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TITLE:

Mouse Footpad Inoculation Model to Study Viral-Induced Neuroinflammatory Responses

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SUMMARY:

The footpad inoculation model is a valuable tool for characterizing viral-induced neuroinflammatory responses in vivo. In particular, it provides a clear assessment of viral kinetics and associated immunopathological processes initiated in the peripheral nervous system.

ABSTRACT:

This protocol describes a footpad inoculation model used to study the initiation and development of neuroinflammatory responses during alphaherpesvirus infection in mice. As alphaherpesviruses are main invaders of the peripheral nervous system (PNS), this model is suitable to characterize the kinetics of viral replication, its spread from the PNS to CNS, and associated neuroinflammatory responses. The footpad inoculation model allows virus particles to spread from a primary infection site in the footpad epidermis to sensory and sympathetic nerve fibers that innervate the epidermis, sweat glands, and dermis. The infection spreads via the sciatic nerve to the dorsal root ganglia (DRG) and ultimately through the spinal cord to the brain. Here, a mouse footpad is inoculated with pseudorabies virus (PRV), an alphaherpesvirus closely related to herpes simplex virus (HSV) and varicella-zoster virus (VZV). This model demonstrates that PRV infection induces severe inflammation, characterized by neutrophil infiltration in the footpad and DRG. High concentrations of inflammatory cytokines are subsequently detected in homogenized tissues by ELISA. In addition, a strong correlation is observed between PRV gene and protein expression (via qPCR and IF staining) in DRG and the production of pro-inflammatory cytokines. Therefore, the footpad inoculation model provides a better understanding of the processes underlying alphaherpesvirus-induced neuropathies and may lead to the development of innovative therapeutic strategies. In addition, the model can

guide research on peripheral neuropathies, such as multiple sclerosis and associated viral-induced damage to the PNS. Ultimately, it can serve as a cost-effective in vivo tool for drug development.

INTRODUCTION:

This study describes a footpad inoculation model to investigate the replication and spread of viruses from the PNS to CNS and associated neuroinflammatory responses. The footpad inoculation model has been intensively used to study alphaherpesvirus infection in neurons[1-3]. The main objective of this model is to allow neurotropic viruses to travel a maximal distance through the PNS before reaching the CNS. Here, this model is used to obtain new insights in the development of a particular neuropathy (neuropathic itch) in mice infected with pseudorabies virus (PRV).

PRV is an alphaherpesvirus related to several well-known pathogens (i.e., herpes simplex type 1 and 2 [HSV1 and HSV2] and varicella-zoster virus [VZV]), which cause cold sores, genital lesions, and chicken pox, respectively[4]. These viruses are all pantropic and able to infect many different cell types without showing affinity for a specific tissue type. However, they all exhibit a characteristic neurotropism by invading the PNS (and occasionally, the CNS) of host species. The natural host is the pig, but PRV can infect most mammals. In these non-natural hosts, PRV infects the PNS and induces a severe pruritus called the “mad itch”, followed by peracute death[5,6]. The role of the neuroimmune response in the clinical outcome and pathogenesis of PRV infection has been poorly understood.

The footpad inoculation model allows PRV to initiate infection in the epidermal cells of the footpad. Then, the infection spreads into sensory and sympathetic nerve fibers that innervate the epidermis, sweat glands, and dermis. The infection spreads by virus particles moving via the sciatic nerve to the DRG within approximately 60 h. The infection spreads through the spinal cord, ultimately reaching the hindbrain when animals become moribund (82 h post-infection). During this time window, tissue samples can be collected, processed, and analyzed for virus replication and markers of the immune response. For instance, histological examination and viral load quantification can be performed in different tissues to establish correlations between the initiation and development of clinical, virological, and neuroinflammatory processes in PRV pathogenesis.

Using the footpad inoculation model, the cellular and molecular mechanisms of PRV-induced pruritus in mice can be investigated. Moreover, this model can provide new insight into the initiation and development of virus-induced neuroinflammation during herpesvirus infections. A better understanding of the processes underlying alphaherpesvirus-induced neuropathies may lead to the development of innovative therapeutic strategies. For instance, this model is useful to investigate the mechanisms of neuropathic itch in patients with post-herpetic lesions (e.g., herpes zoster, shingles) and test novel therapeutic targets in mice for the corresponding human diseases.

PROTOCOL:

All animal experiments were performed in accordance to a protocol (number 2083-16 and 2083-19) reviewed and approved by the Institution Animal Care and Use Committee (IACUC) of Princeton University. This work was done by strictly following the biosafety level-2 (BSL-2) requirements, to which we have a fully equipped lab approved by the Princeton University biosafety committee. The procedures including mouse footpad abrasion, viral inoculation, mouse dissection, and tissue collection were performed in a biological safety cabinet (BSC) in the Princeton University biocontainment animal facility room. Those performing the procedure wore disposable gowns, head cover, eye protection, sterile gloves, surgical masks, and shoe covers.

1. Mouse footpad abrasion

1.1. Anesthetize a C57BL/6 mouse (5–7 weeks old) with isoflurane gas anesthetic, delivered at a dosage of 3% via a small anesthesia system (chamber).

1.1.1. Place the mouse in the surgical plane of anesthesia on its back, prior to inoculation, in the hind footpad. Attach a nose cone with a diaphragm (a slit sufficiently sized to fit the muzzle of the animal) to the mouse and deliver isoflurane gas anesthetic at a constant dosage of 1.5%–2.0%.

1.1.2. Closely monitor the mouse for a response to painful stimulus created by forceful pinching of the toe with forceps.

1.2. Lightly grasp one hind footpad with flat forceps.

1.2.1. Gently abrade the glabrous skin of the hind footpad approximately 20x, between the heel and walking pads, with an emery board (100–180 grit). Do not induce bleeding by abrading too frequently or applying too much pressure.

1.2.2. Using fine forceps, slowly peel off the stratum corneum detached by abrasion to expose the stratum basale.

2. PRV footpad inoculation

2.1. Prepare the virus inoculum diluted to the desired titer in DMEM media containing 2% fetal bovine serum (FBS) and 1% penicillin/streptomycin (**Table of Materials**).

2.1.1. Quantitate the number of plaque-forming units (PFU) in the virus stock on PK15 cells to calculate and standardize the viral dose and dilute the viral inoculum accordingly.

