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Sealing frequency of B104 cells declines exponentially with decreasing transection distance from the axon hillock

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Abstract

Transection of nerve axons (axotomy) leads to rapid (Wallerian) degeneration of the distal portion of the severed axon whereas the proximal portion and the soma often survive. Clinicians and neuroscientists have known for decades that somal survival is less likely for cells transected nearer to the soma, compared to further from the soma. Calcium ion (Ca^{2+}) influx at the cut axonal end increases somal Ca^{2+} concentration, which subsequently activates apoptosis and other pathways that lead to cell death. The same Ca^{2+} influx activates parallel pathways that seal the plasmalemma, reduce Ca^{2+} influx, and thereby enable the soma to survive. In this study, we have examined the ability of transected B104 axons to seal, as measured by uptake or exclusion of fluorescent dye, and quantified the relationship between sealing frequency and transection distance from the axon hillock. We report that sealing frequency is maximal at about 150 μm (μm) from the axon hillock and decreases exponentially with decreasing transection distance with a space constant of about 40 μm . We also report that after Ca^{2+} influx is initiated, the curve of sealing frequency versus time is well-fit by a one-phase, rising exponential model having a time constant of several milliseconds that is longer nearer to, versus further from, the axon hillock. These results could account for the increased frequency of cell death for axotomies nearer to, versus farther from, the soma of many types of neurons.

Keywords

Axotomy; Axolemmal sealing; B104 cells; Calcium influx; Vesicle-mediated repair; Membrane repair

1. Introduction

Neuroscientists and clinicians have known for many decades that transection of dendrites or axons can initiate a Ca^{2+} influx that leads to perikaryal death (Ramón y Cajal, 1928; Schlaepfer and Bunge, 1973; Lucas et al., 1985, 1990; Loewy and Schader, 1977; Yoo et al., 2004; Nguyen et al., 2005; Campbell, 2008; Wolfe et al., 2010; Moe et al., 2015). This influx increases somal Ca^{2+} concentrations, activating calpains and other proteases in

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enzymatic pathways that induce apoptosis or other mechanisms of cell death (Choi, 1988; Tymianski et al., 1993; Ziv and Spira, 1995; Yoo et al., 2004). Cell death occurs more frequently for cells transected nearer to, compared to further from, their cell body (Ramón y Cajal, 1928) due to increased concentrations of somal Ca^{2+} that probably disrupt somal protein synthesis (Yoo et al., 2004; Nguyen et al., 2005).

The same Ca^{2+} influx in all eukaryotic cells that initiates cell death (if left unchecked) also initiates plasmalemmal sealing, which reduces this continuing influx (Krause et al., 1994; Steinhardt et al., 1994; Spaeth et al., 2010, 2012a, 2012b, 2012c; Moe et al., 2015) and thus prevents somal Ca^{2+} concentrations from reaching levels that produce cell death. Ca^{2+} influx activates sealing through multiple parallel and redundant enzymatic pathways that induce vesicles to form and/or migrate to the lesion site where they create a vesicular plug that seals the membrane and reduces Ca^{2+} influx to levels seen in uninjured eukaryotic cells (Krause et al., 1994; Miyake and McNeil, 1995; Eddleman et al., 1997; Blanchette et al., 1999; Jimenez et al., 2014).

Plasmalemmal sealing is a gradual process, taking 10–30 min to complete 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, 2012b, 2012c; Zuzek et al., 2013). Axon-like neurites transected less than 50 μm from their soma seal more slowly at lower frequencies, and are associated with a higher frequency of cell death than those transected greater than 50 μm from their somas (Yoo et al., 2004; Nguyen et al., 2005; Spaeth et al., 2010, 2012a, 2012b, 2012c). The longer sealing times, larger diameters, and shorter Ca^{2+} diffusion their increased frequencies of cell death compared to cells transected farther from their soma (Yoo et al., 2004).

Although the mechanism(s) of plasmalemmal sealing and cell death after neurite transection and the consequences for transections qualitatively nearer vs. further from the perikaryon have been described in some detail (Spaeth et al., 2010, 2012a, 2012b, 2012c), the quantitative nature of the relationship between transection distance vs frequency of plasmalemmal sealing has not been determined. In this article, we have examined quantitative relationships for axon-like neurites of rat B104 cells transected from 2.5 μm to over 400 μm from the axon hillock at 2.5 min to 20 min post- Ca^{2+} addition times (PC times). We report that sealing frequency vs transection distance at any given PC time is best fit by a one phase exponential model that decreases with a space constant (λ) of about 40 μm from a maximum value about 150 μm from the axon hillock to near-zero at the axon hillock. The relationship between sealing frequency and PC time is best fit by a one phase rising exponential model with a time constant (τ) of about 3.6 min for tran-sections <50 μm from the axon hillock and about 2.2 min for transections > 50 μm from the axon hillock.

