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Tunneling Nanotube： a Novel Type of Signal Transmission in The Nervous System*

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Abstract Tunneling nanotubes (TNTs) are F-actin-based thin channel-like structures connecting distant cells, which provide a new route for intercellular communication. Since TNTs are discovered, an increasing number of studies have demonstrated their roles in the transfer of diverse cargoes between connecting cells, including signaling molecules, RNAs, proteins, organelles, and even pathogens, which illustrate the diversity and complexity of TNTs' function. TNTs have been found in various types of cells, including neuronal cells. In the nervous system, the formation of TNTs between neurons or between neurons and astrocytes mediates electrical coupling and the transfer of pathogenic proteins associated with neurodegenerative diseases. Here, we summarized the current results of TNTs in the nervous system, including its formation, regulatory factors, functions, and potential benefits in the treatment of diseases.

Key words tunneling nanotubes (TNTs), nervous system, neurodegenerative diseases
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1 Introduction

Tunneling nanotubes (TNTs) have been regarded as a novel way for intercellular communication since they were discovered by Rustom and colleagues[1]. Distinct from other ways of cell-cell communication, such as gap-junctions or cell-cell contacts between adjacent cells, TNTs are membrane channel structures directly connecting the cytosol of two non-contact cells, which have the potential to mediate long-range intercellular communication. The TNT-like structures have been observed in various cell types[2]. A growing number of researches have revealed that TNTs have powerful functions in intercellular communication. A variety of cellular cargoes, such as proteins, vesicles, mitochondria, Golgi apparatus, and lysosomes have been reported to be transported by TNTs. In addition, pathogens can be transferred through TNTs from infected cells to naïve cells, including prion, HIV, and influenza virus[3-5].

Inclusions of protein aggregates are the pathological hallmark of a number of neurodegenerative diseases, such as Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) [6-7]. Accumulation of protein aggregates has been linked to the dysfunction of neurons, which leads to neuronal loss[8]. The spreading of protein aggregates in the brain is likely to contribute to the progression of neurodegenerative diseases. However, the exact mechanisms of spreading of pathogenic protein aggregates are not fully understood. Recently, numerous studies have demonstrated the role of TNTs in transferring protein aggregates in neuronal cells[4,9-12]. Here in this review, based on the recent literatures, we aim to have a better understanding of the formation and function of TNTs in the nervous system. We will discuss some unsolved problems in

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TNTs so as to promote the follow-up research progress and advance their emerging roles in health and disease.

2 The discovery of TNTs: structural characteristics and potential functions

In 2004, Rustom *et al.*[1] reported a thin membrane structure connecting distant cells, which mediating the transfer of membrane vesicles and organelles. These structures were named as "tunneling nanotubes" (TNTs). As described in this study, the characteristics of TNTs have been summarized into the following aspects: (1) They have a diameter of 50−200 nm; (2) Their length is up to several cell diameters and they rarely have a branched appearance; (3) They do not attach to any substrate; (4) They are sensitive to prolonged light excitation, mechanical stress and chemical stress (such as PFA), which are all leading to rupture, but they are resistant to trypsin-EDTA treatment; (5) They have enriched actin, but not microtubules[13].

Since its discovery, TNTs have been found in various types of cells[14]. With the increase of researches, TNTs exhibit diverse morphology and structural composition. TNTs have been found with a diameter much larger than 200 nm[4,15-16]. In addition, TNTs' length varies from a few microns to hundreds of microns[17]. Initially, TNTs were regarded as actin-enriched structures without microtubules. Studies have shown that F-actin polymerization is critical for TNT formation, whereas depolymerization of F-actin by chemicals or drugs can efficiently suppress the formation of TNTs[18-19]. However, recent works have shown that microtubules are also the common components of some TNTs[16,20-21]. Moreover, intermediate filaments have been found in TNTs[22]. As more research progresses come along, some previous inappropriate descriptions of TNTs have already been excluded, such as length and thickness. In short, the shared features of TNTs include the following aspects: a. They connect at least two cells; b. They do not attach to the substrate, distinguishing them from other cellular protrusions, such as filopodia; c. They contain F-actin[23-24].

