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Review

Long-distance electrical coupling via tunneling nanotubes ☆

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ABSTRACT

Tunneling nanotubes (TNTs) are nanoscaled, F-actin containing membrane tubes that connect cells over several cell diameters. They facilitate the intercellular exchange of diverse components ranging from small molecules to organelles and pathogens. In conjunction with recent findings that TNT-like structures exist in tissue, they are expected to have important implications in cell-to-cell communication. In this review we will focus on a new function of TNTs, namely the transfer of electrical signals between remote cells. This electrical coupling is not only determined by the biophysical properties of the TNT, but depends on the presence of connexons interposed at the membrane interface between TNT and the connected cell. Specific features of this coupling are compared to conventional gap junction communication. Finally, we will discuss possible down-stream signaling pathways of this electrical coupling in the recipient cells and their putative effects on different physiological activities. This article is part of a Special Issue entitled: The Communicating junctions, composition, structure and characteristics.

Contents

1. Introduction

Tunneling nanotubes (TNTs) were discovered a few years ago as conduits for a previously unrecognized form of cell-to-cell communication [1]. TNTs are membranous channels with a diameter of 50–200 nm and form *de novo* between cells up to a distance of several cell diameters. They contain F-actin, in some cases also microtubules [2,3], and have no contact with the substrate. The structural integrity of TNTs is sensitive to mechanical stress, chemical fixation, and even to prolonged light exposure.

Since the discovery of TNTs in pheochromocytoma (PC12) cells, similar structures have been found in a growing number of cell types such as fibroblasts, epithelial cells, immune cells [4,5] or primary neurons and astrocytes [6]. Furthermore, recent studies succeeded in imaging long TNT-like bridges *in vivo* between putative dendritic cells in the cornea of mice [7] and between non-neural ectoderm cells of the midbrain upon neural tube closure in mouse embryos [8]. In addition, TNT-like structures were also detected between malaria parasites [9] and between bacteria growing in biofilms [10]. This suggests that the capability of cells to form TNTs represents a general mechanism of long-distance cell-to-cell communication, which may be conserved during evolution from bacteria to mammals.

Several studies in cell culture models have provided insight into the formation of TNTs. Time-lapse imaging has shown that TNTs form *de novo* within a few minutes by apparently two distinct modes. Firstly, for most studied cell types, which include normal rat kidney (NRK), neural crest (NCC) and immune cells, the vast majority of TNTs formed by dislodgement of abutted cells [11,12]. Secondly, in some other cell types such as PC12 and primary hippocampal neurons, it was evident that TNTs were generated mostly by filopodial interplay [13] (Wang, X., Bukureshtliev, N., Gerdes, HH, unpublished). A

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more detailed investigation of the filopodium-dependent mechanism in HeLa cells revealed that M-Sec promotes the formation of TNTs through the Ral-exocyst pathway [14]. Furthermore, it was reported that stimulation of the Fas signaling pathway induces TNT formation through activation of Rho GTPase [15].

During the past years numerous studies have addressed the intercellular exchange of cargo through TNTs. Using protein markers fused to fluorescent proteins and/or fluorescent dyes, the transfer of cytoplasmic components such as calcium ions, plasma membrane components, endosome-related organelles and mitochondria was shown to involve TNT-like bridges ([4,5], see references therein). Very recently also pathogens such as the human immunodeficiency virus (HIV) [12] and prions [16,17] were found to spread between cells through these tubes. Even though a systematic analysis of generic cargo transferred via TNTs between different cell types has not been performed, the intercellular transfer of cargo along these tracks appears to be a hallmark. It is of note that vesicular carriers moved uni-directionally through TNTs with a speed in the range of actin-dependent transport. In agreement with this, evidence for an actomyosin-dependent transport of vesicular carriers through TNTs was provided [18] and spurs on identification of motor protein(s) involved. Besides a movement in one direction only, a bidirectional microtubule-dependent transport of vesicles was observed for thicker TNTs [2,3].

In addition to the exchange of molecular information, a novel function was recently attributed to TNTs, namely the long-distance electrical coupling of TNT-connected cells [11]. In this review we will survey this new function and its underlying structural basis. Furthermore, we will discuss potential physiological implications of this long range signaling.

