THE EFFECTS OF TONGUE INJECTION OF CTB-SAP ON VENTRAL HYPOGLOSSAL MOTOR NEURONS: A NOVEL MODEL OF ALS

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Introduction and Rationale

- Amyotrophic lateral sclerosis (ALS) is a progressive disorder in which the death of motor neurons leads to a loss of voluntary muscle control.
- Most patients lose the ability to breathe and have to be placed on a ventilator, while many experience dysphagia (swallowing deficits) which often leads to aspiration pneumonia and/or placement of a feeding tube.
- SOD1 transgenic rodents are available for research but take months to develop ALS and are highly variable in the impairment shown (bulbar *vs*. spinal onset).

Can we mimic aspects of the ALS model?

- Nichols et al.[1] showed intrapleural injections of cholera toxin B conjugated to saporin (CTB-SAP) resulted in the targeted death of phrenic motor neurons (~60%; Fig. 1) 7 days later, recapitulating what is observed in SOD1^{G93A} rats.

1

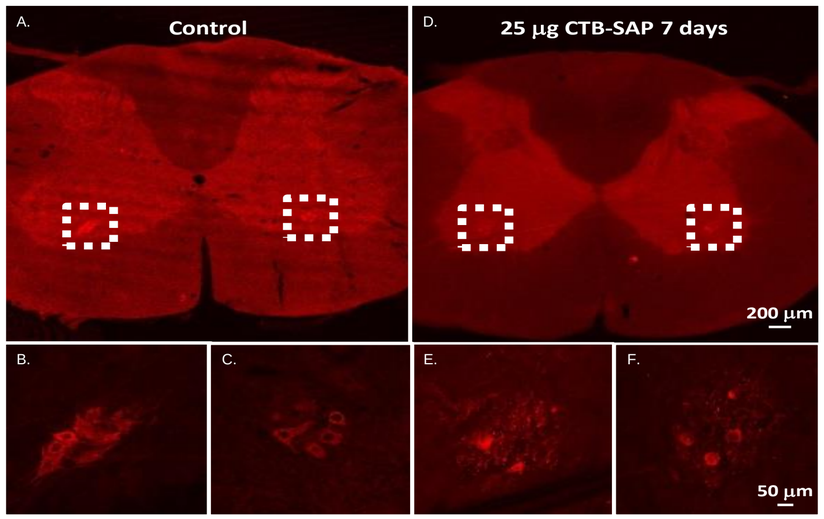

Figure 1. Photomicrographs of the C4 spinal cord of control (A-C) and CTB-SAP injected rats (D-F). A & D were photographed at 4X and the phrenic motor nucleus is outlined in white. At 20X, individual motor neurons can be seen with the number of surviving neurons being markedly reduced in the CTB-SAP injected rats (E,F) relative to the controls (B,C).[1]

- Lever et al.[2,3,4] has shown that end-stage SOD1^{G93A} mice have a decreased lick rate and swallow rate relative to wild type mice (Fig. 2), likely as a result of hypoglossal motor neuron degeneration—Lever et al. has noted vacuolation in the hypoglossal motor nucleus of end-stage mice.

2

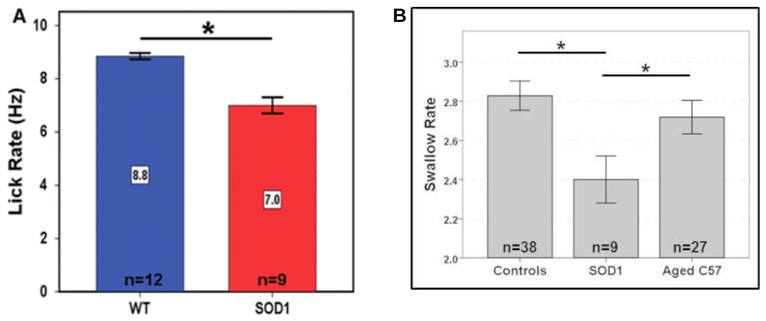

Figure 2. A) End-stage ALS mice show a decrease in lick rate relative to wild type mice.[3] B) End-stage ALS mice show a decrease in swallow rate relative to wild type mice and aged (non-ALS) mice.[4]

- However, as noted above, SOD1 models take months to reach end-stage and are not always consistent in the impairment they exhibit. Thus, an inducible model that mimics swallowing deficits would be advantageous.

Hypothesis

Following genioglossal injection of CTB-SAP, we hypothesize that CTB-SAP will produce a targeted cell death in the ventral hypoglossal nucleus.

Experimental Methods

3 **Genioglossus injection of CTB-SAP or CTB+SAP (control)**

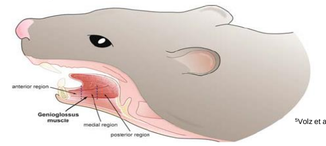

Figure 3. Isoflurane anesthetized adult male rats will be placed in dorsal recumbency to receive a midline injection into the genioglossus muscle on the ventral aspect of the tongue in the middle of the lingual frenulum. In brief, the mouth will be opened and the tongue will be gently protruded using curved forceps. A 26 gauge needle attached to 50 µl Hamilton syringe will be held at a 45-degree angle and inserted to a depth of 8mm (total length of needle); half of the total volume will be expressed and then the needle will be withdrawn halfway to express the remaining volume. Half of the rats (n=10) will be injected with 25µg CTB-SAP + 20µg CTB (to label surviving hypoglossal motor neurons), and the other half will receive 20µg CTB + 25µg SAP (control; n=10).

