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Permalink

https://escholarship.org/uc/item/9v1985gb

Journal

Journal of visualized experiments : JoVE, 2016(109)

ISSN

1940-087X

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Publication Date

2016-03-01

DOI

10.3791/53404

Peer reviewed

Title: A versatile murine model of subcortical white matter stroke for the study of axonal degeneration and white matter neurobiology.

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Abstract

Stroke affecting white matter accounts for up to 25% of clinical stroke presentations, occurs

silently at rates that may be 5-10 fold greater, and contributes significantly to the development of

vascular dementia. Few models of focal white matter stroke exist and this lack of appropriate

models has hampered understanding of the neurobiologic mechanisms involved in injury

response and repair after this type of stroke. Here we present a methodology for the reliable

production of a focal stroke in murine white matter using a local injection of a reversible eNOS

inhibitor. We also present several variations on the general protocol including two unique

stereotactic approaches, retrograde neuronal tracing, as well as fresh tissue labeling and

dissection that greatly expand the potential applications of this technique. These variations allow

for multiple approaches to analyze the neurobiologic effects of this common and understudied

form of stroke.

Introduction

Stroke affecting the subcortical white matter is a common clinical entity, accounting for up to 25% of clinical strokes annually in the US [1]. Ischemic damage to white matter also occurs silently at a significantly higher rate and contributes to the development of vascular dementia [2, 3]. Presently, patients with this form of cerebral ischemia have few, if any treatment choices. Despite the clinical importance of this disease, few clinically relevant animal models exist [4].

We previously described a model of subcortical white matter stroke using ET-1 [5] but have since made several key changes to the experimental protocol and expanded the experimental use of this model [6, 7]. This protocol provides a reliable and modifiable strategy to produce a focal stroke within mouse brain white matter. The major advantages of this model are the use of a chemical eNOS inhibitor (L-Nio) with no known paracrine effects on cellular elements of white matter. In addition, the stereotactic targeting of white matter in the mouse allows the use of any variety of transgenic or knockout strains, greatly expanding the available tools to determine the effect of stroke on brain white matter. Here, we describe two variations on this technique and demonstrate some of the additional variations that can be utilized to enhance our understanding of axonal and white matter damage and repair after stroke.

Protocol

The use of animals in this protocol has been performed in accordance with procedures approved by the University of California Los Angeles Animal Care and Use Committee.

Begin by identifying the target murine population. We have mostly used male wild-type C57/Bl6 mice, however various transgenic or knockout mice can also be used. In some data presented here, we utilized the CNP bacTRAP transgenic mouse expressing EGFP bound to the ribosomal protein L10a under control of the CNP promoter [8]. Note that stereotactic coordinates are based on C57/Bl6 anatomy. We recommend that each user should initially verify localization of the stroke to white matter. Stereotactic coordinates can then be adjusted slightly as needed. In our hands, this method produces a focal white matter lesion approximately 500 microns in diameter and extends approximately 1 mm along the anterior-posterior axis of the corpus callosum.

White matter stroke induction – lateral angled approach

1. Begin by preparing a pulled glass pipette using 0.5 mm capillary tubes. Distal diameter should be between 15-25 microns.

2. Prepare a sterile aliquot of L-Nio (N(5)-(1)-iminoethyl-L-ornithine HCl) (Calbiochem) at 27.4 mg/mL in sterile 0.9% normal saline.

3. Pre-fill the pulled glass pipette with a small volume of L-Nio (2-5 uL) using a horizontal vacuum-assisted approach.

4. Anesthesize mouse using standard isoflurane anesthesia (2L/min inhaled) and place into a stereotactic apparatus equipped with a stereotactic microscope.

5. Adjust the injection arm to 36 degrees.

6. Affix a pulled glass pipette holder to the distal end of a PicoSpritzer and attach to the injection arm.

7. Prepare a sterile surgical field.

8. Mark Bregma as a reference point.

9. Drill a 2 mm ellipitical craniotomy beginning posteriorly at Bregma and extending anteriorly.

10. Remove bone fragments and overlying soft tissue so that the cerebral cortex can be visualized.

11. Affix a pulled glass pipette to the injector arm of the stereotactic apparatus.

12. Align the distal end of the pipette with Bregma and zero the stereotactic coordinates.

13. Advance the pipette to the first A/P and M/L coordinates provided in Table 1.

14. Advance the pipette to the cortical surface and zero the D/V.

15. Slowly pass the pipette into the brain until reaching the first D/V coordinate in Table 1.

16. Using the PicoSpritzer at 20 psi set to 20 msec pulses, inject 100 nL of L-Nio into the brain and wait 5 min to prevent reflux up the pipette track.

