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Building and maintaining the axon initial segment

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The axon initial segment is a unique neuronal subregion involved in the initiation of action potentials and in the control of axonal identity. Recent work has helped our understanding of how this specialised structure develops, not least in identifying possible mechanisms leading to the localisation of the AIS's master organiser protein, ankyrin-G. The most exciting current work, however, focuses on later aspects of AIS function and plasticity. Recent studies have shown that the AIS is subdivided into distinct structural and functional domains, have demonstrated how the AIS acts as a cytoplasmic barrier for axonal transport, and have discovered that the AIS can be surprisingly plastic in its responses to alterations in neuronal activity.

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Introduction

The axon initial segment (AIS) is a highly specialised region of the neuron that clusters cytoplasmic and transmembrane proteins to a section of the axon located near the cell body. To date, the AIS has been attributed two important roles: it acts as the site for action potential initiation and it provides a molecular barrier that helps establish axonal identity. The former role makes the AIS a master integrator of subthreshold synaptic events, converting them into all-or-nothing action potentials. The latter describes its critical structural role in neuronal polarity, where complex protein scaffolds regulate transport along the axon to form a tight boundary with the somatodendritic compartment. Both roles are key to the proper function and development of a neuron and highlight the AIS as a crucial site controlling neuronal polarity and excitability.

For such an important part of a neuron, our knowledge of AIS development, maintenance and subsequent plasticity is relatively poor. However, recent work reviewed here is fast improving this situation. In this review, we focus first on our current knowledge of how the AIS is built during development and the molecules involved in this process. Particularly exciting is the observation that the AIS is not a uniform structure, but is formed of sub-domains of clustered proteins that differ in their molecular composition and corresponding functional roles. We go on to present recent findings on the role of the AIS in setting up and maintaining neuronal polarity, with special emphasis on the role it plays as a selectivity filter for protein trafficking. And we finish with exciting new data that point towards considerable post-developmental AIS modifications — this large, dense neuronal sub-compartment turns out to be surprisingly plastic.

Development of the AIS

After post-mitotic commitment to a neuronal cell fate, the initial development of the AIS is a rather early maturational event, occurring alongside axon/dendrite specification and before the formation of most synaptic connections. In dissociated cultured neurons, the AIS is first detected around three to four days *in vitro* (div) [1,2]. The process of maturation is then relatively rapid, with a large majority of cells showing 'mature' AISs by the start of the second *in vitro* week [1,2]. Nevertheless, developmental modelling and re-modelling of the AIS, as discussed below, can extend a good deal further.

Ankyrin-G

The consensus in the field of AIS development is that the structure is utterly dependent upon the scaffolding protein ankyrin-G (AnkG). In neurons AnkG is targeted with a high degree of specificity to the AIS and nodes of Ranvier, is one of the earliest proteins localised to a segment of proximal axons [1,3], and, through its multiple ankyrin repeats, targets proteins containing AnkG-binding domains to the AIS. These include voltage-dependent sodium and potassium channels, as well as other transmembrane and scaffolding proteins [2–5]. Indeed, transgenic mice lacking AnkG also lack an AIS [6], and cultured neurons where AnkG has been knocked down never develop an AIS [7]. But, although the precise localisation of AnkG to a specific portion of the proximal axon is a crucial step in AIS formation, very little is known about this process. What localises AnkG to the proximal axon in the first place? And how is it restricted to a single discrete zone? One thing that is clear is that these processes are cell-autonomous — in comparison to nodes of Ranvier, where multiple Schwann cell-dependent mechanisms operate to restrict AnkG and other specialised

proteins to node regions [8,9], the AIS can develop perfectly in the absence of any glial influences [10,11].

