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INVITED REVIEW

Repair of traumatic plasmalemmal damage to neurons and other eukaryotic cells

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Abstract

The repair (sealing) of plasmalemmal damage, consisting of small holes to complete transections, is critical for cell survival, especially for neurons that rarely regenerate cell bodies. We first describe and evaluate different measures of cell sealing. Some measures, including morphological/ultra-structural observations, membrane potential, and input resistance, provide very ambiguous assessments of plasmalemmal sealing. In contrast, measures of ionic current flow and dye barriers can, if appropriately used, provide more accurate assessments. We describe the effects of various substances (calcium, calpains, cytoskeletal proteins, ESCRT proteins, mUNC-13, NSF, PEG) and biochemical pathways (PKA, PKC, PLC, Epac, cytosolic oxidation) on plasmalemmal sealing probability, and suggest that substances, pathways, and cellular events associated with plasmalemmal sealing have undergone a very conservative evolution. During sealing, calcium ion influx mobilizes vesicles and other membranous structures (lysosomes, mitochondria, *etc.*) in a continuous fashion to form a vesicular plug that gradually restricts diffusion of increasingly smaller molecules and ions over a period of seconds to minutes. Furthermore, we find no direct evidence that sealing occurs through the collapse and fusion of severed plasmalemmal leaflets, or in a single step involving the fusion of one large wound vesicle with the nearby, undamaged plasmalemma. We describe how increases in perikaryal calcium levels following axonal transection account for observations that cell body survival decreases the closer an axon is transected to the perikaryon. Finally, we speculate on relationships between plasmalemmal sealing, Wallerian degeneration, and the ability of polyethylene glycol (PEG) to seal cell membranes and rejoin severed axonal ends – an important consideration for the future treatment of trauma to peripheral nerves. A better knowledge of biochemical pathways and cytoplasmic structures involved in plasmalemmal sealing might provide insights to develop treatments for traumatic nerve injuries, stroke, muscular dystrophy, and other pathologies.

Key Words: *membrane damage; plasmalemmal sealing; vesicle mediated repair; Ca^{2+}; axon regeneration; neuron*

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Overview

Plasmalemmal damage due to traumatic injuries can range from small membrane holes to complete transections of a cell body (neuronal, muscle fiber, epithelial, *etc.*) or large cytoplasmic process (axon, dendrite, filopodium, *etc.*). Across many biological disciplines, data clearly and consistently demonstrate that rapid (seconds to minutes) repair of plasmalemmal damage to eukaryotic cells is necessary for their survival.

Immediately following traumatic injury, extracellular calcium (Ca^{2+}) quickly diffuses into cells driven by a large (10^3–10^6 M) concentration gradient (Alberts, 2008). Increases in intracellular Ca^{2+} can activate proteases and disrupt many biochemical pathways, thereby inducing apoptosis or other mechanisms of cell death (Schlaepfer and Bunge, 1973; Tymianski et al., 1993; Krause et al., 1994; Steinhardt et al., 1994; Choi, 1988; Bittner and Fishman, 2000; Yoo et al., 2004; Spaeth et al., 2010, 2012a, b, c; Moe et al., 2015).

This same Ca^{2+} influx, that if left unchecked can lead to cell death in all eukaryotic cells, also initiates repair of the plasmalemmal damage, which decreases Ca^{2+} influx and thus prevents somal Ca^{2+} concentrations from reaching levels that produce cell death (Krause et al., 1994; Steinhardt et al., 1994; Spaeth et al., 2010, 2012a, b, c; Moe et al., 2015). Ca^{2+} influx activates plasmalemmal sealing through multiple parallel and redundant enzymatic pathways that induce membranous vesicles to form and/or migrate to the lesion site where they accumulate, interact, and fuse to seal the membrane and reduce Ca^{2+} influx to levels seen in uninjured eukaryotic cells (Krause et al., 1994; Miyake and McNeil, 1995; Eddleman et al., 1997, 1998a, b, 2000; Blanchette et al., 1999; Jimenez et al., 2014).

Rapid repair (sealing) of traumatic plasmalemmal damage is especially important for mammalian neurons that typically do not naturally regenerate their cell body (perikaryon) in post-fetal stages (Cai et al., 2001). The closer to the perikaryon an axon or dendrite is transected, the greater the probability of cell death (Ramón y Cajal, 1928; Lucas et al., 1985, 1990; Yoo et al., 2004; Nguyen et al., 2005; Campbell, 2008; Wolfe et al., 2010). The increase in cell death for cells with axons transected nearer to, compared to further from,

their cell body is likely due to increased concentrations of somal Ca^{2+} (Yoo et al., 2004; Nguyen et al., 2005). Reduction in Ca^{2+} influx following sealing prevents rapid degeneration of severed distal stumps of invertebrate axons, but does not prevent rapid (Wallerian) degeneration of severed distal stumps of mammalian axons (Bittner and Fishman, 2000; Bittner et al., 2000).

Although critically important for understanding regeneration of all eukaryotic cell types, especially neurons as outlined above, plasmalemmal repair of eukaryotic cells has for decades been a topic studied by relatively few laboratories. Recently, plasmalemmal sealing has received much more attention, especially with respect to biochemical mechanisms (Bloom and Morgan, 2011; Moe et al., 2015). This review considers the following important questions: [1] How reliable are different measures used to assess plasmalemmal sealing? [2] What substances or biochemical pathways affect plasmalemmal sealing (**Figure 1**)? [3] What cellular events might account for plasmalemmal sealing (**Figure 2**)? [4] What molecular/subcellular processes might allow vesicles to seal plasmalemmal holes or complete transections? [5] What are the evolutionary origins and subsequent uses of these sealing proteins and mechanisms? [6] What data might account for observations that axonal transections nearer to (versus further from) the perikaryon are associated with higher probabilities of cell death? [7] What might be the basic science or clinical significance of obtaining better data on plasmalemmal sealing mechanisms in neurons and other cell types?

How Reliable Are Different Measures Used to Assess Plasmalemmal Sealing?

