Introduction

Traumatic peripheral nerve injuries often produce significant functional deficits despite optimal medical management. Although the general belief is that the regenerative capacity of the PNS is much more robust after nerve insult relative to a CNS injury, this does not translate in the clinical setting. Segmental nerve injuries such as brachial plexus injuries often are treated with nerve or muscle transfers as reinnervation of the target end organs after primary repair is incredibly poor. Animal studies have shown that nerve repair after prolonged denervation of the muscle yields limited functional recovery. In the clinical arena, muscle undergoes a phenotypic change and is replaced by fibrous and adipose tissue after prolonged denervation. The loss of targets for reinnervation may lead to a down-regulation of trophic factors guiding regenerating axons into their proper end points, leading to end-organ atrophy of the neuromuscular junction. The neuromuscular junction consists of three components: the acetylcholine (Ach) receptor, the terminal axon, and perisynaptic Schwann cells. Recently it was shown that when the nerve is subjected to crush injury, the terminal axon is degraded while perisynaptic Schwann cells continue to maintain contact with acetylcholine receptors. After two weeks, the axon reinnervates the motor end plate as the NMJ then reverts to normal appearance. Multiple studies have focused on examining the gross morphology of muscle fibers after denervation injury. As of yet, no studies have offered a detailed evaluation of the NMJ after prolonged denervation. It is known that assembly and maintenance of the neuromuscular junction depends highly on the interaction between the glycoprotein agrin and its receptor muscle-specific kinase (MuSK). The NMJ matures during development under the direction of signaling mechanisms initiated with binding of agrin to MuSK. Agrin is synthesized by both neurons and perisynaptic Schwann cells at the motor end plate. It is degraded by matrix metalloproteinase 3 (MMP-3), an enzyme also synthesized by perisynaptic Schwann cells. The current study focuses on assessing the NMJ after long-term denervation injury. Levels of agrin and MuSK are quantified at different timepoints to determine if destabilization of the NMJ corresponds to a decrease in the concentration of these proteins. In addition, the effects of prolonged denervation were examined in a knockout mouse for MMP-3 to determine if persistence of agrin at the NMJ would allow for stabilization of Ach receptors following nerve transection.

Materials and Methods

Animal model: Wildtype 129 Sv/Ev and MMP-3 knockout mice were a gift from Dr. Wee Yong at the University of Calgary. Six week old animals were anesthetized with a ketamine/xylazine mixture. A dorsal gluteal-splitting approach was used. A 10mm segment of the right sciatic nerve was removed while the contralateral sciatic nerve was returned to the host bed to serve as a control specimen. Soleus muscles in both wildtype and MMP-3 knockout mice at the following times post injury: 3 days, 7 days, 14 days, 1 month, 4 months (n=4 all time points).

Immunohistochemistry: Whole mount specimens were fixed in 4% PFA solution for 1 hour. Specimens were blocked overnight at 4°C (1% Triton X-100, 4% NGS in PBS). Muscles were then incubated with Alexa 555-conjugated BTX (1:1000) and antibodies to neurofilament (Covance; 1:500) or S100 (Dako; 1:500) for 24 hours at 4°C. Muscle specimens were then washed in PBS and incubated in secondary antibody (Molecular Probes). The muscle was imaged using confocal microscopy. Quantification of endplate area was performed in ImageJ.

Western Blots: Whole gastrocsoleus complexes were harvested from ipsilateral and contralateral sides for wildtype and MMP-3 knockout mice and homogenized according to standard protocol. 50 µg of protein were loaded into 10% SDS gels. Electrophoresis was carried out at 150V for 1 hour and 15 min. Transfer of the protein to nitrocellulose membrane was then performed overnight in 4°C at 30 V. The membrane was then blocked in 2% milk-TBS solution for one hour. Subsequently, mouse monoclonal anti-agnir (Millipore) was applied overnight at 4°C at 1:500 in 2% milk-TBS. The membrane was then placed in 1:1000 HRP conjugated goat anti-mouse mixture for 2 hours. Pierce ECL mixture was used to visualize the resultant protein bands.

Results

Specimens from normal muscle consistently contained Ach receptors with round profiles and multiple perforations which are consistent with the normal phenotype as demonstrated in previously published reports. In contrast, the muscles innervated by the transected nerve demonstrated significant attenuation of Ach receptor profiles. The frequency of these deranged profiles increased in specimens harvested from later time points. In concert with this pattern, the calculated area of the Ach receptors demonstrated a temporal decline. This finding was most prominent in specimens 4 months after injury where these fragmented receptors were encountered with regularity.

Surprisingly, muscles from injured nerves from the MMP-3 knockout mice contain receptor profiles with areas greater than contralateral specimens. Gross inspection of these receptors revealed that they retained the morphology seen in wild-type control specimens. Western blot data demonstrated decrease in agrin in denervated muscles in wildtype animals but an increase in agrin in MMP-3 knockout mice.

Discussion

The components of the neuromuscular junction undergo gradual degredation after prolonged denervation. For the terminal axon and perisynaptic Schwann cells, this process is immediate as the mechanism for Wallerian degeneration is initiated after nerve transection. For the Ach receptor, signs of destabilization are not grossly seen until the later time points, although evidence for this process can be found in loss of receptor area early after injury. The ability of the Ach receptor to resist destabilization after prolonged denervation becomes enhanced after knockout of MMP-3 function. This is likely due to the persistence of agrin at the NMJ as MMP3 is no longer present to remove agrin. The fact that the perisynaptic Schwann is also absent at the NMJ at the 4 month time point in transection models for both wildtype and MMP-3 animals suggests that the derivative maintaining acetylcholine receptors in MMP-3 knockout mice is likely the muscle. Immunoblotting experiments are currently underway to assess the quantity of agrin in both wildtype and MMP-3 knockout mice after transection.

Significance

Taken together, it appears that the NMJ/motor end plate can be stabilized after denervation by rendering MMP3 inactive and thereby allow agrin to remain functional at the NMJ. Further experiments will be aimed towards developing clinically relevant techniques of blocking MMP3 function after traumatic nerve injury.