ORIGINAL RESEARCH

Facial nerve repair utilizing intraoperative repair strategies

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Funding information
U.S. Department of Veterans Affairs Roudebush VA IMMR Young Investigator Award and Career Development Award, Grant/Award Number: IK2RX002688 (C.L.W); Indiana University Collaborative Research Grant (K.J.J)

Abstract

Objectives: To determine whether functional and anatomical outcomes following suture neurorrhaphy are improved by the addition of electrical stimulation with or without the addition of polyethylene glycol (PEG).

Methods: In a rat model of facial nerve injury, complete facial nerve transection and repair was performed via (a) suture neurorrhaphy alone, (b) neurorrhaphy with the addition of brief (30 minutes) intraoperative electrical stimulation, or (c) neurorrhaphy with the addition electrical stimulation and PEG. Functional recovery was assessed weekly for 16 weeks. At 16 weeks postoperatively, motoneuron survival, amount of regrowth, and specificity of regrowth were assessed by branch labeling and tissue analysis.

Results: The addition of brief intraoperative electrical stimulation improved all functional outcomes compared to suturing alone. The addition of PEG to electrical stimulation impaired this benefit. Motoneuron survival, amount of regrowth, and specificity of regrowth were unaltered at 16 weeks postoperative in all treatment groups.

Conclusion: The addition of brief intraoperative electrical stimulation to neurorrhaphy in this rodent model shows promising neurological benefit in the surgical repair of facial nerve injury.

Level of Evidence: Animal study.

KEYWORDS

electrical stimulation, facial nerve, facial nerve injury, nerve repair, polyethylene glycol

1 | INTRODUCTION

Despite the regenerative ability of the peripheral nervous system following injury, complete functional recovery remains elusive. In an effort to improve recovery following facial nerve repair, various strategies, including artificial nerve conduits, stem cell treatments, and delivery of neurotrophic factors, have been employed.[1-3] These strategies aim to improve the number of axons crossing the injury site and improve specificity of regrowth. A major limitation to improving these outcomes is the disruption of the myotopic organization at multiple levels in the central nervous system, which contributes to facial synkinesis.[4-6]

The addition of electrical stimulation (e.stim) to neurorrhaphy has improved functional outcomes and the amount and specificity

of regrowth in multiple models of peripheral nerve injury.[7-9] This results from the ability of e.stim to upregulate expression of pro-regenerative neurotrophic factors, growth promotion, and myelination genes; aiding to accelerate axon regrowth.[7,10-12] Following facial nerve crush in the rat, a single, 30-minute session of e.stim improves functional outcomes.[13,14] Due to its translational potential, we use the same, 30-minute intraoperative e.stim protocol in the current study.

In addition to neurorrhaphy, intraoperative treatment utilizing a fusogen, polyethylene glycol (PEG), has been investigated.[15,16] The PEG treatment protocol developed and characterized by Bittner et al[17] has shown great promise in a rat sciatic nerve injury model, and our laboratory has previously used this protocol in a rat facial nerve injury model.[18] This protocol first bathes the cut ends of the nerve in a hypotonic calcium-free solution, followed by application of an antioxidant, 1% methylene blue (MB) in ddH$_2$O. After microsuture neurorrhaphy, 50% PEG by weight in sterile water is applied to the coaptation site, causing nonspecific fusing of neuronal membranes. Finally, the site is bathed in calcium containing isotonic solution to seal neuronal membranes not previously fused by PEG. This well characterized protocol has ≥98% success rate when these solutions are applied in the correct order.[19] Successive application of these solutions prevents sealing of the nerve endings by expelling vesicles, slows the initiation of injury signaling cascades via inhibiting calcium influx, and potentially lowers the activation energy of membrane fusion by removing the hydration barrier surrounding the axolemma.[15,20] All of which are thought to "prime" the axotomized nerve for fusion potentially restoring some continuity.