Axolemmal/plasmalemmal integrity or sealing is often assumed to be equally validly assessed by making various morphological observations, by measuring membrane potential (V_m) and/or input resistance (R_i) as functional measures of the formation of a barrier to entry of ions (an ionic seal) (Yawo and Kuno, 1983; Lucas et al., 1985; Spira et al., 1993), or by observing the ability of the axolemma to exclude fluorescent dyes or other tracers as a measure of a barrier to entry of larger molecules (Xie and Barrett, 1991). However, as discussed below, data taken from only a single measurement technique often provide a very ambiguous assessment of the effectiveness of restored barriers to entry of ions or molecules at a site of membrane damage.

Morphological observations alone almost always produce ambiguous assessments of seal formation due to a lack of resolution. The presence of a seal has been assessed morphologically by examining phase-contrast images (Yawo and Kuno, 1983) or electron micrographs (Spira et al., 1993; McNeil et al., 2000). However, the former method lacks the resolution necessary to determine whether the plasmalemma is intact, and the latter has not been used to examine whether the axolemma is intact in electron micrographs of sufficient magnification or in more than a few sections through the lesion site. To the best of our knowledge, high power (> 80,000 magnification) electron micrographs of a fortuitous section or 3D reconstruction of many serial sections have never been used to show a continuous membrane barrier -- and would probably be an impossible task.

Electrophysiological measures of membrane potential (V_m) and/or input resistance (R_{in}) by intracellular or patch clamp microelectrodes often provide poor assessments of the state of an ionic seal at a small hole or complete transection, largely due to the distributed cable properties of axons (Krause et al., 1994, 1995). For example, mathematical models of axonal resistance and measurements made from analog axonal equivalent circuits show that V_m and R_{in} are very insensitive to large changes in the resistance of a damaged, but actively sealing, membrane -- even when measured within $1/100^{th}$ of a space constant (λ) from the damage site. Furthermore, V_m and R_{in} depend upon λ, which fluctuates in unpredictable ways during sealing with changes in V_m, permeability of voltage-sensitive ion channels, and the diameter or length of the intact or injured portions of the axon (Krause et al., 1994, 1995). Hence, plots of V_m and R_{in} *versus* time provide ambiguous, inconclusive data on the state of a plasmalemmal seal.

Biophysical measures of injury current density (I_i) at a site of membrane damage by extracellular current probes do not depend upon changes in axonal cable properties (Krause et al., 1994, 1995) and provide accurate assessments of the state of the seal if V_m is also measured to be certain it remains in a normal (control) range, *i.e.*, changes in V_m reflect axonal dysfunction in ion channels and pumps. However, I_i measurements by "vibrating probes" are difficult to obtain especially for smaller mammalian cells, can damage the membrane, and are rarely used (Bittner and Fishman, 2000).

Labeled tracer molecules have been most commonly used to assess barrier formation and are probably the best single method, but are not without pitfalls. Scientists making such measurements need be aware that there is a very non-linear relationship between the size (diameter) of the plasmalemmal damage and the effectiveness of a barrier to diffusion; Ca^{2+} influx is not greatly retarded until holes are reduced to 0.1– 0.2 nm and to about 2 nm to impede 2 kDa dyes or other tracer substances (Krause et al., 1995). Hence, the time course of exclusion of tracer substances during sealing is very dependent on tracer molecular weights. Membranes that show a seal-barrier to larger tracer molecules like horseradish peroxidase-conjugated probes of > 40 kDa might well not show a seal-barrier to Ca^{2+} (Eddleman et al., 1998a,b, 2000; Fishman and Bittner, 2000, 2003). Furthermore, tracer substances of the same molecular weight that have a net neutral charge may be permeable, but tracers having a net negative charge may well be excluded due to negatively charged proteins and other substances at cut axonal ends (Eddleman et al., 1998b, 2000). Smaller, neutrally charged dyes provide the most interpretable and consistent data (Bittner and Fishman, 2000; Fishman and Bittner, 2003; Spaeth et al., 2012a, b, c; Zuzek et al., 2013; McGill et al., 2016).

In summary, a combination of measures such as V_m, I_i, exclusion of positive or uncharged dyes, serial confocal Z sections, and many high-magnification electron micrographs taken through the lesion site are necessary to assess with reasonable confidence the functional and morphological state of a seal. These more-complete multiple-method measures have only

been done for a few invertebrate giant axons. In such cases, no differences were noted in sealing of proximal *versus* distal halves of those giant axons for any functional or morphological measure of axonal sealing (Krause et al., 1994; Eddleman et al., 1997, 1998a, b, 2000; Bittner and Fishman, 2000).

What Substances or Biochemical Pathways Affect Plasmalemmal Sealing? See Figure 1.

Ca^{2+}: From the earliest studies on sealing by assumed formation of membrane barriers (Yawo and Kuno, 1983) through the first reports of vesicular-based sealing (Krause et al., 1994; Steinhardt et al., 1994) to recent articles on sealing phenomena (Jimenez et al., 2014; McGill et al., 2016), Ca^{2+} influx has been reported to be necessary to induce plasmalemmal sealing of small holes or complete transections of any eukaryotic cell (reviewed by Spaeth et al., 2010). A cytoplasmic Ca^{2+} level of about 100 μM induces vesicle formation in invertebrate giant axons (Fishman and Metuzals, 1993; Eddleman et al., 1997, 1998a, 2000). A threshold dependence on Ca^{2+} concentration instead of a graded dependence suggests that injury-induced membranous structures are not solely produced by upregulation of an existing low-level process (*e.g.*, endocytosis). These data agree with other reports that extracellular Ca^{2+} must be raised to at least 100 μM to induce sealing of severed septal neurites in tissue culture (Xie and Barrett, 1991) and that intracellular Ca^{2+} reaches μM to mM levels following axotomy in the lamprey spinal cord (Strautman et al., 1990), squid giant axons (Krause et al., 1994), Aplysia axons *in vitro* (Ziv and Spira, 1995) or large holes in sea urchin eggs (Steinhardt et al., 1994).

This increase in cytoplasmic Ca^{2+} is caused by entry of extracellular Ca^{2+} through the lesion site (Krause et al., 1994; Bittner and Fishman, 2000; Bittner et al., 2000), rather than through voltage-dependent ion channels in the axolemma (Sattler et al., 1996; George et al., 1995) or release of Ca^{2+} from internal stores. Increasing intracellular Na^+ and/or Cl^- levels, or reducing intracellular K^+ concentration does not induce sealing, but may induce formation of some membranous structures (Eddleman et al., 1998a; Bittner and Fishman, 2000) at much higher internal concentrations (> 100 mM) than are needed for induction by Ca^{2+}.