Recent evidence implicates the NFκB inhibitor IκBα in early AIS specification [12•]. IκBα interacts with the transcription factor NFκB and inhibits its translocation to the nucleus. Phosphorylation of specific serine residues on IκBα disrupts the interaction and allows NFκB to travel to the nucleus and control the transcription profile of specific genes. IκBα, together with the kinases responsible for its phosphorylation, is localised to the proximal axon, along with AnkG, in very young cultured neurons, and blocking its phosphorylation prevents the formation of an AnkG-containing AIS. However, although this suggests a role for IκBα in AIS formation, it still does not provide a mechanism for how AnkG position is established. To tackle this issue, more fundamental research into very early stages of axon specification is needed: for example, is AIS formation a necessary consequence of axonal differentiation? Could AnkG and/or IκBα boundaries be set by a combination of cytoplasmic molecular gradients along the axon, which define an optimal region for AIS formation? If so, what is the identity of the substrate(s) used? One attractive possibility is that the cytoskeleton may provide the necessary information. Microtubules in the proximal region of the axon are preferentially tyrosinated (similar to dendrites) and less stable than those in the remainder of the axon, which are preferentially acetylated [13•]. In fact, it is known that microtubule stabilisation is needed for axon formation and drugs that produce this stabilisation result in the formation of multiple axons, even in mature neurons [13•]. However, it is still unclear whether the microtubule network drives AIS formation or vice versa. The interactions between these two complexes have yet to be fully understood.

β-IV-spectrin

AnkG is accompanied at the AIS by another specific scaffolding protein, β-IV-spectrin, which binds to AnkG and, like other spectrins, to the actin cytoskeleton. Mutation studies have shown that the ankyrin-G-binding domain of β-IV-spectrin is crucial for its AIS localisation [2], similar to many other AIS-targeted proteins. *In vivo* studies in β-IV-spectrin KO mice showed that neurons lacked a clear AIS in adult animals, which led to speculation that β-IV-spectrin may also be crucial for AIS development [6,14]. However, RNAi knockdown of β-IV-spectrin expression in cultured neurons does not prevent the formation of (AnkG-based) AISs [7]. The current thinking is that β-IV-spectrin, though not crucial for AIS development, may be vital for maintaining and stabilising the AIS, through its interactions with the cytoskeleton by its actin-binding domains [7,15]. Importantly, quivering mice (qv), which carry a loss-of-function mutation in β-IV-spectrin, show severe auditory and motor neuropathies, including the mistargetting of voltage-gated Na^+ and K^+ channels and a loss of synchronised neuronal responses, further underscoring the importance of this protein in AIS function [16].

