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The Axon Initial Segment, 50 Years Later: A Nexus for Neuronal Organization and Function

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Abstract

The axon initial segment is a highly specialized neuronal compartment, identified almost 50 years ago by the pioneers of electron microscopy. Located in the first 50 μm of the axon, it contains unique cytoskeletal features and concentrates a repertoire of specific scaffold and membrane proteins that assembles just after axon determination. The axon initial segment (AIS) supports two crucial physiological functions of the mature neuron: first, it generates and shapes the action potential. Second, it separates the cell body from the axon, preserving the molecular identity of each compartment. In addition to a diffusion barrier restricting membrane proteins and lipids exchange, an intracellular filter has been proposed that could selectively exclude somatodendritic vesicles and recruit axonal cargoes. Finally, the AIS scaffold is capable of morphological plasticity during development or in response to network activity. These changes directly impact the neuron excitability, allowing an adaptive and homeostatic response. These plastic electrogenic properties, as well as the regulation of protein transport to and from the axon, may have important implications in several neuropathological contexts where the AIS structure is altered. Fifty years after its first characterization, the AIS thus emerges as a nexus for both neuronal organization and physiology.



1. INTRODUCTION

The neuron is the epitome of cell polarity. In 1865, Otto Deiters first described the archetypal neuron: a cell body bearing several “protoplasmic processes” (dendrites) and a single “axis cylinder process” (axon) (Craig & Banker, 1994; Deiters & Guillery, 2013). As hypothesized later by Ramon y Cajal as the “law of dynamic polarization” in 1889 (Sotelo, 2003), this asymmetry supports the directionality of signal transfer in the brain: the somatodendritic compartment receives synaptic inputs, and the axonal compartment sends signals toward downstream cells in the form of action potentials (Craig & Banker, 1994). The advent of neuronal cell culture allowed deciphering the respective molecular compositions of the somatodendritic and axonal compartments (Craig & Banker, 1994) and the mechanisms underlying the emergence of polarity during neuronal development (Caceres, Ye, & Dotti, 2012; Cheng & Poo, 2012). Later studies have focused on how neuronal polarization arises in vivo, where extracellular cues complement cell-autonomous stochastic events (Lewis, Courchet, & Polleux, 2013; Namba et al., 2015). After the initial axon specification, the neuron grows elaborate dendritic and axonal ramifications and maintains this polarized architecture using a multitude of molecular transport, sorting, and diffusing mechanisms (Kapitein & Hoogenraad, 2011; Lasiecka, Yap, Vakulenko, & Winckler, 2009).

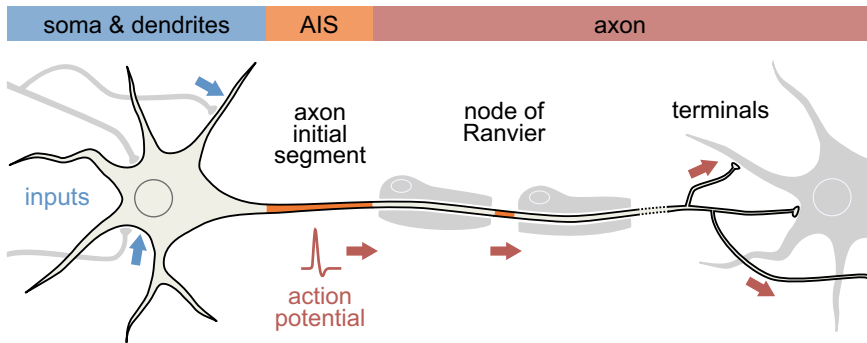


Figure 1 *The axon initial segment (AIS).* The neuron is a polarized cell that integrates synaptic inputs over the cell body (soma) and dendrites (on the left, blue (dark gray in print versions)). The AIS (orange (light gray in print versions)) located along the first 20–60 μm of the axon, realizes the final integration of inputs and the initiation of the action potential (red (darker gray in print versions)) that propagates along the axon toward presynaptic terminals that contact downstream neurons (on the right, red (darker gray in print versions)). The axon can be myelinated by Schwann cells or oligodendrocytes (along the axon in gray), and the action potential is regenerated at nodes of Ranvier between myelin sheets (red (darker gray in print versions)).

Strategically located between the somatodendritic and axonal compartments, the axon initial segment (AIS) occupies the first 20–60 μm of the axon (Figure 1). It is present in almost all vertebrate neuronal types, forming a unique compartment with specific morphological features and protein components (Chang & Rasband, 2013). The AIS ensures two functions that are crucial for the neuron physiology: it generates the action potentials, and separates the axon from the cell body to maintain its molecular identity (Leterrier & Dargent, 2014). I will detail how AIS components and organization were determined along the years, before outlining the mechanisms of its formation and maintenance. After reviewing its role in electrogenesis and polarity maintenance, I will discuss its recently discovered plasticity and alterations in disease.



2. THE AIS MORPHOLOGY: EARLY ELECTRON MICROSCOPY WORKS

The emergence of the axon from the cell body was first described by Kölliker in 1849 and pictured by Deiters in 1865 (Clark, Goldberg, & Rudy, 2009; Deiters & Guillery, 2013). The first precise descriptions from electron microscopy works defined the AIS as the axonal portion immediately

following the hillock, usually 20–60 μm long, stopping at the beginning of the myelin sheath in myelinated neurons. The AIS has a 0.5–2 μm diameter and presents unique morphological features: a dense layer undercoating the plasma membrane, a grouping of microtubules into fascicles, and a sharp decrease in the number of ribosomes (Conradi, 1966; Kohno, 1964; Palay, Sotelo, Peters, & Orkand, 1968; Peters, Proskauer, & Kaiserman-Abramof, 1968). On close examination, the undercoat is connected with an external density on the other side of the plasma membrane, suggesting a dense complex of submembrane and membrane proteins (Palay et al., 1968). The undercoat is tripartite, with a 7.5-nm granular layer connecting the plasma membrane to a 7.5-nm-thick lamina that lies above 35-nm deep filamentous tufts (Chan-Palay, 1972). In pyramidal cortical neurons and cerebellar Purkinje cells, microtubules form 5 to 8 fascicles that group together 3 to 10 microtubules. In a fascicle, microtubules are ~ 40 nm apart (microtubule center to center) and connected by protein bridges (Kohno, 1964; Palay et al., 1968; Peters et al., 1968) of unknown molecular identity. Finally, most of these early works emphasized the presence of axoaxonic, symmetric synapses: these postsynaptic specializations are devoid of the AIS undercoat and often associated with cisternal organelles (Figure 2(A) and (B)). Besides synaptic appositions, the AIS is also specifically contacted by microglia processes in the cortex (Baalman et al., 2015).



3. THE AIS MOLECULAR COMPONENTS AND THEIR INTERACTIONS

3.1 The Ankyrin G-Bound Scaffold

Over the years, studies have identified molecular components of the AIS. The canonical protein complex in the AIS consists in ion channels and cell adhesion molecules (CAMs) anchored to a specialized ankyrin/spectrin scaffold that is connected to both actin filaments and microtubules. The central interacting hub of this complex is ankyrin G (from the Greek *ankyra*, anchor), which interacts with all other components and is often designated as the AIS “key organizer” (Figure 2(C) and Figure 3(A), Rasband, 2010). Ankyrin G is the third member of the ankyrin family in mammals that also includes ankyrin R (part of the ankyrin/spectrin complex in red blood cells, Bennett & Stenbuck, 1979) and ankyrin B (originally described as the “brain” ankyrin, Davis & Bennett, 1984). Ankyrin G was first detected using anti-ankyrin R antibodies in an ankyrin R-deficient

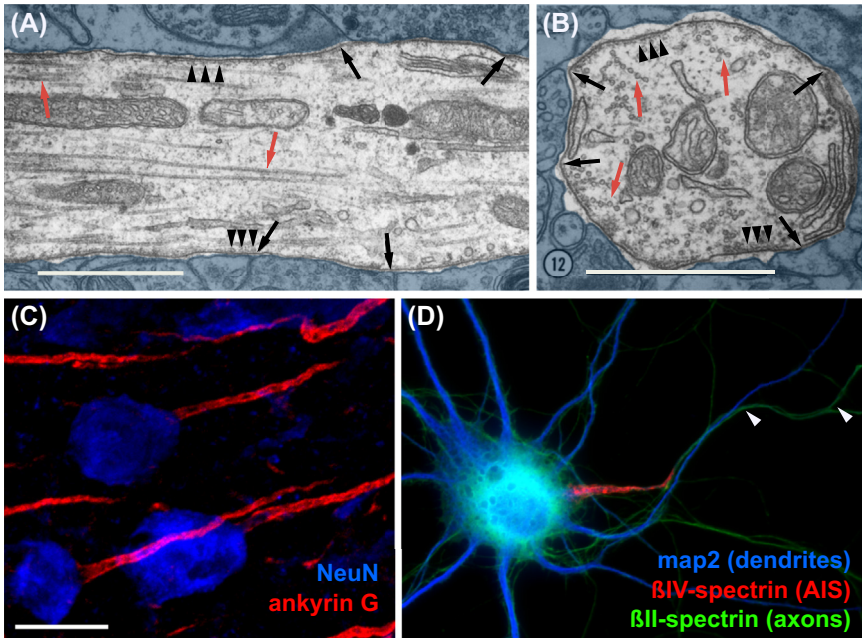


Figure 2 *Morphology of the axon initial segment (AIS).* (A) Electron microscopy image of a longitudinal section through the AIS of a rat hippocampal CA3 neuron. The microtubule fascicles are visible (red arrows) supporting the transport of organelles and vesicles. The dense undercoat lines the plasma membrane (arrowheads) and stops at axoaxonic synaptic contacts present on the AIS (top and bottom membrane, between arrows). Scale bar, 1 μm . (B) Electron microscopy image of a cross section through the AIS of a rat hippocampal CA3 neuron. Microtubule fascicles are seen as string of beads (red arrows). The dense undercoat can sometimes be seen as a row of small dots (arrowheads). The undercoat stops at sites of cisternal organelles apposition (arrows). Scale bar, 1 μm . ((A) and (B) are reproduced from (Kosaka, 1980) with permission from Springer.) (C) Brain cortical section from an adult rat showing neurons (labeled with NeuN, blue) and their initial segments (ankyrin G, red). Scale bar, 10 μm . (D) Rat hippocampal neuron after 15 days in culture, labeled for the somatodendritic marker map2 (blue) the AIS scaffold $\beta\text{IV-spectrin}$ (red) and the distal axon scaffold $\beta\text{III-spectrin}$ (green, arrowheads). Scale bar, 20 μm . (See color plate)

mice (Kordeli & Bennett, 1991; Kordeli, Davis, Trapp, & Bennett, 1990). The cloning of ankyrin G and the generation of specific antibodies demonstrated ankyrin G localization at the AIS and nodes of Ranvier (Kordeli, Lambert, & Bennett, 1995). By contrast, ankyrin B is found along the whole shaft of unmyelinated axons (Boiko et al., 2007; Chan, Kordeli, & Bennett, 1993) and clusters around paranodes in myelinated fibers (Chang et al.,