We previously evaluated the addition of PEG to neurorrhaphy following facial nerve transection in the rat, and found it provided no functional or anatomical benefits beyond suturing alone.[18] However, other combinatorial treatments have been beneficial.[8,14,21] Thus, we hypothesized that the addition of brief intraoperative e.stim to neurorrhaphy would improve functional and anatomical outcomes, and the combination of e.stim and PEG would further improve outcomes. To test these hypotheses, functional and anatomical outcomes were quantified in a rat model of facial nerve injury.

2 | MATERIALS AND METHODS

2.1 | Overview of methods

In total, 23 male Wistar Rats (250-450 g; Envigo) were used: 3 uninjured, 4 suture neurorrhaphy, 8 suture neurorrhaphy plus e.stim, and 8 suture neurorrhaphy plus e.stim and PEG. The IACUC of Indiana University School of Medicine approved all experimental protocols. Data were collected from 18 July 2016, through 1 April 2017. For all procedures, 2.5% isoflurane in 98% oxygen at a rate of 1 L/min was used for anesthesia. Nine millimeter wound clips were used to close surgical sites and 1 mL of buprenorphine hydrochloride was administered immediately postsurgery. All surgical procedures were performed by a trained head and neck surgeon whom had 3 weeks of practice surgeries prior to performing the surgeries for the study.

2.2 | Surgical groups

2.2.1 | Facial nerve axotomy and neurorrhaphy

A 1.5-cm postauricular incision was made on the animal's right side to expose the main extratemporal trunk of the facial nerve. Once 5 to 6 mm of the main truck was exposed and freed from surrounding tissue, complete transection was performed 2.5 to 3 mm distal from its exit at the stylomastoid foramen (Figure 1). Neurorrhaphy was performed using 10-0 nylon suture (AROSurgical), and care was taken to prevent excess tension on the nerve. These measures also prevented fraying and swelling of the nerve, preventing protrusions from the suture site, allowing for close apposition of the epineurium.

2.2.2 | Addition of brief electrical stimulation to neurorrhaphy

Following exposure of the facial nerve, fine wire electrodes were carefully hooked around the nerve 2 mm proximal to the intended axotomy site and formed into a loop encircling the nerve. Stimulator (Pulse generator; Tektronics AFG1022 Arbitrary Function Generator, Tektronics & Stimulus isolator; Analog Stimulus Isolator 200, A-M Systems) test pulses were administered to confirm correct placement of the electrodes by visually observing facial twitches. Immediately following neurorrhaphy, the nerve was stimulated (100 μs charge-balanced square waves; peak-to-peak amplitude 3 V) in a continuous 20 Hz train for 30 minutes.[7,22] Following stimulation, electrodes were removed, and the surgical site closed.
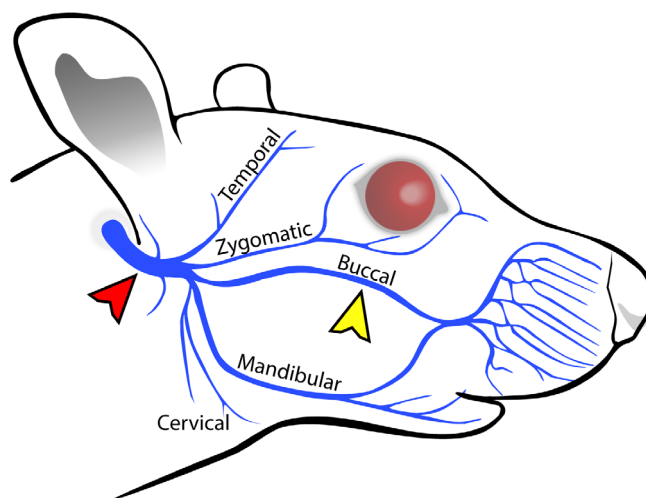

FIGURE 1 Anatomy of the rat facial nerve and surgical sites. Anatomy of the rat facial nerve. The five main branches form after the facial nerve exits the stylomastoid foramen. Red arrowhead indicates location of facial nerve transection and neurorrhaphy. Yellow arrowhead indicates the site of retrograde tracer labeling. Adapted from Hohman et al. JAMA Facial Plas Surg. 2014;16 (1):20-24. doi:10.1001/jamafacial2013.1431