2. TNT-dependent electrical coupling

2.1. Analysis of TNT-dependent electrical coupling

The narrow cylindrical membrane tube of TNTs encloses a bundle of actin fibers and a cytoplasmic sleeve. Because the conductance of cytoplasm is about eight orders of magnitude higher than that of its surrounding membrane [19], induced ionic currents should be expected to run along the TNT towards the connected cell. For artificial nanotubes, which were pulled out from liposomes with a similar diameter and length as TNTs, the transfer of electrical current was shown [20]. By using optical membrane-potential measurements combined with mechanical stimulation, we addressed the TNT-dependent propagation of electrical signals in living cells [11]. When one NRK cell of the TNT-connected pair was mechanically depolarized in the presence of the voltage-sensitive dye $DiBAC_4(3)$, the fluorescence of both stimulated and connected cells increased synchronously (Fig. 1). Neighboring cells without physical connections to the stimulated cell did not show any change in fluorescence signal. This excluded the spread of depolarization by diffusion of molecular signals from the stimulated to neighboring cells and clearly demonstrated a TNT-dependent electrical coupling between remote cells. Whole-cell patch-clamp recordings from TNT-connected NRK cell pairs confirmed these data: when voltage steps were applied to one cell of a TNT-connected pair, fast electrical coupling with an average conductance of 566 ± 129 pS was recorded [11]. Both directions of measurement showed a similar conductance indicating non-rectifying, bi-directional electrical coupling. Notably, cell pairs that were connected by two TNTs, displayed higher conductance values compared to cell pairs connected by one TNT of similar length. This implies that multiple TNT-connections can operate as parallel conduits for the spread of electrical signals between cells.

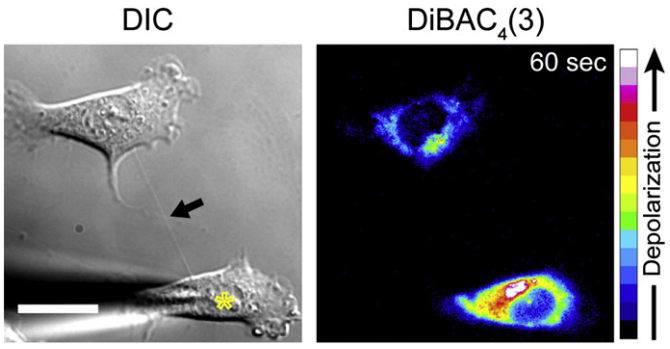

Fig. 1. TNT-dependent electrical coupling between NRK cells. The DIC image shows two NRK cells connected by a TNT (*arrow*). One cell (*asterisk*) was depolarized by mechanical stimulation using microinjection. The $DiBAC_4(3)$ fluorescence was recorded before and 60 s after stimulation. The pseudo-colored intensity image generated by subtracting the two images shows the increase in fluorescence intensity (depolarization) in both TNT connected cells 60 s after stimulation. Color bar indicates relative level of depolarization. Scale bar = 20 μm.

Another important feature of TNT-dependent coupling was the decrease in conductance with increasing length of the TNTs. This indicates a passive spread of current through TNTs. Notably, spreading of a depolarization signal over a ~60 μm long TNT decreased ~10-fold in strength as compared to a ~10 μm long TNT [11]. This implies that the effective distance to trigger downstream effects in TNT-connected NRK cells is limited to a few cell diameters. However, since the regression of the signal amplitude along the length of the TNT mainly depends on the diameter of the TNT, thicker TNTs of other cell types may have higher conductivity and allow longer effective distances of signal transduction. Evidence for a variation in the sleeve size of TNTs was indeed found in several cases since fluorescent dyes were shown to enter into TNTs of THP-1 [21] but not NRK cells [11]. Nevertheless, the conductance of TNTs is likely to be far less as compared to that of physical contacts accomplished by filopodia/lamellopodia. In the case of NRK cells, on average a ~15-fold less conductance was measured as compared to filopodia/lamellopodia (8.8 ± 2.1 nS) [11].

