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▶ **To cite this version:**

Chiara Zurzolo. Tunneling nanotubes: Reshaping connectivity. Current Opinion in Cell Biology, 2021, 71, pp.139-147. 10.1016/j.ceb.2021.03.003 . pasteur-03934820

HAL Id: pasteur-03934820

https://pasteur.hal.science/pasteur-03934820

Submitted on 11 Jan 2023

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Current Opinion in Cell Biology

Tunneling nanotubes: Reshaping connectivity

Chiara Zurzolo

Abstract

Tunneling nanotubes (TNTs), open membranous channels between connected cells, represent a novel direct way of communication between distant cells for the diffusion of various cellular material, including survival or death signals, genetic material, organelles, and pathogens. Their discovery prompted us to review our understanding of many physiological and pathological processes involving cellular communication but also allowed us to discover new mechanisms of communication at a distance. While this has enriched the field, it has also generated some confusion, as different TNT-like protrusions have been described, and it is not clear whether they have the same structure–function. Most studies have been based *on* low-resolution imaging methods, and one of the major problems is the inconsistency in demonstrating the capacity of these various connections to transfer material between cells belonging to different populations. This brief review examines the fundamental properties of TNTs. In adult tissues, TNTs are stimulated by different diseases, stresses, and inflammatory signals. 'Moreover', based on the similarity of the processes of development of synaptic spines and TNT formation, we argue that TNTs in the brain predate synaptic transmission, being instrumental in the orchestration of the immature neuronal circuit.

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Current Opinion in Cell Biology 2021, **71**:139–147

This review comes from a themed issue on **Membrane Trafficking**

Edited by **Judith Klumperman** and **Thomas Pucadyil**

For a complete overview see the Issue and the Editorial

Available online 15 April 2021

https://doi.org/10.1016/j.ceb.2021.03.003

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Introduction

In 2004, Gerdes and colleagues reported a previously unrecognized form of cell-to-cell communication: long, membranous protrusions connecting distant cells in culture named tunneling nanotubes (TNTs) [1]. These cellular extensions are unique as they allow the transfer of various-sized cargoes, from small molecules (e.g. calcium ions) and macromolecules (nucleic acids, proteins, etc.) to entire organelles (vesicles, lysosomes, mitochondria, autophagosomes, etc.) between connected cells [2]. The connection of multiple cells by TNTs leads to the formation of functional cellular networks [3].

Gerdes' seminal paper defined TNTs very clearly: "TNTs are open-ended channels mediating membrane continuity between connected cells" [1] (See Box 1). In this definition resides the revolutionary concept that has shaken the cell biology field and incited great debate amongst the scientific community [4]. This discovery challenged the very dogma of cells, the basic structural, functional, and biological unit of all known organisms, as distinct entities. Indeed, the discovery of TNTs established the concept of super-cellularity [5]. Super-cellularity allows for the rapid balance of metabolic needs, nutrients, nucleic acids, as well as stress factors and organelles through long-range exchanges between cells not immediately in contact; thus, TNTs facilitate fast and specific responses between cells regardless of distance and diversity of tissue. Another mechanism that allows rapid communication between distant cells is represented by the synapse that is exclusive to the brain; rapid and fast communication between brain areas and between the brain and the rest of the body is assured by long specialized cellular protrusions called axons. Through a synaptic connection, the axons propagate electrochemical signaling to downstream populations, which can be very distant from the input-originating cell.

Following a survey of recent literature, I will argue that TNTs represent an early feature of cells (See Box 2). In the specific case of neuronal cells, TNTs would predate brain development and synaptic transmission, as we know it. This hypothesis is based on the eukaryotic presence of TNTs in nondifferentiated cell-states showing this 'presynaptic' ability for fast and specific long-distance communication. Thus, in many ways and in more specific cases that I will analyze below, this discovery has the potential to rewrite some pages of biology text books.

Box 1. TNT variety and definition

According to the original definition, "TNTs are open-ended channels mediating membrane continuity between connected cells" [1] (Figure 1). TNTs are membrane protrusions directly connecting cells that do not derive from cell division. In tissue culture, they fulfill the following criteria:

1 Have a diameter between 50 and 700 nm (with an average of 200 nm)
2 Have a length from tens up to several hundred microns (average between 20 and 100 μm)
3 Hover on top of the substratum and are very dynamic
4 Contain actin*
5 Provide cargo transport
6 Establish continuity of the plasma membrane of the connected cells
7 Are open-ended

*Regarding the cytoskeletal composition, in addition to actin, TNT-like protrusions have also been shown to contain microtubules [6]. We have excluded this from the specific characteristics of *bona fide* TNTs as the tubulin-containing structures are thicker than average TNTs and appear to be closed-ended [6]. Some cells have been shown to contain both types of connections: actin only and actin and microtubules [7,8]. Nevertheless, their functionality seems to be rather disrupted by the use of drugs that depolymerise actin rather than tubulin [7,9].

Since their discovery, multiple studies have reported the presence of TNTs between cell types of various origins *in vitro*, namely epithelial, endothelial, mesenchymal, neuronal, muscle and immune [6]. The TNTs described up-to-date in the various conditions and cellular contexts show high variability in their morphology, in terms of length, thickness and cytoskeleton content [6]. This morphological heterogeneity, the lack of molecular targets for TNT identification and structural characterization, combined with low-resolution imaging methods used to date [10] are major limitations in TNT investigation. More clarity is needed in addressing the existence of either one or several types of *bona fide* TNTs and whether different structures allow for different cellular functions.

A major shortcoming of current studies is the inconsistency in assessing or demonstrating the capacity of these various connections to transfer material between cells belonging to different populations. As the distinguishing property of TNTs, compared to other similar structures, is their transfer capacity, the term TNT should be used *only* when this condition is fulfilled and experimentally demonstrated [10]. Indeed, the use of ambiguous nomenclature (e.g.; nanoscale conduit, interpericyte (IP)-TNTs, Ras homolog enriched in the striatum (Rhes)-Tunnels, microtubule (MT)-nanotubes or simply nanotubes [6,7,11,12], in the last 10 years has diverged from the original and precise definition of TNTs given by Gerdes and collaborators [1]; this necessitates better technical approaches and more rigor in the study of TNTs.