2014; Ogawa et al., 2006). The largest isoform of ankyrin G is a 480-kDa protein (4377 amino acids for human ankyrin G) composed of different domains (Bennett & Lorenzo, 2013): a membrane-binding domain that contains 24 ankyrin repeats and binds to ion channels and CAMs in the AIS; a spectrin-binding domain that interacts with β -spectrin; a serine-rich domain; a ~ 2300 amino acids unstructured tail (sharing homology with i-connexin/titin); and a carboxyterminus containing a death domain. A 270 kDa isoform of ankyrin G is also present in the AIS that results from a splicing event that removes the last 1900 amino acids of the tail. The common domains shared by these two AIS-specific ankyrin G isoforms are the serin-rich domain and the tail, which are missing in the smaller 190 kDa ankyrin G found in the brain, kidney, and heart (Kordeli et al., 1995). The ternary structure of several ankyrin domains has been resolved (Liu, Zhang, & Wang, 2014; Wang, Wei, et al., 2014; Wang, Yu, Ye, Wei, & Zhang, 2012). Ankyrin G labeling is found in close apposition to the plasma membrane (Iwakura, Uchigashima, Miyazaki, Yamasaki, & Watanabe, 2012; Le Bras et al., 2013). This membrane association is important for ankyrin G localization at the AIS, as shown by the role of palmitoylation at cystein 70 in the membrane-binding domain (He, Jenkins, & Bennett, 2012). In contrast, membrane association of distal axon ankyrin B is inhibited by intramolecular interactions (Abdi, Mohler, Davis, & Bennett, 2006; He, Tseng, & Bennett, 2013).

In the AIS, the spectrin that interacts with ankyrin G is β IV-spectrin (Figures 2(D) and 3(A), Berghs et al., 2000). The full-length β IV-spectrin $\Sigma 1$ isoform is a 280-kDa protein (2564 amino acids for human β IV-spectrin $\Sigma 1$) that contains: two adjacent aminoterminal calponin-homology domains that bind actin; 17 consecutive triple helical repeats; a charged domain specific to β IV-spectrin; and a carboxyterminus containing a pleckstrin-homology domain (Bennett & Baines, 2001). In rodents and probably in humans, a shorter β IV-spectrin $\Sigma 6$ isoform (140 kDa) is also present at the AIS: it lacks the aminoterminal half of the protein, including actin-binding sites and the first nine triple helical repeats (Komada & Soriano, 2002). The canonical spectrins exist as heterotetramers of two α -spectrins and two β -spectrins, with lateral antiparallel α - β dimerization combined with end-to-end interaction between α dimers. Although β IV-spectrin can interact with α II-spectrin (Uemoto et al., 2007), no α -spectrin has been specifically identified at the AIS, in contrast to the α II-spectrin/ β II-spectrin assembly found along the distal axon and at paranodes (Galiano et al., 2012; Ogawa et al., 2006). Ankyrins interact

with the 15th helical repeat of β -spectrins (Davis et al., 2009; Kennedy, Warren, Forget, & Morrow, 1991; Yang, Ogawa, Hedstrom, & Rasband, 2007) via their spectrin-binding domain composed of two ZU5 (*ZO-1* and *unc5*) and one UPA (*unc5*, *PIDD*, and *ankyrin*) modules (Ipsaro, Huang, & Mondragón, 2009; Mohler, Yoon, & Bennett, 2004; Wang et al., 2012). In their recent work uncovering the specific role of 480-kDa ankyrin G at the AIS, Vann Bennett's group uncovered a potentially phosphorylated serine in the specific tail domain that is critical for β IV-spectrin interaction and recruitment to the AIS (Jenkins et al., 2015). The possible interplay between this tail motif and the spectrin-binding domain remains to be clarified.

Beside its linkage with actin via β IV-spectrin, ankyrin G is also linked to the microtubule cytoskeleton via the *end binding* (EB) proteins EB1 and EB3 (Leterrier, Vacher, et al., 2011). EB proteins are dimers that associate with growing microtubule plus tips in interphase cells (+TIPs) and coordinate the assembly of the others +TIPs such as CLASPs and CLIPs (Akhmanova & Steinmetz, 2010). On electron microscopy images of the AIS, microtubule fascicles often appears apposed to the membrane undercoat, suggested a link between these structures (Nakajima, 1974; Westrum & Gray, 1976). Interestingly, ankyrin B was initially found to bind brain microtubules (Bennett & Davis, 1981; Davis & Bennett, 1984), using tubulin preparations that are rich in EB proteins (Sweet et al., 2011). At the AIS, ankyrin G binds to the hydrophobic pocket of the EB dimer, preventing binding to +TIPs. This results in a high local concentration of EB and its binding along the microtubules, leading to a stable, filamentous labeling (Leterrier, Vacher, et al., 2011; Nakata & Hirokawa, 2003). The EB-binding site on ankyrin G is unknown, but the tail domain of the 480-kDa ankyrin G notably contains six EB-binding SxIP motifs, making it a strong candidate for EB binding (Jiang et al., 2012). It is likely that several other microtubule-associated proteins have a specific role at the AIS: nuclear distribution element-like 1 (Ndel1/Nudel), a cytoskeleton regulator, concentrates at the AIS (Bradshaw et al., 2008; 2011; Pei et al., 2014; Sasaki et al., 2000). Antibodies against phosphorylated I κ B α label a microtubule-associated epitope along the AIS (Sanchez-Ponce, Tapia, Muñoz, & Garrido, 2008; Schultz et al., 2006), but the target is not I κ B α itself (Buffington, Sobotzik, Schultz, & Rasband, 2012). Finally, an autoantibody found in a patient suffering from paraneoplastic encephalomyelitis results in microtubule-associated labeling along the AIS, but its target is unknown (Shams'ili, de Leeuw, Hulsenboom, Jaarsma, & Smitt, 2009).

3.2 Ankyrin G-Bound Membrane Proteins: CAMs and Ion Channels

As the central hub of the complex, ankyrin G interacts with a range of membrane proteins that concentrate at the AIS ([Figure 3\(A\)](#)). CAMs of the L1-CAM family represent up to 1% of the membrane proteins in the brain ([Bennett & Baines, 2001](#)). The 186-kDa isoform of neurofascin (NF-186) as well as the *neuronal cell adhesion molecule* (NrCAM) is specifically concentrated at the AIS and nodes of Ranvier ([Davis, Lambert, & Bennett, 1996](#)). Their cytoplasmic tail interacts with several sites along the inner groove of the ankyrin G membrane-binding domain ([Michaely & Bennett, 1995](#); [Wang, Wei, et al., 2014](#)). Relatively to the diverse roles of L1-CAMs in cellular interactions and neuronal growth, not much is known so far about the specific role of NF-186 and NrCAM at the AIS. NF-186 recruits several extracellular matrix components such as aggrecan, brevican ([Hedstrom et al., 2007](#)), tenascin R, and versican that concentrate in the AIS in vitro and in vivo ([Brückner, Szeöke, Pavlica, Grosche, & Kacza, 2006](#); [Frischknecht et al., 2009](#); [John et al., 2006](#)).

