ALTERATIONS IN THE ULTRASTRUCTURE OF SYNAPTIC JUNCTIONS IN THE MOTOR CORTEX OF WEAVER-SYNDROME CATTLE

by

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INTRODUCTION

The term Weaver-syndrome is descriptive of a specific motor dysfunction in Brown Swiss cattle; it has been observed as familial and affects both sexes. The affected animals appear normal until they reach 6 to 8 months of age, at which time they develop an abnormal, swinging gait. This steadily worsens, with the animals first losing control of movement and later the ability to stand. They become recumbent and thus unmanageable 18 to 36 months after signs are first observed.

A previous study has demonstrated gross pathological changes in the muscles of the loin and hindquarter, including atrophy and discoloration.[1] Histologic lesions identified in these muscles include a reduced number of normal fibers and increased amounts of adipose and connective tissue. No cellular hypertrophy was noted. The cerebral cortex, midbrain, cerebellum, spinal cord, sciatic and femoral nerves all appeared normal with light microscopic examination.

Similar clinical signs of progressive muscular dysfunction exist in diseases of other species, such as Weaver mice.[2] These clinical manifestations have been traced to disorders of the central nervous system. Because modulation of neuronal control of motor function takes place at the synapse, an ultrastructural investigation of synaptic junctional complexes in the cerebral cortex was undertaken. In a preliminary study we found evidence of alterations in the Weaver-syndrome synapses, particularly in the motor region of the cerebral cortex.[3]

The current study was undertaken to evaluate more animals, focusing on the motor cortex. Electron microscopy allows study of specific

anatomic substructures of the synaptic junction, so an ultrastructural approach was employed in these attempts to elucidate the role of the central nervous system (CNS) in the Weaver-syndrome.

MATERIALS AND METHODS

Experimental Protocol

Four Brown-Swiss cattle with clinical manifestations of the Weaver-syndrome were studied. These animals ranged in age from 12 to 36 months, included a steer, two heifers and a cow, and ranged in weight from 750 to 1200 pounds. Two normal animals were studied, a 24-month-old, 1250-pound Holstein steer and a 40-month-old, 850-pound Guernsey cow. All Weaver animals and one normal cow were euthanitized by barbiturate injection followed by exsanguination. The remaining normal animal was sacrificed at a local abbatoir using a stun gun and exsanguination.

The brain was removed from each animal immediately. Areas of the cerebral cortex associated with primary motor function, the anterior and posterior sigmoid gyri adjacent to the cruciate sulcus, were identified and dissected free.[4] Within 15 minutes of death these tissue slices, < 3mm thick, were placed in 5% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.3 and approximately 650 mOsmol. Thirty minutes later samples approximately $0.5mm^3$ were microdissected out and transferred to fresh cacodylate, buffered 2.5% glutaraldehyde, for overnight fixation.

Preparation of Samples for Electron Microscopy

All tissues were rinsed three times in the sodium cacodylate buffer. One set of tissues was then post-fixed in buffered 1% osmium tetroxide (OsO_4) and dehydrated through a graded ethanolic series then acetone. A second set of tissues was rinsed with distilled water before ethanol dehydration, then stained with ethanolic phosphotungstic acid (E-PTA) for 2 hours.[5] All tissues were embedded in Epon/Araldite.

Thin sections of the embedded tissues were cut on a Sorvall MT-2 ultramicrotome using a diamond knife. Sections from OsO_4 samples were stained with ethanolic uranyl acetate followed by triple lead stain.[6] All sections were carbon coated.

Prior to examination in the electron microscope, the grids were coded to provide a blind study with respect to the diseased versus normal control animals. Sections were examined with a Philips EM 301 and micrographs were taken for measurement of ultrastructural features of the synaptic junctional complexes. All micrographs for these measurements were made at a final magnification (photography and printing) of 89,300x.

Measurements

Measurements of approximately equal numbers of synapses from each animal were made on those micrographs having measurable synaptic junctional complexes. Each synapse from a total of 276 synapses measured was assigned a number and all data from a given synapse were thus subsequently identified. Specific ultrastructural features were measured using a hand-held magnifier with a metric-ruled reticle. Measurements included: the number of presynaptic clear vesicles (SV),

the mean diameter of the clear vesicles (SVDM), the number of coated vesicles (CV), the number of presynaptic dense projections (PDP), the height of the presynaptic dense projections (PPHT), the peak-to-peak distances of the presynaptic dense projections (PPPK), the width of the junctional cleft (CL), the width of the postsynaptic density (PD) and the length of the postsynaptic density (PDLN).