2.2.3 | Addition of electrical stimulation and PEG to neurorrhaphy

For repairs utilizing PEG, a 50 wt% PEG solution (33.35 kDa; Sigma Aldrich) was prepared in sterile water immediately before surgeries. Prior to axotomy, test pulses were performed as previously described to confirm the correct placement of stimulation electrodes. Once confirmed, electrodes were removed, and the area was rinsed with a calcium-free solution (Plasma-Lyte; Baxter International, Inc.). Following transection, the area was rinsed again with a calcium-free solution, and two drops of 1% MB (Fisher Science) in ddH$_2$O were applied to both ends of the axotomized nerve. To remove residual MB and improve visibility, the area was rinsed again with the calcium-free solution. After neurorrhaphy PEG solution was applied to the suture site for 60 to 90 seconds. The area was then rinsed 5 to 6 times with a calcium containing solution (Lactated Ringer's; Hospira).[23,24] Last, e.stim was then applied as outlined in the previous section.

2.3 | Functional outcome measures

Axotomy was visually confirmed immediately postsurgery by observing ipsilateral facial paralysis, consisting of complete loss of vibrissae movement, posterior orientation of vibrissae, and loss of eye blink reflex.[25] Beginning 1 week postoperatively, animals were monitored by a single blinded observer weekly for 16 weeks to evaluate recovery of eye blink reflex and vibrissae function. Functional recovery was scored on a 3-point scale (1 = no recovery; 2 = partial recovery; 3 = complete recovery, indistinguishable from uninjured side). Eye blink reflex was tested by delivering a brief direct stream of air to the eye using a rubber bulb syringe. A score of 2 was considered recovery for eye blink reflex, which is further defined as the ability to close the eye more than halfway owing to voluntary muscular contraction. Vibrissae function was monitored by stabilizing animals' heads with a rodent restraint cone (AIMS, Inc.) with the end cut, allowing the head to emerge and the animal to whisk freely. A score of 2 was considered recovery for vibrissae functional recovery and was defined as the ability of the vibrissae to move synchronously during consecutive whisks.

2.4 | Retrograde tracing

Buccal branch labeling was performed 16 weeks postoperatively. A 1-cm incision was made 5 mm dorsal to the maxilla to expose the buccal branch. Once freed from the surrounding tissue, the buccal branch was transected, and the proximal end bathed in 2 μL of 4% fluorogold (Fluorochrome, LLC) (Figure 1). A small piece of absorbable gelatin (Gelfoam; Pfizer) was placed on the proximal end of the transected nerve to sequester fluorogold to the area.

2.5 | Tissue analysis

One week after branch labeling, animals were anesthetized using a mixture of ketamine and xylazine hydrochloride and perfused with phosphate-buffered saline solution followed by 4% paraformaldehyde (PFA). The brain and brainstem were removed, postfixed in 4% PFA for 12 hours, and transferred to 30% sucrose solution until sectioned. The pons from each animal was cryo-sectioned at 20 μm and mounted on microscope slides (Superfrost slides; Thermo Scientific). Five slide sets were collected from each animal and one slide from each set was Nissl stained to visualize the facial motor nucleus (FMN) and prevent double counting of motoneurons. A map of the rat FMN was used to assess myotopic organization (Figure 2A).[26] The middle seven stained sections from each animal were used for quantification. All image processing and counting was performed by the same blinded individual using photo editing software (Photoshop CS6; Adobe). The number of motoneurons per section, number of motoneurons with buccal branch projections, and number of motoneurons with misguided projections were quantified.

2.6 | Data analysis

The statistical program "R" was used to perform one-way analysis of variance using the Tukey post hoc test for all behavioral and anatomical outcomes. A P value <.05 was considered statistically significant.