2. Methods

2.1. Cell culture

B104 cells, a rat hippocampal neuroblastoma line, were cultured as previously described (Spaeth et al., 2010, 2012a, 2012b, 2012c; Zuzek et al., 2013). Unlike PC-12 cells or other commonly used neuronal cell lines, B104 cells do not require the addition of nerve growth

factor or other supplements to differentiate. B104 neurites are bipolar and have many properties typical of axons such as action potentials, smooth endoplasmic reticulum, and neurotransmitter release. After 24 h of differentiation, axons of B104 cells can vary from a few μm to over 500 μm in length, with most ranging from 20 μm to 150 μm long. B104 cells have often been used as an in vitro model system to study neuronal function (Bottenstein and Sato, 1979; Toda et al., 1999; Yoo et al., 2003, 2004; Nguyen et al., 2005; Miller et al., 2006).

B104 cells were grown in 75 cm^2 vented cap, tissue culture flasks containing 1:1 Dulbecco's Modified Eagle's Medium and Ham's F12 supplement with 10% fetal bovine serum in a humidified incubator that was maintained at 37 °C, 5% CO_2 . This growth medium was replaced every two days until the flask reached 80% confluency. Cells were then either subcultured into a new flask or seeded at a density of 5000 to 15,000 cells/ cm^2 on poly D-lysine coated (5 $\mu\text{g}/\text{ml}$) cell culture plates (60 $\times$ 15 mm, Falcon). The next day, plated cells were differentiated in serum-free 1:1 Dulbecco's Modified Eagle's Medium and Ham's F12 supplement for 24 h. Stock populations of B104 cells were subcultured at most 35 times to mitigate the effects of senescence.

2.2. Axon transection and microscopy

Glass knives were created by pulling 1 mm diameter microcapillary tubes with a micropipette puller (Sutter Instrument CO, USA), and breaking the pipettes at their tips. The glass knives were mounted on a micromanipulator (Narishige Instruments) attached to a Zeiss ICM-405 phase contrast microscope. Cell culture plates containing 100,000 to 300,000 differentiated B104 cells were gently rinsed twice and filled with Ca^{2+} and Mg^{2+} free Dulbecco's phosphate buffered saline (DPBS $^{-/-}$, Hyclone). To transect B104 axons, a cell culture plate was placed on the microscope stage and the glass knife was lowered onto the surface of the plate using the micromanipulator. The microscope stage was moved parallel to the knife's edge to transect some B104 axons, etching a visible "transection line" on the surface of the plate. Axons of 500–800 cells were transected on each plate within 10 min after adding the DPBS $^{-/-}$. Note that most cells on a plate were *not* transected.

After transection, DPBS $^{-/-}$ medium was replaced with DPBS medium containing Ca^{2+} and Mg^{2+} (DPBS $^{+/+}$) to initiate the sealing process, as previously reported [The addition of Ca^{2+} to the extracellular medium initiates plasmalemmal sealing in all eukaryotic cells (Krause et al., 1994; Blanchette et al., 1999; Detrait et al., 2000a, 2000b; Yoo et al., 2003; Spaeth et al., 2010)]. Cell culture plates containing transected B104 axons were returned to the CO_2 incubator and allowed to sit for either 2.5, 3.75, 5, 10, 15, or 20 min post DPBS $^{+/+}$ addition, i.e., for 2.5–20 min post Ca^{2+} addition times (PC times: Spaeth et al., 2010). At each given PC time, the dye-free DPBS $^{+/+}$ medium was replaced with DPBS $^{+/+}$ medium containing 9 nM Dextran conjugated Texas Red (3 kDa, Molecular Probes, D3328). After 15 min, the DPBS $^{+/+}$ containing Texas Red Dextran was washed out with dye-free DPBS $^{+/+}$.

B104 cells were observed with a Zeiss Axio Vert.A1 microscope fitted with phase contrast and fluorescent optics, an excitation/emission filter of 595/615, a Zeiss AxioCam MRm camera and an eyepiece reticle (Klarmann Rulings, KR-407). Representative images of transected cells were acquired and their brightness and contrast were adjusted using Adobe

Photoshop to enhance visibility. Images of the eyepiece reticle were also acquired using an iPhone 6S camera.

Individually-identified cells with axons that intersected a transection line or stopped near the transection line and aligned with an anuclear, distal axonal segment on the opposite side of the transection line were counted as transected. Cells transected more than once were not counted. The fluorescent state, a Yes/No value, of each uniquely-identified transected cell was recorded. The distance between the transection site and the axon hillock for each cell was recorded using the eyepiece reticle in 2.5 μm increments. We measured transection distance from the axon hillock because the rough endoplasmic reticulum in axons does not extend beyond this point, and other landmarks (e.g. nucleus) had varied locations in different cells. Curved axons or axons that extended outside the microscope field of view were measured in sequential segments, using membrane irregularities and cellular debris as markers. Typically, 60–120 cells could be counted on each plate in 30–40 min before photo-bleaching began to affect dye detection.

2.3. Analyses of sealing frequency versus transection distance

To determine sealing frequencies at any given PC time, cells were grouped by transection distance into different bins using various “binary” or “multinary” methods, and the frequency of cells that excluded Texas Red in each bin was calculated. For the binary groups, B104 cells were grouped into “transected $\leq 50 \mu\text{m}$ or $> 50 \mu\text{m}$ from the axon hillock. For the multinary groups, various methods (see Results) were used to generate a set of bin widths starting at $0 \mu\text{m}$ from the axon hillock. An outlier test was applied to both multinary and to binary data sets: Any cell culture plate with transected cells exhibiting a sealing frequency more than three standard deviations from the weighted mean for that given transection range and counting method was excluded from analysis. In total, 4 of 120 plates were excluded.

