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Induced Pseudomembrane Enrichment in Long Nerve Allograft Reconstruction

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ABSTRACT

Introduction/Aims: Long nerve defects are typically reconstructed with autograft or processed acellular nerve allograft (PNA). PNA is convenient and avoids donor morbidity but lacks the neurotrophic environment of autograft. Increased levels of neurotrophic factors have been identified in pseudomembranes induced around silicone implanted between nerve ends. This study aimed to determine if pseudomembrane can be reliably induced around silicone implanted between nerve ends, and if this enhances regeneration of PNA inset within using a staged technique.

Methods: Lewis rats ($n = 24$) underwent resection of a 15-mm sciatic nerve. The defect was filled with a silicone tube ($n = 12$) (MA) or the nerve ends were secured to a muscle bed ($n = 12$) (NMA). After 4 weeks, the silicone was replaced with PNA threaded within the pseudomembrane tunnel. In both groups, PNA was used to reconstruct the nerve defect. Weekly neuromotor assessment was performed with sciatic function index (SFI). At 16 weeks, muscle recovery was assessed, and nerve samples were obtained for histomorphometry.

Results: The MA group's average normalized muscle weight was 46.25% versus the NMA group's 33.19% ($p < 0.05$). The MA group's average normalized muscle girth was 78.25% versus the NMA group's 60.73% ($p < 0.05$). Axon counts, g-ratio, and muscle force were not statistically different. At Week 15, the MA group had a significantly higher average SFI: −82.25 versus the NMA group −95.03 ($p < 0.05$).

Discussion: PNA inset within induced pseudomembrane sheath enhanced muscle reinnervation. A staged membrane enhancement technique may be effective for improving PNA efficacy in peripheral nerve injury reconstruction.

1 | Introduction

When direct approximation of nerve ends in peripheral nerve injuries is not possible, the defect must be bridged utilizing one of three available tools: conduits, processed acellular nerve allograft (PNA), or nerve autograft. Nerve autograft is the conventional gold standard offering superior neurosupportive qualities but requiring a donor site, which decreases surgical efficiency and increases surgical morbidity [1, 2]. PNA lacks many of the growth factors and supportive cells found in normal nerve

Abbreviations: ANA, acellular nerve allograft; MA, PNA plus induced membrane group; NMA, PNA without induced membrane enhancement; PL, paw length; PNA, processed acellular nerve allograft; SFI, sciatic function index; TS, toe spread.

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tissue [3–6], potentially compromising the neurotrophic environment in comparison to nerve autograft [7–9]. Enhancement of this neurotrophic potential should increase the value of PNA as a more reliable nerve autograft substitute.

Most experimental strategies to enhance PNA have focused on artificially augmenting intraneural neurotrophic factors or directly seeding the graft with neurosupportive cells [10–22]. However, the physiological interactions between growth factors and neurons are complex, and counterintuitively, simply adding local factors can inhibit axon elongation [23–25]. Furthermore, while increasing the population of supportive cells within the graft via cell seeding has shown promise in small animal studies [20, 26–30], overpopulating the same trophic-poor environment seems unlikely to effectively alter the fate of these cells.

The two-stage induced membrane technique originally described by Masquelet et al. has been an effective and widely accepted method for osseous defect reconstruction [31, 32]. The success of this technique has been attributed to increased concentrations of vital growth factors directly related to the membrane [31–34]. This induced membrane technique could be translated to nerve reconstruction applications to improve vascularity and enhance the local neurotrophic environment. There are clinical situations in which a staged technique may be practical including nerve reconstruction delay to allow dampening of the inflammatory scar phases of nerve bed healing while encouraging improved vascularity and neurotrophic factor concentrations at the future graft site [33, 35–37]. Furthermore, there are patients with multi-nerve, large segment, or large nerve defects that simply cannot be fixed with available autograft or other conventional surgical strategies. Anecdotal reports from the military include poly-trauma patients with multiple extremity amputations severely limiting autologous nerve graft supply [38]. Scenarios like these may benefit from staged PNA-based repairs.