To calculate the mean diameter of clear vesicles for a given synapse, the diameter was measured either: (1) for each of 10 randomly selected vesicles; or, (2) for all vesicles when less than 10 were present at a synapse. In assessing the widths of the cleft and of the postsynaptic density, a mean value was taken from measurements at three sites across the junction.

Statistical Analyses

All data were entered into an IBM 370/158 computer for subsequent evaluation. Statistical analyses included analysis of variance, the Student's t-test for significance, and Duncan's Multiple Range test.[7,8] Significance levels are reported with results.

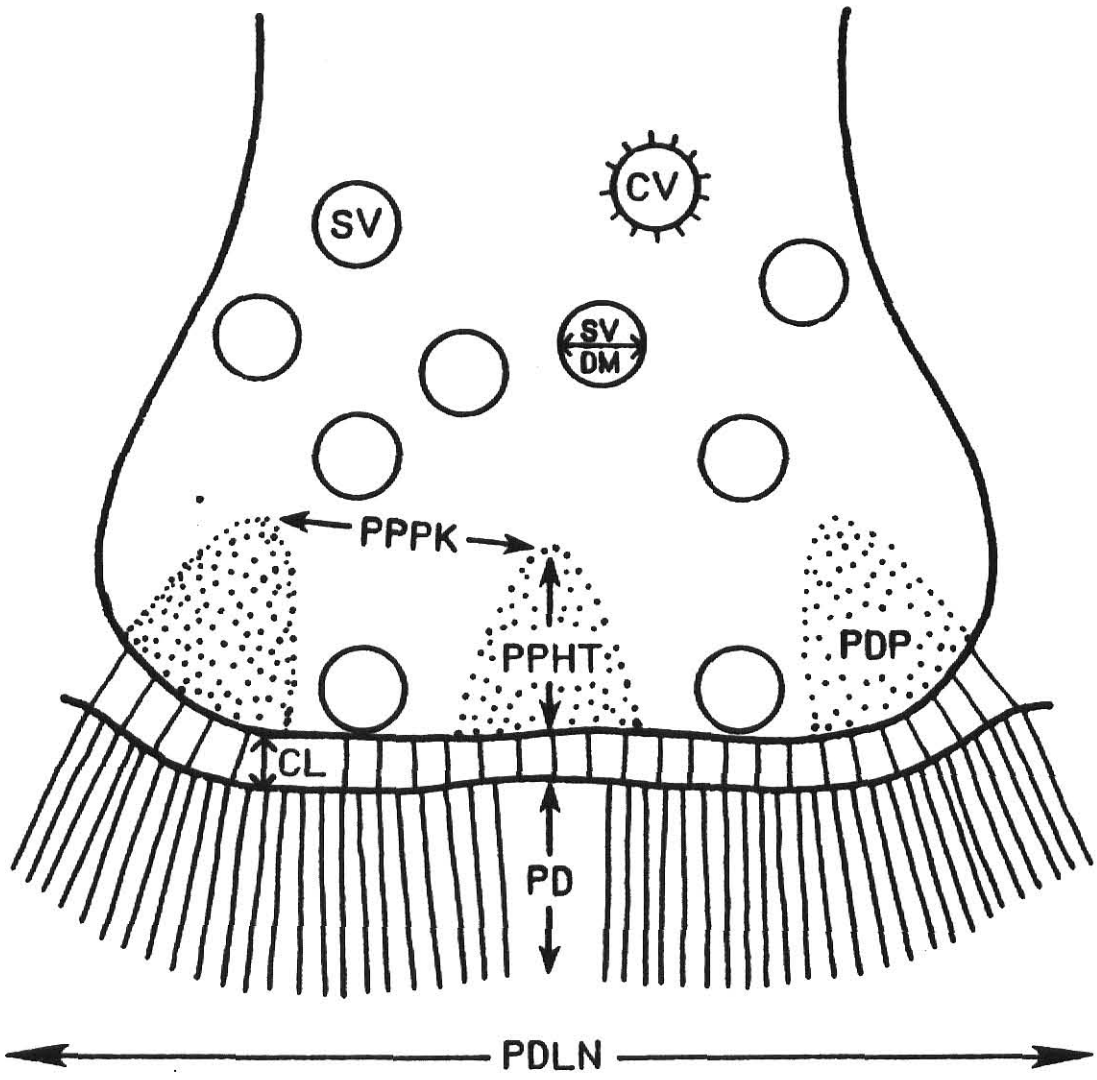
RESULTS

Measurements of the Paramembranous Densities

Several aspects of the presynaptic dense projections were measured, including the number of projections, the height of each one, and the distance between the peaks of adjacent projections (Fig. 1). The mean values for these measurements are given (Table 1). Significant differences were noted in several parameters. The mean height of the

PLATE I

FIGURE 1 Diagrammatic representation of a synaptic junctional complex and its measureable components. Cleft width (CL), coated vesicle (CV), thickness of postsynaptic density (PD), presynaptic dense projection (PDP), postsynaptic density length (PDLN), presynaptic dense projection height (PPHT), peak-to-peak distance (PPPK), synaptic vesicle (SV), and synaptic vesicle diameter (SVDM).


SV
CV
SV
DM
PPPK
PPHT
PDP
CL
PD
PDLN

projections was less in the Weaver-syndrome brains than in normal brains ($p < .001$), stained with OsO_4 or E-PTA. Similarly, the peak-to-peak distances were significantly smaller in the Weaver animals ($p < .001$), again with either stain.

The length and the thickness of the postsynaptic densities were measured and mean values are presented (Table 2). The density lengths were quite variable within each group, and no significant differences in these measurements were found between Weaver-syndrome and normal brains. However, the thickness of the postsynaptic densities was significantly smaller in the Weaver-syndrome tissue ($p < .0001$), stained with both OsO_4 and E-PTA.

Measurements of Synaptic Vesicles and Cleft