3 | RESULTS

3.1 | Functional outcomes

Among the 20 male rats that underwent injury, two died prior to recovering from anesthesia, one from the "Suture + e.Stim" group, and one from the "Suture + E.Stim + PEG" group. Of the 18 remaining rats, ipsilateral facial paralysis was seen immediately postsurgery. Passive eyelid movement due to retraction of the eye may be mistaken for active orbicularis oculi-induced closure. To prevent this misinterpretation, slow-motion video analysis was used. Once the recovery criteria for eye blink reflex was achieved (see Section 2), blink on the injured side remained slower than the uninjured side and was frequently accompanied by incomplete eyelid opening after a blink. Visible vibrissae movement typically began 2 to 3 weeks postoperatively and consisted of uncoordinated vibrissae oscillations. Upon reaching the criteria for whisking recovery (see Section 2), whisks remained slower and of smaller amplitude compared to the uninjured side.

Neurorrhaphy alone resulted in a mean (SD) time to eye blink recovery of 9.0 (2.0) weeks, and a mean (SD) time to whisking recovery of 13.0 (1.4) weeks. The addition of e.stim to neurorrhaphy significantly improved time to eye blink recovery 4.3 (1.2) weeks (Tables 1 and 2), and mean time to whisking recovery 5.6 (4.5) weeks (Tables 1 and 2). The addition of e.stim and PEG to neurorrhaphy did not significantly
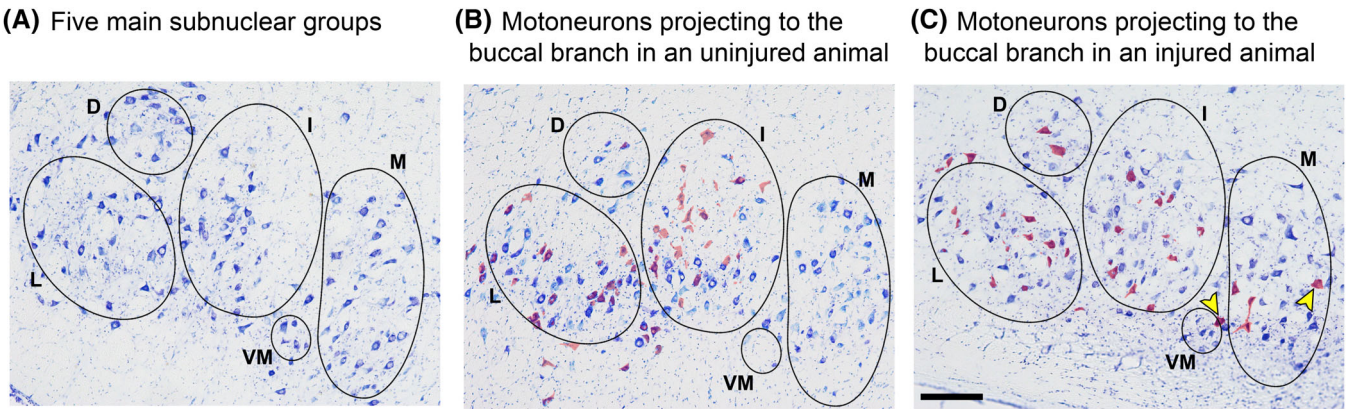
FIGURE 2 Organization of the rat facial motor nucleus. A, Nissl-stained rat facial motor nucleus is subdivided into the following five main subnuclear groups: lateral (L), dorsal (D), intermediate (I), ventromedial (VM), and medial (M). B, Overlay of motoneurons projecting to the buccal branch (red) from an uninjured animal. C, Overlay of motoneurons projecting to the buccal branch (red) from an injured animal; yellow arrowheads indicate motoneurons misguiding projection to the buccal branch. Scale bar = 200 μm