17. Slowly withdraw the pipette and repeat steps 14-18 at the second and third set of coordinates provided in Table 1.

18. After the final injection, cover the craniotomy with bone wax and close the scalp wound with VetBond.

19. Inject 0.1 cc of 1% Marcaine into the wound margins to prevent local pain associated with the scalp incision.

20. Return the animal to housing and supply post-operative antibiotics (0.48 mg/mL
trimethoprim-sulfamethoxazole) in the drinking water for 5 days.

White matter stroke induction – posterior angled approach

1. Perform steps 1-12 as in the lateral angled approach protocol, except adjust the injection
   arm of the stereotactic setup to 45 degrees oriented anterior to posterior.

2. Advance the pipette to the first A/P and M/L coordinates provided in Table 2.

3. Complete remaining steps 14-20 as in the lateral angled approach protocol.

Retrograde neuronal labeling

1. Prepare a sterile aliquot of L-Nio at 54.8 mg/mL in 0.9% normal saline.

2. Prepare a sterile aliquot of 20% Fluororuby (or 20% biotinylated dextran amine or 2%
   Fluorogold) (Fluorochrome, Inc.) in 0.9% normal saline.

3. Dilute together 1:1 for final concentrations of 27.4 mg/mL L-Nio and 10% Fluororuby.

4. Perform the stroke protocol as above using this solution.

5. Visualize the natively fluorescent tracer in tissue section.

Tissue processing for immunofluorescence

At an appropriate post-stroke interval ranging from 3 hrs to 14 days after stroke, mice are
euthanized via isoflurane overdose, transcardially perfused with PBS followed by 4%
paraformaldehyde and brains removed, post-fixed in 4% PFA for 24 hrs and cryoprotected in
30% sucrose for 48 hrs. Forty micron floating sections and antibody processing are performed as
previously described [5-7].

Tissue processing for protein or RNA analysis

At an appropriate post-stroke interval ranging from 3 hrs to 14 days after stroke, mice are euthanized and the brain freshly dissected.

1. Using a brain block, 2-3 mm slabs containing the stroke are prepared and placed in to cold dissection buffer containing protease inhibitors and RNase inhibitors.

2. Under a dissecting microscope, identify the white matter underlying motor cortex in the injected hemisphere. At longer post-stroke intervals, the region *may* be visually identified by focal necrosis and myelin pallor. At earlier post-stroke intervals, injection of L-Nio mixed with 1 uL of 10% Fast Green (Sigma) can allow visual identification of the stroke (Figure 4A).

3. Under guidance of a dissecting microscope and using a fresh scalpel, carefully dissect the region of white matter containing the stroke, removing overlying cortex and underlying striatum as desired.

4. Homogenize the tissue thoroughly and follow additional protocols for the isolation of protein and/or RNA.

Representative Results

Using the model presented, the white matter underlying forelimb sensorimotor cortex can reliably be targeted. This chemically induced stroke model produces focal axonal and myelin loss, astrocytosis, and microgliosis (Figure 1), as is typically seen in human lacunar infarcts. By using three injections, a clinically useful model is established with early impairment on forelimb motor tasks [6] and a significant portion of brain tissue experiences ischemia that immunohistochemical, immunofluorescent, and biochemical techniques are feasible and reliable at the quantitative level. At early time points (hours) after stroke induction, changes in axonal molecular organization can be detected (Figure 2). At 7 days, the stroke completes its maturation to a circumscribed area of axonal loss (Figure 1a). By 7 days, the average infarct size is 10.5 mm3 and will have an elliptical shape. Further growth in the size of the infarct is rarely seen beyond 7 days.

The addition of a simultaneous injection of dextran amine results in significant neuronal labeling in layer 5 and layer 6 neuronal cell bodies that have axons projecting through the region of stroke (Figure 3). Overlying cortical neurons that are not damaged by the sham aspects of the procedure (passage of fine needle), undergo distal axonal damage and can be identified by the inclusion of a tracer with the L-Nio preparation. This approach was used to demonstrate dynamic changes in the axon initial segment after stroke [7].

While the region of tissue affected by the stroke limits the use of other common techniques such as TTC staining, the white matter stroke region can be identified in fresh tissue. The addition of a

common dye such as Fast Green produces an identifiable region of tissue that can be dissected under a dissecting microscope (Figure 4). Once dissected this tissue can be used for protein analysis with western blot or immunoprecipitation, or for RNA isolation and analysis (Figure 4C). Through the use of various transgenic mouse lines, a variety of innovative approaches can be used to study the neurobiology after focal white matter stroke including cell fate mapping studies, laser capture microdissection, and translating ribosomal affinity purification (Figure 4C).