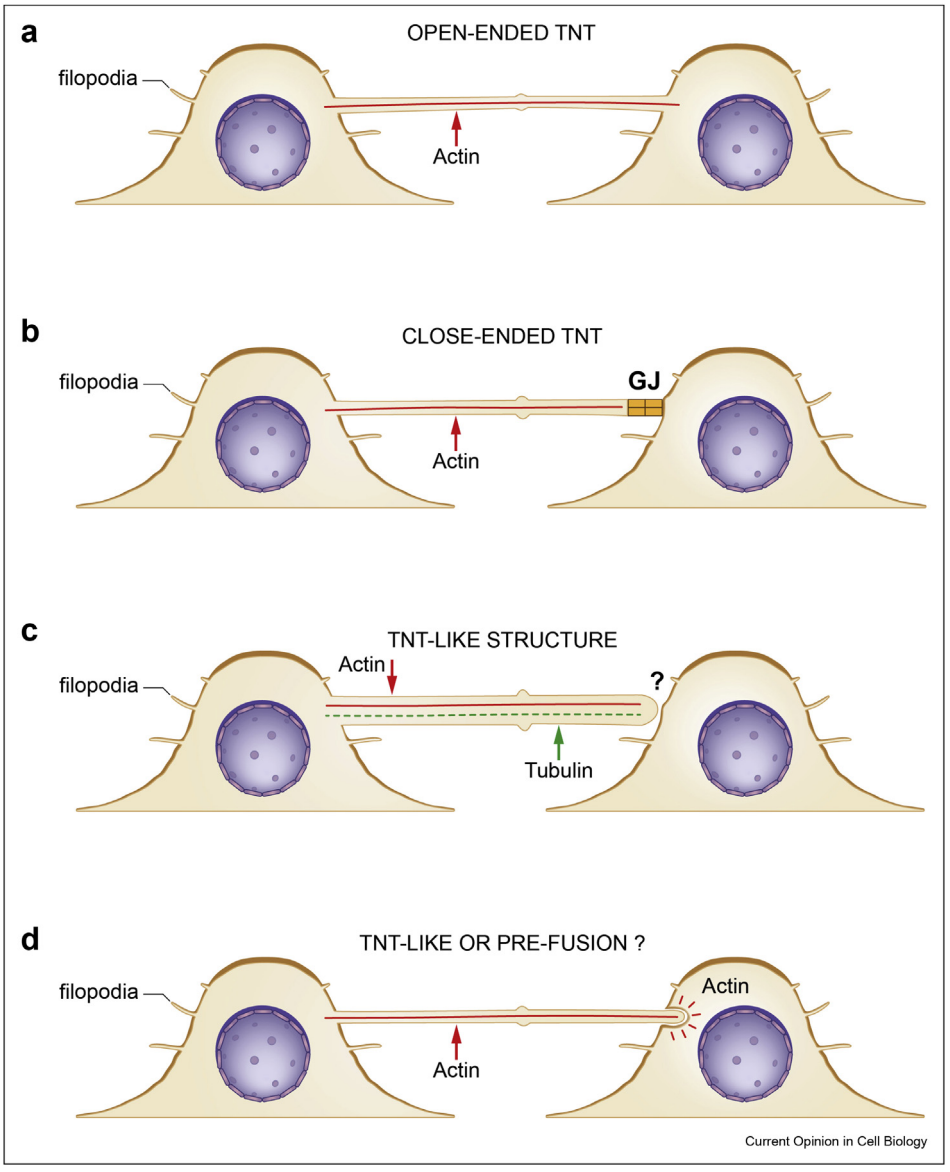
Nevertheless, this does not mean that protrusions that do not fulfill the aforementioned characteristics are not important nor involved in intercellular communication. On the contrary, the discovery of TNTs has aroused a new interest into the exploration of new remote communication mechanisms [6,7,11–13]. Yet, consistent reporting is crucial in new discoveries. When the membranous protrusions do not ensure direct communication between cells (i.e., they do not enable the formation of an open tunnel, but rather resemble closed filopodia-like protrusions), or when transfer capacity is not verified, a more appropriate appellation or the general term 'TNT-like' should be used (Figure 1).

Open-ended TNTs and the mystery of fusion

Questions still linger on the open- or close-endedness of TNTs. This can be addressed by studying their ultrastructure using electron microscopy (EM)-based approaches. Yet, conventional embedding and dehydration methods for sample preparation in transmission and scanning EM (TEM and SEM, respectively) are not suitable for preserving the fragile structure of TNTs and their actin cytoskeleton [14–16]. The cryo-correlative light EM (cryo-CLEM) approach, albeit technically quite challenging, is the best option to preserve the membrane structures identified to date by fluorescence microscopy (FM) and live-cell imaging [14]. In practice, cryo-CLEM, cryo-electron tomography (ET) and focused ion beam SEM (FIB-SEM) have revealed that the structure of TNTs is very different from filopodia as TNTs can form bundles of thin parallel tubes (named individual TNTs, iTNTs) in human and mouse neuronal cells. These iTNTs contain actin bundles and vesicles and are held together by linkers containing N-Cadherin [14]. In addition, FIB-SEM data also shows that iTNTs can be open-ended and closed-ended (Figure 1); this technique can be further improved to better resolve the membrane structure at the contact site, for example, by using cryo-FIB-SEM [17]. Insofar as these advanced EM microscopy approaches are technically demanding, and require specific, costly instruments and trained personnel, there is a lack of studies to date, which have tried to approach this problem [10]. In light of the continuous improvement and larger availability of technologies [18], these issues will be fully addressed in different physio-pathological conditions and diverse tissues in the near future. Notwithstanding, the use of live and quantitative fluorescent and super-resolution microscopy can discriminate TNTs from other structures with a reasonable margin of error [10,16].

