Microglial density and morphology after respiratory motor neuron death in a novel neurodegenerative rat model

KAYLIE A. CANDA AND NICOLE L. NICHOLS

Department of Biomedical Sciences, University of Missouri, Columbia, MO 65211

Abstract

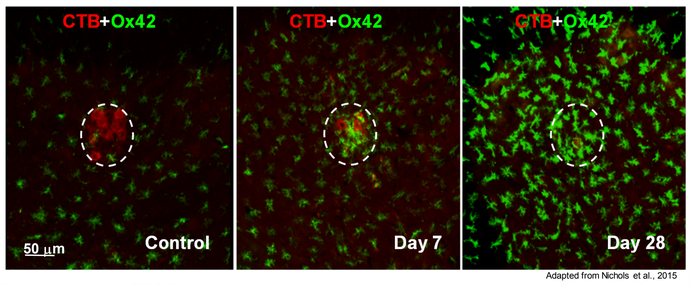
Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease causing impaired respiratory function and death due to loss of motor neurons (*e.g.,* phrenic and intercostal) innervating inspiratory muscles. Increased microglia corresponds with motor neuron death, but whether this increase is beneficial or harmful remains unknown. Microglia can exist in multiple states including resting (ramified) and activated (amoeboid) morphology. In a novel rat model of respiratory motor neuron death induced by intrapleural injections of cholera toxin B conjugated to saporin (CTB-SAP), increased microglial density was seen in the phrenic motor nucleus. However, less is known about microglia in the intercostal region. The aim of this study is to analyze microglial density in the intercostal motor nucleus, as well as microglial morphology in the phrenic and intercostal motor nuclei. We hypothesize that there will be an increase in microglial density in the intercostal motor nucleus in CTB-SAP rats, as well as amoeboid microglial morphology in the phrenic and intercostal motor nuclei. Spinal cord sections from CTB-SAP rats were stained for microglia using immunohistochemistry, visualized using confocal microscopy, and are being analyzed using ImageJ and IMARIS. We have observed increased intercostal microglial density in 7 (not 28) day (d) CTB-SAP rats ($p<0.05$ *vs.* controls); however, we expect that microglia from all CTB-SAP rats will have an amoeboid morphology. These results would indicate increased microglial activation in areas controlling inspiration, but we suggest that microglia may play a differential role in intercostal function (*i.e.*, harmful at 7d since density increased and beneficial at 28d since density decreased).

Rationale

1 A. Intrapleural cholera toxin B fragment conjugated to saporin (CTB-SAP) mimics aspects of neuromuscular disorders and neurodegenerative diseases

Variables	25 μg CTB-SAP, 7d	25 μg CTB-SAP, 28d
Phrenic motor neuron survival	~40%	~40%
Phrenic motor output	Decreased > 50%	Decreased > 50%
Respiratory function	Decreased	Decreased

B. Microglial density is increased in the phrenic motor nucleus in CTB-SAP treated rats



Adapted from Nichols et al., 2015

Figure 1: A. Intrapleural CTB-SAP injections mimic aspects of neuromuscular disorders and neurodegenerative diseases including the amount of phrenic motor neuron survival, phrenic motor output, and respiratory function. **B.** Microglial density (green) is increased near CTB(+) phrenic motor neurons (red) in the phrenic motor nucleus (denoted by dashed, white circle) in CTB-SAP treated rats.

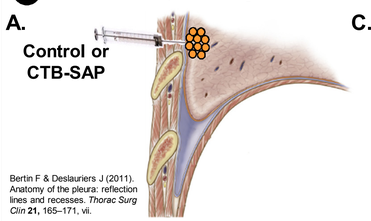
It remains unknown how microglial density is affected in the intercostal motor nucleus or how microglial morphology is affected in either the phrenic or intercostal motor nuclei of CTB-SAP treated rats.

Hypothesis

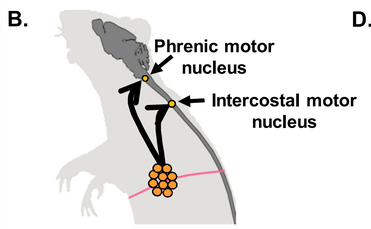
In CTB-SAP treated rats, there will be an increase in microglial density in the intercostal motor nucleus, as well as amoeboid morphology in both the phrenic and intercostal motor nuclei.

