Establishing Immunohistochemistry Staining Patterns for Canine Degenerative Myelopathy

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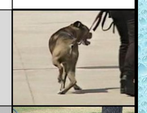
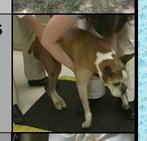
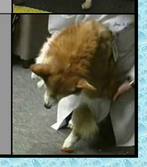
INTRODUCTION

Canine Degenerative Myelopathy (DM) is a neurodegenerative disease caused by a gene mutation in *superoxide dismutase 1 (SOD1)*, which leads to the accumulation of aggregates in motor nuclei of the spinal cord and brain. The clinical signs of DM are similar to some forms of *SOD1*-associated amyotrophic lateral sclerosis (ALS), leading to the proposal of DM as a possible animal model for ALS. The adult onset of DM progresses in stages from hind limb proprioceptive ataxia to general muscle wasting, difficulty with tongue movements and swallowing (dysphagia), and front limb paralysis. Previous work in our lab used Hematoxylin and Eosin (H&E) staining of DM-affected hypoglossal nucleus (motor nucleus for tongue movement) to identify subtle evidence of neurodegeneration. The goal of our current project is to use immunohistochemistry (IHC) methods to evaluate for histopathological biomarkers of neurodegeneration in DM and further establish canine DM as a disease model for ALS.

Table 1: Stage of neurological signs in DM dogs

Stage	Neurologic Signs	
1 Early	**UMN Paraparesis** • Progressive general proprioceptive ataxia • Asymmetric spastic paraparesis • Intact spinal reflexes	
2 Early	**Nonambulatory Paraparesis to Paraplegia** • Mild to moderate loss of muscle mass • Reduced to absent spinal reflexes in pelvic limbs • +/- urinary and fecal incontinence	
3 Late	**LMN Paraplegia to Thoracic Limb Paresis** • Signs of thoracic limb paresis • Flaccid paraplegia • Severe loss of muscle mass in pelvic limbs • Urinary and fecal incontinence	
4 Late	**LMN Tetraplegia and Brain Stem Signs** • Flaccid tetraplegia • Difficulty with swallowing and tongue movements • Reduced to absent cutaneous trunci reflex • Generalized and severe loss of muscle mass • Urinary and fecal incontinence	

GOALS and OBJECTIVES

- **Establish reliable IHC staining protocols for canine neural tissue.**
- **Evaluate IHC in brainstem of DM-affected dogs for validating canine DM as a disease model for ALS.**

METHODS

- Samples acquired at time of euthanasia from both DM and control dogs (age-matched)
- Immersion fixation of brainstem tissue in 10% neutral buffered formalin for 3-20 months, then post-fixed in fresh formalin for 3-5 days
- Tissues processed in paraffin and sectioned at 10 µm.by microtome
- Performed IHC (ChAT and Vimentin)

ACKNOWLEDGMENTS

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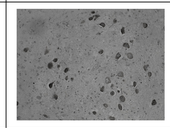
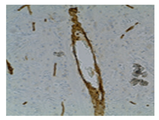
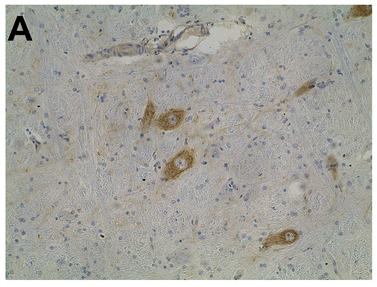
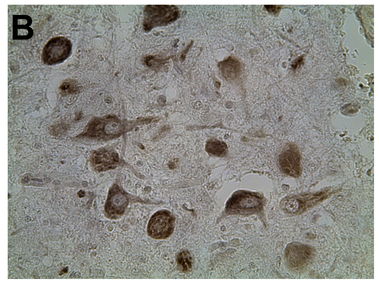
RESULTS

