Formalin-Induced Neuropathic Pain in Mice

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Abstract and/or Background

Neuropathic pain is a chronic pathology of the nervous system (Finnerup et al., 2021), often related to inflammatory conditions, including multiple sclerosis (MS) and Guillain-Barre syndrome (GBS) (Ubogu, 2015). In vitro and vivo studies have investigated the role of proinflammatory cytokines, chemokines, and other inflammatory mediators within the spinal cord (SC) for the development and persistence of neuropathic pain (Tsuda, 2017; Gwak, 2012). In vivo studies have established a formalin-induced pain model in rats to be comparable to spinal nerve ligation (SNL) and induce persistent spinal inflammation (Salinas-Abarca, 2017). It remains unknown whether a formalin-induced pain model can induce neuropathic pain in mice. Our research aims to investigate whether a formalin-induced pain model can induce persistent neuropathic inflammatory pain in mice and to study consequent in vivo mechanisms. Our research, as outlined in **Figure 1**, will modify this existing protocol in C57BL/6 mice; following formalin injection, mice will be assessed for pain sensitivity over a two-week period and sacrificed to collect whole SC's for the following: 1) flow cytometry to detect immune cell infiltration; 2) histological observations using Luxol Fast Blue (LFB) and Golgi stains for qualitative analysis of myelin and neuron morphology and distribution; 3) immunohistochemical markers for glia activation and neuronal pathology, such as Glial Fibrillary Acidic Protein (GFAP) and Ionized Calcium Adaptor Protein 1 (Iba1) and Neuronal Nuclear protein (NeuN). Determining the efficacy of formalin-induced pain models in mice provides potential in other animal models including experimental autoimmune encephalomyelitis (EAE), which simulate MS as an inflammatory demyelinating disease (Gold, 2006). Our research investigates the efficacy of a formalin-induced pain model to induce neuropathic pain in mice and studies the consequent molecular mechanisms to develop novel targeted therapeutics for inflammatory neuropathic pain condition.

Introduction and/or Research Question

Methods

Mouse Treatment: Eight-week-old healthy male or female BALB/c mice were kept on a 12h light/dark cycle with food and water ad libitum. Mice were injected with 20-µl formalin created by diluting formaldehyde to 5% in PBS, was injected into the plantar surface of the mouse hind paw. Pain was assessed over a two-week period using the Von Frey test, then the mice were anesthetized and sacrificed through intracardial perfusion of a solution of 0.9% cold saline and then quickly followed with 1% Paraformaldehyde (PFA) in 0.1 M Phosphate-Buffered Saline (PBS) with a pH of 7.4. The entire spinal cord was collected for use in flow cytometry and histology.

Flow cytometry: The meninges were removed from the spinal cords and the tissue was minced in Hank's Balanced Salt Solution (HBSS) treated with trypsin and collagenase in Dulbecco's modified eagle's medium (DMEM) then passed through a 40µm cell strainer and resuspended in ACK lysing buffer. Cells were washed with PBS and fluorescence-activated cell sorting (FACS) buffer, incubated for 10 minutes with Fc Block, then for another 30 minutes in FACS buffer with antibodies against CD45, CD40, CD19, CD27, CD3, CD4, and CD8, or isotype control, to detect infiltrating immune cells. The cells were then fixed with 4% paraformaldehyde in PBS. Data was gathered using a Flow Cytometer and analyzed using FlowJo software.

Histology: The freshly dissected spinal cord sections intended for LFB staining were put into a 1% PFA for 24 h at 4°C for fixation. For histological analysis, the tissues underwent gradual dehydration: 70% ETOH/24 hours, 96% ETOH/20 minutes, 96% ETOH/20 minutes, 100% ETOH /30 minutes, and finally xylol/30 minutes. The tissues were then embedded in blocks of paraffin cassettes at 56°C for 2 hours. For sectioning, the tissues were cut into 8-µm sections using a microtome and prepared for staining. The tissues were rehydrated using Xylol/30 seconds, alcohol: absolute/30 seconds, 96%/30 seconds, 70%/30 seconds, and distilled water/30 seconds. The slides were then placed in 0.1% LFB solution/2 minutes and rinsed with distilled water. Coverslips were placed onto the slides with Omnimount and then visualized with a Leica Aperio microscope. For tissues that were stained with the Golgi method, the fresh spinal cord sections were placed into a 5% PFA solution for at least 2 days at 21°C at a pH of 7.4. After fixation, the spinal cord tissues were placed into a freshly made chromating solution with 3% potassium dichromate, 4% PFA, 2% glutaraldehyde, and PBS with a pH of 7.4. The solution was changed every 24 hours, and the tissues were infiltrated by the chromating solution for 48 hours. The spinal cord tissue was then washed multiple times with a 2% silver nitrate/ distilled water solution, and then incubated in silver nitrate for 48 hours. During the 48 hour staining period, the spinal cord tissue was covered in aluminum foil to prevent light exposure. After the staining period ceased, the tissue blocks were then sectioned at 50 µm, and mounted on to slides with Omnimount. The slides were visualized using a Leica Aperio microscope.