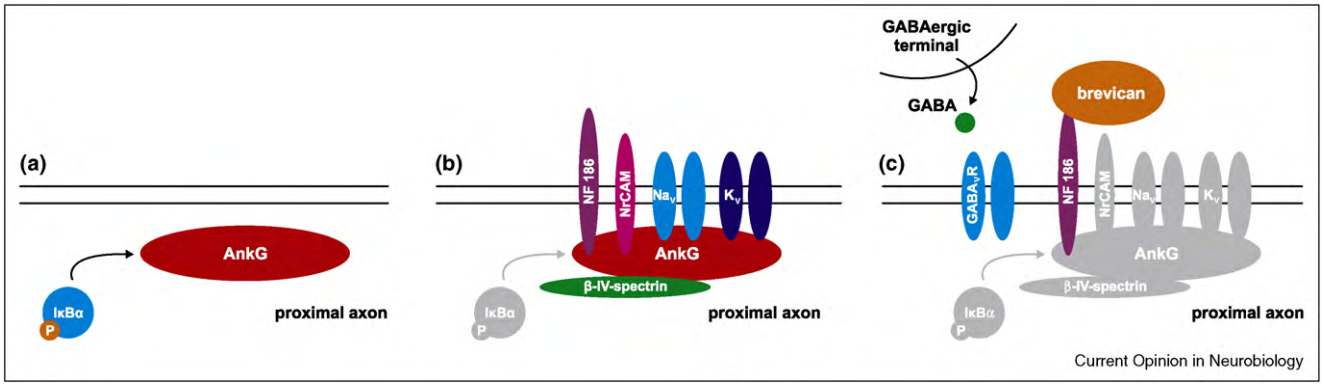
Na_v channels

The AIS owes its functional role as the site of action potential initiation to the high concentration of voltage-gated sodium (Na_v) channels that cluster there [17]. Sodium channels are recruited to the AIS following the appearance of AnkG [3], by means of an intracellular AnkG-binding domain present in the cytoplasmic II–III loop [4,18]. In fact, along with this specific AnkG-binding site, the II–III loop of all alpha channel subunits also contains a membrane internalisation sequence that removes sodium channels from compartments other than the AIS [19]. In this way, Na_v channels use two motifs, working in concert, to localise themselves to AnkG-containing zones [4,19]. However, the AnkG-binding site found in Na_v channels is not specific and also allows binding to ankyrin-B, a scaffolding protein found throughout the length of the axon. How, then, do sodium channels localise specifically to the AIS? Recent evidence found that specific residues in the II–III loop are phosphorylated by protein kinase CK2 (CK2), an enzyme localised specifically to the AIS in neurons. Blocking CK2 activity disrupted Na_v channel AIS localisation, suggesting phosphorylation plays an important role for AnkG-specific Na_v channel interactions [20]. However, the mechanisms producing CK2's AIS localisation remain unknown. To complicate matters further, there is also evidence showing that knocking down Na_v channels disrupts AISs in some cell types [21] but not others [7], suggesting that Na_v channels may be an important structural component for AIS development/maintenance, but only in certain tissues. This view is further complicated by developmental changes that occur in Na_v channel subunit content at the AIS. In retinal ganglion cells, for example, $Na_v1.2$ subunits are predominantly found in early development, but are gradually supplemented by increased expression of $Na_v1.6$ subunits as the AIS matures [22,23]. Whether this simply reflects changes in overall cell expression from one subunit to another, or whether there are developmental changes in AnkG or other protein–protein interactions at the developing AIS to allow more $Na_v1.6$ binding, is currently unclear. Finally, alpha Na_v channel subunits co-localise and are modulated by accessory beta subunits and modulatory proteins such as FGF14. Compromising these associated proteins is known to affect neuronal firing characteristics for example [24,25], but any developmental role for beta subunits or atypical FGFs in AIS formation is presently unclear.

Other voltage-gated channels

Although action potentials are initiated at the AIS through the action of Na_v channels, they are shaped and modulated by local voltage-gated K^+ (K_v) and Ca^{2+}

Figure 1



Simplified stages in AIS development. **(a)** During and shortly after axon specification, phosphorylated IκBα in the proximal axon is needed for localisation of AnkG. **(b)** AnkG, now localised to a single band in the proximal axon, binds to and localises β-IV-spectrin, Na_v and K_v channels, and transmembrane proteins, amongst other molecules. **(c)** NF186 is then required for the development of a specialised brevican-containing AIS ECM, and for the formation of AIS-specific GABAergic synapses.

channels [26]. A recent study has implicated T-type and R-type Ca^{2+} channels in the generation of single and complex spikes using a pharmacological approach [27•], but little is known about the mechanisms of localisation at the AIS, nor their developmental time course. Rather more clear is the role played by specific AIS K_v channels, which can directly modulate action potential width and repetitive spiking [26,28,29]. KCNQ2/3 channels share the same AnkG-binding site as Na_v1 channels [5], although this sequence likely evolved some time after the appearance of the Na_v–AnkG-binding site in early chordates [30]. The two channel types may therefore compete for limited AnkG space within the AIS, but whether KCNQ channels also possess the membrane removal sequences and/or CK2 phosphorylation sites that are necessary for targeting sodium channels to the AIS is presently unclear. Interestingly, the localisation of KCNQ2/3 heteromer requires the presence of an AnkG motif on the KCNQ3 subunit, but not on the KCNQ2 subunit, indicating subunit specificity in AIS targeting [31]. Finally, K_v1 channels appear to use different mechanisms for AIS localisation that involve other scaffolding proteins, such as PSD-93, since an RNAi knockdown of this protein disrupted K_v1 channel AIS clustering [32]. How PSD-93 is localised to the AIS, though, is presently not known.

