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Rapid morphological fusion of severed myelinated axons by polyethylene glycol

(nerve regeneration/axolemmal fusion/cytoplasmic repair/*Lumbricus terrestris*)

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ABSTRACT **We are able to morphologically fuse the severed halves of an invertebrate-myelinated axon by application of polyethylene glycol (PEG) to closely apposed cut ends. Morphological fusion of the medial giant axon (MGA) of the earthworm *Lumbricus terrestris* is defined as axoplasmic and axolemmal continuity in serial longitudinal sections of MGAs taken through the fusion site as viewed with light or electron microscopes. Morphological continuity is also shown by the transfer of Lucifer yellow dye between apposed MGA segments fused with PEG, but not between apposed MGA segments in normal or hypotonic saline without PEG application. PEG-induced MGA fusion rates can be as high as 80–100% with an appropriate choice of PEG concentration and molecular mass, tight apposition and careful alignment of the cut ends, and treatment with hypotonic salines containing reduced calcium and increased magnesium. A variant of this technique might produce rapid repair of severed mammalian-myelinated axons.**

Neurons are unique among metazoan cell types in having long cytoplasmic processes (axons). Nerve axons typically degenerate within 12–48 hr when severed from their cell bodies in mammals, many other vertebrates, and some invertebrates (1–4). In the mammalian central nervous system such myelinated axons usually do not regenerate. Outside the central nervous system, myelinated axons can regenerate but may take months to years to reach denervated tissues, and the regenerated connections are often not correct. Repair (if any) occurs by the slow (1–2 mm/day) outgrowth of axonal processes regenerating from proximal stumps to contact denervated target cells. This repair of some invertebrate axons and many vertebrate axons is complicated (5, 6) by a myelin sheath, defined as tight wrappings of glial cell membranes around individual axons. We have employed polyethylene glycol (PEG), a substrate used by molecular biologists to fuse plasma membranes of cells suspended in aqueous medium (7–9), to repair the cut ends of an invertebrate-myelinated central nervous system axon in the earthworm. Our procedure takes about a minute to morphologically reconnect the cytoplasm and plasma membranes of two severed axonal segments.

MATERIALS AND METHODS

Earthworms, *Lumbricus terrestris*, were obtained from a local wholesale bait distributor. The animals were maintained in plastic trays containing moist soil and peat moss at 13°C to promote a more favorable fatty acid ratio for membrane fusion (10, 11).

Operations. After anesthesia [4% (wt/vol) Chlorotone (Kodak)], adult earthworms 100–150 segments in length were pinned on a paraffin dish containing 50–100 μM carbachol in an earthworm saline (40.1 mM NaCl/10 mM Na_2SO_4/25 mM sodium acetate/0.5 mM K_2SO_4/3.0 mM $CaCl_2$/0.5 mM $MgCl_2$/1.25 mM Tris/1.5 mM Hepes, pH 7.4). The ventral nerve cord (VNC) was dissected free from 50–75 body segments and pinned to its approximate normal length on a Sylgard-filled dish containing saline. The pH was maintained at 7.4 and 50–100 μM carbachol was added to reduce contraction of smooth muscle fibers within the VNC. When the VNC is pinned dorsal side up, the medial giant axon (MGA) is clearly visible when viewed through a dissecting microscope at ×25–×100.

About 400 MGAs were completely severed under direct visual observation by cutting the entire VNC with an ultrafine pair of iridectomy scissors or a microknife made from a fine shard of a single-edged razor (Gillette Blue Blade). After such axonal severance with complete VNC transection, severed MGAs, and other axons protruded from the cut ends of the VNC in various orientations. In other cases ($n > 150$), MGAs were severed by placing the razor shard on the VNC with the MGAs dorsal side up and pressing hard so that the MGAs and other axons visibly retracted on either side of the blade, but allowing the tough outer collagen sheath of the VNC to remain intact. After axonal severance with an intact VNC sheath, the cut ends of all severed axons remained approximately in their original orientations and were tightly apposed as the outer sheath retracted back to its original length upon removal of the blade.

