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·Review·

Axon guidance factor netrin-1 and its receptors regulate angiogenesis after cerebral ischemia

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Neurogenesis and angiogenesis play important roles in functional recovery after ischemic stroke. When cerebral ischemia occurs, axon regeneration can compensate for the loss of apoptotic neurons in the ischemic area. The formation of new blood vessels ameliorates the local decrease in blood supply, enhancing the supply of oxygen and nutrients to newly-formed neurons. New blood vessels also act as a scaffold for the migration of neuroblasts to the infarct area after ischemic stroke. In light of this, researchers have been actively searching for methods to treat cerebral infarction. Netrins were first identified as a family of proteins that mediate axon guidance and direct axon migration during embryogenesis. Later studies have revealed other functions of this protein family. In this review, we focus on netrin-1, which has been shown to be involved in axon migration and angiogenesis, which are required for recovery after cerebral ischemia. Thus, therapies targeting netrin-1 may be useful for the treatment of ischemic stroke.

Keywords: netrin-1; angiogenesis; cerebral ischemia; neuronal recovery

Introduction

Stroke, especially acute ischemic stroke (which accounts for 87% of stroke cases), is a major cause of mortality and disability worldwide[1]. Ischemic stroke is primarily caused by blockage of blood vessels in the brain due to thrombi or cardiogenic emboli. The affected tissue loses its supply of oxygen and glucose, with immediate disturbance of function. Research regarding therapies for stroke has long focused on neuroprotective agents. However, clinical use of the only approved drug, tissue plasminogen activator, is limited due to the short time-window of administration and the potential for life-threatening hemorrhage[2]. Safer and more effective treatment strategies for stroke are urgently needed. The vasculature in the adult brain is stable under normal conditions; however, it responds to ischemia through angiogenesis, which drives the formation of new blood vessels. Angiogenesis has been identified as a potential pathway to promote the recovery of neuronal function.

Members of the netrin protein family act as bifunctional axon-guidance cues that regulate migration during neuronal development. Netrins either attract or repel pathfinding axons, depending on the identity of the receptors. Netrin-1 was the first to be purified and is the best-characterized member. In addition to its involvement in guiding axon migration during embryonic development, netrin-1 functions in organ formation[3-6], tumorigenesis[7], inflammation[8, 9], and anti-apoptotis[10], as well as being a potential biomarker for renal injury and certain cancers[11, 12]. It also promotes the recovery of neuronal function after cerebral ischemia in animal models[13-16]. Importantly, a growing number of studies have focused on elucidating the effects of netrin-1 on angiogenesis. In this review, we discuss the implications of the actions of netrin-1 in angiogenesis after cerebral ischemia.

Structures and Functions of Netrin-1 and Its Receptors

Netrins are laminin-related proteins with highly-conserved structures. The N-terminus is composed of two domains that are similar to laminin domains V and VI. The domain V-like region contains three epidermal growth factor (EGF)-like repeats. The C-terminal domain contains binding sites for membrane glycolipids and extracellular matrix components, such as heparin sulfate proteoglycans, integrin α3β1, and integrin α6β4[17, 18]. Three secreted netrins (netrin-1, -3, and -4) and two glycosylphosphatidylinositol (GPI)-anchored membrane-bound netrins (netrin-G1 and -G2) have been identified in mammals[19, 20]. In netrin-1, -3, -G1, and -G2, the N-terminus is homologous to the laminin γ chain; in netrin-4 (also called β-netrin), the N-terminus is more similar to the laminin β chain[17] (Fig. 1A and B).