Various aspects of the vesicle population were evaluated using only the OsO_4 stain because E-PTA does not stain vesicle membranes definitively.[9] Clear vesicles were assessed both in number and by measuring the diameter (Table 3). No significant differences were noted between Weaver-syndrome and normal animal tissues. The number of coated vesicles was measured, and the ratio of coated to clear vesicles calculated (Table 3). Again, no differences were observed.

The width of the synaptic cleft was also measured; mean values are given (Table 3). No differences in cleft width between Weaver-syndrome and normal animals were noted.

Micrographs of synapses from normal and Weaver-syndrome motor cortices demonstrate the synaptic alterations described above (Fig. 2). A schematic representation of these changes is also shown (Fig. 3).

TABLE 1 Mean values for measurements of aspects of presynaptic dense projections in synaptic junctional complexes in normal and Weaver-syndrome animals.

	OsO_4		E-PTA	
	Normal	Weaver	Normal	Weaver
Number	2.44	2.55	3.0	2.96
Height	67.0nm	60.5nm	70.1nm	61.2nm
	($p < .001$)		($p < .001$)	
Peak-to-peak Distances	165.5nm	112.0nm	114.2nm	93.5nm
	($p < .001$)		($p < .001$)	

TABLE 2 Mean values for measurements of aspects of postsynaptic thickenings in synaptic junctional complexes in normal and Weaver-syndrome animals.

	OsO_4		E-PTA	
	Normal	Weaver	Normal	Weaver
Length	428.7nm	344.6nm	365.7nm	336.0nm
Thickness	62.5nm	53.2nm	58.6nm	48.0nm
	($p < .0001$)		($p < .0001$)	

TABLE 3 Mean values for measurements of aspects of vesicles and synaptic clefts in Weaver-syndrome and normal bovine brains.

	OsO_4		E-PTA	
	Normal	Weaver	Normal	Weaver
Synaptic Vesicle (SV) Number	27.0	17.7	NA	NA
Synaptic Vesicle Diameter	34.9nm	34.0nm	NA	NA
Coated Vesicle (CV) Number	0.92	1.15	NA	NA
$\frac{\text{CV Number}}{\text{SV Number}}$	.114	.089	NA	NA
Cleft width	11.9nm	12.4nm	21.3nm	21.7nm

PLATE II

FIGURE 2 Synapses in normal bovine motor cortex (A & B) and in Weaver-syndrome motor cortex (C & D), stained with either OsO_4 (A & C), or E-PTA (B & D). Note the shorter and more closely spaced presynaptic dense projections (PDP) and the thinner postsynaptic density (PD) in the Weaver synapses. x108,900.