In cases of axonal severance with complete VNC transection, the two cut ends of the VNC with protruded MGAs were physically separated and reoriented with glass micropipettes or minutenadelen to appose the severed MGAs as tightly as possible without distorting them. Pins were placed in nerve roots arising from the severed VNC segments several millimeters from the potential fusion site to hold the MGAs in place. In cases of axonal severance with an intact VNC sheath, the cord was left undisturbed after lesioning. After both types of lesion, the normal saline (175 milliequivalents/liter) was usually replaced by a hypotonic saline (120–160 milliequivalents/liter) containing NaCl reduced to 13.9–28.1 mM, $CaCl_2$ reduced to 0–0.5 mM, and $MgCl_2$ raised to 3.5–5 mM.

PEG Application. A glass micropipette (25- to 100-μm tip diameter) filled with 50% (wt/wt) PEG (1–10 kDa; Baker) in distilled water was positioned over the potential site of fusion between two apposed MGA segments and the PEG was extruded by using positive pressure. The PEG solution was visible because of its greater density compared to the surrounding saline and because food coloring was often added. After 30–60 sec of PEG application, negative pressure was applied to the PEG-containing pipette and the hypotonic bath saline was replaced with several washes of normal saline.

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Abbreviations: MGA, medial giant axon; PEG, polyethylene glycol; VNC, ventral nerve cord; LY, Lucifer yellow.

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Histological/Ultrastructural Procedures. Within 1–60 min after an attempted fusion, one group ($n > 40$) of MGAs were fixed by replacing the earthworm saline with 2.5% (vol/vol) glutaraldehyde in 150 mM sodium cacodylate (pH 7.4) for 1 hr. The MGAs were then post-fixed in 2% (wt/vol) OsO_4 in sodium cacodylate, sequentially dehydrated in ethanol, and embedded in Spurrs (12). Thick (0.5–1 μm) and thin (0.1–0.2 μm) sections were cut longitudinally through the fusion site and examined with a light microscope (Zeiss ICM-405) or electron microscope (Hitachi H-300 or Siemens 1A), respectively.

Within 30–85 min after an attempted fusion, other ($n > 50$) groups of MGAs were iontophoretically injected on one side of the fusion site with Lucifer yellow CH (LY) by using 4-Hz/0.2-sec duration/25-nA pulses of hyperpolarizing current. Such pulses change membrane potential by only a few millivolts in these giant axons and hence do not produce axolemmal damage (13). LY-injected MGAs were fixed in buffered 4% (wt/vol) paraformaldehyde, dehydrated, cleared with methyl salicylate, and observed using a transmitted-light fluorescent microscope.

RESULTS

We chose to use the earthworm MGA (Fig. 1) for this study because of its large diameter (50–100 μm), its long length (10–15 cm in adult animals), its easy visibility in the VNC (14), and the ready availability of invertebrate animals needed for large numbers of initial trials. Furthermore, the MGA develops from individual axons each having a cell body in a segmental ganglion; these axons make electrotonic (gap) junctions often with axons arising from cell bodies in the adjacent rostral and caudal ganglia. With increasing ontogenetic age, these gap junctions break down, resulting in cytoplasmic and axolemmal fusion between about 60% of adjacent MGA segments in adult animals (15, 16). This natural ability of the MGA to fuse during development might render the axon more susceptible to PEG fusion. Finally, MGAs are surrounded by a multilayered myelin sheath similar to vertebrate myelin in its tight wrapping, spiral arrangement, and ability to increase the conduction velocity of action potentials (14–16).

When PEG was not applied to the cut ends, severed MGAs never fused ($n > 275$). The cut ends of severed MGAs sealed off within minutes in normal saline as viewed in light (Fig. 1*C*) or electron micrographs. Axoplasm did not leak from the cut ends of MGAs, as reported for severed ends of squid (17) or cockroach (18) axons. LY injected into severed MGAs in normal salines remained only within the cut segment (Fig. 1*D*). MGA resting potentials were near normal when recorded a few millimeters from the cut ends of severed MGAs in normal saline. If two severed MGAs in transected VNCs were aligned and mechanically held together for up to 30 min, the cut ends usually became misaligned in the absence of PEG application. Finally, when severed MGAs remained apposed in a VNC with an intact outer sheath, the axonal ends collapsed, became visually opaque when viewed through a dissecting microscope, and immediately separated when the sheath was cut at the lesion site.