Consistent with its role as the action potential initiation site, the AIS contains a high concentration of voltage-gated sodium channels ([Catterall, 1981](#); [Wollner & Catterall, 1986](#)). These sodium channels are found at a 5- to 50-fold higher density than in the dendrites or distal axon, based on quantitative immunohistochemical or electrophysiological evidence ([Kole et al., 2008](#); [Lorincz & Nusser, 2010](#); but see [Colbert & Pan, 2002](#); [Fleiderovich, Lasser-Ross, Gutnick, & Ross, 2010](#)). The dominant isoform of sodium channels at the AIS of mature neurons is Nav1.6 in rodent ([Jenkins & Bennett, 2001](#); [Kress, Dowling, Eisenman, & Mennerick, 2010](#); [Lorincz & Nusser, 2008](#); [2010](#); [Van Wart, Trimmer, & Matthews, 2007](#)) and human neurons ([Inda, DeFelipe, & Muñoz, 2006](#); [Tian, Wang, Ke, Guo, & Shu, 2014](#)). Nav1.2 is more abundant in the immature AIS and gradually complemented by Nav1.6 during development ([Boiko et al., 2003](#); [Osorio et al., 2010](#)). In mature neurons of the cortex and hippocampus, Nav1.2 localizes in the proximal part of the AIS, and Nav1.6 is more concentrated along the distal part ([Hu et al., 2009](#); [Lorincz & Nusser, 2010](#); [Tian et al., 2014](#); [Wimmer, Reid, Mitchell, et al., 2010](#)). Nav1.1 channels have also been detected along the proximal part of the AIS in cortical interneurons ([Ogiwara et al., 2007](#); [Tian et al., 2014](#); [Van Wart et al., 2007](#); [Wimmer et al., 2015](#)), motoneurons ([Duflocq, Le Bras, Bullier, Couraud, & Davenne, 2008](#)), and cerebellar Purkinje cells ([Xiao, Bosch, Nerbonne, & Ornitz, 2013](#)). Nav channel concentration is due to

their interaction with ankyrin G (Srinivasan, Elmer, Davis, Bennett, & Angelides, 1988) that occurs between a motif in the intracellular loop II–III of the channels (Garrido et al., 2003; Gasser et al., 2012; Lemaillet, Walker, & Lambert, 2003) and ankyrin repeats in the ankyrin G membrane-binding domain (Srinivasan, Lewallen, & Angelides, 1992; Wang, Wei, et al., 2014).

At the AIS, the Nav1.1/1.2/1.6 α subunits are often associated with auxiliary β subunits (O'Malley & Isom, 2015). These immunoglobulin loops containing CAMs associate with the α pore-forming subunit via noncovalent interaction (for β 1 and β 3) or disulfide bonds (for β 2 and β 4). Depending on the neuronal type, the AIS can exhibit a concentration of β 1 (Brackenbury et al., 2010; Wimmer et al., 2015), β 2 (Chen et al., 2012), or β 4 (Buffington & Rasband, 2013; Miyazaki et al., 2014) subunits. These subunits have been reported to interact with several other AIS components, including NrCAM, NF-186 (McEwen & Isom, 2004), and ankyrin G (Malhotra et al., 2002). Intracellular homologous factors of fibroblast growth factors FGF12 (Wildburger et al., 2015), FGF13 (Goldfarb et al., 2007), and FGF14 (Laezza et al., 2007; Lou et al., 2005; Xiao et al., 2013) also interact with Nav α subunits at the AIS, where they modulate their electrophysiological properties. In addition to voltage-gated sodium channels, ankyrin G also binds to potassium channels KCNQ2 and KCNQ3 (Kv7.2 and Kv7.3) that are concentrated at the AIS (Devaux, Kleopa, Cooper, & Scherer, 2004; Klinger, Gould, Boehm, & Shapiro, 2011; Pan et al., 2006; Rasmussen et al., 2007; Sanchez-Ponce, DeFelipe, Garrido, & Muñoz, 2012). The ankyrin G-binding motif, present in multiple copies in the tetrameric channel, is homologous to the sodium loop II–III motif (Pan et al., 2006), but is likely to have appeared independently (Hill et al., 2008), and binds to an overlapping site in the ankyrin G membrane-binding domain (Xu & Cooper, 2015).

3.3 AIS Synapses and Cisternal Organelles

As noted in the early electron microscopy works, the AIS often contains postsynaptic specializations characterized by an interruption of the AIS undercoat (Jones & Powell, 1969; Peters et al., 1968) sometimes located on axonic spines (Figure 3(B), Kosaka, 1980; Sloper & Powell, 1979; Westrum, 1970). These are GABAergic synapses and their density and repartition depend on the neuronal types studied: cerebellar Purkinje cells AIS only have rare synapses on the proximal AIS (Iwakura et al., 2012; Somogyi & Hámori, 1976), whereas pyramidal neurons from the cortex

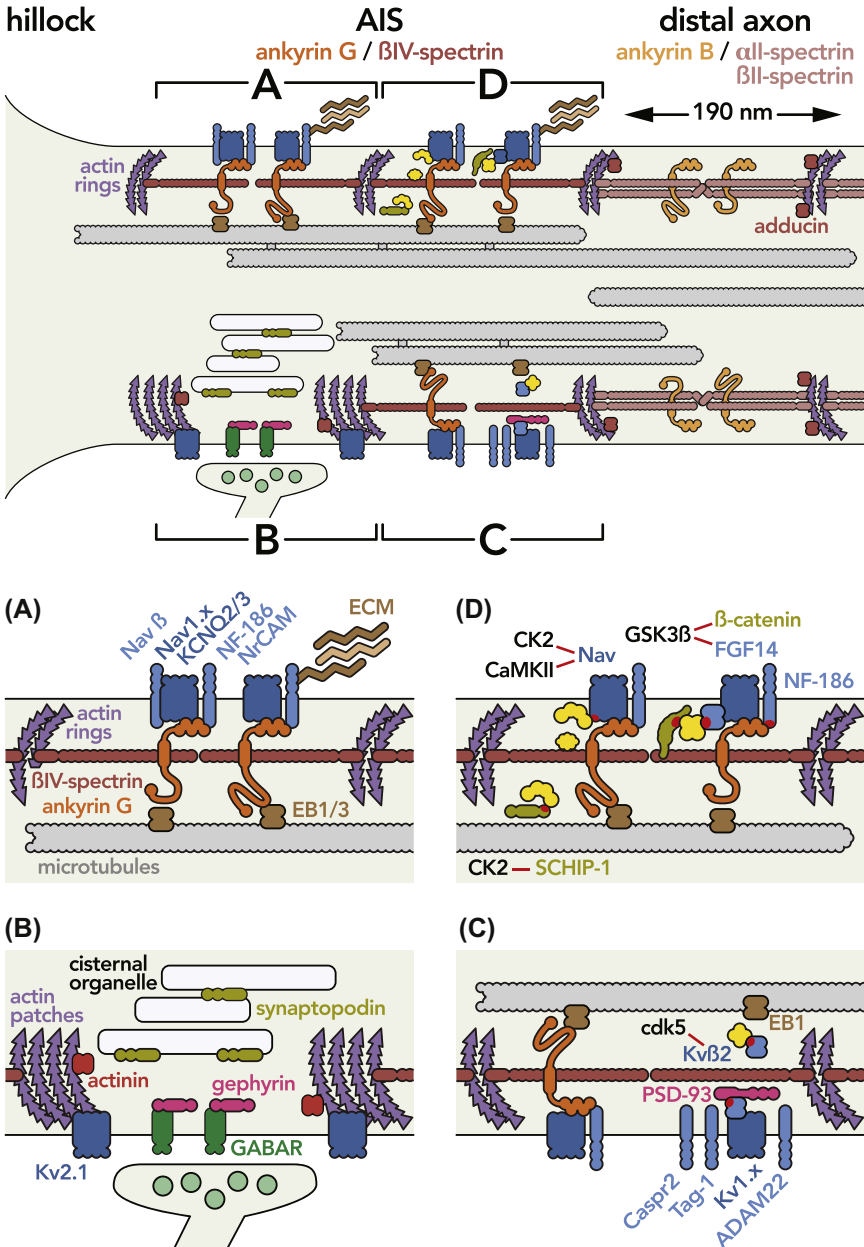


Figure 3 Components of the AIS and their modulation. The AIS, characterized by its ankyrin G/ β IV-spectrin scaffold sits between the axon hillock (left) and the distal axon (right), which contains an ankyrin B/ β III-spectrin scaffold. Actin rings are regularly found (190 nm period) along the AIS and distal axon, capped by adducin (left). (A) The canonical AIS complex. Actin rings are connected by dimers of β IV-spectrin, that

are contacted by 30–200 synapses, with a higher density on the distal AIS (Fish, Hoftman, Sheikh, Kitchens, & Lewis, 2013; Kosaka, 1980; Sloper & Powell, 1979). In the cortex, AIS GABAergic innervation is ensured by specialized axoaxonic interneurons called chandelier cells (DeFelipe, Hendry, Jones, & Schmechel, 1985; Inan et al., 2013; Lewis, 2011; Somogyi, Freund, & Cowey, 1982; Tai, Janas, Wang, & Van Aelst, 2014). GABAergic AIS innervation is also present in hippocampal cultures (Brünig, Scotti, Sidler, & Fritschy, 2002; Burkarth, Kriebel, Kranz, & Volkmer, 2007; Muir & Kittler, 2014). AIS synapses contain GABA $\alpha 2$ and to a lesser extent $\alpha 1$ receptors (Brünig et al., 2002; Christie & De Blas, 2003; Nusser, Sieghart, Benke, Fritschy, & Somogyi, 1996) anchored to gephyrin (Burkarth et al., 2007; Panzanelli et al., 2011; Tretter et al., 2008). Concentration of NF-186 is important for proper innervation of the AIS in Purkinje cells (Ango et al., 2004) and hippocampal neurons (Burkarth et al., 2007; Kriebel et al., 2011).