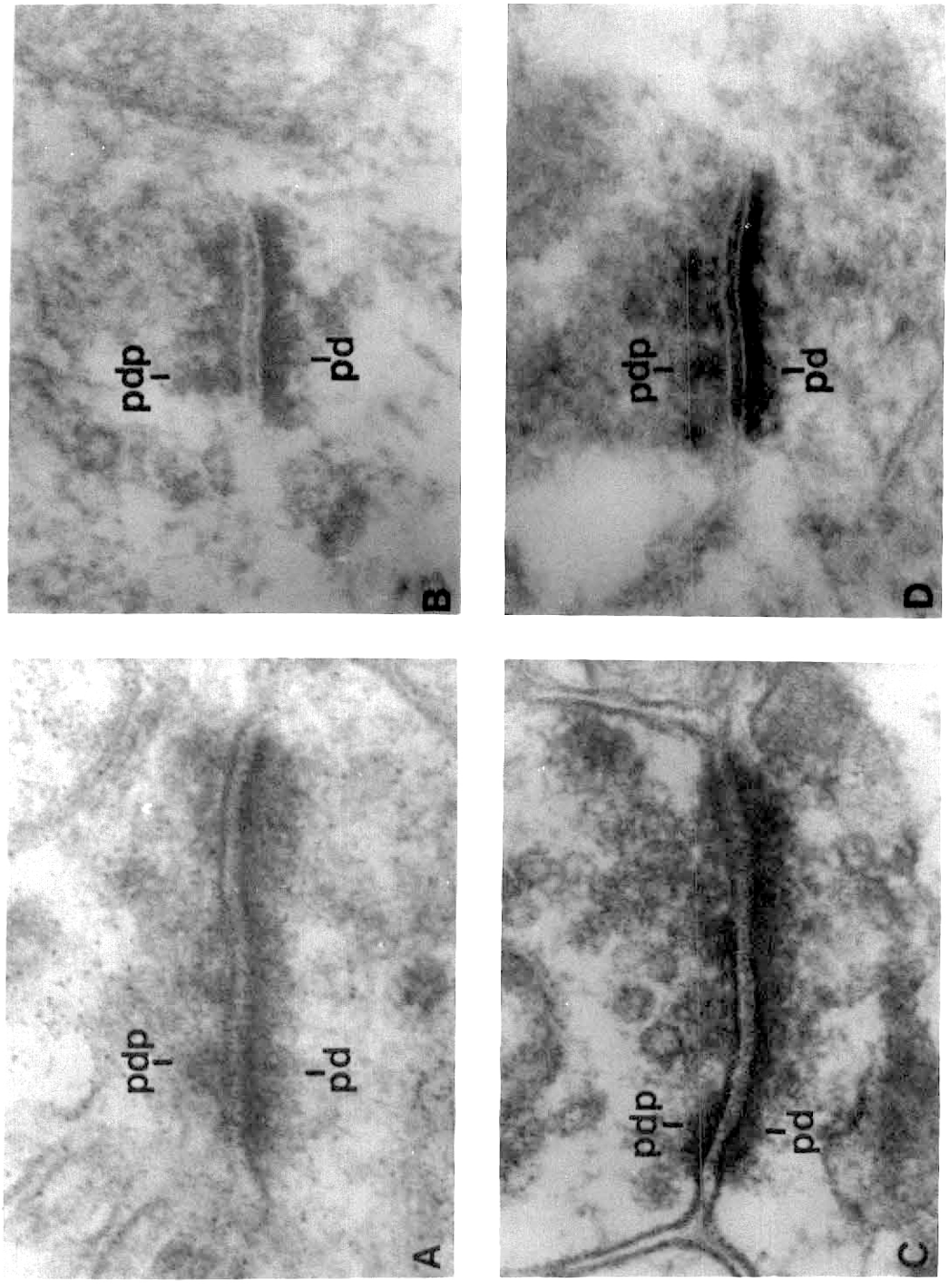
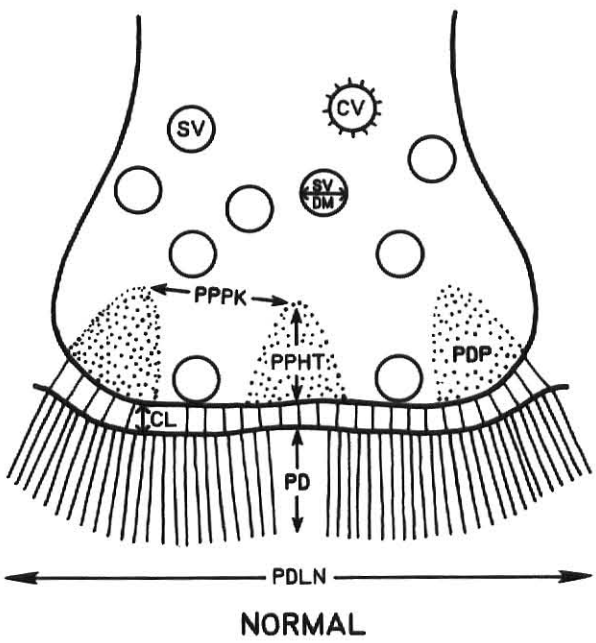
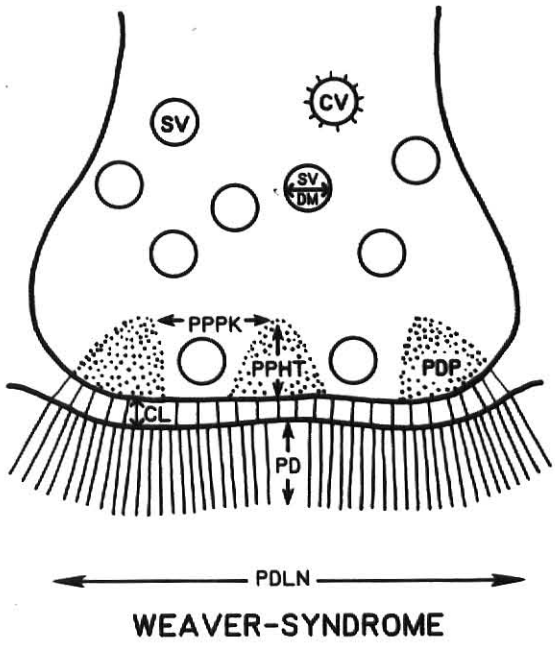