TNTs have powerful functions for intercellular communication. Membrane vesicles and organelles have been detected inside TNTs since the original report[1]. Over the past few years, a variety of cytoplasmic components were found to be transported intercellularly *via* TNTs, including mitochondria, vesicles, lysosomes, Golgi apparatus, proteins, possibly RNA granules, and even pathogens (such as viruses and bacteria) [25-26]. TNTs mediated signaling transport or organelles transfer plays an important role in both physiological and pathological conditions[27]. In physiological conditions, TNTs are involved in cell reprogramming, repair of mitochondrial dysfunction, senescence, angiogenesis, and differentiation[15,28-33]. Under pathological conditions, TNTs promote the progression of diseases, such as neurodegenerative diseases, AIDS, and cancers[3,12,34-35]. Here, we summarize the existing literatures on the formation and role of TNTs in the nervous system as well as the potentials in neurological diseases.

3 TNTs in the nervous system

3.1 Formation and molecular regulators of TNTs

TNTs have been observed in a variety of cell types, including cell lines and primary cells dissected from nerve tissue[36]. Time-lapse imaging has shown that TNTs are formed by at least two ways: a. Separating from two contact cells; b. One cell forms a filopodium-like structure and then extends to reach another cell to form TNTs. Both forms of TNT formation are found in the cells of neuronal culture[13,37]. These two forms do not interfere with each other and they can coexist in the same cell type[38].

Increased TNT formation has been observed when cells are under stress, such as oxidative stress, serum deprivation, pathogenic protein aggregation, and infection with pathogens[14]. However, no universal molecular mechanism regulating TNT formation has been revealed so far. Recent studies have uncovered several genes/pathways that regulate TNT formation in the nervous system (Figure 1 and Table 1). Actin polymerization is required for TNT formation; thus it is not surprising that proteins regulating actin dynamics may modulate TNT formation. Cdc42, a Rho family of GTPase, has been reported to regulate actin polymerization and promote TNT formation by regulating Arp2/3 and WASP in macrophages[39]. However, in neuronal CAD cells, Cdc42, acting as a negative regulator, inhibited TNT formation and TNT-mediated vesicle transfer *via* Cdc42/IRSp53/VASP network. Epidermal growth

factor receptor pathway 8 (Eps8), another actin regulator, increased TNT formation dependent on its bundling activity[40]. The role of Eps8 in regulating TNT formation has also been reported in cancer cells[41]. Myosin Ⅹ is an actin-based motor protein with ATPase activity, which regulates filopodia formation and plays an important role in neurite outgrowth and axon guidance[42-43]. In addition, myosin Ⅹ could regulate the extension of actin by transporting VASP to the tips of filopodia to compete with actin capping proteins[44]. TNTs can arise from filopodium-like structures, suggesting that myosin Ⅹ may play a role in regulating TNT formation. Indeed, a study has found that TNTs arose from a subset of myosin Ⅹ -driven filopodia in neuronal CAD cells. Myosin Ⅹ could promote the formation of TNTs, and its F2 subdomain of FERM domain was essential to promote TNT formation[38]. Wnt pathway, a classical signaling pathway involved in diverse cellular processes, has also been found to regulate the formation of TNTs. The activated Wnt/Ca^{2+} pathway promoted the formation of TNTs in primary neurons (by Wnt5a) and neuronal CAD cells (by Wnt7a) by modulating the interaction between βCaMKII and actin cytoskeleton, which facilitating actin polymerization and stabilization of TNTs[45]. In addition to actin polymerization, vesicular trafficking has been revealed to be involved in the formation of TNTs[46-47]. Rab GTPases are master regulators of intracellular membrane trafficking, which surely plays a role in the traffic of vesicles[48]. Rab GTPases also regulate cytoskeleton dynamics[49]. These results suggest that Rab GTPases may be involved in regulating the formation of TNTs. Indeed, Rab8a and Rab11a have been reported to promote TNT formation in primary Schwann cells[50]. Furthermore, a latest research confirmed that Rab11a-Rab8a cascade promoted TNT formation in neuronal CAD cells, and the formation of TNTs was regulated by the downstream effector of Rab8a, VAMP3[51].