Peculiar cisternal organelles, similar to the spine apparatus found in a subset of dendritic spines, are often seen associated with AIS synapses on electron microscopy images (Kosaka, 1980; Peters et al., 1968). These cisternal organelles are calcium stores (Benedeczky, Molnár, & Somogyi, 1994) enriched in synaptopodin, inositol 1,4,5-triphosphate receptor 1 (IP(3)R1), α -actinin, and annexin 6 (Figure 3(B), Antón-Fernández, Rubio-Garrido, DeFelipe, & Muñoz, 2015; Bas Orth, Schultz, Müller,



interact with the ankyrin G scaffold. Nav α subunits and KCNQ2/3 ion channels are concentrated by their anchoring to ankyrin G. Also anchored to ankyrin G are CAMs (NF-186 and NrCAM) that bind ECM components (aggrecan, brevican, tenascin R, versican), whereas Nav β subunits are linked to their α counterpart. Ankyrin G is in turn bound to microtubules via the EB end binding proteins EB1 and EB3. (B) The AIS synapse. GABAergic synapses contain $\alpha 2$ and $\alpha 1$ GABA receptors (GABAR), anchored by gephyrin. Apposed to the synapse are cisternal organelles rich in synaptopodin. The synapses are often associated with actin patches rich in actinin, and membrane clusters of Kv2.1. (C) The distal AIS complex. In certain neuronal types, potassium channels Kv1.2 and Kv1.1 are concentrated along the distal portion of the AIS. These are associated with the CAMs Caspr2 and Tag-1, the metalloprotease ADAM22, and the PSD-93 scaffold. Kv $\beta 2$ subunits associate with Kv1 channels, and regulate their delivery via their phosphorylation by cdk2/5 that detach them from the microtubule-associated EB1. (D) Modulation of component interactions by phosphorylation: a number of kinases can phosphorylate AIS components to regulate their interactions. CK2 phosphorylates Nav channels, modulating their binding to ankyrin G. CK2 also phosphorylates SCHIP-1 to modulate its interaction with ankyrin G. CaMKII is bound to β IV-spectrin and also phosphorylates Nav channels. GSK3 β phosphorylates both FGF14 (a Nav channel partner) and β -catenin at the AIS. (See color plate)

Frotscher, & Deller, 2007; Sanchez-Ponce, Blázquez-Llorca, DeFelipe, Garrido, & Muñoz, 2012; Sanchez-Ponce, DeFelipe, Garrido, & Muñoz, 2011). Clustering of potassium channels Kv2.1 at the AIS occur in proximity to these cisternal organelles (King, Manning, & Trimmer, 2014; Sarmiere, Weigle, & Tamkun, 2008). Interestingly, these sites of Kv2.1 clustering have been implicated in endoplasmic reticulum—plasma membrane junction (Fox et al., 2015), suggesting a protein trafficking role for the actin-rich assembly of GABAergic synapses and cisternal organelles. These complexes correspond to gaps in the ankyrin G/ β IV-spectrin undercoat (King et al., 2014; Peters et al., 1968). This does not rule out a role for the ankyrin-based scaffold in their assembly, as perisynaptic 480-kDa ankyrin G organizes GABAergic synapses around the cell body and AIS of mature neurons (Tseng, Jenkins, Tanaka, Mooney, & Bennett, 2015). This is done via interaction with GABARAP, which was shown to concentrate at the AIS when expressed in a transgenic mouse model (Koike et al., 2013).

3.4 The Distal AIS Complex and Other AIS Components

Another distinct, but less well-characterized complex is found along the distal AIS (Figure 3(C)). This complex is found in cortical neurons and motoneurons, but not in cerebellar Purkinje cells (Lorincz & Nusser, 2008) and includes voltage-gated potassium channels Kv1.1 and Kv1.2 (Dodson, Barker, & Forsythe, 2002; Duflocq, Chareyre, Giovannini, Couraud, & Davenne, 2011; Goldberg et al., 2008; Inda et al., 2006; Kirizis, Kerti-Szigeti, Lorincz, & Nusser, 2014; Lorincz & Nusser, 2008; Sanchez-Ponce, DeFelipe, et al., 2012; Trimmer, 2015). They are associated with the CAMs Caspr2 (*contactin-associated protein-like 2*) and Tag-1 (*transient axonal glycoprotein-1*, Ogawa et al., 2008), as well as the metalloprotease ADAM22 (*A disintegrin and metalloprotease domain 22*, Ogawa et al., 2010). These proteins lack known interactions with ankyrin G, but Kv1.1/1.2 channels bind to PSD-93 (Ogawa et al., 2008) as well as the cytoplasmic subunit Kv β 2 in the distal AIS (Vacher et al., 2011).

Over the years, several other membrane and cytoplasmic proteins have been reported to localize or act at the AIS. Voltage-gated calcium channels Cav2.1 (P/Q-type current) and Cav2.2 (N-type) concentrate along the AIS of cortical neurons (Yu, Maureira, Liu, & McCormick, 2010). Modulation of the action potential occurs at the AIS via Cav3.1/3.2 (T-type) channels (Bender & Trussell, 2009; Bender, Ford, & Trussell, 2010; Bender, Uebele, Renger, & Trussell, 2012), but no AIS immunolocalization has been reported. Serotonergic receptors 5HT1a have been found to localize

along the AIS of cortical neurons in humans (Cruz, Eggan, Azmitia, & Lewis, 2004; Czyrak, Czepiel, Maćkowiak, Chocyk, & Wędzony, 2003; DeFelipe, Arellano, Gómez, Azmitia, & Muñoz, 2001) and could inhibit action potential generation via spillover from soma-liberated serotonin (Cotel, Exley, Cragg, & Perrier, 2013). Regarding cytoplasmic proteins, SCHIP1 (*Schwannomin interacting protein 1*) has been found to localize at the AIS, thanks to its interaction with ankyrin G (Martin et al., 2008; Papandréou et al., 2015). The endocytic protein amphiphysin II/BIN1 was found to localize very specifically to the AIS, but this finding was not replicated or pursued (Butler et al., 1997). Finally, an antibody against a phosphorylated form of β -catenin was found to preferentially label the AIS of neurons in cultures or brain sections (Tapia et al., 2013), as well as antibodies against the E3 ubiquitin ligase MDM2 (*murine double minute 2*, Zhao, Wang, Xu, & Zhu, 2014).



4. THE MOLECULAR STRUCTURE OF THE AIS

To precisely understand the AIS functions, it is necessary to determine the precise arrangement of AIS components. Super-resolution microscopy now allows studying the organization of protein complexes down to a few tens of nanometers in situ (Maglione & Sigrist, 2013) and has provided decisive insights into the organization of the axon and AIS. The flexible nature of helical repeats allows spectrin to act like a spring, with length of tetramers varying between 60 and 200 nm depending on biological contexts (Brown et al., 2015). In erythrocytes, a hexagonal lattice is formed by spectrins with a 160-nm radial extent (Byers & Branton, 1985; Liu, Derick, & Palek, 1987; Nans, Mohandas, & Stokes, 2011). In axons, extension of the α II/ β II tetramer connected to actin on both sides results in a striking periodic lattice along the axon plasma membrane, with longitudinal spectrins connecting rings of adducin-capped actin filaments (Xu, Zhong, & Zhuang, 2013). Actin rings and spectrin epitopes are thus regularly spaced every 190 nm (Xu et al., 2013; Zhong et al., 2014), a period corresponding to the purified spectrin tetramer length observed by electron microscopy (Bennett, Davis, & Fowler, 1982; Glenney, Glenney, & Weber, 1982). Accordingly, ankyrin B (that interacts with the center of the spectrin tetramer) is found as regularly spaced clusters between the actin rings (Xu et al., 2013). This regular actin/ β II-spectrin submembrane lattice could provide both flexibility and resistance to the axon, with rigid rings connected by flexible linkers, like a vacuum cleaner hose (Brown et al., 2015; Lai & Cao, 2014; Morris, 2001). In support to this hypothesis,

Caenorhabditis elegans axonal β -spectrin/UNC-70 forms a prestressed lattice important for touch sensation (Krieg, Dunn, & Goodman, 2014), and mutants lacking β -spectrin/UNC-70 have fragile axons that break when the animal moves (Hammarlund, Jorgensen, & Bastiani, 2007).

In the AIS, early electron microscopy work detailed the membrane undercoat as being made of three radial layers, and presenting a longitudinal periodicity of ~ 70 nm (Chan-Palay, 1972). Similar to the actin/ β II-spectrin lattice in the distal axon, super-resolution microscopy resolves β IV-spectrin and actin as regularly spaced clusters with a 190-nm period (Figure 3, D'Este, Kamin, Göttfert, El-Hady, & Hell, 2015; Leterrier et al., 2015; Xu et al., 2013; Zhong et al., 2014). β IV-spectrin aminoterminal localizes along actin bands, and β IV-spectrin carboxyterminus bands are found in the void between actin bands, showing that the AIS undercoat is formed by head-to-head dimers of β IV-spectrin connecting rings of actin filaments (Leterrier et al., 2015). Whether these filaments are associated with adducin is unsure, given the low adducin immunolabeling in the AIS (Jones, Korobova, & Svitkina, 2014). Sodium channels are also periodically clustered between actin rings, and it was hypothesized that this could have an influence on action potential generation at the AIS (Rasband, 2013; Xu et al., 2013). NF-186 clusters also localize between actin rings (D'Este et al., 2015; Leterrier et al., 2015). Scanning electron microscopy coupled to immunogold labeling was recently used to visualize individual proteins inside the AIS and showed ankyrin G and β IV-spectrin were visible as elongated rods (120–160 and 40–80 nm long, respectively) that formed a dense coat around AIS microtubules (Jones et al., 2014). The discrepancy between the ~ 60 nm length of individual β IV-spectrin proteins seen by electron microscopy, and the 190-nm periodicity of β IV-spectrin observed by super-resolution microscopy could be due to the presence of the $\sum 6$ isoform, that is shorter and does not bind actin, or to refolding of spectrins during sample processing for electron microscopy.

Sodium channels, NF-186, and the carboxyterminal side of β IV-spectrin all bind to the aminoterminal part of ankyrin G, which is also periodically organized in the submembrane complex (Leterrier et al., 2015; Zhong et al., 2014). Strikingly, no periodic pattern is detected for the carboxyterminus of ankyrin G (Leterrier et al., 2015; Zhong et al., 2014). This could be due to the presence of the 480- and 270-kDa isoforms that have tails of different lengths, or to a localization of the ankyrin G carboxyterminus away from the periodic submembrane lattice. Radial mapping of the ankyrin

G epitopes using super-resolution microscopy supports the latter hypothesis, showing that the ankyrin G carboxyterminus localizes below the submembrane complex, 35 nm deeper than its aminoterminal part (Leterrier et al., 2015).