2.4. Statistical analyses

The relationship between transection distance (x axis) and sealing frequency (y axis) was characterized at each PC time by fitting a one-phase exponential model described by the equation $Y = Y_0 + Y_{\text{max}} * e^{-x/\lambda}$, where Y_0 is the Y-intercept, Y_{max} is the maximum sealing frequency for that PC curve, x is transection distance, and λ is the space constant (the distance required for the predicted sealing frequency to reach 63% of the plateau). Linear regressions were applied to the same data set as an alternative hypothesis. The F-test for regression and Akaike’s Information Criterion (AIC) were used to compare the goodness of fit of different curves for exponential and linear models.

The effects of PC time on sealing frequency were identified by comparing data at each PC time in a pairwise fashion using two-tailed Cochran–Mantel–Haenszel Chi-Squared (CMH X^2) tests for independence. These tests integrate multiple 2×2 X^2 analyses that differ by a nominal replicate variable to calculate a significant difference between sealing frequencies. The independent and dependent variables used in the 2×2 comparisons were PC time and dye uptake; the replicate variable differentiating the comparisons were transection distance bins. The relationship between PC time and sealing frequency was investigated by fitting

one-phase exponential and linear regression models to the sealing frequencies of cells in each 12-width optimized transection distance bins at different PC times. In the one-phase exponential model, τ is the time required for the predicted sealing frequency to reach 63% of the plateau. The F-test for regression was used to compare the goodness of fit of different curves for exponential and linear models.

To compare multinary data to previously reported binary data (Spaeth et al., 2010; Zuzek et al., 2013), measurements collected using the multinary method in the present study were transformed to mimic the way data were collected using the binary method. The binary sealing frequency of cells transected $<50 \mu\text{m}$ and $50 \mu\text{m}$ from their hillocks were calculated from the multinary data and used for comparison against the sealing frequency of $<50 \mu\text{m}$ and $50 \mu\text{m}$ binary transection data as previously reported. One-phase exponential models were fit to plots of PC time vs sealing frequency plots created by this multinary to binary transformation. CMH X^2 tests were used to compare the curves, using PC time as the replicate variable, and individual data points were compared with Fisher's exact test.

3. Results and discussion

3.1. Assessing transected axon length and dye uptake for B104 cells

Fig. 1 shows B104 cells with transected (Fig. 1A,D–I) or intact (uncut) axons (Fig. 1B,C) after treatment with Texas Red Dextran at 10 min PC and subsequent washing with DPBS +/- (see Methods). Images of the same cell(s) were taken using both phase contrast (Fig. 1A,B,D,F,H) and fluorescent imaging (Fig. 1C,E,G,I). Transection lines (asterisks) made by the glass knives are clearly visible under both phase contrast and fluorescent microscopy.

Fig. 1A shows a typical transected B104 cell located below the eyepiece reticle having shorter vertical lines $2.5 \mu\text{m}$ apart and longer lines $25 \mu\text{m}$ apart. The transection site is marked by a thick arrow in this and all other panels. The axon hillock is the point where the axon inflects between a mostly constant to increasingly thicker width, and is marked by arrowheads in this and all other panels. The nearest and farthest edges of the nuclear membrane from the transected axon are marked with thin arrows. The authors' measurements of the distance between this cell's transection site and axon hillock varied from 30 to $35 \mu\text{m}$ ($33.5 \pm 2 \mu\text{m}$ Standard Deviation, $n = 4$) and the nearest edge of the nuclear membrane to the transection site from 55 to $60 \mu\text{m}$ ($57 \pm 1.75 \mu\text{m}$). This variation in measuring transection distance introduces higher percentage error at distances nearer to than further from the axon hillock. If sealing frequency is related to transection distance, a $5 \mu\text{m}$ error at a $10 \mu\text{m}$ transection distance would produce a greater error in sealing frequency than a $5 \mu\text{m}$ error at $150 \mu\text{m}$.

Fig. 1B shows an untransected cell with an intact plasmalemma viewed with phase contrast that did not take up the membrane impermeable fluorescent dye, Texas Red Dextran, as viewed with fluorescence microscopy (Fig. 1C). The white dotted lines in this and other panels outline each cell as viewed under phase-contrast. Fig. 1D shows a transected cell in phase contrast that took up Texas Red Dextran (Fig. 1E), i.e., the cell did not seal its cut, open end. Fig. 1F shows a transected cell in phase contrast and fluorescent imaging (Fig. 1G) that did not take up dye, i.e., sealed its cut end. The transection line occasionally cut

B104 cell somas directly (Fig. 1H,I). Some of these cells sealed their extensively damaged plasmalemma and did not take up dye added at 10 min PC (Fig. 1I).

3.2. Controls for dye uptake and photobleaching

To control for the possibility that some uncut cells took up Texas Red Dextran, control plates of differentiated B104 cells were prepared like other 10 min PC plates, but these cells were not transected. Of the 1533 cells counted on these control plates, 6 cells (0.4%) fluoresced. Uncut cells took up Texas Red Dextran so rarely they did not significantly affect any results. These quantitative data confirmed qualitative observations in other publications that undamaged cells did not take up membrane impermeable fluorescent dyes (Steinhardt et al., 1994; Ballinger et al., 1997; Spaeth et al., 2010).