pdp
pd
A
pdp
pd
B
pdp
pd
C
pdp
pd
D

PLATE III

FIGURE 3 Schematic representation of normal vs. Weaver-syndrome motor cortex synapses. Note the smaller presynaptic dense projections with closer peaks, and the thinner postsynaptic density in the Weaver synapses.



NORMAL



WEAVER-SYNDROME

Intragroup Comparisons

All measurements were subject to intra-group comparisons as well as comparing diseased animal measurements to those from normal animals. The measurements of each synapse were assessed relative to other synapses from the same study group to determine if any significantly aberrant synapses were present and weighting the means. No such effects were found to be present. Similarly, the means and standard deviations of each measurement for each animal were calculated and compared to other animals in the same study group (diseased or normal) to pick out significant differences between animals; none were found.

DISCUSSION

The present study demonstrated alterations in the ultrastructure of synaptic junctions in the motor cortex of Weaver-syndrome cattle. As compared to normal bovine motor cortex synapses, the Weaver synapses have smaller and more closely spaced presynaptic dense projections, as well as less thick postsynaptic densities. The functional significance of these structural alterations is not known; however, several theories present themselves.

The presynaptic dense projections have been described as a three-dimensional trigonal grid, thought to serve a guiding function in the movement of synaptic vesicles to the cleft area for neurotransmitter release.[10] With the decreased size of this grid in the Weaver synapses, there may be impedence of this vesicle movement. Abnormal motor function could result from impaired vesicle movement and transmitter release.

The second ultrastructural alteration in Weaver cerebral cortex, the narrowed postsynaptic density, suggests other possibilities. Current theories suggest that the postsynaptic density provides stabilization of postsynaptic neurotransmitter receptors.[11] If the anchorage of receptors at the postsynaptic density becomes impaired, again the efficiency of neurotransmission would be impaired with obvious effects on the motor system innervated.