Our laboratory has previously shown that tissue samples harvested from an analogously induced peri-neural pseudomembrane tube formulated around an implanted silicone tube retained for 4 weeks demonstrated significantly elevated levels of multiple neurogenic growth factors [35]. This study aimed to determine if a pseudomembrane can be reliably induced around silicone implanted between free nerve ends in a nerve injury model. Furthermore, we aimed to determine if this pseudomembrane enhances regeneration of inset PNA using a two-staged nerve reconstruction technique. We hypothesized that this growth factor-rich pseudomembrane would enhance the microenvironment around implanted PNA and lead to improved nerve regeneration.

2 | Methods

In compliance with our institution's animal utilization committee and in accordance with national guidelines, 24 male Lewis rats were divided into two cohorts: PNA plus induced membrane (MA) and PNA without membrane enhancement (NMA). All surgical procedures were performed under aseptic conditions with the aid of surgical microscope visualization. Anesthesia was induced with 3% isoflurane administered via nose cone inhalation and maintained with 2%–3% isoflurane. A standard bicep femoris-semitendinosus muscle splitting approach was used to expose the sciatic nerve; experimental limb laterality was alternated in each subject. The design and timeline of surgical proceedings is detailed in Figure S1.

At 0 weeks, 15 mm of sciatic nerve were excised in all subjects. For the MA group ($n = 12$), a 2-cm sterile 6-French (2 mm outer diameter) silicone pediatric foley catheter (Teleflex Medical, Annacotty, Limerick, Ireland) was interposed in the nerve defect and secured to the adjacent epineurium with 9-0 nylon suture (AROSurgicals, Newport Beach, California) similar to graft interposition in nerve reconstruction. In the NMA group ($n = 12$), the cut nerve ends were sutured directly to the adjacent soft tissue bed in line with the in situ nerve with 9-0 nylon sutures (Figure S2) for ease of identification during second-stage surgery.

PNA was processed and sterilized for this experiment by Axogen Inc. (Alachua, FL) using nerve allograft obtained from a genetically distinct strain of rats (Sprague–Dawley) and utilizing a proprietary detergent and enzymatic process analogous to the preparation of commercially available human PNA (Avance graft, Axogen Inc., Alachua, FL). The PNA was shipped frozen to our lab where it was stored at subfreezing temperatures (−80°C) until the time of usage and thawed prior to implantation.

After 4 weeks to allow induced pseudomembrane formation, all animals were re-anesthetized, and the previous surgical sites re-opened. Care was taken to protect the pseudomembrane and small incisions were made through the pseudomembrane at the distal and proximal attachments of the foley. For both groups, the sciatic nerve stumps were identified, mobilized, and sharply freshened. For the MA group, 20-mm PNA was sutured to one end of the silicone foley so that as the silicone tube was pulled free from the pseudomembrane tunnel, the graft was pulled into the tunnel (so that it was contained within the pseudomembrane). The graft was sutured to the nerve stumps and pseudomembrane was closed with 9-0 nylon sutures. In NMA subjects, 20 mm PNA was inset between the nerve stumps using 9-0 nylon sutures. All surgical wounds were irrigated and closed with 4-0 nylon (AROSurgicals, California). Postoperative pain was monitored and controlled using buprenorphine 0.5 mg/kg subcutaneously.

At weekly intervals, starting prior to surgery and after appropriate acclimation, video pawprint images were obtained from rats traversing a transparent plank. Six measurements were obtained for each timepoint to calculate SFI using ImageJ software (imagej.nih.gov). Toe spread was determined between the first and fifth toes (1–5, TS) and between the second and fourth toes (2–4, intermediate TS), and normalized using the paw length (PL) for each foot (Figure S3).