While the mechanism of TNT formation has been the subject of a few studies that have identified the critical role of actin remodeling and membrane trafficking [19,20], little effort has been put into investigating the molecular mechanisms of fusion that must occur

Figure 1



Schematic representation of the main types of open- and closed-ended TNTs and alike protrusions observed *in vitro*. **(a)**. Classical thin open-ended TNTs supported by filamentous actin. **(b)**. Closed-ended actin supported TNTs allowing transfer of electrical signals and small molecules through a Gap Junction placed at one end. **(c)**. Thicker TNT-Like structures containing both actin filaments and microtubules, closed-ended. These structures allow communication at the tip (e.g., exchange by receptor-mediated signaling or by vesicle budding/uptake, not depicted, but indicated by '?'). **(d)**. Closed-ended TNT-like structures poking into the connecting cell or prefusion event of a classical TNT. The actin depicted in the receiving cell has not been observed but is hypothesized to be present by analogy with Drosophila myoblast fusion (Original schematic from Diego Cordero-Cervantes, modified by Beatrice de Cougny, "Service Communication Institutionnelle et Image de l'Institut Pasteur").

between the tip of a nascent TNT and the plasma membrane of the opposing cell, or between the membranes of two TNTs coming from opposite directions (Figure 1). The discovery showing that neuronal cell TNTs often correspond to a bundle of iTNTs, connected through N-Cadherin linkers [14], may hint at this fusion mechanism [21], in which case these iTNT bundles would be the precursors of thicker single tubes. Cell adhesion molecules (CAMs), like N-Cadherin can promote membrane juxtaposition that allows mixing of the lipids and initiation of the fusion pore formation [22]. CAM function, however, must be differently regulated beyond its role in the formation of cell junctions in order to avoid the formation of a classic junctional complex and a massive fusion of adjacent cells, which establish junctional complexes (e.g., epithelial

tissue or neuromuscular junctions). A revealing example comes from studies of *Drosophila* myoblast fusion where a genetic screen has identified CAMs, adaptor proteins, actin cytoskeletal regulators, and vesicle trafficking proteins (reviewed in Ref. [22]). More specifically, Chen and collaborators have elegantly uncovered the critical role of the actin cytoskeleton in cell–cell membrane fusion. They showed that, while the fusion-competent myoblast (FCM) drills actin polymerization-mediated fingerlike protrusions into the founder cell, the latter builds a stiffer actin cortex to resist the invasive forces. They propose that following CAM-mediated contact, the mechanical interactions between the two fusion partners push the two cell membranes close enough to promote fusion and that spectrin functions as a mechanosensor of the shear forces at the fusion synapse [22,23]. Similar invasive actin protrusions have been found in mammalian cell fusion sites [24], and many of the molecules involved in the fusion of *Drosophila* myoblasts are also present in mammals [25].

Strikingly this is also reminiscent of the images we obtained by FIB-SEM; we observed closed-tip TNTs invaginating within recipient cells [14] (Figure 1). It is conceivable to hypothesize that the combination of adhesion and mechanical forces is also involved in TNT tip fusion. These premises open the TNT field of study to further exciting exploration. Assessing the role of actin and other molecules shown to be involved in cell-to-cell fusion may give interesting hints on the mechanism of fusion in TNTs.

Box 2. TNT purpose and origin

Over the last 15 years, TNTs have been shown to transfer organelles, pathogens, ions, genetic material, and misfolded proteins [8,15,21,26–28] and proposed to participate in a range of physiological processes such as differentiation, tissue regeneration, and immune responses [3]. Current data suggest that TNTs are minimally present in adult tissues (perhaps excluding the immune system) in physiological conditions [3,29]. Rather, it appears that they are stimulated by different diseases, stresses, and inflammatory stimuli and may be implicated in disease transmission [3,5,13]. For example, several pathogens have evolved to adapt, or even hijack, TNTs to amplify their capacity for infection and to spread toxicity between connected cells [8,21]. We unequivocally demonstrated that TNTs transfer prions and other amyloid proteins (e.g., tau and α-synuclein involved in Alzheimer's and Parkinson's diseases, respectively) between neuronal cells *in vitro* [30,31] and proposed that they may play a major role in the progression of neurodegenerative diseases [26]. Conversely, TNTs are involved in metabolic rescue mechanisms by transferring healthy second messengers, mitochondria or lysosomes, into damaged cells [27]. This rescue mechanism, in the case of cystinosis [32], contributes to disease rescue, yet, it may promote the progression of several cancers [33–35].

The observation that diverse pathogens like viruses, bacteria, and prions can exploit TNTs and TNT-like structures to invade eukaryotic cells is compelling; it implies these structures may have an ancient evolutionary origin and a function in intercellular communication that has hitherto been underestimated. Emerging evidence supports the existence of various TNT-like connections (called cytonemes, signaling filopodia, MT-TNTs) [6,29] in embryonic development. Here, they play a role in signaling, as well as material exchange between distant cells; they determine cell synchronization, positioning, and fate in the embryo [36]. The most prominent feature distinguishing these other types of cell protrusions from TNTs is that they are closed-ended. Thus, while TNTs allow the transfer of cargoes through establishing cytoplasm continuity the latter allows signal transduction and transfer of proteins through a receptor-dependent mechanism. Open-ended TNTs have not been found in the embryo [29]. Given their electrical coupling and signaling capacity, TNTs in the embryo may regulate morphogenic migratory activity [37], and contribute to synchronizing cell divisions and programmed cell death by exchanging cell death signals. As shown *in vitro* in the case of muscle cells and cardiomyocyte differentiation from stem cells, TNTs may also contribute to cell fate determination in the embryo [38]. Whether TNTs coexist *in vivo* with other protrusions and whether some of these other 'specialized filopodia' connections could indeed be true TNTs needs further investigation.

The case of the brain

One of the most difficult questions asks whether classical, open-ended TNTs exist in living organisms. In addition to developmental biology, this quest is particularly interesting in the brain. In its complexity, the brain is a fascinating organ where synaptic transmission is very reminiscent of TNT-mediated signaling. Interestingly, signaling mediated by cytonemes in the embryo shares morphological and molecular characteristics with neuronal synapses; thus, the creation of the term morphogenetic synapsis has been coined [36].

Exciting new data shows that decapentaplegic (DPP) signaling at the air sac primordium ASP, the precursors to the dorsal thoracic air sacs found in adult Drosophila, could be mediated by functional glutamatergic synapses on cytonemes [39]. This is consistent with the presence of an evolutionarily conserved mechanism for cellular communication that might be functioning in different tissues and contexts. Is it possible that TNTs facilitate a form of presynaptic communication at a distance in the brain? Furthermore, is it possible that the establishment of TNT connections contributes to the shaping of mature neuronal circuits?

