Published in final edited form as:

J Neurosci Res. 2017 March ; 95(3): 863–866. doi:10.1002/jnr.23849.

Robinson and Madison have published no data on whether polyethylene glycol fusion repair prevents reinnervation accuracy in rat peripheral nerve

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Significance

Polyethylene glycol (PEG)-fusion technologies have been reported to rapidly, effectively, and permanently restore lost behavioral functions within days to several weeks after transecting or ablating portions of mammalian sciatic nerves, the latter injuries best repaired by allografts of viable sciatic nerves. PEG-fusion must result in inappropriate fusions of surviving distal, proximal and/or graft motor and sensory axons having different muscles of origin and/or receptive fields than their proximal axonal connections. Somehow, these inappropriately PEG-fused axons that do not exhibit Wallerian degeneration and/or axons re-innervating target tissues by outgrowth (after at least several weeks) must be responsible for the extensive behavioral recovery at 3 days to 8 weeks. Neither we nor Robinson and Madison have yet published any data on the accuracy of any reinnervation of motoneurons after successful PEG-fusion of a PNS nerve. Accurate data on cellular/molecular mechanisms of axonal specificity and plasticity are important to understand the reasons for the success of PEG-fusion technologies that could produce a paradigm shift in the treatment of traumatic PNS nerve injuries.

Robinson and Madison (2016) have recently published a paper entitled “Polyethylene Glycol Fusion Repair Prevents Reinnervation Accuracy in Rat Peripheral Nerve” in the *Journal of Neuroscience Research*. Unfortunately, the title of the paper reflects only one of many possible interpretations of their data. That is, (1) the lack of critical controls in methodologies, and (2) limitations in the experimental design of their protocols, lead them to (3) incorrect conclusions that are unfortunately reflected in (4) their very inappropriate and inaccurate title. Specifically:

Background

Previously published papers (Bittner et al., 2012, 2016a,b; Sexton et al., 2013; Ghergherehchi et al., 2015; Riley et al., 2015) that are directly relevant to their paper, demonstrate that PEG-fusion is accomplished by a specific sequence of surgical procedures and bioengineered solutions as follows: Complete transections of PNS nerves in rats cause severed axonal ends to separate by 1–3 mm. [Sciatic nerves are often used, Robinson and Madison use femoral.] A 5–10 mm segment of a sciatic nerve can also be ablated from a host animal followed by its autografted reinsertion or by insertion of a slightly longer

allograft from a donor animal. All cut ends of severed nerves are carefully-trimmed so they can be closely re-apposed with micro-sutures. Following a single transection or “doubly cut” graft, the severed nerve ends are carefully re-aligned, but that alignment is never perfect at the axon-to-axon level. Individual proximal and distal cut ends of specifically identified axons cannot ever be perfectly aligned, even at apposed cut ends of a single transection. That is, even the largest (myelinated) axons in rats and other mammals are ~20µm in diameter and cannot be uniquely identified at 10–20× in operating microscopes. The alignment problem is further compounded by the facts that the average myelinated axon is about 5µm in diameter, myelin scatters light, and unmyelinated axons may be as small as ~0.2µm in diameter. Axon-to-axon “correct” alignment of a donor *allograft* with the host nerve is completely impossible because the allograft and host nerves do not have the same number of unidentified axons.

Bioengineered PEG-fusion solutions are then applied sequentially: First, the injury site is bathed in Ca^{2+} -free hypotonic saline to open cut axonal ends and expel vesicles. Methylene blue, an antioxidant that inhibits new vesicle formation, is then directly applied to the injury site for 1–2min. Next, PEG (2–5kD 50% w/w in distilled water) is directly applied to the surgically repaired lesion site for 1–2min to non-specifically join (connect, fuse) closely apposed axonal or other membranes. Finally, PEG-fused axons are bathed in isotonic Ca^{2+} -containing saline to seal any remaining axolemmal disruptions. That is, PEG-fusion does not completely repair (anneal) closely apposed axonal ends. The subsequent exposure of the imperfectly-fused damaged axonal regions to an isotonic solution containing physiological levels of Ca^{2+} seals these plasmalemmal holes by Ca^{2+} -induced vesicles that interact to plug (seal) the holes, i.e., a process we have called “PEG-sealing” to be contrasted with “PEG-fusion” (Bittner et al., 2012, 2016a,b; Ghergherehchi et al., 2016; Riley et al., 2015; Spaeth et al., 2012. See Bittner et al, 2016c for an extensive review of PEG-sealing, especially Figure 2).