NOTE: In this study, virulent strain of PRV (PRV-Becker) was used at a dose of 8 x 10^6 PFU. This dose was optimized in a previous preliminary experiment to ensure that all inoculated animals showed clinical symptoms at 82 h post-inoculation (hpi).

2.1.2. Keep the virus inoculum on ice and gently mix before use.

2.2. Add a 20 µL droplet of virus inoculum (8 x 10^6 PFU) onto the abraded footpad (topical administration). Carry out mock inoculations (medium only) in parallel.

2.3. Gently rub 10x with the shaft of a needle to facilitate adsorption of the virus. Avoid scratching with the needle point. Repeat this step every 10 min.

2.4. Keep the mouse under anesthesia for 30 min until the abraded footpad is dry.

2.5. After stopping anesthesia, monitor the mouse until it can maintain sternal recumbency and place it in an individual cage for clinical follow-up and sampling.

3. Mouse dissection and tissue collection

3.1. At appropriate times after infection, euthanize the mouse by the asphyxiation method (CO_2)**.**

NOTE: In this study, the humane endpoint (when animals begin to exhibit terminal symptoms) for PRV-Becker infected mice is 82 hpi. Euthanize control animals in parallel.

3.2. Place the mouse ventral side facing up on a surgical mat using needles/pins. Secure the limbs of the mouse with pins to a surgical foam board. Wet the ventral side of the mouse with 70% ethanol to minimize fur contamination.

3.3. Pinch the external layer of fur and skin using forceps and make a small initial incision using fine scissors near the urethral opening.

3.3.1. From this opening, continue the incision on the mid ventral side up to the chin.

3.3.2. Extend two lateral incisions towards the extremities of the forelimbs and hind limbs.

3.3.3. Separate the skin from the underlying muscular layer and pin down to the side.

3.3.4. Open the abdominal cavity and incise up to the base of the thorax. Complete the opening of the abdominal cavity by making two transversal incisions.

3.3.5. Open the thoracic cavity by cutting the diaphragm and ribs on both lateral sides.

NOTE: Cutting the sides of the ribcage prevents the risk of cutting the heart.

3.3.6. Collect exposed organs, including heart, lungs, spleen, pancreas, liver, kidneys, and bladder in 1.5 mL microtubes. Keep the tubes on ice.

3.3.7. Cut the abraded footpad between the heel and walking pads and place the piece of tissue in a tube as described in step 3.3.6.

3.4. Place the mouse dorsal side up and wet the fur with 70% ethanol. Cut down the skin layer to expose the vertebral column.

3.4.1. Make a small incision in the region of the pelvis. Strip the skin from the hind limbs towards the head.

3.4.2. Remove the legs and arms by cutting with scissors parallel with and close to the spinal column on both sides.

3.4.3. Cut the head off at the base of the skull (C1–C2 level).

3.4.4. Cut open the skull with scissors from the foramen magnum to the frontal bone.

3.4.5. Pull open the skull in lateral directions using forceps.

3.4.6. Gently scoop out the brain using forceps and proceed as described in step 3.3.6.

3.5. Clean the spinal column by cutting muscle, fat and soft tissues using curved scissors. Cut the tail. Place the intact spinal column in a 15 mL tube containing sterile ice-cold phosphate-buffered saline (PBS). Keep tubes on ice until further dissection of the spinal cord and DRG.

4. Spinal cord and DRG extraction

NOTE: Extract spinal cord and DRG from the vertebrae column directly following mouse dissection. The protocol for spinal cord and DRG extraction has been adapted from a previous publication[7].

4.1. Remove the vertebrae column from ice-cold PBS tube and remove remaining soft tissues, which becomes easier after refrigeration.

4.2. Make three transverse cuts in the column through the vertebrae (T1, L1, and S2 levels) using a razor blade. Place the three pieces in a 15 mm Petri dish containing sterile ice-cold PBS. Keep track and mark origin of segments for later analysis.

4.3. Take one segment and secure it between forceps, dorsal side facing up. Make a single, longitudinal cut down through the midline using a razor blade, splitting the column in two equal halves.

4.4. Place both halves in a new Petri dish containing new sterile ice-cold PBS. Ensure the correct orientation of the segment to be able to distinguish left and right sides.

4.5. Under a dissecting microscope, gently peel the spinal cord out of the vertebrae column from each half in a rostral to caudal direction using fine forceps.

4.6. Collect both spinal cord halves in 1.5 mL microtubes containing 500 µL of sterile ice-cold PBS. Keep the tubes on ice.

4.7. Gently remove the meninges from one side of the spinal column to the other to expose the DRG.

4.8. Remove each DRG individually by grasping the exposed spinal nerve and pull it gently out of the spinal column. Place the collected DRG in a 15 mm Petri dish containing ice-cold PBS. Harvest all ipsilateral and contralateral DRG separately from the spinal column segment and proceed as described in step 4.5.

NOTE: The DRG is visible as a round transparent structure along the white spinal nerve.

4.9. Repeat steps 4.3–4.7 using the two other spinal column segments within 30–45 min.

4.10. Centrifuge all tubes at high speed (17,900 *x g*) for 3 min at 4 °C.

4.11. Aspirate supernatant and flash-freeze tubes in liquid nitrogen. Store tubes at -80 °C for further tissue homogenization.

4.12. Alternatively, fix and section the cleaned DRG and other mouse tissues for histopathological analyses and/or immunofluorescence staining after step 4.9.

5. Tissue homogenization

5.1. Thaw the tubes containing frozen tissue samples on ice.

5.2. Weigh 100 mg of tissue and place it in a 2 mL microcentrifuge tube containing a sterile steel bead and 500 µL of RIPA buffer containing 0.5 M EDTA (pH = 8.0), 1 M Tris-HCl (pH = 8.0), 5 M NaCl, 10% SDS, and protease cocktail inhibitor tablets.

5.3. Disrupt tissues at room temperature (RT) using a homogenizer at 20 cycles/s for 2 min, followed by a 1 min waiting period, then 20 cycles/s for 2 min.

5.4. Centrifuge the tube at high speed (17,900 *x g*) for 10 min at RT.

5.5. Collect supernatant (500 µL) in a new tube and store it at -20 °C until performing ELISA and qPCR to quantify inflammatory markers and viral loads, respectively.