TABLE 1 Recovery of function

	Mean number of weeks for recovery (SD)	95% Confidence interval	SE of group mean	No. (%) of animals reaching recovery in 16 wk
Function				
Eye blink recovery				
Suture (n = 4)	9.0 (2.0)	4.0-13.9	±1.2	4 (100.0)
Suture + E.Stim (n = 7)	4.3 (1.2) *	3.1-5.6	± 0.5	5 (71.4)
Suture + E.Stim + PEG (n = 7)	6.0 (2.3)	3.6-8.4	±0.9	6 (85.7)
Whisking recovery				
Suture (n = 4)	13.0 (1.4)	10.8-15.3	±0.7	3 (75.0)
Suture + E.Stim (n = 7)	5.6 (4.5) *	0.1-11.2	± 2.0	6 (85.7)
Suture + E.Stim + PEG (n = 7)	7.3 (4.5)	2.7-12.0	± 1.8	6 (85.7)

Abbreviations: SD, standard deviation; SE, standard error; E.Stim, electrical stimulation; PEG, polyethylene glycol.
*$P < .05$, Tukey's post hoc compared to Suture group.

TABLE 2 Functional outcomes

Group comparisons	Significance
Multiple comparisons (ANOVA and Tukey HSD)	
Eye blink recovery (ANOVA; P = .03)	
Suture vs Suture + E.Stim	.02
Suture vs Suture + E.Stim + PEG	.25
Suture + E.Stim vs Suture + E.Stim + PEG	.42
Whisking recovery (ANOVA; P = .04)	
Suture vs Suture + E.Stim	.04
Suture vs Suture + E.Stim + PEG	.11
Suture + E.Stim vs Suture + E.Stim + PEG	.75

Abbreviations: ANOVA, analysis of variance; E.Stim, electrical stimulation; PEG, polyethylene glycol.

improve either time to eye blink recovery 6.0 (2.3) weeks, or time to whisking recovery 7.3 (4.5) weeks compared to neurorrhaphy alone (Tables 1 and 2). Not all animals reached recovery by the end of the functional assessment period. Of interest, the percentage of rats reaching eye blink recovery (4 of 4 [100%], 5 of 7 [71.4%], and 6 of 7 [85.7%]) and whisking (3 of 4 [75%], 6 of 7 [85.7%], and 6 of 7 [85.7%]) at 16 weeks postoperative did not favor any surgical group. (Table 1).