At 12 weeks post PNA reconstruction, all rats were re-anesthetized, and bilateral sciatic nerves and gastrocnemius muscles were exposed. Each limb was secured to a platform via placement of Kirschner wires through the femoral condyle and the distal tibia, and the gastrocnemius tendon was coupled to

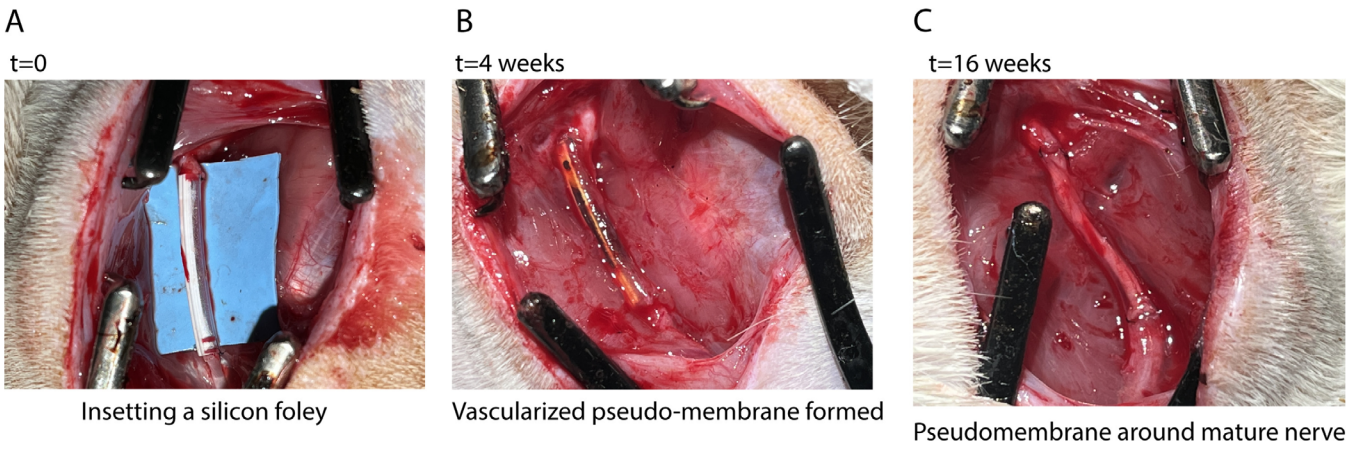

FIGURE 1 | Pseudomembrane formation. (A) Timepoint 0 weeks, nerve defect with interposed silicone foley. (B) Timepoint 4 weeks, silicone foley with pseudomembrane formation. Note thick membrane and vascular environment. (C) Timepoint 16 weeks (12 weeks post nerve reconstruction), mature PNA nerve reconstruction within pseudomembrane. Note thick membrane integrity and vascularity have been preserved.