Netrin receptors mainly include members of the DCC (deleted in colorectal cancer) family (including neogenin), the UNC5 (uncoordinated 5) protein family, and DSCAM (Down syndrome cell-adhesion molecule). All of these transmembrane receptors belong to the immunoglobin (Ig) superfamily (Fig. 1C)[19]. DCC mediates axon attraction, whereas UNC5 homodimers and UNC5-DCC heterodimers mediate axon repulsion[17]. The extracellular portion of DCC family members contains four Ig and six fibronectin type 3 (FNIII) domains, and the intracellular region consists of three highly-conserved domains (P1, P2, and P3) that play important roles in intracellular signal transduction. The extracellular region of UNC5 contains two Ig domains, followed by two thrombospondin (TSP) type-I modules. Its intracellular region contains a ZU5 domain of undetermined function, a DCC-binding site, and a death domain that is associated with apoptotic signaling. In vertebrates, the UNC5 family is composed of four members, UNC5A, B, C, and D[20]. Of these, UNC5B, expressed during early blood-vessel formation, is the most important and is implicated in netrin-1-regulated angiogenesis[21]. It is expressed in the semicircular canals and retina, as well as in the epiphysis, thalamus, and placenta[22-24]. UNC5B has been reported to act as a pro- or anti-angiogenic receptor in different studies[25-28]. DSCAM is a type I transmembrane protein that contains 10 Ig domains and six FNIII repeats in its extracellular domain[19, 20]. In addition, the membrane-associated, G-protein-coupled adenosine A2b receptor functions as a netrin-1 receptor. However, it is unclear whether the A2b receptor is involved in netrin-1-mediated axon-guidance signaling[29-31]. In epithelial cells, binding of the netrin-1 C-terminus with integrins α3β1 and α6β4 provides cues for axon adhesion and migration[32].

When combined with different receptors, netrins provide chemotropic guidance cues. Receptors for secreted netrins include DCC, neogenin, UNC5, and DSCAM. GPI-anchored membrane proteins bind the netrin G ligands NGL-1 and NGL-2[20].

Netrin-1 is currently the best-studied of the netrins. Initially, the UNC-6 protein was identified in *Caenorhabditis elegans* as a guide for cell and axon migration[33]. Thereafter, a homolog of UNC-6, netrin-1, was purified from embryonic chick brain. The root 'netr' originates from a Sanskrit word meaning 'one who guides'[34]. Netrin-1 is expressed in the developing and mature nervous systems, including the spinal cord, cerebellum, visual system, olfactory system, substantia nigra, corpus striatum, ganglionic eminence, and internal capsule[35-37]. It is also expressed in the lung, pancreas, placenta, and mammary gland[24, 38-40]. Netrin-1 acts as a chemoattractant or chemorepellent for migrating cells and axons in the developing central nervous system. It also plays a crucial role in the survival of neurons expressing UNC5 and DCC[41]. Netrin-1 is important in oligodendrocyte development[42]. Netrin-1 silencing in mice is associated with developmental disorders of the spinal cord, corpus callosum, and hippocampus, leading to lethal developmental defects of the nervous system[43].

Netrin-1 and Angiogenesis

Axon guidance factors primarily belong to one of the four receptor/ligand families[44-46]: the roundabout (Robo) receptors bind Slit ligands; neuropilins bind semaphorins or vascular endothelial growth factor (VEGF); Ephs bind ephrins; and UNC5 and DCC bind netrins. These receptors are expressed on neurons and endothelial cells (ECs). They regulate neuronal and vascular development, as well as tumor angiogenesis, by binding to their corresponding ligands[47].

The formation of new blood vessels occurs by vasculogenesis, angiogenesis, and arteriogenesis[48]. The