3.2 | Motoneuron survival

Following peripheral nerve injury, motoneuron survival in Wistar rats is ≥75%.[18,27,28] We found 86% motoneuron survival in all surgical groups compared to uninjured controls (mean 122.3 motoneurons per tissue section; 95% confidence interval [CI] = 117.5-127.1; $P = .01$). While mean (SD) motoneuron survival in the "Suture" 117.3 (9.6) (95% CI = 107.7-126.9; $P = .00$), "Suture + E.Stim" 125.2 (10.0) (95% CI = 15.2-135.2; $P = .03$), and "Suture + E.Stim + PEG" 121.8 (10.1) (95% CI = 111.8 -131.9; $P = .01$) groups significantly decreased compared to uninjured controls 142.6 (25.2), we found no difference
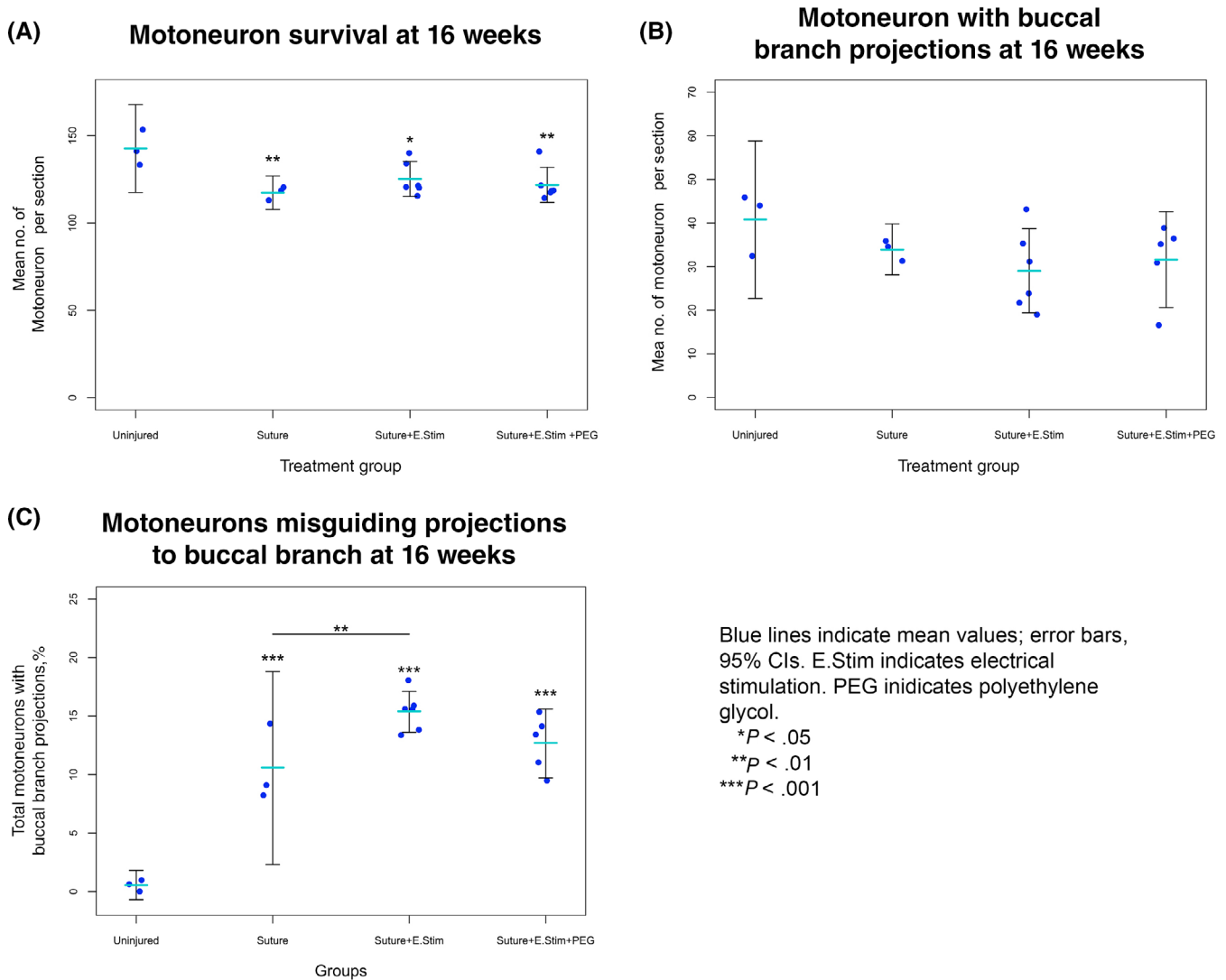
(A)

Motoneuron survival at 16 weeks

(B)

Motoneuron with buccal branch projections at 16 weeks

(C)

Motoneurons misguiding projections to buccal branch at 16 weeks

Blue lines indicate mean values; error bars, 95% CIs. E.Stim indicates electrical stimulation. PEG inidicates polyethylene glycol.

$*P < .05$
$**P < .01$
$***P < .001$

FIGURE 3 F Anatomical outcomes. Blue lines indicate mean values; error bars, 95% CIs. $*P < .05$, $**P < .01$, $***P < .001$. E.Stim, electrical stimulation; PEG, polyethylene glycol

between the "Suture" group compared to either the "Suture + E.stim" (95% CI = 115.2-135.2; $P = .60$) or "Suture + E.Stim + PEG" (95% CI = 111.8-131.9; $P = .90$) groups (Figure 3A). These results indicate that none of our surgical interventions provided benefit nor produced adverse effects on motoneuron survival.

3.3 | Amount and specificity of regrowth

Amount of regrowth was assessed by quantifying the number of motoneurons routing projections to the buccal branch via fluorogold labeling at 16 weeks postoperative (Figure 2B and C). We found no significant differences in the mean number motoneurons with buccal branch projections between groups (95% CI = 28.4-37.1; $P = .18$) (Figure 3B). These data suggest that brief electrical stimulation with or without the addition of PEG produce no beneficial or adverse effects on the amount of regrowth compared to neurorrhaphy alone.

