Microglial density and morphology following CTB-saporin induced respiratory motor neuron death

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Abstract

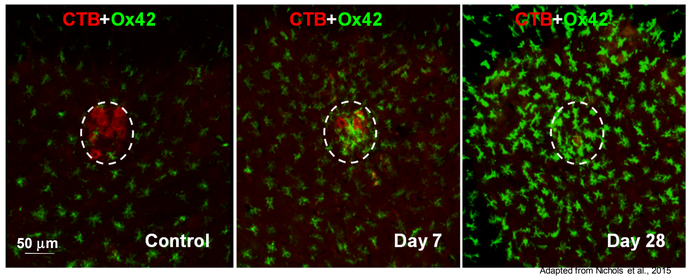
Motor neuron death and increased microglia (resident immune cells in the CNS) are observed in many neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS). Loss of motor neurons (*e.g.,* phrenic and intercostal) innervating inspiratory muscles (*e.g.,* diaphragm and intercostal muscles) leads to impaired respiratory function and eventually death in ALS. In addition, increased microglial density corresponds with motor neuron death, but whether this increase is beneficial or harmful remains unknown. Microglia can exist in multiple states including resting (ramified morphology) and activated (amoeboid morphology). Increased microglial density is also observed in the phrenic motor nucleus in a novel rat model of respiratory motor neuron death induced by intrapleural injections of cholera toxin B conjugated to saporin (CTB-SAP); however, less is known about microglia in the intercostal motor nucleus. In this study, microglial density and morphology in the intercostal motor nucleus as well as microglial morphology in the phrenic motor nucleus will be analyzed. Cervical and thoracic spinal cord sections containing the phrenic and intercostal motor nuclei from CTB-SAP treated rats will be stained for microglia using immunohistochemistry techniques, visualized using confocal microscopy, and analyzed using ImageJ and IMARIS software. We hypothesize that there will be an increase in microglial density in the intercostal motor nucleus, as well as amoeboid microglial morphology in both the phrenic and intercostal motor nuclei. If the data support our hypotheses, this would indicate increased microglial activation in areas controlling inspiration and suggest that microglia may play a role in respiratory function.

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 and tissue preparation:

A and 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 and 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.

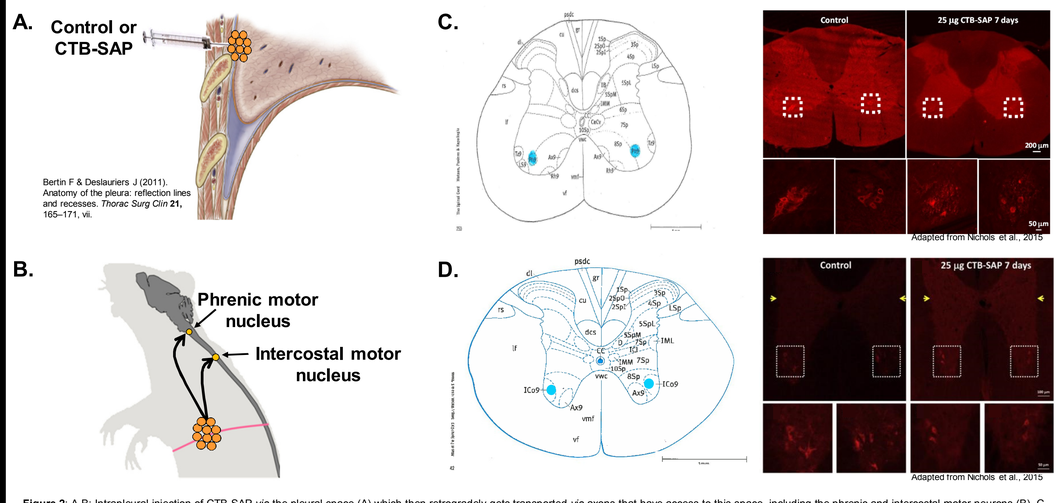


Figure 2: A-B: Intrapleural injection of CTB-SAP *via* the pleural space (A) which then retrogradely gets transported *via* axons that have access to this space, including the phrenic and intercostal motor neurons (B). C-D: CTB-SAP injection results in decreased phrenic motor neuron survival (C) and 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 .