To test for the possibility that photobleaching affected results, cells were grouped into quartiles of time according to the order they were counted for each plate. The sealing frequencies of cells in each plate were calculated for each author for each quartile, and a two-tailed paired t-test was used to compare the sealing frequencies of each quartile. The sealing frequency of cells in the fourth quartile ($68.6 \pm 16.9\%$, $n = 715$) of one author (but no other author) was borderline significantly higher than their first quartile ($61.9 \pm 15.8\%$, $n = 725$; $p = 0.052$), a result consistent with photobleaching. Hence, the fourth quartile of all observations made by this author were removed.

To further control for photobleaching, the fluorescence states of identified transected cells on 10 min PC plates were noted and counted for 10 min. The plate was then indirectly illuminated for 40 min. Afterwards, the originally transected cells were identified and their fluorescence state again noted. The status of all the originally identified transected cells were unchanged, although the cells fluoresced more dimly.

3.3. Multinary grouping methods for transected B104 cells

To plot the relationship between transection distance and sealing frequency at 10 min PC, data from B104 cells were grouped by transection distance into bins with uniform widths of $2.5 \mu\text{m}$ (Fig. 2A: the reticle's smallest measurement), $10 \mu\text{m}$ (Fig. 2B), or $20 \mu\text{m}$ (Fig. 2C). Note in Fig. 2B–H that horizontal bars associated with each data point show the upper and lower bounds in μm of that bin. No bars are displayed in the $2.5 \mu\text{m}$ grouping method because the width of the bin is equal to the width of the symbols. The mean transection distance for the cells contained in any bin, shown by the open circles, usually does not equal the midpoint of the bin. For example, the mean transection distance for all the cells in the bin from 0 to $20 \mu\text{m}$ in Fig. 2C is $12.49 \mu\text{m}$, not $10 \mu\text{m}$.

The sealing frequency of adjacent $2.5 \mu\text{m}$ bins often showed large increases or decreases, almost certainly due to small numbers of cells ("sampling number") in a given bin. For example in Fig. 2A, the $2.5 \mu\text{m}$ -wide bin for 202.5 to 205 μm contained only two cells with a sealing frequency of 100%. Other nearby bins had 0–4 cells and sealing frequencies between 0 and 100%. Generating bins with $10 \mu\text{m}$ (Fig. 2B) or $20 \mu\text{m}$ (Fig. 2C) widths increases the sampling number per bin and generally reduces sampling variation at all transection distances. However, since axons of most B104 cells were $\leq 150 \mu\text{m}$ long, bins at transection distances $\geq 150 \mu\text{m}$ often continued to have much variation due to small sampling number.

For example, the 10 μm bin from 200 to 210 μm contains 13 cells (Fig. 2B), the 20 μm bin (Fig. 2C) from 200 to 220 μm contains 21 cells, and one cell from 400 to 420 μm . In contrast, 10 μm and 20 μm bins between 0 and 60 μm from the axon hillock each contain over one hundred cells each.

As two alternative methods, data were grouped into 19 or 12 transection distance bins with widths that increased with transection distance. Starting from the axon hillock, the 19 width-optimized group (Fig. 2D) had twelve bins of 5 μm width, two of 10 μm , one of 20 μm , two of 25 μm , one of 100 μm , and one bin containing all the remaining cells >240 μm from the axon hillock. The 12 width-optimized group (Fig. 2E) starting from the axon hillock had two bins of 5 μm width, five of 10 μm , two of 20 μm , two of 25 μm , and one bin containing all the remaining cells 150 μm from the axon hillock. The variation in sealing frequency between adjacent bins was greatly reduced in these “width-optimized” bins compared to the uniform-width bins. The 19 width-optimized bin algorithm sometimes produced bins with sampling numbers equal to or less than thirty at 10 min PC (A sampling number of 30 is the standard minimum for statistical tests). The 12-width optimized algorithm had higher sampling numbers at all PC times, and thus less variation between adjacent bins compared to the 19-width optimized method. The bin with the smallest sampling number at all PC times extended from 125 to 150 μm at 15 min PC, and included 18 cells.

Because all these methods sometimes produced bins with sampling numbers less than 30, a “post-hoc” grouping algorithm (Fig. 2F) was developed. This algorithm repeatedly extended bins from the furthest to nearest transection distance, ensuring that each bin contained at least 40 cells by adjoining adjacent bins that failed to reach 40 cells. These plots produced similar amounts of variation to the 12 width-optimized plots at all PC times.

As a final type of multinary grouping method, an “n-optimized” grouping algorithm was developed. This algorithm used a computer optimization program that altered the boundary of each bin to minimize sampling size variation across all bins at each PC time. The algorithm was applied using 20 bins (Fig. 1G), and 10 bins (Fig. 1H). The 20 bin n-optimized method had low sampling numbers at PC times other than 10 min, and variation similar to the 19 width optimized method. The 10 bin n-optimized method produced plots with similar amounts of variation to the post-hoc grouping algorithm and the 12 width optimized algorithm, and created bins with sampling numbers greater than 40 at all PC times.