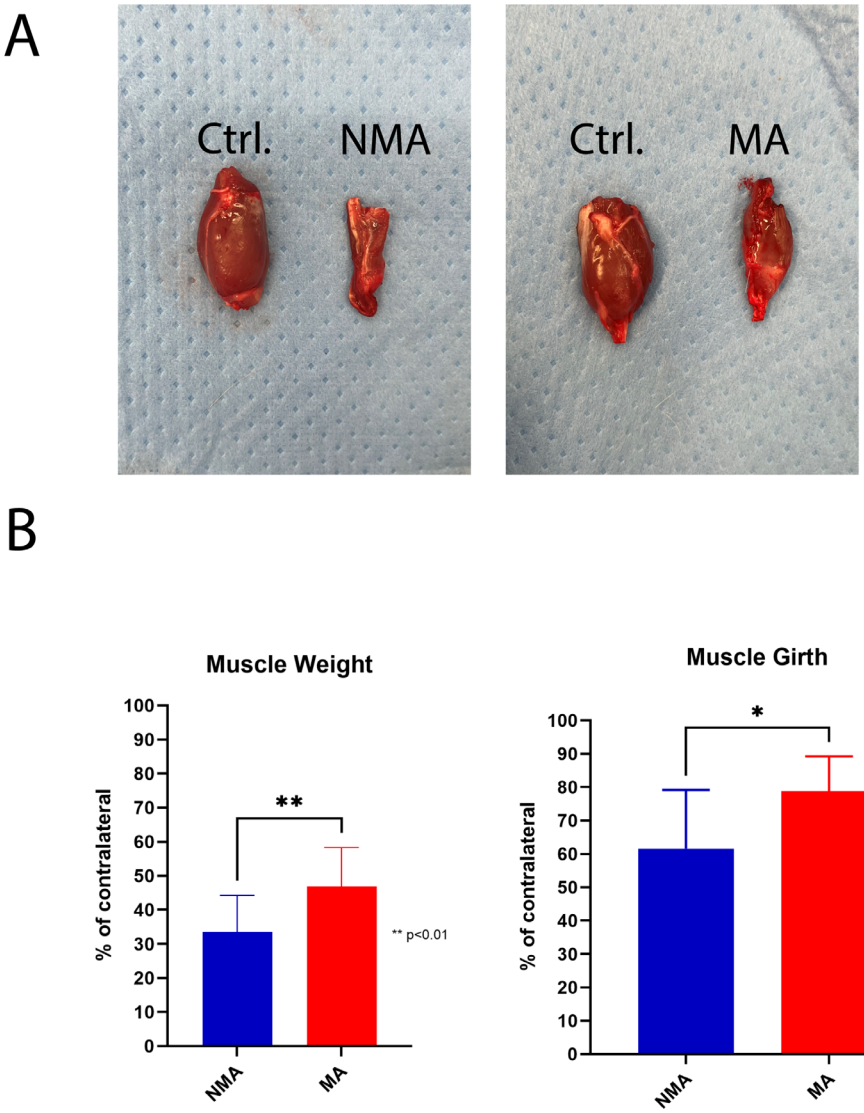

FIGURE 2 | Muscle morphology. Comparison of (A) Nonmembrane PNA control group (NMA) and membrane PNA experimental group (MA) average muscle gross appearance. (B) Weight and girth represented as the percentage of nonsurgical contralateral limb (*$p < 0.05$, **$p < 0.05$).

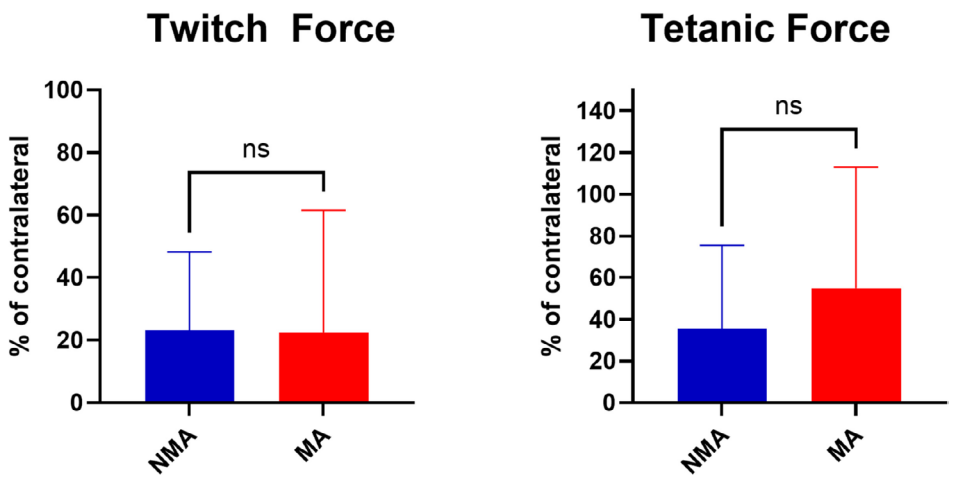

FIGURE 3 | Muscle force. Comparison of NMA and MA average muscle force after single twitch and tetanic stimulation represented as the percentage of nonsurgical contralateral limb.