Elevated intracellular Ca^{2+}, rather than any other ion (*i.e.*, Mg^{2+}) or injury *per se*, is the necessary trigger to form injury-induced membranous structures and an axonal barrier to various dyes and ions in invertebrate (Krause et al., 1994; Steinhardt et al., 1994, Blanchette et al., 1999) and mammalian cells (Detrait et al., 2000a, b; Reddy et al., 2001; Idone et al., 2008), including neurons (for reviews, see Bittner and Fishman, 2000; McNeil et al., 2000; Spaeth et al., 2010; Moe et al., 2015). Hence, sealing time courses are measured by post-calcium influx (PC) times and not post-transection times (Eddleman et al., 2000; Detrait et al., 2000a, b; Yoo et al., 2003, 2004; Spaeth et al., 2010, 2012a, b, c; McGill et al., 2016). However, TRIM proteins: Cai et al., 2009) or polyethylene glycol (PEG: Spaeth et al., 2010 see below) are exceptions to Ca^{2+} dependence and can artificially induce plasmalemmal sealing in the absence of extracellular Ca^{2+}.

In summary, Ca^{2+} influx initiates the endogenous sealing process and Ca^{2+} affects many substances and pathways involved in sealing, as discussed in subsequent paragraphs and topic headings.

Calpains and other proteases

Calpain and other proteases induce plasmalemmal sealing in mammalian, other vertebrate and invertebrate neurons and other cell types (Xie and Barrett, 1991; Krause et al., 1994; Godell et al., 1997; Yoo et al., 2003; Spaeth et al., 2010). According to electrical or dye-exclusion measures, exogenous calpain induces sealing in squid giant axons (Godell et al., 1997). When other proteases which are not as dependent on Ca^{2+} as calpain for activation (*e.g.*, bromelian, papain, trypsin, dispase, chymotrypsin) are added to divalent-free salines or Ca^{2+}-free salines, crayfish giant axons usually seal. Dispase can induce sealing in Ca^{2+}-free salines, but uniquely requires Mg^{2+} (Godell et al., 1997). These observations are in agreement with other reports (Xie and Barrett, 1991) that several proteases (papain, dispase, and trypsin) enhance sealing in severed mammalian septal axons in tissue culture. These proteases could increase sealing *via* cleavage of cytoskeletal elements, such as actin and microtubules, perhaps changing cell shape (see below), removing cytoskeletal barriers to vesicular seal formation, and/or by cleaving proteins anchoring vesicles in place so that they may then undergo exocytosis to the damage site.

Cytoskeletal proteins

Cell shape, as controlled dynamically by the cytoskeleton following traumatic injury, also plays an important, albeit indirect, role in plasmalemmal sealing. That is, although the cytoskeleton undergoes changes to narrow the size of the plasmalemmal hole in severed axons, "collapse and fusion" of opposing plasmalemmal leaflets does not occur (Krause et al., 1994; Eddleman et al., 1997, 1998a, 2003). However, not all cells respond similarly to cytoskeletal modifications. For example, various stabilizers of microtubules (20 mM taxol) or destabilizers of F-actin (6 μg/mL cytochalasin E) -- compounds that should stabilize or reduce axonal diameter -- do not significantly affect the sealing of crayfish giant axons (Krause et al, 1994). In contrast, taxol, cytochalasin E, and phalloidin inhibit sealing, and colchicine promotes sealing of mammalian septal axons (Xie and Barrett, 1991). F-Actin and myosin-2 help close plasmalemmal holes in frog oocytes and Drosophila embryos (Mandato and Bement, 2001; Abreu-Blanco et al., 2012).

In brief, although changes in the cytoskeleton affect plasmalemmal sealing, the precise role of a given cytoskeletal element appears to differ among various cell types.

The endosomal sorting complex required for transport (ESCRT)

ESCRT, known to be involved in budding in yeast and fission of single and multi-vesicular bodies in mammalian endosome, has recently been reported to seal small (< 100 nm wide) plasmalemmal holes generated by mechanical- or laser-induced micro-punctures, or by membrane deforming toxins (Jimenez et al., 2014). Given the small diameters of these plasmalemmal wounds and the known role of ESCRT

proteins and adaptors in endosomal processes, ESCRT proteins probably have auxiliary roles in complete transections or large plasmalemmal holes caused by traumatic injury (Moe et al., 2015).

Isomers of substances involved in synaptic transmission, Golgi vesicular trafficking, and/or other interactions of membrane-bound organelles

Since 1994, several laboratories studying plasmalemmal sealing have reported that isomers of substances involved in synaptic transmission (calmodulin, synatobrevin, synaptotagmin, synaptophilin, syntaxin, SNAP-25), Golgi vesicular trafficking (Snap-25 isomers, NSF), and/or other interactions of membrane-bound organelles (annexins, kinesin, ESCRT) affect plasmalemmal sealing (Steinhardt et al., 1994; reviewed by Spaeth et al., 2010 (supplement); Moe et al., 2015). These and other substances are involved in several parallel, redundant, and interrelated biochemical pathways initiated by Ca^{2+} influx to induce or enhance plasmalemmal sealing. The parallel pathways identified to date are as follows:

The PKA pathway

Damage-induced Ca^{2+} influx activates Ca^{2+}-dependent adenylate cyclases that increase cAMP levels and activate PKA. PKA is a protein kinase with a diverse array of cellular functions, including activating proteins involved in neurotransmission and exocytosis (Spaeth et al., 2010, 2012a, b, c; Zuzek et al., 2013). The PKA sealing pathway utilizes SNARE, SNAP-25, syntaxin, synaptotagmin and other proteins involved in vesicle-mediated neurotransmitter release, but not proteins involved in Golgi vesicular trafficking consistent with other data (Yoshihara et al., 2000).

