The Triple Functional Domain Protein Trio with Multiple Functions in the Nervous System
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Summary
Neuronal development, including neurogenesis, neuronal migration, axon outgrowth and guidance, dendritic arborization and synaptic formation, requires proper regulation of cytoskeletal rearrangement. Abnormal regulation of these processes results in neurodevelopmental disorders. Rho GTPases and their regulators, including Rho GEFs and GAPs are implicated in the regulation of such diverse processes. Recently, mutations in a GEF Trio were identified to be associated with various neurodevelopmental disorders, including schizophrenia, autism, bipolar disorder, and intellectual disability. The functions of Trio have been previously widely studied in neuronal development for many years. Here, we provide a comprehensive overview of the diverse functions of the triple functional domain protein Trio in neuronal development, emphasizing the genetic evidence in neurite outgrowth, axon guidance, dendritic arborization, and synaptic transmission obtained from the worm, fly and mice, as well as cultured primary neurons. We also discuss the mutations identified in TRIO gene in individuals with neurodevelopmental disorders, mainly focusing on the mutation-function relationship underlying these diseases.

Introduction
Rho GTPases, especially the widely studied Rac1, Cdc42, and RhoA play essential functions in neuronal development, including neurogenesis, neuronal migration, axon outgrowth and guidance, dendritic arborization and synaptic formation.1,2 Mutations or functional dysregulation of these Rho GTPases often lead to neurodevelopmental disorders, including schizophrenia, autism, bipolar disorder, and intellectual disability.3-6 Biochemically, Rho GTPases cycle between GTP-bound active state and GDP-bound inactive state and are mainly regulated by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs).7 Specifically, GEFs activate Rho GTPases by accelerating the exchange of GDP for GTP; GAPs inactivate Rho GTPases by promoting the hydrolysis of GTP to GDP; and GDIs block Rho GTPases activation by sequestering them in the GDP-bound state.8 In recent years, human mutations identified in such a GEF Trio and its functional studies have broadened our understanding of the functions Rho GTPases and their regulators played in neuronal development and neurodevelopmental disorders.

Human Trio was firstly identified over 20 years ago by yeast interaction-trap assay in an attempt to find the interacting proteins involved in the signaling pathway mediated by LAR (leukocyte common antigen-related protein), which is a transmembrane protein tyrosine phosphatase and is proposed to regulate cell-matrix interactions.9
The full name of Trio is **Triplet functional domain** because mammalian Trio contains three enzymatic domains: An N-terminal GEF (GEF1) domain, a C-terminal GEF (GEF2) domain and a protein serine/threonine kinase (PSK) domain located at the most C-terminal. The two GEF domains activate the Rho family GTPases via accelerating GTP/GDP exchange, while the function of the PSK domain is still unknown and less studied, but structurally, this domain has a high sequence similarity to calcium/calmodulin dependent PSKs, such as myosin light chain kinase (MLCK).

Trio orthologs in invertebrates, such as *C. elegans* UNC-73 and *Drosophila* trio lack the kinase domain, indicating that new functions of the multi-domain protein may emerge in vertebrates, although human Trio does not show any kinase activity to a number of artificial substrates by *in vitro* assays. In developing rodent brain, multiple isoforms of Trio are expressed. The expression of the kinase domain-containing full-length Trio is extremely low in the nervous system with other shorter isoforms. Both of the two shorter isoforms, Trio9L and Trio9S (also known as TrioD and TrioA, respectively) contain both GEF1 domain and GEF2 domain, and express as the most abundant isoforms in the brain, thus the most abundant isoforms show the highest similarities to their orthologs in worm and fly. An even shorter isoform Trio8 (also known as TrioC) that contains only GEF1 domain but no GEF2 domain is cerebellum-specific, highlighting its role in cerebellar development.

This review briefly discusses the biochemical and cell biological properties of Trio GEFs before focusing on the genetic evidence of Trio’s role in neuronal development and synaptic transmission. We will further discuss the mutations identified in individuals with neurodevelopmental disorders.