2.2. Dependence on gap junctions

Electrical cell-to-cell coupling via TNTs implies cytoplasmic continuity between the connected cells. In a recent study the structural basis for this continuity was addressed. The lack of membrane mixing between TNT-connected NRK cells ruled out a model where TNTs are continuous with the membranes of both cells [11]. Instead, only one end of the TNT displayed membrane continuity, whereas the other exhibited a membrane border, which was immuno-positive for Cx43 (Fig. 2). The same findings were made for other cell types and suggest that interposed gap junctions are essential requirements for a TNT-dependent electrical coupling of cells (Table 1). In further support of this model patch-clamp analysis revealed that the TNT conductance was voltage-sensitive, which has been identified as a characteristic feature of Cx43-containing gap junctions [22]. Furthermore, the gap junction blocker meclofenamic acid led to a reduction of the TNT-dependent coupling and finally PC12 cells, which do not express gap junctions, did not show electrical coupling. In addition, we noticed that the number of electrically competent TNTs is determined by the abundance of functional gap junctions, especially in those cells containing a low number of functional gap junctions, such as neural crest cells (Table 1).

Co-localization studies between TNTs and Cx43 revealed two different classes of TNTs: those that lack connexins and thus do not display electrical coupling, and those that possess connexins and participate in electrical coupling. In the latter, Cx43 signals were most frequently expressed at only one end of a TNT connection, consistent with a membrane interface at that side. In some cases Cx43 signals were observed at both ends of the TNT and may have originated from two or more closely neighboring TNTs, which could not be resolved microscopically. Given the conductance of about 61 pS for a single Cx43 channel [23], the maximally observed conductance of 1.3 nS for one TNT connection between NRK cells suggests that at least an equivalent of ~20 open channels were present at this contact site. Because the contact area of a TNT is likely to be the limiting factor for recruitment of connexons, the greatest possible number may be

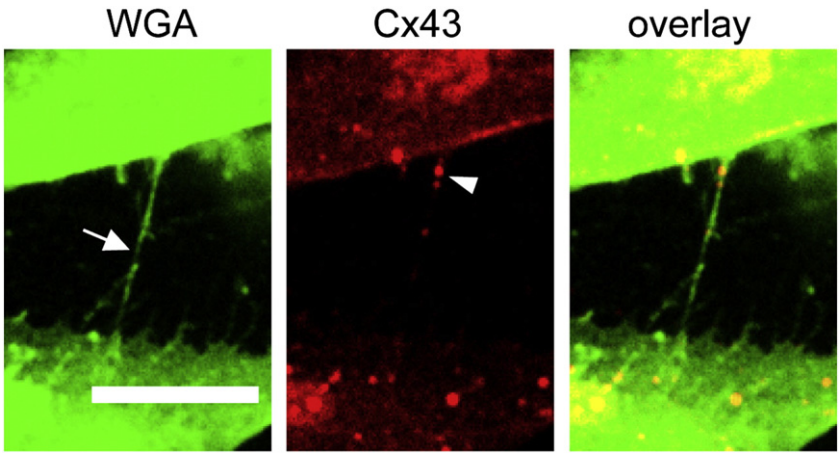

Fig. 2. Cx43 localizes with TNTs. NRK cells were fluorescently labeled using Alexa Fluor 488 wheat germ agglutinin (WGA, *green*) and anti-Cx43 (*red*). The confocal images show a TNT (left, *arrow*) between two cells and distinct signals of Cx43 immuno-labeling (middle, *arrowhead*) at one end of the TNT. Scale bar = 20 μm.

significantly higher than 20. However, even then a TNT connection will have far less gap junctions as compared to those of abutted cells, which can incorporate up to several thousand connexons [24].

2.3. Peculiarity of TNT-dependent electrical coupling

The combination of TNTs and gap junction channels has major advantages over other types of cell–cell communication. In comparison to conventional gap junctions, TNTs accomplish a long-range electrical signaling between two cells with a distance up to ~100 μm. Furthermore, electrical signals propagated via TNTs are presumably more selective than those passing through gap junctions of abutted cells. This is reasoned from the relatively low amplitude of TNT-dependent signals, which is likely to affect only the connected cell. Gap junction-dependent signals, however, are on average much stronger due to the relatively low resistance of the gap structure as compared to TNTs, and can propagate consecutively through many gap junction-connected cells.

In comparison to other forms of long distance intercellular communication involving secretion of signaling molecules and their subsequent receptor-mediated uptake by neighboring cells, the transfer of electrical signals via TNTs is more selective and occurs much faster, i.e. within milliseconds. Although electrical synapses also transfer bidirectional electrical current over long distances, they are only present between neuronal cells. Like in TNT connections, these synapses consist of connexon channels associated with long membrane tubes, namely axons and dendrites. However, pivotal differences in the efficiency and strength of electrical signal transmission are evident between these two structures. Both axons and dendrites have much less resistance than TNTs due to a >10-fold larger diameter. In addition, axons generate action potentials through energy-consuming activation of voltage-gated sodium channels, contrasting the passive flow of current through TNTs, and allow saltatory signal propagation due to the myelin coat. Together, this results in transmission of electrical signals with high amplitude across long distances spanning the size of whole organs and between parts of the body.