The measurements made and noted as significant in these animals suggest an altered state of synaptic maturity.[12] Whether this reflects delayed neural maturation in specific brain areas or altered neuronal populations is unknown. Synapses are known to undergo turnover in response to injury, and thus it is possible that these ultrastructural alterations in Weaver-syndrome synapses reflect abnormal synaptogenesis resulting from developmental lesions elsewhere in the nervous system.[11]

Finally, it must be considered that there are a variety of neuronal types in the motor cortex.[13] The assumption cannot be made that the distribution of the neuronal types is the same in Weaver-syndrome cattle as in normal animals. It is, in fact, possible that one entire neuronal population is dropped from the Weaver-syndrome motor cortices. This could result in skewed data, and reflect a loss of one specific neuronal function. Additionally, a specific type of asymmetric synapse, e.g. axo-axonal, dendro-dendritic, somato-dendritic, somato-somatic, etc., may drop out of the population. Thus it is possible that a shift in the relative proportions of different synaptic types in the Weaver cattle motor cortex could account for the alterations in junctional features noted here.

Synaptic alterations differing ultrastructurally from those reported here were noted in our preliminary investigations of synapses in brains of Weaver cattle.[3] The pre-synaptic dense projections were smaller in the Weaver cortex, but in addition we found that the mean vesicle diameters were smaller and the clefts were narrower in these animals. Also, the qualitative observation was made that Weaver synaptic junctional complexes appeared to have increased numbers of coated vesicles. The disparity between these findings and the current results can be understood in view of the synapse populations being studied. In the earlier pilot project, samples were taken from a variety of areas in the cerebral cortex, with the most striking alterations in synaptic ultrastructure being seen in the motor cortex. Thus, the decision was made that further studies would focus on that brain region of normal and Weaver-Syndrome cattle.

Two different stains, chosen for their enhancement of complementary features of the synaptic junction, were used on tissue samples in this study. Osmium tetroxide gives optimal staining of membranes of synaptic vesicles and junctions, allowing for accurate measurement of vesicle diameters as well as synaptic cleft width. Conversely, ethanolic phosphotungstic acid does not stain membranes but gives selective enhancement of paramembranous densities, allowing for optimal measurements of these structures. It is important to clarify, however, that all changes in Weaver synapses were observed with both stains. Thus, the experimental design using these two staining procedures allowed for two independent verifications of our findings within the scope of this project.

It is clear that alterations at CNS synapses could result in impaired neuromuscular functions. This could be as simple as decreased neuronal firing resulting in a disuse atrophy, or could be of much greater complexity. However, the relationship of these synaptic changes to the specific progression of clinical signs in the Weaver-syndrome animal remains to be elucidated.

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ABSTRACT

The Weaver-syndrome affects Brown Swiss cattle with signs of motor dysfunction including abnormal gait and eventual inability to stand. An ultrastructural study of synapses of the motor cortex was made comparing four adult Weaver-syndrome cattle to two normal animals. The animals were sacrificed and tissues were fixed immediately, with subsequent staining with either osmium tetroxide or ethanolic phosphotungstic acid. Micrographs of synaptic junctional complexes were made and specific ultrastructural features were measured. Measurements included presynaptic clear vesicle number and diameter; coated vesicle number; presynaptic dense projection number, height, and peak-to-peak spacing; cleft width; and postsynaptic density width and length. Means were calculated for each and statistical analyses of variance were made.

Several alterations were noted in the paramembranous densities at the synaptic junction in tissues from the Weaver-syndrome cattle. The mean height of the presynaptic dense projections was decreased ($p < .001$), and the peak-to-peak distance was significantly smaller ($p < .001$) in the Weaver animals. In addition, the mean thickness of the postsynaptic density was significantly decreased ($p < .0001$) in the Weaver tissues.

These changes in the paramembranous densities of motor cortex synapses in Weaver-syndrome cattle may be linked to the clinical manifestations of the disease in one or more ways. The presynaptic dense projection function of vesicle-guidance to the cleft for transmitter release may be impaired in these animals, or the postsynaptic neurotransmitter receptors may be less stable due to the

altered density there. The ultrastructural alterations may reflect lesion-induced synaptic turnover from injury elsewhere in the CNS. Alternatively, there may be a loss of a specific cell population of the motor cortex in Weaver animals.