In brief, microsuture repairs closely and non-specifically appose distal and proximal cut ends of motor and sensory axons of unknown motor units or receptor field specificity in *all* transection/autograft/allograft + suture experimental protocols with or without the addition of PEG.

Successful PEG-fusion must be confirmed by an independent measure. Within minutes after applying a PEG-fusion technology, we analyze compound action potentials (“CAPs”) from the entire sciatic nerve initiated extracellularly proximal to the lesion site and recorded extracellularly distal to the lesion site (see Figure 4 in Bittner et al., 2016b, for a detailed schematic). Electromyogram recordings are another possible method to demonstrate that a PEG-fusion operation was successful. Successful PEG fusion is also demonstrated by much more rapid and complete return of behavioral recovery at one or more post-operative weeks than is obtained by microsutures. Even the best surgeons occasionally fail in a PEG-fusion trial, so each animal must be individually identified, tested immediately post-repair by CAPs and behaviorally weekly thereafter.

As with any surgical procedure as discussed in previous papers (Bittner et al., 2016a,b; especially see Fig.5; Ghergherehchi et al., 2016; Riley et al, 2015), the outcome of the PEG-

fusion surgical procedure depends on a set of variables, many unknown or very hard to precisely control, such as the skill of the surgeon, the exact nature of the lesion and its repair, patient (rat or human) health, age, genetic composition, emotional state, etc. Because regenerating outgrowths at 1–2mm/day cannot have yet reached distal denervated muscle masses, any significant increase in restoration of lost behaviors within 3 weeks must almost-certainly be due to survival of PEG-fused distal axons, i.e., prevention of Wallerian degeneration.

Much of the substantial, permanent recovery that occurs within 2–3 weeks after PEG-fusion must be due to prevention of Wallerian degeneration of axons in distal stumps following single transects or allografts following ablations, as reinnervation by outgrowth has not yet had time to occur. Reinnervation by outgrowths at ~1mm/day could provide additional functional recovery after 4 or more weeks following simple transections. Additional functional recovery by reinnervation would be expected to provide much less, if any, improvement many weeks after complete ablations (Bittner et al., 2012, 2016a, 2016b, Ghergherehchi et al., 2016; Riley et al., 2015; Sexton et al., 2012).

Consequently, PNS tracer-labeled axons distal to a PEG-fusion repair site at a post repair time of 8 weeks could be either **(a)** original surviving axons successfully PEG-fused or **(b)** reinnervating axons that grew to the label site for whatever reason, perhaps because they were unsuccessfully PEG-fused. If one is going to study the innervation specificity of regrowing, reinnervating axons, then labeling methodologies and protocols MUST be able to reliably distinguish original surviving axons from newly reinnervating axons. If this is not attempted or accomplished, then simple logic demands that one cannot take data from both surviving and reinnervating axons and then claim the data are only from reinnervating axons.

(1) Problems with Methodology

Robinson and Madison (2016) did not examine CAPs (or use any other method) to assess restoration of continuity of (non-specifically) repaired axons across the lesion site immediately after PEG-fusion in their 1–4 day protocols or their 8 week protocols in tracer-assessed animals. Hence, they do not know which, if any, animals might have at least some axons successfully PEG-fused. We note that their tracer data in Figure 2 suggest that at least some axons were successfully PEG-fused as presumably any axonal regrowth after a few days would be at most several mm, and could not have been tracer-labeled by muscle injections several cm away. They argue that some of this tracer may be extra-axonal, but their illustrations in Figure 2 show no such apparent difference and hence they do not know if any label is intra-axonal or extra-axonal. Nevertheless, although some of their results may be ambiguous, the authors used no protocol that could have enabled them to determine whether they labeled an original PEG-fused surviving axon or a reinnervating axon that regrew to the site where label was applied.