In addition to cytoskeleton regulatory factors, some genes or pathways involved in cell signaling regulate TNT formation. In an earlier study, Wang *et al*.[9] found that oxidative stress (H_2O_2 treatment) and serum depletion induced TNT formation in rat primary astrocytes and neurons. Further study suggested that the formation of these TNTs was dependent on the activation of p53 along with its target genes, EGFR, Akt, PI3K, and mTOR, contributing to the formation of TNTs[9]. Furthermore, the results showed that p53 activation efficiently induced the overexpression of M-Sec, a well-known inducing factor of TNT formation. Thus, it is proposed that M-Sec might be the downstream effector for p53, EGFR, or Akt/PI3K/mTOR, triggering F-actin polymerization and mediating the formation of TNTs. In this study, TNTs have been observed to form invariably from stressed cells toward the unstressed cells. Follow-up studies from the same group revealed that S100A4 acted as a navigator to determine the direction of TNTs. In stressed cells, p53 activated caspase-3, which led to S100A4 cleavage, thus creating a concentration gradient between stressed (low) and target (high) cells. Then, the concentration gradient of S100A4 guided the formation of TNTs along with its receptor, RAGE[52]. So far, S100A4 is the only reported guidance factor for TNT formation. Furthermore, a latest research showed that Rhes, a brain-enriched GTPase/SUMO E3-like protein, induced the biogenesis of TNT-like cellular protrusions in striatal neuronal cells. The full-length wild-type Rhes could be transported in these TNT-like tunnels so that these structures were referred to as “Rhes tunnels” in this study. Further work demonstrated that the SUMO E3-like domain of Rhes was required to promote TNT formation, and the mutation of Ser33 in the N-terminal GTPase domain abrogated Rhes' activity in promoting the formation of TNT-like Rhes tunnels. These results indicate that both the GTPase domain and the SUMO E3 ligase domain of Rhes coordinate to induce TNT-like Rhes tunnels[53].

Although several genes/pathways have been found to be involved in the regulation of TNT formation, it is not difficult to find that these results were all obtained from *in vitro* experiments and have not been verified *in vivo*. Besides, the expression of these genes is not limited to TNTs, and they cannot be recognized as specific molecular markers to identify TNTs. Currently, it is still incapable of distinguishing TNTs from other cellular protrusions by labeled molecular marker, which hinders the development of experiments *in vivo*. Therefore the identification of specific markers for TNTs can greatly promote progress in this field.

3.2 Potential physiological role of TNTs in the nervous system

TNTs have been reported to play a role in various physiological processes by transferring diverse cargoes[2,23]. Electrical coupling is one of the important physiological functions of TNTs, which has been studied in neurons. In 2010, the electrical coupling by TNTs was firstly discovered by Wang *et al*.[54] in NRK cells, HUVEC, HEK293 cells, and quail neuronal crest cells (NCC). The depolarization of the TNTs-connected cells activated low-threshold voltage-gated Ca^{2+} channels, thus leading to an increase in intracellular Ca^{2+} levels. Further studies demonstrated that the electrical coupling was dependent on gap junction, connexin 43 (CX43) proteins. Since neuronal PC12 cells did not express CX43, the TNTs formed by PC12 cells could not occur electrical coupling. The electrical coupling in the nervous system mediated by TNTs was also reported by the same group. This work demonstrated that immature hippocampal neurons and adult astrocytes could be electrically coupled through TNTs, which was dependent on CX43 expression. With the neuronal differentiation, the expression of CX43 in neurons decreased, and the TNTs formed between neurons and astrocytes did not mediate the electrical coupling any more[37]. The activation of low-threshold voltage-gated Ca^{2+} channels was also observed in neurons and astrocytes. Calcium signals have been suggested to be the important regulators for proliferation, migration, and differentiation of neurons[55]. In addition, astrocytes play a significant role in maintaining brain hemostasis and participating in guiding neuronal progenitor cell migration[56]. These results suggest that TNTs-mediated electrical coupling between astrocytes and neurons might be a signaling mechanism to regulate early brain development. Moreover, the radial glia extends radial fibers to guide the migration of embryonic neurons from the ventricular region to the cortical plate, in which gap junction proteins Connexin 26 and 43 are required[57]. Combined these results, it can be speculated that the presence of TNTs in the nervous system provides an efficient way for information processing, especially in the early stages of neural development.

3.3 TNTs in neurological diseases

TNTs are multifunctional structures, which have been well-studied in many types of cells, including cancer cells, immune cells, and neuronal cells. TNTs play an important role in both physiological and pathological conditions[27]. Although the physiological functions of TNTs have not been well studied, most studies are currently focusing on the role of TNTs in diseases including neurological disorders. Here we summarize two aspects of TNTs' role in neurological diseases according to the functions by transferring mitochondria and pathogens (Figure 1 and Table 2).