Besides the differences in propagation of electrical signals, molecules may diffuse through TNTs to different degrees as compared to gap junctions (Table 2). In this context, Cx43-positive TNT connections of several cell lines do not display Cascade Blue dye (MW = 548) coupling, which was shown to freely diffuse through gap junctions of abutted cells [11]. Furthermore, calcium ions do not pass through Cx43-positive TNTs of different cell types [11,25]. However, Watkins and colleagues showed both dye coupling and calcium flux through TNTs connecting myeloid-lineage dendritic cells or monocytes. In addition, they found that the transport was gap junction-independent [21]. These data suggest that different cell types form diverse TNTs with distinct properties. Another important difference between TNTs and gap junctions concerns the intercellular transfer of organelles, which was observed for TNTs, but not for gap junctions (see below).

3. Formation of electrical coupling-competent TNTs

Current knowledge suggests that the presence of gap junction channels in TNTs is essential for TNT-dependent electrical coupling. Although it has not been addressed how these channels interpose into TNT connections, it can be assumed that their recruitment is strongly influenced by the way TNTs form (Fig. 3). In the case of cells that generate TNTs by dislodgement, it is intriguing to speculate that electrically competent TNTs form at sites of gap junctions. When cells move away from each other, membrane tubes are pulled out at these anchorage points. During progressive breakup of the gap junction structure, a subpopulation of the gap junction channels remains intact and becomes an integral part of the emerging TNT connection. This model is supported by data showing that numerous Cx43-positive nanotubes developed at gap junctions of dislodging NRK cells or astrocytes (Wang, X., Bukureshtliev, N., Gerdes, HH, unpublished). Despite the efficient entry of gap junction channels into TNT structures, some remain Cx43-negative. In for example NRK cells approximately 20% of the formed TNTs do not

Table 1
Analysis of electrical coupling between various cell types.

Cell type	Cx43	Electrical coupling ratio of cells	
		TNT-connected	Abutted
NRK [11]	(+)	80%	96%
HEK293 [11]	(+)	52%	95%
HUVEC [11]	(+)	71%	96%
NCC [11]	(+)	20%	23%
PC12 [11]	(−)	0	0
Neuron-astrocytes[a]	(+)	(+)	(+)
ARPE 19[b]	(+)	(+)	(+)

(+) detected; (-) not detected.
[a] Wang, X, Bukroeshtliev, NV, Gerdes, HH, unpublished data.
[b] Wittig D, Wang X, Walter C, Gerdes HH, Funk RHW, Roehlecke C, unpublished data.

Table 2
Comparison of gap junction- and TNT-dependent communica.

	Gap junction	TNT	
Electrical coupling[a]	(+)	(+) [11]	(−) [11]
Ca^{2+} flux	(+)	(+) [14, 21]	(−) [11, 12, 25]
Dye coupling	(+)	(+) [21]	(−) [11]
Organelle transfer	(−)	(+) [1, 11]	(−)[b]

(+) detected, (−) not detected, references for the respective studies on TNTs are given in superscript.
[a] See also Table 1.
[b] Not addressed.

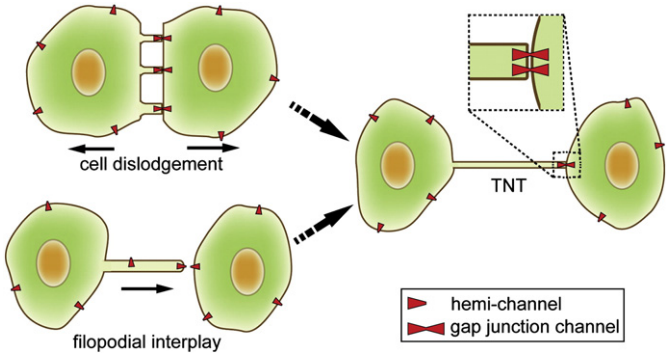

Fig. 3. The biogenesis of TNTs with interposed gap junction channels. The schematic representation illustrates two different mechanisms of TNT formation with interposed gap junction channels. When two cells move apart from each other, short TNT-like membrane protrusions with gap junction channels form between the cells (top, left). During progressive dislodgement most of them disappear and one TNT with gap junction channels (enlarged box) at one-end remains. Alternatively, filopodia containing hemi-channels prolong toward another cell (bottom, left). When the filopodium reaches the target cell, the hemi-channels at the tip of the filopodium dock to hemi-channels of the target cell and form functional heterotypic gap junction channels (enlarged box).

