Vacuolated Anterior Horn Cells in Wobbler Mouse
Motor Neuron Disease: Peripheral Axons and Regenerative Capacity

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Abstract. We investigated whether vacuolated cervical anterior horn cells of the wobbler mouse maintain axons to the periphery, and if these morphologically abnormal neurons are capable of supporting axonal regeneration. Using retrograde axonal transport, we applied horseradish peroxidase (HRP) to peripheral nerves or muscles and with electron microscopy sought evidence for perikaryal labeling in vacuolated neurons in 23 wobbler mice. When HRP was injected into forelimb muscles, 12 of 36 vacuolated neurons became positively labeled indicating that these neurons have axons in continuity with the periphery. In regeneration studies, after nerve crush at the brachial plexus, 23 out of 85 vacuolated neurons were labeled after HRP application at the elbow level. However, after a sufficient regeneration period, none of the 36 vacuolated neurons were labeled if HRP was applied in muscles below the elbow. In all experiments, morphologically normal neurons were always labeled. Our studies indicate that some vacuolated neurons of wobbler mice not only maintain axons into the periphery, but are also capable of supporting regeneration. However, the overall function of these vacuolated neurons appears marginal compared with the majority of morphologically normal neurons in this motor neuron disease.

Key Words: Axons; Axotomy; Motor neurons; Peroxidase (HRP); Regeneration; Retrograde axonal transport; Wobbler mice.

INTRODUCTION

The wobbler mouse has an autosomal-recessive motor neuron disease clinically characterized by progressive paralysis, atrophy, and contracture, predominantly in forelimb muscles (1, 2). Pathologically, anterior horn cells (AHC) undergo perikaryal vacuolar degeneration which is pathognomonic of this disease (1–5). At a given age and in a given transverse section of cervical spinal cord, however, only 4% or less of AHC exhibit vacuolar degeneration; most AHC appear morphologically normal (6). Detailed morphological analyses of AHC from perikaryon to axon terminal at different ages have shown active regeneration at the ventral root, suggesting that regeneration originated from the axon after it had undergone focal axonopathy in the course of early perikaryal degeneration (5, 7). In fact, axonal regeneration, although abnormal, does take place after nerve crush in this animal, but studies have suggested that regenerated axons were almost entirely derived from the morphologically normal AHC (8). It is not known whether vacuolated AHC are able to support axonal regeneration or whether they are premorbid neurons. In this study, we investigated to what extent the axon of the vacuolated neuron is connected with the periphery and whether this morphologically abnormal neuron is capable of sustaining axonal regeneration.

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Fig. 1. A. An unstained 1-μm-thick section shows two heavily labeled morphologically normal neurons and unlabeled normal neurons (arrows). Small dark round spots are photographic artifacts. B. Another semithin section shows one labeled normal AHG and vacuolated neuron (arrowhead). It is unclear whether vacuolated neurons are labeled with HRP.

MATERIALS AND METHODS

Wobbler mice and normal littermates used as controls were obtained from the Cleveland Clinic Wobbler Mouse Colony (the original breeding pairs were supplied by Dr. W. G. Bradley, University of Vermont and Dr. C. Hanson, National Institute of Health (5, 8)). The animals were studied at three months of age and all studies were performed in the forelimb system as in the previous investigation of axonal regeneration (8). All surgical procedures and fixative perfusions were performed under halothane anesthesia.

Anatomical Axonal Connection: To determine if the axons of vacuolated neurons extend distally into the periphery as far as the brachial plexus and up to the forelimb muscles, we injected 30% HRP (Type-VI, Sigma, St. Louis) prepared in Tris buffer, pH 8.6, distally, and after a delay (24 hours in all the experiments) sought evidence for its presence proximally in the vacuolated neurons (see below). In two wobbler mice, three major nerve trunks were ligated at the level of the brachial plexus (approximately 6 mm from the spinal cord) and the HRP was microinjected through a 50 μm tip diameter glass pipette into the endoneurium with a Picospitzer (General Valve, New Jersey). In six wobbler mice, approximately 7 μl of HRP was injected into the forelimb flexor muscles (two locations) and in extensor muscles (one location) below the elbow. Studies were done bilaterally in most animals. In a few animals, the injection was done only in one side; the uninjected side was used as a control (see below).