Along the distal axon, 220- and 440-kDa isoforms of ankyrin B are likely to have distinct roles, with the shorter 220 kDa ankyrin B primarily needed for axonal transport (Lorenzo et al., 2014). Finally, actin rings may not be the sole form of actin present in the AIS and axon: $\sim 1\text{-}\mu\text{m}$ actin patches have also been described in the AIS (Jones et al., 2014; Watanabe et al., 2012), and a system of actin “hot spots” and labile longitudinal filaments named “actin trails” have been identified along the whole axon (Ganguly et al., 2015). Actin patches have been proposed to help filtering vesicular transport through the axon (Watanabe et al., 2012; see below), or assigned to AIS synapses (D’Este et al., 2015). Orientation of actin filaments is crucial to the filtering mechanism and is still debated, with different results depending on the technique used (Jensen et al., 2014; Jones et al., 2014). Importantly, disruption of actin impacts the nascent AIS (Song et al., 2009; Xu & Shrager, 2005), but the mature AIS scaffold is resistant to actin depolymerization (Jones et al., 2014; Li et al., 2011; Sanchez-Ponce, DeFelipe, et al., 2011).



5. AIS ASSEMBLY AND MAINTENANCE

5.1 Timing and Relation to Axon Formation

Accumulation of ankyrin G and β IV-spectrin is detected in cortical neurons right after birth in rodents (Galiano et al., 2012; Jenkins & Bennett, 2001) and primates (Cruz, Weaver, Lovallo, Melchitzky, & Lewis, 2009), with reports of AIS-positive labeling detected as soon as E19–E20 in rats (Berghs et al., 2000; Gutzmann et al., 2014). Ankyrin G is first detected at the distal part of the AIS and accumulates toward the soma in cortical neurons (Galiano et al., 2012). In mouse motoneurons, ankyrin G restriction to the AIS begins at E11.5, after being detected along the whole axon (Le Bras et al., 2013). In hippocampal culture, ankyrin G and spectrin appear at the AIS after around two days in culture, are detected in all neurons after 4–6 days (Boiko et al., 2003; Hedstrom et al., 2007; Yang et al., 2007), and progressively accumulate over the weeks (Jones et al., 2014). In all cases, AIS assembly occurs after axon specification and is not required for the initiation of neuronal polarity, which occurs normally in ankyrin

G-depleted neurons (Galiano et al., 2012; Hedstrom et al., 2007; Jenkins & Bennett, 2001; Zhou et al., 1998).

5.2 Ankyrin–Spectrin Trafficking and Clustering

Time course analysis of protein concentration at the AIS in wild-type or component-depleted neurons have delineated the hierarchical “inside-out” sequence of AIS assembly. Ankyrin G is the main AIS organizer: it appears early at the AIS and is responsible for the recruitment of its partners. Depletion of ankyrin G results in the complete absence of an AIS (Dzhashiashvili et al., 2007; Hedstrom et al., 2007; Jenkins & Bennett, 2001; Sobotzik et al., 2009; Zhou et al., 1998). The initial concentration of ankyrin G in the AIS depends on the interplay between the proximal ankyrin G/ β IV-spectrin and the distal ankyrin B/ α II/ β II-spectrin scaffolds (Galiano et al., 2012). In cortical neurons, ankyrin B/ α II/ β II-spectrin are first expressed and invade the distal part of the axon, due to transport via a kinesin-2 (KIF3/KAP3) complex (Galiano et al., 2012; Takeda et al., 2000). Ankyrin G is expressed later and accumulates along the plasma membrane not yet occupied by ankyrin B, resulting in a concentration along the proximal axon (Galiano et al., 2012). Ankyrin G is transported into the axon by a kinesin-1 motor (KIF5) that binds its membrane-binding domain (Barry et al., 2014). In addition, the specific domains found in the 270- and 480-kDa isoforms (serin-rich domain and tail) help restricting ankyrin G expression to the proximal axon (Zhang & Bennett, 1998).

Ankyrin B could also have a role in β II-spectrin transport to the axon, as its 220-kDa isoform is necessary for axonal transport (Lorenzo et al., 2014), and neurons lacking ankyrin B have more β II-spectrin in dendrites than in the axon (Zhong et al., 2014). The β II-spectrin periodic scaffold, as well as the AIS morphology, are not altered in ankB $^{-/-}$ neurons (Lorenzo et al., 2014). This suggests that ankyrin B is more important for transport than for the assembly of submembrane scaffolds in distal axons, in contrast to ankyrin G in the AIS. Accordingly, the distal periodic scaffold would primarily be formed by β II-spectrin. Interestingly, β II-spectrin seems to be present as a periodic lattice in the proximal axon and nascent AIS, before being gradually replaced by β IV-spectrin (Zhong et al., 2014). β IV-spectrin is recruited by ankyrin G in the AIS (Yang et al., 2007), and is not necessary for AIS formation (Hedstrom et al., 2007; Lacas-Gervais et al., 2004; Yang, Lacas-Gervais, Morest, Solimena, & Rasband, 2004). However, prolonged absence of β IV-spectrin leads to a progressive destabilization of the AIS in vivo, with a decreased clustering of ankyrin G and ion channels

(Lacas-Gervais et al., 2004; Uemoto et al., 2007; Yang et al., 2004). Similarly, knockdown of the EB1/EB3 link between ankyrin G and microtubules does not prevent AIS formation, but progressively decreases AIS components concentration (Leterrier, Vacher, et al., 2011).

As both ankyrin G (480/270 kDa) and β IV-spectrin ($\sum 1/\sum 6$) have long and short isoforms at the AIS, an interesting question is the respective role of these isoforms in the AIS formation and maintenance. Recently, Vann Bennett's group has developed tools to address the specific roles of 480- and 270-kDa ankyrin G at the AIS (Jenkins et al., 2015). Using knockout mice lacking both isoforms coupled with rescue by transfection in cultured neurons, they showed that 480-kDa ankyrin G is necessary and sufficient for AIS formation. One should thus consider 480-kDa ankyrin G as the main AIS ankyrin G isoform, the shorter isoform being unable to concentrate on its own in the AIS (Jenkins et al., 2015). The shorter β IV-spectrin $\sum 6$ isoform has been shown to be necessary for AIS maintenance in addition to the $\sum 1$ isoform (Uemoto et al., 2007), but whether the two isoforms have a different role at the molecular level is unknown.

5.3 Recruitment of Membrane Proteins and Role in Maintenance

Shortly after the concentration of ankyrin G and β IV-spectrin, membrane protein partners (CAMs and ion channels) start to accumulate at the AIS (Hedstrom et al., 2007; Jenkins & Bennett, 2001). Ankyrin G is directly responsible for the recruitment of most of its membrane protein partners: Nav α and β subunits, KCNQ2/3 channels, NF-186 (Ango et al., 2004; Buffington & Rasband, 2013; Hedstrom et al., 2007; Jenkins & Bennett, 2001; Zhou et al., 1998). Several mechanisms have been proposed to drive the accumulation of AIS membrane proteins: (1) nonselective insertion followed by selective endocytosis from outside the AIS, (2) nonselective insertion followed by diffusion and capture by the AIS scaffold, and (3) direct insertion in the AIS, with eventual cotransport of scaffolds and membrane proteins (Leterrier, Brachet, Dargent, & Vacher, 2011).

Nonspecific delivery followed by selective endocytosis from the somatodendritic compartment was originally put forth to explain the AIS localization of the Nav channels, based on the study of CD4 fused to intracellular domains of Nav1.2 (Fache et al., 2004; Garrido et al., 2001). A first endocytosis motif in the Nav1.2 carboxyterminus drives axonal expression by triggering somatodendritic-selective endocytosis (Garrido et al., 2001). A second one, located near to the ankyrin-binding motif in

the intracellular loop II-III, is necessary for restricting surface localization to the AIS (Fache et al., 2004). Recently, the use of full-length Nav1.6 constructs confirmed that the ankyrin-binding motif was necessary and sufficient for AIS concentration (Gasser et al., 2012). The diffusion-trapping mechanism is supported by the ankyrin G-dependent immobilization of Kv-Nav chimeras and full-length channels at the AIS (Akin, Solé, Dib-Hajj, Waxman, & Tamkun, 2015; Brachet et al., 2010). However, pulse-chase surface labeling experiments suggest that insertion of the full-length Nav1.6 occurs directly at the AIS, with fewer nonselective delivery events followed by endocytosis or diffusion (Akin et al., 2015). This direct insertion is consistent with the proposed cotransport of Nav channels together with ankyrin G by kinesin-1 motors (Barry et al., 2014). It is possible that both selective endocytosis/diffusion trapping and direct insertion can occur, depending on the type of sodium channel and the developmental stage, Nav1.2 being expressed earlier than Nav1.6. Axonal targeting of KCNQ2/3 channels depends on multiple motifs in the carboxyterminal tail (Chung, Jan, & Jan, 2006), and an ankyrin G-binding motif homologous to the Nav channel motif directs their AIS concentration (Pan et al., 2006; Rasmussen et al., 2007). Less is known about Kv1 trafficking to the distal AIS complex: axonal targeting occurs via the Kv β 2 cytoplasmic subunit that binds the Kv1 T1 domain (Gu, Jan, & Jan, 2003; Rivera, Chu, & Arnold, 2005), kinesin-2, and EB1 (Gu et al., 2006; Vacher et al., 2011). Kv1 are thought to be anchored by PSD-93 at the AIS, but PSD-93 depletion impairs targeting only in vitro, not in vivo (Duflocq et al., 2011; Ogawa et al., 2008; 2010), and the accumulation of Kv1 depends on ankyrin G (Sanchez-Ponce, DeFelipe, et al., 2012). Nonspecific delivery followed by selective endocytosis is also an axonal targeting mechanism for CAMs like L1/NgCAM (*neuron-glia cell adhesion molecule*, Wisco et al., 2003) and has been shown to target NF-186 to the AIS via a doublecortin-dependent mechanism (Yap et al., 2012). Downstream immobilization of NF-186 at the AIS depends on ankyrin G binding (Boiko et al., 2007; Garver, Ren, Tuvia, & Bennett, 1997; Zhang, Davis, Carpenter, & Bennett, 1998).