The Epac pathway

Damage-induced Ca^{2+} dependent increases in cAMP activate Epac 1&2, which are guanine exchange factors with cAMP binding sites of lower affinity than PKA (de Rooij et al., 1998). During plasmalemmal sealing, Epac isoforms activate proteins involved in both vesicle-mediated release of neurotransmitters and Golgi vesicular trafficking. These data from hippocampal B104 cell cultures are consistent with reports that Epac increases neurotransmission in rat hippocampal slices (Ster et al., 2009) and PC12 cells (Hatakeyama et al., 2007) by activating SNAP-25 isoforms (Vikman et al., 2009) and other proteins important for Ca^{2+}-dependent vesicle trafficking possibly by increasing trans-Golgi vesicular trafficking *via* Rap1 (de Rooij et al., 1998).

The DAG, Munc-1, PKC pathway

Sealing is enhanced by diacyl-glycerol (DAG) pathways that likely converge onto cAMP pathways and activate some isomers of protein kinase C (PKC) (Zuzek et al., 2013). PKC isomers, like PKA, activate a wide array of proteins involved in diverse cellular processes, including proteins involved in neurotransmission and exocytosis. PLC, DAG, some PKC isoforms and downstream proteins specifically activated by PKC (MARCKS) also contribute to plasmalemmal sealing. cAMP might activate some PKC isoforms, thereby demonstrating that PKC and PKA pathways converge at multiple points in their respective pathways to contribute to sealing (Zuzek et al., 2013).

The cytosolic oxidation pathway

Although often described as toxic, cytosolic oxidation (*e.g.*, by H_2O_2, or loss of cytosolic reducing potential) enhances Ca^{2+}-dependent plasmalemmal sealing *via* proteins involved in neurotransmitter release, but not Golgi vesicular trafficking (Spaeth et al., 2012a). These data are consistent with reports that the antioxidant melatonin (Rojas et al., 2009) decreases plasmalemmal sealing (Spaeth et al., 2010, 2012c) and that H_2O_2 increases vesicle-mediated processes, such as long term potentiation in hippocampal (Thiels et al., 2000) or spinal neurons (Smith et al., 2013). A family of Tri-Partite Motif (TRIM) proteins activated by cytosolic oxidation also increases vesicle number and vesicle trafficking to repair damaged muscle cells (Cai et al., 2009), although the role of TRIM proteins in neuronal plasmalemmal repair has not be demonstrated.

The PKA, Epac and oxidation pathways of sealing all converge on N-ethylmaleimide (NEM) sensitive factor (NSF)

Sealing pathways involving vesicle interactions all require membrane fusion typically mediated by the NSF, *i.e.*, NEM inhibits NSF-induced membrane fusion (Garcia et al., 1995). Simultaneous activation of the PKA, Epac and oxidation sealing pathways does not overcome NEM inhibition of NSF, consistent with the hypothesis that all these biochemical pathways converge on NSF (Spaeth et al., 2010, 2012a, b, c; Zuzek et al., 2013). In addition to a role in membrane fusion, NSF is also involved in endocytotic pinching off of vesicles from the plasmalemma (Garcia et al., 1995). Endocytosis of nearby undamaged membrane may be an important source of vesicles for plasmalemmal repair (Eddleman et al., 1997; see following section).

In brief, Ca^{2+} entry at sites of plasmalemmal damage has three major roles: (1) Ca^{2+} entry elevates axoplasmic [Ca^{2+}], which induces or facilitates processes (*e.g.*, endocytosis, vesiculation, myelin delamination and fusions) necessary for the rapid repair of axolemmal damage (Ballinger et al., 1997; Eddleman et al., 1997, 1998a) and (2) Ca^{2+} entry activates Ca^{2+}-dependent proteases (*e.g.*, calpain), which promote processes (*e.g.*, vesicular interactions) that are essential for axonal seal formation. (3) Ca^{2+} entry initiates processes (*e.g.*, calpain and other proteases) that lead to cell death by apoptosis and other pathways (see subsequent sections for additional detail).

Polyethylene glycol (PEG)-sealing

PEG bypasses all substances and all endogenous sealing pathways described above to rapidly (within milliseconds) and directly induce plasmalemmal sealing by removing waters of hydration at closely apposed membranes at severed axonal ends and small holes, thereby allowing membrane lipids in plasmalemmal leaflets to collapse, fuse, and seal cut ends or to spread and seal smaller plasmalemmal holes (Lee and Lentz, 1999; Lore et al., 1999; Bittner and Fishman, 2000; Lentz, 2007). That is, none of the substances listed above that induce, enhance, inhibit, or prevent endogenous sealing

(including Ca^{2+}) have any detectable effect on PEG-sealing of B104 cells *in vitro* or rat sciatic axons *ex vivo* or *in vivo* (Spaeth et al., 2012b). Furthermore, sealing probability increases sigmoidally from 0% to 100% with increasing PEG concentration, as might be expected of a substance that directly induces plasmalemmal sealing (Spaeth et al., 2012b).

What Cellular Events Might Account for Plasmalemmal Sealing? See Figure 2.

Textbooks that briefly discussed plasmalemmal repair (*e.g.*, Kandel and Schwartz, 1985) typically reported that transected axons (**Figure 2A**) sealed by complete collapse of the severed axolemmal leaflets that rapidly fused to form a continuous membrane barrier (**Figure 2B**). No references were cited. Until observations were made on invertebrate axons (Krause et al., 1994), sea urchin eggs, and mammalian epithelial cells (Steinhardt et al., 1994), this intuitive assumption was by far the most commonly proposed mechanism in journal articles for axolemmal sealing following transection of various invertebrate (Krause and Bittner, 1990; Spira et al., 1993; Gallant, 1998) or mammalian axons (Xie and Barrett, 1991). As one alternative mechanism, Yawo and Kuno (1983) proposed that membrane of unspecified origin formed a partition-like structure de novo near the cut end (**Figure 2C**) to seal transected cockroach giant axons. However, in the absence of a fusogen like PEG, plasmalemmal collapse and fusion should not occur based on biochemical and energetic properties of hydrophobic lipid interactions (Lentz, 2007).