Axonal Regeneration of Vacuolated Neurons: We also utilized retrograde axonal transport of HRP to determine if vacuolated neurons were capable of sustaining axonal regeneration. The forelimb nerves of seven wobbler mice were crushed at the brachial plexus. The crush technique, which was identical to that used in previous studies, produced a complete axotomy (8). Two weeks after the crush, the median and ulnar nerves were transected at the level of the elbow, approximately 10–12 mm distance from the original crush site to investigate regeneration from plexus to elbow. Horseradish peroxidase (HRP) was directly applied to the distal end of transected nerves for 20 minutes. Leakage of HRP was prevented by a thin layer of paraffin placed underneath the nerve.

In four other wobbler mice, HRP was injected into forelimb muscles four weeks after brachial plexus crush to determine if there had been regeneration from plexus to forearm muscles beyond the elbow. We arbitrarily chose intervals of two and four weeks following crush after
Fig. 2. Normal AHC in the wobbler mouse. The HRP was transported from the forelimb muscle. Tetramethyl benzidine-reacted HRP is characteristically an irregular multi-layered crystalline structure (arrowheads). N: Nucleus. Bar = 0.5 $\mu$m.

our experience in previous regeneration studies (8), and so as to assure that even the most slowly regenerating axons would reach the desired point.

Tissue Processing and Analyses: All animals were perfused with aldehyde fixative (2% glutaraldehyde and 1% paraformaldehyde) through an intracardiac catheter, 24 hours after the HRP injection. The cervical spinal cord from C5 through T1 level was cut into serial 75-$\mu$m-thick sections on a Lancer vibratome. Floating sections were treated with tetramethyl benzidine (TMB) chromogen techniques (9, 10). The sections were postfixed with 1% OsO$_4$ and embedded in Spurr resin according to the method described by Carson and Mesulam (11). In each animal, approximately 30–40 blocks were made and unstained 1-$\mu$m-thick sections were examined in order to identify vacuolated AHC accurately.

In unstained semithin sections, positive HRP labeling, if present in normal neurons, was distinct whereas the presence of label in vacuolated neurons was often uncertain (Fig. 1). Sections containing vacuolated AHC in the proximity of labeled normal neurons (we presumed that the vacuolated neurons belong to the same motor pool of labeled normal neurons) were chosen for ultrathin sectioning. Most sections (approximately ten ultrathin sections in each experiment) were stained with lead citrate and uranium acetate; these, plus unstained ones were viewed on a Philips 410 electron microscope (EM) (Netherlands). The number of AHC (vacuolated-unlabeled and vacuolated-labeled) were counted separately on EM examinations.

Control Studies: In each experiment, at least one normal littermate was studied in identical fashion in order to assure that the technique was producing clear positive labeling in normal AHC.

The uninjected side of a few wobbler animals served as negative controls. Additional measures were taken in four wobbler mice to exclude the possibility of false-positive HRP reactions: 1) an animal without HRP injection, to examine the intrinsic peroxidase reaction, which was considered unlikely in TMB reaction (10); 2) remote HRP injection (in hindlimb),
Fig. 3. A typical vacuolated neuron has several HRP granules. The HRP was injected in the muscle, indicating this particular neuron has a viable axonal connection to the muscle. Bar = 5 μm. Inset shows a higher view of a typical HRP granule. Bar = 0.5 μm.

to see the reaction to blood-borne HRP; 3) application of HRP to the intact forelimb nerve, to examine unexpected HRP entry; and finally 4) HRP application at the cut end of the forelimb nerve at the elbow only one day after nerve crush, to exclude endoneurial transport of HRP.