We fit linear and one-phase exponential models to the 10 min PC data. The R^2 (coefficient of determination) of the linear model (not shown) varied from 0.071 to 0.623. The R^2 of the one-phase exponential model was higher for each grouping method, varying from 0.329 to 0.972. The F-test of regression showed that the one-phase exponential model was a better fit regardless of grouping method ($p < 0.01$). AIC tests (see methods) similarly indicated that the one-phase exponential model was a better fit compared to the linear model (AIC relative likelihood >97%). The R^2 in the linear model changed significantly with grouping method, but the curves generated by the exponential model were weakly affected by grouping method (Fig. 1I). Each curve lay within the 95% confidence band of every other curve. Because the 12-width optimize model featured variation and goodness of fit comparable to

the best of any other method and was simple to apply, further analyses were limited to this grouping method for which many results are listed in Table 1.

3.4. Sealing frequency and transection distance at different PC times

Figs. 3 and 4 show the mean sealing frequencies of B104 axons transected at 2.5 min PC, 3.75 min PC, 5 min PC, 10 min PC, 15 min PC, and 20 min PC plotted using the 12 bin-width optimized algorithm. As for Fig. 2, the horizontal bars in Fig. 3 represent the upper and lower boundaries of each bin and the open circles show the mean transection distance of all cells in each bin. The gray arrows point to bins with sampling numbers between 30 and 40. The black arrows point to bins with sampling numbers less than 30. At all PC times, the sealing frequency of all curves started at a plateau value at about 150 μm and decreased exponentially with decreased transection distance to a value that was greater than 0 (Y intercept). Because of the non-linear relationship, changes in transection distance nearer the axon hillock have a greater effect on sealing frequency compared to changes further from the axon hillock (Figs. 3,4).

Linear and one-phase exponential models were applied to the datasets shown in Figs. 3 and 4. The F-test for regression and the AIC test showed that the one-phase exponential model was a better fit compared to the linear model (Table 1). All of the plateau sealing values (p in Fig. 3) increased or remained constant with increased PC time, except for 3.75 min PC. The λ (Table 1; defined in Methods) did not show a consistent relationship with PC time. The predicted sealing frequency of the models at zero transection distance (Y_0 in Fig. 3) for the one-phase exponential model were not significantly different from zero at 2.5 or 3.75 min PC, but were significantly different from zero at all other times (Table 1). The Y-intercepts increased with increasing PC time. The exponential model thus predicts that B104 cells should seal at longer PC times if transected at the axon hillock, as we have observed (Fig. 1H,I).

Fig. 4 shows sealing frequency vs transection distance for six PC times. CMH X^2 tests were used to compare entire curves in a pairwise fashion. Each curve was usually significantly different from every other curve (12/15 comparisons; $p < 0.01$ in each case. See Table 2). The three curves not significantly different from each other were the 2.5 vs 3.75 min PC curves (Fig. 3A,B; $p = 0.989$), the 5 vs 10 min PC curves (Fig. 3C,D; $p = 0.196$), and the 10 vs 20 min PC curves (Fig. 3D,F; $p = 0.058$). Among the comparisons that produced significant differences, in nearly every case (11/12 comparisons), the curve at the higher PC time had a higher sealing frequency than the curve at the lower PC time. The one exception was that the sealing frequency for the 15 min PC curve was significantly ($p < 0.001$) higher than the sealing frequency for the 20 min PC curve (Fig. 3E,F). Thus, as a general trend, sealing frequency increased with PC time (Fig. 4).

All these curves show that sealing frequency decreases exponentially from a maximum plateau value at about 150 μm from the cell body to a Y intercept greater than zero. The λ characterizing the decay of sealing frequency varied from 25 to 50 μm at different PC times with no obvious trend relating PC time to λ .

3.5. PC time and sealing frequency

The sealing frequency of each 12 width optimized bin was plotted against six PC times (Fig. 5A–F). Note that in Fig. 5A–D three bins are plotted per panel, e.g., 0–5 μm , 30–40 μm , and 80–100 μm bins in Fig. 5A. In Fig. 5E and F, all 12 bins are plotted and labeled 1–12 according to their increasing distance from nearest to furthest from the axon hillock. A linear model (dotted lines) or one-phase exponential model (solid lines) were fit to the data (Fig. 5A–F). An F-test for regression showed there was no significant difference between the goodness of fit of the linear and one-phase exponential models vs transection distance at any specific PC time.

($p > 0.05$ in each case). However, the R^2 values of the one-phase exponential model were always greater than the R^2 values of the linear models for each transection distance bin. Such a result is highly significant (2×2 Chi-Squared test), suggesting that the relationship between sealing frequency and PC time is best fit by a one-phase exponential model, as previously used (Spaeth et al., 2010, 2012a, 2012b, 2012c), although they did not compare their goodness of fit to other models.

Table 3 shows the plateau sealing frequency, Y-intercept (Y_0) sealing frequency, and time constant (τ) for each of the curves in Fig. 5F alongside their ordinal ranks in parentheses, listed 1–12 from nearest to furthest (Bin#) or lowest to highest (Plateau, Y_0 , and τ). There is a strong relationship between the midpoint of each bin and the plateau sealing frequency of each bin's curve (Fig. 6A, $R^2 = 0.954$). Similarly, a significant correlation was found between bin number the ordinal rank of their plateau sealing frequencies (Fig. 6B, $p = 0.001$). There is also a strong relationship between the Y_0 sealing frequency of each bin's curve and the midpoint of the curve's transection distance bin (R^2 of 0.574, $\lambda = 54.25 \mu\text{m}$), and a significant correlation between Y_0 ordinal rank and bin number ($R^2 = 0.584$, $p = 0.004$). Cardinal or ordinal plots of τ vs. bin # showed a (non-significant, $p > 0.05$) trend from nearest to farthest bin in each case.