As a second alternative mechanism to account for plasmalemmal sealing, Fishman and Metuzals (1993) proposed that injury-induced vesicles that rapidly formed and accumulated at sites of axolemmal damage of squid giant axons might somehow be involved in axonal repair (**Figure 2D**). Although not widely discussed in most textbooks, vesicle-based plasmalemmal sealing mechanisms have been documented in over 100 published papers from over 20 different laboratories (for reviews see McNeil et al., 2000; Bittner and Fishman, 2000; Fishman and Bittner, 2003; Spaeth et al., 2010; Moe et al., 2015). As described in greater detail in following sections, single or multilayer injury-induced vesicles formed by plasmalemmal endocytosis or from other membranous structures such as smooth endoplasmic reticulum, axonal transport vesicles, mitochondria, lysosomes, and myelin delaminations are all involved in plasmalemmal sealing of small holes or complete transections (**Figure 2D**).

In unmyelinated plasmalemma of neurons or other cell types, Ca^{2+} influx at small holes or complete transections induces single-walled vesicles to arise from the plasmalemma and adjacent supporting cells (*e.g.*, glia) by endocytosis and induces smooth endoplasmic reticulum to break up. Ca^{2+} influx also induces pre-existing membranous structures such as subcortical vesicles (*e.g.*, in various oocytes), axonal transport vesicles, mitochondria, or lysosomes to migrate toward the wound site (**Figure 2D**; Krause et al., 1994; Steinhardt et al., 1994; Eddleman et al., 1997; Ballinger et al., 1997; Bittner and Fishman, 2000; Reddy et al., 2001). All such membranous structures interact to form what has been termed called a "vesicular plug" that gradually (seconds to minutes) forms an effective diffusion barrier that restores the influx of progressively smaller molecules to levels exhibited by an intact plasmalemma (**Figure 2D**) (Krause et al., 1994; Steinhardt et al., 1994). In myelinated axons, Ca^{2+} influx at the lesion site also induces myelin delaminations that rapidly form multilayered membranous structures that migrate to the lesion site and contribute to the vesicular plug (Ballinger et al., 1997; Eddleman et al., 1997; Lore et al., 1999; Lichstein et al., 2000). Transected ends of the plasmalemma in neurons (Krause et al., 1994; Eddleman et al., 2003; Fishman and Bittner, 2003) or other cells such as muscle fibers (Moe et al., 2015) constrict and shorten within minutes after axotomy, but do not completely collapse and fuse. Scanning electron micrographs (Detrait et al., 2000b; Eddleman et al., 2003) of the cut distal and proximal ends of crayfish giant axons have tightly-packed vesicles of widely-differing size associated with an ionic seal having high electrical resistance and dye barrier that forms amidst the packed vesicles.

By 24 hours post-transection, a continuous axolemma is observed at distal and proximal cut ends (Lichstein et al., 2000). From 1–10 days post-transection, vesicular structures at proximal and distal cut ends gradually disappear (**Figure 2D3**), and neuritic outgrowths arise from sealed proximal axonal ends whose internal micro-architecture appears very normal (Eddleman et al., 1997; Lore et al., 1999).

What Molecular/Subcellular Processes Might Allow Vesicles to Seal Plasmalemmal Holes or Complete Transections?

Immediately following plasmalemmal damage, membrane-bound structures interact to form a diffusion barrier and eventually restore a continuous plasmalemma (**Figure 2D**). However, the exact mechanism(s) for these phenomena are as yet unknown for any cell (Moe et al., 2015). Possible molecular/subcellular mechanisms for forming a diffusion barrier to ions and larger molecules (*e.g.*, tracers of any molecular weight) include:

a. Contraction of the plasmalemma by cytoskeletal elements that repair complete transections because the cut ends narrow, but never completely collapse (**Figure 2D**) (Krause et al., 1994; Eddleman et al., 2003; Bittner and Fishman, 2000; Fishman and Bittner, 2003). Cytoskeletal contraction might seal small holes, albeit this result has not yet been demonstrated (see Moe et al., 2015 for a recent review) and ESCRT proteins might close small holes (Jimenez et al., 2014).

b. Vesicle/plasmalemma interactions or fusions such as externalization of membrane by ESCRT-mediated budding or internalization of membrane by endocytosis that both could remove and thereby seal a small hole in the plasmalemma (Jimenez et al., 2014; Moe et al., 2015).

c. Denser packing of vesicles as observed for small holes or complete transection of invertebrate giant axons that would create a longer, narrower diffusion path (**Figure 2D1**; Krause et al., 1994; Eddleman et al., 1997, 1998a, b, 2003; Bittner and Fishman, 2000; Fishman and Bittner, 2003).