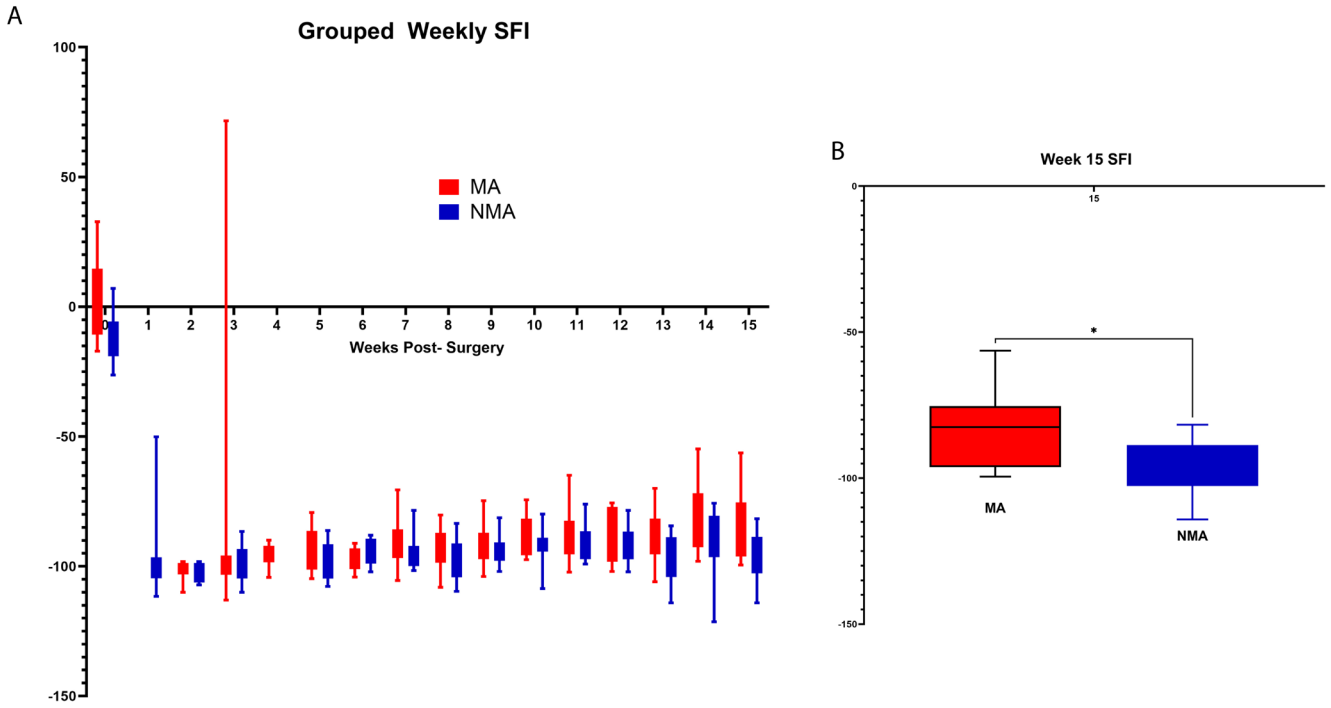

FIGURE 4 | Sciatic function index (SFI). Comparison of NMA and MA SFI. (A) Weekly calculation of SFI based on gait analysis. (B) Week 15 SFI for ease of visualization (*$p < 0.05$).

a force transducer (AD Instruments, Sydney, Australia) using 2-0 silk suture (AROSurgicals, Newport Beach, California). A stimulating electrode was placed 5 mm distal to the sciatic notch on the sciatic nerve and proximal to the nerve reconstruction. Single square pulses of 0.2 ms duration were applied while gradually increasing the strength of the stimulus (up to 2 V) until a maximum muscle contraction was achieved, and muscle tension was optimized based on the Blix curve. Twitch contraction (5 V, 0.2 ms) and tetanic contractions (5 V, 0.2 ms, 130 repeats) were measured and recorded using the AD Instruments Power Lab system (Sydney, Australia). Three contractions separated by 3 min of rest time were averaged for each tetanic stimulation. Bilateral gastrocnemius muscles and sciatic nerves were harvested for morphologic and histomorphologic assessment.

Nerve specimens were postfixed in 4% paraformaldehyde containing glutaraldehyde and sodium cacodylate buffer, at 4°C. Samples obtained 5 mm distal to the nerve reconstructions were resin embedded, sectioned, and stained with toluidine blue to allow for axon counting. Using AxonDeepSeg software (2018 NeuroPoly, École Polytechnique, Université de Montréal, Montreal, Quebec, Canada), a blinded observer obtained average axon counts across 6–8 high-powered fields (40×). The average axon count per high-powered field was multiplied by the