Mitochondria, as the powerhouse of the cell, play a key role in the maintenance of normal physiological functions of cells and the progression of diseases. Mitochondrial dysfunction in neurons leads to brain damage which is usually caused by hypoxic-ischemia[58-59]. Lower levels of oxygen increase the production of ROS, and then impair mitochondrial function, thus triggering neuronal death. Since mitochondria are the most common cargoes of TNTs, different studies have reported that TNT-mediated mitochondrial transport can effectively improve cell survival[60-62]. Thus TNT-mediated mitochondrial transport is likely to help to reduce cell injury caused by hypoxic-ischemia. *In vitro* experiments indicated that mitochondria could be transferred from mesenchymal stem cells (MSCs) to $CoCl_2$-induced PC12 cells *via* TNTs, resulting in ameliorating mitochondrial dysfunction and reducing cell injury of PC12 cells[63]. Mitochondria have been reported to be transferred by TNTs from multipotent mesenchymal stem cells (MMSCs) to ischemic injured astrocytes or neuron-like PC12 cells, restoring the bioenergetics of the recipient cells and stimulating their proliferation[64]. These results suggest that increasing TNT formation by genetic manipulation or medication may have a beneficial effect by preventing hypoxic-ischemic brain injury.

The most commonly studied function of TNTs in diseases is to mediate the transfer of pathogens, including prions, bacteria, and viruses[2]. The pathological hallmark of a number of neurodegenerative diseases is the intracellular or extracellular inclusions of protein aggregates in the brain[7]. The spreading of protein aggregates in the brain has been proposed to be associated with the pathological progression of neurodegenerative diseases. Numerous studies have shown that disease-related protein aggregates could be intercellularly transferred by TNTs, contributing to their spreading

anatomically for the underlying pathology[10-11,65]. In an *in vitro* experiment, PrP^{Sc} (an infectious form of prions) was transferred between neuronal CAD cells *via* TNTs. PrP^{Sc} could also be transferred from bone marrow-derived dendritic cells to primary neurons, which may be further transferred to the central nervous system[4]. Astrocytes are one of the earliest sites where prion accumulation occurs. These prion-infected astrocytes might be responsible for disease propagation by causing neuronal damage[66-67], but the exact mechanism is still unclear. A recent study offers a possible explanation by showing that astrocytes could transfer PrP^{Sc} to neurons by forming TNTs between them[68]. Another example of protein aggregates spreading from cell to cell by TNTs is α-synuclein. It has been reported that α-synuclein fibrils inside lysosomal vesicles were efficiently transferred between neuronal cells through TNTs, and the soluble α-synuclein was then seeded to form aggregates by transferred fibrils in the recipient cells[11]. TNT-mediated α-synuclein transfer was also observed by other groups in neuronal CAD cells, primary neurons, astrocytes, SH-SY5Y cells, and primary brain pericytes from patients with Parkinson's disease[12,45,69]. These results provide a clue for how non-neuronal cells play a role in disease progression. Furthermore, mutant huntingtin has been reported to promote TNT formation, which in turn provides an efficient mechanism for its transfer in neuronal cells and primary neurons[10]. Recently, Rhes, a brain-enriched GTPase/SUMO E3-like protein, was demonstrated to promote TNT formation in mouse striatal neurons, thus facilitating the transfer of mHTT[53]. Most importantly, experimental evidence has been obtained from different disease-associated proteins including Aβ, TDP-43, and tau, supporting TNT-mediated transfer for pathogenic protein aggregates[9,35,65,70]. Together, these results suggest that TNTs may serve as a common mechanism for disease progression in neurodegenerative disorders, thus providing a potential target for the treatment of these devastating diseases.

3.4 Potential role of TNTs in the treatment of neurological diseases

Reviewing the above experimental results, we find that TNTs, as one of the highly efficient transmission routes of pathogens or pathogenic proteins, play a great role in promoting the progression of neurological diseases. Therefore, a hypothesis is proposed whether inhibiting the formation of TNTs can delay the disease progression and reverse the disease status. Although there is no *in vivo* study so far, some clues and circumstantial evidence can be retrieved from *in vitro* studies which can provide a reference for future *in vivo* research.