Once formed, the AIS is strikingly stable, as shown by the slow decrease of protein content after shRNA transfection (Hedstrom, Ogawa, & Rasband, 2008), the persistence of live-cell NF-186 labeling over 10 days (Schafer et al., 2009), and the negligible recovery of Nav1.6 in FRAP experiments (Akin et al., 2015). The low turnover of AIS membrane proteins seems in turn to stabilize the whole compartment. NF-186 is not

necessary for AIS formation (Dzhashiashvili et al., 2007; Hedstrom et al., 2007; Zonta et al., 2011), but conditional ablation of NF-186 suggests that it is recruiting NrCAM to the AIS, and helps maintaining the AIS integrity over time (Zonta et al., 2011). It is not known if Nav channels have a role in stabilizing the AIS assembly, but in cultured motoneurons where the AIS appears rapidly (within 2 days), Nav depletion hinders its assembly (Xu & Shrager, 2005).

5.4 Modulation of Component Interactions

Protein interactions within the AIS are dynamically modulated via multiple phosphorylation events (Figure 3(D)). The first example of such a mechanism is the regulation of the NF-186 binding to ankyrin G: an unknown tyrosine kinase phosphorylates NF-186 at its FIGQY motif, resulting in decreased binding to ankyrin G and decreased anchoring at the AIS (Boiko et al., 2007; Garver et al., 1997). Another example is the regulation of the interaction between Nav channels and ankyrin G by protein kinase CK2: CK2 enhances Nav channels binding to ankyrin G by phosphorylating the binding motif on Nav channels in vitro and in vivo (Bréchet et al., 2008; Hien et al., 2014). This strengthened interaction helps sodium channels accumulation at the AIS (Bréchet et al., 2008; Gasser et al., 2012) by enhancing their immobilization (Brachet et al., 2010). The analogous KCNQ2/3 ankyrin-binding motif can also be phosphorylated by CK2, strengthening ankyrin binding (Xu & Cooper, 2015), whereas Nav1.8 (which is normally not expressed in the CNS) has a phosphomimetic motif that natively binds to ankyrin G with a high affinity (Monterisino et al., 2014). CK2 is concentrated in the AIS in a Nav-dependent manner (Bréchet et al., 2008; Hien et al., 2014), and CK2 inhibition inhibits AIS formation and maintenance (Sanchez-Ponce, Muñoz, & Garrido, 2011). CK2 also phosphorylate SCHIP1, regulating its binding to ankyrin G (Papandréou et al., 2015). It would be interesting to determine if CK2 directly phosphorylates ankyrin G to modulate its function, as it was shown for ankyrin R in erythrocytes (Ghosh, Dorsey, & Cox, 2002) and for the *Drosophila* neuronal ankyrin 2 (Bulat, Rast, & Pielage, 2014). Another kinase that is likely to regulate the AIS structure is cyclin-dependent kinase 5 (cdk5). In mammalian neurons, cdk5 is present in the AIS and modulates the transport of potassium channels Kv β 2 subunits by regulating their interaction with EB1 (Vacher et al., 2011). In *Drosophila*, cdk5 controls the length of an AIS-like complex enriched in ANK1, actin and ion channels (Trunova, Baek, & Giniger, 2011). Another kinase, Ca²⁺/calmodulin-dependent

protein kinase CaMKII, is present in the AIS where it associates with β IV-spectrin and phosphorylates Nav channels (Hund et al., 2010). Glycogen synthase kinase GSK3 β participates in sodium channel targeting to the AIS by regulating its interaction with FGF14 (Shavkunov et al., 2013), and phosphorylates β -catenin, helping AIS maintenance (Tapia et al., 2013).



6. ELECTROGENESIS AT THE AIS

In the next two parts, I will focus on the physiological functions of the AIS. The role of the AIS in neuronal electrogenesis has been thoroughly reviewed (Bender & Trussell, 2012; Clark et al., 2009; Kole & Stuart, 2012; Kress & Mennerick, 2009) and will only be briefly summarized here. Early experimental work (Araki & Otani, 1955; Coombs, Curtis, & Eccles, 1957; Edwards & Ottoson, 1958; Fatt, 1957), as well as modeling studies (Dodge & Cooley, 1973; Mainen, Joerges, Huguenard, & Sejnowski, 1995), suggested the proximal axon as the site for action potential generation. Over the last decades, accumulating evidence has confirmed this for a repertoire of neuronal types: excitatory neurons in the neocortex (Hu et al., 2009; Kole, Letzkus, & Stuart, 2007; Meeks & Mennerick, 2007; Palmer & Stuart, 2006; Popovic, Foust, McCormick, & Zecevic, 2011; Shu, Duque, Yu, Haider, & McCormick, 2007), hippocampus (Kress et al., 2010; Kress, Dowling, Meeks, & Mennerick, 2008; Schmidt-Hieber, Jonas, & Bischofberger, 2008), and basal ganglia (Atherton, Wokosin, Ramanathan, & Bevan, 2008); cerebellar Purkinje cells (Foust, Popovic, Zecevic, & McCormick, 2010; Khaliq & Raman, 2006; Palmer et al., 2010); and parvalbumin and somatostatin-expressing inhibitory interneurons of the neocortex (Li et al., 2014). In addition to the Nav channels density, the low action potential threshold at the AIS could be due to specific biophysical properties of the channels (Colbert & Pan, 2002; Fleidervish et al., 2010; Hu et al., 2009), with a possible role for their interaction with ankyrin G (Shirahata et al., 2006). Importantly, differences in AIS localization, sodium channels density, and subtype content influence the neuron intrinsic excitability (Kress et al., 2010; Kuba & Ohmori, 2009; Kuba, Ishii, & Ohmori, 2006; Wang, Wang, Yu, & Chen, 2011). Notably, the localization of the AIS emergence from a basal dendrite rather than the cell body, which occurs in a subset of hippocampal principal cells, specifically modifies the sensitivity to inputs from this dendrite (Thome et al., 2014). Other ion channels at the AIS modulate the action potential

initiation and shape. KCNQ2/3 channels generate the I_M current, restraining intrinsic firing and neuronal excitability (Brown & Passmore, 2009; Guan, Higgs, Horton, Spain, & Foehring, 2011; Shah, Migliore, Valencia, Cooper, & Brown, 2008; Tran, Gavrilis, Cooper, & Kole, 2014). Kv1 channels can modulate the firing threshold (Cudmore, Fronzaroli-Molinieres, Giraud, & Debanne, 2010; Kole et al., 2007) and are important for shaping the action potential waveform (Bialowas et al., 2015; Dodson et al., 2002; Kole et al., 2007; Rowan, Tranquil, & Christie, 2014; Shu, Yu, Yang, & McCormick, 2007) and dampening excitability in interneurons (Campanac et al., 2013; Goldberg et al., 2008). Regarding calcium channels, Cav2.1 (P/Q-type) and Cav2.2 (N-type) channels have been shown to allow calcium entry into the AIS during electrical activity in cerebellar Purkinje cells (Callewaert, Eilers, & Konnerth, 1996) and cortical neurons (Yu et al., 2010). In auditory brainstem interneurons, Cav3.1/3.2 channels (T-type) modulated by dopamine release regulate complex spiking patterns (Bender et al., 2010; 2012; Bender & Trussell, 2009).

Another aspect of electrogenesis is the role of GABAergic synapses along the AIS. It was hypothesized that these synapses could exert a strong inhibitory control on action potential generation (Jones & Powell, 1969). It was later found that GABAergic axoaxonic synapses could have an excitatory effect on cortical pyramidal cells due to an increased local chloride concentration (Khirug et al., 2008; Molnár et al., 2008; Szabadics et al., 2006; Woodruff, Xu, Anderson, & Yuste, 2009). However, the issue is still debated, with numerous examples of inhibitory action of these synapses (Dugladze, Schmitz, Whittington, Vida, & Gloveli, 2012; Glickfeld, Roberts, Somogyi, & Scanziani, 2009; Veres, Nagy, Vereczki, András, & Hájos, 2014; Wang, Hooks, & Sun, 2014) suggesting that AIS synapses could either have an inhibitory or excitatory effect depending on the neuronal type and network state.



7. THE AIS AND THE MAINTENANCE OF AXONAL IDENTITY

7.1 Maintenance of Axonal Identity by the AIS

The other important role of the AIS is to separate the somatodendritic compartment from the axon, maintaining the axonal molecular identity and thus the overall neuronal polarity. In fact, this polarity maintenance function is likely to have appeared early during evolution,

before the clustering of ion channels via ankyrin-binding and the electrogenic properties (Rolls & Jegla, 2015). Accordingly, concentration of ankyrin in the proximal axon has been described in *Drosophila*, forming a proto-AIS (Trunova et al., 2011). In *C. elegans*, there is no clustering of the ankyrin homolog UNC-44, but it has a role in regulating proper microtubule organization and axonal sorting (Maniar et al., 2012), and organelles access to the axon is regulated (Edwards et al., 2013). Mammalian neurons lacking an AIS progressively acquire dendritic properties along the axon (Rasband, 2010). After AIS disassembly triggered by depletion of ankyrin G, the proximal axon of cultured neurons becomes positive for the somatodendritic marker map2, with the formation of ectopic spines bearing excitatory postsynaptic specializations (Hedstrom et al., 2008). Furthermore, membrane proteins that are restricted to the somatodendritic domain such as β 1-integrin can enter the axon in AIS-deficient neurons (Franssen et al., 2015). In vivo, mice lacking ankyrin G in Purkinje cells also show ectopic spines and a perturbation of the proximal axon identity (Sobotzik et al., 2009).