d. Vesicle to vesicle attachments, *e.g.*, desmosomes, adher-

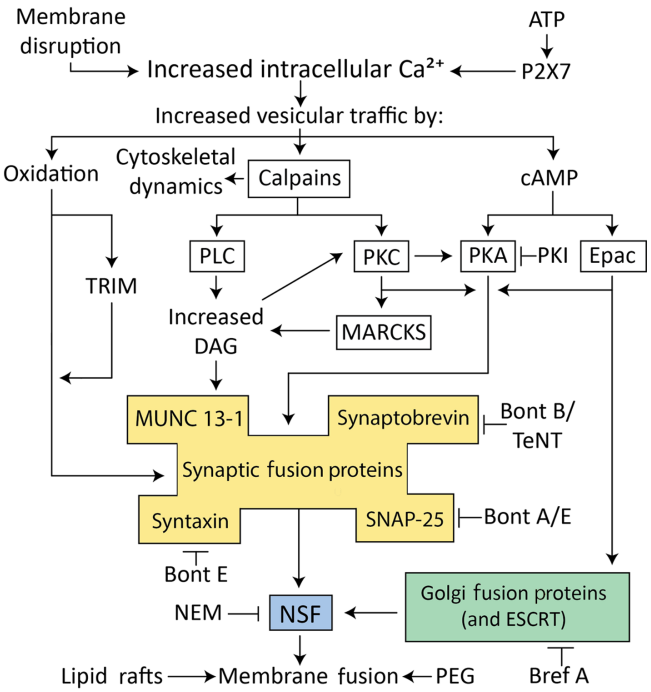

Figure 1 Sealing pathway schematic for multiple proteins and pathways involved in vesicle mediated repair of plasmalemmal damage
See text for references. Ca^{2+} influx at a site of plasmalemmal damage activates Ca^{2+} binding proteins, many of which increase vesicular traffic. The P2X7 protein, activated by ATP, also increases intracellular Ca^{2+}. To date, three parallel sealing pathways have been identified that involve 1) oxidation, 2) calpains, and 3) cAMP (whose levels are mediated by Ca^{2+} activated adenylate cyclases). (1) The oxidation pathway activates vesicle formation, synaptic membrane fusion proteins (labeled in yellow), and Tri-partite motif proteins (TRIM proteins). (2) Calpains cleave cytoskeletal elements to free vesicles bound to the cytoskeleton, phospholipase C (PLC) and regulatory subunits of PKC isomers. PLC activation, in turn, cleaves the membrane lipid PIP2 into second messengers IP3 and diacylglycerol (DAG). DAG binding to regulatory subunits activates some PKC isomers. Ca^{2+} and DAG activate additional PKC isomers and the membrane fusion protein MUNC-13. (Note: not all PKC isomers are implicated in plasmalemmal sealing). PKC phosphorylates various isomers of synaptic membrane fusion proteins, and the membrane bound protein MARCKS. MARCKS increases DAG production, thereby enhancing PKC activity. (3) cAMP activates PKA and Epac. PKA directly phosphorylates SNARE proteins, such as SNAP-25 (inhibited by Botulinum toxin (BonT) A/E), synaptotagmin (inhibited by BonT B/TeNT), and syntaxin (inhibited by BonT E). PKA likely does not activate isomers of SNARE proteins required for Golgi membrane fusion (labeled in green) and ESCRT proteins that are inhibited by the fungal toxin Brefeldin A. In contrast, Epac (the guanine exchange factor) activates SNARE proteins in both the Golgi and synaptic membrane fusion pathways that converge at N-ethylmaleimide sensitive factor (NSF: labeled in blue). Finally, both lipid rafts and PEG produce membrane fusion, bypassing NSF.

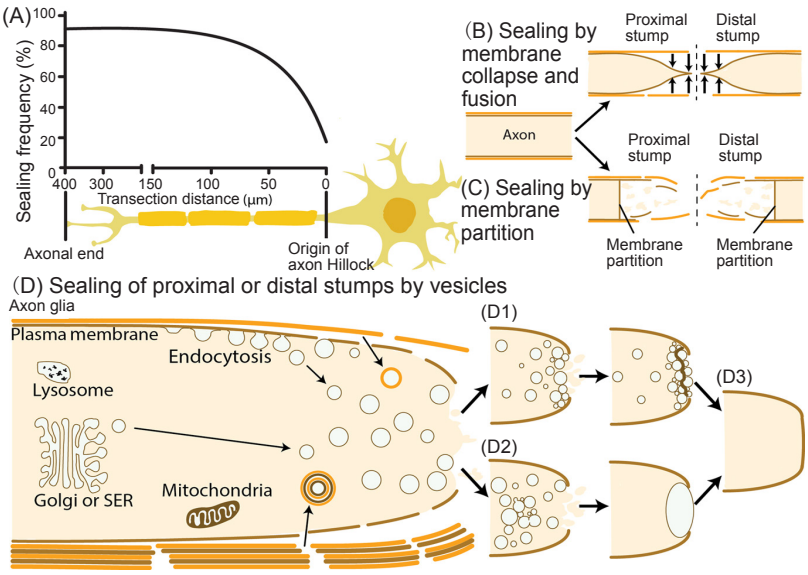

Figure 2 Schematic of plasmalemmal sealing probabilities (A) and mechanisms (B–D).
(A) Probability (sealing frequency) of plasmalemmal sealing of proximal stump decreases exponentially with decreasing distance of transection site from axon hillock. (B–D) Sealing of transected proximal and distal stumps by (B) plasmalemmal collapse and fusion, (C) formation of a new plasmalemmal partition, or (D) accumulation of vesicles or other membrane-bound structures that arise by plasmalemmal endocytosis, breakup or budding of the Golgi apparatus, smooth endoplasmic reticulum (SER), adaxonal (non-myelinating) glial membrane, or myelin sheath delaminatyion. Lysosomes and mitochondria can also contribute to the accumulation of membrane bound structures at a hole or complete transection of a cytoplasmic process (axon, muscle fiber, dendrite, *etc.*). These vesicular structures form a vesicular plug and pack tighter and tighter and/or fuse with each other and the plasmalemma (D1) or "wound vesicle" (D2). The vesicles are eventually resorbed and/or integrated to form a continuous membrane with the morphological and barrier characteristics of an undamaged plasmalemma (D3).

ens junctions, gap junctions that have not yet been reported, but also not explicitly searched for (**Figure 2D1**; Bittner and Fishman, 2000; Fishman and Bittner, 2003).

e. Various forms of vesicle-to-vesicle fusion to form larger vesicles or multi-layered vesicles (**Figure 2D**; Ballinger et al., 1997; Eddleman et al., 1997a,b, 1998; Terasaki et al., 1997; Bittner and Fishman, 2000; Togo, 2004; Moe et al., 2015).

f. Exocytosis of vesicles including very large (wound) vesicles that increase the area of the plasmalemma or completely "patch" a hole or transected end in one exocytotic event (**Figure 2D2**; Terasaki et al., 1997; McNeil et al., 2000; Alberts, 2008; Cai et al., 2009).

Mechanisms a and b by themselves might seal a small (< 100 μm) hole, but not larger holes or complete transections (Bittner and Fishman, 2000; Fishman and Bittner, 2003; Moe et al., 2015) - - - but could be used in addition to mechanisms c–f that could seal larger holes or complete transections. Mechanisms a–f are all part of gradual, continuous formation of a barrier formed by a vesicle plug.