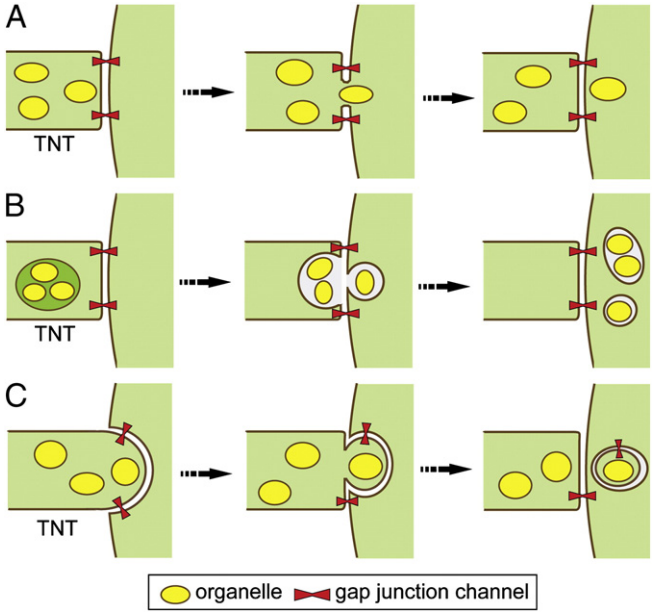

Fig. 4. Proposed models for the intercellular transfer of organelles via TNTs. (A) Transient membrane fusion model. The tip of the TNT fuses with the plasma membrane of target cell and forms a transient pore to allow organelle transfer from the TNT into the target cell. (B) Multivesicular body fusion model. The membrane of the multivesicular body fuses with the membrane at the tip of the TNT to release its intraluminal organelles, which are subsequently endocytosed by the target cells to form double-layered vesicles. (C) Phagocytosis model. When a TNT attaches to the plasma membrane of a neighboring cell, a phagocytotic activity is induced and results in the uptake of the tip of the TNT and the organelles inside. The organelles enter the target cell as three membrane-layered vesicles.

contain Cx43 as evidenced by the absence of Cx43 immuno-staining and electrical coupling [11]. This subpopulation may have failed to recruit connexons during cell dislodgement. Alternatively, it is possible that connexons were initially also targeted to these TNTs but were subsequently removed by internalization into the connected cell due to short half-life of Cx43, which is of only a few hours at the plasma membrane of cells [26]. Furthermore, it is conceivable that gap junctions are preferred sites of TNT formation between dislodging cells. Although the connexons alone may not provide enough adhesion force to pull out membrane tubes at the contact site, the joined presence of cadherins at these sites may support the tight membrane association by homophilic interactions [27].

In the case of cells which form electrically competent TNTs by filopodial interplay, it is likely that hemi-channels are located at the tips of outgrowing filopodia, which upon contacting neighboring cells, dock to their counterparts at the plasma membrane of opposing cells to form functional gap junction channels. The probability of successful channel formation at TNT-specific junctions is likely to increase with an increasing expression level of connexins [28]. Although gap junction channels certainly represent an important module of TNTs facilitating electrical coupling, it should be noted that they are not essential for TNT formation. This is evidenced by PC12 cells, which form TNTs by filopodial interplay, but do not express connexins.

Although to date only Cx43 as by far the most widely expressed connexin family member has been found in TNTs, other members might also integrate into TNT structures, in particular since co-expression of two or more types of connexins is the standard for most cells [29]. Another unsolved but intriguing question is whether functional heterotypic gap junction channels can form between a TNT and the connected cell. Such a possibility could provide an enormous potential for long distance cell-to-cell communication between different cell types.