Table 2: Immunohistochemistry (IHC) Staining Methodology

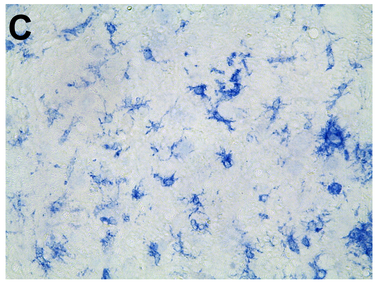
IHC	Antigen	Purpose	Antibody Used	IHC Results
Calcitonin Gene Related Peptide (CGRP)	Motor neurons that express CGRP (a neuropeptide)	Pathological motor neurons should be CGRP-positive at early stages of disease and completely degenerate by disease end-stage.	*Primary:* Anti CGRP (Abcam, Mouse Monoclonal, Primary Dilution 1:400) *Secondary:* Vectastain ABC Kit Mouse IgG	In Progress
Choline Acetyltransferase (ChAT)	Motor neurons that express acetylcholine transferase	Pathological motor neurons should have high or moderate ChAT staining that is associated with cholinergic dysfunction, leading to motor neuron death at disease end-stage.	*Primary:* Anti ChAT (EMD Millipore Corp., Goat Whole Antisera, Primary Dilution 1:400) *Secondary*: Vectastain ABC Kit Goat IgG	
Glial Fibrillary Acidic Protein (GFAP)	Astrocytes	Astrocyte activity is increased in ALS	*Primary:* Anti GFAP (Novus Bio. Goat Column Purified Polyclonal, Primary Dilution 1:400) *Secondary:* Vectastain ABC Kit Goat IgG	In Progress
Ionized Calcium Binding Adaptor Molecule 1 (IBA1)	Microglia	Microglia activity is increased in ALS	*Primary:* Anti IBA-1 (Wako Pure Chem., Rabbit Column Purified Polyclonal, Primary Dilution 1:400) *Secondary*: Vectastain ABC Kit Rabbit IgG	In Progress
Nucleoporin p62	p62 aggregates in motor nuclei	Stains protein aggregates containing autophagy markers	*Primary:* Anti SQSTM1/p62 (Abcam, Rabbit Column Purified Polyclonal, Primary Dilution 1:100) *Secondary*: Vectastain ABC Kit Rabbit IgG	In Progress
Superoxide Dismutase 1 (SOD1)	SOD1 aggregates in motor nuclei	SOD1 mutant protein aggregates are seen in DM and some forms of ALS	*Primary:* Anti SOD1 Cu/Zn ((Enzo Life Sci., Rabbit Column Purified Polyclonal, Primary Dilution 1:200) *Secondary:* Vectastain ABC Kit Rabbit IgG	In Progress
Vimentin	Vimentin protein contained in glial cells and endothelial cells of CNS	Establish proper fixation levels of canine neural tissues. Overfixed tissue will not stain with Vimentin. Strong staining occurs in ALS-affected neural tissue compared to weaker staining in non-disease neural tissue..	*Primary:* Anti Vimentin (DAKO, Mouse Monoclonal, Primary Dilution 1:200) *Secondary:* Vectastain ABC Kit Mouse IgG	

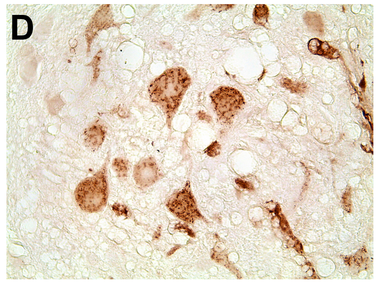
IHC Staining Examples





Representative sections from canine brainstem tissue are shown in Images A and B to the left. Image A shows ChAT staining (brown) with Hematoxylin counterstaining (blue). Image B shows ChAT nickel staining of motor neurons (brown). In both images, ChAT staining is appropriately localized to the cytoplasm of motor neurons in canine brainstem tissue.





Representative sections from murine brainstem tissue are shown in Images C and D to the left. Image C demonstrates IBA1 staining of microglia. Image D shows CGRP staining in motor neurons. We are in the process of validating these two antibodies in canine brainstem tissue.

CONCLUSIONS

- **Few antibodies are validated for use in canine neural tissue.**
- **We have developed an IHC assay for vimentin and ChAT antibody staining in formalin-fixed neural tissue from dogs.**

FUTURE DIRECTIONS

- **Further IHC staining in the hypoglossal nucleus and other brainstem nuclei involved in swallowing is needed to identify specific markers of dysphagia in DM/ALS.**
- **Dual staining of CGRP and ChAT will identify whether Canine DM displays similar patterns of motor neuron vulnerability as seen in ALS.**
- **Vibratome sectioning (thick sections) will facilitate more effective IHC staining using free-floating methods.**
- **Analysis of thick sections using design-based stereological methods will permit accurate estimations of cell counts and measurements using bright field and fluorescent microscopy.**