NOTE: For qPCR, collect all samples with RNase-free material (i.e., beads, tubes, etc.) and disrupt tissues with RNA lysis buffer containing 1% betamercaptoethanol (**Table of Materials**).

6. Preparation and immunofluorescence staining of frozen DRG sections

6.1. Tissue processing

6.1.1. Fix the dissected and cleaned DRG in 1% paraformaldehyde (PFA) for 2 h at RT.

6.1.2. Transfer the tissue into a 15 mL tube with 10% sucrose in PBS. Incubate overnight at 4 °C.

6.1.3. Transfer the tissue into a 15 mL tube with 20% sucrose in PBS. Incubate overnight at 4 °C.

6.1.4. Transfer the tissue into a 15 mL tube with 30% sucrose in PBS. Incubate overnight at 4 °C.

6.1.5. Embed tissues in OCT using a cryomold and place blocks in dry ice for rapid freezing.

6.1.6. Store the samples at -80 °C until use.

6.2. Cryosectioning preparation

6.2.1. Remove the frozen block from -80 °C and allow it to equilibrate in the cryostat chamber temperature for 30 min.

6.2.2. Cryosection the DRG at a thickness of 15 µm and mount the sections onto slides.

6.2.3. Use a pen to draw a hydrophobic circle around the slide-mounted tissue.

6.2.4. Keep the slides at 4 °C until staining.

6.3. Immunofluorescence staining

6.3.1. Wash the DRG sections 3x in PBS for 10 min at RT.

6.3.2. Incubate the sections with 100 µL of desired primary antibody (e.g., mouse antibody against PRV glycoprotein B) for 1 h at 37 °C. Dilute the primary antibody in PBS containing 10% negative goat serum.

6.3.3. Repeat step 6.3.1.

6.3.4. Incubate the sections with 100 µL of desired secondary antibody (goat anti-mouse Alexa Fluor 488) for 50 min at 37 °C. Dilute secondary antibody in PBS only.

6.3.5. Add 100 µL of DAPI (4',6-diamidino-2-phenylindole) dye diluted in PBS onto the section. Further incubate samples 10 min at 37 °C.

6.3.6. Repeat step 6.3.1.

6.3.7. Mount the samples using a fluorescence mounting medium onto glass slides and secure and cover with coverslip.

7. Preparation and H&E staining of paraffin-embedded tissue sections

7.1. Fix the tissue in 10% formalin for 24 h at 4 °C.

7.2. Perform standard processing schedule for paraffin embedding and sectioning of fixed tissues. Transfer fixed tissues to 70% ethanol and process through upgraded alcohols to xylene followed by paraffin, as previously described[8].

NOTE: Use polyester sponges to keep small tissue samples such as DRG inside cassettes.

7.3. Perform standard deparaffinization followed by H&E staining of tissue sections, as previously described[9].

REPRESENTATIVE RESULTS:

The mouse footpad inoculation model allows for characterization of the immunopathogenesis of alphaherpesvirus infection in vivo, including replication and spread of the infection from the inoculated footpad to the nervous system and the induction of specific neuroinflammatory responses.

In this study, we first abraded the mouse hind footpad and either mock-inoculated or inoculated the abraded region with a virulent strain of PRV (PRV-Becker). The site of abrasion was visible in the control footpad. A crust was formed at the abrasion site as part of the healing process (**Figure 1**, black arrow). In contrast, mice inoculated with PRV showed severe inflammation at the humane endpoint (82 hpi), characterized by swelling of the footpad and redness.

Following footpad inoculation with the virulent PRV-Becker strain, mice began showing clinical signs at 72 hpi, characterized by swelling of the inoculated footpad and increasingly frequent tremors. By 82 hpi, PRV-Becker infected mice showed constant tremors in the inoculated leg and distinctive PRV symptoms, including intense scratching and biting of the foot. Severe inflammation was also observed in the footpad. The inflammatory response induced during PRV infection was then further characterized, including the infiltration of immune cells into tissues.

Histopathological examination of several tissues was performed, including the inoculated footpad and DRG, followed by H&E staining of paraffin-embedded tissue sections. Epidermal necrosis and severe dermal inflammation (edema and fibrin) were observed in PRV-infected foot sections **(Figure 2A, panel b**). The epidermis, dermis, and connective tissues of infected mice showed a massive infiltration of neutrophils (identified by multilobed nuclei) marked with black arrows. Footpads of control mice were normal (**Figure 2A, panel a**). PRV-infected DRG showed minimal neuronal necrosis and mixed inflammation in infected mice while the DRG of control mice were normal (**Figure 2B, panels a and b**). The mixed inflammation infiltrate consisted mainly of neutrophils and lymphocytes.

Next, the kinetics of inflammatory cytokine production in mouse tissue after PRV footpad inoculation were established. Levels of specific inflammatory cytokines were quantified (i.e, interleukin-6 [IL-6] and granulocyte-colony stimulating factor [G-CSF]) from several tissues collected and homogenized from control and PRV-infected mice. The results demonstrated a significant increase of G-CSF levels in the footpad and DRG compared to controls at 7 hpi and 82 hpi (**Figure 3A**). Significant G-CSF levels were observed at 82 hpi in spinal cord, brain, heart, and liver tissue of PRV-infected mice compared to controls. Moreover, significant IL-6 levels were detected in all tissues of PRV-infected mice compared to controls starting at 24 hpi (**Figure 3B**).

The footpad inoculation model was further used to investigate PRV replication and spread from the inoculated footpad to the PNS and CNS and potential correlations with neuroinflammatory response development. PRV loads were quantified in several homogenized tissues by qPCR to amplify PRV DNA. DNA concentration was then converted to PFU, as previously described[10]. PRV loads were detected in the footpad starting at 24 hpi (~1 x 10^4 PFU/mg of tissue) and in DRG starting at 60 hpi (~1 x 10^3 PFU/mg of tissue; data not shown).