Furthermore, with respect to methods used (or *not* used), behavioral tests of functional recovery provide the only appropriate assessment of the success of any PEG-fusion trial. In our experience, about 30% of student surgeons and 70% of board-certified MDs successfully learn to use PEG-fusion protocols so that their PEG-fusions are successful in >80% of their

trials by behavioral measures. Each animal must be assessed individually, preferably weekly, by a behavioral measure. Behavioral measures need be correlated with morphological measures for each animal to demonstrate that successful PEG fusions are associated with retardation or prevention of Wallerian degeneration of PEG-fused axons (or reinnervation outgrowth) that are responsible for rapid return of behavior. As one example, after successful PEG-fusion of allografts, much behavioral recovery in paw placement occurs within 3 days to 3 weeks, a rate not explainable by hypothesizing regeneration by outgrowth of less than centimeters per day (Riley et al., 2015). As another example, we have examined axon diameters in PEG-fused, negative control, and unoperated nerves. Successfully PEG-fused nerves as assessed by behavioral measures have significantly larger axons inside their epineural sheaths than negative controls (Ghergherehchi et al., 2015; Riley et al., 2015; Bittner et al., 2016a,b). However, Robinson and Madison have made no attempt to assess functional, morphological or behavioral recovery for any tracer-labeling data, yet they interpret their results as having direct implications for functional recovery.

(2) Problems with protocols

In addition to problems with methods that render their data interpretation highly questionable, Robinson and Madison used experimental protocols that did not enable them to distinguish between tracer-labeled axons that were **(a)** originally present and continuously survived after being successfully PEG-fused or **(b)** were more recently present due to reinnervation outgrowth. They assume without evidence in Figures 4 and 5 that all tracer-labeled transected axons at 8 weeks post-PEG-fusion repair arose by reinnervation outgrowth from severed proximal stumps and ignore the possibility from previously-published data cited above that many (maybe most or all) tracer-labeled axons could be original, successfully PEG-fused, axons that never underwent Wallerian degeneration.

After successful PEG-fusion as demonstrated by CAP conduction but prior to any functional recovery, we have found that motoneuron labeling is present in the spinal cord by 5 days post-injury (Fig. 1). Such retrograde-transport tracer label cannot be due to “extra-axonal” labeling or to regenerating axons reaching a muscle in the lower leg 30–50 mm distal to the site of PEG-fusion repair. In Figure 1, proximal axons of motoneurons that do not normally innervate the anterior tibialis were connected to surviving distal anterior tibialis motor axons in a successfully PEG-fused nerve. These connectivity changes must be correlated with an original surviving axon, not a newly reinnervating axon.

(3) Problems with conclusions and title

In brief, Robinson and Madison (2016) made no effort to determine whether PEG-treatment was successful in any animal by any electrophysiological, morphological, or behavioral method. Since Robinson and Madison attempted no such correlations, they have no basis for correlating PEG-fusion status with anything, much less with the reinnervation accuracy of any axon regenerating by outgrowth in any animal nor with any functional or behavioral implication. Robinson and Madison conclude that PEG-fusion prevents reinnervation accuracy by outgrowth. In fact, from their data, reinnervation accuracy by outgrowth could be 0%, 100%, or anything in between because they do not know whether any axon they

observed and then reported as appropriate or inappropriately connected in their study reached an injection site by outgrowth or was an original, surviving PEG-fused axon. Thus, the authors have presented no evidence for:

(4) the inappropriate and inaccurate title of their paper. If their tracer methods are indeed valid, then Robinson and Madison have shown that motoneurons in animals with attempted PEG-fusion exhibit an unusual labeling pattern in the spinal cord. How that pattern relates to reinnervation specificity or surviving axon plasticity, much less functional or behavioral recovery, need be determined by future publications.

