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Polyethylene glycol restores axonal conduction after corpus callosum transection

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Abstract
Polyethylene glycol (PEG) has been shown to restore axonal continuity after peripheral nerve transection in animal models. We hypothesized that PEG can also restore axonal continuity in the central nervous system. In this current experiment, coronal sectioning of the brains of Sprague-Dawley rats was performed after animal sacrifice. 3Brain high-resolution microelectrode arrays (MEA) were used to measure mean firing rate (MFR) and peak amplitude across the corpus callosum of the *ex-vivo* brain slices. The corpus callosum was subsequently transected and repeated measurements were performed. The cut ends of the corpus callosum were still apposite at this time. A PEG solution was applied to the injury site and repeated measurements were performed. MEA measurements showed that PEG was capable of restoring electrophysiology signaling after transection of central nerves. Before injury, the average MFRs at the ipsilateral, midline, and contralateral corpus callosum were 0.76, 0.66, and 0.65 spikes/second, respectively, and the average peak amplitudes were 69.79, 58.68, and 49.60 μV, respectively. After injury, the average MFRs were 0.71, 0.14, and 0.25 spikes/second, respectively and peak amplitudes were 52.11, 8.98, and 16.09 μV, respectively. After application of PEG, there were spikes in MFR and peak amplitude at the injury site and contralaterally. The average MFRs were 0.75, 0.55, and 0.47 spikes/second at the ipsilateral, midline, and contralateral corpus callosum, respectively and peak amplitudes were 59.44, 45.33, 40.02 μV, respectively. There were statistically differences in the average MFRs and peak amplitudes between the midline and non-midline corpus callosum groups ($P < 0.01$, $P < 0.05$). These findings suggest that PEG restores axonal conduction between severed central nerves, potentially representing axonal fusion.

Key Words: *nerve regeneration; polyethylene glycol; nerve repair; axonal fusion; central nerve injury; axonal conduction; corpus callosum; neural regeneration*

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Introduction
Polyethylene glycol (PEG) has been extensively investigated for its potential role in axonal fusion after peripheral nerve injury (Bittner et al., 2012, 2016; Sexton et al., 2012, 2015; Riley et al., 2015; Bamba et al., 2016). The proposed mechanism for axonal fusion is PEG-induced lipid bilayer fusion by removing the hydration barrier surrounding the axolemma and reducing the activation energy required for membrane fusion. Despite the success in peripheral nerve injury, there has been little investigation into PEG fusion in the central nervous system (Borgens and Shi, 2000; Shi, 2013).

PEG has traditionally been used to produce hybridomas *via* membrane fusion. PEG has been used to fuse crayfish and giant earthworm axons and improvement in axonal fusion is found when axonal endings are exposed to a calcium free saline solution (Bittner et al., 1986; Krause and Bittner, 1990). In an axonal injury without PEG, axonal endings seal after an influx of calcium, preventing axonal fusion (Yoo et al., 2003). When severed axonal endings are exposed to calcium-free hypotonic saline and an antioxidant (*i.e.*, methylene blue or melatonin), vesicle-mediated sealing is decreased, keeping membrane leaflets open. PEG is then applied to artificially induce closely apposed membranes of severed axonal ends to flow into each other and fuse. This produces a partial repair of the plasmalemmal membranes that are then perfused with calcium containing saline, which causes vesicles to accumulate and seal remaining holes at the injury site.

Given the capability of PEG to produce hybridomas and fuse peripheral axons, we suspect its nerve fusion capabilities are not specific to peripheral nerves. Though peripheral nerve regeneration is slow after injury, the capability to regenerate provides peripheral nerve injury with a large advantage compared to central nerve injury. The capability to fuse central nerves could represent a revolutionary treatment of central nerve injury. In this study, we will examine the capability of PEG to promote axonal fusion after corpus callosum transection. Of note, this study is a preliminary study of PEG's capability to restore axonal conduction after central nerve transection.

Materials and Methods

Ethics statement

All experimental procedures were approved by and performed in accordance with the standards set forth by the Institutional Animal Care and Use Committee (IACUC) at Vanderbilt University (Protocol Number M13079).