**Trio is a GEF regulating multiple Rho GTPases**

The biochemistry and the downstream cell biological functions of the two GEF domains of Trio have been extensively studied. The earliest work has proved *in vitro* that both the two GEF domains are functional. GEF1 domain is responsible for the activation of Rac1, while GEF2 domain is specific for RhoA activation. The activity and specificity were further confirmed by evidence obtained from cell culture experiments. Overexpression of GEF1 domain triggers JNK activation by MAPK pathway and produces membrane ruffles, both of which are the result of Rac1 activation. On the other hand, overexpression of GEF2 domain leads to the formation of stress fibers, which is the marker of RhoA activation. This observation of cytoskeleton rearrangements induced by Trio GEF domains is consistent with the later result that GEF1 domain enhances cell migration. Another Rho GTPase RhoG might also be a direct target of Trio GEF1 domain, because GEF1 domain preferentially binds RhoG(T17N) but not Rac1(T17N) in a yeast two-hybrid system, and expressing dominant-negative RhoG completely abolishes Trio GEF1 function while dominant-negative Rac1 or Cdc42 only partially blocks Trio GEF1 signaling. This Trio GEF1/ RhoG pathway is also involved in NGF induced PC12 cell neurite outgrowth process, as Trio protein accumulates in the soluble fraction of PC12 cells after NGF stimulation. Moreover, the function of Trio in neurite outgrowth of PC12 cells is completely blocked by dominant-negative RhoG, while only partial or mildly inhibition is observed by dominant-negative Rac1 or Cdc42. These results from the early *in vitro* studies suggest that Trio might indirectly activate Rho GTPases Rac1 or Cdc42 through the direct activation of RhoG.

**Trio in neuronal development of invertebrate**

Trio’s function and the underlying mechanism in neuronal development have been extensively studied in invertebrates. In *C. elegans*, UNC-73 is the ortholog of both Trio and Kalirin (a Trio paralog). The expression of UNC-73 in the nervous system is highest, and mutations in *UNC-73* show abnormalities in axon extension, neuronal migration, and growth cone turning. SLT-1 and UNC-6 are the *C. elegans* orthologs of mammalian Slit-1 and Netrin-1, and SAX-3 and UNC-40 are the orthologs of mammalian Robo and DCC. UNC-73 was found to increase the surface expression of SAX-3 and UNC-40 to enhance SLT-1 and UNC-6 mediated pathways, respectively. In addition, UNC-73 is negatively regulated by CRML-1, the *C. elegans* ortholog of mammalian CARMIL. CAML-1 inhibits the GEF activity of UNC-73, thus regulating SAX-3 level in the growth cone. UNC-73 is also involved in mediating UNC-6 induced inhibition of filopodia protrusion in axon repulsion of VD neurons. Recently, UNC-73 was shown to determine the direction specificity of neuritogenesis of bipolar PLM neurons, in which UNC-73 activates both CED-10/Rac1 and MIG-2/RhoG to regulate anterior neurite extension, while the posterior extension is regulated by TIAM-1.
above. Moreover, Trio is enriched in the mushroom body of *Drosophila*, and controls the development of mushroom body
d54. Besides axon extension and guidance, *Drosophila*
Trio also functions in dendritic arborization in PNS sensory
neurons. Trio mutants or knockdown display reduced
dendritic branching and increased branch length, while
overexpression of Trio displays the opposite phenotypes26,
27. Of noted, the GEF2 domain is also involved in the
regulation of dendritic arborizations, in a manner that this
domain interacts with Rho1 to restrict dendritic extension
and higher-order branching27. Thus, Trio may function in
both axon and dendrite development through both GEF
domains.

**Trio in neuronal development of vertebrate**

*In vitro* functions of Trio in axon outgrowth and guidance
have also been extensively studied in cultured mammalian
cells. In PC12 cells, Trio and Kidins220 co-localize at the
tips of neurites, activating Rac1 and mediating NGF induced
neurite outgrowth28. Trio is implicated to be a microtubules
plus-end-tracking protein (+TIP), and interact with EB1
in the mouse hippocampal neurons. EB1 enhances Trio’s
activity to Rac1 by recruiting to the microtubules to
form a Nav1-Trio complex, which is required for neurite
outgrowth29. Trio’s activity is also negatively regulated in
the hippocampal neurons. The binding of proNGF with p75
neurotrophin receptor - SorCS2 complex will dissociate the
binding of Trio with this complex, resulting in decreased
Rac1 activity, which further leads to growth cone collapse30.