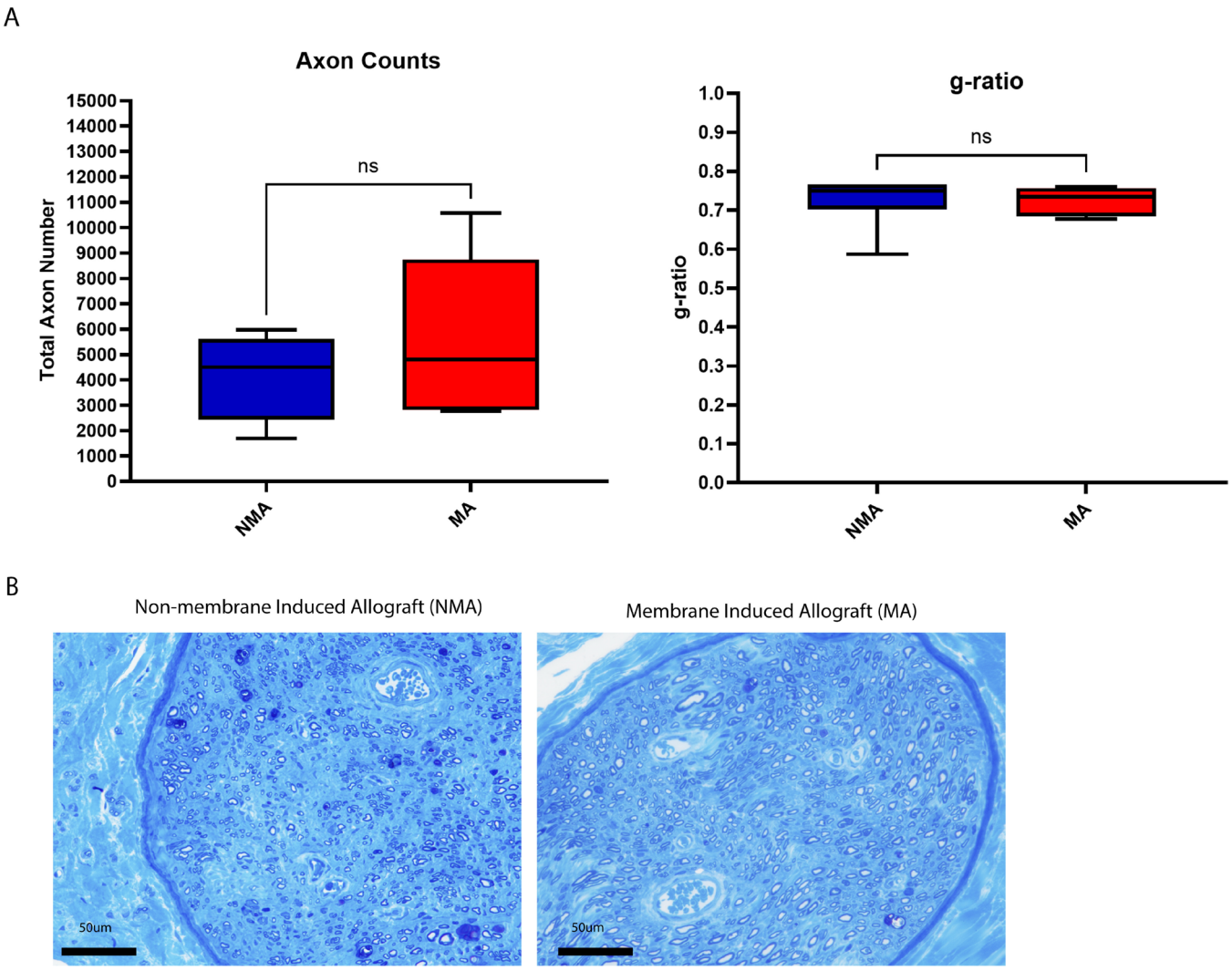

FIGURE 5 | Nerve histology. (A) Comparison of NMA and MA postcoaptation axon counts and G-ratio at Week 16 (12 weeks post PNA reconstruction). (B) Representative histology slides of NMA and MA nerves. Indicator bar measures 50 um.

appropriate factor as determined by the cross-sectional area of the specimen based on a 4× image.

