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Mechanisms of Neurovascular Dysfunction in Acute Ischemic Brain

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Abstract

The neurovascular unit is now well accepted as a conceptual framework for investigating the mechanisms of ischemic stroke. From a molecular and cellular perspective, three broad mechanisms may underlie stroke pathophysiology – excitotoxicity, oxidative stress and inflammation. To date, however, most investigations of these basic mechanisms have focused on neuronal responses. In this mini-review, we ask whether these mechanisms of excitotoxicity, oxidative stress and inflammation can also be examined in terms of non-neuronal interactions in the neurovascular unit, including the release of extracellular vesicles for cell-cell signaling.

Keywords

Neurovascular unit; stroke; neuronal cell death; neuroprotection; extracellular vesicles; cell-cell interaction

1. INTRODUCTION

Stroke is one of the most serious life-threatening diseases in the developed countries [1]. Over the past two decades, advances in molecular and cellular biology have defined many potential mechanisms and targets for stroke. But it has been very difficult to translate these gains in basic knowledge into true clinical applications [2, 3]. Therapeutic options for stroke patients remain limited to reperfusion with tissue plasminogen activator or mechanical catheter-devices.

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CONFLICT OF INTEREST

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There are many reasons why neuroprotection trials have mostly failed [4, 5], but one major reason may be related to the idea that stroke damages not only neurons but also all cell types in the neurovascular unit (NVU), i.e. neurons, glia and vascular cells. Cell-cell interactions between neurons, glia (astrocytes, microglia, and oligodendrocytes), vascular compartments (endothelial cells, pericytes, and smooth muscle cells), and extracellular matrix underlies central nervous system (CNS) homeostasis. For signaling purposes, a large array of endogenous molecules such as cytokines, growth factors, chemokines, and microparticles (MPs) are proposed to be essential mediators within this modular concept (Fig. 1). Hence, any disruption of these complex interactions may lead to NVU dysfunction during neurodegenerative conditions including brain ischemia. In this regard, therapeutic approaches for stroke should aim to not only protect neurons but also rescue all cell types and restore function in the entire NVU [4, 6].

Research at the molecular and cellular level, as well as in animal models, have revealed many interesting mechanisms and targets for neuroprotection. After ischemic stroke, the initial loss of blood flow in the central core regions is very severe, so neuronal cell death rapidly occurs due to lack of oxygen and glucose. However, in the surrounding penumbral areas, energy loss is relatively moderate or mild and it has been proposed that brain cells here are potentially salvageable. Within the evolving penumbra, neurons slowly die because a complex cascade of cell death mechanisms becomes triggered. Broadly speaking, these mechanisms can be grouped into general categories involving excitotoxicity, oxidative stress and inflammation [7]. To date, however, dissection of these overall mechanisms has mostly been centered on neuronal responses. In this mini-review, we attempt to explore how cell-cell interactions within the NVU, especially in non-neuronal cells, can modify and amplify these pathophysiologic mechanisms by affecting the ability of NVU components to regulate signaling mediators such as glutamate, free radicals, growth factors, cytokines, chemokines and extracellular vesicles.

2. EXCITOTOXICITY

Glutamate is a major excitatory neurotransmitter in the mammalian brain. It contributes to cellular function, synaptic plasticity, cell death and survival, neuronal development, and learning and memory [8]. Under normal conditions, neurons are exposed to small amount of glutamate to maintain their function. Although exposure to low-dose glutamate does not lead to neuronal damage [9, 10], excessive levels of glutamate cause excitotoxicity by over-stimulating various glutamate receptors of neurons, such as N-methyl-D-aspartate (NMDA) receptors, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors and kainate receptors of ionotropic receptors, and metabotropic receptors. The calcium overload by excessive glutamate exposure gives neurons oxidative stress, mitochondrial dysfunction, calpain activation, and DNA fragmentation [11]. Following an ischemic insult, the subsequent ATP loss causes ionic imbalance of certain ion, such as sodium, potassium, calcium and chloride ions [12]. This imbalance inhibits the uptake of glutamate by glutamate transporters, and even causes the reversal of the transporters, which induce over-accumulation of extracellular glutamate [13].