The location of the AIS immediately suggests that it maintains the molecular identity of the axon by physically separating it from the somatodendritic compartment. Indeed, the AIS regulates protein mobility between the soma and axon by two distinct mechanisms: on the one hand, the AIS assembles a surface diffusion barrier that keeps the somatodendritic and axonal membrane proteins from mixing. On the other hand, the AIS acts as an intracellular traffic filter that exclude somatodendritic protein from entering the axon, and could help to recruit axonal proteins. The diffusion barrier mechanism is well established, and is consistent with the progressive proximo-distal perturbation of axonal identity in AIS-defective neurons (Rasband, 2010). By contrast, the intracellular filter was more recently proposed (Song et al., 2009) and is still hotly debated (Jenkins et al., 2015; Leterrier & Dargent, 2014).

7.2 The AIS Membrane Diffusion Barrier

In the AIS, membrane proteins and lipids do not freely diffuse through the AIS, but are slowed down or immobilized, resulting in the segregation of membrane components. Initially proposed by Dotti and Simons (Dotti & Simons, 1990), this hypothesis was first demonstrated by showing that fluorescent lipids could not diffuse from the axon to the cell body

(Kobayashi, Storrie, Simons, & Dotti, 1992). This result was challenged by later studies, either theoretical (Futerman, Khanin, & Segel, 1993) or experimental (Winckler & Poo, 1996). However, confirmation that the AIS formed a diffusion barrier for axonal proteins was firmly established by measuring the tractability of beads attached to different transmembrane proteins (Winckler, Forscher, & Mellman, 1999). The capacity of the diffusion barrier to also immobilize membrane lipids was conclusively confirmed by video-rate tracking of fluorescent or nanogold-tagged lipids (Nakada et al., 2003). Although a few FRAP studies have been inconclusive (Fukano, Hama, & Miyawaki, 2004; Howarth et al., 2003), the existence of the diffusion barrier has since been confirmed using single-particle tracking of proteins labeled with quantum dots: VAMP2 (Song et al., 2009) or nonpolarized ion channel constructs (Brachet et al., 2010).

An intact AIS structure is necessary for the existence of the diffusion barrier. Nakada et al. showed correlation between the AIS formation and the immobilization of lipids in the proximal axon (Nakada et al., 2003). Furthermore, AIS disassembly via ankyrin G depletion leads to the disappearance of the diffusion barrier (Song et al., 2009). The diffusion barrier is also impaired by actin disruption (Nakada et al., 2003; Winckler et al., 1999). According to the classical “fences and pickets” model, the submembrane lattice (fences) coupled with its high density of bound transmembrane proteins (pickets) is able to slow all lipids and proteins diffusion (Kusumi et al., 2005). In the AIS, the picket role for membrane proteins bound to ankyrin G is confirmed by the immobilization of ion channels and CAMs at the AIS (Angelides, Elmer, Loftus, & Elson, 1988; Boiko et al., 2007; Brachet et al., 2010; Nakada et al., 2003). However, the periodic AIS lattice is a particular case, as the submembrane actin rings (fences) and membrane proteins anchored by ankyrin G (pickets) are separated by the 90 nm length of the β IV-spectrin protein (Xu et al., 2013). It is likely that ankyrin G-bound ion channels and CAMs (rather than the actin/ β IV-spectrin lattice) are the primary source of the diffusion barrier because a similar periodic actin/ β II-spectrin lattice is present along the distal axon, where surface diffusion is unhindered. An interesting result in this regard is the role of β IV-spectrin, which selectively restricts the diffusion of L1 at the AIS compared to the distal axon. This could suggest a protein-specific component to the diffusion barrier, or a partial compensation by β II-spectrin in β IV-spectrin knockout mice (Nishimura, Akiyama, Komada, & Kamiguchi, 2007).

7.3 Intracellular Filter: Role of Ankyrin G and Actin

In addition to the surface diffusion barrier, it has been proposed that the AIS also assembles an intracellular filter for both cytoplasmic diffusion and vesicular transport. In 2009, Moo-Ming Poo's lab showed that cytoplasmic protein diffusion was hindered at the AIS in a size-dependent manner (Song et al., 2009). In addition, vesicular transport through the AIS was found to be slower than in the distal axon and successful axonal entry to depend on the high transport efficacy of axonal kinesins. Both processes depend on actin and ankyrin G, leading the authors to propose an intracellular "molecular sieve" model. The sieve (average pore size of 13 nm) would sterically slow diffusion and transport in the AIS, helping in the maintenance of neuronal polarity (Song et al., 2009). The diffusion barrier for intracellular proteins has been confirmed (Sun, Wu, Gu, Liu, et al., 2014) and its disruption could be the basis of the Map2 invasion of the proximal axon in AIS-depleted neurons (Hedstrom et al., 2008; Jenkins et al., 2015). In contrast, the existence and mechanism of a vesicular transport filter are currently under question. Several subsequent reports did not detect a slowing down of axonal vesicles through the AIS (Jenkins et al., 2015; Petersen, Kaech, & Banker, 2014), questioning the validity of the physical sieve model. Although accumulation of mitochondria at the AIS entrance has been described (Li et al., 2004), the generally unhindered passage of large organelles into the axon also argues against this model.

It is nonetheless accepted that a selective transport occurs at the axon entrance, with specific exclusion of somatodendritic proteins (Al-Bassam, Xu, Wandless, & Arnold, 2012; Burack, Silverman, & Banker, 2000; Petersen et al., 2014). However, a specific role of the AIS scaffold in this process is disputed. Somatodendritic vesicles and organelles did not significantly enter the axon in ankyrin G-depleted neurons (Jenkins et al., 2015). Regarding a distinct role for AIS actin in this process, Don Arnold's lab has demonstrated the targeting of somatodendritic proteins via myosin V binding (Lewis, Mao, Svoboda, & Arnold, 2009). Myosin VI, which travels in the opposite direction on actin filaments, conversely helps axonal targeting (Lewis, Mao, & Arnold, 2011). This leads to an "AIS actin filter" model where oriented actin patches and filaments in the AIS can stop vesicular transport of somatodendritic vesicles harboring myosin V motors (Al-Bassam et al., 2012; Watanabe et al., 2012). In addition to submembrane actin rings, it is clear that the AIS and distal axon can contain intracellular actin filaments and patches (D'Este et al., 2015; Ganguly et al., 2015; Watanabe et al., 2012). In the AIS, the interplay between this actin-based filter and the

ankyrin G/ β IV-spectrin scaffold remains to be determined. Others have argued that the main sorting mechanism occurs upstream in the cell body, when vesicles are packaged and bud from the Golgi apparatus (Fariás et al., 2012; Petersen et al., 2014), with actin disruption resulting in disorganized packaging and mistargeting of proteins in all compartments (Petersen et al., 2014). It is likely that both mechanisms are complementary, the evolution of these partially redundant processes allowing a low rate of mistargeted proteins.

7.4 Microtubule and Vesicular Transport through the AIS

Long-range vesicular transport is ensured by microtubules in neuronal cells (Coles & Bradke, 2015; Kapitein & Hoogenraad, 2015; Prokop, 2013). In the axon, the uniform polarity of microtubules (plus-end out) leads to specific directional transport by kinesins (anterograde) and dynein (retrograde) (Baas & Lin, 2011; Kevenaar & Hoogenraad, 2015). As kinesins can transport both somatodendritic and axonal cargoes, there must be some cues that allow motors to specifically find and walk along microtubules that enter the axon (Hirokawa & Tanaka, 2015). The kinesin-1 (KIF5) head is selectively targeted to axons (Jacobson, Schnapp, & Banker, 2006; Nakata & Hirokawa, 2003; Nakata, Niwa, Okada, Perez, & Hirokawa, 2011) and its nonprocessive mutant accumulates selectively along microtubules in the axon hillock, suggesting the existence of a specific recruitment cue (Nakata & Hirokawa, 2003). Microtubules in the AIS and proximal axon are thought to be stable (Baas, Ahmad, Pienkowski, Brown, & Black, 1993; Hammond et al., 2010) and they are enriched in posttranslational modifications such as acetylation, detyrosination, polyglutamylolation, and possibly polyamination (Hammond et al., 2010; Konishi & Setou, 2009; Song et al., 2013; Tapia, Wandosell, & Garrido, 2010). Kinesin-1 preferably binds to highly modified, stabilized MTs (Cai, McEwen, Martens, Meyhofer, & Verhey, 2009; Dunn et al., 2008; Reed et al., 2006), suggesting that these modifications drive the recruitment of kinesin-1 to the AIS. Stabilizing all neuronal microtubules using taxol abolishes kinesin-1 targeting to the axon (Hammond et al., 2010; Nakata & Hirokawa, 2003), but no consensus exist yet on whether acetylation (Reed et al., 2006; Tapia et al., 2010), detyrosination (Konishi & Setou, 2009), or a combination of multiple modifications (Hammond et al., 2010) have a specific impact on kinesin-1 binding. The concentration of the microtubule minus-end associated protein CAMSAP2 at the proximal AIS could also play a role in the selective recruitment of axonal cargoes (Yau et al., 2014).

