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Review

Functional implications of axon initial segment cytoskeletal disruption in stroke

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Axon initial segment (AIS) is the proximal part of the axon, which is not covered with a myelin sheath and possesses a distinctive, specialized assembly of voltage-gated ion channels and associated proteins. AIS plays critical roles in synaptic integration and action potential generation in central neurons. Recent evidence shows that stroke causes rapid, irreversible calpain-mediated proteolysis of the AIS cytoskeleton of neurons surrounding the ischemic necrotic core. A better understanding of the molecular mechanisms underlying this "non-lethal" neuronal damage might provide new therapeutic strategies for improving stroke outcome. Here, we present a brief overview of the structure and function of the AIS. We then discuss possible mechanisms underlying stroke-induced AIS damage, including the roles of calpains and possible sources of Ca^{2+} ions, which are necessary for the activation of calpains. Finally, we discuss the potential functional implications of the loss of the AIS cytoskeleton and ion channel clusters for neuronal excitability.

Keywords: axon initial segment; stroke; Ca^{2+}; calpain; spreading depression; sodium channels

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Introduction

Stroke is the fourth most common cause of death and the leading cause of disability in adults[1]. Stroke-associated neurological abnormalities partly depend on neuronal loss in the primary damage area. It is widely recognized that aberrant neuronal circuit function in regions surrounding the stroke necrotic core could be responsible for the development of post-stroke neurological deficits, seizures, and psychiatric and cognitive disabilities[2]. Whereas most research over the past decade has focused on dendritic dysfunction as a consequence of ischemic stroke, recent morphological evidence indicates that stroke causes rapid, irreversible calpain-mediated proteolysis of the AIS cytoskeleton of neurons on the periphery of ischemic territory[3]. The AIS, an approximately 40- to 60-μm-long segment devoid of myelin and adjacent to the cell body[4], represents a highly specialized structure[5-8] that plays critical roles in synaptic integration and action potential (AP) generation. Under most conditions, central neurons are strongly biased to initiate AP in the AIS[9] rather than in the nodes of Ranvier[10] or in the dendrites[11]. Excitable properties of the AIS are largely determined by its electrotonic interactions with the somatodendritic compartment[12-14] and by distinctive patterns of density and characteristics of voltage-gated ion channels. These active and passive parameters appear to be highly plastic under physiological conditions[6, 15-17]. The focus of this review is to summarize the recent findings on the mechanisms of stroke-induced AIS disruptions and to discuss their implications for AIS ion channel function and for neuronal excitability.

AIS structure

Early morphological studies defined AIS as the proximal part of the axon where the neuronal membrane is not covered with a myelin sheath. Many recent studies, however, redefine AIS as the proximal portion of the axon which possesses a distinctive, specialized assembly of cytoskeletal and scaffold proteins[18]. The main scaffold protein that is responsible for the anchoring of ion channels in the AIS is Ankyrin G (AnkG)[19]. The loss of AnkG causes disassembly of the AIS structure[20]. AnkG has a molecular mass of 480 kDa and is composed of a membrane-binding domain, a spectrin-binding domain, a serine-rich domain, a long tail (2200 amino acids) and a carboxy-terminal domain. In comparison to AnkG 480 kDa, the second longest isoform of AnkG found at the AIS, AnkG 270 kDa, lacks the last 1900 amino-acids of the tail[21]. AnkG is one of the earliest proteins to take part in the formation of the AIS[22]. The mechanism of AnkG clustering is not fully understood. Several studies have concluded that an important regulator of the transcription factor NF-κB, IkBα, is also present at the early stages of AIS assembly[23]. The phosphorylation of IkBα by the

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IKK complex releases NF-κB. Interference in this sequence could cause damage to AnkG clustering. Another mechanism associated with AnkG clustering within the AIS is the absence of AnkB, αII-spectrin, or βII-spectrin[24]. These proteins appear in developing axons prior to AnkG and spread from the distal part of the axon towards the proximal part. AnkG begins to accumulate in the proximal part of the axon, a segment where AnkB, αII-spectrin and βII-spectrin are absent. AnkG is also attached to microtubules through EB1/EB3[25]. This stabilizes the AIS and perhaps also regulates vesicular transport[26].