Astrocytes are key players in the regulation of glutamate in CNS brains. They express several glutamate transporters such as glutamate-aspartate transporter (GLAST, EAAT 1 in human) and glial glutamate transporter 1 (GLT-1, EAAT 2 in human) [14]. After astrocytes take up glutamate, glutamate is converted to glutamine by astrocytic glutamine synthase followed by being released from the astrocytes [15]. The released glutamine is then removed from the extracellular space into neurons [16]. After that, neurons convert glutamine back to glutamate and proceed to release it into synapse [17, 18]. Ischemic conditions disrupt this glutamate-glutamine cycle [19]. Rossi DJ *et al.* suggested that transporter-mediated extracellular glutamate homeostasis failed dramatically under ischemic conditions and that the astrocytic transporters were the major source of extracellular glutamate to trigger the death of neurons [13]. The reverse uptake of glutamate in astrocytes was demonstrated by the evidence of increased vesicular and non-vesicular release of glutamate in the extracellular space [20]. NMDA receptors are proposed as the predominant glutamate receptor for neuronal death because of the high permeability of calcium. Indeed, multiple NMDA receptor subtypes, such as NR2Aand NR2B-containing NMDA receptors, have different function in epileptogenesis and ischemic stroke [21, 22]. Reverse glial glutamate uptake may trigger neuronal cell death through preferential activation of extra-synaptic NR2Bcontaining NMDA receptor-related pathways [23]. Furthermore, astrocytes may also assist neurons in terms of energy regulation [24]. Because astrocytes are less susceptible than neurons to ischemic conditions, astrocytes may try to protect neurons through glutamate uptake, glycogen hydrolysis to lactate for energy, and conduction of protective molecules through gap junctions, even under the ischemic conditions. In addition, glutamate activates metabotropic glutamate receptors (mGluRs) in astrocytes, which releases arachidonic acid derivatives to modulate cerebral blood flow (CBF). Under stroke conditions, both competitive and non-competitive NMDA receptor antagonists increase in CBF while attenuating injury. Therefore, NMDA receptor antagonists may also contribute to neuroprotection through augmenting CBF in affected brain areas [25]. Taken together, these observations suggest that restoring astrocyte function in terms of glutamate handling and energy regulation may provide potential therapeutic targets for ischemic stroke.

Oligodendroglial lineage cells, which are one of the main cell types in cerebral white matter, also express AMPA and kinate glutamate receptors. AMPA/kainate receptor blockade protects cultured oligodendrocytes from hypoxic injury [26]. Recently, it was reported that oligodendrocytes also expressed functional NMDA receptors containing NR1, NR2C and NR3 subunits in ischemia [27]. NMDA receptors have higher glutamate affinity than AMPA or kinate receptors. Hence, oligodendrocytic NMDA receptors could contribute to brain injury, especially white matter damage, which occurs when the glutamate level is elevated in stroke. Because mature oligodendrocytes myelinate axons to support axonal signal transduction, oligodendrocyte-neuron interaction should be very important in maintaining normal neuronal function. Indeed, oligodendrocytes can signal to neurons via myelin-axon interactions [28, 29]. Mouse models of oligodendrocyte injury have demonstrated axonal loss without considerable demyelination [30, 31], suggesting that oligodendrocytes support axonal survival through a myelinindependent mechanism [32]. Moreover, oligodendrocytes may serve as a principal metabolic supplier of lactate, which is integral for axonal energy support through monocarboxy-late transporter 1 [33]. In addition, oligodendrocyte-derived

trophic factors promote neuron survival and axon outgrowth *in vitro* [34]. Hence, oligodendrocyte death by glutamate toxicity may eventually lead to neuronal damage and dysfunction.

