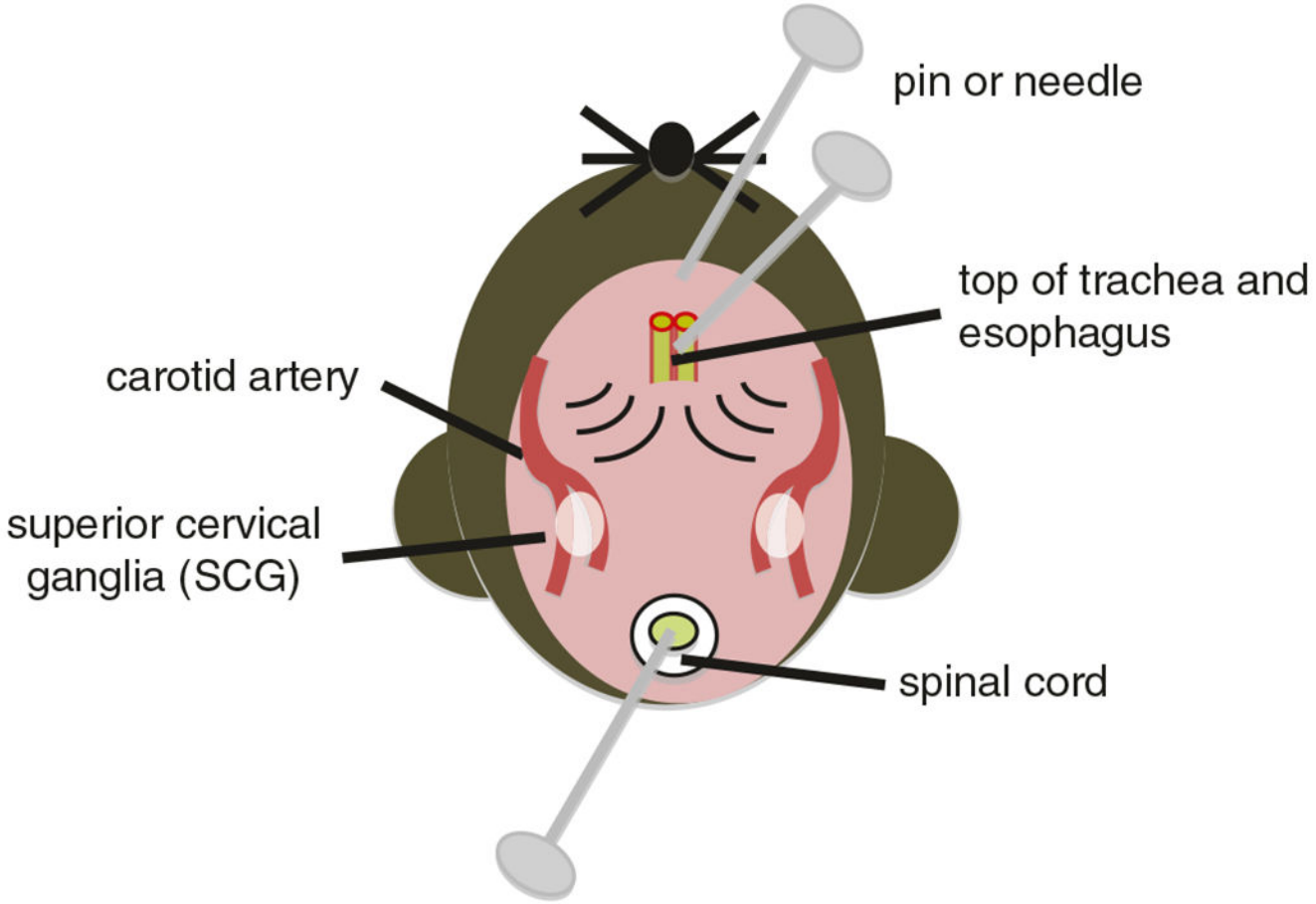

Fig. 1.

Landmarks used to locate the superior cervical ganglia. Schematic representation of a prenatal rat head viewed from the ventral side showing the recommended placement of three pins or syringe needles used to immobilize the head and to pin back the esophagus and trachea (described in Subheading 3.1). The superior cervical ganglion is an almond-shaped, semitransparent structure that appears almost colorless compared to surrounding tissues and is found above the branch point of the carotid arteries