We first determined what parameters might facilitate the fusion of severed MGA segments. MGA segments were apposed in various concentrations of PEG, dimethyl sulfoxide, calcium, and ionic strength of the bath saline. PEG molecular mass was varied from 1 to 10 kDa. Changes in dimethyl sulfoxide and PEG concentration or PEG molecular mass reportedly have rather unpredictable effects on the fusion of different cell types (7, 8), including nerve cell bodies (9). Reduced calcium minimizes protease activation (19, 20) and prevents sealing of axonal cut ends (17, 18). Hypotonic salines reportedly eliminate or rearrange intramembrane particles and increase fusion frequency (8). We observed that hypotonic salines also caused the collapsed ends of a severed MGA to open and to extrude axoplasm, which resulted in closer apposition of the axolemma and myelin sheath of two MGA segments.

In these initial experiments, the application of 50% PEG in distilled water to the lesion site sometimes ($n = 15/86$) resulted in adherence of the cut MGA ends and apparent MGA fusion when viewed through a dissecting microscope. A higher ($n = 111/131$) rate of adhesion and apparent axonal continuity was obtained using hypotonic salines (70–90% of normal tonicity) with reduced calcium and increased magnesium concentrations. The highest success rate (equaling 100% in some trials) was obtained by exposing MGAs to solutions having 0 mM calcium, 3.5 mM magnesium, and a total ionic strength of 151 mM and then applying 50% PEG (4 kDa) to the cut ends for 30–60 sec. Hence, this procedure was used as our "PEG application in hypotonic saline" in all experiments described below.

In one experimental trial using 10 severed MGAs in completely transected VNCs, the cut ends of all 10 MGAs remained connected after PEG application in hypotonic saline and subsequent return to normal saline. All 10 MGAs appeared continuous when viewed through a dissecting microscope. Serial longitudinal thick sections taken of these axons showed apparent cytoplasmic and membrane continuity (Fig. 1*G*) in 8 of the 10 fusion attempts. Longitudinal thick sections showed that 2 of the 10 MGAs had obvious (≥3 μm) gaps in the axolemma and myelin sheath in all sections taken through the fusion site. As a control, the severed halves of 8 other MGAs were apposed and placed for 5–10 min in hypotonic saline, but no PEG was applied to the cut ends. None of the axonal ends adhered after return to normal saline. Longitudinal sections taken of 4 such control MGAs showed that the cut ends had collapsed, thereby sealing off the MGA.

Three of the eight PEG-fused axons described above were examined ultrastructurally by taking sequential longitudinal thin sections through the fusion site (Fig. 2). Each MGA showed axoplasmic continuity at the fusion site with gaps of 1–20 μm between a line of amorphous osmiophilic densities (Fig. 2 *A* and *C*). No section had any continuous axoplasmic barrier. Compared to normal axons in which neurotubules, neurofilaments, and smooth endoplasmic reticulum within the axoplasm were oriented parallel to the long axis of the axon (15, 16), these cytoplasmic structures were disrupted for 0.5–2 mm on either side of the fusion site. In some sections the axolemma was obviously present at the fusion site and for many (10–100) micrometers on either side of the fusion site. However, axolemmal gaps of 0.1–1 μm were occasionally seen at the fusion site (Fig. 2 *A*, *D*, and *E*). Axolemmal gaps were not continuous in sequential sections; that is, axolemmal gaps appeared to represent individual axoplasmic holes 0.1–1 μm in maximum diameter. The frequency and length of such holes decreased with increasing distance up to 1 mm from the fusion site. The axolemma was usually continuous ≥1 mm from the fusion site in axons exposed to PEG (Fig. 2*B*). Similar gaps were occasionally seen in nonfused MGAs, presumably due to fixation or sectioning artifacts. Glial membranes of the myelin sheath were rather disrupted for several millimeters on either side of the site of axoplasmic and axolemmal fusion (Fig. 2 *A*, *C–E*). In other trials ($n > 20$), similar histological and ultrastructural data were obtained from fused MGAs from completely transected VNCs (Fig. 1*G*) or from fused MGAs in VNCs having an intact outer sheath (Fig. 1*E*).