Our hypothesis is that TNTs could serve as a nonsynaptic mechanism for intercellular communication; they are instrumental in the orchestration of the immature neuronal circuit and partially responsible for mature synapse-based circuitry.

Open-ended TNTs and closed-ended TNTs in brain development

The possibility that open-ended TNTs may exist in the developing brain is supported by several facts. For example, the similarity of processes involving actin

remodeling and filopodia intercalation in spine development and TNT formation [20,40].

The actin regulator Eps8 is a potent inducer of TNTs in neuronal cells [41]. The formation of dendritic spines is regulated by Eps8 through its capping and bundling activity [42]. In the brain, Eps8 is localized postsynaptically in the dendritic articulations of cerebellar granule neurons and in axons of cultured hippocampal neurons, where it regulates the formation of filopodia. Finally, Eps8 knockout (K/O) mice display immature spines and impaired cognitive functions, and Eps8 levels are reduced in the brains of patients affected by autism [42]. Altogether these data support a possible role of EPS8 in the regulation of TNT formation during brain development.

The Wnt/Ca2+ pathway regulates TNTs in primary neurons in culture in early brain development through Ca2+/calmodulin-dependent protein kinase II (CaMKII) [43]. βCaMKII has been shown to regulate synaptic plasticity in the hippocampus and the cerebellum through actin remodeling [44]. The Wnt pathway is also involved in the formation of cytonemes. However, the downstream players are different. While the downstream activation of beta-catenin regulates cytonemes, TNTs are stimulated by the Ca-calmodulin pathway [43,45]. This suggests that cytonemes and TNTs are, in fact, different structures that might have diverse developmental functions. Thus, it will be interesting to investigate the role of Eps8 and Wnt in the formation of presynaptic TNT-like protrusions during brain development.

One of the earlier discovered TNT inducers in neuronal cells that further supports our hypothesis that TNT plays a role in shaping brain circuitry during development is Myosin10 (Myo10) [46]. Myo10 is highly regulated during nervous system development and exists in two forms [47], the full-length and the 'headless' isoform; the 'headless' isoform lacks motor activity and is differently regulated during development [47,48]. In cortical neurons, these two forms seem to have opposite effects on axon outgrowth, as full length and headless Myo10 respectively stimulate and reduce axons [48]. Headless Myo10 also suppresses the filopodia-inducing activity of the full-length protein when coexpressed in cultured fibroblasts [48]. Coexpression of both isoforms does not block TNT induction in neuronal cells, thus highlighting regulation differences of filopodia and TNTs [46]. Knocking-out (KO) or knocking-down (KD) Myo 10 in cortical neurons of embryonic mouse brain impairs axon initiation and contralateral branching/targeting. Similar axon deficits are detected in Netrin-1-KO or netrin receptor Deleted in Colorectal Cancer (DCC)-KD cortical neurons [49]. DCC and neogenin (NEO1) have different affinities for full-length Myo10 and the short headless form in filopodia elongation (mediated only by DCC, thus different Myo10 expression levels in the developing brain could be a mechanism for regulating TNTs during development. Intriguingly Myo10 forms complexes with N-Cadherin adhesion molecules in order to allow neuronal radial migration [50] during neocortical brain development. Together with our recent data showing a possible role of N-Cadherin in TNTs, it would be interesting to assess whether these two molecules cooperate in the formation of TNTs in the brain.

TNTs could be instrumental in brain development due to their capacity for transmitting electrical signals. Indeed, a subset of 'closed-ended' TNTs facilitate electrical coupling between cells through gap junctions (GJ), in particular connexin 43 (Cx43), at the tip of the protrusion and the recipient cell [2,37].

Communication through GJ is a major signaling mechanism in early brain development as neural progenitors are not excitable by chemical synapses [51,52]. These structures could therefore play a role in neuronal migratory processes via the propagation of depolarization signals to synchronize actin remodeling during brain development [2,37]. During corticogenesis, connexin-43 and connexin-26 are required to ensure that glia guide the proper migration of embryonic neurons from the ventricular region to the cortical plate [53]. It would be interesting to assess whether glial guidance could be mediated by TNTs that are electrically coupled to gap junctions. Furthermore, connexin-36-dependent dye coupling has been shown to occur between mature neurons separated by more than 100 μm [54]. These observations have not been followed up by subsequent studies; however, they could be explained by the intervention of TNTs as they span over such distances.

TNTs in the adult brain in health and diseases

Recently protrusions comparable to closed-ended TNTs have been described in the retina of a mouse *in vivo* [11]. In this elegant study, the authors showed the existence of interpericyte bridges called interpericyte tunneling nanotubes (IP-TNTs) that mediate bidirectional transfer of Ca2+ between distant pericytes on two separate capillary systems, forming a functional network in the vasculature plexus. This network allows a spatial–temporal regulation for the rapid redistribution of a limited amount of blood between connected capillary systems through IP-TNTs; the regulation phenomenon is called neurovascular coupling and enables more blood flow to run towards more active areas of the brain. Human pericytes were previously shown to connect to neuronal cells *in vitro* through TNTs and exchange both α-synuclein aggregates and electrical signals [55]. Furthermore, TNT-like protrusions originating from pericytes, connecting to other pericytes or endothelial

cells, have been shown to connect distant vessels in the fetal cortex and glioblastoma explants. Thus, they may play an essential role in the early phases of both physiological conditions and in tumor angiogenesis in the brain [56].

Interestingly Cx43, together with growth-associated protein 43 (GAP-43), appears to be responsible for the functional networking between glioma patient-derived stem cells (GSCs) implanted in a mouse brain [57]. Cells were interconnected by tumor microtubes (TMs), long and thick membrane protrusions containing actin and microtubules, which are able to propagate ion fluxes. Tumors of higher malignant grades formed more TMs and were more resistant to irradiation, while knockdown of Cx43 resulted in a decrease in TM-mediated connections and increased sensitivity to radiotherapy [57]. TMs are clearly distinct from TNTs [35] yet, these data support the role of protrusion-mediated signaling in the brain other than synaptic connections. Furthermore, our recent data indicate that organoids from Glioblastoma (GBM) patient stem cells (GSCs) can form TMs but also classical TNTs that allow the exchange of entire organelles like mitochondria between interconnected cancer cells [35]. TNTs appear prior to TMs in these organoids, suggesting that the TNT-mediated network may be instrumental in the formation of TMs and the invasive therapy-resistant GBM phenotype observed *in vivo* in mice xenografts.