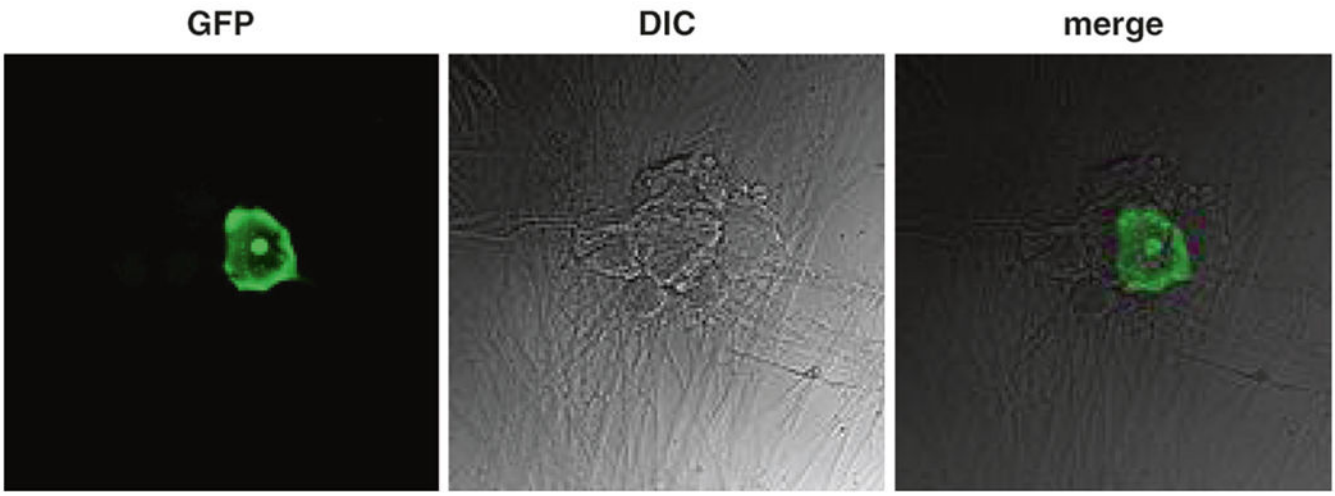

Fig. 2.

Reactivation of latent HSV-1 GFP-Us11 after inhibition of neuronal PI3-kinase. Green fluorescence (GFP) and differential interference contrast (DIC) imaging of latently infected neurons undergoing reactivation. Neurons were prepared and cultured as outlined in Subheadings 3.1 and 3.2 and then infected with wild-type HSV-1 (HSV-1 GFP-Us11) at an m.o.i. of 1, as described in Subheading 3.3. Cultures were maintained in medium containing 100 μM acyclovir for a further 7 days to allow the virus to establish latency. To elicit reactivation, the culture media with acyclovir was removed and the neurons treated with 20 μM LY294002, a potent phosphatidylinositol 3-kinase inhibitor [4]. After 3 days, the cultures were fixed with phosphate-buffered saline containing 4 % paraformaldehyde and 20 % sucrose and examined by fluorescent microscopy using a Zeiss LSM 510 META confocal microscope. A small cluster of neuronal cell bodies is shown, and one of the neurons displays robust EGFP-Us11 expression indicative of the onset of viral genome amplification and productive reactivation

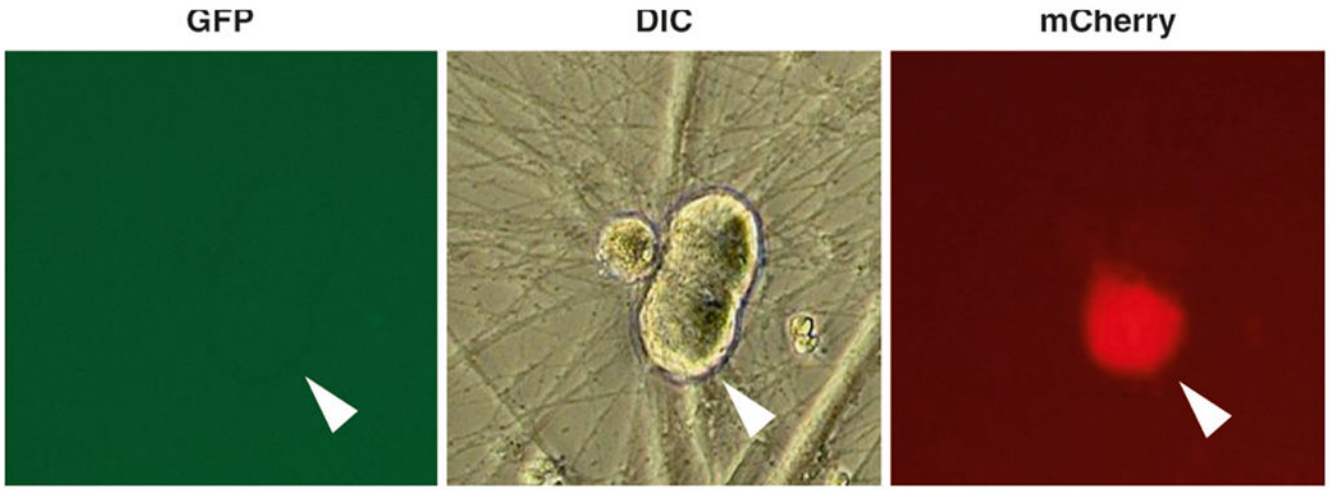

Fig. 3.

Monitoring lentiviral infection of HSV-1-infected SCG neurons. Light microscopy analysis of live SCG neurons that have been infected with HSV-1 GFP-Us11 in the presence of 100 μM acyclovir, as described in Subheading 3.3. After allowing the virus to establish latency over 5 days, the cells were coinfected (Subheading 3.5) with a lentivirus that constitutively expresses an shRNA against a viral lytic gene product and mCherry, a red fluorescent protein used as a visual marker of lentivirus-infected cells. The cell bodies of two neurons are shown 2 days after addition of the lentivirus, with obvious accumulation of mCherry in one of the neurons (*white arrowhead*). Note the absence of any GFP signal, consistent with the lack of HSV-1 reactivation under these conditions