Results from Trio global knock out mouse provided
the first genetic evidence of Trio in neural development31.
Trio-knockout embryos die between E15.5 and birth with
abnormal brain development including disorganized
dentate gyrus cells in the hippocampal formation and
mitral cell layer in the olfactory bulb. In addition, Trio also
controls the neuronal clusters in the hindbrain, as Trio-
knockout mice also show abnormal cellular organization
in both the inferior olivary nuclei (ION) and facial motor
nuclei (fMN) where Cadherin-11 is expressed and found in a
complex with Trio32. To explore the *in vivo* functions of Trio
during cerebellar development, Trio conditional knockout
mice were generated33. The authors first crossed Trio flox
mice with Nestin-Cre to drive Trio deletion in the nervous
system. Neural-specific knockout mice live longer than the
decayed cortex, and Trio is found to be indirectly interacted
with DCC, leading to the conclusion that Trio mediates
Netrin-1 induced Rac1 activation in axon outgrowth
and guidance. In Trio deficient spinal cord, commissural
axon projections are defective with defasciculation in
the floor plate, and dorsal spinal cord explant reduces
neurite extension elicited by Netrin-1, consistent with
observations from cortical cultures36, and CGN cultures33.
However, although Rac1 activity is dramatically decreased
after the ablation of Trio, Rac1 is still activated further by
Netrin-1 stimulation in the cerebellum37, suggesting in the
cerebellum, Trio is not exclusively required for Rac1
activation upon Netrin-1 stimulation. In fact, other known
GEFs, such as DOCK18038 and Tiam139 play compensatory
roles in mediating Netrin-1 induced Rac1 activation.
This inconsistency with cortex perhaps stems from the
different intrinsic properties between these two tissues. In
addition, cultured Trio-deficient CGNs also lost the neurite
outgrowth responsiveness elicited Semaphrin-6A, but not by
NGF or glutamate33, suggesting Trio may underlie some
other signal transduction pathway downstream of guidance
cues and growth factors beyond Netrin-1. A recent study
demonstrated that Trio is also involved in Slit2 induced
RhoA activation during the development of the ventral
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Hsc70 (heat shock cognate protein 70). Hsc70 enhances Trio GEF activity toward Rac1 and increases axon length in cortical neurons. Hsc70 interacts with Trio through the N-terminal domain and GEF1 domain, and regulates Trio localization in the growth cone periphery upon Netrin-1 stimulation.

To study the function of each GEF domain of Trio and their downstream signaling in neurite outgrowth, mice with specific knockout of each GEF domain were attempted to be generated. Although gene targeting strategies that would not produce frame-shift were applied, mutant Trio with deletion of fragments in each of the two GEF domain were actually not expressed37, preventing the authors to study the functions of each GEF using this genetic approach. Mice with GEF1 domain knockout are distinguishable from the previous Trio knockout mice. However, multiple splicing isoforms are expressed in the cerebellum12, 13, especially the Trio8 isoform that contains only GEF1 domain but with no GEF2 domain, the GEF2 domain knockout strategy actually results in mice expressing only Trio8 in the cerebellum, thus, the function of GEF2 domain or the longer isoforms (Trio-FL, Trio9L and Trio9S) has been studied using this model. Studies using these GEF2 knockout mice demonstrated that both the neurite length and the growth cone morphology are not distinguishable from control neurons, suggesting GEF2 domain does not participate in the neurite outgrowth of CGNs. However, the Netrin-1 induced neurite elongation is abrogated, implying that the GEF1 domain and its N-terminal sequences are not solely responsible for mediating Netrin-1 signaling. In addition, to study the downstream pathway the GEF1 domain is involved in neurite outgrowth in CGNs, the authors first analyzed the phenotype of Rac1-deficient CGNs isolated from Rac1 knockout mice. Surprisingly, neurite length of Rac1-deficient CGNs is normal, but the growth cone displays a phenotype of reduced area and filopodia number. On the other hand, knockdown of Cdc42 produces a significantly reduced neurite length and constitutively-active Cdc42 restores the neurite outgrowth defect. The authors concluded that at least in CGNs, Cdc42 rather than Rac1 mediates Trio’s function in neurite outgrowth.