Another important scaffold protein found in the AIS is βIV-spectrin[27]. βIV-spectrin binds to AnkG and is considered essential to maintaining the proper organization of the AIS, but not to its development. The βIV-spectrin Σ6 isoform is the main isoform found in the AIS, although it lacks the actin-binding domain[19]. A recent study using super-resolution fluorescence microscopy revealed the structure of the axon cytoskeleton[28]. Circumference ring-like structures composed of actin were found to repeat every ~180 nm. Spectrin is formed of a hetero-tetrameric complex of two αII-spectrins and two βII-spectrins, with the β-spectrins bound to the actin/adducin filaments[28, 29]. A study by Xu *et al*[28] revealed that both the β-spectrins that are present in the axon and the βIV-spectrin that is present in the AIS form the same repetitive periodic structures at intervals of ~180 nm.

NrCAM and NF186 are adhesion molecules that appear in the late stages of AIS assembly[18]. They bind to AnkG through their FIGQY tyrosine domain when the domain is not phosphorylated[30]. NF186 has an important role in the formation of GABAergic synapses with axo-axonal Chandelier cells[31]. The AIS GABAergic synapses are believed to be inhibitory, although there is evidence of their surprising excitatory effect[32]. NrCAM and NF186 also play a critical role in the function of AIS as a "diffusion barrier", for maintaining neuronal polarity[26]. AnkG can also bind to GABAa receptor-associated protein (GABARAP) and stabilize the GABAergic synapses[33].

Ion channels

Ca^{2+} channels

The presence of calcium channels in the AIS membrane has been appreciated for the past 20 years[34, 35], but only in recent years has their function begun to receive attention. Using two-photon fluorescence imaging, electrical recording and pharmacological tools, Bender and Trussell[36] found that the AISs of dorsal cochlear nucleus cartwheel interneurons contain T- and R-type voltage-gated Ca^{2+} channels. They also demonstrated that blockade of the Ca^{2+} channels delays spike generation and causes the AP voltage threshold to rise. In a different study, Bender and Trussell found that dopamine released from dopaminergic synapses can reduce calcium influx to the AIS by ~30% via activation of G-protein coupled dopamine type 3 receptors[37]. AIS of L5 pyramidal neurons of the ferret prefrontal cortex were shown to contain a different set of Ca^{2+} channels, P/Q and N[38], which respond to both sub- and supra-threshold depolarization.

K^+ channels

$K_v7.2$- and $K_v7.3$-containing channels present in the AIS and nodes of Ranvier generate slowly activating, non-inactivating M current that is active at a near-threshold range of voltages[39]. The channels bind to AnkG at the same motif as voltage-gated Na^+ channels. The central axons also contain $K_v1.1$ and 1.2 channels whose localization mechanism does not depend on AnkG binding, but rather on PSD-93 and Caspr2[40], which are present in the juxtaparanodes[41]. When both $K_v1.1$ and 1.2 channels are present in the AIS of a cell, they show similar patterns of distribution along the AIS[42]. Thus, the density of these channels in L2/3 and CA1 pyramidal neurons gradually increases towards the distal part of the AIS, whereas in the AIS of L5 and CA3 pyramidal neurons, the channels are evenly distributed[42]. The $K_v1.1$ and 1.2 channels give rise to rapidly activating, low-threshold, slowly inactivating, outward potassium current[43, 44]. A subthreshold depolarization can inactivate a portion of these channels and cause a significant broadening of the axonal APs which, in turn, enhances the AP elicited Ca^{2+} influx in the proximal axonal terminals thereby increasing the probability of synaptic release[45, 46]. $K_v2.2$ and K_v3 found in principal cells of the medial nucleus of the trapezoid body are considered to have a role in the regulation of inter-spike intervals during repetitive firing[47]. The presence of Ca^{2+}-activated BK channels in the paranodes of cerebellar Purkinje cells has been recently reported[48]. These channels are characterized by rapid activation and deactivation kinetics and by allosteric gating that depend on the local intracellular Ca^{2+} concentration.

Na^+ channels

The functionally critical features of AIS excitability are largely determined by the density patterns and characteristics of voltage-gated Na^+ (Na_v) channels. Na_v channel density is relatively high in the proximal axon and in the nodes of Ranvier in both excitatory and inhibitory neurons. There remains some controversy, however, as to what extent the density of functional Na^+ channels is greater in the AIS than in the soma[10, 49-52]. Within the AIS and in the nodes, these channels are anchored to AnkG by the binding motif in the II/III loop of the α subunit[53]. In other areas of the cell, the Na_v channels are prone to elimination by endocytosis[54]. Phosphorylation of the Na_v binding motif of AnkG by protein kinase CK2 (an enzyme located specifically in the AIS) regulates the interaction between these two important proteins[55].