Comparative analysis of muscle weight and girth, SFI, muscle contractile force, and nerve histomorphology was performed using Student's *t*-test with $p < 0.05$ considered statistically significant.

3 | Results

At 4 weeks postimplantation, a uniform induced pseudomembrane reliably formed in all 12 experimental test subjects (Figure 1). This membrane was stable enough to facilitate PNA reconstruction.

3.1 | Muscle Weight and Girth

Both muscle girth and weight were significantly higher in the MA group compared to NMA rats at 12 weeks post-reconstruction (Figure 2). The induced membrane group's average normalized muscle girth was 78.25% ± 10.38% and the NMA group's was 60.73% ± 17.4% ($p < 0.05$). The induced membrane group's average normalized muscle weight was 46.25% ± 11.49% and the NMA group's average was 33.19% ± 10.71% ($p < 0.05$).

3.2 | Muscle Strength Testing

In the NMA group, the average normalized twitch muscle force was 23.05% ± 25.19%, while the mean normalized tetanic muscle force was 35.50% ± 40.03%. In the MA group, the average normalized twitch muscle force was 22.44% ± 39.09%, while the average normalized tetanic muscle force was 54.91% ± 58.06%. These differences were not statistically different (Figure 3).

3.3 | Sciatic Function Index

At Weeks 0–14, no significant difference in SFI between MA and NMA groups was found (Figure 4). At the final Week 15 evaluation, a significant difference was observed between groups in SFI measurement ($p < 0.05$).

3.4 | Nerve Histology

Average axon counts were 4146.50 ± 1750.15 in the NMA group and 5654.33 ± 3227.14 in the MA group. The G-ratio was found to be 0.727 ± 0.069 in the NMA group and 0.725 ± 0.035 in the MA group. These differences were not statistically different (Figure 5).

4 | Discussion

Using a two-stage induced pseudomembrane nerve reconstruction technique, we successfully generated pseudomembrane sheaths surrounding silicone tubes in 12 of 12 experimental subjects. Moreover, this membrane was durable and appeared to withstand manipulation and intramembranous nerve reconstruction. Supportive of our hypothesis, the PNA inset within this pseudomembrane did seem to support more robust nerve regeneration as evidenced by greater muscle hypertrophy and higher SFI scores compared to PNA alone.

Though utilizing different target muscles and acellular nerve allograft generated utilizing a different tissue processing protocol than used in our study, Saheb-Al-Zamani et al. reported reinnervated extensor digitorum longus muscles normalized weights of 36% and 65% following 20 mm acellular nerve allograft and isograft reconstruction versus our reinnervated gastrocnemius normalized muscle weights of 33% and 46% following 20-mm PNA allograft without and with pseudomembrane formation respectively [7]. Moore et al. evaluated the recovery of 14 mm nerve defects repaired with PNA at 6 and 16 weeks. At 16 weeks, these subjects demonstrated 57.57% muscle weight and 22.25% motor force recovery [39]. Whitlock et al. further evaluated PNA versus isograft controls in 14- and 28-mm defect lengths. Comparable recovery of muscle weight (34% and 40%) was noted between groups across the 14-mm defect, but only 16% muscle weight and < 6% muscle force recovery was noted following 28-mm PNA reconstruction [40].

Zadegan et al. utilized a similar two-staged technique to the one employed in this study and performed motor testing at 12 weeks post autograft reconstruction in a rodent model. They found a significantly improved 56.2% motor recovery in membrane-enhanced subjects compared to 42.7% recovery in control animals. However, they were unable to demonstrate significant enhancement of functional muscle recovery during gait analysis at 3-, 6-, and 15-week time points compared to control subjects [36, 37]. Similarly, we were unable to elicit significant enhancement of functional muscle recovery via SFI gait analysis from Weeks 1 to 14. However, in the final week 15 analysis, we did find a significant enhancement of SFI measurements in the experimental group compared to controls. As has been observed by Contreras et al. and others, this may be attributed to delayed functional regeneration in the nerve allograft [41].