Following injury, the myotopic organization of the FMN is disrupted, contributing to facial synkinesis.[29] This results from regenerating axons being misguided to the incorrect nerve branch and subsequent musculature.[30] In uninjured animals, motoneurons from the lateral, dorsal, and intermediate subnuclei project to the buccal branch (Figure 2B). Following injury and repair, motoneurons in the ventromedial and medial subnuclei also project to the buccal branch, disrupting this myotopic organization (Figure 2C). Our data show a significant disorganization in all surgical groups compared to uninjured controls (95% CI = 9.0-17.7; $P < .01$) (Figure 3C). The addition of e. stim to neurorrhaphy resulted in greater disorganization compared to neurorrhaphy alone (95% CI = 13.6-17.1; $P = .01$).

It should be noted that buccal branch labeling was suboptimal in one animal from the "Suture" group, one animal from the "Suture + E. Stim" group, and two animals from the "Suture + E.Stim + PEG" group. Data from these animals were not included in amount and specificity of regrowth quantification.

4 | DISCUSSION

Multiple studies have shown the efficacy of e.stim to promote recovery after peripheral nerve injury. Following sciatic nerve transection 1 hour of e.stim accelerates axon regeneration, improves specificity of regrowth, and temporally compresses "staggered regeneration" times.[7,31] Additionally, 30 minutes of e.stim following facial nerve crush improves functional outcomes.[13,14] To our knowledge, the current study is the first to show that as little as 30 minutes of intraoperative e.stim is needed to improve functional outcomes following transection and neurorrhaphy. The well-characterized PEG fusion protocol used by Bittner, Ghergherehchi, Riley, and Mikesh has improved functional and anatomical outcomes in the sciatic nerve injury model.[19,24,32,33] Bittner et al[19] reports that using this protocol, even unskilled surgeons, after some training, have a high success rate (≥98%) of PEG fusion. However, the favorable outcomes due to PEG fusion can be impaired by excess tension on the nerve.[34-36]

PEG fusion is commonly evaluated immediately following PEG application by measuring compound action potentials (CAPs).[19,32] However, there are multiple issues with immediate evaluation of CAPs. First, axons distal to the injury do not fragment until 37 hours after transection, still allowing for conduction of electrical current.[37] Clinically this is evident as the nerve distal to the injury is identifiable via electrical stimulation for up to 72 hours.[36,38] Second, small amounts of solution(s) in the surgical field can conduct electrical current.[38] To circumvent these issues, Salomone et al[39] measured compound muscle action potentials (CMAPs) following PEG fusion, and showed no electrophysiologic differences until 6 weeks postoperatively. Due the limitations in-vivo with measuring CAPs immediately following repair, and differences in CMAPs not appearing until 6 weeks post-operatively, we concur with Bittner et al[17] that behavioral outcomes are the best measure of success for a treatment. To completely separate the rat facial nerve from surrounding tissue(s) and solutions in the surgical field, excess tension would need to be applied to the nerve, undeniably impacting regeneration and recovery. Due to high success rate of PEG fusion, issues with measuring CAPs immediately postrepair, and the harmful effects of applying excess tension on the nerve, we did not measure CAPs immediately following PEG fusion.

Despite our previous study showing PEG provided no neurological benefits in our model of nerve injury, we hypothesized that combining e.stim with PEG would further improve outcomes, due to PEG's ability to limit injury signaling cascades, and e.stim accelerating axonal regeneration. However, we found that this combinatorial treatment hindered the functional benefits provided by e.stim alone.

Anatomically, recovery is impacted by neuronal death, impaired regrowth, poor specificity of regeneration, and aberrant branching, three of which were evaluated here.[4] Following peripheral nerve injury neuronal death is minimal in the rat, and we found no adverse effects of our intraoperative treatments.[6,27,28] At 16 weeks postoperatively the amount of regrowth was similar between uninjured controls and all surgical groups.