Animals

Sprague-Dawley rats were housed in groups (n = 3/group) and supplied with water and food (Purina Rodent Diet) at all times. Animal cages were inspected daily to ensure animal health and cleanliness of housing by both the Vanderbilt Veterinary Staff and research personnel.

Preparation of artificial cerebral spinal fluid (aCSF)

aCSF was made according to the following specifications: 119 mM NaCl/26.2 mM $NaHCO_3$/2.5 mM KCl/1 mM NaH_2PO_4/1.3 mM $MgCl_2$/10 mM glucose/2.5 mM $CaCl_2$. Solution was filter sterilized at 0.22 μm, maintained over ice, and oxygenated by gas infusion of 95% O_2/5% CO_2 for a minimum of 30 minutes prior to application to brain slices. Fresh aCSF was made daily as experiments required.

Brain slice preparation

Female Sprague-Dawley rats (n = 3, age of 6 months) were anesthetized *via* inhaled isoflurane (2%). The animals were euthanized *via* intracardiac injection of euthasol (pentobarbital solution), and their brains were harvested. Brain slices were obtained using hand-held razors to obtain 2 mm-thick coronal sections. The brains were maintained in pre-oxygenated aCSF over ice and were sectioned coronally. Brain sections were then placed in fresh chilled pre-oxygenated aCSF for 30 minutes. During this time, aCSF was continually infused with 95% O_2/5% CO_2 and maintained over ice. Following incubation, the coronal sections were transferred to the APS-MEA chip, and pre-injury electrophysiological measurements were recorded. Under loupe magnification, the corpus callosum was sharply transected. The cut ends of the corpus callosum were left in alignment and post-injury measurements were obtained. Control measurements were taken prior to PEG placement. After post-injury recordings, PEG (50% w/v; 3.35 kDa, Sigma Aldrich, St. Louis, MO, USA) was applied over the cut ends of the corpus callosum, incubated for 1 minute, and subsequently washed away with aCSF. Post PEG recordings were taken immediately.

High density active-pixel sensor recordings

We used an active-pixel sensor multi-electrode array system (APS-MEA) (3Brain, Wadenswil, Switzerland) to measure mean firing rate (MFR) and peak amplitude across the corpus callosum of the *ex vivo* brain slices. The APS-MEA, extensively described elsewhere (Imfeld et al., 2008) consists of a microelectrode array chip and an amplification system designed to provide simultaneous extracellular recordings from 4,096 electrodes at a sampling rate of 7.7 kHz. The APS-MEA also consists of a complementary metal oxide semiconductor (CMOS)-based Charged Coupled Devices (CCD) monolithic chip modified such that pixels were designed to sense electrical voltage variations induced by electrogenic tissues. Each square pixel measures 21 × 21 μm^2, and the array was integrated with an electrode pitch of 42 μm. There was a 64 × 64 array configuration of pixels with an active area of 7.22 mm^2 and a pixel density of 567 pixel/mm^2. The three on-chip amplification stages provided a global gain of 60 dB, with a 0.1–5 kHz band-pass filter. This bandwidth was adapted to record slow LFP signals as well as fast APs. Acquisition was controlled by the software BrainWave (3Brain Gmbh, Wadenswil, Switzerland). MFR and peak amplitude were taken at the ipsilateral, midline, and contralateral corpus callosum. All measurements were made in duplicate.

Statistical analysis

Student's t-tests were employed to specifically compare the differences between groups. All P values were two-tailed where appropriate, and significance was determined at $P < 0.05$. All analyses were performed using IBM SPSS Statistics 23.0 software (IBM Corporation, Armonk, NY, USA).

Results

Color mapping

Figure 1 demonstrates a color map of the electrophysiological signal recorded using APS-MEA. Signal across the corpus callosum was measured in the *ex vivo* brain slices (**Figure 1B**). After complete transection of the corpus callosum, the signal was no longer present (**Figure 1C**). Following application of PEG to the injury site, the signal was restored (**Figure 1D**).