Despite our positive findings, muscle contraction forces and axon counts were not statistically different between groups. Variances in some data points could be explained by inconsistencies in surgical technique, lack of precision in outcomes measures, and biologic variability between animals. While efforts to preserve the pseudomembrane's vascularity appeared successful, the limitations in the available surgical field space and the size of the target nerves did offer some technical challenges (both smaller than would be expected in humans). Retained perfusion was not assessed and inadvertent disruption could further account for some discrepancies and variability. Another possibility is that full regenerative capacity of PNA may not have been evident at our 12-week endpoint. In their study of ANA versus autograft reconstruction of 15-mm rat sciatic nerve defects, Contreras et al. found effective but delayed regeneration in the nerve allograft subgroup. Muscle recovery was absent in 1/3rd of subjects at 13 weeks post-reconstruction but became evident at the 17-week endpoint. Postcoaptation axon counts were however similar between ANA and autograft groups at 17 weeks [41]. Interestingly, delay in observed recovery may be related to the extra time necessary for Schwann cell repopulation. Furthermore, we utilized artificial intelligence-based axon analysis software. This approach has been previously validated and mean axon counts of the NMA groups were reassuringly similar to previously collected data across a 20-mm rodent PNA utilizing more conventional counting techniques [42].

Despite overall encouraging results, our study suffered limitations to be further explored. Length and thickness are variables thought to negatively correlate with the neurogenic potential of PNA and any therapeutic benefit derived from the induced pseudomembrane technique might be more evident as these parameters are increased. Difficult to achieve in a rodent model, this future research would necessitate the use of larger animals. Additionally, in our model, the local environment around the PNA was not compromised by injury as may be seen in the clinical setting. Physiologic advantages of the two-staged nerve reconstruction technique might be more evident when applied to a scarred or damaged tissue bed. Print length, a major variable in SFI measurements, can be affected by gait velocity not measured in this study, which may have affected SFI calculations. The aims of our study were to determine the feasibility and overall effect of a two-stage induced pseudomembrane technique on enhancing nerve regeneration. Future in-depth evaluations including muscle histology and histomorphometry, nerve graft growth factor quantification, and graft vascularity analysis may provide further valuable insights. Finally, our study is based on a 4-week pseudomembrane maturation timepoint as described in Masquelet's original studies [32]. The optimal timing for pseudomembrane maturation for nerve regeneration has not been defined.

There are scenarios in which a staged PNA-based reconstructive technique may be more practical and beneficial to outcomes, such as multiple extremity injuries with limited autograft supply or wounds with hostile tissue beds. In these settings, full demarcation of the injury, dampening of local inflammatory response, and debridement of scar and contamination are necessary to optimize recovery. Furthermore, a stage induced membrane strategy may improve vascularity and neurotrophic factor concentrations at the PNA implantation site [33, 35–37]. While excessive delays in nerve reconstruction would be deleterious to recovery by reducing Schwann cell support, decreased neuromuscular junction receptivity, and muscle atrophy and fatty replacement, a relatively brief 4 weeks to allow membrane maturation would be unlikely to substantially affect axonal regeneration [43–45]. Overall, the results of our study are favorable and

suggest that a staged membrane enhancement technique may be an effective and viable option for enhancing PNA reconstruction.

Author Contributions

Omar A. Protzuk: conceptualization, investigation, funding acquisition, writing – original draft, methodology, validation, visualization, writing – review and editing, software, formal analysis, project administration, data curation, supervision, resources. **Mariam A. Samuel:** data curation, formal analysis, writing – original draft, investigation, software. **Kriston R. Seward:** investigation, data curation, formal analysis, software. **Christopher A. Keshishian:** investigation, data curation, software, formal analysis. **Geetanjali S. Bendale:** conceptualization, investigation, funding acquisition, writing – original draft, writing – review and editing, software, formal analysis, supervision, resources, data curation, methodology. **Jonathan E. Isaacs:** conceptualization, investigation, writing – original draft, writing – review and editing, methodology, funding acquisition, project administration, supervision, resources.

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Ethics Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Conflicts of Interest

Omar A Protzuk has received research support in grant funding from the American Foundation for Surgery of the Hand for this study. Jonathan E Isaacs has other contracted academic research with Axogen Inc. Nerve graft materials were provided by Axogen Inc. The remaining authors have no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.