Glutamate function was originally described in terms of neuronal neurotransmission. But as discussed in the preceding paragraphs, it is now known that this signaling system applies to many other cell types in the CNS. So in the context of stroke and brain injury, excitotoxicity mechanisms should operate in multiple cell types. Besides astrocytes and oligodendrocytes, glutamate transporters also exist in pericytes [35], and over-activation of ionotropic glutamate channels can trigger apoptosis in cerebral endothelium as well [36, 37]. Excitotoxic cascades are not only a neuronal phenomenon but comprise all compartments in the neurovascular unit. Taken together, suppression of glutamate toxicity under ischemic conditions would promote neuronal survival. However, clinical application of glutamate receptor antagonists have been tested without successful results [13, 38]. One possible reason why glutamate antagonists were not successful would be partly because the treatable target focused on only neuronal NMDA glutamate receptors [38]. To achieve effective neuronal protection, we may need to consider how to control the glutamate-mediated excitotoxicity through non-NMDA receptors in neurons as well as glutamate receptors/transporters in non-neuronal brain cells.

3. OXIDATIVE STRESS

Reactive oxygen species (ROS) are associated with many diseases, including ischemic stroke and other neurodegenerative diseases. Ischemic insults produce an excess amount of free radicals, especially in the reperfused ischemic regions [39, 40]. Oxidative stress causes neuronal cell death by attacking key cellular components. For example, ROS open mitochondrial permeability transition pore, and subsequently cause mitochondrial swelling, resulting in necrosis [41]. ROS also initiate apoptosis through activating p53 and p38 MAPK [42]. Hence, protecting neurons from oxidative stress have been considered as a promising therapeutic approach for stroke. Indeed, a great number of antioxidants were evaluated in clinical trials. For example, a radical-spin trap NXY-059 improved disability significantly 3 months following stroke [43]. However, in the next SAINT-II trial, the compound showed no significant effects [3]. Nevertheless, research of oxidative stress in ischemic stroke field continues to reveal novel possibilities for antioxidant therapies [44]. In this section, we will discuss how non-neuronal cells regulate oxidative stress within the NVU compartment.

Astrocytes possess a large capacity of endogenous antioxidants. The release of antioxidants, such as glutathione and superoxide dismutase (SOD), from astrocytes may play an important role in maintaining and enhancing neuronal survival [45–49]. In this regard, nuclear factor-erythroid 2 related factor 2 (Nrf2) is a promising target to attenuate brain damage and neurological deficits following stroke [50]. Nrf2 is the redox-sensitive transcription factor for phase II defense enzymes and antioxidant stress proteins, and oxidative stress induces nuclear translocation and binding of the antioxidant response element in the promoter of protective genes [50]. Indeed, astrocytic Nrf2 mediates several gene expressions to represent a major contributors of endogenous defense system for neurons against oxidative stress [51–

54]. In a rat stroke model, Nrf2 expression was higher in peri-infarct region than ischemic core [55]. In addition, transient ischemia activated Nrf2 in astrocytes both *in vivo* and *in vitro* and increased the expression of Nrf2 target genes, which contribute to neuroprotection [54]. Metallothioneins (MTs) in astrocytes also play essential roles in protecting neurons against ROS stress. MTs are a family of metal binding proteins and have multiple function including the regulation of metal concentration and detoxification of heavy metal ions [56]. MT overexpressed mice showed significant reduction of infarct size and better motor function after transient middle cerebral artery occlusion [57]. In turn, both MT-I- and MT-II-deficient mice developed larger infarcts and worse neurological outcomes compared to wild-type mice [58]. Interestingly, MT was shown to be transferred from astrocytes to neurons in response to injury [59]. These MTs from astrocytes may protect neurons from oxidative stress [60]. These findings suggest that intercellular communications between astrocytes and neurons exist, and because of that, neurons are guarded by astrocytes against oxidative stress.