Electrophysiology measurements

Prior to injury, MFRs were 0.76, 0.66, and 0.65 spikes/second at ipsilateral, midline, and contralateral corpus callosum, respectively. After midline corpus callosum transection, MFRs were 0.71, 0.14, and 0.25 spikes/second at the ipsilateral, midline, and contralateral corpus callosum, respectively. Following application of PEG, there were spikes in MFR at the injury site and contralaterally. MFRs were 0.75, 0.55, and 0.47 spikes/s at the ipsilateral, midline, and contralateral corpus callosum, respectively (**Figure 2A**). At the injury site (midline), MFRs in PEG-treated brain slices were statistically superior to those in the control brain slices ($P < 0.01$).

Prior to injury, the average peak amplitudes were 69.79, 58.68, and 49.60 μV at ipsilateral, midline, and contralateral corpus callosum, respectively. After midline corpus callosum transection, peak amplitudes were 52.11, 8.98, and 16.09 μV at ipsilateral, midline, and contralateral corpus callosum, respectively. Following application of PEG, there were spikes in peak amplitude at the injury site and contralaterally. Peak amplitudes were 59.44, 45.33, 40.02 μV at the ipsilateral, midline, and contralateral corpus callosum, respectively (**Figure 2B**). At the injury site (midline), the peak amplitudes in PEG-treated brain slices were statistically superior to those in the control brain slices ($P < 0.05$). Distal to the injury site (contralateral site), the peak amplitudes in PEG treated brain slices were statistically superior to those in the

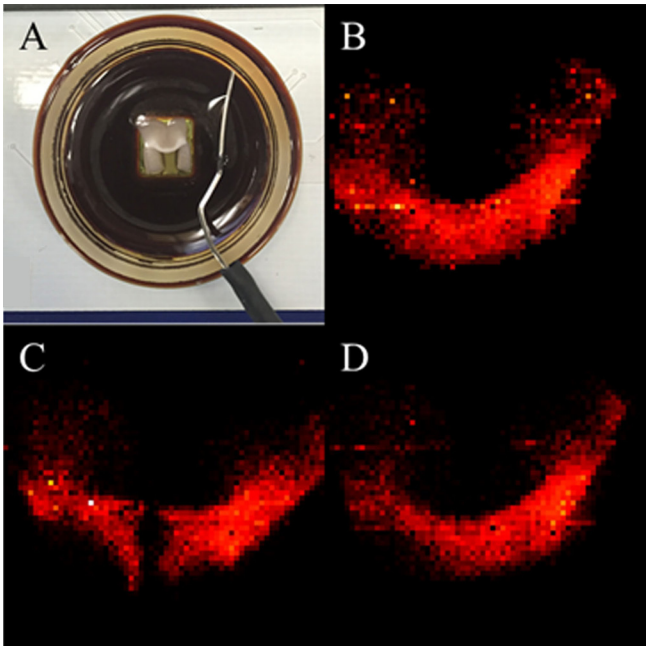

Figure 1 A color map of the electrophysiological signal recorded using active-pixel sensor multi-electrode array system in rat brain slices.
(A) A rat brain slice on microarray electrode chip. Electrophysiological recordings of (B) normal control brain slice, (C) control transected brain slice, and (D) polyethylene glycol (PEG)-treated transected brain slice. The red coloring in the image reflects electrical activity. There is a clear lack of activity at the injury site of the control transected brain slice (C). There is electrical activity at the injury site after PEG application (D).