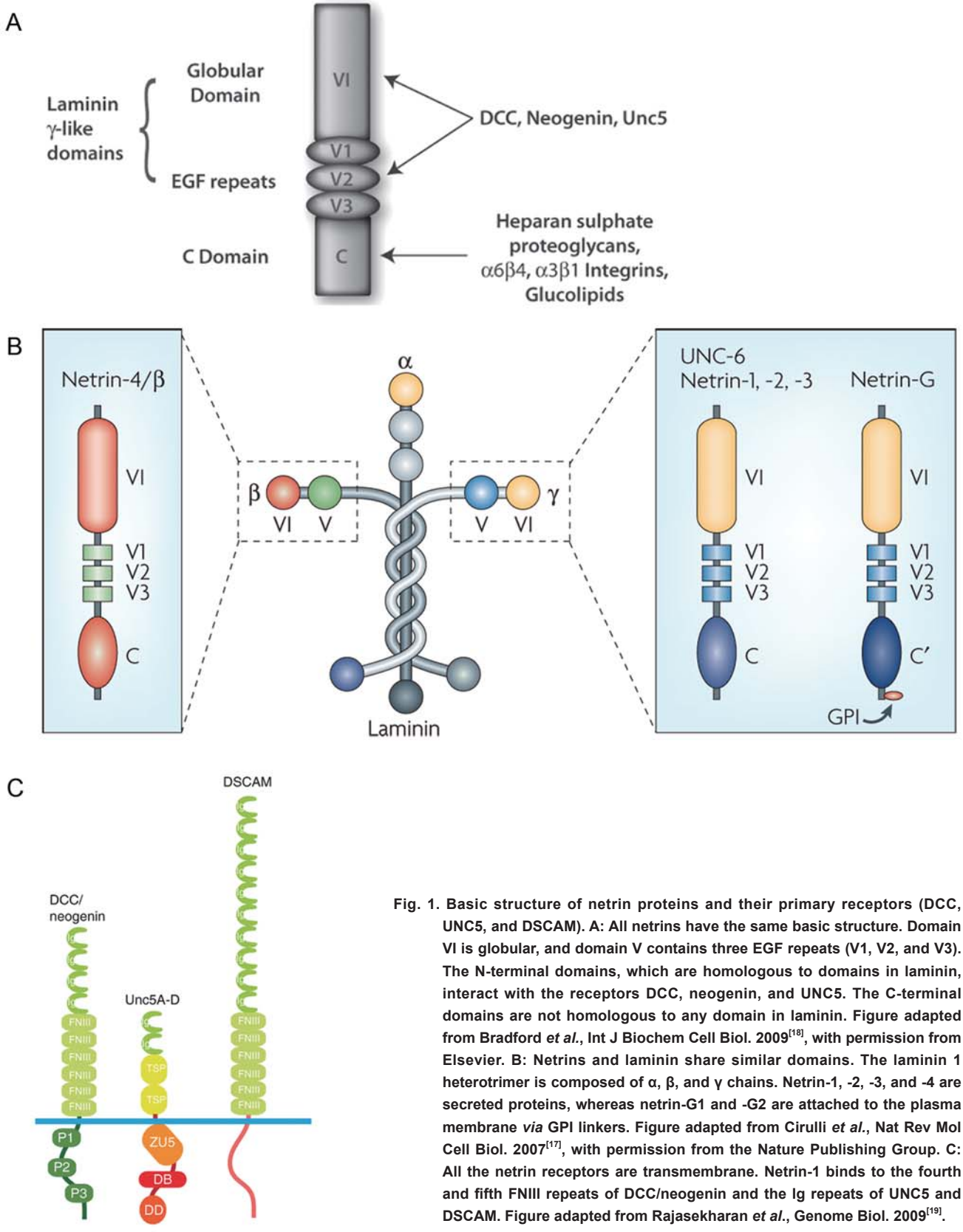

Fig. 1. Basic structure of netrin proteins and their primary receptors (DCC, UNC5, and DSCAM). A: All netrins have the same basic structure. Domain VI is globular, and domain V contains three EGF repeats (V1, V2, and V3). The N-terminal domains, which are homologous to domains in laminin, interact with the receptors DCC, neogenin, and UNC5. The C-terminal domains are not homologous to any domain in laminin. Figure adapted from Bradford *et al.*, Int J Biochem Cell Biol. 2009[18], with permission from Elsevier. B: Netrins and laminin share similar domains. The laminin 1 heterotrimer is composed of α, β, and γ chains. Netrin-1, -2, -3, and -4 are secreted proteins, whereas netrin-G1 and -G2 are attached to the plasma membrane *via* GPI linkers. Figure adapted from Cirulli *et al.*, Nat Rev Mol Cell Biol. 2007[17], with permission from the Nature Publishing Group. C: All the netrin receptors are transmembrane. Netrin-1 binds to the fourth and fifth FNIII repeats of DCC/neogenin and the Ig repeats of UNC5 and DSCAM. Figure adapted from Rajasekharan *et al.*, Genome Biol. 2009[19].