In a moribund state (82 hpi), PRV was detected in the footpad, DRG, spinal cord, and brain, with the highest concentration of PRV in DRG (~1 x 10^5 PFU/mg of tissue; **Figure 4**). PRV infection of DRG was confirmed by indirect immunofluorescence staining of DRG cryosections. PRV infection was detected in DRG neurons using anti-PRV gB antibody. PRV glycoprotein gB was expressed during late stages of infection in the cytoplasm of infected cells. As expected, the cytoplasmic expression of PRV gB (green) was confirmed in infected DRG, while no gB was expressed in control samples (**Figure 5**). Cell nuclei were identified with DAPI staining (blue).

FIGURE LEGENDS:

Figure 1: Representative images of mouse right hind paws after PRV inoculation. Mice are either mock-inoculated or inoculated with PRV in the abraded right hind footpad. PRV-inoculated footpad shows signs of inflammation, including redness and swelling at humane endpoint (82 hpi). The footpad of control mice appears normal with a dark red crust at the abraded site, indicating that the wound is healing. Black arrows indicate the site of abrasion. This figure has been modified from a previous publication[11].

Figure 2: Histopathological findings in footpad and DRG after PRV footpad inoculation. Hematoxylin and eosin (H&E) staining of (**A**) mouse inoculated footpads and (**B**) and ipsilateral DRG from control (panel a) and PRV-infected (panel b) mice at 82 hpi. Histopathological manifestations observed in PRV-infected tissues (epidermal and neuronal necrosis and neutrophil infiltration) are absent from all examined mock-infected mice. Results are representative of three biological replicates for a given type of tissue. Black arrows indicate representative areas of inflammation with immune cell infiltration. Scale bars (50 µm) are indicated for each picture. This figure has been modified from[11].

Figure 3: Kinetics of inflammatory cytokine production in homogenized mouse tissues after PRV footpad inoculation. (**A**) G-CSF and (**B**) IL-6 protein levels detected in PRV-infected (red) and control (black) mouse homogenized tissues at different hpi. Protein levels are quantified by ELISA and expressed as picogram (pg) per milligram (mg) of homogenized tissue (n = 5 per group, *$p < 0.05$, ns = not significant). This figure has been modified from a previous publication[12].

Figure 4: Quantification of PRV genome in mouse homogenized tissues. PRV DNA is quantified in homogenized mouse tissues by qPCR using PRV UL54 primers. PRV loads are expressed as plaque forming units (PFU) per mg of tissue. PRV DNA is detected only in the foot, DRG, spinal cord, and brain (and not in other tissues), n = 10 per group. Dotted line shows the detection limit. This figure has been modified from a previous publication[11].

Figure 5: Assessment of PRV infection in DRG neurons by immunofluorescence staining. Confocal Z-stack images of mock- and PRV-infected DRG neurons after immunofluorescence staining using a mouse antibody specific for PRV gB (green). Cell nuclei are stained with DAPI (blue, panels a and c). Panel d shows several PRV-infected neurons expressing gB (white arrows). No gB expression is detected in the control DRG sections (panel b). Scale bars (50 µm) are indicated for each picture.

DISCUSSION:

The footpad inoculation model described here is useful to investigate the initiation and development of neuroinflammatory responses during alphaherpesvirus infection. Moreover, this in vivo model is used to establish the kinetics of replication and spread of alphaherpesvirus from the PNS to CNS. This is an alternate to other inoculation models, such as the flank skin inoculation model, which relies on deep dermal scratching[13], or the intracranial route, which directly introduces the virus into the CNS[14-16]. As a result, with the footpad model, it is possible to obtain a more detailed assessment of viral kinetics of replication and spread with associated local and distant immunopathological processes in the nervous system[11,12].

In this protocol, the abrasion of the footpad and subsequent viral inoculation are crucial steps. Indeed, the stratum corneum needs to be completely removed after adequate abrasion in order to expose the stratum basale to viral inoculum for successful infection. However, the abrasion must be gentle and not induce bleeding, as this helps prevent infection of the blood

circulation. The detached stratum corneum can be visualized by H&E staining under light microscopy. The corneocytes present in the stratum corneum are flat, eosinophilic cells that lack nuclei. The volume of the viral inoculum (20 µL droplet) has been optimized to ensure that the droplet stays on the footpad and covers the abraded site. Gently rubbing the inoculum droplet onto the abraded footpad is essential for efficient viral penetration. It is recommended to wait until the footpad is completely dry to stop the anesthesia and place the animal in a new cage. This step will avoid the mouse from licking the viral inoculum off the abraded footpad. It is recommended to process a maximum of three mice at once, using a nose cone that is set to expose them simultaneously to anesthesia.

While performing the mouse dissection, it is important to make cuts parallel to the vertebrae column, which prevents damage to the spinal cord and associated DRG. It also is suggested to remove as much fat, muscle, and soft tissue as possible to reduce accidental cutting into the spinal column and facilitate a better grip on the column segment with forceps before cutting down the midline.

It is also recommended to perform transverse cuts through the vertebrae column between discs in order to produce cleaned column segments and limit the risk of damaging DRG pairs. The meninges surrounding the spinal cord and covering the DRG must be completely removed to facilitate the identification and extraction of DRG. The DRG should be removed carefully from the spinal column without damage from the forceps. It is important that DRG remain intact for H&E and immunofluorescence staining. Timing is critical for efficiency of this in vivo experiment, and the mouse dissection and spinal cord/DRG extraction should be performed consecutively in order to collect tissue that is as fresh as possible.

The tissue homogenization method described here has been optimized to ensure efficient disruption of a large number of heterogenous tissue samples. It is paramount to standardize the amount of tissue used, which allows direct comparison of ELISA and qPCR results between samples. For instance, it is suggested to weigh 100 mg of tissue for each homogenization procedure. Each tissue sample must be homogenized in its entirety, and leftovers should not be freeze-thawed. It is important to autoclave the steel beads and forceps before use to avoid any contamination during homogenization. A volume of 500 µL is optimal to ensure complete homogenization of a 100 mg tissue sample. It is important to note that this limited volume allows only for the processing of three to four ELISA kits per sample.

Using the footpad inoculation model, it was demonstrated that PRV infection in mice induces severe inflammation, characterized by neutrophil infiltration in the footpad and DRG. High concentrations of inflammatory cytokines G-CSF and IL-6 were also detected in many homogenized tissues using ELISA. In addition, a strong correlation was found between PRV gene and protein expression (by qPCR and IF staining) in DRG and the production of both pro-inflammatory cytokines.