Above studies on Trio’s role in neuronal development focusing on its regulation of cytoskeletal dynamics, however, membrane trafficking is also key for neuronal development and offers the membrane materials for growth and receptors for guidance. Since Trio is associated with trans-Golgi network and immature secretory granules and regulates secretion in neuroendocrine cells43, the possibility that Trio may be involved in membrane trafficking during neurite outgrowth was also explored44. Using subcellular fractionation and immunofluorescence, a pool of Trio with Golgi localization was identified in the developing cerebellum and cultured CGNs. Trio-deficient CGNs exhibit abnormal membrane trafficking, and the membrane vesicles that destined to the growth cone display an increased turning frequency as well as a decreased running distance, without any change in velocity of movements. Further, the authors showed that activities of Rab GTPases members, both Rab8 and Rab10 are decreased in Trio-deficient CGNs, and constitutively-active Rab8 or Rab10 rescues the neurite outgrowth defect. Biochemically, Trio interacts with Rab GEF Rabin8 to regulate its phosphorylation and GEF activity. Thus, this study suggests the possibility of the interaction of two GEF families in the coordination of both cytoskeletal dynamics and membrane trafficking during neuronal development.

**Trio in synaptic transmission**

Besides the function of Rho GTPases elucidated in axon outgrowth and guidance, they are also implicated in the modulation of dendritic spines and synaptic function. Most importantly, Rac1 plays an essential role in the maintenance of morphology and density of dendritic spines45-47, thus Rac1 GEFs must be involved in this process. It is known that Kalirin-7 is highly enriched in the postsynaptic densities48, and overexpression of Kalirin-7 in cortical neurons results in increased numbers of spine-like structures49. However, Kalirin-depleted neurons show brain region-specific phenotype that knockout of Kalirin results in significantly decreased spine density in the frontal cortex but not in the hippocampus50, suggesting Trio may play redundant...
functions in the maintenance of spine morphologies. Actually, a recent study demonstrated that both Trio and Kalirin play postsynaptic roles in AMPAR up-regulation in hippocampus CA1 pyramidal neurons\(^5\). The authors observed that overexpression of either Kalirin-7 or Trio-9 in CA1 pyramidal neurons produces a significant increase in AMPAR-mEPSC amplitude, but not in NMDAR-mEPSC amplitude. This postsynaptic function of Rac1 GEFs is specific for Kalirin and Trio since another Rac1 GEF Tiam1 does not produce a similar result. Conversely, the knocking down of either Kalirin-7 or Trio-9 results in a \~60\% reduction of AMPAR-mEPSC amplitude and a moderate reduction of NMDAR-mEPSC amplitude. Strikingly, knocking down both Kalirin-7 and Trio-9 results in nearly complete elimination of both AMPAR- and NMDAR-mEPSCs. This loss of synaptic strength is attributed to the loss of synapses since a \~80\% reduction of dendritic spines is observed in neurons lacking of both Kalirin-7 and Trio-9. The authors further suggested that phosphorylation of both Kalirin and Trio by CaMKII mediates CaMKII induced LTP. The function of Trio on the postsynaptic regulation is complex and controversial. The effect of Trio knockdown in AMPAR- and NMDAR-mEPSC amplitude is consistent with the result in Trio-knockout mice\(^3\). However, a different phenotype of dendritic spine density is observed, that a significant increase of spine density is induced after Trio knockout in both cortical neurons and hippocampal CA1 neurons\(^4\). There is also evidence supporting that down-regulating of Trio reduces AMPAR endocytosis thus increase surface AMPAR, since they observed less internalized GluA1 after Trio knockdown\(^5\), however, not consistent with others, they observed increased AMPAR-mEPSC amplitude after Trio knockdown in the meantime. So far, what is clear is that Trio’s function seems specific to AMPAR rather than NMDAR. Regardless of the differences observed by different groups, the dendritic spine induction role of Trio-Rac1 axis is negatively regulated by a phospholipid-binding protein SESTD1, in a manner that SESTD1 interferes the interaction between Trio and Rac1\(^5\). Overexpression of SESTD1 in hippocampal neurons significantly reduces dendritic spines while knocking down of SESTD1 induces a significant increase in spine density in hippocampal neurons. This observation further supports the positive role of Trio in the synaptic transmission.

