Structural alterations at the neuromuscular junctions of matrix metalloproteinase 3 null mutant mice

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Abstract

Matrix metalloproteinases are important regulators of extracellular matrix molecules and cell-cell signaling. Antibodies to matrix metalloproteinase 3 (MMP3) recognize molecules at the frog neuromuscular junction, and MMP3 can remove agrin from synaptic basal lamina (VanSaun & Werle, 2000). To gain insight into the possible roles of MMP3 at the neuromuscular junction, detailed observations were made on the structure and function of the neuromuscular junctions in MMP3 null mutant mice. Striking differences were found in the appearance of the postsynaptic apparatus of MMP3 null mutant mice. Endplates had an increased volume of AChR stained regions within the endplate structure, leaving only small regions devoid of AChRs. Individual postsynaptic gutters were wider, containing prominent lines that represent the AChRs concentrated at the tops of the junctional folds. Electron microscopy revealed a dramatic increase in the number and size of the junctional folds, in addition to ectopically located junctional folds. Electrophysiological recordings revealed no change in quantal content or MEPP frequency, but there was an increase in MEPP rise time in a subset of endplates. No differences were observed in the rate or extent of developmental synapse elimination. In vitro cleavage experiments revealed that MMP3 directly cleaves agrin. Increased agrin immunofluorescence was observed at the neuromuscular junctions of MMP3 null mutant mice. These results provide strong evidence that MMP3 is involved in the control of synaptic structure at the neuromuscular junction and they support the hypothesis that MMP3 is involved in the regulation of agrin at the neuromuscular junction.

Introduction

The matrix metalloproteinases are a family of more than twenty enzymes that are responsible for the degradation or modification of extracellular matrix molecules. Each enzyme will preferentially degrade unique matrix molecules, yet individual matrix molecules can be degraded by multiple MMPs. Most MMPs are secreted in an apoenzymatic form and must be activated through proteolysis for themselves to become active (VanWart & Birkedal-Hansen, 1990). The activity of matrix metalloproteinases is kept in check by a family of inhibitors of matrix metalloproteinases (reviewed in Reynolds, 1986; Sternlicht & Werb, 2001; Nagase & Brew, 2002). Proper activity of MMPs is important for the overall integrity of the extracellular matrix, and MMPs are important in regulating cell-cell signaling.

One important signaling molecule at the neuromuscular junction is agrin. Agrin is a heparan sulfate proteoglycan that is a component of the synaptic basal lamina. Mice that lack agrin, or its receptor MuSK, fail to form neuromuscular junctions and die at birth (Gautam et al., 1996; DeChiara et al., 1996). Agrin induces the aggregation of synapse specific molecules, including AChRs-and AChE, while also directing the formation of postjunctional folds (Wallace et al., 1989; Cohen et al., 1997). Treatment of cultured myotubes with agrin results in the aggregation of AChRs on the surface of the myotubes, and removal of agrin results in the rapid dispersal of these agrin-induced AChR aggregates (Wallace, 1988). Strong evidence supports the hypothesis that agrin is produced by the motor neuron, where it is transported to the synapse and released (McMahan, 1990; Reist et al., 1992). Once released agrin binds with high affinity to the gamma-1 chain of laminin in the synaptic basal lamina and simultaneously interacts with MuSK to induce the formation of the postsynaptic apparatus on the surface of the muscle fiber (Glass et al., 1996; Kammerer et al., 1999). Thus, it is of critical importance to understand the mechanisms that control agrin binding to synaptic basal lamina.
Matrix metalloproteinases have been shown to be present throughout the nervous system. In the peripheral nervous system it has been shown that MMPs are present in the nerve sheaths, and that the expression of these MMPs is altered following nerve injury (LaFleur et al., 1996; Kherif et al., 1998; Hughes et al., 2002). Previously, we reported that MMP3 was localized to the frog neuromuscular junction, and that purified recombinant MMP3 removed agrin from synaptic basal lamina (VanSaun & Werle, 2000). To gain insight into the possible roles of MMP3 at the neuromuscular junction we made detailed observations on the structure and function of the neuromuscular junctions in MMP3 null mutant mice. The MMP3 null mutant mice had an increased density of AChRs; increased number and size of postsynaptic folds; abnormal miniature endplate potentials; and an increased agrin immunofluorescence at their neuromuscular junctions. These results provide strong evidence that matrix metalloproteinase 3 is involved in the control of synaptic structure at the neuromuscular junction, and further support the hypothesis that MMP3 is involved in the regulation of agrin at the neuromuscular junction.

