*Journal of Food Protection, Vol. 71, No. 11, 2008, Pages 2307–2311*

**Research Note**

# An Evaluation of a Method for the Detection of Sensory Ganglia in Product Derived from Advanced Meat Recovery Systems†

**SCOTT HAFNER,[1]* MARY T. SUTTON,[1] JOSEPH HILL,[1] PATRICK C. McCASKEY,[1] AND LYNDA COLLINS KELLEY[2]**

[1]*U.S. Department of Agriculture, Food Safety and Inspection Service, Office of Public Health Science, and* [2]*U.S. Department of Agriculture, Food Safety and Inspection Service, Office of Food Defense and Emergency Response, Russell Research Center, P.O. Box 6085, Athens, Georgia 30604, USA*

MS 07-634: Received 6 December 2007/Accepted 13 July 2008

## ABSTRACT

A method is described for the identification of dorsal root ganglia (DRG)–associated sensory neurons within advanced meat recovery (AMR) product derived from bovine vertebral columns. This method relies on the unique microanatomy of sensory neurons and immunohistochemical staining, primarily for glial fibrillary acidic protein. Sensory neurons are variably sized unipolar neurons, exhibiting a single-cell process that is rarely seen in histologic sections. These neurons are surrounded by a prominent ring of glial fibrillary acidic protein–positive satellite cells that produce a distinctive and readily identifiable staining pattern in histologic sections. Fragmented DRG were detected to the 0.25% level in samples of ground beef or nonvertebral-origin AMR product spiked with these sensory ganglia. Similarly examined commercially produced nonvertebral-origin AMR product ($n = 157$) did not contain sensory ganglia, while 3.3% of vertebral-origin AMR product ($n = 364$) contained fragmented DRG.

The meat product extracted from the bones of beef carcasses by advanced meat recovery (AMR) systems has been the subject of numerous federal regulations since it was defined as ''meat'' in 1994 *(9).* Since AMR systems use pressure to separate the meat that remains attached to bones after hand deboning *(10),* and vertebral columns are often used as source material, there were concerns that spinal cord and/or dorsal root ganglia (DRG) could be included in AMR product derived from bovine vertebral columns *(9).* An immunohistochemical method for the detection of spinal cord in AMR product was developed that in part utilized antibodies to glial fibrillary acidic protein (GFAP) to detect central nervous system–specific astrocyte processes *(6).*

In naturally occurring clinical cases of bovine spongiform encephalopathy, altered prion protein has only been detected in the central nervous system; however, experimental inoculation studies have suggested that the DRG of cattle older than 30 months may also accumulate this altered protein, probably by centripetal spread from the spinal cord *(7).* After the discovery of bovine spongiform encephalopathy in the United States, vertebral columns of cattle older than 30 months were no longer allowed in the AMR process, but AMR product continued to be derived from the vertebral columns of cattle younger than 30 months *(9).* Because spinal cord and DRG are not expected to be present in a product labeled ''meat,'' the AMR product derived from cattle younger than 30 months continued to be examined for spinal cord, and methods to identify DRG in this product were evaluated. Presently, establishments with a history of being unable to produce AMR product without incorporating spinal cord or DRG are not allowed to utilize vertebral columns as source material *(9).*

DRG are focal aggregates of sensory neurons located in nerves that pass through the intervertebral foramina, passages that allow nerves from the spinal cord to extend to peripheral tissues. DRG are located within or immediately adjacent to these foramina *(4, 5, 8).* The purpose of the present study was to evaluate an immunohistochemical method for the identification of sensory neurons derived from bovine DRG in AMR product.

## MATERIALS AND METHODS

**Preparation of control tissues.** Peripheral nerves, DRG, spinal cord, and paravertebral sympathetic ganglia were removed from bovine vertebral columns. These tissues were fixed in 10% neutral buffered formalin, routinely processed for histology, and embedded in paraffin blocks. Sections 4- to 6-μm thick were cut from paraffin blocks, placed on charged glass slides, deparaffinized, and rehydrated. Some sections were then stained with hematoxylin and eosin (H&E), and some were left unstained for use as immunohistochemical controls.