3.6. Comparing current multinary to previous binary data

To assess how our multinary data compared to previously published binary data, we transformed our multinary data into binary data by grouping B104 cells into two transection distance bins, $<50 \mu\text{m}$ and $\geq 50 \mu\text{m}$ from the axon hillock, and calculated the sealing frequency for each binary bin for each of six PC times. Our “transformed binary” sealing frequency and the binary data sealing frequency reported by Spaeth et al. (2010) and Zuzek et al. (2013) were plotted on the same panels, and one-phase exponential models with $Y_0 = 0$ were fit to each data set (Fig. 7A,B).

As Fig. 7A illustrates, our transformed binary PC time vs sealing frequency curve for cells transected $<50 \mu\text{m}$ from their axon hillocks was significantly different from the curve reported by Spaeth et al. (2010; $p < 0.001$, CMH χ^2 test). Similarly, our transformed binary curve for cells transected $\geq 50 \mu\text{m}$ from their axon hillocks (Fig. 7B) was also significantly different from both the curves reported by Spaeth et al. (2010; $p < 0.001$) and the curve reported by Zuzek et al. (2013; $p < 0.01$). Furthermore, the curves reported by Spaeth et al.

(2010) and Zuzek et al. (2013) for cells transected 50 μm from the hillocks (Fig. 7B) were significantly different from each other ($p < 0.001$).

The τ for our PC time vs sealing frequency curve of cells transected 50 μm from their axon hillocks was 2.20 min (Fig. 7B), a value very similar to the 2.16 min τ reported by Spaeth et al. (2010) and the 2.79 min τ reported by Zuzek et al. (2013). Our PC time vs sealing frequency curve of cells transected 50 μm featured a τ of 3.55 min (Fig. 7A). This value is less than the 6.54 min reported by Spaeth et al. (2010), but in agreement with Spaeth et al. (2010) this value is greater than the τ for cells transected 50 μm from their axon hillocks.

We used Fisher's exact test to assess differences between our transformed binary sealing frequencies and the binary sealing frequencies previously reported by Spaeth et al. (2010) or Zuzek et al. (2013) of cells transected $<50 \mu\text{m}$ (Fig. 7C) or 50 μm (Fig. 7D) from their axon hillock at specific PC times. There was no significant difference between the sealing frequency of our $<50 \mu\text{m}$ transformed multinary data and the $<50 \mu\text{m}$ binary data reported by Spaeth et al. (2010; Fig. 7C) at either 2.5 min PC ($p = 0.21$) or 5 min PC ($p = 0.28$).

However, our $<50 \mu\text{m}$ transformed binary data had significantly lower sealing frequency than data reported by Spaeth et al. (2010; Fig. 7C) at both 10 min PC ($p < 0.001$), and 20 min PC ($p < 0.001$).

There was no significant difference between our transformed binary 50 μm sealing frequencies reported and those reported by Zuzek et al. (2013; Fig. 7D) at 5 min PC ($p = 0.18$) or 10 min PC ($p = 0.37$). However, our 50 μm binary sealing frequencies were significantly higher than those reported by Zuzek et al. (2013; Fig. 7D) at 2.5 min PC ($p < 0.05$), and significantly lower at 20 min PC ($p < 0.001$). There was no significant difference between our 50 μm binary data and that of Spaeth et al. (2010; Fig. 7D) at 2.5 min PC ($p = 1.0$). Our 50 μm binary sealing frequencies were significantly lower than those of Spaeth et al. (2010; Fig. 7D) at 5 min PC ($p < 0.001$), 10 min PC ($p < 0.001$), and 20 min PC ($p < 0.001$). There was no significant difference between the 50 μm binary sealing frequencies reported by Spaeth et al. (2010) and those reported by Zuzek et al. (2013) at 2.5 min PC ($p = 0.165$), and 20 min PC ($p = 0.087$). However, the 50 μm binary sealing frequencies reported by Spaeth et al. (2010) were significantly higher than those reported by Zuzek et al. (2013) at 5 min PC ($p < 0.001$), and 10 min PC ($p < 0.05$).

The differences between the sealing frequencies or τ presented herein vs those reported by Spaeth et al. (2010) or Zuzek et al. (2013) may be due to differences in experimental protocol. Spaeth et al. (2010) transected selected, individual B104 cells using small, targeted cuts, while in Zuzek et al. (2013) and this paper, cells were cut randomly using long, straight line cuts. Randomly cutting cells provides higher sampling numbers but a wider variation in transection distances than selecting cells and aiming to produce a more limited range of transection distances. Additionally, Zuzek et al. (2013) performed transections in the presence of a fluorescein-conjugated dextran, then assessed PC sealing by adding a second dye, Texas red conjugated dextran. In contrast, Spaeth et al. (2010) and our study used a single-dye technique. Charge differences between fluorescein, an anion, and Texas Red, a zwitterion, could introduce errors in identifying transected cells (Eddleman et al., 1998).