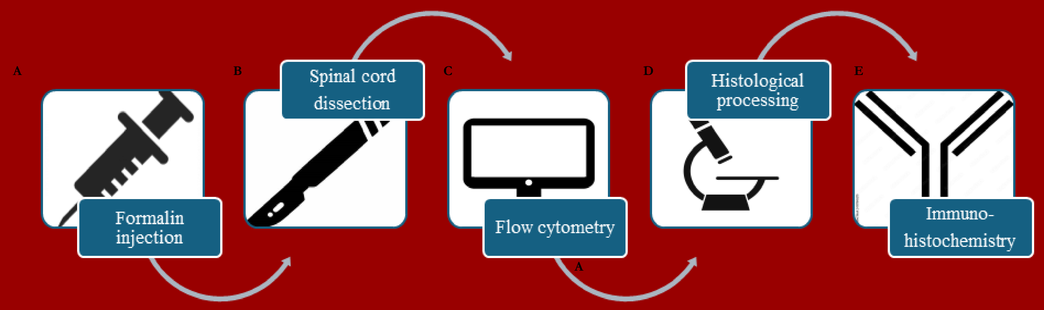

Figure 1. Formalin Induced Pain and Processing of Spinal Cord Tissue
Above represents the general outline of the methods used within this study. **A)** Formalin is injected into the mice to potentially induce a CNS inflammatory response. The mice were analyzed for pain symptoms for 2 weeks before being sacrificed. **B)** The mice were sacrificed and the spinal cord was removed for processing. **C)** Flow cytometry of the spinal tissue was conducted and used to quantify the number of immune cells present within the spinal cord tissue. **D)** Histologic processing accounted for the staining of the spinal tissue with two differential stains, LFB and The Golgi Method. **E)** Immunohistochemistry was utilized to analyze glial activation and neural pathology to detect signs of inflammation.

Results and/or Conclusion

The expected results pertain that immune cells will be present within the spinal tissue due to the prolonged inflammation and immune stimulation. Thus, deeming a successful crossing of formalin through the blood brain barrier to initiate an immune/inflammatory response. Therefore, our conclusion is that the formalin pain model can be applied to murine models to initiate a pain response within the CNS.

Future Work

1. This will be useful to observe neural pathways in response to inflammation and pain. Morevoer, this research can supplement future studies in which the focus is to understand the pathways to treat patients with chronic pain such as individuals with MS or GBS.

References and/or Acknowledgments

Finnerup, N. B., Kuner, R., & Jensen, T. S. (2021). Neuropathic pain: From mechanisms to treatment. Physiological Reviews, 101(1), 259–301. https://doi.org/10.1152/physrev.00045.2019

Gold, R. (2006). Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. Brain, 129(8), 1953–1971. https://doi.org/10.1093/brain/awl075

Gwak, Y. S., Kang, J., Unabia, G. C., & Hulsebosch, C. E. (2012). Spatial and temporal activation of spinal glial cells: Role of gliopathy in central neuropathic pain following spinal cord injury in rats. Experimental Neurology, 234(2), 362–372. https://doi.org/10.1016/j.expneurol.2011.10.010

Salinas-Abarca, A. B., Avila-Rojas, S. H., Barragán-Iglesias, P., Pineda-Farias, J. B., & Granados-Soto, V. (2017). Formalin injection produces long-lasting hypersensitivity with characteristics of neuropathic pain. European Journal of Pharmacology, 797, 83–93. https://doi.org/10.1016/j.ejphar.2017.01.018

Tsuda, M., Koga, K., Chen, T., & Zhuo, M. (2017). Neuronal and microglial mechanisms for neuropathic pain in the spinal dorsal horn and anterior cingulate cortex. Journal of Neurochemistry, 141(4), 486–498. https://doi.org/10.1111/jnc.14001

Ubogu EE (2015) Inflammatory neuropathies: pathology, molecular markers and targets for specific therapeutic intervention. Acta Neuropathologica 130:445–468. https://doi.org/10.1007/s00401-015-1466-4