The Na^+ channel is composed of large, pore-forming α subunits and four smaller auxiliary β subunits[56]. Four of the α subunits (encoded by SCN1A–SCN3A and SCN8A) are considered to be responsible for the $Na_v1.1$–$Na_v1.3$ and $Na_v1.6$ channels in central neurons[57]. Auxiliary β-subunits in CNS are encoded by SCN1B–4B genes[57]. The AIS of central neurons contain a mixture of these subtypes[42], and in most neurons, they are not evenly distributed along the AIS. This causes great diversity in AIS sodium current among different neurons[58]. In addition, this can play a role in the difference in AP initiation in different neuronal types, along with the unique

cable properties of the AIS[12]. In most neurons, the main Na_v channel in the AIS and nodes of Ranvier is $Na_v1.6$, and it is present in all the major neuron types of the neocortex, hippocampus, cerebellum and olfactory bulb[42]. In L5 cortical and in CA2/3 hippocampal pyramidal neurons, $Na_v1.2$ channels are present in the proximal part of the AIS while $Na_v1.6$ channels almost exclusively populate the distal part of the AIS[42, 51, 59]. Other study suggests, however, that the distribution of $Na_v1.6$ within the AIS is more uniform[42]. $Na_v1.1$ channels are found in the proximal part of the AIS of the parvalbumin (PV)- and somatostatin (SST)-positive GABAergic interneurons[60] of the neocortex and cerebellum[42], in retinal ganglion cells[61] and in spinal cord motor neurons[62]. Mutations in the genes encoding the $Na_v1.1$ channels can cause Dravet syndrome and other types of infantile epilepsy[63]. In both PV and SST interneurons, $Na_v1.6$ channels were found in the distal part of the AIS, whereas $Na_v1.2$ channels were found in the proximal part of the AIS only in SST-positive INs[60]. A recent study showed that, in human cortical pyramidal cells, the pattern of Na^+ channel distribution is similar to that in rodents[64]. The primary difference was a clustering of $Na_v1.2$ channel in some nodes of Ranvier in addition to the $Na_v1.6$ channels.

Na_v channels generate three functionally different currents: (1) I_{NaT}, transient Na^+ current responsible for the AP upstroke generation; (2) I_{NaP}, non-inactivating, persistent Na^+ current, that is generated predominately in the proximal part of the axon[65] and plays a role in the amplification of PSPs[66], repetitive firing[67] and in bursts generation[68]; and (3) I_{NaR}, resurgent Na^+ current that in some neurons facilitates generation of repetitive APs[69].

AIS plasticity

Recent studies[16, 70] (for review see[6, 15]) revealed that the AIS of central neurons is more dynamic than previously thought. Thus, chronic increase in the firing rate of cultured hippocampal neurons result in AIS relocation away from soma[16], while chronic sensory deprivation causes AIS elongation in the chick auditory brainstem[70]. Similar to previously described forms of long-term plasticity of intrinsic excitability[71], it was reported that the plastic changes in the AIS require cytoplasmic Ca^{2+} elevation[72]. AIS plasticity was proposed to be bidirectional and homeostatic[17]. The mechanism of AIS plasticity is not fully understood. Most likely, it is related to changes in the intra-axonal boundaries between AnkG and AnkB[24]. AnkG overexpression has been shown to increase AIS length in cultured hippocampal neurons, whereas AnkB overexpression causes the opposite. The activity-dependent relocation of the AIS in dissociated hippocampal neurons[16] requires L-type channel-mediated Ca^{2+} influx and calcium-sensitive phosphatase calcineurin activation[72]. The functional consequences of AIS plasticity for neuronal excitability are not completely clear. They have to be viewed in a framework of more general biophysical model of AP initiation incorporating the details of Na^+ channel distribution, characteristics and electronic interactions between the trigger zone and neighboring compartments[12]. Interestingly, AIS plasticity has also been found in models of stroke, demyelination, epilepsy, Angelman syndrome, *etc.* (for review see[73]). Some of these disease-related forms of AIS plasticity will be discussed in the following sections.