IHC Protocol

DAY 1

- Select 6 sections containing either the phrenic or intercostal motor nucleus as shown in Figure 2 for each animal (8 animals per group).
- Wash tissue in 1X PBS for 3 X 5 min. on shaker at RT.
- Incubate tissue in blocker solution (1X PBS + 0.2% Triton + 5% normal donkey serum) for 1 hour on shaker at RT.
- Incubate tissue in primary antibody solution (1X PBS + 0.1% Triton + 5% normal donkey serum + antibody against CTB (goat polyclonal, 1:2000, Calbiochem, Billerica, MA) and Cd11b (mouse monoclonal, 1:500, Bio-Rad, Hercules, CA) overnight at 4°C on shaker.

DAY 2

- Wash tissue in 1X PBS for 3 X 5 min. on shaker at RT.
- Incubate tissue in secondary antibody solution (1X PBS + 0.1% Triton + 5% normal donkey serum + donkey anti-goat Alexa-Fluor 555 (polyclonal, 1:1000; Invitrogen, Carlsbad, CA) and donkey anti-mouse Alexa-Fluor 488 (polyclonal, 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, and coverslip.
- Covered slides are stored at -4°C until imaging and quantification of staining is performed using a Leica DM4000 microscope at 20x magnification and ImageJ, respectively.

3 IMARIS software to quantify microglial projections

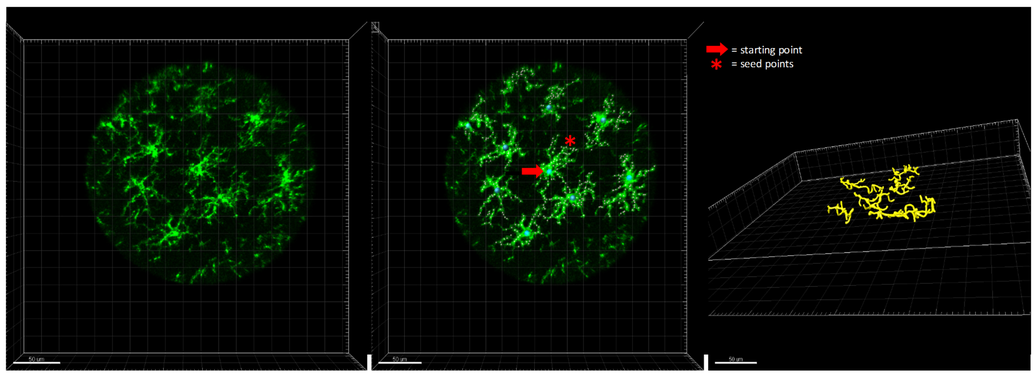


Figure 3: Visual representation of the skeleton and masking process that is being used to quantify microglial projections. In other words, we are quantifying average branch length of microglial projections, average number of branches projecting from the microglial cell body, and average number of end-points 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 microglia branching.

Expected Results

- **We expect to see increased microglial density in the intercostal motor nucleus of CTB-SAP treated rats *vs*. controls.**
- **Amoeboid microglial morphology (*e.g.*, decreased number and length of branches) is expected to be seen in the intercostal motor nucleus and phrenic motor nucleus of CTB-SAP treated rats *vs*. controls.**

Implications & Future Directions

- **Increased microglial density and amoeboid morphology would suggest increased microglial activation in the intercostal and phrenic motor nuclei, areas that control inspiration.**
- **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 the phrenic and intercostal 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
4. Sengul, G. et al., *Atlas of the Spinal Cord* 2012

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