**DRG mixtures.** Chilled (not frozen) but unfixed DRG were dissected from bovine vertebral columns, trimmed free of peripheral nerves, and finely minced with a razor blade. The DRG were weighed, and specific aliquots were added to ground beef or AMR product of nonvertebral origin to make proportionate mixtures of 0.25, 0.50, 1.0, 2.0, 5.0, and 10.0% DRG (wet weight/wet weight). Each of these spiked mixtures was placed in an individual plastic

* Author for correspondence. Tel: 706-546-3606; Fax: 706-546-3589; E-mail: Scott.Hafner@FSIS.USDA.gov.

† Trade names and/or commercial products are mentioned solely to provide specific information, and this does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Downloaded from http://meridian.allenpress.com/doi/pdf/10.4315/0362-028X-71.11.2307 by guest on 26 May 2022

bag, which was manipulated to partially mix the contents. Both nonvertebral-origin AMR and ground beef without added DRG were used as negative controls (0% mixtures).

**Histochemical and immunohistochemical procedures.** From each of these mixtures (0, 0.25, 0.50, 1.0, 2.0, 5.0, and 10.0% DRG), four aliquots of material sufficient to create a surface approximately 1.5 by 2 cm were placed in histology cassettes and fixed in 10% neutral buffered formalin for 24 h. These tissues were routinely processed for histology and embedded in paraffin wax. Two sections 4- to 6-μm thick were cut from each paraffin block and placed on charged slides. This resulted in two slides from each block (eight slides per level). Four of the slides from each mixture (0 through 10%) were stained with H&E, and four slides from each mixture were immunostained with antibodies to GFAP. The morphology and GFAP staining characteristics of the fragmented ganglia in spiked mixtures were verified by comparison to similarly immunostained DRG. Positive controls for GFAP immunohistochemistry were spinal cord, DRG, and peripheral nerves; negative-control sections lacked the primary antibody (GFAP), but were otherwise treated identically.

Replicate sections were obtained from some paraffin blocks, placed on charged slides, deparaffinized, rehydrated, and immunostained with *Griffonia simplicifolia* isolectin B4 (IB4) or antibodies to tyrosine hydroxylase (TH). Sections of control tissues (DRG, sympathetic ganglia) were similarly immunostained for GFAP, IB4, and TH. Negative-control sections lacked the primary antibody or lectin, but were otherwise treated identically.

Specifically, slides containing sections of paraffin-embedded material were deparaffinized with xylene (three times), and rehydrated in 100% ethanol (three changes) and 95% ethanol (three changes). Sections were rinsed with distilled water, and antigen retrieval with citrated buffer was performed according to instructions, using a microwave (Antigen Retrieval-Citra, BioGenex, San Ramon, Calif.). Sections were rinsed with buffer (Tris-buffered saline plus Tween, Signet Laboratories, Dedham, Mass.) and the primary antibody or biotinylated lectin was applied to the section. Antibodies included GFAP (prediluted, but additionally diluted 1:10; monoclonal clone GA-5, BioGenex), which was incubated for 30 min at room temperature and TH (polyclonal, diluted 1:250; AB 152, Chemicon International, Temecula, Calif.), which was incubated at 4°C for 2 h. IB4 (diluted 1:200; B1205, Vector Laboratories, Burlingame, Calif.), a biotinylated lectin, was incubated on sections at room temperature for 2 h. After incubation, sections were again rinsed with buffer, and the primary antibody or lectin detected using a streptavidin–biotin–alkaline phosphatase detection kit (BioGenex Supersensitive Multilink Kit) according to instructions, with Fast Red (BioGenex) as the chromogen. Sections were rinsed with buffer and counterstained with Gill's hematoxylin (Anatech, Ltd., Battle Creek, Mich.) for 15 s, then rinsed with acidic tap water, blued in Scott's tap water for 15 to 60 s, rinsed again in tap water, and drained. Two to five drops of Crystal Mount Aqueous/Dry mounting media (Biomeda Corp., Burlingame, Calif.) were applied to each section, and slides were dried horizontally in a 60 to 70° C oven for 10 to 30 min. Slides were allowed to cool, each was quickly dipped into xylene, and sections were covered with glass coverslips, using a permanent mounting medium.