Dilsizoglu *et al*.[18] reported that tolytoxin, a cyanobacteria macrolide that targets actin by inhibition of its polymerization, significantly inhibited the formation of TNTs in neuronal cells (SH-SY5Y), and the transfer of α-synuclein fibrils as well. Besides, tolytoxin at low nanomolar concentrations can specifically reduce TNT formation without affecting filopodia and inducing cell death or dramatic morphological changes. These results suggest that inhibition of TNTs is feasible and tolytoxin may be a candidate drug, but further research is needed.

Everything could be a double-edged sword, and so do TNTs. TNTs can not only transfer pathogenic particles as a connecting pipe but also be utilized as an alternative strategy to treat neurological diseases. As mentioned above, TNTs could mediate the transfer of mitochondria from healthy cells to injured cells, which preventing neuronal cell death *in vitro*[63-64]. Therefore, it is reasonable to propose that promoting connections between damaged neurons and surrounding healthy cells through TNTs *in vivo* may play a beneficial role in repairing damaged brain tissue. At present, the biggest challenge in the treatment of neurological diseases is how to facilitate drugs to cross the blood-brain barrier (BBB) more efficiently. Engineered nanoparticles (NPs) can effectively cross BBB and have a promising application targeting lesions in the central nervous system (CNS) to deliver drugs at the proper position. However, how NPs can be transmitted quickly between neurons after crossing the BBB is still unknown. Tosi *et al*.[71] demonstrated that NPs can be transferred between glial cells and neurons in an F-actin-dependent manner *via* TNTs. Increasing TNTs by M-Sec can then promote NPs transmission. A recent study also confirmed that TNTs could be used as a new strategy for NPs delivery in the brain[72].

4 Conclusions and perspectives

Recent studies have revealed the important role of TNTs in physiological and pathological conditions.

TNTs as a novel type of intercellular communication, their formation, functions, and the regulatory factors in the nervous system are documented and summarized in Figure 1. Combined with existing results, TNTs appear to be a universal mechanism for the progression of neurological diseases, which promote the transmission of pathological proteins between neurons and other non-neuronal cells.

Nonetheless, our understanding of TNTs is inadequate and there are still many challenging questions worth of investigation. The molecular mechanisms of TNT formation are not fully understood. In particular, currently identified genes/pathways regulating TNT formation cannot be used as specific markers for TNTs because they are not limited to TNTs but widely expressed in cells, thus hindering the development of *in vivo* experiments. In addition, TNTs play a double-edged role in neurological diseases because of their featured function. The proper regulation of TNT formation is so complex that should be well considered in disease progression. A better understanding of TNTs is surely beneficial for the treatment of neurological disorders.