timing of vasculogenesis is during embryonic development, when the endothelial precursors, or angioblasts, assemble and differentiate into ECs to form the vascular plexus. Angiogenesis is the subsequent sprouting of new capillaries from pre-existing blood vessels. Arteriogenesis is the process of stabilizing the new vessels, and involves surrounding the vessels with pericytes and vascular smooth muscle cells, as well as remodeling *via* increased blood flow[48, 49]. Blood vessels in adults are generally in a quiescent state. New vessel formation primarily occurs under pathological conditions through angiogenesis. Research may provide new targets for the treatment of diseases associated with excessive angiogenesis, such as cancer and retinopathy, as well as diseases associated with insufficient angiogenesis, such as coronary heart disease and ischemic stroke. Angiogenesis involves degradation of the extracellular matrix by proteolytic enzymes, and the proliferation, differentiation, and migration of ECs[50, 51]. ECs maintain high plasticity and extend filopodia after stimulation by angiogenic signals[49]. Apoptosis of ECs impedes vascular regeneration; thus, the inhibition of apoptosis is an important mechanism for EC survival and a key step in angiogenesis[50]. According to Castets *et al*.[25], netrin-1 is a survival factor for ECs. When it binds to UNC5B, the action of the downstream apoptotic signal serine/threonine kinase DAPK (death-associated protein kinase) is inhibited, and then the UNC5B-dependent EC apoptosis is blocked. In contrast, EC apoptosis is induced when netrin-1 is not bound to UNC5B. In zebrafish, vascular sprouting defects induced by netrin-1 silencing are reversed by inhibition of caspase activity, UNC5B silencing, or DAPK silencing[25]. Therefore, it can be inferred that netrin-1 promotes angiogenesis by inhibiting EC apoptosis.

Park *et al*.[52] demonstrated that netrin-1 is involved in the development of the nervous and vascular systems during mouse embryonic development. Mice lacking netrin-1 exhibit blood-vessel defects, suggesting that netrin-1 facilitates vascular development. Another study showed that UNC5B deficiency results in placental arteriole dysplasia, leading to embryonic death[26]. Therefore, the receptor for netrin-1, UNC5B, is essential for the process of placental vascular formation.

Importantly, netrin-1 mediates angiogenesis by promoting adhesion between ECs and vascular smooth muscle cells. Netrin-1 enhances the reaction of vessels with VEGF, thereby stimulating angiogenesis. Therefore, some researchers have proposed that netrin-1 is also a pro-angiogenic factor[52]. Highly-purified netrin-1 stimulates EC proliferation, migration, and tube-formation *in vitro*, with non-significant expression levels of UNC5B, DCC, and neogenin, suggesting that the process does not depend on netrin-1 receptors[53]. These findings indicate that netrin-1 is a pro-angiogenic factor that can act independently of its receptors.

Consistently, Fan *et al*.[54] showed that netrin-1 stimulates the proliferation and migration of human cerebral ECs. In the adult brain, netrin-1 hyperstimulation facilitates focal angiogenesis. Newly-formed vessels induced by netrin-1 contain an intact EC monolayer surrounded by multiple cell layers. Nguyen *et al*.[55] demonstrated that netrin-1 induces angiogenesis by increasing endothelial nitric oxide production *via* a DCC-dependent ERK1/2-eNOS feed-forward mechanism in aortic ECs. All these studies suggest that netrin-1 is a pro-angiogenic factor, albeit with some discrepancies (Table 1).

However, in some cases, netrin-1 has also been found to block angiogenesis. For example, Lu *et al*.[27] reported that netrin-1 reduces endothelial migration and filopodial extension, and this is mediated by signaling through UNC5B. UNC5B is a repulsive netrin-1 receptor expressed by endothelial tip cells of the vascular system during mouse embryonic development. Disruption of the *Unc5b* gene in mice leads to excessive vessel branching, confirming that netrin-1 inhibits angiogenesis *via* the UNC5B receptor. Likewise, Larrivee *et al*.[28] demonstrated that deletion of the *Unc5b* gene ameliorates the netrin-1-mediated inhibition of angiogenesis. Netrin-1 repels ECs when UNC5B is expressed, with angiogenesis being suppressed throughout this process. Both studies imply that netrin-1 functions as an anti-angiogenic factor when acting in concert with UNC5B (Table 1).