**Commercially produced AMR samples.** Two-pound samples of AMR product that were at least partly derived from vertebral columns ($n = 364$) and 2-lb samples of AMR product of nonvertebral origin ($n = 157$) were collected, chilled, and shipped to the laboratory for examination. All AMR product was derived from the bones of cattle that were younger than 30 months. Each 2-lb sample was obtained by combining four approximately 0.5-lb subsamples collected routinely during production. Four portions, approximately 2 by 2 by 1 cm each, were obtained from four randomly selected areas in each 2-lb sample. Each of these small meat patties was placed into a labeled plastic tissue cassette, wrapped with filter paper, and fixed in 10% neutral buffered formalin. These tissues were routinely processed for histology, and were then embedded in blocks of paraffin wax. Two sections, 4 to 6 μm thick, were cut from each of the four paraffin blocks; these sections were placed on charged glass slides. One section from each block was stained with H&E and the other section immunostained with antibodies to GFAP. Additional replicate sections were obtained from some blocks and immunostained for IB4 and/or TH. Sections were examined by light microscopy. Results were analyzed using the *Z*-test for differences in two proportions.

## RESULTS

Microscopic examination of H&E-stained sections of bovine DRG and sympathetic ganglia verified the distinct morphologic differences between bovine sensory and sympathetic neurons. In sensory neurons of DRG, the single-cell processes were rarely observed exiting the cells, and therefore, neurons almost universally appeared round (Fig. 1). Nuclei of these neurons were most often centrally located and ranged from 12 to 20 μm in diameter. Neurons ranged in size from 30 to 80 μm in diameter, and there was some variation in the staining intensity of neuronal cytoplasm, with the cytoplasm of large neurons lightly eosinophilic in H&E stains. Each sensory neuron was encircled by a ring of low cuboidal satellite cells separated from the neuron by a perineuronal space. Satellite cells were surrounded by a connective tissue capsule. The interneuronal spaces in these ganglia were filled with axons.

Sympathetic neurons from the paravertebral ganglia were most often 30 to 40 μm in diameter with eccentrically located 12- to 15-μm nuclei. These neurons were distinctly multipolar (Fig. 2) with numerous cell processes exiting neuron cell bodies. Neuronal cytoplasm was often a deep gray blue on H&E stains and occasionally contained brown pigment. Each sympathetic neuron was surrounded by an irregularly shaped perineuronal space, a flattened layer of satellite cells that were at times unapparent, and an irregularly shaped capsule.

Examination of DRG immunostained for GFAP revealed each neuron to be surrounded by a prominent ring of GFAP-immunopositive satellite cells (Fig. 3), while satellite cells of sympathetic neurons stained poorly with GFAP. In DRG stained with antibodies to tyrosine hydroxylase, most neurons did not exhibit any staining, with only occasional small neurons exhibiting faint staining. Sympathetic neurons were nearly diffusely moderately to markedly immunostained by antibodies to TH, which accentuated their multipolar character (Fig. 4). In sections of DRG stained with IB4, a significant percentage (roughly a third) of DRG neurons that were predominantly small to medium sized (30 to 40 μm in diameter) exhibited a multifocal punctate cytoplasmic staining (Fig. 5), while in similarly immunostained sympathetic ganglia only rare (1 of 100 or fewer) neurons exhibited any staining. Endothelial cells of blood vessels were diffusely immunostained with IB4.

Downloaded from http://meridian.allenpress.com/doi/pdf/10.4315/0362-028X-71.11.2307 by guest on 26 May 2022