RESULTS

Axonal Connection: Axonal connections between the periphery (brachial plexus) and the vacuolated neurons were easily identified. Detailed counting of AHC under EM was not performed in this experiment. Axonal connections between forelimb muscles and vacuolated neurons were also present. The amount of HRP in normal-looking neurons was sufficient to identify the positive labeling in semithin sections (Fig. 1), but HRP labeling in vacuolated neurons was uncertain in semithin sections and required EM examination (Fig. 1). With intramuscular injection, four of six wobbler mice had labeled vacuolated AHC, whereas all animals had positive labeling in normal neurons (Fig. 2). In this study, 12 of 36 vacuolated neurons had positive HRP labeling (Fig. 3). The possibility that some neurons may have been recounted on EM cannot be excluded, although we tried to avoid such occurrences.

Regeneration from Brachial Plexus to the Elbow: In semi-thin sections there was labeling of normal AHC in all animals. With EM examination, four out of seven wobbler mice had positive labeling in vacuolated neurons (Fig. 4). Among a total of 85 vacuolated AHC examined, 23 were found to be labeled with HRP. The amount of labeling in each vacuolated neuron varied and there was no clear difference in the amount of HRP per neuron between the axonal connection and regeneration studies.
Fig. 4. A vacuolated neuron is similarly labeled with HRP (arrowheads) which was directly applied at the elbow, two weeks after nerve crush at the brachial plexus, confirming that the regenerated axon had reached to the elbow. Bar = 5 μm. Inset shows a higher view of a HRP granule (arrow). Bar = 0.5 μm.

Regeneration from Brachial Plexus to Forelimb Muscles: In semithin sections, there were scattered, clearly positive, morphologically normal AHC; however, the number of labeled neurons appeared to be fewer than in other experiments. A total of 36 vacuolated neurons were examined and none was labeled, whereas morphologically normal AHC were labeled in EM studies (Fig. 5).

Control Studies: In none of the negative control studies was there positive labeling of morphologically normal or vacuolated AHC by EM examination, indicating that labeling found in our studies resulted from retrograde axonal HRP transport from the various injection sites.

DISCUSSION

Anatomical Integrity and Retrograde Transport: Previously, it was not known whether morphologically abnormal neurons in the wobbler mouse ever maintained anatomical axonal connection with the periphery. Our experiments showed that vacuolated anterior horn cells (AHC) do indeed connect to target muscles. We found that vacuolated neurons not only maintain anatomical connections (axons) with the periphery, but they also had the ability to transport HRP in a retrograde fashion, suggesting that they are functionally viable in this respect (12). It is likely that some axons of these vacuolated neurons ended in neuromuscular junctions, since HRP normally undergoes endocytosis at the presynaptic axon terminals in intact muscles (13). However, it is also possible that HRP entered intramuscular axons (13) previously identified in wobbler mice (5, 7) undergoing focal axonopathy and axonal regeneration.

Fig. 5. Only morphologically normal AHC is found labeled in the regeneration study from the brachial plexus to the muscle. There are numerous HRP granules. Bar = 5 μm. Inset shows a higher view of a typical HRP granule (arrow). Bar = 1 μm.

We could not determine from our study what proportion of vacuolated AHC had normal axonal connections with the periphery. Although roughly one third of the vacuolated neurons examined were labeled with HRP injected in muscle, this cannot be considered to represent an accurate proportion, since our sampling method was largely qualitative. Moreover, the fact that some animals had no labeled vacuolated neurons suggests that the degree of peripheral axonal connection may vary greatly among affected animals. It appears that only a proportion of vacuolated AHC have functional axonal connection to the muscles, while the rest may have significantly "shorter" axons or nonfunctioning axons.

Axonal Regeneration: The existence of the peripheral axonal connection allowed us to analyze axonal regeneration of the vacuolated neurons after nerve crush at the brachial plexus. The HRP technique was particularly suited for investigating regeneration in the individual neuron. Our studies clearly showed that vacuolated neurons were capable of regenerating after a traumatic axotomy.