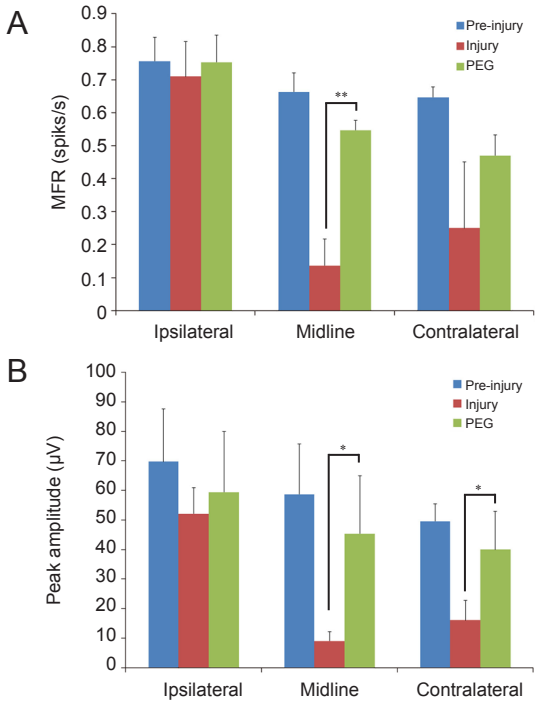

Figure 2 Electrophysiology measurements in rat corpus callosum.
(A) MFR of brain slices pre- and post-injury. (B) Peak amplitudes of brain slices pre- and post-injury. As demonstrated in (A), PEG-treated brain slices had higher MFRs compared to non-PEG treated slices (**P < 0.01). As demonstrated in (B), PEG-treated brain slices had higher peak amplitude compared to non-PEG treated slices at the injury site (*P < 0.05) and contralaterally (*P < 0.05). Student's t-test was used. Data are expressed as the mean ± SD (n = 3 rats/group). All measurements were made in duplicate. MFR: Mean firing rate; PEG: polyethylene glycol.

control brain slices (P < 0.05).

Discussion

PEG fusion represents a potentially revolutionary treatment of peripheral nerve injury, and given the inability of the central nervous system to regenerate, its applicability to central nerves could have an even larger impact. Our study demonstrated the restoration of conduction across the tramsfected corpus callosum after PEG application, and this finding provides evidence that PEG fusion is applicable to the central nervous system.

Previous studies regarding PEG use for treatment of spinal cord injury have suggested that PEG can facilitate membrane repair (Shi, 2013). Nehrt et al. (2010) found that PEG enhanced axolemmal resealing in guinea pig spinal cords. PEG has a neuroprotective role in central nerve injury and is thought to decrease apoptosis, reduce oxidative stress, and increase tissue sparing (Luo and Shi, 2007; Kwon et al., 2011; Siddiqui et al., 2015). Baptiste et al. (2009) treated central nerve injury through systmic and local administration of PEG. The mechanism underlying the neuroprotection of PEG is unclear as the existing evidence has demonstrated that PEG alone has limited neuroprotective potential (Ditor et al., 2007; Kwon et al., 2009).

Glial and extracellular environment in the central nervous system is not beneficial for cell growth (Yiu and He, 2006). The peripheral nervous system is more amenable to growth and regeneration, and thus this may be a more welcoming environment for PEG fusion. Previously, our lab focused solely on the applicability of PEG fusion to repair of peripheral nerve injury. The mechanism underlying PEG axonal fusion is not clearly understood. The immune response to PEG fusion has not been investigated, but PEG has been found to have anti-inflammatory properties (Ackland et al., 2010). Exploring the surrounding immune response in PEG fusion at both the central and peripheral levels is needed to better understand this interesting process.

Restoration of signal across severed nerves after PEG application potentially represents axonal fusion by PEG. Bittner et al. (1986) were the first to found that PEG application restored action potentials across severed medial giant axons in crayfish. Our findings mirror these initial discoveries of PEG's fusogenic properties and potentially represent a novel finding in the central nerve injury. However, in-depth studies are needed.

It is unlikely that there is an alternative explanation for our findings. One potential explanation may be that the presence of PEG allows conductivity not otherwise present. This could explain the restoration of signal after PEG application. However, an argument against this would state that the activity on color map of signals is confined to the area of interest, as opposed to a diffuse activity reading one would expect if PEG solution transmits signals. Additionally, PEG

was washed away after its application onto the nerve injury site. For those reasons, it is reasonable to accept our findings that the role of PEG in the central nerve injury may extend beyond just neuroprotection and includes fusogenic properties.

Currently, both central and peripheral nerve injury is devastating due to the lack of rapid effective treatment. PEG fusion could revolutionize both types of injuries as it provides a fast reconnection of severed nerves. Our initial evidence presented here supports that axonal continuity is restored with PEG. However, more investigation is needed to understand PEG fusion in central nerves. If PEG fusion is applicable clinically, it represents a novel therapy of nerve injury.

Author contributions: *RB, DCR, RBB and ACP designed this study, performed the experiments, and analyzed experimental data. RB wrote the paper. ACP was responsible for critical analysis of this study. RBS and WPT participated in experimental design and provided critical analysis of this study. All authors approved the final version of this paper.*
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