The upregulation of Myo10 in the brain during nerve regeneration following sciatic axotomy or following peripheral nerve injury [58,59] suggests its role in resuming TNT formation in the adult brain. This is consistent with the fact that TNTs are stimulated by different stresses and inflammatory stimuli. Typically, amyloid aggregates increase the number of TNTs, and Myo10 overexpression appears to increase the spreading of prions and amyloid proteins between neurons [26]. Further studies should investigate whether Myo10 is upregulated in the brain in neurodegenerative diseases.

Consistent with this hypothesis, our recent data indicate that Eps8 overexpression also increases α-synuclein spreading between neurons, while we have shown that this is reduced in primary neurons derived from βCaMKII K/O mice (*Pepe* et al., *unpublished*, [43]). Exciting recent data indicate that neuron dedifferentiation is one of the mechanisms of neurodegeneration in early-onset familial Alzheimer's disease [60]. Because TNTs are more abundant in immature neurons, as well as neuronal precursors, this could be again a mechanism for disease progression [43].

Overall, these results allow us to postulate that both open-ended and closed-ended TNTs might have a fundamental role in brain development. This hypothesis extends the landscape for the investigation of TNT-mediated communication in brain function and dysfunction. I would suggest paying particular focus on their possible role in neurodevelopmental disorders, neurodegenerative diseases, and cancer.

Conclusions and perspectives

In 2004, the seminal discovery of TNTs as enabling a novel mechanism of cell-to cell communication challenged my understanding of many physio-pathological processes. TNT-mediated communication is already proven *in vitro* in multiple events/conditions, demonstrating their tremendous potential in many fields (immunology, virology, cancer, neuroscience, development, therapy). Yet, no important undertaking comes without challenges. On the one hand, we need to discover the molecular, structural, and biophysical characteristics of TNTs *in vitro* to enable our understanding of how they facilitate cell communication. On the other hand, we need to demonstrate their occurrence *in vivo*.

This review outlines that TNTs most likely exist during development and likely play a major role in the brain. This nonsynaptic intercellular mechanism of communication may be instrumental in orchestrating neuronal migration and in establishing functional neural circuits. While the construction of the synaptic-mediated circuitry may replace TNT-mediated communication in most of the brain, TNTs might still be present in selected areas during adult life. Although the previous hypothesis is speculative, we definitively know that TNTs can be exacerbated/highjacked in diseases (e.g., cancer, Neurodegenerative Diseases, infections) in response to stress and inflammatory signals [1,5,26,34].

In 1906, Camillo Golgi and Santiago Ramón y Cajal shared the Nobel prize in « recognition of their work on the structure of the nervous system » (https://www.nobelprize.org/prizes/medicine/1906/golgi/lecture/; https://www.nobelprize.org/prizes/medicine/1906/cajal/lecture/. Yet, the two scientists did not agree on the fundamentals; Golgi believed that brain cells were physically connected, allowing the brain to function as a whole (reticulon theory), while Cajal believed that brain cells worked as individual units (the neuron theory). Cajal had the unique intuition that they communicated through juxtapositions (later called synapses by Sherrington); he was correct, and this built a modern neuroscience principle on which tremendous advances have been based. Conversely, science works two ways, so in the light of these new TNT discoveries, should we go back and reconsider?

Conflict of interest statement

Nothing declared.

Acknowledgements

I would like to thank Martha Bowler, Diego Cordero-Cervantes, Michael Henderson, Harshawandhan Khare, and Olga Korenkova for their critical reading of the manuscript. I also thank all the present and past members of the lab for their continued support, excellent work, and stimulating discussion, and Reine Bouyssie for her superb assistance and devotion to the lab. TNT research in my lab is supported by grants from Equipe FRM (Fondation pour la Recherche Médicale) 2014 (DEQ 20140329557), Agence Nationale de la Recherche (ANR 16 CE160019-01 NEURO-TUNN), PSL-QLife Institut de convergence (ANR-17-CONV-0005 Q-LIFE), (PIA)-Inception (ANR-16-CONV-0005) and Cancéropôle Île-de-France grants (2018-1-PL-03-IP-1).

References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. Rustom A, Saffrich R, Markovic I, Walther P, Gerdes HH: **Nanotubular highways for intercellular organelle transport**. *Science* 2004, **303**:1007–1010.

2. Abounit S, Zurzolo C: **Wiring through tunneling nanotubes–from electrical signals to organelle transfer**. *J Cell Sci* 2012, **125**:1089–1098.

3. Ariazi J, Benowitz A, De Biasi V, Den Boer ML, Cherqui S, Cui H, Douillet N, Eugenin EA, Favre D, Goodman S, *et al.*: **Tunneling nanotubes and gap junctions-their role in long-range intercellular communication during development, health, and disease conditions**. *Front Mol Neurosci* 2017, **10**:333.

4. Baker M: **How the Internet of cells has biologists buzzing**. *Nature* 2017, **549**:322–324.

5. Rustom A: **The missing link: does tunnelling nanotube-based supercellularity provide a new understanding of chronic and lifestyle diseases?** *Open Biol* 2016, **6**:160057.

6. Yamashita YM, Inaba M, Buszczak M: **Specialized intercellular communications via cytonemes and nanotubes**. *Annu Rev Cell Dev Biol* 2018, **34**:59–84.

7. * Crespo AC, Mulik S, Dotiwala F, Ansara JA, Sen Santara S, Ingersoll K, Ovies C, Junqueira C, Tilburgs T, Strominger JL, *et al.*: **Decidual NK cells transfer granulysin to selectively kill bacteria in trophoblasts**. *Cell* 2020, **182**:1125–1139. e1118.
Nanotube transfer of the antimicrobial peptide granulysin (GNLY) allows human maternal decidual NK (dNK) cells to protect against infection of *Listeria monocytogenes* (Lm) while leaving the maternal–fetal barrier intact. This immune defense is not restricted to pregnancy. Although it is not clear whether these are bonafide TNTs, authors show that the transfer depends on actin and not on microtubules.