In contrast to astrocytes, endothelial cells could be a major source of ROS under pathological conditions due to ample amount of Nox [61]. Nox is a catalytic subunit of NADPH oxidases, whose identified isoforms are Nox1, 2, 4, and 5 [62]. Endothelial cells also produce nitric oxide (NO), which is essential to maintain vascular tone. NO improves cerebral blood flow during and after ischemic onset. However, NO together with superoxide would form the much more powerful oxidant peroxynitrite [63] to attack neighboring cells including neurons under acute phase of stroke. Additionally, an inflammatory cytokine tumor necrosis factor-α (TNF-α), which is released after brain ischemia, significantly increases intracellular level of ROS in endothelial cells [64]. Under normal conditions, cerebral endothelial cells nourish neighboring neurons through growth factor release. But under pathological conditions, those endothelial-derived ROS may in turn induce neuronal cell death.

The cerebral white matter consists of abundance of oligodendrocytes and lipid-rich contents of myelin sheath, and intrinsic antioxidant properties in the white matter are relatively low. Therefore, cerebral white matters can be also an enormous source of ROS. Hence, under ischemic conditions, axons in the white matter may be damaged due to oligoden-drocyte-derived ROS release. In addition, oligodendrocytes and their precursors themselves appeared to be relatively susceptible to oxidative stress [65, 66]. As noted, oligodendrocytes support neuronal function in the white matter via myelin-axon interactions. Moreover, precursor cells of oligodendrocytes exist even in adult white matters to repair axonal function after injury through differentiating mature oligodendrocytes. Therefore, ROS accumulation in the white matter after stroke may cause neuronal (axonal) damage in both direct and indirect manners.

In general, excessive production of ROS may be damaging in stroke and a wide range of CNS disorders. But it is becoming evident that like other mediators in the neuroscience of disease, ROS can be detrimental or beneficial, depending on concentration and cellular context [67]. For example, low levels of ROS promote OPC migration [68] and oligodendrocyte differentiation and myelin formation [69], and homeostatic levels of nitric oxide may support EPC function [70]. Recently, there is an important movement to more

carefully define not only sources but also different types of radicals [71]. Not all free radicals are the same. Investigating these different cellular and chemical categories will be critical as we continue to dissect these mechanisms in the neurovascular unit for the future development of diagnostics and therapeutics.

4. INFLAMMATORY RESPONSES

Focal ischemia induces a potent inflammatory response within a few hours after onset of ischemia. Inflammation after ischemic insults composes reperfusion injury, and is an important part of stroke [72]. The insufficient blood supply after initial ischemic insult causes the development of coagulation cascades, which induce impact-induced shear stress and cellular damages in endothelial cells primarily. Expressions of adhesion molecules, such as P-selectin are increased in platelets and endothelial cells as early as 15 minutes after ischemic onset, and then, pro-inflammatory cascade is promptly initiated [73]. Endothelial cells are the major component of blood-brain barrier (BBB), and inflammatory cascades including platelet aggregation and leukocyte adhesion lead to BBB breakdown [73]. Once BBB is disrupted, various blood immune cells infiltrate into ischemic area [74–76]. These migrated immune cells (along with injured brain cells) produce inflammatory mediators, resulting in promoting neuronal death [77].

Microglial cells, which derive from bone marrow stem cells, are the resident immune cells of the CNS. Basically, resting microglial cells scan the environment, and play an active part in ischemic stroke because of their very low threshold of activation [78]. Neurons have been thought as victims of activated microglia (i.e. activated microglia are known as phagocytes of neurons after ischemia)[79]. Wake H. *et al*. examined the mechanisms of direct and activity-dependent connections between microglia and neuronal synapses. *In vivo* two-photon imaging analysis demonstrated that the duration of these interactions was prolonged in ischemic brain [80]. Under normal conditions, healthy neurons actively protect themselves from phagocytosis by displaying “don’t-eat-me” signals (CD200, CD47, and CD22 etc.) on their surface [81, 82]. Microglia have receptors for these signal molecules, and with these receptors’ activation, microglial phagocytosis is maintained at low level. However, under ischemic conditions, reduction of these “don’t-eat-me” signals exacerbates microglial activation to accelerate neuronal damage.