Discussion

A number of prior models of subcortical stroke have been described including focal injections of endothelin-1 into the internal capsule, subcortical white matter and striatum in the rat [9-11] and mouse [5, 12]. More recent models of small focal strokes have utilized cholesterol microemboli injection in the carotid artery [13] and photothrombotic occlusion of a single penetrating arteriole [14]. Each of these models has both advantages and disadvantages [4]. The presently described model produces a lesion that has a number of characteristics that mimic human lacunar infarction including axonal abnormalities and loss, myelin degradation, a focal necrotic core and a clinical deficit that is minimal and demonstrates fairly rapid recovery dependent on age [6]. Targeting murine white matter allows a wide variety of genetic manipulations that can support mechanistic studies.

Focal injection of ET-1 using the approach described here has been reported but endothelin-1 was shown to have direct paracrine effects on oligodendrocyte differentiation and maturation [15, 16] confounding the study of post-stroke white matter biology. In contrast, the L-Nio approach targets endothelial cells alone while producing an identical lesion and eliminates any confounding paracrine effects on the cells involved in injury response. L-Nio is not directly cytotoxic and the selected dose was determined by preliminary dose escalation experiments (data not shown). The posterior angled approach was developed to more precisely undercut axons from primary motor cortex producing the maximal behavioral deficit that can be attributed to white matter injury. The lateral angled approach also damages motor cortex axons but extends more laterally and involves axons underlying primary sensory cortex.

The stroke lesion expands fairly rapidly over the first 24 hrs. By seven days, the size of the infarct is maximal and we have not observed significant lesion growth beyond that time. Additional cellular events and axonal degeneration will occur beyond this initial stage but the infarct size as measured by the necrotic core will not change significantly in the absence of any intervention.

Co-injection of neuroanatomical tracers at the time of stroke identifies neurons experiencing ischemic axonal injury. Using either the lateral or posterior angled approach, the neuronal cell bodies with damaged axonal projections remain unharmed. This creates a useful model to study the effect of ischemic axotomy on central nervous system neurons. We have utilized primarily retrograde tracers including dextran amine and fluorogold, which both show excellent uptake by stroke-damaged axons.

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Figure legends

Figure 1. Focal white matter stroke using both lateral and posterior angled approaches. Immunofluorescent labeling for neurofilaments (A, red) demonstrates the degree of axonal loss seven days after stroke using the lateral approach. Using the posterior angled approach, the white matter stroke lesion is targeted just above the lateral ventricle (B, C) and shows intense microglial (B) and astrocytic reactivity (C). Two astrocyte intermediate filament markers, vimentin (red) and glial fibrillary acidic protein (GFAP, green), both reveal changes in morphology of white matter (fibrous) astrocytes after stroke (C). Scale bars = 500 μm.

Figure 2. White matter stroke alters axonal microdomain organization. Within 3 hours of white matter stroke induction, axonal microdomain organization at the node, marked by beta-IV spectrin (red) and at the paranode, marked by contactin-associated protein (caspr, green), is disrupted (B). Contralateral white matter axons show regular nodal, paranodal, and juxtaparanodal organization (A & C) while the ipsilateral white matter shows nodal and paranodal elongation that is typical of axons with lost axoglial contact (B & D). Scale bar = 5 μm.

Figure 3. Retrograde neuronal labeling with white matter stroke identifies individual neurons with axonal damage. Co-injection of fluorescent dextran amine (red) at the time of stroke induction allows identification of individual neurons with axons injured by stroke. Most of the labeling occurs in axons within Layer 5 and 6 neurons in primary sensorimotor cortices overlying the stroke. Scale bar = 500 μm.

Figure 4. Microdissection of white matter stroke lesions for use in biochemical and transcriptional assays. Co-injection of Fast Green at the time of stroke induction allows early identification of the injured region at 24 hrs (A, upper panel). Immunoblotting for specific axonal proteins can be performed to demonstrate reductions in the region of stroke alone (A, lower panel). At longer time points, the region of white matter stroke can be focally dissected without specific labeling (B). PCR for oligodendrocyte-specific genes using dissected regions from the CNP bacTRAP mouse [17] that expressed GFP-tagged ribosomes in mature oligodendrocytes. IL = ipsilateral; CL = contralateral; c = control; s = stroke.

Table 1. Stereotactic coordinates for lateral angled approach

Injection	Anterior/Posterior	Medial/Lateral	Dorsal/Ventral
1	0.22	0.22	-2.10
2	0.70	0.15	-2.16
3	1.21	0.15	-2.18

Table 1. Stereotactic coordinates for posterior angled approach

Injection	Anterior/Posterior	Medial/Lateral	Dorsal/Ventral
1	-0.75	-0.96	-2.10
2	-1.00	-0.96	-2.05
3	-1.25	-0.96	-2.00