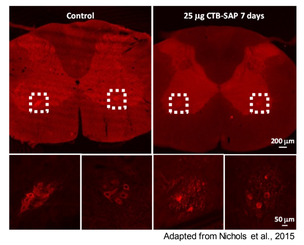
Materials & Methods

2 Bilateral, intrapleural injection, tissue preparation, and IHC:

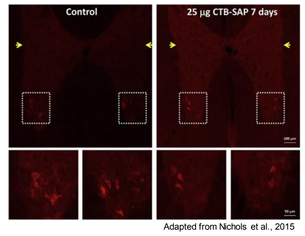


Bertin F & Deslauriers J (2011). Anatomy of the pleura: reflection lines and recesses. *Thorac Surg Clin* **21**, 165–171, vii.





Adapted from Nichols et al., 2015



Adapted from Nichols et al., 2015

Figure 2: **A & B.** 25 μg Cholera toxin B conjugated to saporin (CTB-SAP; Advanced Targeting Systems) or control (cholera toxin B (CTB; Calbiochem) not conjugated to saporin (SAP; Advanced Targeting Systems); CTB+SAP) was bilaterally, intrapleurally injected into adult male Sprague-Dawley rats (**A.**), which was then retrogradely transported along the phrenic and intercostal nerves to the cell bodies of phrenic and intercostal motor nuclei (**B.**). **C & D:** 7 and 28 day treated control and CTB-SAP treated rats were then perfused with 4% paraformaldehyde, the cervical and thoracic spinal cords containing the phrenic and intercostal nuclei, respectively, were isolated and sectioned at 40 μm using a freezing-sliding microtome, and then sections from C4 (**C.**) and T2-T7 (**D.**) were prepared for immunohistochemistry (IHC). CTB-SAP injection results in decreased phrenic motor neuron survival (**C.**) and intercostal motor neuron survival (**D.**); representative CTB(+) staining for the phrenic and intercostal motor nuclei (white squares in top panels of C and D, respectively) is shown at higher magnification in the bottom panels (**C and D**, respectively) for 7 day control and CTB-SAP treated rats.

DAY 1 **IHC protocol for microglia**

- Select 6 sections containing either the phrenic or intercostal motor nuclei for 8 animals/group.
- Wash tissue in 1X PBS for 3 X 5 min. on shaker at RT and then incubate tissue in 1X PBS + 0.2% Triton + 5% normal donkey serum for 1 hour on shaker at RT.
- Incubate tissue in 1X PBS + 0.1% Triton + 5% normal donkey serum + antibody against CTB (goat, 1:2000, Calbiochem) and Cd11b (mouse 1:500, Bio-Rad) O/N at 4°C on shaker.

DAY 2

- Wash tissue in 1X PBS for 3 X 5 min. on shaker at RT.
- Incubate tissue in 1X PBS + 0.1% Triton + 5% normal donkey serum + donkey anti-goat Alexa-Fluor 555 and donkey anti-mouse Alexa-Fluor 488 (both 1:1000; Invitrogen) for 2 hours on a shaker in the dark at RT.
- Wash covered tissue in 1X PBS for 3 X 5 min. on shaker at RT.
- Mount tissue on glass slides, apply anti-fade, coverslip, and store at -4°C until imaging and quantification of staining is performed using a Leica DM4000 microscope at 20x magnification and ImageJ.

3 IMARIS software to quantify microglial projections

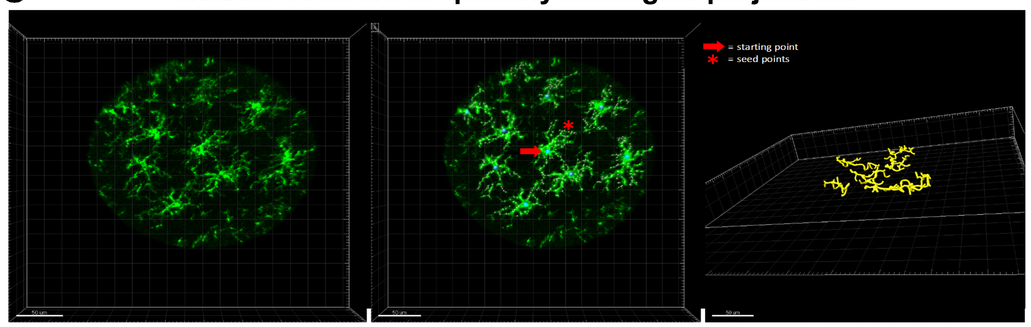


Figure 3: Visual representation of the skeleton and masking process that is being used to quantify microglial projections. In other words, we are quantifying average branch length of microglial projections, average number of branches projecting from the microglial cell body, and average number of end-points present on the branches of microglial projections to determine if CTB-SAP treated rats have microglia that have decreased branch length, number and end-points which would suggest morphology indicative of the activated microglial state (*i.e.*, amoeboid morphology). Left image: ROI for mask of Cd11b(+) cells (*i.e.*, microglia). Center image: depiction of how branching quantification is conducted with branching starting points (denoted by arrow) and seed points for tracing branching (denoted by *). Right image: 3D representation of microglial branching.