Resolution differences may also have created differences in measurements of transection distance.

4. Conclusion

Membrane sealing and cellular survival following axon transection depends on Ca^{2+} induced membrane barrier formation via vesicle generation, trafficking, and fusion near the injury site (Krause et al., 1994; Steinhardt et al., 1994; Yoo et al., 2003, 2004; Nguyen et al., 2005; Spaeth et al., 2010, 2012a, 2012b, 2012c; Jimenez et al., 2014; Moe et al., 2015). The results presented herein consistently show that neuronal sealing frequencies are significantly influenced by the exact distance of the transection site from the axon hillock and soma. For B104 cells, sealing frequency decreases in a one phase exponential with a λ of about 40 μm from a maximum plateau value at about 150 μm from the axon hillock. The relationship between sealing frequency and PC time is a rising one phase exponential with a τ of 3.6 min nearer to the axon hillock and 2.2 min further from the axon hillock. These data confirm qualitative observations made for decades (Ramón y Cajal, 1928; Lucas et al., 1985, 1990) and more recent semi-quantitative observations (Spaeth et al., 2010, 2012a, 2012b, 2012c). Our data provide a more precise relationship between transection distance from the axon hillock, time, and sealing frequency.

All these observations are consistent with a hypothesis that longer sealing times, larger axonal diameters and shorter Ca^{2+} diffusion distances to the soma as the site of proteins synthesis are the cellular mechanisms responsible for the increased frequency of cell death for transections nearer to, vs farther from, the soma (Yoo et al., 2004; Nguyen et al., 2005; Spaeth et al., 2010, 2012a, 2012b, 2012c). Using data presented herein, the mechanisms underlying the relationships between transection distance, sealing frequencies, calcium influx and cell death can be more accurately tested.

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Abbreviations:

AIC	Akaike's Information Criterion
Ca^{2+}	calcium ion
CMH χ^2	Cochran–Mantel–Haenszel Chi-Squared (test)
DPBS	Dulbecco's phosphate buffered saline
DPBS+/+	Ca^{2+} and Mg^{2+} containing DPBS
DPBS–/–	Ca^{2+} and Mg^{2+} free DPBS
μm	micrometers
min	minutes

n-optimized	number of cells/bin optimized
PC times	post-Ca ²⁺ addition times
R²	coefficient of determination
λ	space constant
τ	time constant

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Fig. 1. Transected (A,D–I) or intact B104 cells (B,C) in phase contrast (A,B,D,F,H) or fluorescence (C,E,G,I) microscopy. Eyepiece reticle (A) measures transection site (thick arrows in all panels) of a cut B104 axon 33.5 μ m from its axon hillock (arrow heads in all panels). Nuclear membrane edges are marked with thin arrows. Asterisk identifies transection line. Dotted lines outline the B104 cells in fluorescent images (C,E,G,I). Unsealed cell (E) took up Texas Red Dextran and the sealed cell (G) does not.

Fig. 2.

Plots of B104 cell sealing frequency (%) vs transection distance (μm) at 10 min PC for different grouping methods (see text). Grouping methods are: (A) 2.5 μm bins, (B) 10 μm bins, (C) 20 μm bins, (D) 19 width-optimized bins, (E) 12 width-optimized bins, (F) post-hoc grouping algorithm bins, (G) 20 N-optimized algorithm bins, (H) 10 N-optimized algorithm bins. (I) Best-fit one-phase exponential curves for all grouping methods (A–H); the curves lie within each other's 95% confidence bands. Open circles plot average (mean) sealing frequency vs average transection distance for each bin. Horizontal bars show upper and lower bounds of transection distance for each bin. R^2 value is given in the key.

Fig. 3.

Plots of B104 cell sealing frequency (%) vs transection distance (μm) at (A) 2.5 min PC, (B) 3.75 min PC, (C) 5 min PC, (D) 10 min PC, (E) 15 min PC, and (F) 20 min PC. Horizontal bars show upper and lower transection distance bounds of each bin. Solid lines show best-fit one-phase exponential curves. Gray arrows point to bins with $30 \leq n \leq 40$. Black arrows point to bins with $n < 30$. R^2 values are given in key. Y_0 and small horizontal bars mark Y -intercepts. P marks plateau sealing frequency. Small ticks mark these values on the Y -axes.

Fig. 4.

One-phase exponential best-fit curves for B104 cell sealing frequency (%) vs transection distance (μm) at 2.5 min PC (purple), 3.75 min PC (olive green), 5 min PC (black), 10 min PC (red), 15 min PC (blue), 20 min PC (green). R^2 value is given in the key.

Fig. 5.