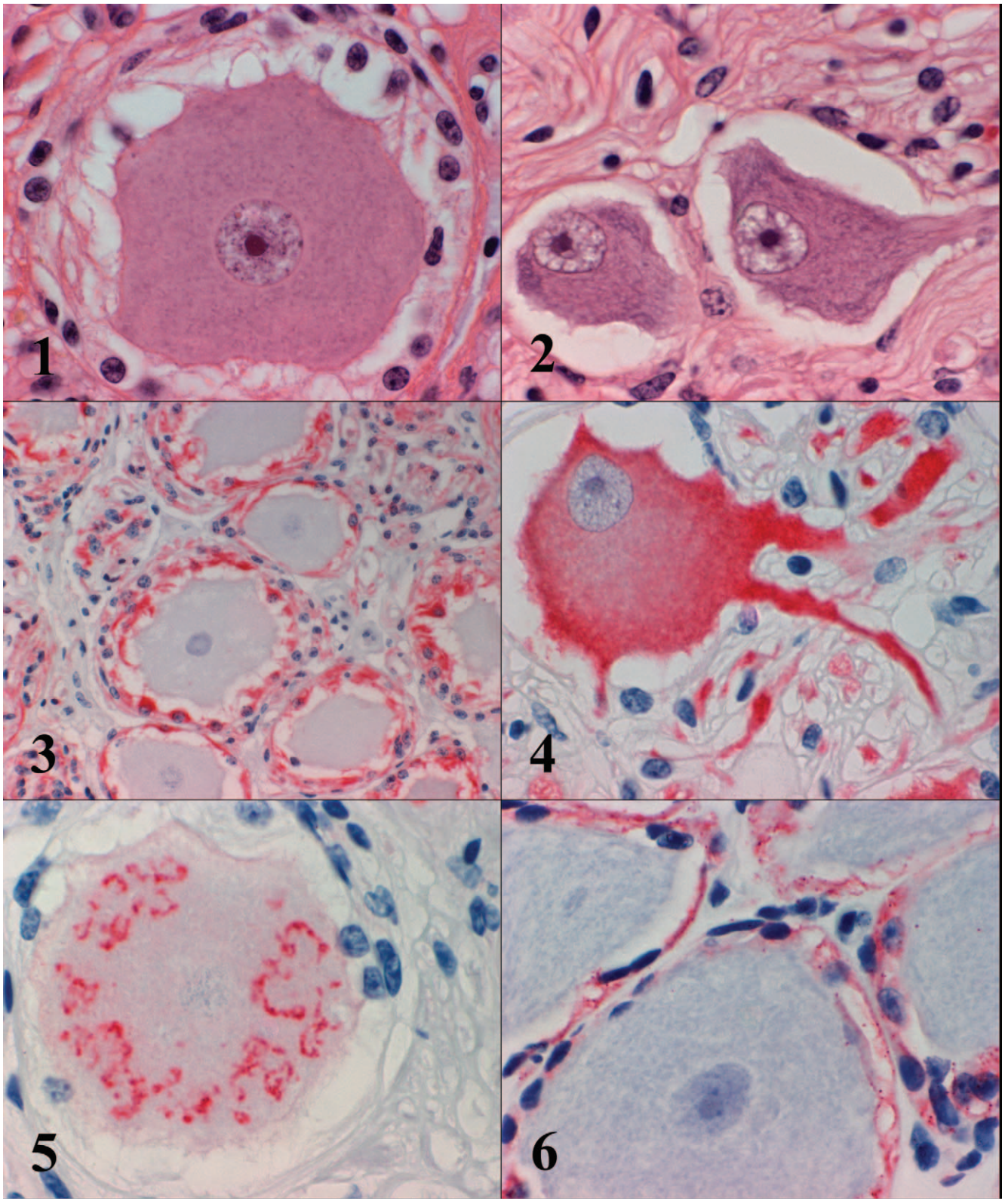


FIGURE 1. *Sensory neurons of a DRG lack visible cell processes, contain centrally located nuclei, and are surrounded by cuboidal satellite cells (H&E staining, magnification ×1,000).*

FIGURE 2. *Neurons of sympathetic ganglia are angular, contain eccentrically located nuclei, and exhibit cell processes (H&E staining, magnification ×1,000).*

FIGURE 3. *DRG neurons are surrounded by GFAP-positive satellite cells (GFAP immunostaining, magnification ×400).*

FIGURE 4. *The multipolar nature of sympathetic neurons is accentuated by antibodies to tyrosine hydroxylase (immunostain for TH, magnification ×1,000).*