8. * Souriant S, Balboa L, Dupont M, Pingris K, Kviatcovsky D, Cougoule C, Lastrucci C, Bah A, Gasser R, Poincloux R, *et al.*: **Tuberculosis exacerbates HIV-1 infection through IL-10/STAT3-dependent tunneling nanotube formation in macrophages**. *Cell Rep* 2019, **26**:3586–3599. e3587.
TB is the most common co-infection of HIV-1 and is a leading cause of AIDS-related deaths. The authors report that TNTs are key players in the IL-10/STAT3 signaling axis, which increases virus spread in human macrophages in a TB context. They report the existence of thin and thick (tubulin-positive) TNTs between macrophages and provide functional data on transfer.

9. Wang Y, Cui J, Sun X, Zhang Y: **Tunneling-nanotube development in astrocytes depends on p53 activation**. *Cell Death Differ* 2011, **18**:732–742.

10. ** Cordero Cervantes D, Zurzolo C: **Peering into tunneling nanotubes—the path forward**. *EMBO J* 2021 Mar 1, e105789, https://doi.org/10.15252/embj.2020105789. Epub ahead of print. PMID: 33646572.
Extensive review focused on TNT structure, formation-related pathways, and biological function. It pinpoints gaps and dichotomies found across the field, discusses the methods employed to date, and suggests the application of new technologies to better study these unique structures.

11. ** Alarcon-Martinez L, Villafranca-Baughman D, Quintero H, Kacerovsky JB, Dotigny F, Murai KK, Prat A, Drapeau P, Di Polo A: **Interpericyte tunnelling nanotubes regulate neurovascular coupling**. *Nature* 2020, **585**:91–95.
Using multidisciplinary approaches, this elegant study describes nanotube-like processes that connect two pericytes on separate capillary systems, forming a functional network in the mouse retina, including in living animals. These structures are Cx43-positive, evocative of CX43-based gap junctions, and are similar to closed-ended TNTs.

12. Sharma M, Subramaniam S: **Rhes travels from cell to cell and transports Huntington disease protein via TNT-like protrusion**. *J Cell Biol* 2019, **218**:1972–1993.

13. Chinnery HR, Keller KE: **Tunneling nanotubes and the eye: intercellular communication and implications for ocular health and disease**. *BioMed Res Int* 2020, **2020**:7246785.

14. ** Sartori-Rupp A, Cordero Cervantes D, Pepe A, Gousset K, Delage E, Corroyer-Dulmont S, Schmitt C, Krijnse-Locker J, Zurzolo C: **Correlative cryo-electron microscopy reveals the structure of TNTs in neuronal cells**. *Nat Commun* 2019, **10**:342.
For the first time, sets up a pipeline for the ultrastructural analysis of TNTs by cryo-CLEM tomography and FIB-SEM in neuronal cells. Demonstrates that TNTs can be formed by a bundle of tubes (iTNTs) that can be open-ended at both ends. Shows membrane compartments, including mitochondria inside the TNT lumen, likely moving on actin cables.

15. * Kolba MD, Dudka W, Zareba-Koziol M, Kominek A, Ronchi P, Turos L, Chroscicki P, Wlodarczyk J, Schwab Y, Klejman A, *et al.*: **Tunneling nanotube-mediated intercellular vesicle and protein transfer in the stroma-provided imatinib resistance in chronic myeloid leukemia cells**. *Cell Death Dis* 2019, **10**:817.
CLEM and tomography reveal that TNTs between stromal and leukemic cells contain vesicles and are continuous with the cell body of one of the connected cells. Different from neuronal cells, these structures are formed by a single tube. SILAC reveals a nonautonomous proteome indicating transfer of specific sets of proteins with a potential role in survival and adaptation.

16. Dubois F, Benard M, Jean-Jacques B, Schapman D, Roberge H, Lebon A, Goux D, Monterroso B, Elie N, Komuro H, *et al.*: **Investigating tunneling nanotubes in cancer cells: guidelines for structural and functional studies through cell imaging**. *BioMed Res Int* 2020, **2020**:2701345.

17. Guo J, Wang G, Tang W, Song D, Wang X, Hong J, Zhang Z: **An optimized approach using cryofixation for high-resolution 3D analysis by FIB-SEM**. *J Struct Biol* 2020, **212**:107600.

18. Stuart DI, Subramaniam S, Abrescia NG: **The democratization of cryo-EM**. *Nat Methods* 2016, **13**:607–608.

19. * Bhat S, Ljubojevic N, Zhu S, Fukuda M, Echard A, Zurzolo C: **Rab35 and its effectors promote formation of tunneling nanotubes in neuronal cells**. *Sci Rep* 2020, **10**:16803.
One of the few study addressing the contribution of membrane trafficking pathways to TNTs. It demonstrates the role of Rab 35 in TNT formation, highlighting, as shown before for Rab8 and Rab 11, the involvement of the recycling pathway and the signaling specificity of TNTs formation compared to other protrusions.

20. ** Ljubojevic N, Henderson MJ, Zurzolo C: **The ways of actin: why tunneling nanotubes are unique cell protrusions**. *Trends Cell Biol* 2021, **31**:130–142, https://doi.org/10.1016/j.tcb.2020.11.008.
Unique in its content, this review addresses the role of actin regulators in the formation of TNTs and suggests potential players in comparison with other actin-based protrusions.

21. Jansens RJJ, Van den Broeck W, De Pelsmaeker S, Lamote JAS, Van Waesberghe C, Couck L, Favoreel HW: **Pseudorabies virus US3-induced tunneling nanotubes contain stabilized microtubules, interact with neighboring cells via cadherins, and allow intercellular molecular communication**. *J Virol* 2017, **91**. e00749-17.

22. Lee DM, Chen EH: **Drosophila myoblast fusion: invasion and resistance for the ultimate union**. *Annu Rev Genet* 2019, **53**:67–91.