Our result clarified questions raised in previous studies. First, we had noted active spontaneous axonal regeneration in wobbler ventral roots (5, 7), i.e. axonal regeneration in a primary perikaryal degenerative disorder—defined as a neuronopathy (14). We had speculated (5, 7) that vacuolated neurons might be able to regenerate spontaneously in response to focal axonal disease in the course of early perikaryal vacuolar degeneration. The current study supports the idea that even a neuron undergoing progressive degeneration can support active axonal regeneration. Second, we have demonstrated that axonal regeneration occurs in morphologically normal neurons in response to traumatic axotomy, but the overall rate is impaired and
individual regenerative capacity may range widely, from near normal to near absent (8). Using axonal transport and quantitative histological techniques, we had not been able to determine whether vacuolated neurons represent the end of this spectrum or whether they have no regenerative capacity. We have now confirmed that the axon from the vacuolated neuron can indeed regenerate after axotomy. The vacuolated neurons seen in our axotomized animals represented a primary pathological change and did not result from axotomy since the number and distribution of the vacuolated AHC, and their ultrastructural changes did not differ from those seen in unoperated wobbler spinal cords (5, 7). Furthermore, in another study Andrews et al (2) found no evidence of vacuolar degeneration secondary to axotomy.

Although we found that axons from vacuolated neurons could regenerate, only a small proportion of vacuolated AHC appeared to have such an ability. The axonal regeneration to the muscle seemed more difficult than that to the elbow, not only in vacuolated neurons, but also in morphologically normal neurons. Nevertheless, axonal regeneration occurring in these neurons was not a simple expression of axonal sprouting, but in fact was a true axonal elongation (8), which required sustained metabolic supply from the cell body (15).

Perikaryal vacuolar degeneration in wobbler AHC occurs in close association with the endoplasmic reticulum (4, 5), suggesting that RNA metabolism and protein synthesis are abnormal in wobbler AHC (16, 17). Despite such abnormalities, our study indicates that vacuolated AHC maintain some fundamental neuronal functions, such as protein synthesis, and fast and slow transport of newly synthesized materials for axonal elongation (15, 18). How well these individual functions are retained in the abnormal neurons remain to be determined.

The previous studies of slow axonal transport (19) and axonal regeneration (8) in the wobbler mouse have shown that biochemical abnormalities must involve normal-looking neurons, suggesting that the morphological abnormality follows the biochemical changes. Therefore, morphological abnormality is certainly not synonymous with functional normality. The present study indicates that the morphological abnormality accompanies functional impairment, but not a complete loss of function.

In human motor neuron disease, it is not known whether all the remaining AHC have functionally viable axons to the periphery. The existence of such axons is closely associated with the underlying process of neuronal and axonal degeneration which has been recently investigated (20). Histologically, axonal regeneration has been well recognized in human motor neuron disease and occurs in two locations: in the main peripheral nerve trunks (20, 21) and, less frequently, in the ventral roots (21 and Mitsumoto and Gambetti, unpublished observations). Although in human disease the primary process that triggers axonal regeneration is unclear, it is likely that focal axonopathy resulting from neuronal degeneration may stimulate axonal regeneration as in wobbler mice. The other frequently observed form of regeneration in human motor neuron disease is collateral sprouting which occurs in muscle—the reinnervation of a denervated muscle fiber (21, 22). The degree of this reinnervation has been associated with more slowly progressive disease and is expressed electrophysiologically as an increased fiber density (23, 24) and histologically as muscle fiber type grouping (25). Recently, a circulating factor that interferes with in vitro collateral reinnervation has been identified in patients with amyotrophic lateral sclerosis (26, 27). A clarification of the mechanisms of progressive neuronal degeneration and regeneration may have important therapeutic implications. We hope that further analyses in wobbler motor neuron disease will provide a better understanding of the mechanisms involving motor neuron degeneration in general.

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