Plots of B104 cell sealing frequency (%) vs PC time (min) for each of 12 width-optimized bins. (A) 0–5 μm , 30–40 μm , and 80–100 μm bins. (B) 5–10 μm , 40–50 μm , and 100–125 μm bins. (C) 10–20 μm , 50–60 μm , and 125–150 μm bins. (D) 20–30 μm , 60–80 μm , and 150–415 μm bins. Linear model (dotted lines) and one-phase exponential model (solid lines) were fit to the data. (E,F) Composite of linear (E) and exponential (F) curve fits. In E and F, the numbers mark the bins in order of increasing distance from the axon hillock, i.e., 1 marks the 0–5 μm bin, 2 the 5–10 μm bin, 3 the 10–20 μm bin, 4 the 20–30 μm bin, 5 the

30–40 μm bin, 6 the 40–50 μm bin, 7 the 50–60 μm bin, 8 the 60–80 μm bin, 9 the 80–100 μm bin, 10 the 100–125 μm bin, 11 the 125–150 μm bin, and 12 the 150–420 μm bin.

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Fig. 6. Sealing frequency plateau values versus transection distances taken from Fig. 5F and plotted as cardinal values (A) or ordinate numbers, lowest to highest (B).

Fig. 7.

Binary sealing frequencies (%) at different PC times (min) and transection distances (μm) as reported herein, by Spaeth et al. (2010), or by Zuzek et al. (2013). Multinary data were transformed into binary (A,C) $<50 \mu\text{m}$ or (B,D) $\geq 50 \mu\text{m}$ transection distances. (A,B) Solid curves show one-phase exponential fit through origin with time constants (τ) in the key. (C,D) Sample size (number, n) shown at bottom and sealing frequency shown at top of each bar. Sealing frequencies were compared for all PC times (A,B; CMH χ^2) and at each PC

time (C,D; Fisher's exact test). The black lines linking curves (A,B) and bars (C,D) show each comparison. Asterisks above each line: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

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Table 1

Best-fit coefficients, constants and standard errors for linear and one-phase exponential regression for B104 cell sealing frequency vs transection distance at PC times.

	Constant or Coefficient	2.5	3.75	5	10	15	20
1	Linear Model						
2	Slope	0.27 ± 0.08	0.22 ± 0.06	0.22 ± 0.07	0.30 ± 0.07	0.24 ± 0.06	0.26 ± 0.05
3	Significance compared to zero	0.006	0.004	0.008	0.002	0.003	< 0.001
4	Y-Intercept (% sealing)	29.4 ± 6.7	31.8 ± 5.2	48.7 ± 5.9	46.5 ± 6.7	59.6 ± 5.6	50.5 ± 5.0
5	Significance compared to zero	0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
6	Goodness ofFit (R ²)	0.554	0.608	0.520	0.623	0.607	0.712
7	One-Phase Exponential						
8	Plateau (% sealing)	69.1 ± 5.1	65.8 ± 5.5	79.1 ± 2.1	91.1 ± 2.8	93.5 ± 3.9	93.8 ± 4.8
9	Space Constant (µm)	35.2 ± 10.4	42.8 ± 15.3	25.7 ± 4.3	34.6 ± 5.1	29.1 ± 8.0	50.0 ± 11.9
10	Y-intercept (% sealing)	2.1 ± 7.9	13.7 ± 6.6	18.2 ± 4.7	15.7 ± 4.4	31.8 ± 7.4	31.6 ± 4.8
11	Significance compared to zero	0.801	0.068	0.004	0.006	0.002	< 0.001
12	Goodness ofFit (R ²)	0.897	0.870	0.962	0.972	0.905	0.940
13	F-Test for Regression (p value)	< 0.001	0.002	< 0.001	< 0.001	< 0.001	< 0.001
14	AIC one-phase exponential Relative Likelihood	99.84%	98.85%	> 99.99%	> 99.99%	99.79%	99.93%

Table 2

P-values for pairwise CMH X^2 comparisons of transection distance vs sealing curves at different PC times.

PC time	2.5	3.75	5	10	15	20
2.5	N/A	0.969	<0.001	<0.001	< 0.001	<0.001
3.75		N/A	<0.001	<0.001	<0.001	<0.001
5			N/A	0.196	<0.001	0.008
10				N/A	<0.001	0.058
15					N/A	<0.001
20						N/A

Table 3

Coefficients and constants for the best-fit curves in Fig. 5F. Curves are described both with ordinal bin number (column 1) and the twelve-width optimized boundaries (column 2). Ordinate values for the plateau sealing frequency (column 3), Y-intercept sealing frequency (Y_0 , column 4), and time constants (τ , column 5) are given in parentheses. [†]Values are equal only after rounding, and their ordinate position were determined before rounding.

Bin #	Boundaries	Plateau	Y_0	τ
1	0 to 5	28.8 (1)	-33.0 (2)	2.04 (2)
2	5 to 10	45.7 (2)	-11.4(4)	3.33 (6)
3	10to 20	60.0 (3)	11.9 (6)	5.16 (10)
4	20 to 30	66.5 (5)	-12.6 (3)	3.32 (5)
5	30 to 40	65.3 (4)	-35.8 (1)	1.99 (1)
6	40 to 50	74.2 (6) [†]	-5.9 (5)	2.94 (4)
7	50 to 60	74.2 (7) [†]	55.5 (12)	6.06 (12)
8	60 to 80	82.8 (8)	26.1 (7)	3.40 (8)
9	80 to 100	87.0 (9)	40.6 (11)	2.47 (3)
10	100 to 125	91.5 (10)	40.0 (10)	3.34(7)
11	125 to 150	98.0 (12)	29.3 (8)	4.75 (9)
12	150 to 420	95.4(11)	39.9 (9)	5.47 (11)