This model is suitable to compare the kinetics of viral replication/spread as well as neuroinflammatory responses among different alphaherpesvirus infections. For instance,

regarding VZV, the restricted host-specificity and lack of clinical disease have limited the use of animal models[17]. Therefore, the mouse footpad PRV inoculation model may represent a new animal model for the study of the cellular and molecular mechanisms responsible for neuropathic pruritus in patients with post-herpetic lesions. Based on the similarities in clinical signs, pathogenesis, and genomes between VZV and PRV, it is believed that this mouse model will enhance the understanding of VZV pathogenesis and lead to the developments in innovative therapeutic strategies.

Finally, the model will guide research on peripheral neuropathies, such as multiple sclerosis and associated viral-induced damage to the PNS[18]. The pathogenesis of several neurotropic viruses (i.e., rabies virus, poliovirus, West Nile virus, Zika virus), which are known to infect the PNS, can also be studied using this model[19-22]. The footpad inoculation model can be used as a possible cost-effective tool for drug development. For instance, it may serve as a platform to screen and test the efficacy of anti-inflammatory and antiviral drugs designed to prevent viral-induced peripheral neuropathies.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

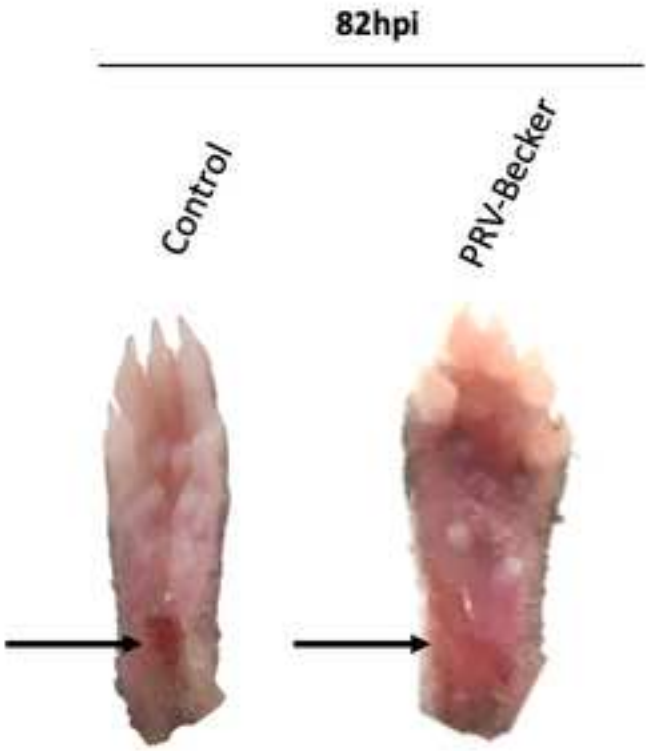

Figure 2

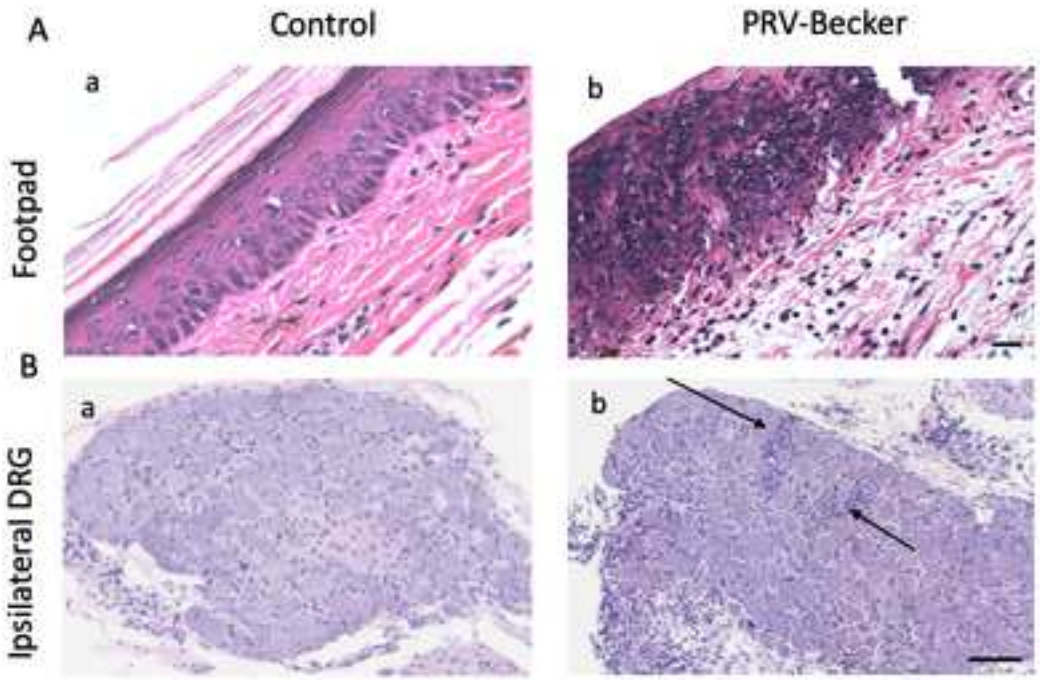

Figure 3

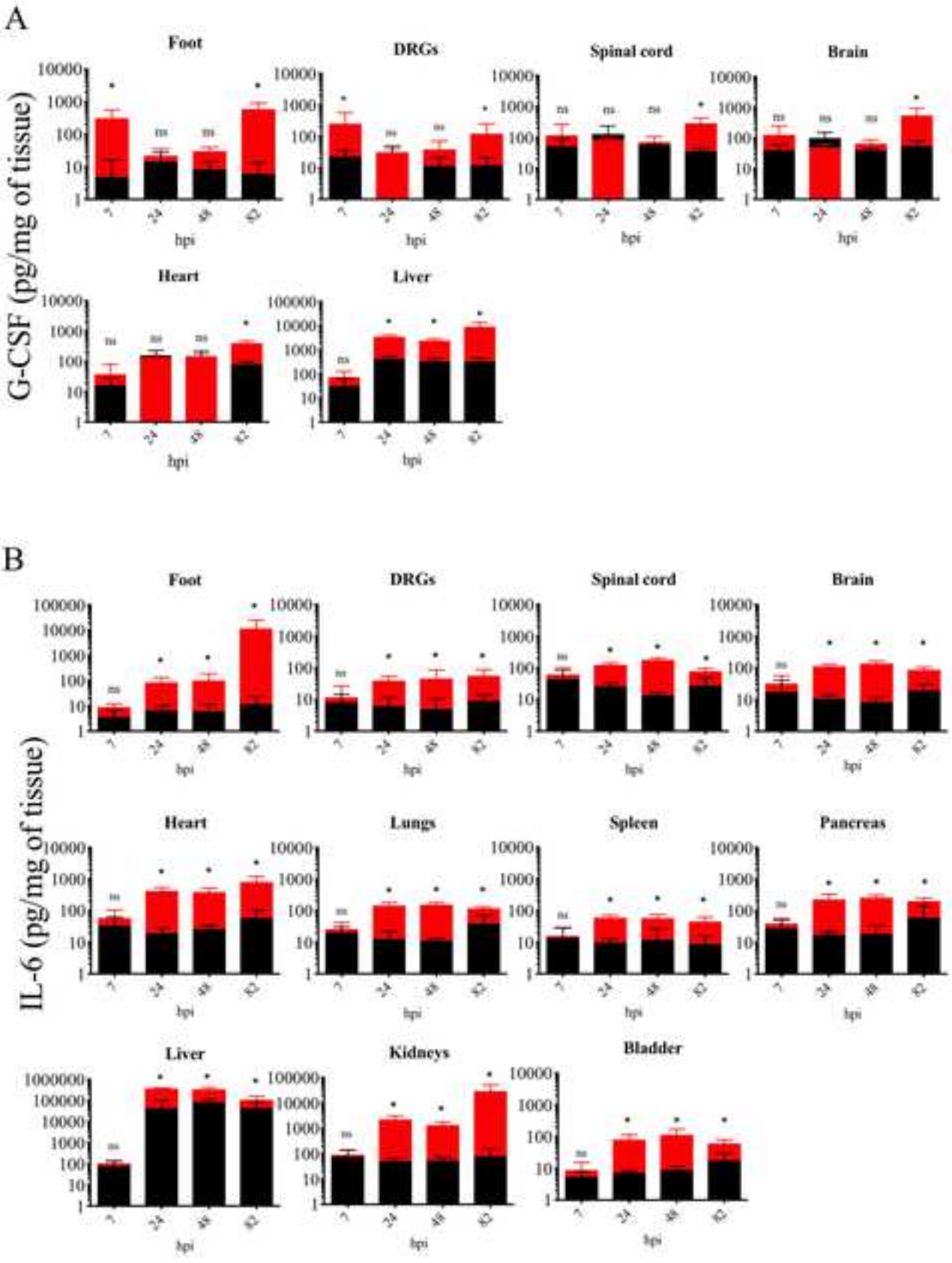

Figure 4

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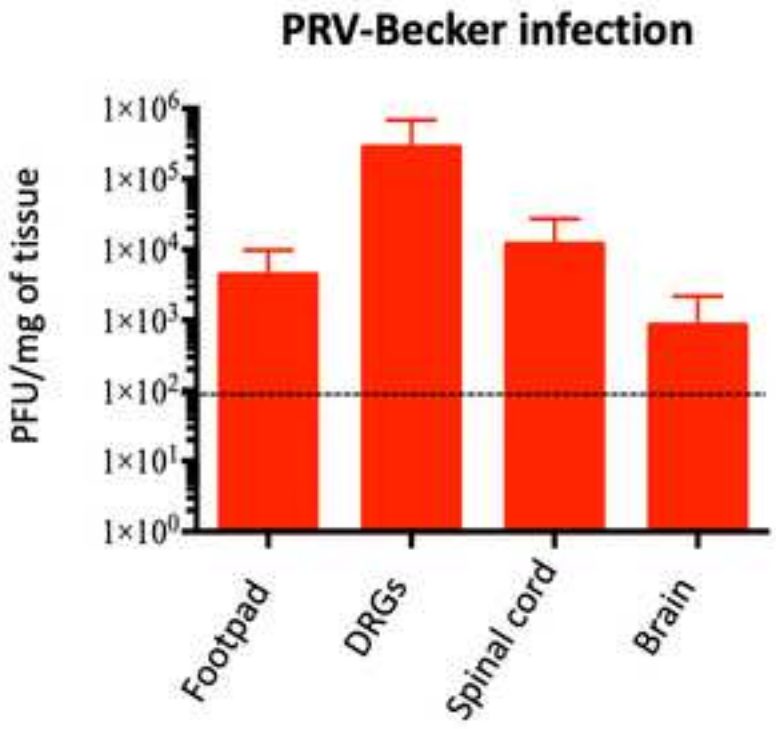