Trio also functions presynaptically, since the frequencies of AMPAR- and NMDAR-mEPSC are also decreased in Trio knockout mice. The decreased frequencies may be attributed to the abnormal presynaptic release of neurotransmitters since an increased paired-pulse ratio is also observed in Trio-deficient neurons\(^5\). The function of Trio in regulating neurotransmitter release is supported by its presynaptic localization. In addition to dendritic spines, Trio localizes in the presynaptic terminals and interacts with presynaptic active zone proteins Piccolo and Basson\(^6\), thereby regulating cytoskeletal assembly at the presynaptic active zone required for synaptic vesicles fusion. In C. elegans, UNC-73/Rho axis affects locomotion by modulating the dense core vesicle-mediated releasing of neuropeptides or neuromodulators in motor neurons\(^5\). Genetic evidence in Drosophila suggests Trio is required for synaptic growth at the neuromuscular junction (NMJ)\(^5,6\). In fly motor neurons, Rac activity-induced NMJ overgrowth is dependent on an intact BMP signaling which regulates Trio expression. Heterozygotic trio mutant showed an over 50\% reduction in Rac-induced increase in synapse number\(^5\). As expected, a significant reduction of synaptic boutons numbers is also observed in trio null mutant larvae and is rescued by re-expression of Trio in motor neurons, suggesting Trio functions presynaptically for synapse growth at NMJ. In addition, Trio may also regulate NMJ growth through the activation of diaphanous. In motor neurons of diaphanous mutant fly, the synapse growth is impaired. Diaphanous is a rho effector and plays functions in both maintenances of the presynaptic actin cytoskeleton and modulating the behavior of dynamic pioneer microtubules\(^7\). Thus, Trio functions presynaptically at least through the regulation of both actin and microtubules dynamics. It remains to be elucidated that whether Trio is involved in presynaptic neurotransmitter release by a similar mechanism underlying endocrine cell exocytosis\(^4,5,8\) or membrane trafficking during neurite outgrowth\(^4\).

**Trio in neural disorders**

The essential roles Trio plays in neuronal development and synaptic transmission directly implicate that Trio mutations in humans may lead to several neural diseases. The identification of Trio mutants in human individuals has emerged since the whole exom sequencing (WES) was applied in clinical diagnostic settings. The first report identified two de novo missense mutations p.Asp1368Val and p.Thr2563Met in two patients, respectively\(^9\). Both patients are with severe intellectual disability, regardless of that they also carry another de novo mutations in other known intellectual disability genes, PDHA1 and TCF4, respectively. Genomic microarray, which is routinely used to detect copy-number variations (CNVs) in the clinical evaluation of intellectual disability and developmental delay, further identified a 235-kb deletion in TRIO locus in an individual with developmental delay and facial dysmorphisms\(^10\). A follow-up study using molecular inversion probes further identified three additional mutations in TRIO in additional individuals with mild to borderline intellectual disability\(^11\). These mutations include two nonsense mutations, p.Trp1376* and p.Arg217* and a frameshift truncating mutation Asp1251Valfs*11. Another truncating mutation p.Gln1489Argfs*11 resulting in TRIO degradation was identified in a girl, as well as her father...
and uncle. These three family members presented global developmental delay and mild learning difficulties. Interestingly, this family was previously reported to have Stoll syndrome, characterized by digital abnormalities and micrognathia, suggesting Trio might also be responsible for the syndrome phenotype. Three other de novo missense mutations were also simultaneously reported in the children recruited to the Deciphering Developmental Disorders (DDD) study. Among these mutations, p.Arg1428Gln and p.Pro1461Thr locate in the GEF1 domain and significantly reduce the GEF activity toward Rac1, while the p.Asn1080Ile mutation locates in the spectrin repeats and shows no altered GEF activity toward Rac1. This study implicates that the mutations in Trio GEF1 domain result in intellectual disability and microcephaly, and the mutation in the spectrin repeats may also contribute to intellectual disability. More comprehensive studies identified several other de novo mutations in autism spectrum disorder, schizophrenia, intellectual disability, and epileptic encephalopathy. Most of these mutations are discovered in the GEF1 domain, but several missense mutations are also found in the spectrin repeats and the GEF2 domain. Interestingly, five of the six mutations in the GEF1 domain are predicted to disrupt Rac1 activation, while the mutation p.Asp1368Val is experimentally validated to increase the GEF activity toward Rac1. In fact, transfection of Trio with mutation p.Lys1431Met or p.Pro1461Thr results in the mutation p.Asp1368Val is experimentally validated to increase AMPAR-eEPSC amplitude, while transfection into rat hippocampal CA1 pyramidal neurons results in significant increases of AMPAR-eEPSC amplitude and dendritic spine density, suggesting mutations in the GEF1 domain impact glutamatergic neurotransmission in both hypomorphic and hypermorphic manner, by decreasing and increasing the GEF activity to Rac1, respectively. On the other hand, a rare sequence variant p.Met2145Thr in the GEF2 domain significantly increases the GEF activity toward RhoA. Although the function of this variant in neuronal development and synaptic transmission is not studied, individual baring this mutation has bipolar disorders. In addition, besides the mutations in the GEF domains, another mutation such as p.Glu883Asp in the spectrin repeats is also found to be associated with autism, further confirms that some mutations in the spectrin repeats may also interfere with Trio’s function.