We note that one mechanism (**Figure 2D2**: exocytotic patching in one final step by a wound vesicle) has often been proposed to seal large holes or complete transections (Terasaki et al., 1997; McNeil et al., 2000; Alberts, 2008), -- but has much evidence against its actual occurrence. This

proposed mechanism arose from morphological observations that Ca^{2+}-containing sea water is injected deep within the cytoplasm of sea urchin eggs -- or within extruded cytoplasm -- immediately induces a large bilayer vesicle (wound vesicle) to form around the sea water (Terasaki et al., 1997; McNeil et al., 2000). One of the authors (McNeil) has often proposed that such large wound vesicles presumably formed by vesicle-vesicle fusions then seal plasmalemmal holes or complete transections in one final step termed "patching". However, because of lack of supporting data, serious problems exist for wound vesicle patching as an explanation for plasmalemmal sealing in any cell, much less its acceptance as a general mechanism for sealing of traumatic injuries to all eukaryotic cells (Bittner and Fishman, 2000; Eddleman et al., 2003; Fishman and Bittner, 2003): (1) Morphological, confocal, or EM studies have never reported such a large wound vesicle naturally forming at the lesion site in sea urchin eggs, invertebrate giant axons, or any other preparation that all exhibit many vesicles of smaller to larger size packed internally and externally at the lesion site. (2) Functional measures of plasmalemmal sealing (V_m, R_{in}, I_i, tracer molecule exclusion) all report gradual, continuous changes in each measure and not the stepwise sudden change that should occur with a large opening suddenly patched in one step by a wound vesicle in an exocytotic event to seal a plasmalemmal lesion.

In contrast, all morphological and functional measures published to date are consistent with a hypothesis that plugs of membrane-bound substances gradually restore each measure of plasmalemmal sealing (including dye uptake) to control values found in intact membranes (Krause et al., 1994; Eddleman et al., 1997, 1998; Lore et al., 1999; Detrait et al., 2000a, b; Yoo et al., 2004; Nguyen et al., 2005; Spaeth et al., 2012a, b, c; *c. f.* Moe et al., 2015). The formation of more junctional complexes between membranous structures or a tighter packing density of membranous structures would be expected to produce a more restricted diffusion pathway for a given tracer or ionic current. Differences in the nature of the interactions among membranous structures could also partially account for differences in the times for exclusion of the same tracer, *e.g.*, specific dextran-conjugated fluorescent dyes or ions. More specifically, the time course of sealing would be expected to differ for different cells. Based on the concept of a vesicular plug, we predicted, and confirmed the following hypotheses:

Sealing should be much faster (*e.g.* seconds) if cells have an abundance of preformed vesicles located just below the plasmalemma
For example unfertilized and undamaged sea urchin eggs (Steinhardt et al., 1994; Terasaki et al., 1997) seal much faster compared to cells or axons of similar diameter that do not have such cytoplasmic elements in extreme abundance (Bittner and Fishman, 1990; Krause et al., 1994).

Sealing should be faster in smaller cells with smaller lesions *versus* larger cells with larger lesions
Sealing in squid giant axons (Bittner and Fishman, 2000; Fishman and Bittner, 2003) occurs much more slowly than sealing of the tiny, single neurites of B104 cells (McGill et al., 2016) or PC12 cells (Yoo et al., 2004).

Sealing should be faster for cells placed in higher extracellular Ca^{2+} concentrations versus lower concentrations: This result clearly occurs for cells in culture where the extracellular Ca^{2+} concentration can be carefully controlled (Yoo et al., 2003, 2004; Nguyen et al., 2005; Spaeth et al., 2012a; McGill et al., 2016).

Sealing should be faster for higher molecular weight tracers than for lower molecular tracers
This result clearly occurs in B104 cells in culture (Detrait et al., 2000a, b; Yoo et al., 2004) and in crayfish giant axons both *in vivo* and *ex vivo* (Krause et al., 1994; Eddleman et al., 1998, 2000; Detrait et al., 2000a).

In all protocols outlined above, graphs/plots of the extent and or probability of sealing should vary continuously and monotonically with time after initiating Ca^{2+} influx
This result is observed in both *in vitro* (Terasaki et al., 1997; Detrait et al., 2000b; Eddleman et al., 1998, 2000; Yoo et al., 2003, 2004; Spaeth et al., 2010, 2012a, b, c; Zuzek et al., 2013; Moe et al., 2015) and *in vivo* (Krause et al., 1994; Godell et al., 1997; Terasaki et al., 1997; Togo, 2004).

What Are the Evolutionary Origins and Subsequent Uses of These Sealing Proteins and Mechanisms?

Eddleman et al. (1997) and Bittner and Fishman (2000) hypothesized that a very conservative evolution of mechanisms and substances for sealing plasmalemmal damage has occurred in neurons and other eukaryotic cells because plasmalemmal damage was an existential problem faced by early-evolved eukaryotic cells. Hence, the first evolved role of membrane fusion proteins was to seal plasmalemmal damage. Membrane fusion proteins were likely then co-opted in eukaryotic evolution for use in intracellular vesicular trafficking or for exocytosis or for communication among intracellular organelles (*e.g.*, components of the Golgi apparatus). Subsequently, as proposed by Sudhof and Rothman (2009), some of these substances and mechanisms were then co-opted for neurotransmitter release.

The details of this early-evolved mechanism for plasmalemmal repair can now vary with the type of lesion, the configuration of the injured plasmalemma, the type of injured cell and its relation to nearby cells, and the proximity of intracellular membranous structures to the lesion (Eddleman et al., 1997). Nevertheless, there are many common themes: Plasmalemmal damage is repaired by accumulations of hydrophobic membranous structures from any readily available source having substances and/or structures not typically found in intact plasmalemma. The substances/structures in, or portions of, the "temporary-seal membrane" are then gradually removed or replaced with substances/structures in undamaged plasmalemmal membrane once a good functional seal is established (**Figure 2**).

What Data Might Account for Observations that Axonal Transections Nearer to (*versus* further from) the Perikaryon Are Associated with Higher Probabilities of Cell Death?

Neuroscientists and clinicians have known for many decades that perikaryal survival decreases for neurons whose axons or dendrites are transected nearer to the soma, compared to further from the soma (Ramón y Cajal, 1928; Lucas et al., 1985, 1990; Yoo et al., 2004; Nguyen et al., 2005; Campbell, 2008; Wolfe et al., 2010). Ca^{2+} influx at cut axonal ends increases somal Ca^{2+} concentrations that probably lead to the disruption of somal protein synthesis, and trigger various pathways that can lead to cellular apoptosis (Choi, 1988; Tymianski et al., 1993; Ziv and Spira, 1995; Yoo et al., 2004; Nguyen et al., 2005).