Figure 5

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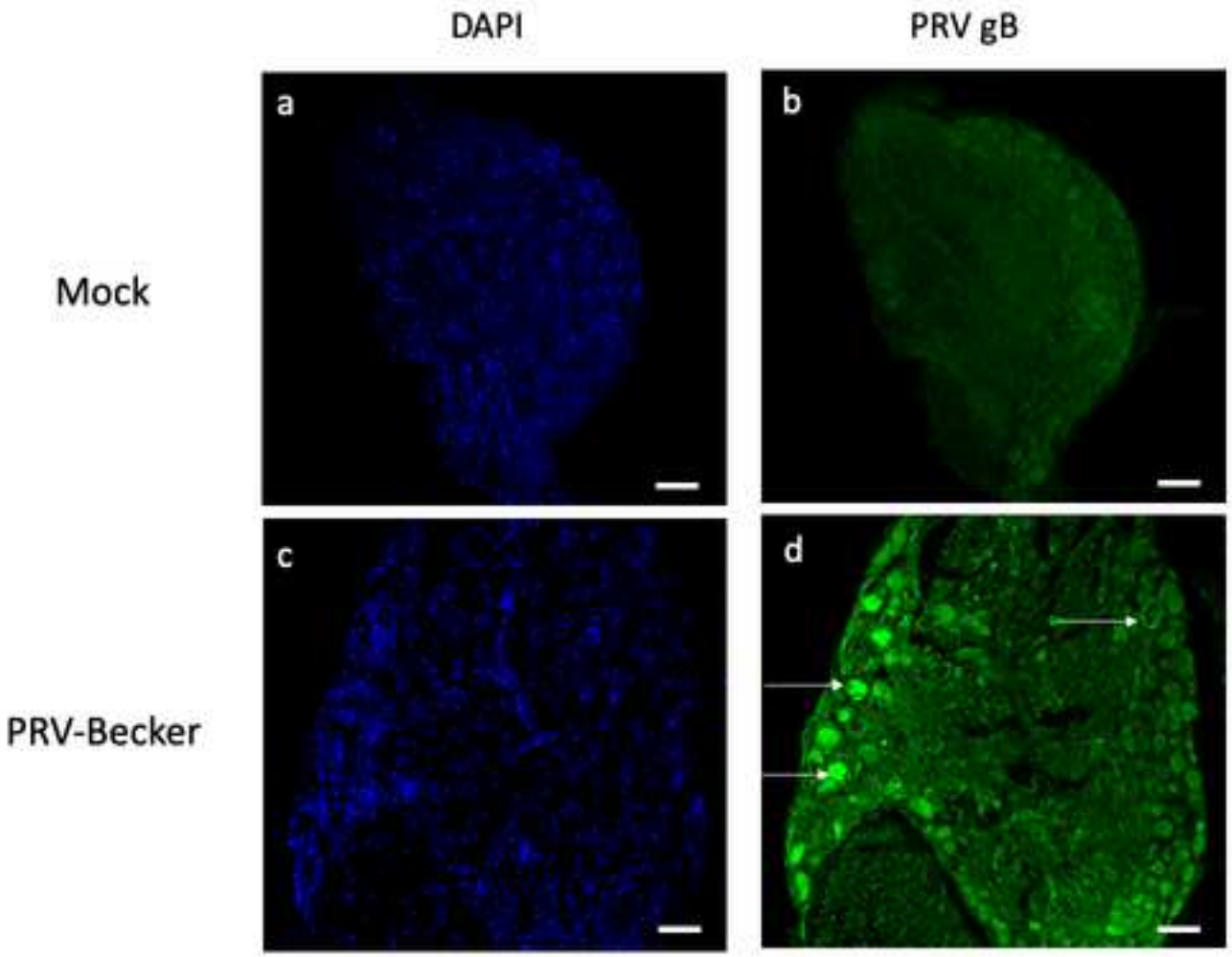

Figure 5


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Figure 1


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Figure 2

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Figure 3


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Figure 4

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Figure-4.svg

Name of Material/ Equipment
Antibody anti-PRV gB
Aqua-hold2 pap pen red
Compact emery boards-24 count (100/180 grit nail files)
Complete EDTA-free Protease Inhibitor Cocktail
C57BL/6 mice (5-7 weeks)
DAPI solution (1mg/ml)
Disposable sterile polystyrene petri dish 100 x 15 mm
Dulbecco's Modified Eagle Medium (DMEM)
Dulbecco's Phophate Buffer Saline (PBS) solution
Fetal bovine serum (FBS)
Fine curved scissors stainless steel
Fluoromount-G mounting media
Formalin solution, neutral buffered 10%
Isothesia Isoflurane
Microcentrifuge tube 2ml
Microtube 1.5ml
Negative goat serum
Penicillin/Streptomycin
Precision Glide needle 18G
Razor blades steel back
RNA lysis buffer (RLT)
Stainless Steel Beads, 5 mm
Superfrost/plus microscopic slides
Tissue lyser LT
Tissue-Tek OCT
488 (goat anti-mouse)

Company	Catalog Number
Made by the lab	
Fisher scientific	2886909
Revlon	
Sigma-Aldrich	11836170001
The Jackson Laboratories	
Fisher scientific	62248
Sigma-Aldrich	P5731500
Hyclone, GE Healthcare life Sciences	SH30022
Hyclone, GE Healthcare life Sciences	SH30028
Hyclone, GE Healthcare life Sciences	SH30088
FST	14095-11
Fisher scientific	0100-01
Sigma-Aldrich	HT501128
Henry Schein	NDC 11695-6776-2
Denville Scientific	1000945
SARSTEDT	72692005
Vector	S-1000
Gibco	154022
BD	305196
Personna	9412071
Qiagen	79216
Qiagen	69989
Fisher scientific	12-550-15
Qiagen	69980
Sakura	4583
Life Technologies	A11029

Comments/Description

1/500 dilution

1/1000 dilution

1/2000 dilution

To:

JoVE

Editorial office

January 16th, 2020

Revised version of JoVE61121

"Mouse footpad inoculation model for the study of viral-induced neuroinflammatory responses"

Dear Editor, Vineeta Bajaj,

Please consider our revised version of the manuscript as a research article for publication in *JoVE*. We would like to acknowledge the reviewers for their comments and suggestions that improved the quality of the present manuscript.

We have attached our response to the reviewers' comments as well as a revised version of the manuscript with changes.

We are looking forward to your evaluation.

Sincerely,

Dr. Kathlyn Laval

Prof. Dr. Lynn Enquist

Editorial comments:
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
The manuscript has been thoroughly proofread.

2. Please include a single line space between each step, substep, and note of the protocol.
This has been corrected in the text.

3. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example Bain non-breathing circuit diaphragm, etc.
Commercial language has been removed from the manuscript.

4. Please reword 318-321 as it matches with previously published literature.
This paragraph has been rewritten.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc. The Protocol should contain only action items that direct the reader to do something.
This has been corrected in the text. Usage of phrases such as "could be," "should be," and "would be" have been removed from the protocol section. All safety procedures and use of hoods have been mentioned in lines 114-122.

7.Please ensure you answer the "how" question, i.e., how is the step performed?
This has been addressed in the protocol section.

8. 1.1: Age, sex, strain of mice used for the experiment?
This has been added in the text in line 127 and in the table of materials and reagents.

9. 1.3: How do you visually identify each of the layers?
The detached stratum corneum can be visualized by hematoxylin and eosin staining under light microscopy. The corneocytes present in the stratum corneum are flat, eosinophilic cells that lack nuclei. This comment has been added in the text in lines 457-459.