4 Intercostal Microglial Density Results

Microglial density is increased in the intercostal motor nucleus of CTB-SAP treated rats

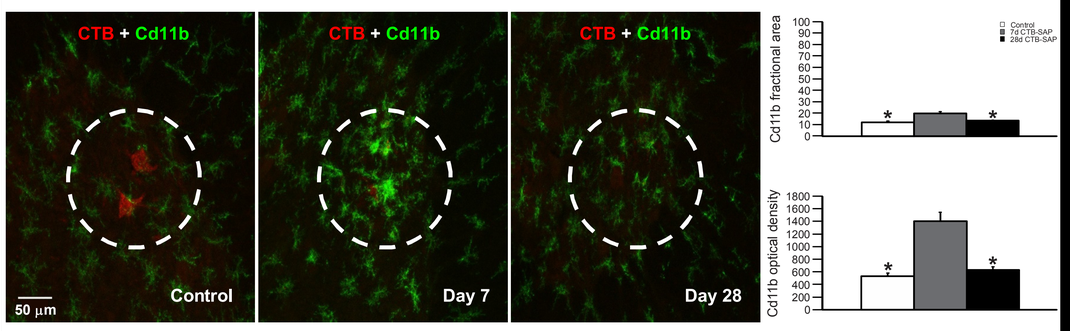


Figure 4: Representative photomicrographs from a control (**left image**) and CTB-SAP treated rats (day 7, **middle image**; day 28, **right image**), where microglial density (Cd11b; green) is increased near CTB(+) intercostal motor neurons (red) in the intercostal motor nucleus (denoted by dashed, white circle) in 7 day CTB-SAP treated rats. 7 day CTB-SAP treated rats have increased Cd11b fractional area (**top graph**) and optical density (**bottom graph**) in the intercostal motor nucleus in comparison to control and 28 day CTB-SAP treated rats (* = $p<0.05$).

IMARIS Results

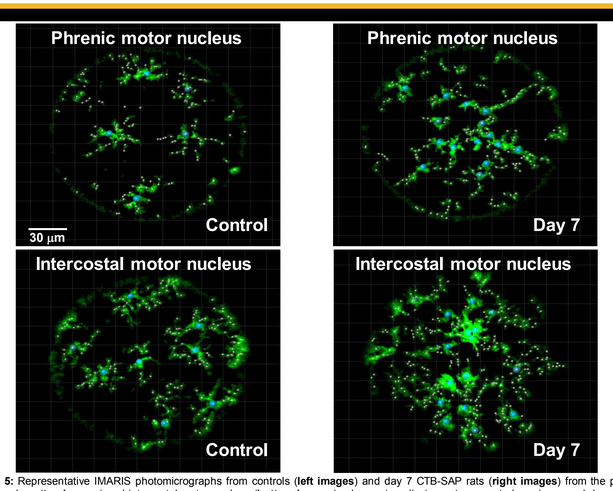


Figure 5: Representative IMARIS photomicrographs from controls (**left images**) and day 7 CTB-SAP rats (**right images**) from the phrenic motor nucleus (**top images**) and intercostal motor nucleus (**bottom images**), where microglia (green) appear to have increased density and amoeboid morphology in both motor nuclei. Next, we will quantify microglial morphology and expect to observe decreased number and length of branches (indicative of amoeboid morphology) in the intercostal and phrenic motor nuclei of CTB-SAP treated rats *vs.* controls.

Implications & Future Directions

- Increased microglial density and amoeboid morphology suggest microglial activation in the intercostal and phrenic motor nuclei.
- Increased microglial activation in these regions could suggest that microglia may play a role in respiratory function. Since respiratory function is impaired in patients that suffer from respiratory motor neuron death (*e.g.*, ALS), knowing the role microglia play can contribute to knowledge of the disease process and potential avenues of therapy.
- Future directions will be focused on understanding which factors are produced by microglia in these motor nuclei in CTB-SAP treated rats, and whether these factors impact breathing.

References

1. Nichols, N.L. et al. *Exper. Neurol.* 2015
2. Bertin, F. & Deslauriers, J. *Thorac. Surg. Clin.* 2011
3. Hanisch U.K. & Kettenmann H. *Nat. Neurosci.* 2007

Acknowledgements

Project supported by:
University of Missouri College of Veterinary Medicine Committee on Research (COR) Grant,
AVMA/AVMF 2nd Opportunity Research Scholarship, and the Spinal Cord Injury and Disease Research Program (**SCIDRP**)

Veterinary Research Scholars Program
University of Missouri