Other brain cells than microglia may also contribute to inflammatory responses to cause neuronal damage after ischemia. Following the onset of ischemia, a variety of molecules are released from the intracellular part of dead/damaged cells. These endogenous molecules are called danger-associated molecular patterns (DAMPs), and are regarded as activators of microglia and infiltrating peripheral immune cells [83]. Among DAMPs, high mobility group box 1 (HMGB1) has been relatively well investigated to understand the pathophysiology under ischemic conditions [84, 85]. Most brain cell types along with microglia and peripheral immune cells express HMGB1 receptors, such as advanced glycation end products (RAGE), toll-like receptor-2 (TLR-2), and TLR-4 [86, 87]. Once these receptors are activated, several pro-inflammatory cytokines are released to accelerate inflammatory responses. For example, activated microglia by HMGB1 contribute to post-ischemic inflammation by secreting TNF-α, ineteleukin-1β (IL-1β), ROS, and many pro-

inflammatory cytokines [88]. Reactive astrocytes are also harmful after stroke in producing pro-inflammatory cytokines, such as IL-6, TNF-α, IL-1β and interferon-γ [89]. In addition, reactive astrocytes form glial scars, which inhibit axon function [89]. Pericytes, which exist intermittently along the wall of capillaries to support endothelial function, such as BBB tightness, are also known to release proinflammatory cytokines after injury [90, 91]. Released cytokines can promote inflammatory responses via inducing further release of DAMPs. These vicious cycles may accelerate inflammatory reactions and exacerbate neuronal loss.

Taken together, suppression of inflammatory responses remains a promising therapeutic approach in acute phase. Unlike other stress cascades, such as oxidative stress and glutamate excitotoxicity, inflammation occurs over hours and continue for days or weeks [92]. This wider therapeutic time window may provide potential opportunities for anti-inflammatory therapy for ischemic stroke [93]. However, it may be important to recognize that some mediators/responses in neuroinflammation may have biphasic characteristics, i.e. deleterious at acute phase but beneficial at chronic remodeling phase after brain injury [94, 95]. For example, matrix metalloproteinases represent a highly conserved component of inflammation post-stroke [96–98]. Early elevations in MMPs may be deleterious by degrading blood-brain barrier integrity and overall neurovascular function [99]. But during stroke recovery, MMPs may be essential for neurovascular plasticity and remodeling [100]. Therapeutic approaches that target inflammation should be carefully titrated so as not to interfere with the beneficial endogenous mechanisms of remodeling within the NVU.

5. EXTRACELLULAR VESICLES AND NEUROVASCULAR UNIT IN ISCHEMIC STROKE

As mentioned, various kinds of endogenous molecules are secreted from cells within the NVU. In this regard, extracellular vesicles (ECVs) have attracted our attentions on understanding the detail mechanisms of trophic coupling within this conceptual framework. A large amounts of ECVs are found within or out of eukaryotic cells [101]. They are called either MPs or exosomes according to their origins, sizes, and release mechanisms. MPs were first described in 1967 as “platelet dust” [102], whereas exosome was first named in 1980s as 5’-nucleotidase activity-containing vesicles [103]. Recently, microvesicles (MVs) is generally used for vesicles with limited diameters regardless of their origins and innate characteristics. However, as new contents and function in ECVs emerge, the simple distinction based on their size does not represent a complete explanation of ECVs any longer. It has been confirmed that MVs contain necessary signaling to exert physiological and pathogenic effects. Indeed, lipids, proteins, and nucleic acids in the vesicles are considered to have pivotal roles in cellular communication and contribute to pathogenesis in a broad scope of diseases including tumor, immunity disorders, cardiovascular diseases, neurodegeneration, infectious diseases, renal diseases and blood diseases [104–108]. For example, in the case of diabetic patients, the losses of endothelial micro RNA (miRNA) in MPs, which are involved in the endothelial function, are related to the impairment of peripheral angiogenic signaling [109].