High accuracy/specificity of target reinnervation may not be as important as we and others have assumed. As one example, when an “expendable” PNS nerve is transected near its target and sutured to the cut edge of another PNS nerve that has a more important target function, subsequent restoration of behavior has been correlated with much plasticity of CNS synapses (Lou et al., 2006). What is now needed is clear documentation of the effects of axonal regrowth/reinnervation or axonal preservation on restoration of lost behaviors.

As described above, PEG-fusion does not selectively reconnect distal and proximal portions of severed motor or sensory axons, much less individually-identifiable axons, especially following ablations and insertions of autografts and donor allografts. Rather, much plasticity must occur in the spinal cord or brain that might include: (1) distal portions of sensory or motor axons could be re-specified by the PEG-fused proximal portion; (2) distal portions of sensory or motor axons could re-specify spinal connections as directed by the PEG-fused proximal segment; (3) collateral outgrowths from surviving distal and motor axons could within 2 weeks make new peripheral connections. Furthermore, reinnervation by outgrowth is not prevented by PEG-fusion and could make appropriate or inappropriate (followed by plasticities 1–3 above) connections that all contribute to behavioral recovery starting some weeks post PEG-fusion (Bittner et al., 2012, 2016a,b; Ghergherehchi et al., 2015; Riley et al., 2015).

Identifying specific cellular mechanisms like 1–3 above for post PEG-fusion behavioral recovery requires appropriate controls, immediate and continuous measures of electrophysiological or functional recovery, and appropriate experimental design for the questions posed. Pre-labeling specific sensory or motor axons in the sciatic nerve, and then relabeling them after transection with or without PEG-fusion with alternative tracers should identify some connective plasticity. For example, if proximal motor axons become fused to distal sensory axons, does collateral sprouting occur from the motor axons to allow for reinnervation of the target musculature? Labeling projections of specific sets of sensory axons and dendritic architectures of the motoneurons in intact versus PEG-fused nerves could show much morphological plasticity. As one example, if a given population of proximal motor axons become fused with distal motor axons innervating a different target muscle, do the motoneurons adopt a dendritic architecture appropriate to the new target? In contrast to the inappropriate conclusion based on no relevant data that PEG-fusion should lead to behavioral recovery poorer than microsuturing (Robinson and Madison, 2016), PEG fusion has produced rapid, effective, and permanent repair of transected or ablated sciatic nerves in rats (Bittner et al., 2015a). PEG-fusion protocols could produce a paradigm shift to

rapidly restore many behaviors lost by axonal severance in clinical procedures, a result not obtained by any other chemical or surgical treatment published to date.

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Figure 1. Retrograde labeling of spinal motoneurons shows altered connectivity after successful PEG-fusion

Motoneurons were labeled with horseradish peroxidase conjugated to the cholera toxin B subunit (BHRP) injected into the anterior tibialis muscle in the lower leg. Darkfield digital micrographs of transverse hemisections through the spinal cords of an intact control animal (A) and an animal with a PEG-fusion repair of a transected sciatic nerve at 5 postoperative days (B). In the intact control animal, labeling was located in column 4 of the L3 spinal segment, the normal location for motoneurons innervating the anterior tibialis. In the PEG-fusion repaired animal, labeling was located in column 6 of the L6 spinal segment; these motoneurons typically innervate the flexor digitorum brevis muscle of the foot but now project to the anterior tibialis. (C) CAP conduction was confirmed in the intact sciatic nerve before cut-severance (initial). After cut-severance and microsuture repair (post-severance), CAP conduction was abolished through the lesion site. Following PEG-fusion repair (post-PEG fusion), CAP conduction through the lesion site was restored.