In conclusion, it remains controversial whether netrin-1 promotes or inhibits angiogenesis. The precise role may depend on experimental conditions, animal models, and types of ECs or vessels examined. Besides, it is unclear which netrin-1 receptors mediate specific functions. According to Yang *et al*.[56], the concentration of netrin-1 affects its impact on vessels: low doses appear to promote

Table 1. Effects of netrin-1 and its receptors on angiogenesis

Effects	Receptors involved
Pro-angiogenic effect	
Inhibit EC apoptosis[25]	UNC5B
Stimulate EC Proliferation, migration, and TF[53]	Independent of UNC5B, DCC, neogenin/--
Increase endothelial NO production[55]	DCC
Promote vascular development[52]	UNC5B/-----
Facilitate focal angiogenesis in adult[54]	------
Anti-angiogenic effect	
Block EC migration[27]	UNC5B
Repel EC[28]	UNC5B
Dual function	------

EC, endothelial cell; NO, nitric oxide; TF, tube formation; -----, unknown.

angiogenesis, whereas higher doses inhibit it *in vitro*. Taken together, the purity and concentration of netrin-1, and the specific type of receptor expressed in a given cell type, may be important for defining the role of this protein in angiogenesis.

Angiogenesis after Cerebral Ischemia

The process of angiogenesis—the sprouting of new capillaries from pre-existing blood vessels—participates in neuronal recovery after ischemic stroke. In the early 1990s, Krupinski *et al.*[57] analyzed brain tissues from 10 patients, and found that the number of microvessels, particularly in the penumbra, is significantly increased after cerebral ischemic stroke. The microvessel density correlates with the long-term survival of patients. Sbarbati *et al.*[58] found that microvessels form 2 weeks after middle cerebral artery occlusion (MCAO) in rats, implying that microvessel formation mediates the recovery of blood flow and helps to compensate for the collateral circulation after permanent MCAO.

Interruption of cerebral blood flow rapidly triggers the transcription of angiogenesis-related genes. Using cDNA analysis, Hayashi *et al.*[59] examined the expression levels of 96 angiogenesis-related genes after transient MCAO (tMCAO) in mice, and found that 42, 29, and 13 genes are upregulated at 1 h, 1 day, and 21 days, respectively, after transient occlusion. Beck *et al.*[60] found that the mRNA expression of angiogenin-2, which promotes angiogenesis, is upregulated after 6 h of occlusion in MCAO rats. These changes lead to the synthesis of pro-angiogenic proteins such as VEGF. The expression levels of both VEGF and endostatin increase after cerebral ischemia, although they play opposite roles in angiogenesis[61]. These studies suggest that pro- and anti-angiogenic factors work together to regulate angiogenesis.

Pro-angiogenic factors promote EC proliferation. Initially, microvessels form in the boundary zone of the ischemic region, increasing the supply of oxygen and nutrients. Gradually, the newly-formed vessels grow and increase the cerebral blood volume. However, whether angiogenesis leads to the formation of an intact and functional vessel network in the ischemic zone after stroke needs to be determined[62].

With regard to the role of angiogenesis after cerebral ischemia, Yu *et al.*[63] proposed that the major function of ischemia-stimulated vessel formation is to eliminate necrotic tissue and debris *via* macrophages. Wei *et al.*[64] demonstrated that angiogenesis helps to restore blood flow in the ischemic zone by interacting with the arteriole collateral circulation established after cerebral ischemia, thereby improving the long-term recovery of neurological function in rats. Angiogenesis after ischemic stroke protects damaged tissue by supplying nutrition for neuronal remodeling and improving the metabolism of surviving neurons.

In addition, mounting evidence indicates that the vasculature acts as a scaffold for neuroblasts in the subventricular zone (SVZ) to migrate to the infarct area after ischemic stroke[65-67]. SVZ-derived neuroblasts assemble around blood vessels adjacent to the infarct area, migrate along the vessels, and finally reach the infarct zone. The ECs secrete stromal-derived factor-1α (SDF-1α) to attract neuroblasts expressing CXCR4, the receptor for SDF-1α. These neuroblasts gradually differentiate into mature neurons, and new blood vessels supply nutrition to the newly-developed neurons[68]. Migrating neuroblasts pass through areas of vascular remodeling and regeneration, suggesting that angiogenesis assists neurogenesis, and that both processes promote functional recovery after ischemic stroke. Taken together, angiogenesis helps to restore neuronal function after ischemic stroke, which may offer useful insights into new treatment approaches for the condition[62, 68, 69].