10. 2.1: How did you determine the viral dose?
The viral dose was optimized in a preliminary experiment. Briefly, we inoculated mice with several virus doses (10^5, 10^6, 10^7 and 10^8 PFU). We selected the appropriate dose that ensured all inoculated mice showed clinical symptoms at 82hpi. A note has been added in the text in lines 156-159.

11. 3.1: How was euthanasia performed in your experiment?

The euthanasia was performed by CO2 asphyxiation as mentioned in lines 176-177.

12. 7.2, 7.3: Citation?
Two citations have been added in the text.

13. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Relevant protocol for video recording has been highlighted in blue in the text.

14. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in Figure Legend, i.e. "This figure has been modified from [citation]."
Please find attached two links to the editorial policy of PlosPathogens and Journal of Virology, which apply the CC by license:
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15. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol.
This section has been addressed in lines 453-465.
b) Any modifications and troubleshooting of the technique
This section has been addressed in lines 467-476, 480-488.
c) Any limitations of the technique
This section has been addressed in lines 476-478.
d) The significance with respect to existing methods
This section has been addressed in lines 447-451.
e) Any future applications of the technique
This section has been addressed in lines 496-510.

16. Please sort the materials table in alphabetical order.
The materials table has been sorted in alphabetical order.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
This study described a method to infect mice with PRV (pseudorabies virus) using a footpad model, which can be used to examine neuro-inflammation and neuro-pathogenesis. The details described will be useful not only for PRV, but also for other alpha-herpesvirinae subfamily models, HSV-1 for example. The details of dissecting the tissues and assays that can be used are very well explained. With that said, there are a few minor issues that should be resolved. These issues are summarized below.

Major Concerns:

No major concerns.

Minor Concerns:

1. Could the authors provide data about what the kinetics of virus replication is following infection with PRV (Becker strain) following footpad infection? This would be helpful for readers to understand where 82 hours after infection was in the model presented. For example, was this prior to or after acute infection?
Following footpad inoculation, PRV-Becker was first detected in the inoculated footpad at 24 hpi (approx. 10^4 PFU/mg of tissue). No PRV was detected in DRGs at 24 and 48 hpi. Low PRV load (< 10^3PFU/mg of tissue) was detected in DRGs at 60 hpi. At moribund state (82 hpi), PRV was detected in higher loads in the DRGs (10^5 PFU/mg of tissue) and spinal cord (10^4 PFU/mg of tissue) than in the hindbrain (10^2 PFU/mg of tissue). The incubation period of PRV infection using this model is approx.70-72 hpi (See answer question 2). These results have been described in detail in two studies (Laval *et al.*, 2018; Laval *et al.*, 2019).
Additional information has been provided in the text in lines 393-396.

2. A more in-depth description of the neurological symptoms that occur prior to encephalitis would be useful for readers.
Following footpad inoculation with virulent PRV-Becker strain, after an incubation period of 70-72 hpi mice began showing clinical signs characterized by swelling of the inoculated footpad and frequent tremors. By 82hpi, PRV-Becker infected mice showed constant tremors in the inoculated leg and distinctive PRV symptoms, such as intense scratching and biting of the foot, resulting in severe inflammation of the footpad. However, PRV-Becker infection did not cause encephalitis as the virus barely reached the hindbrain by 82hpi. In contrast, PRV-Becker infection caused a systemic inflammatory response that killed the infected animal. A detailed description of the clinical symptoms was provided in a previous published study (Laval *et al.*, 2018).

Additional information has been provided in the text in lines 365-369.

Reviewer #2:

Manuscript Summary:
This manuscript authored by Laval et al. proposes a very detailed protocol to perform footpad inoculation of neuro-invasive viruses in mouse and study neuro-inflammatory response to the infection. This method is of high interest for all the community working with neuro-virulent pathogens. The protocol is complemented with an example of infection with the virulent strain of pseudorabies virus (PRV-Becker). The manuscript is very well written and detailed. At this stage, only few elements are missing to make it totally suitable for publication:

Major Concerns:
None

Minor Concerns:

1) Line139: "emery board", since numerous emery boards exist with different abrasive properties please specify which one should be preferentially used.

We used compact emery boards 24 counts and 100-180 grit nail files from Revlon. This has been specified in the table of Materials and Reagents.

2) Line 142: paragraph 1.2.2. a picture would really help the ready to visualize how the stratum granulosum should be peeled off.
This step will be covered in the video recording.

3) Line 228: paragraph 4.7. Here a schematic would be very helpful for those who have never collected DRG.
This step will be clearly covered in the video recording.

4) Paragraph 4.8. Authors suggest that the procedure 4.7. can be repeated multiple times. However, it is not mentioned how long the procedure can be performed before the degradation of the samples. This should be specified.
This has been specified in the text in lines 262-263.

5) Paragraph 4.11., authors propose fixation as an alternative use of the collected samples. Is it directly after procedure 4.7.or after 4.9. ? Please specify.
This has been specified in the text in line 270.

6) Paragraph 5.4.: It is unclear why centrifuging at 4°C while disrupting at room temperature just before.
This mistake has been corrected in the text. Centrifuging at RT is fine.

7) Paragraph 5.5.: For RT-qPCR it should be specified that all samples should be collected with RNase free material (beads, tubes, etc) and eventually disrupted with a more appropriate RNA lysis buffer containing 1% betamercaptoethanol (RLT, RA1 or equivalent).
This has been added in the text in lines 287-290 and in the table of Materials and Reagents.

8) Line 278, while we all know DAPI the abbreviation should be totally written before. Could Authors specify the concentration as well? I guess 1µg/ml?
The abbreviation of DAPI has been fully written in the text in line 333 as well as the concentration used (See table of materials and reagents).

9) Paragraph 7.3. Numerous "standard deparaffinization" procedures exist. Could the author at least specify if deparaffinization requires toluen, xylen or if new dewaxing compounds can be used as well or if they alter tissues notably DRG?
This deparaffinization procedure requires xylene. Additional information has been provided in the text in lines 346-349.

10) Have the authors observed superinfection (bacterial or other) after the inoculation? If so, could an antiseptic treatment be locally applied? Which one authors would recommend?
PRV inoculation has been performed on the abraded footpad of approximately 200 mice and so far, we never observed superinfection (bacterial or other).