As a further test of morphological continuity between two PEG-fused MGA segments, LY was iontophoretically injected into one MGA segment 30–85 min after PEG-induced fusion. In nonsevered control MGAs ($n > 20$), LY remained within the axoplasm after injection and did not label adjacent

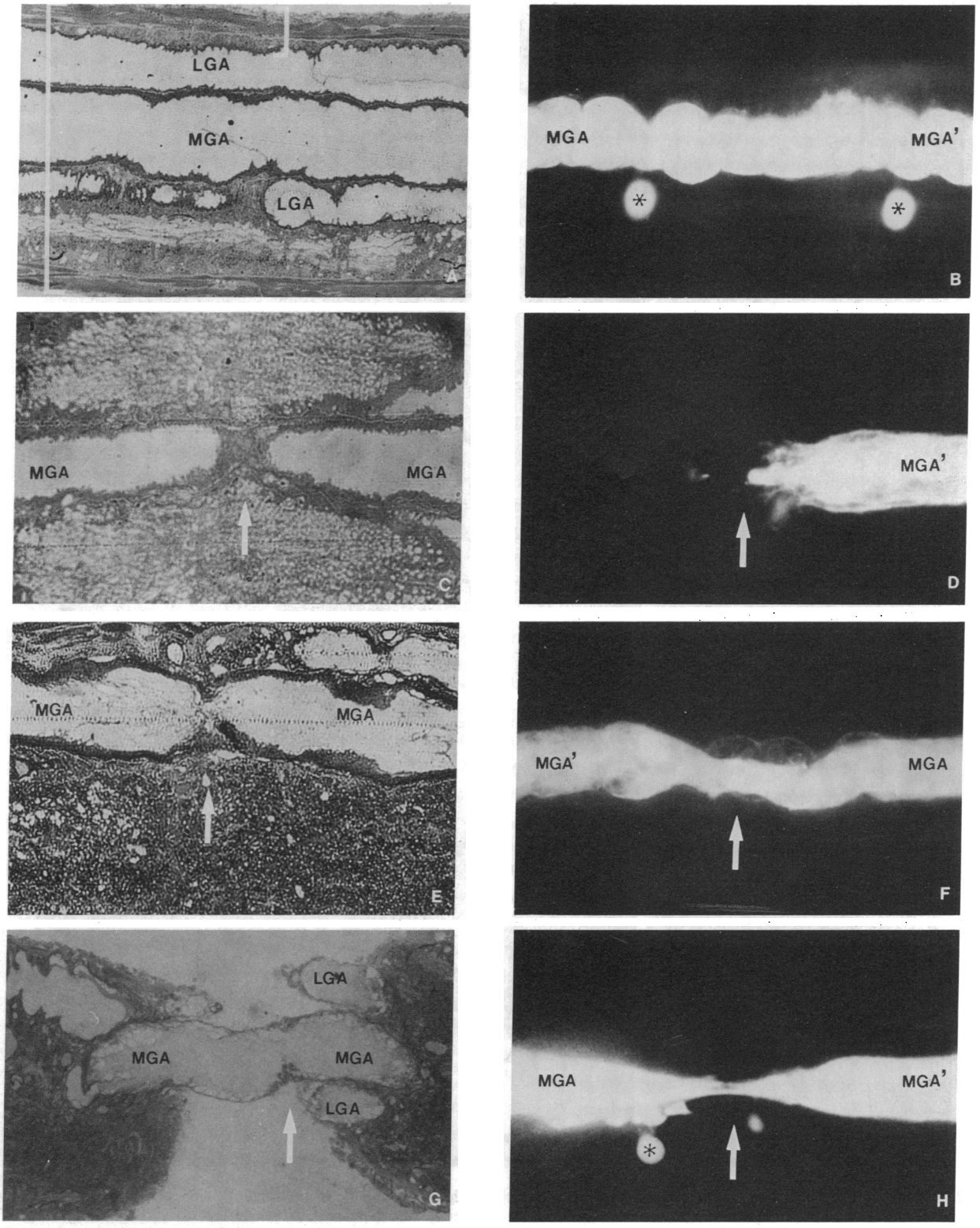

FIG. 1. Light micrographs of thick sections of MGAs from *ex vivo* VNCs cut longitudinally perpendicular to the dorsal–ventral axis (*Left*) and LY-filled MGAs in whole mounts (*Right*). Each figure is from a different preparation. (*A* and *B*) Control MGAs. In *A*, the adjacent lateral giant axons (LGAs) are labeled in the VNC ([) and the outer sheath (]) of the VNC. In *B*, LY injected into an MGA (MGA') within minutes diffused to all portions of the injected MGA' and MGAs in adjacent body segments, as well as to MGA cell bodies (*). (*C* and *D*) Completely severed MGAs in VNCs with intact sheaths exposed to hypotonic saline but not to PEG. Note complete transection (arrow) of the MGA in *C*. LY injected into the right half (MGA') of a severed MGA did not cross the lesion site (arrow in *D*). (*E* and *F*) Completely severed MGAs in VNCs with intact sheaths exposed to hypotonic salines and PEG. Note cytoplasmic continuity at original severance site (arrow in *E*). LY injected into the left-hand MGA segment (MGA' in *F*) diffused across the original fusion site (arrow) and heavily labeled the right-hand MGA segment (MGA). (*G* and *H*) Completely severed MGAs in completely severed VNCs fused with hypotonic salines and PEG. Arrow marks fusion site of MGA segments that protruded from the VNC (*G*). Note the cut ends of nonfused lateral giant axons (LGAs) have sealed over. LY injected into the right-hand portion of the fused MGA (MGA' in *H*) diffused across the fusion site (arrow in *H*) and heavily labeled the left-hand portion (MGA). (All photographs at ×175.)