23. ** Duan R, Kim JH, Shilagardi K, Schiffhauer ES, Lee DM, Son S, Li S, Thomas C, Luo T, Fletcher DA, *et al.*: **Spectrin is a mechanoresponsive protein shaping fusogenic synapse**

architecture during myoblast fusion. *Nat Cell Biol* 2018, **20**: 688–698.
By an integrated and multidisciplinary approach demonstrates that spectrin is required for myoblast fusion and accumulates in response to shear deformation at the fusogenic synapse. Reveals a function of spectrin as a mechanoresponsive protein with larger general implications.

24. Randrianarison-Huetz V, Papaefthymiou A, Herledan G, Noviello C, Faradova U, Collard L, Pincini A, Schol E, Decaux JF, Maire P, *et al.*: **Srf controls satellite cell fusion through the maintenance of actin architecture**. *J Cell Biol* 2018, **217**: 685–700.

25. Brukman NG, Uygur B, Podbilewicz B, Chernomordik LV: **How cells fuse**. *J Cell Biol* 2019, **218**:1436–1451.

26. Victoria GS, Zurzolo C: **The spread of prion-like proteins by lysosomes and tunneling nanotubes: implications for neurodegenerative diseases**. *J Cell Biol* 2017, **216**:2633–2644.

27. Wang X, Gerdes HH: **Transfer of mitochondria via tunneling nanotubes rescues apoptotic PC12 cells**. *Cell Death Differ* 2015, **22**:1181–1191.

28. Gousset K, Schiff E, Langevin C, Marijanovic Z, Caputo A, Browman DT, Chenouard N, de Chaumont F, Martino A, Enninga J, *et al.*: **Prions hijack tunnelling nanotubes for intercellular spread**. *Nat Cell Biol* 2009, **11**:328–336.

29. Korenkova O, Pepe A, Zurzolo C: **Fine intercellular connections in development: TNTs, cytonemes, or intercellular bridges?** *Cell Stress* 2020, **4**:30–43.

30. Abounit S, Bousset L, Loria F, Zhu S, de Chaumont F, Pieri L, Olivo-Marin JC, Melki R, Zurzolo C: **Tunneling nanotubes spread fibrillar alpha-synuclein by intercellular trafficking of lysosomes**. *EMBO J* 2016, **35**:2120–2138.

31. Chastagner P, Loria F, Vargas JY, Tois J, I Diamond M, Okafo G, Brou C, Zurzolo C: **Fate and propagation of endogenously formed Tau aggregates in neuronal cells**. *EMBO Mol Med* 2020, **12**, e12025.

32. * Goodman S, Naphade S, Khan M, Sharma J, Cherqui S: **Macrophage polarization impacts tunneling nanotube formation and intercellular organelle trafficking**. *Sci Rep* 2019, **9**: 14529.
Investigates macrophage-mediated TNT formation and intercellular trafficking in coculture with cystinotic cells and *in vivo* following transplantation in mice. Data support the induction of ticker TNT-like protrusions by cystinotic cells but also highlight some discrepancy between *in vivo* and *in vitro* results, warning about the risk of oversimplification from *in vitro* models.

33. * Hanna SJ, McCoy-Simandle K, Leung E, Genna A, Condeelis J, Cox D: **Tunneling nanotubes, a novel mode of tumor cell-macrophage communication in tumor cell invasion**. *J Cell Sci* 2019, **132**, jcs223321.
Shows that the formation of heterotypic TNTs between macrophages and breast tumor cells results in stimulation of an invasive tumor cell phenotype depending on EGF-EGFR signaling. Using an *in vivo* zebrafish model demonstrates that tumor cell invasion is enhanced when tumor cells are co-injected with control macrophages, while it is reduced in the presence of TNT-defective macrophages.

34. * Kretschmer A, Zhang F, Somasekharan SP, Tse C, Leachman L, Gleave A, Li B, Asmaro I, Huang T, Kotula L, *et al.*: **Stress-induced tunneling nanotubes support treatment adaptation in prostate cancer**. *Sci Rep* 2019, **9**:7826.
Shows that androgen receptor (AR) pathway inhibition (a common treatment for this cancer) induces Eps8-facilitated TNT production that is exploited for cancer cell survival. Propose a model where stress-induced TNT formation could be a mechanism for intercellular communication, stress adaptation, and treatment resistance in prostate cancer.

35. * Pinto G, Saenz-de-Santa-Maria I, Chastagner P, Perthame E, Delmas C, Toulas C, Moyal-Jonathan-Cohen E, Brou C, Zurzolo C: **Patient-derived glioblastoma stem cells transfer mitochondria through tunneling nanotubes in tumor organoids** 2021, **478**(1):21–39, https://doi.org/10.1042/BCJ20200710.
Shows for the first time coexistence of TNTs and MTs in tumor organoids from Glioblastoma patients derived stem cells. Proposes that both protrusions contribute to the establishment of a functional network in glioblastoma, which is essential for tumor survival and progression.

36. Gonzalez-Mendez L, Gradilla AC, Guerrero I: **The cytoneme connection: direct long-distance signal transfer during development**. *Development* 2019, **146**, dev174607.

37. Gerdes HH, Rustom A, Wang X: **Tunneling nanotubes, an emerging intercellular communication route in development**. *Mech Dev* 2013, **130**:381–387.

38. Wang T, Xu Z, Jiang W, Ma A: **Cell-to-cell contact induces mesenchymal stem cell to differentiate into cardiomyocyte and smooth muscle cell**. *Int J Cardiol* 2006, **109**:74–81.

39. ** Huang H, Liu S, Kornberg TB: **Glutamate signaling at cytoneme synapses**. *Science* 2019, **363**:948–955.
Genetic, histological, and functional evidence show that components of the presynaptic and postsynaptic compartments of neuronal synapses, as well as Ca^{2+} influx, are essential for cytoneme-mediated signaling in the Drosophilaair sac primordium. Proposes that cytoneme contacts are functional glutamatergic synapses and that glutamate-dependent signaling is also present in nonneuronal epithelial cells.

40. ** Ozel MN, Kulkarni A, Hasan A, Brummer J, Moldenhauer M, Daumann IM, Wolfenberg H, Dercksen VJ, Kiral FR, Weiser M, *et al.*: **Serial synapse formation through filopodial competition for synaptic seeding factors**. *Dev Cell* 2019, **50**:447–461.e448.
Using live imaging and computational modeling, this paper investigates the relationship between filopodial dynamics and synapse assembly. A 'winner takes all' distribution of synaptic seeding factors renders one filopodium at a time synaptogenic, thereby pacing development and ensuring robust connectivity.