As discussed in previous sections, the same Ca^{2+} influx in eukaryotic cells that initiates cell death (if left unchecked) also initiates plasmalemmal sealing that reduces the influx, thereby preventing somal Ca^{2+} concentrations attaining levels producing cell death. Plasmalemmal sealing is a gradual process, that can take 5–15 minutes to complete in hippocampal-derived B104 cells in tissue culture depending in part on axon diameter, which is larger near the axon hillock, (Krause et al., 1994; Yoo et al., 2003; Spaeth et al., 2010) and on which enzymatic pathways are initiated (Spaeth et al., 2010, 2012a, b; Zuzek et al., 2013). Axons transected less than 50 μm from their soma seal more slowly with lower probability versus those transected greater than 50 μm from their somas (Yoo et al., 2004; Nguyen et al., 2005; Spaeth et al., 2010, 2012a, b, c). Longer sealing times, larger diameters, and shorter Ca^{2+} diffusion distances of cells transected nearer to their soma are associated with their increased frequencies of cell death compared to cells transected farther from their soma (Yoo et al., 2004).

McGill et al. (2016) quantified the relationship between sealing frequency, as measured by dye exclusion, and transection distance from the axon hillock. Sealing frequency was maximal for transections ≥ 150 μm from the axon hillock and decreased exponentially from 150 μm with a space constant of about 40 μm. Furthermore, the relationship of sealing frequency versus time was well-fit by a one-phase, rising exponential model having a time constant of several milliseconds that was longer for transections nearer to, versus further from, the axon hillock. All these observations are consistent with the hypothesis that longer sealing times, larger axonal diameters and shorter Ca^{2+} diffusion distances relative to the perikaryon as the site of proteins synthesis are the primary mechanisms responsible for an increased frequency of cell death following axonal transections made nearer to, versus farther from, the cell body of many types of neurons (Yoo et al., 2004; Nguyen et al., 2005; Spaeth et al., 2010, 2012a, b, c).

What Might Be The Clinical Significance of Data on Plasmalemmal Sealing Mechanisms in Neurons and Other Cell Types?

Altering the ability of neurons to seal their plasmalemma

Modulating pathways that affect sealing may have neuroprotective effects. For example, damage-induced increases in $[cAMP]_i$ leading to increased activation of cAMP-dependent proteins (PKA, Epac) enhances plasmalemmal sealing *in vitro* (**Figure 1**) and *ex vivo* (**Figure 2C**, **D**) -- and therefore almost certainly *in vivo*. Furthermore, both PKA and Epac increase survival and axonal outgrowth following injury, even in non-permissive growth substrates of the adult central nervous system (Cai et al., 2001), possibly by first ensuring rapid plasmalemmal repair. Increasing plasmalemmal sealing decreases the adverse effects of treadmill climbing in normal mice and in a mouse model of muscular dystrophy (Bansal and Campbell, 2004). Therefore, a greater understanding of how PKA, Epac and other pathways affect sealing could improve our understanding of how these molecules are involved in neuronal survival following traumatic nerve injury.

Furthermore, in contrast to natural sealing of a damaged plasmalemma, PEG rapidly (<< 1 second) and directly "artificially" seals ("PEG-seals") plasmalemmal damage *in vitro* and *in vivo* and does not utilize any previously reported pathways for membrane fusion in eukaryotic cells (Spaeth et al., 2010, 2012a, b, c). Data on PEG and other substances that promote or reduce axolemmal repair have been used to develop and improve a well-defined sequence of bioengineered solutions in combination with micro-sutures to reconnect (PEG-fuse) the distal and proximal ends of transected PNS axons. Such PEG-fused axons that were completely transected can rapidly (within days) and at times nearly completely, restore lost behavioral functions (Bittner et al., 2012, 2015b; Ghergherehchi et al., 2015; Riley et al., 2015), largely because transected distal portions of PEG-fused axons do not undergo Wallerian degeneration.

Plasmalemmal sealing, Wallerian degeneration, and PEG

Distal or otherwise anucleated portions of transected axons in mammals and birds degenerate in 3–6 days (Wallerian degeneration). In contrast, some axons in amphibians and fish, and many axons in most invertebrate phyla, then often survive for months to years (for reviews, see Bittner, 1991; Bittner et al., 2000, 2015b). The distal portions of transected mammalian axons seal according to the same time course and substances that affect sealing of other membranes in all eukaryotes studied to date (Spaeth et al., 2012a; Bittner et al., 2012, 2015b; Ghergherehchi et al., 2015). That is, Wallerian degeneration in mammals is not due to an inability of the axolemma to seal or to prevent Ca^{2+} influx. Rapid Wallerian degeneration is also not due *per se* to higher mammalian body temperatures (compared to "lower" vertebrates) because severed distal axons in several strains of mutant mice do not degenerate for many weeks (Lunn et al., 1989; Tsao et al., 1999). Rather, it is possible that rapid Wallerian degeneration of mammalian axons is an active process due to phagocytosis by Schwann cells and macrophages, perhaps due to loss of an inhibitory substance supplied by fast transport in intact axons. Cooling severed distal stumps or injections of cyclosporine retard Wallerian degeneration, perhaps by slowing phagocytosis (Sea et al., 1995; Sunio and Bittner, 1997). Successful PEG-fusion of a distal or even allografted segment prevents their Wallerian degeneration (Riley et al., 2015; Bittner et al., 2015b), perhaps because fast transport and/or a phagocytic inhibitory signal are rapidly restored.

PEG-fusion protocols that rapidly connect proximal and distal portions of severed axons could produce a paradigm shift in clinical treatments of traumatic injuries to peripheral nerves. The ability to PEG-fuse severed axons in autografts and allografts to rapidly restore behavioral deficits might dramatically alter functional outcomes for patients with mutilated extremities or oncologic resections. For example, the long-term function of a salvaged, but denervated hand is especially dismal and limb amputation often occurs if a segment of a major nerve is lost (Campbell, 2008; Wolfe et al., 2010). PEG-fusion technologies might also repair spinal lesions in which the primary deficit is severance of spinal axons in dorsal, ventral and/or lateral columns of white matter (Bittner et al., 2015a).

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