4 **Location of the hypoglossal nucleus**

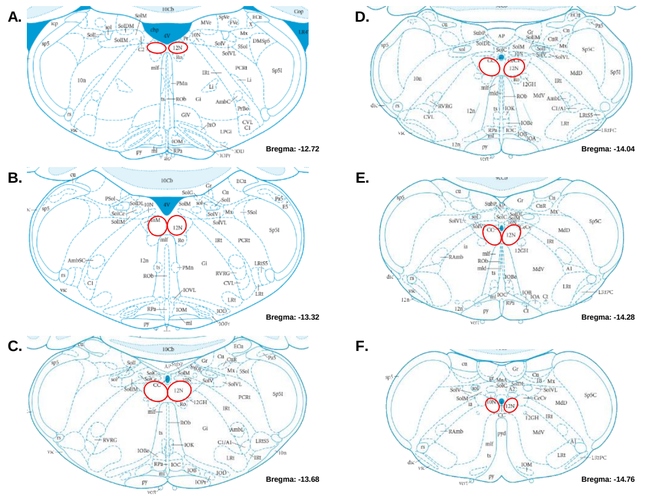

Figure 4. The hypoglossal nucleus extends throughout the length of the medulla, but its size and shape changes along the rostral-caudal axis as shown in these diagrams from The Rat Brain in Stereotaxic Coordinates, 5th ed.[5] The hypoglossal nucleus is labelled 12N and is outlined in red. The sections are arranged in order with A being the most rostral and F the most caudal.

5 **IHC Protocol**

DAY 1
- Select 6 sections containing the hypoglossal nucleus as shown in Figure 4 for each animal.
- Wash tissue in 1X PBS 3 X 5 minutes 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 cholera toxin B subunit (CTB; goat polyclonal, 1:2000, Calbiochem; Billerica, MA) overnight at 4°C on a shaker.

DAY 2
- Wash tissue in 1X PBS 3 X 5 minutes 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 (1:1000; Molecular Probes, Eugene, OR)) for 2 hours on a shaker in the dark at room temperature.
- Wash covered tissue in 1X PBS 3 X 5 minutes on shaker at RT.
- Mount tissue on glass slides, apply anti-fade, and coverslip.
- Covered slides will be stored at -4°C until quantification of staining is performed using a Leica DM4000 microscope at 20x magnification.

Figure 5. As soon as the physiology studies are completed, the rats will be perfused with paraformaldehyde. The brain and spinal cord will be removed, stored in sucrose, cut into 40 µm sections with a freezing-sliding microtome, and stored at -20° C. They will then be stained by immunohistochemistry, mounted on slides, and stored at -4° C until quantification can be performed.

6

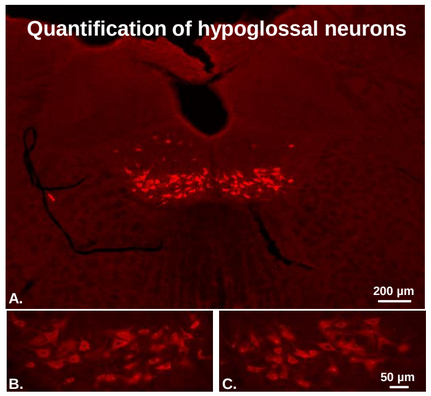

Figure 6. A. Photomicrograph (5X) of the hypoglossal nucleus from a rat receiving a midline genioglossal injection of CTB. The hypoglossal nucleus can be divided into ventral and dorsal compartments. Motor neurons in the ventral compartment (outlined in yellow) supply the protrusor muscles of the tongue and those in the dorsal compartment (outlined in white) supply the retrusor muscles. The genioglossus is a protrusor muscle so we expect to see an effect primarily in the ventral compartment, but all 4 compartments outlined will be counted. Counting will be done at 20X where the neurons can be easily seen as in B) left ventral compartment and C) right ventral compartment.

Expected Results

- Rats receiving tongue injections of CTB-SAP will have fewer surviving hypoglossal motor neurons compared to control treated rats.
- Others members of our team will be conducting lick rate and swallowing studies to determine if CTB-SAP tongue injected rats show a similar deficit as that observed in ALS mice.
- In addition, *in vivo* neurophysiology will be used to measure hypoglossal and phrenic motor output to show that hypoglossal, not phrenic, motor output is decreased following CTB-SAP tongue injections.

Implications

- We hope this model will aid researchers studying dysphagia associated with ALS by enabling them to rapidly produce test subjects with predictable deficits.
- This model should produce animals with none of the other clinical signs associated with ALS, which makes it ideal to study treatments aimed specifically at dysphagia.

References

1. Nichols, N.L. et al. *Exper. Neurol.* 2015
2. Lever, T.E. et al. *Dysphagia.* 2009
3. Lever, T.E. et al. *Dysphagia.* 2010
4. Lever, T.E. et al. *J. Vis. Exp.* 2015
5. Volz, L.M. et al. *Dysphagia.* 2007
6. Paxinos, G. and C. Watson. The Rat Brain in Stereotaxic Coordinates, 5th ed. 2005

Acknowledgements

University of Missouri College of Veterinary Medicine

University of Missouri College of Veterinary Medicine Committee on Research (COR) grant

Richard Wallace Faculty Incentive Grant

Special thanks to Erika Murphy, Sarah Stern, and Taylor Craig for performing behavioral studies and Miles Tanner for performing perfusions.

Veterinary Research Scholars Program
University of Missouri