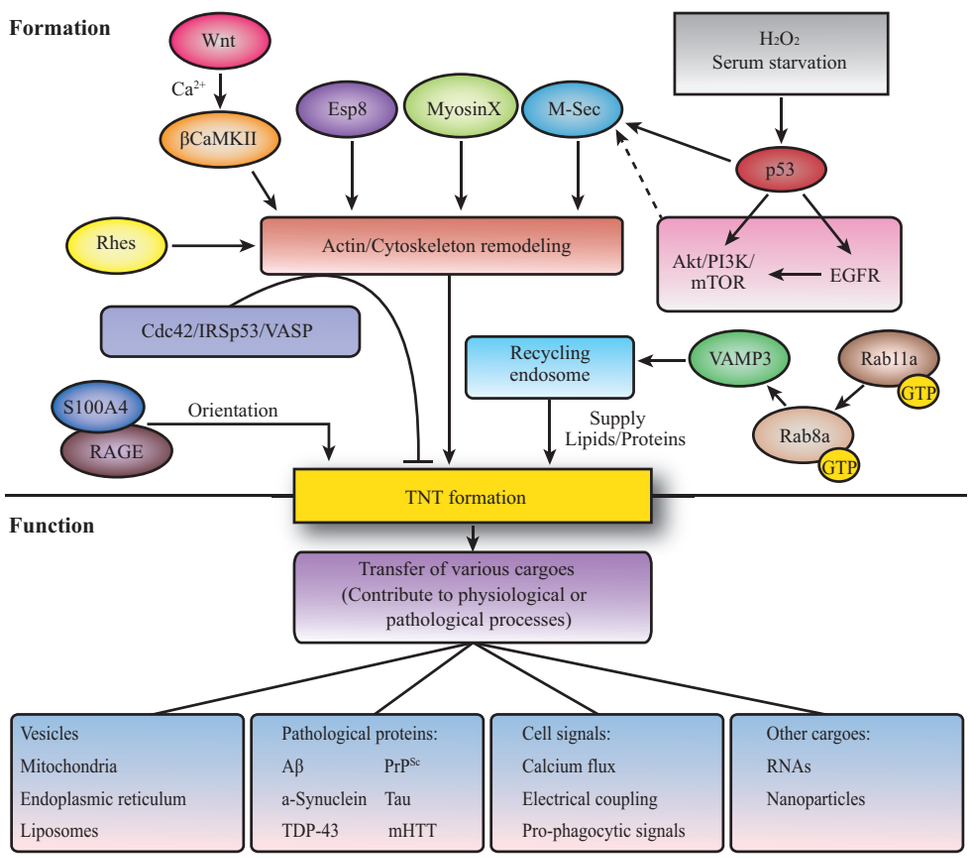

Fig. 1　Schematic molecular mechanisms in the regulation of TNT formation and potential functions of TNTs in the nervous system

Representative signaling molecules described in the text are shown in different colored shapes, which play a regulatory role in TNT formation.

Table 1　Genes/pathways involved in the formation of TNTs in the nervous system

Genes/Pathways	Cell types	References
p53/EGFR/Akt/mTOR	Rat primary astrocytes and neurons	[9]
Myosin X	Neuronal CAD cells	[38]
CDC42/IRSp53/VASP and Esp8	Neuronal CAD cells	[40]
Rab8a and Rab11a	Primary Schwann cells	[50]
Rab11a/Rab8a/VAMP3	Neuronal CAD cells	[51]
Wnt /Ca^{2+}/CAMKII	Neuronal CAD cells	[45]
Rhes	$STHdh^{Q7/Q7}$ cells	[53]

Table 2 Cargoes of TNTs in the nervous system

Proposed function	Cell types	References
Transfer of vesicles or organelles		
Vesicles	PC12 cells	[1]
	Neuronal CAD cells	[38]
	Neuronal CAD cells	[51]
Mitochondria	PC12 cells	[20]
	Schwann cells	[50]
	MSCs to PC12 cells	[64]
	MSCs to PC12 cells	[63]
Endoplasmic reticulum	SH-SY5Y cells	[73]
Liposomes	U87, NHA cells	[74]
Transfer of proteins		
Aβ	Rat primary astrocytes and neurons	[9]
mHTT	Neuronal CAD cells， primary neurons	[10]
	$STHdh^{Q7/Q7}$ cells	[53]
α-Synuclein	Neuronal CAD cells	[11]
	Primary mouse cortical neurons	[45]
	SH-SY5Y cells	[69]
	Astrocytes	[12]
PrP^{Sc}	Neuronal CAD cells	[4]
	Neuronal CAD cells	[75]
	Astrocytes to neurons	[68]
Tau	Neuronal CAD cells, primary neurons	[35]
	Neuronal CAD cells	[70]
Rhes	$STHdh^{Q7/Q7}$ cells	[53]
TDP-43	U251 cells	[65]
Transmission of cell signals		
Calcium flux	SH-SY5Y	[73]
Electrical coupling	Astrocytes to neurons	[37]
Pro-Phagocytic Signals	PC12 cells	[76]
Transfer of other cargoes		
RNAs	Schwann cells	[50]
Nanoparticles	Glial cells and neurons	[71]
	Primary astrocytes and neurons	[72]

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隧道纳米管：神经系统中的新型细胞间交流结构*

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摘要　隧道纳米管（tunneling nanotubes，TNTs）是基于细胞骨架尤其是纤维状肌动蛋白形成的细胞间管道样结构，其功能主要是介导广泛的细胞间物质交换，包括各种信号分子、RNA、蛋白质、细胞器甚至病原体，在生理和病理过程中都发挥重要作用. 各种细胞类型中均发现有TNTs的形成，尤其在神经元细胞和神经胶质细胞中得到广泛关注. 神经元细胞间或神经元细胞与星形胶质细胞间形成的TNTs，能够介导电耦合，还参与神经退行性疾病相关致病蛋白质的转移和/或传播，进而在神经系统发育和疾病进展中发挥作用. 本文简要总结了在神经系统细胞间形成TNTs的研究进展，包括调节其形成的分子机制、功能和在神经系统疾病治疗中的潜在优势.

关键词　隧道纳米管，神经系统，神经退行性疾病
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