Cell adhesion molecules, extracellular matrix and synapses

In contrast to development at nodes of Ranvier, where glial-dependent cell adhesion molecules direct the initial stages in maturation [8,9], extracellular signalling molecules such as NrCAM and NF186 appear relatively late at the AIS [1,3,7], and are dependent upon AnkG for their localisation. Both NrCAM and NF186 bind to AnkG through a FIGQY motif in their cytoplasmic domain [18,33], and whilst RNAi knockdown of either protein does not affect the development of an AnkG-based AIS in cultured hippocampal neurons, AnkG knockdown prevents either CAM from localising to the proximal axon [7]. NF186 does appear vital, however, for recruiting extracellular components to the AIS. At relatively late stages in development the AIS is surrounded by a dense extracellular matrix (ECM) of unique composition, including the chondroitin sulphate proteoglycan brevican, which shows preferential AIS localisation [34]. NF186 binds to brevican through its extracellular domain, and is necessary for its AIS targeting [7]. NF186 is also vital for localising specific synaptic inputs to the AIS. In many projection neuron cell types *in vivo*, the AIS is specifically targeted by GABAergic inputs from specialised interneurons, with current controversy existing over whether this privileged input at the site of action potential initiation is actually inhibitory or excitatory [35–37]. Regardless of their function, GABAergic inputs to cerebellar Purkinje cell proximal axons precisely follow the distribution of NF186, which in turn is dependent upon the localisation of AnkG [38].

The initial formation of the AIS therefore follows quite a clear sequence (Figure 1). First, just after or during axon specification, and in a phosphorylated-IκBα-dependent manner, AnkG localises to a domain in the proximal axon. Through various ankG-binding sites, β-IV-spectrin, Na_v channels, K_v channels, NrCAM and NF186, amongst other proteins [39] are all then recruited to form the basic AIS intracellular/membrane complex. Finally, NF186 directs the formation of an AIS-specific ECM, and targeted GABAergic synaptic inputs.

Sub-domains within the AIS

Recent studies have shown that the AIS is not a uniform structure. Its molecular composition, and in consequence its functional role, can change significantly between its

proximal and distal compartments. What is more, this subdivision within the AIS varies according to cell type: different neurons possess AISs that are subdivided in different ways, allowing for a high degree of specificity and variety in the way cells initiate, propagate and shape an action potential [40•,41]. One of the clearest examples of this AIS subdivision is in different isoforms of Na_v alpha subunits. The $Na_v1.1$ channel, when expressed in neurons, is usually found in a small, tightly localised band at the very proximal edge of the AIS [23,40•,42]. This contrasts markedly with the distribution of $Na_v1.6$ channels, which do not co-localise with $Na_v1.1$ subunits, and which instead increase in density towards the distal end of the AIS [23,40•]. An important recent study described a similar subdivision between proximal $Na_v1.2$ and distal $Na_v1.6$ subunits in the AIS of cortical pyramidal neurons [43••]. Through a combination of patch–clamp recordings and mathematical simulations, these authors went on to show that the $Na_v1.2$-containing and $Na_v1.6$-containing portions of the AIS subserve distinct functional roles. The low voltage threshold of $Na_v1.6$ channels makes the distal AIS the site of action potential initiation, a role backed up by other recent imaging, recording and modelling studies [17,43••,44,45]. In contrast, the concentration of higher threshold $Na_v1.2$ channels in the proximal AIS ensures backpropagation of action potentials into the somatodendritic compartment [43••]. AIS subdivision can therefore allow the structure to play multiple distinct roles in action potential firing.

As well as different Na_v classes targeting to different AIS regions, recent immunohistochemical evidence reveals that in certain cell types $K_v1.1$ and $K_v1.2$ channels also preferentially target the distal AIS. There, they always co-localise with each other, and with $Na_v1.6$ [40•], suggesting that these channels could play a specialised role in the control of AP initiation.

Developmentally, how do neurons build a subdivided AIS? All Na_v subunits appear to use the same conserved II–III loop sequence to bind AnkG, and AnkG, along with all other AIS scaffolding proteins described thus far, is present along the entirety of the AIS. Different preferential binding partners could underlie AIS subdivision, then, but no candidate domains nor any possible partners have yet been identified. Different channel subtypes could also compete with each other for AIS space, but this would not on its own produce two distinct, discrete AIS zones, and besides, $Na_v1.1$ channels are still restricted to proximal AIS in RGCs even when $Na_v1.6$ expression levels are very low [23]. The molecular and developmental mechanisms of AIS sub-localisation should form a very interesting field of study for the future.