41. Delage E, Cervantes DC, Penard E, Schmitt C, Syan S, Disanza A, Scita G, Zurzolo C: **Differential identity of Filopodia and Tunneling Nanotubes revealed by the opposite functions of actin regulatory complexes**. *Sci Rep* 2016, **6**:39632.

42. Menna E, Zambetti S, Morini R, Donzelli A, Disanza A, Calvigioni D, Braida D, Nicolini C, Orlando M, Fossati G, *et al.*: **Eps8 controls dendritic spine density and synaptic plasticity through its actin-capping activity**. *EMBO J* 2013, **32**: 1730–1744.

43. * Vargas JY, Loria F, Wu YJ, Cordova G, Nonaka T, Bellow S, Syan S, Hasegawa M, van Woerden GM, Trollet C, *et al.*: **The Wnt/Ca(2+) pathway is involved in interneuronal communication mediated by tunneling nanotubes**. *EMBO J* 2019, **38**, e101230.
Shows TNTs between immature cortical neurons and demonstrate the role of the Wnt-CaMKII mediated pathway forTNT formation and spreading of α–synuclein aggregates. Opens the field to further investigations.

44. Borgesius NZ, van Woerden GM, Buitendijk GH, Keijzer N, Jaarsma D, Hoogenraad CC, Elgersma Y: **betaCaMKII plays a nonenzymatic role in hippocampal synaptic plasticity and learning by targeting alphaCaMKII to synapses**. *J Neurosci* 2011, **31**:10141–10148.

45. Mattes B, Dang Y, Greicius G, Kaufmann LT, Prunsche B, Rosenbauer J, Stegmaier J, Mikut R, Ozbek S, Nienhaus GU, *et al.*: **Wnt/PCP controls spreading of Wnt/beta-catenin signals by cytonemes in vertebrates**. *Elife* 2018, **7**, e36953.

46. Gousset K, Marzo L, Commere PH, Zurzolo C: **Myo10 is a key regulator of TNT formation in neuronal cells**. *J Cell Sci* 2013, **126**:4424–4435.

47. Sousa AD, Berg JS, Robertson BW, Meeker RB, Cheney RE: **Myo10 in brain: developmental regulation, identification of a headless isoform and dynamics in neurons**. *J Cell Sci* 2006, **119**:184–194.

48. Raines AN, Nagdas S, Kerber ML, Cheney RE: **Headless Myo10 is a negative regulator of full-length Myo10 and inhibits axon outgrowth in cortical neurons**. *J Biol Chem* 2012, **287**:24873–24883.

49. Yu HL, Peng Y, Zhao Y, Lan YS, Wang B, Zhao L, Sun D, Pan JX, Dong ZQ, Mei L, *et al.*: **Myosin X interaction with KIF13B, a crucial pathway for netrin-1-induced axonal development**. *J Neurosci* 2020, **40**:9169–9185.

50. Lai M, Guo Y, Ma J, Yu H, Zhao D, Fan W, Ju X, Sheikh MA, Malik YS, Xiong W, *et al.*: **Myosin X regulates neuronal radial migration through interacting with N-cadherin**. *Front Cell Neurosci* 2015, **9**:326.

51. Belousov AB, Fontes JD, Freitas-Andrade M, Naus CC: **Gap junctions and hemichannels: communicating cell death in neurodevelopment and disease**. *BMC Cell Biol* 2017, **18**:4.

52. Rozental R, Srinivas M, Gokhan S, Urban M, Dermietzel R, Kessler JA, Spray DC, Mehler MF: **Temporal expression of neuronal connexins during hippocampal ontogeny**. *Brain Res Brain Res Rev* 2000, **32**:57–71.

53. Elias LA, Wang DD, Kriegstein AR: **Gap junction adhesion is necessary for radial migration in the neocortex**. *Nature* 2007, **448**:901–907.

54. Allison DW, Ohran AJ, Stobbs SH, Mameli M, Valenzuela CF, Sudweeks SN, Ray AP, Henriksen SJ, Steffensen SC: **Connexin-36 gap junctions mediate electrical coupling between ventral tegmental area GABA neurons**. *Synapse* 2006, **60**:20–31.

55. Dieriks BV, Park TI, Fourie C, Faull RL, Dragunow M, Curtis MA: **alpha-synuclein transfer through tunneling nanotubes occurs in SH-SY5Y cells and primary brain pericytes from Parkinson's disease patients**. *Sci Rep* 2017, **7**:42984.

56. Errede M, Mangieri D, Longo G, Girolamo F, de Trizio I, Vimercati A, Serio G, Frei K, Perris R, Virgintino D: **Tunneling nanotubes evoke pericyte/endothelial communication during normal and tumoral angiogenesis**. *Fluids Barriers CNS* 2018, **15**:28.

57. Osswald M, Jung E, Sahm F, Solecki G, Venkataramani V, Blaes J, Weil S, Horstmann H, Wiestler B, Syed M, *et al.*: **Brain tumour cells interconnect to a functional and resistant network**. *Nature* 2015, **528**:93–98.

58. Plantman S, Zelano J, Novikova LN, Novikov LN, Cullheim S: **Neuronal myosin-X is upregulated after peripheral nerve injury and mediates laminin-induced growth of neurites**. *Mol Cell Neurosci* 2013, **56**:96–101.

59. Tanabe K, Bonilla I, Winkles JA, Strittmatter SM: **Fibroblast growth factor-inducible-14 is induced in axotomized neurons and promotes neurite outgrowth**. *J Neurosci* 2003, **23**:9675–9686.

60. * Caldwell AB, Liu Q, Schroth GP, Galasko DR, Yuan SH, Wagner SL, Subramaniam S: **Dedifferentiation and neuronal repression define familial Alzheimer's disease**. *Sci Adv* 2020, **6**, eaba5933.

Shows that human induced pluripotent stem cell (hiPSC)-derived neurons from donor patients with early-onset familial Alzheimer's disease dedifferentiate to a precursor-like state with signatures of ectoderm and nonectoderm lineages.