AIS damage in stroke

Using immunohistochemistry and immunoblot analysis in a rat MCA occlusion model of stroke, Schafer *et al* (2009)[3] found that stroke, 3–72 h from induction, causes massive damage to the AIS, including proteolysis of AnkG and βIV-spectrin. In addition, proteolysis of αII-spectrin in the distal part of the axon was observed. There was also proteolysis of Na_v channels but only 12 h after stroke and on smaller scale. The proteolysis of scaffold proteins was assumed to be caused by Ca^{2+}-dependent cysteine protease-calpain. In an *in-vitro* oxygen glucose deprivation (OGD) model, calpain inhibitors, but not the inhibitors of other proteases, reduced the damage to the AIS scaffold proteins. The researchers also showed that this process is not linked to cell death. Although the nodes of Ranvier have a similar structure to that of the AIS, surprisingly, they were not disrupted in this model. In a similar model of diffused axonal injury and axotomy, a significant local, Ca^{2+}-dependent, calpain proteolysis of scaffold proteins, cytoskeleton[74] and Na_v channels[75] was found. Interestingly, in addition to calpain inhibitors, proteolysis was also blocked by the Na^+ channel blocker TTX, suggesting a role for partially proteolyzed Na^+ channels in the axonal Ca^{2+} overload[76]. Long-term consequences of stroke on AIS structure was studied in cortical neurons two weeks after photo-thrombosis[77]. AIS length of L5 pyramidal neurons in the vicinity of the stroke necrotic core was shortened by ~15% while the overall intensity of the immunofluorescence for AnkG and βIV-spectrin was not significantly different. These changes in AIS structure are, most likely, the result of functional remodeling of the peri-stroke neuronal circuits, rather than calpain-mediated AIS damage.

In all the above models, the damage to the AIS was mediated by calpain activation. Two subtypes of calpain, μ-calpain (calpain I) and m-calpain (calpain II), are considered to be active during neuronal injury[78]. One of the main differences between the two calpain subtypes is that activation of m-calpain requires extremely high elevation in intracellular Ca^{2+} (400–800 μmol/L), while μ-calpain is significantly activated when Ca^{2+} rises to 3–50 μmol/L[79]. Calpain also has a major role in degradation of other axonal cytoskeletal and scaffold proteins during ischemia such as microtubules, neurofilaments and αII-spectrin (reviewed in[80]). The source of the pathological Ca^{2+} elevation that would be sufficient to activate calpains is one of the most important aspects of the AIS damage mechanism.

Ca^{2+} overload

In the center of an ischemic territory, the countdown to neuronal death starts with the onset of anoxic depolarization, approximately 1–5 min after the onset of ischemia[81], when the neuronal cation outflux by the ATP-dependent pumps fails

to compensate for the cation influx of sodium and calcium[82]. Excessive release of glutamate begins a short time after the start of oxygen and glucose depravation (<15 s)[81]. In the post-synaptic membrane, the massive release of glutamate activates AMPA and NMDA receptors[83, 84]. Influx of Na^+ and Ca^{2+} ions via the glutamate receptors causes depolarization and activates Ca^{2+} signaling mechanisms. AMPA receptors are usually impermeable to Ca^{2+} because of the presence of the GluR2 subunit[85]. Under ischemic conditions, there is an increase in AMPA-mediated Ca^{2+} flux through GluR2-lacking AMPA receptors[86].

Another plausible source of Ca^{2+} in stroke is influx via the channels belonging to the Transient Receptor Potential (TRP) family, in particular the TRPM2 and TRPM7 channels[87, 88]. TRPM2 channels are thought to be activated by reactive oxygen species (ROS), nitric oxide (NO), and ADP-ribose (ADPR)[89]. TRPM7 channels can be activated through low extracellular divalent cations, ROS, and pH changes[64]. All of the above are present in neuronal injury areas. TRPM2 is a cation, non-selective ion channel that is permeable to Ca^{2+}, Na^+ and K^+ ions. TRPM7 is characterized by high permeability to divalent cations such as Ca^{2+}, Mg^{2+} and Zn^{2+}[90]. Down regulation of TRPM7 in hippocampal CA1 neurons lowered the Ca^{2+} current through TRPM7 channels and prevented delayed neuronal death[91].

In the last decade, several studies have implicated members of recently discovered Pannexin channel subfamily in neuronal injury and death[92]. Pannexins are non-selective, large-pore channels[93]. The first study that investigated Pannexin 1 channels in an OGD model found them passing a large inward current after 10 min of OGD[94]. Their activation is likely due to NO and ROS[95]. An increase in Pannexin 1 open probability was attributed to NMDA receptor activation. Namely, activation of Src family kinases (SFKs) by NMDA receptors causes phosphorylation of Pannexin 1 resulting in its activation[96]. It is still unclear, however, to what extent Pannexin activation contributes to neuronal injury in *in vivo* stroke. One study using a MCA occlusion model showed that down-regulation of Pannexin 1 and 2 causes a significant decrease in infarct volume[97]. Another study using an anoxic model in hippocampal slices, however, found no effect of pharmacological blockade of Pannexins on the development of anoxic depolarization. Moreover, the anoxic depolarization was completely abolished by bath application of glutamate and GABA receptor blockers[98].