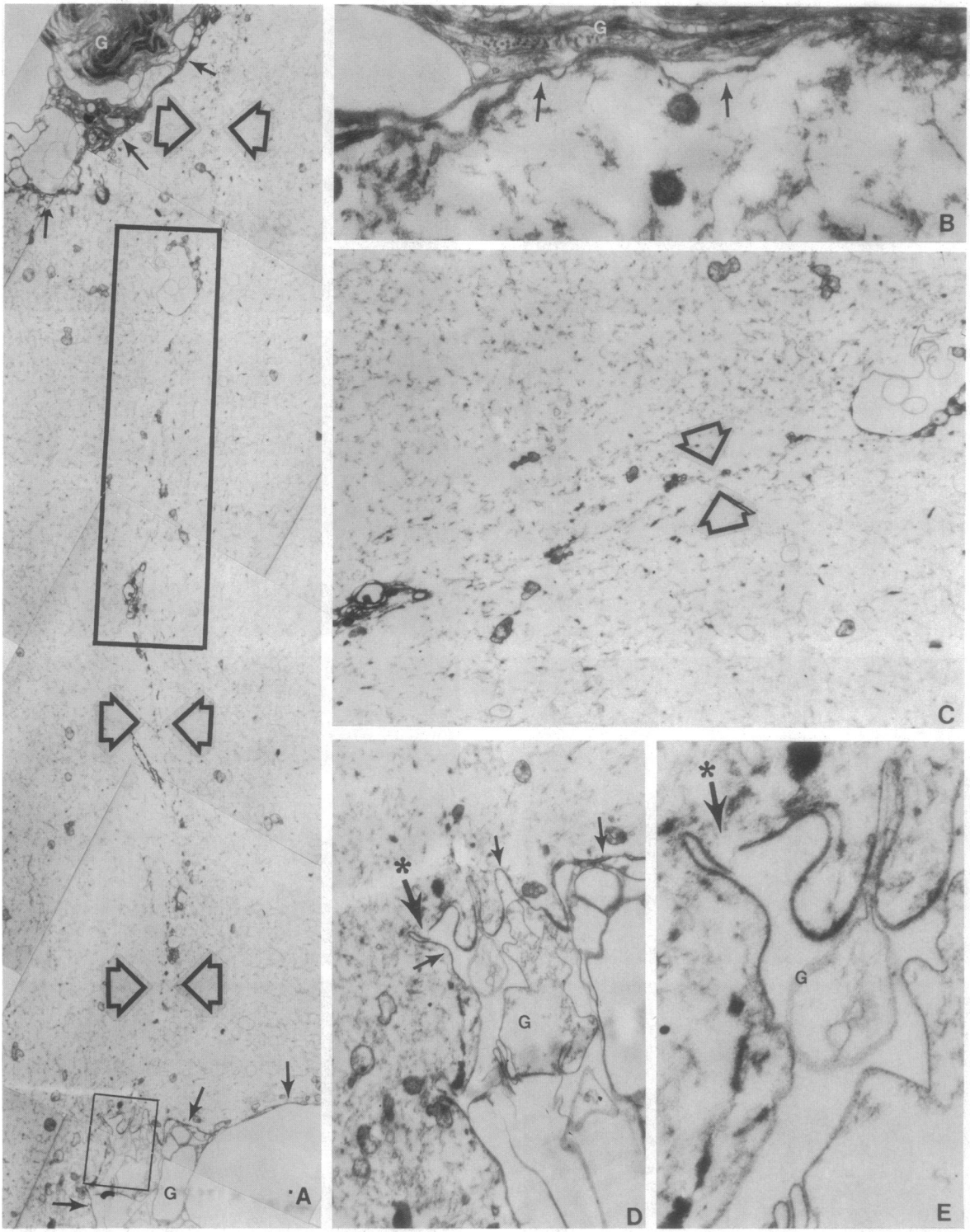

FIG. 2. Electron micrographs of an MGA fused by PEG application in hypotonic saline in a completely transected VNC and fixed within minutes after PEG fusion. (*A*) Longitudinal section through fusion site perpendicular to the dorsal–ventral axis oriented as in Fig. 1. The fusion site is marked by large arrows facing each other in the axoplasm of each MGA segment. Note the absence of any continuous axoplasmic barrier at the fusion site. The axolemma is marked by small arrows and is discontinuous for about 0.2 μm at one site within the small boxed area. Such axolemmal discontinuities decreased in frequency at greater distances from the fusion site. G, glial sheath. (×2200.) (*B*) Axoplasm, axolemma (small arrows pointing to axoplasmic face), and glial sheath (*G*) in PEG-fused axon several millimeters rostral to the site of the fusion. (×6000.) (*C*) Enlargement of axoplasmic area boxed with thick lines in *A* (rotated about 90° clockwise) showing cytoplasmic continuity between apposed MGA segments (apposed arrows) at fusion site. (×4000.) (*D*) Enlargement of axonal–glial interface boxed with thin lines in *A*. Arrows point to axolemma from axoplasmic direction. Arrow with asterisk is a 0.2- to 0.3-μm discontinuity in axolemma at fusion site. G, glial sheath. (×5400.) (*E*) Further enlargement of region of axoplasmic discontinuity at fusion site (arrow with asterisk). (×13,700.)

sheath structures (Fig. 1*B*). In completely transected VNCs, LY did not cross to the opposite MGA (n = 8) when two severed MGAs were apposed in hypotonic salines without PEG application. When PEG was applied to severed, apposed MGAs in normal saline with no exposure to hypotonic saline, LY injected into one MGA segment did not diffuse to

label the apposed MGA segment (n = 5). When PEG was applied to severed MGAs apposed in hypotonic saline, LY injected into one MGA segment crossed the fusion site and heavily labeled the opposite MGA segment in three of eight cases (Fig. 1*H*).