4. Dual function of TNTs?

The intercellular exchange of organelles via TNTs has been documented for numerous cell types, which express connexins. In the case of NRK cells our data suggests that at least 80% of all TNTs facilitate electrical coupling [11] and about 50% accomplish the transfer of endocytic organelles [18]. This suggests that some TNTs are engaged in both activities and raises the question as to how organelles cross the membrane interface between TNT and connected cell. Several mechanisms are conceivable to explain the transmission of organelles into target cells. One model proposes that organelle transfer is facilitated by a transient fusion of the membrane at the tip of the TNT with the membrane of the connected cell (Fig. 4A). This theory resembles transient membrane fusions proposed in the "kiss and run" model of secretory granule exocytosis [30]. Furthermore, it is in agreement with experimental data obtained for TNTs of PC12 cells, which transfer endocytic organelles, but do not facilitate electrical coupling and thus possess a membrane interface to the connected cells. Interestingly, in transmission electron micrographs some TNTs display a continuous membrane to both connected cells [1,31]. These open-ended TNTs could reflect transient structures to allow short-lived organelle transfer and may only be detectable by electrophysiological means if the observation time is long enough. Although the similarity of TNTs between PC12 and NRK cells is striking, and suggests a direct comparison, it should be noted that connexons are only present at the TNT interface membrane of NRK cells and thus may change the capability of membrane fusion.

Another model of organelle transfer involves the fusion of multivesicular bodies with the membrane at the tip of the TNT to release their luminal vesicles. Subsequently these vesicles could be endocytosed by the target cell and thus would contain two membrane layers (Fig. 4B). A third model proposes that intercellular organelle transfer is accomplished by phagocytotic uptake of the tip of a TNT by the target cell [32] or the formation of double-membrane gap junction vesicles at the gap site through an endocytotic process [33] (Fig. 4C). In both cases organelles enter the target cells as free riders composed of three membrane layers. An important task for future research will be to investigate these models and the underlying regulatory mechanisms.

5. Potential physiological implications of TNT-dependent electrical coupling

An important function of the plasma membrane potential is to regulate ion channels through the voltage-sensing helix in the transmembrane domain [34]. In this respect, we showed that TNT-mediated electrical coupling could activate low voltage-gated calcium channels in a threshold-dependent manner in recipient HEK293 cells. This finding suggests that other voltage-gated ion channels may also be

activated through TNT-mediated depolarization. Similarly, TNT-mediated electrical coupling may affect other downstream pathways, including the modulation of activity of small-molecule transporters and the activation of enzymes such as voltage-sensitive phosphatase [35], phosphatidyl inositol-3-OH kinase [36] or protein kinase A [37]. Taken together, these examples suggest a model, in which transient, long distance electrical signals initiate diverse chemical signals and produce intensive effects on cell behavior.

The possible down-stream signaling pathways enable us to hypothesize on the potential roles of electrical coupling during various physiological processes via TNT-like structures. During chick embryonic development it has been demonstrated by *in vivo* studies that within a stream of migratory neural crest cells (NCC), cells are connected by thin cellular processes of up to 100 μm in length [38]. Our study on neural tube explants from quail showed that a subpopulation of migrating NCC is electrically coupled via TNTs [11]. It is tempting, therefore, to speculate that fast electrical signaling between NCC via thin cellular extensions could be crucial to ensure synchronization of their migratory activity within the expanded tissues. Moreover, the connection of migratory cells by thin filopodia-like structures during sea urchin gastrulation suggests a role in cell-to-cell communication associated with signaling and patterning [39]. This may also include electrical signaling between distant cells. Last but not least, TNT-like structures were recently monitored between cells from neural folds and shown to form bridges across the gap during neural tube closure in mammals [8]. Also here a synchronizing effect through long distance electrical signaling may be anticipated.

Another interesting physiological process, where TNT-dependent electrical signaling could be implicated is wound healing. F-actin-rich membrane extensions connecting opposing cells at wound sites have been observed, as well as the activation of PI3 kinase during wound healing and a membrane depolarization at the leading edge of wounds [36,40,41]. These findings may suggest that membrane extensions/TNT-like structures propagate depolarization signals over long distances to synchronize the observed F-actin remodeling by activation of downstream signaling cascades during healing.

Finally, given the wide distribution of TNTs across cell types, it is interesting to speculate that the presence of TNTs in brain could add an additional level of complexity to information processing. In particular the passive flow of small electrical currents between different neurons, or different branches of their dendritic trees, or even between neurons and astrocytes could provide instructive communication cues. In summary, these examples suggest electrical coupling-competent TNTs as intriguing cues for complex long distance cell–cell interaction and synchronization during development and homeostasis of multi-cellular organisms.

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