There are also other channels and exchangers that are considered to be activated during ischemia and are permeable to Ca^{2+} such as L-type Ca^{2+} channels, acid-sensing ion channels (ASICs),Na^+/Ca^{2+} exchangers (NCX) and P2X7 channels[99] sensitive to extracellular ATP concentrations (reviewed in Ref[100]). P2X7 channels are considered to be activated in neuronal injury due to high extracellular concentrations of ATP and its metabolites under ischemic conditions[99], but the activation is not sufficient to cause neuronal death alone[101]. A recently published study found that P2X7 receptor activation reduced the density of AnkG and Na_v channels in the AIS, whereas inhibition of these receptors resulted in the opposite[102]. Interestingly, in a MCA occlusion model similar to that in Schafer *et al*[3], inhibition of P2X7 receptors prevented the AIS disruption.

In summary, all the above mechanisms may play a role in AIS Ca^{2+} elevation during ischemia. It remains to be studied, however, whether Ca^{2+} elevation in the proximal axon is due to direct influx, whether the relevant channels and transporters are localized in the AIS, whether intracellular Ca^{2+} stores are involved, or whether it reflects lateral Ca^{2+} diffusion from the soma.

Peri-infarct depolarization

Whereas terminal depolarization in the ischemic core is associated with neuronal death[82], recent studies revealed the existence of persistent waves of transient depolarization spreading in the peri-infarction, penumbra areas[2]. These waves spread at velocity of 2–6 mm per minute causing significant Ca^{2+} and Na^+ elevations, loss of excitability, neuronal swelling, an increase in the interstitial K^+ concentration, dendritic damage and massive release of excitatory neurotransmitters such as glutamate[2]. Spreading depolarization, on one hand increases the tissue's metabolic demand and on the other hand could decrease the blood supply due to "inverse" neurovascular coupling[103]. The mismatch between the metabolic demand and supply could contribute to the expansion of the damage to the tissues that did not suffer from direct ischemia and thus cause the stroke to grow in the first hour following the cerebrovascular event[104]. The damage to penumbra could be reversible (in the first six hour) and the long-term outcome of the stroke can be dramatically improved with the fast revascularization of penumbra[105]. A recent study demonstrated that somatosensory activation of the cortex, apparently by increasing the metabolic demand in the penumbra, could trigger spreading depolarization waves in penumbral "hot zones"[106]. The mechanisms of spreading depolarization wave generation remain unclear.

Functional implications

Evidence by Schafer *et al*[3] in a rodent MCA occlusion model indicates that stroke causes a massive degradation of scaffold proteins of the AIS, but not of the nodes of Ranvier. Interestingly, the degradation of scaffold proteins was not associated with a massive immediate degradation of the Na_v channels. As discussed in the first section, Na_v channel anchoring and assembly is AnkG-dependent[20]. What are the possible functional consequences of the AIS proteolysis? The fate of the AIS Na_v channels remains uncertain. One possibility is that the channels undergo proteolysis and become non-functional, as described in the traumatic brain injury model[75]. It is noteworthy that distinctive Na^+ channel α-subunits have different susceptibility to calpain cleavage, raising a possibility that the extent of channel proteolysis may vary over the AIS length reflective of the nonhomogeneous channels distribution. Moreover, if indeed the main source of the AIS Ca^{2+} elevation is by diffusion from the soma, there might be a

Ca^{2+} and proteolysis gradient over the AIS length, similar to that in the axotomy model[74]. Because the AP trigger zone is located in the distal part of the AIS[11, 12, 44], detailed knowledge of the extent of the proteolysis at this location is necessary for understanding the effect of stroke on neuronal excitability. The middle AIS section might contribute to AP generation by decreasing the capacitive load on the trigger zone. It also plays a critical role in AP backpropagation by providing current, which starts the regenerative depolarization wave in the soma and dendrites[12]. Damage to the channels in the middle part of the AIS, therefore, can increase the AP threshold and suppress the AP backpropagation and spike timing dependent plasticity (STDP). Destruction of the entire AIS, especially the distal part, could make neurons in the stroke penumbra less responsive to synaptic input and thus be partially responsible for the circuit dysfunction and neurological deficits in stroke.

Another possibility is that following cleavage, the channels remain functional while detached from the scaffold. Detached Na^+ channels are expected to diffuse laterally in the membrane and accumulate either in the soma or in the nodes of Ranvier where the scaffold is intact. The presence of partially proteolyzed Na^+ channels in the nodes might significantly affect their excitable properties, especially if proteolysis led to modification of channel gating[76].

In summary, understanding the functional consequences of AIS damage in the stroke penumbra may help to identify potential pharmacological targets for preventing secondary neuronal damage and limiting stroke complications.

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