Methods

ACETYLCOLINE RECEPTOR DENSITY

Diaphragm muscle was dissected from control and MMP3 null mutant mice (John Mudgett; Merck Research Labs). Muscles were pinned onto Sylgard coated dishes and fixed in cold 1% paraformaldehyde in 0.13 M phosphate buffer at pH 7.5 for fifteen minutes. The muscles were then washed with 0.13 M phosphate buffer with 5% normal goat serum (PBS/NGS) for fifteen minutes. Rhodamine α-bungarotoxin used at 1 ng/µl in PBS/NGS was applied to the muscles for half an hour to stain for acetylcholine receptors. The muscles were then washed in PBS/NGS for one hour. After washing the muscles were post fixed in ice cold 95% ETOH. The diaphragm muscles were cut out and mounted on slides with an anti-fade medium. Images of endplates were acquired using rhodamine optics on a Zeiss confocal microscope. Images of endplates were acquired using a Zeiss confocal microscope. AChR staining was used to calculate the total volume of endplate for ten endplates for each muscle each day (thirty endplates total per treatment group per day). Volume measurements were calculated using 3D for LSM software (Zeiss). Volume measurements were then transferred to StatView and graphed. Statistical analysis was performed using ANOVA.

SYNAPSE ELIMINATION

To determine the rate of synapse elimination, diaphragm muscles were removed from three littermate control and MMP3 null mutant mice at days 6, 9, 12, and 15 after birth. Muscles were pinned onto Sylgard coated dishes and fixed in cold 0.13 M PBS pH 7.5 with 1% paraformaldehyde for fifteen minutes. To block nonspecific sites, muscles were incubated in PBST (0.1% Triton)/NGS and then stained with primary neurofilament heavy chain antibodies 1:1000 (Chemicon) and secondary FITC conjugated donkey anti-rabbit 1:200 for visualization of axon terminals. Endplates were stained with rhodamine α-bungarotoxin 1 ng/µl. After post fixation in ethanol, muscles were infiltrated with anti-fade medium (80% glycerol, 20% 0.5 M phosphate buffer pH 8.0, and 2% N-propyl gallate) and mounted on slides. For each muscle, more than one hundred endplates were analyzed, counting only endplates with discernable innervation. The number of endplates with more than one innervating axon for each of three muscles was summed and divided by the total number to get the percentage of polyneuronal innervation at each time point. The error was calculated using the standard error of the proportion.

ELECTRON MICROSCOPY

Three control (129/c57bl6 hybrid; Jackson Labs) and three MMP3 null mutant mice age 52 days and weight matched, were perfused using 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. After perfusion, the endplate region from the diaphragm was dissected, rinsed with 0.1 M PBS and then post fixed in 1% OsO4 in 0.1 M cacodylate for 45 min. The tissue was washed in water, dehydrated in an ethanol dilution series, and washed with propylene oxide before infiltrating it with half propylene oxide and half araldite overnight while rotating. Polymerization of the araldite was achieved by incubation at 100 °C overnight. After polymerization, the area of interest in the tissue was cut out and mounted on a dowel. Sections were cut at 80 nm, grid stained and observed in a JEOL microscope. Photographic negatives of the endplates were scanned at 600 dpi and analyzed using NIH Image Software. The number of primary junctional folds were counted. A primary junctional fold is defined as a fold which opens up into the synaptic cleft, or muscle sarcolemma. The length of the junctional folds were measured by measuring a line drawn along the lamina densa.