FIGURE 5. *Some of the sensory neurons of DRG are marked by immunostains using IB4 (magnification ×1,000).*

FIGURE 6. *Satellite cells of DRG neurons in commercially produced AMR product are also brightly stained with antibodies to GFAP (GFAP immunostaining, magnification ×1,000).*

Downloaded from http://meridian.allenpress.com/doi/pdf/10.4315/0362-028X-71.11.2307 by guest on 26 May 2022

Sensory neurons were detected in all levels (0.25, 0.50, 1.0, 2.0, 5.0, and 10.0%) of nonvertebral AMR product or ground beef spiked with added DRG. These fragmented ganglia were not present in the nonvertebral-origin AMR product or ground beef without added DRG (0% level).

Twelve of the 364 samples of vertebral-origin AMR product contained fragments of sensory ganglia that were compatible with DRG. None of the 157 nonvertebral-origin AMR samples contained DRG. The differences in the presence of DRG fragments between these two populations were significant ($P < 0.05$). DRG fragments in AMR samples were generally smaller than the fragments in prepared mixtures, and neurons were mildly distorted and less well preserved, but the distinctive GFAP staining of satellite cells was readily identifiable (Fig. 6). Replicate sections of some AMR product containing DRG fragments were immunostained with IB4 and a portion of the neurons present displayed the typical punctate cytoplasmic staining pattern; these neurons were not immunostained with antibodies to TH.

## DISCUSSION

Ganglia composed of sensory neurons are the DRG associated with the vertebral column and trigeminal and other ganglia located within or immediately adjacent to the skull *(4, 5, 8).* All sensory ganglia (with the exception of vestibulocochlear ganglia) contain distinctive neurons with a unipolar morphology not seen in other peripheral nerve ganglia *(8).* Sensory neuron ganglia found in AMR product derived from vertebral columns are therefore fragmented DRG. Satellite cells surrounding DRG in commercially manufactured, vertebral-origin AMR product were also stained brightly by this immunohistochemical method (Fig. 6). Other nerve ganglia likely to be present in vertebral-origin AMR products are either peripheral nerve ganglia or, more commonly, paravertebral sympathetic ganglia; neurons of both of these ganglia are readily differentiated from sensory neurons. Neurons also are found in the gray matter of the spinal cord, but these multipolar neurons are readily differentiated from those of peripheral nerve ganglia, as there is no organization into ganglia.

Sensory neurons of DRG are a heterogeneous population that sense pain, pressure, and other stimuli *(8).* These neurons exhibit a marked disparity in size and cytoplasmic staining intensity, but, because of their unipolar character, are uniformly round in histologic sections. This shape may be distorted by the pressure of the AMR process, and a sufficient number of neurons need to be present in the fragmented ganglia to demonstrate both the characteristic immunostaining and typical sensory neuron morphology. DRG were not left intact by the AMR process, and fragments generally contained only 5 to 20 neurons. Mincing the DRG added to spiked mixtures was intended to mimic this shearing, but the fragments generated in this manner were nearly always larger than those found in the commercial AMR product. Neurons in this AMR product were less well preserved and more distorted than the neurons in the spiked samples, but the typical neuronal morphology and distinctive GFAP staining were still readily identifiable. Since the sensitivity of the method is dependent on both the number and volume of DRG fragments present, the sensitivity described for AMR product spiked with DRG will not directly extrapolate to commercially prepared AMR product (the method may be more or less sensitive). Adding the ganglia intact to control tissues would decrease the sensitivity and not emulate the AMR process. The method is entirely specific, however, as DRG are directly visually identified by both morphology and typical immunohistochemical staining, a finding reinforced by the detection of DRG fragments only in vertebral origin, commercially produced AMR product. Preservation of this microscopic morphology is paramount; samples should not be frozen (freezing destroys cell membranes) or subjected to excessive manipulation (blending) prior to fixation. This method is only suitable for fresh or chilled product and would likely not be satisfactory for further processed or cooked product.

Sympathetic neurons from the paravertebral ganglia exhibit a more limited size range and are distinctly irregular in shape. Nuclei are generally centrally located in DRG neurons and are often eccentrically located in sympathetic neurons *(1).* In addition to these distinct morphologic characteristics, the satellite cells that surround DRG neurons are much more prominent and stain more intensely with GFAP than do those that surround neurons of paravertebral sympathetic ganglia.