Netrin-1 Promotes Angiogenesis after Cerebral Ischemia

The expression levels of netrin-1 and its receptors change after injury to the nervous system. In rats subjected to MCAO, netrin-1 and UNC5B are expressed in neuronal perikarya in the peri-infarct area, and DCC is expressed in perivascular astrocytes[70]. In a similar study, netrin-1 was reported to be expressed in neurons in the peri-infarct region, DCC in neurons and astrocytes, and neogenin in ECs in the infarct area[71]. The expression levels of netrin-1 and its receptors peak at 14 days after MCAO. Because netrin-1 promotes axon migration, the spatial and temporal similarities between the netrin-1 and DCC expression indicate that they are simultaneously involved in axon migration.

Neogenin may also play a role in angiogenesis. The temporal overlap of netrin-1 and neogenin expression implies that axon regeneration and angiogenesis occur concomitantly. Neurons and vessels share similar signaling pathways that mediate differentiation, maturity, and migration. They also have similar parallel branching structures[72]. Therefore, neurogenesis and angiogenesis may influence each other after cerebral ischemia.

Netrin-1 is involved in the functional recovery of neurons after cerebral ischemia. Bayat *et al.*[13] found that hippocampal administration of exogenous netrin-1 significantly improves spatial memory and enhances synaptic plasticity in a dose-dependent manner 24 h after global cerebral ischemia secondary to cardiac arrest in rats. Wu *et al.*[14] reported that the presence of netrin-1 decreases the infarct size and the number of apoptotic neurons in MCAO mice, suggesting that it has protective effects after cerebral ischemia. Liu *et al.*[70] showed that netrin-1 and its receptors may be involved in remodeling the peri-infarct neuronal circuitry after treadmill exercise, suggesting that exercise encourages neuronal survival in the infarct region by regulating the netrin-1/UNC5B signaling pathways.

Lu *et al.*[15] constructed an adeno-associated viral netrin-1 vector (AAV-NT-1), and delivered it into mouse brain after tMCAO. Netrin-1 expression increased and neurobehavioral outcomes significantly improved at 7 days after tMCAO in the mice with AAV-NT-1 transduction compared to controls. They therefore proposed that netrin-1 promotes functional recovery after cerebral ischemia, and that netrin-1 gene transfer could be used to treat cerebral ischemic diseases.

Our research group has been actively investigating the role of netrin-1 after cerebral ischemia. Recently, we demonstrated that the administration of exogenous netrin-1 protects neurons by attenuating secondary apoptosis in the ventroposterior thalamic nucleus (VPN) ipsilateral to a focal cerebral infarction in rats. This process may depend on the UNC5H2 receptor. Insufficient expression of endogenous netrin-1 may cause secondary damage in the VPN after ischemic stroke[16].

Other recent studies have explored the effects of netrin-1 overexpression using the AAV-NT-1 vector. Sun *et al.*[73] reported that netrin-1 overexpression notably increases the peri-infarct vessel density and promotes the migration of immature neurons to the infarct territory. Netrin-1 overexpression also assists in the recovery of motor function after cerebral infarction in rats. Lu *et al.*[74] found that netrin-1 overexpression contributes to functional recovery by diminishing the infarct size and promoting angiogenesis after mouse tMCAO.

Therefore, netrin-1 appears to play an important role in functional recovery. Although the underlying mechanism

remains unclear, the current evidence suggests that netrin-1-mediated angiogenesis is one such mechanism. Future studies are needed to clarify its role in promoting angiogenesis after cerebral ischemia.

Prospects

Ischemic stroke has high mortality and disability, and numerous studies have attempted to find effective therapies for this condition. Netrin-1 promotes axon migration and regeneration, inhibits neuronal apoptosis, and facilitates angiogenesis in the infarct area, increasing the blood supply to ischemic tissues and improving the prognosis. Current studies of netrin-1 have focused on the cellular and animal levels, and its role in the promotion or inhibition of angiogenesis remains controversial. Thus, future studies addressing the seemingly contradictory pro-angiogenic and anti-angiogenic effects of netrin-1 are sorely needed. Nevertheless, netrin-1 may be a potential therapeutic target for the promotion of neuronal recovery following ischemic stroke. To date, the functions of other members of the netrin protein family have not been fully elucidated, and therefore are critically in need of investigation.

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