**Trio’s function beyond the nervous system**

Besides those studies on the nervous system, Trio is also implicated to function in other cellular processes. In endocrine cells, Trio is activated by Cdk5, further lead to Rac1 activation and cytoskeleton changes to mediate the subsequent hormone exocytosis and release. Application of GEF1 domain-specific inhibitor to AtT-20 cells leads to a decrease of hormone-releasing and overexpression of the GEF1 domain leads to an accumulation of hormone molecules. In skeletal muscle, Trio is implicated to function in myoblast fusion. Trio interacts with cadherins at adherens junctions and Trio knockdown blocks M-cadherin mediated adhesion, which is required for myoblast fusion. On the other hand, Cadherin-11 is required for Xenopus cranial neural crest cells migration through binding with Trio and activation of Rac. Cadherin-11/Trio/Rac1 signaling also functions in breast cancer cell migration under the control of HOXC8. The interaction of Trio and cadherin also takes part in the adherens junctions of vascular endothelial cells, where VE-cadherin activates Rac1 through binding with Trio to increase endothelial resistance. Endothelial Trio also helps form a docking site required for promoting leukocyte transendothelial migration migration. Trio’s function in enhancing cell migration is also implicated in some tumor or normal cells. Trio/Rac1 is identified to function counteracting with MgRacGAP during cytokinesis in dividing cells, and transducing mitogenic signals from GPCR to control cell growth. Trio/RhoG axis plays a role in the formation of circular dorsal ruffles, which is associated with cell migration, micropinocytosis and receptor internalization. These above numerous studies are all on Trio GEF1 domain, while concerning on Trio GEF2/RhoA, the studies were countable. Trio GEF2/RhoA signaling is involved in the activation of Shroom3, whose activity is required for the shape-changing process called apical constriction, a process accompanied by lens pit invagination during eye development. Another study is that Echo30 virus infection activates Trio GEF2/RhoA, further leads to neuronal cell death.

**Concluding remarks**

It has been over 20 years since the Trio protein was identified and studies on Trio have been switched from early biochemistry and cell biology to human genetics. Much progress has been made in the biochemical properties and genetic functions during development and physiology, however, many questions still remain to be answered. For example, why are there expressed multiple isoforms in developing brain and different brain regions? Does Trio in all neuronal types present the same functions and regulations? What determines Trio in discrimination of various extracellular signaling? What are the in vivo functions and substrates of the unexplored kinase domain? Future detailed studies are needed to understand the function of Trio in neuronal development and related disorders in various regions and different time, and to develop promising therapeutic strategies in treating these diseases.

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Conflict of Interest

The authors declare no conflict of interest.

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