Immunostaining of neurons for the enzyme tyrosine hydroxylase provides good training for the recognition of sympathetic neurons as these neurons are brightly immunopositive, and the multipolar nature of the neurons is accentuated. Tyrosine hydroxylase is the rate-limiting enzyme in the production of noradrenalin, which is used as a neurotransmitter by sympathetic neurons. Some DRG neurons may stain faintly with antibodies to tyrosine hydroxylase, as a small percentage of these neurons are dopaminergic, and tyrosine hydroxylase is also essential in the conversion of tyrosine to dopamine *(2).* The binding of *Griffonia simplicifolia* isolectin B4 to surface galactosyl residues *(3)* may also help to differentiate between DRG neurons and paravertebral sympathetic neurons, as a significant subpopulation of small DRG sensory neurons exhibit cytoplasmic staining, whereas very few sympathetic neurons exhibit staining. These adjunct analyses were most helpful in initially documenting the ability of morphology and GFAP staining to discriminate between suspect ganglia, but were not considered necessary once criteria were established.

In summary, we describe an immunohistochemical method for the detection of the sensory neurons of DRG in comminuted meat products. This method utilizes the unique morphology and staining characteristics of sensory neurons to differentiate fragmented ganglia containing these neurons from other peripheral nerve ganglia.

## ACKNOWLEDGMENTS

The authors thank Alice Self, Brenda King, Kathy Wortham, and Barbara Stancel for the preparation of numerous histochemical and immunohistochemical stains.

## REFERENCES

1. Aughey, E., and F. L. Frye. 2001. Comparative veterinary histology with clinical correlates. Iowa State University Press, Ames.

Downloaded from http://meridian.allenpress.com/doi/pdf/10.4315/0362-028X-71.11.2307 by guest on 26 May 2022

2. Brumovsky, P., M. J. Villar, and T. Hökfelt. 2006. Tyrosine hydroxylase is expressed in a subpopulation of small dorsal root ganglion neurons in the adult mouse. *Exp. Neurol.* 200:153–165.
3. Fang, X., D. Laiche, S. McMullan, C. Berry, S. G. Waxman, K. Okuse, and S. N. Lawson. 2006. Intense isolectin-B4 binding in rat dorsal root ganglion neurons distinguishes C-fiber nociceptors with broad action potentials and high Nav1.9 expression. *J. Neurosci.* 26: 7281–7292.
4. Getty, R. (ed.). 1975. Sisson and Grossman's the anatomy of domestic animals, 5th ed. W. B. Saunders, Philadelphia.
5. Jenkins, T. W. 1978. Functional mammalian neuroanatomy, 2nd ed. Lea & Febiger, Philadelphia.
6. Kelley, L. C., S. Hafner, P. C. McCaskey, M. T. Sutton, and K. A. Langheinrich. 2000. An evaluation of methods for the detection of spinal cord in product derived from advanced meat recovery systems. *J. Food Prot.* 63:1107–1112.
7. Masujin, K., D. Matthews, G. A. H. Wells, S. Mohri, and T. Yokoyama. 2007. Prions in the peripheral nerves of bovine spongiform encephalopathy-affected cattle. *J. Gen. Virol.* 88:1850–1858.
8. Summers, B. A., J. F. Cummings, and A. de Lahunta. 1995. Veterinary neuropathology. Mosby-Year Book, St. Louis.
9. U.S. Department of Agriculture, Food Safety and Inspection Service. 2004. Meat produced by advanced meat/bone separation machinery and meat recovery (AMR) systems. Interim final rule. *Fed. Regist.* 69:1874–1885.
10. Willemsen, B. J. J. 1994. Separation of residual meat attached to bones, p. 39–43. *In* Proceedings of the 47th annual Reciprocal Meat Conference. American Meat Science Association, Chicago.

Downloaded from http://meridian.allenpress.com/doi/pdf/10.4315/0362-028X-71.11.2307 by guest on 26 May 2022