In another set of trials using severed MGAs in VNCs with an intact outer sheath (Fig. 1*E*), LY injected into an MGA segment never crossed to the apposed MGA (n = 8) placed in hypotonic saline without PEG application (Fig. 1*D*). In two of these cases, LY did diffuse in the outer sheath across the lesion site to label nuclei in the sheath of the apposed MGA, but LY did not label the axoplasm of the apposed MGA. When PEG was applied in the absence of hypotonic saline, 1/5 cases showed LY transfer to the apposed MGA. When PEG was applied in the presence of hypotonic saline, LY injected into an MGA segment transferred across the fusion site to heavily label the apposed MGA in 12/13 cases (Fig. 1*F*). Axonal repair was often so complete that LY-labeled fused axons (Fig. 1*F*) looked very similar to LY-labeled control axons (Fig. 1*B*).

Finally, an examination of all ($n > 90$) histological and LY data of PEG-fused MGAs in hypotonic salines showed that the rate (80–100%) of MGA morphological fusion was consistently high in different trials if the outer VNC sheath remained intact compared to the rate (0–80%) of MGA fusion in different trials after complete VNC transection.

DISCUSSION

Axonal regeneration is classically associated with relatively long outgrowths from severed proximal stumps which reinnervate target tissues in many vertebrates and some invertebrates (1–4). However, functional reconnection of many invertebrate axons has now been shown to occur through activation of surviving distal stumps by relatively short outgrowths from severed proximal stumps (1). Although distal stump activation has been suspected to occur by morphological fusion in several instances (1, 21–23), only one uncontested example exists for membrane fusion as a naturally occurring mechanism (21). In most cases, functional regeneration by distal stump activation occurs by way of gap junctions, chemical synapses, or ephaptic current spread (1). We now report morphological evidence that PEG in combination with hypotonic salines can fuse severed segments of the myelinated MGA in the earthworm. This technology takes only a minute to reconnect the severed axonal ends.

Our current rate (80–100%) of successful morphological fusion using PEG in combination with hypotonic salines and reduced calcium is much greater than reported previously (0.5–7%) for a nonmyelinated axon using PEG in the absence of such pretreatments (24). Our present study also suggests that careful alignment of severed axonal halves and tight apposition of severed segments significantly enhance the rate of successful morphological fusion of single axons by PEG. Ideally, return of axonal function might always be associated with return of structural continuity; e.g., action potentials (as a measure of axolemmal function) and fast axonal transport (as a measure of axoplasmic function) would always traverse the fusion site. However, the trauma of axonal severance and PEG application might sufficiently disrupt intramembrane particles (8), cytoskeletal organization (7, 8), internal calcium concentrations (25), axonal–glial relationships (5, 6), ATP levels (25), etc., to block axonal conduction and/or fast transport. In several trials, a few MGAs have conducted action potentials through the lesion site within the first hour after fusion. The ability of action potentials to conduct through the fusion site increased with time in the previous report of fusion of single nonmyelinated giant axons from crayfish (24). Hence, allowing additional time, supplying ATP, etc., may also increase the return of axolemmal and cytoplasmic function in myelinated earthworm MGAs morphologically fused with PEG after hypotonic saline pretreatment.

We hope that a variant of this procedure might be used for the rapid reconnection of severed myelinated axons in mammals. Reconnection in mammals is more difficult because many small-diameter axons lie parallel to each other in peripheral nerve trunks or central nervous system nerve tracts. To be completely successful, reconnection would require exceedingly precise alignment of these bundles of severed axons followed by the simultaneous fusion of all cut ends. However, the alignment of cut ends might not need to be absolutely precise to produce some behavioral recovery since adjacent axons in mammals often have very similar receptive fields or innervate similar target cells. Furthermore, the survival or successful regeneration of only 10% of a bundle of mammalian peripheral or central nervous system axons has often been reported to produce significant behavioral recovery (2, 3). Hence, significant behavioral recovery in mammals (including humans) might occur if PEG application in hypotonic salines were able to fuse 10% of all severed axons in the severed halves of a carefully aligned nerve bundle.

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