An interesting alternative is the possible role of GTP remnants along AIS MTs, resulting from incomplete hydrolysis of tubulin-GTP along microtubules (Dimitrov et al., 2008). Interestingly, these remnants are enriched along the AIS microtubules and could trigger the recruitment of kinesin-1 (Nakata et al., 2011). There could be an interplay between these GTP remnants and EB proteins concentrated in the AIS (Leterrier, Vacher, et al., 2011), as EB proteins preferentially interact with GTP-tubulin when interacting with the dynamic plus ends (Maurer, Fourniol, Bohner, Moores, & Surrey, 2012). Furthermore, the link between ankyrin G and microtubules via EB proteins or other partners could help organize microtubules along the AIS, indirectly driving the recruitment of axonal vesicles. Microtubule organization by the AIS scaffold is further suggested by the lack of fascicles in ankyrin G-deficient Purkinje cells (Sobotzik et al., 2009) and is consistent with the recent discovery of *Drosophila* giant ankyrins that organize microtubules in synapses (Koch et al., 2008; Pielage et al., 2008) and the axonal shaft (Bennett & Walder, 2015; Stephan et al., 2015).



8. AIS MORPHOLOGICAL PLASTICITY AND ALTERATION IN DISEASE

8.1 Developmental and Activity-Dependent Plasticity

AIS components have been identified over the years, uncovering an intricate assembly of cytoskeleton, scaffold, and membrane proteins, analogous in some ways to the postsynaptic scaffold (Sheng & Hoogenraad, 2007). In line with this analogy, recent work has unraveled the morphological plasticity of the AIS, with its position and content regulated by developmental, physiological, and pathological processes (Yoshimura & Rasband, 2014). These changes profoundly influence the AIS electrogenic properties. AIS length and position vary during development (Cruz, Lovallo, Stockton, Rasband, & Lewis, 2009; Galiano et al., 2012) and this can tune sensitivity to inputs, as exemplified by the relationship between AIS morphology and the development of the visual sensory cortex (Gutzmann et al., 2014) or avian auditory neurons (Kuba, Adachi, & Ohmori, 2014). Moreover, two studies have uncovered how that neuronal activity can change the AIS location as a new mechanism of slow homeostatic adaptation (Grubb & Burrone, 2010; Kuba, Oichi, & Ohmori, 2010). Depriving inputs to chick brainstem auditory neurons leads to a longer AIS associated with enhanced excitability (Kuba et al., 2010). Elevating neuronal activity in cultured hippocampal neurons leads to a slow displacement of the AIS toward the distal axon, which leads to a lower

excitability (Grubb & Burrone, 2010), a finding recently confirmed using channelrhodopsin-expressing neurons in hippocampal slices (Wefelmeyer, Cattaert, & Burrone, 2015). Interestingly, GABAergic synapses stay close to the cell body, resulting in increased GABA receptors mobility (Muir & Kittler, 2014) and increased inhibition of action potential firing (Wefelmeyer et al., 2015). In inhibitory dopaminergic interneurons, this homeostatic plasticity is reversed: elevated activity leads to longer initial segments that are closer to the soma, resulting in increased excitability (Chand, Galliano, Chesters, & Grubb, 2015).

8.2 Potential Mechanisms for AIS Plasticity and Alteration

How does a compartment as stable as the AIS profoundly rearrange or disassemble in a matter of hours? The proposed mechanisms for the AIS morphological plasticity are diverse and incomplete, but calcium signaling seems to be the common denominator. In line with the presence of calcium stores (cisternal organelles) and calcium channels at the AIS, calcium-dependent pathways can have a profound impact on the AIS stability. Calmodulin binding drives the assembly of KCNQ2/3 heteromers and thus their targeting to the AIS (Liu & Devaux, 2014). AIS relocation in response to elevated activity depends on Cav1 (L-type)-mediated calcium entry and on the activation of calcineurin, a calcium- and calmodulin-dependent phosphatase implicated in synaptic plasticity and memory (Evans et al., 2013). Finally, fast AIS disassembly in response to ischemia depends on scaffold proteolysis by calcium-dependent cysteine protease calpain, which degrades ankyrin G and β IV-spectrin (Schafer et al., 2009). Interestingly, calpain is also involved in AIS partial disassembly triggered by ATP-activated purinergic P2X7 receptors leading to calcium entry (Del Puerto et al., 2015).

8.3 Alterations of the AIS in Disease

Beyond physiological adaptation, the AIS is also affected in a vast range of pathological conditions. Complete disassembly has been observed in different *in vivo* and *in vitro* models of neuronal injury (Schafer et al., 2009). Subtle alterations in AIS length have been detected after stroke (Hinman, Rasband, & Carmichael, 2013), and small changes in AIS location have been measured in mouse and rat models of epilepsy (Harty et al., 2013). Axotomy caused by mild traumatic brain injury preferentially happens at the AIS (Greer, Hånell, McGinn, & Povlishock, 2013), and less impacted neurons have a lengthier AIS (Baalman, Cotton, Rasband, & Rasband, 2013). A mouse model for Angelman syndrome (caused by deletion of the ubiquitin ligase UBE3A gene) exhibits upregulated ankyrin G and Nav1.6 channels

resulting in longer initial segments, an alteration that can be rescued by genetically reducing the Na/K-ATPase $\alpha 1$ (Kaphzan, Buffington, Jung, Rasband, & Klann, 2011; Kaphzan et al., 2013). AIS innervation by GABAergic synapses and associated presence of 5HT1a receptors is perturbed in the cortex of schizophrenic patients (Cruz et al., 2004; Cruz, Lovallo, 2009; Woo, Whitehead, Melchitzky, & Lewis, 1998). AIS alteration could be a common feature in nervous disease and injury and a possible target for therapeutic intervention.

A detailed account of the pathologies associated with defects or mutations in AIS components is beyond the present scope and this subject has been reviewed recently (Buffington & Rasband, 2011; Hsu, Nilsson, & Laezza, 2014), so I will only briefly summarize them and outline most recent works. Due to the role of AIS ion channels in the initiation of action potentials, a large number of mutations in sodium or potassium channels present at the AIS (Nav1.1, Nav1.2, Nav1.6, KCNQ2/3) result in epileptic conditions in humans (Wimmer, Reid, So, Berkovic, & Petrou, 2010). Ankyrin G has been repeatedly associated with risks of schizophrenia and bipolar disorder (Leussis, Madison, & Petryshen, 2012), and some mechanistic insights were recently obtained. AnkG downregulation has behavioral effects in mice (Leussis et al., 2013): variation in ankyrin G mRNA expression was found in bipolar patients (Wirgenes et al., 2014) and could result from the deregulation of the miR-34 microRNA (Bavamian et al., 2015). Identification of patients with heterologous disruption of ankyrin isoforms or homozygous truncation in 480-kDa ankyrin G revealed a range of intellectual disabilities (Iqbal et al., 2013). However, it is not known if these are the consequences of ankyrin G role at the AIS or caused by alternative mechanisms such as GABAergic synapse organization by 480-kDa ankyrin G (Tseng et al., 2015), or synapse maintenance (Smith et al., 2014) and control of neurogenesis (Durak et al., 2015; Paez-Gonzalez et al., 2011) by the smaller 190-kDa isoform.

The AIS could also be the place of axonal transport perturbations that are present in several neurodegenerative diseases (Encalada & Goldstein, 2014; Millecamps & Julien, 2013). Ankyrin G has been diversely implicated in Alzheimer's disease (León-Espinosa, DeFelipe, & Muñoz, 2012; Sanchez-Mut et al., 2013; Santuccione et al., 2012). In particular, mouse models of Alzheimer's disease have decreased ankyrin G levels at the AIS due to upregulation of the miR-342-5p microRNA (Sun, Wu, Gu, & Zhang, 2014). This downregulation of ankyrin G results in an altered intracellular diffusion filter, with easier axonal entry of large cytoplasmic proteins

(Sun, Wu, Gu, Liu, et al., 2014). By contrast, motor neurons of amyotrophic lateral sclerosis (ALS) mouse models show an accumulation of neurofilaments and mitochondria in the AIS (Sasaki, Warita, Abe, & Iwata, 2005), consistent with organelle accumulation and proximal axon swelling in ALS patients (Sasaki & Iwata, 1996). Finally, a recent work suggests that demyelinating diseases such as multiple sclerosis implicate defects in axonal transport resulting in gray matter damage (Calabrese et al., 2015; Dutta et al., 2011; Hares et al., 2013; Sorbara et al., 2014). Subtle alterations in the AIS morphology were observed in demyelinated cortical neurons (Hamada & Kole, 2015), and it would be interesting to examine the possible AIS alterations occurring in multiple sclerosis.



9. CONCLUSION

The AIS is a unique neuronal subcompartment that serves crucial neurophysiological functions. Recognized for a long time as the site of action potential initiation site, it was merely seen as a static assembly of scaffold and channels. Similar to what happened for the synapse in the past two decades, the AIS has now been revealed as a dynamic assembly, capable of morphologic plasticity in physiological and pathological conditions, resulting in profound effects on the neuron excitability. Moreover, cell biology studies uncovered the essential role of the AIS in the maintenance of axonal identity, with insights into its regulation of protein mobility and trafficking. Several key questions remain: What is the exact nature of the vesicular sorting that occurs at the AIS? What are the structural mechanisms of the AIS morphological plasticity? Is the organizing role of ankyrin G at the AIS implicated in neuropsychiatric disorders where it is recognized as a major risk factor? Thanks to the growing interest for the AIS among neurobiologists, the coming years will undoubtedly be very exciting, providing numerous answers and even more numerous new questions.

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