Following neurorrhaphy of a mixed nerve, motoneurons preferentially regrow into motor pathways if given equal access to motor and sensory pathways via preferential motor reinnervation (PMR). PMR is evident 8 weeks postoperatively when misguided motoneuron projections to sensory pathways are pruned, resulting in improved specificity.[40,41] The addition of e.stim accelerates PMR to 3 weeks after repair.[7] 8 weeks after repair, when PMR has occurred, Robinson et al[16] found that PEG impaired specificity of regrowth, likely due to non-specific PEG fusion "locking" axons in place, limiting pathway sampling. PMR results from the specificity of trophic support from sensory or motor specific Schwann cells.[42,43] There is no know distinction between trophic support from Schwann cells in different motor branches. Thus, the known mechanism for improved specificity of regrowth in mixed nerves is not present in purely motor nerves, such as the rat facial nerve. Since e.stim accelerates regrowth, and PMR is not present in purely motor nerves, this is likely why we found more non-specific regrowth in our e.stim group, and improved recovery times.

Due to the above differences in the specificity of regrowth in mixed-nerves verses motor nerves; we suggest clinicians evaluate the efficacy of treatments such as e.stim and PEG on a case-by-case basis. The evidence supporting the addition of e.stim to neurorrhaphy is favorable in both mixed and motor nerves, however, the efficacy of PEG is still debated. We previously found that PEG provided no benefit beyond suturing alone in a motor nerve, and Robinson et al[16] reported impaired specificity of regrowth in a mixed nerve when PEG was applied. However, other groups report PEG being a potential panacea. Based on our findings, intraoperative e.stim proximal seems to hasten functional recovery, though longer-term follow-up is necessary to determine if overall improvement is better compared to suture neurorrhaphy alone.

4.1 | Limitations

For functional outcome measures, the observer was blinded; however, this approach to scoring does not remove all subjectivity. Other studies have used more advanced opto-electric systems to assess functional recovery, which would remove any subjectivity from the functional evaluations used here.[44,45] As previously mentioned in the discussion section, we did not directly assess axon fusion via CAP measurement, which is a limitation of the current study. Future studies using this injury model should assess axon fusion by measuring CMAPs. Since no time course was performed to evaluate regrowth, we cannot determine the temporal effects our treatments. We also did not histologically evaluate the repair site. To date, no studies using PEG have done so; thus, we cannot confirm or deny that PEG may "lock" axons in place limiting their ability to sample possible pathways. Last, due to anatomical constraints in our model, and proactively limiting tension on the nerve, the musculature underlying the facial nerve was undoubtedly stimulated during e.stim, which may contribute to improvements in these animals.

5 | CONCLUSION

The addition of brief, 30 minute, intraoperative e.stim to neurorrhaphy in this rodent model improved functional outcomes, which is consistent with other models of peripheral nerve injury. The addition PEG provided no further neurological benefit, and negated the benefits e.stim provided. The encouraging improvements e.stim provided in the absence of PEG, should be corroborated by clinical trials with long-term follow-ups. Future studies should evaluate whether e. stim alters long-term facial synkinesis in other injury models.

AUTHOR CONTRIBUTIONS

Chandler L. Walker and Kathryn J. Jones had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Taha Z. Shipchandler, Kathryn J. Jones, Chandler L. Walker. Acquisition, analysis, or interpretation of data: Brandon L. Brown, Morgan M. Sandelski, Sarah M. Drejet, Elizabeth M. Runge, Chandler L. Walker. Drafting of manuscript: Brandon L. Brown. Critical revision of manuscript for important intellectual content: Taha Z. Shipchandler, Kathryn J. Jones, Chandler L. Walker. Obtained funding: Kathryn J. Jones, Chandler L. Walker. Administrative, technical, or material support: Brandon L. Brown, Morgan M. Sandelski, Sarah M. Drejet, Elizabeth M. Runge. Study supervision: Taha Z. Shipchandler, Kathryn J. Jones, Chandler L. Walker.

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