MVs/MPs may contribute to NVU function/dysfuntion after ischemic stroke. Almost all the cell types composing NVU can produce ECVs [110], and therefore, they may mediate the inter-cellular communication within the NVU. Thus far, endothelial-derived MPs (EMPs) have been prominently researched. EMPs are highly organ-dependent [111], and are suggested as markers of endothelial dysfunction [112]. Production of EMPs is known to be stimulated by mediators such as TNF-α [113], thereafter increased EMPs convey proteases of matrix metalloproteinase family or mRNA that may promote recovery via angiogenesis under certain circumstances [114, 115]. Circulating EMPs account for about 17.1% of total MPs. Although EMPs have been categorized into groups according to their surface markers detected by flow cytometry (Table 1) [113], more studies are needed for clarification. Due to sampling limitations, EMPs profiling within ischemic region is still difficult to be completed, however, the correlation of circulating EMPs phenotype with intracranial and extracranial arterial stenosis was already identified [116]. Hence, circulating EMPs may be used as prognostic marker for acute ischemic patients when analyzed within 18.5–51.8 hours after ischemic onset [117].

Other NVU component cells may be involved in the ECV phenomena after injury. As noted, astrocytes communicate with surrounding cells by direct contact and by releasing soluble factors. But they also shed ECVs that may induce angiogenesis and apoptosis [118, 119]. Within larger ECVs shed from astrocytes, mitochondria and other membranous organelles were identified [120]. Oligodendrocytes also secrete exosomes to balance myelin proteins and lipids and to relieve cell stress. These secreted exosome-like vesicles can inhibit morphological differentiation and myelin sheath formation [121]. Moreover, some exosomes released from oligodendrocytes would be selectively transferred from oligodendrocytes to microglia [122]. Research in NVU and ECVs is relatively new, and therefore, mechanisms by which ECVs from non-neuronal cells affect neuronal survival/ function are still mostly unknown. But recently, conditioned media from neuronal cells was shown to dramatically reduce the release of exosomes from oligodendrocytes [123]. Hence, deeper comprehension of ECV roles on cell-cell signaling in the NVU may hopefully lead to novel therapeutic strategies to protect neurons and all other NVU components against ischemic stress.

6. CONCLUSIONS

The concept of NVU provides an integrated framework for understanding cerebral function and dysfunction in both normal and pathological conditions. In this regard, salvage of only neurons is insufficient for treating stroke or other neurodegenerative diseases. Because cell-cell interactions are essential for proper functioning of the NVU, function and crosstalk between all cell types must be rescued. In this minireview, we have tried to summarize and re-interpret the major mechanisms of stroke pathophysiology in the context of cell-cell signaling in the NVU. A deeper understanding of these signals and substrates will be required for further development of stroke therapies.

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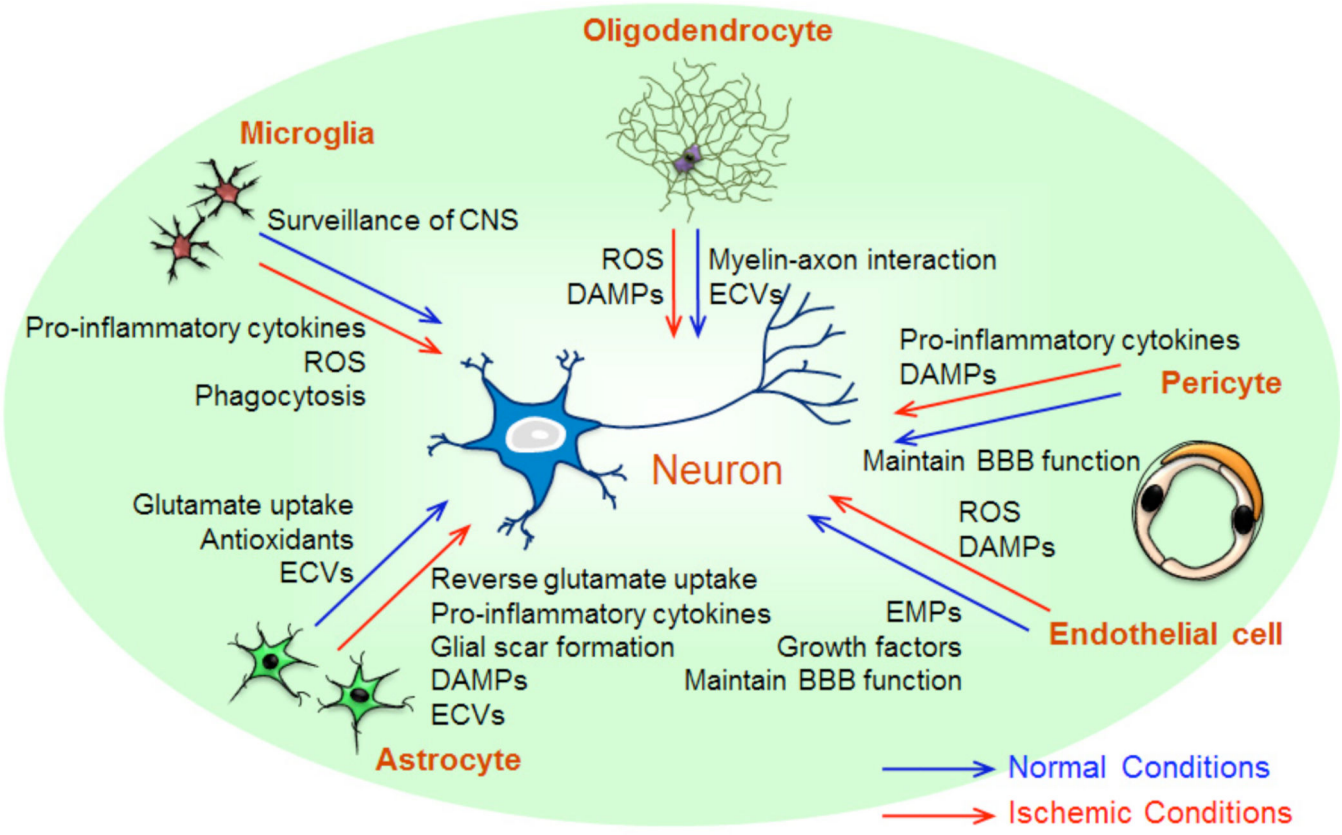

Fig. (1).

Neurovascular Unit (NVU) is composed with neurons, glia (astrocytes, microglia, and oligodendrocytes), vascular components (endothelial cells, pericytes, and smooth muscle cells), and extracellular matrix. Non-neuronal components have cell-cell interactions with neurons through endogenous molecules, such as cytokines, growth factors, chemokines, and microparticles. Neuronal function is maintained on the basis of mutual interactions, but after ischemic stroke, non-neuronal compartments may lead to neuronal damage, resulting in overall NVU/brain dysfunction.

Table 1

Categorization of EMPs According to their Surface Markers Recognized by Flow Cytometry

EMP Name	Surface Markers Recognized by Flow Cytometry
E^+ EMP	$CD105^+$, $CD41a^-$, $CD45^-$
C^+ EMP	$CD105^+$, $CD144^+$
PS^+ EMP	$CD105^+$, PS^+, $CD41a^-$
I^+ EMP	$CD105^+$, $CD54^+$, $CD45^-$

E^+ EMP: endoglin-positive EMP; C^+ EMP: specific endothelial EMP expressing VE-cadherin and endoglin; PS^+ EMP: EMP expressing phosphatidylserine; I^+ EMP: EMP expressing ICAM-1.