A role for the AIS in neuronal polarity

The precise complex of proteins that makes up the AIS seems designed to bring together high densities and particular arrangements of voltage-gated ion channels, along with specific synaptic input, to enable action potential initiation, propagation and modulation. But the protein matrix that evolved to enable precise control over cell excitability (cf. [30]) may have a very different secondary function in delimiting the boundary between major neuronal compartments. Previous findings have shown that the AIS presents a significant barrier for the movement of molecules along the plasma membrane — proteins that diffuse freely within somatic or axonal membrane become more restricted as they pass through the AIS (e.g. [46]). A recent study has shown that this barrier role for the AIS extends to molecular movement within the axonal cytoplasm. Song *et al.* [47••] introduced different sized fluorescent dextrans into cultured hippocampal neurons and found that diffusion from the soma to the axon was restricted for large (70 kDa) molecules. Importantly, they showed that the emergence of this axonal 'filter' occurred at ~5 div, a time when the AIS forms. A combination of RNAi and pharmacology showed that the filter was dependent upon AnkG and F-actin; this, along with its location and the developmental time course over which it appears, strongly implicates the AIS in a filtering role. Furthermore, the authors went on to show that the ability of vesicles carrying membrane proteins to pass efficiently through the AIS depends on the transport efficacy of specific KIF motor proteins. Whereas KIF5 normally transports VAMP2-containing vesicles efficiently through the AIS, KIF17 driven vesicles that contain dendritic proteins such as NR2B do not cross the AIS. Interestingly, when the cargo-binding domains for KIF5 and KIF17 were swapped, KIF5 was able to carry NR2B across the AIS more efficiently [47••].

The presence of a selective cytoplasmic filter in the proximal axon raised the possibility that the AIS may be actively involved in differentiating somatodendritic from axonal compartments. Indeed, data from two recent studies — one *in vitro* and one *in vivo* — suggest that this is precisely the case. Hedstrom *et al.* [48] targeted the knockdown of AnkG in cultured hippocampal neurons at a stage when the AIS had already formed. They found not only that this loss dismantled the AIS entirely, but also that the longest neuronal process — normally the axon — now showed multiple dendritic characteristics, including MAP2 expression and excitatory synapses that contacted dendritic-like spine protrusions. Very similar results were seen in cerebellar-specific AnkG KO mice, where the basal processes of Purkinje cells, normally smooth axons, developed dendrite-like spine protrusions that received bona fide synaptic contacts [49]. Together, these results suggest that the AIS plays an important role in conferring axonal identity. Furthermore, recent work on the axonal regeneration of severed axons has provided some interesting findings on neurite identity: severing an axon within 35 μm of the soma resulted in a nearby

dendrite changing its identity to become an axon, whereas a more distal cut caused the re-growth of the same axon [13•]. Although not explored, an intriguing possibility is that this 35 μm cut-off point represents the AIS, which, when removed, induces the re-specification of axonal identity to a dendrite.

These intriguing results raise a number of questions. Is the AIS, as a membrane and cytoplasmic diffusion barrier, the structure that precisely delineates where dendrites stop and the axon begins? If so, given that the molecular complex comprising the AIS often starts tens of microns away from the soma, is the stretch of neurite (including the axon hillock) between the cell body and the AIS actually axonal at all? Are different cellular compartments created by the AIS-like dendritic 'hotspots' that exist in certain neurons that lack an axon [50]? And, on the other hand, how does clear axon–dendrite specification occur in the neurons of species that lack Na_v-dense AISs, such as *C. elegans* or *Drosophila* (cf. [51])? In all likelihood the AIS is probably a major contributing factor towards neuronal polarity, rather than the one mechanism which defines the dendritic–axonal boundary. After all, the basal processes in AnkG knockout Purkinje cells still look far more like Purkinje cell axons than like the characteristic planar dendritic tree of this cell type [49], and it is possible to dissociate axon specification from AIS formation, as with blockade of IκBα phosphorylation, for example [12•]. The precise interplay between axonal identity and AIS formation will undoubtedly be a focus of much work in the near future.

AIS plasticity

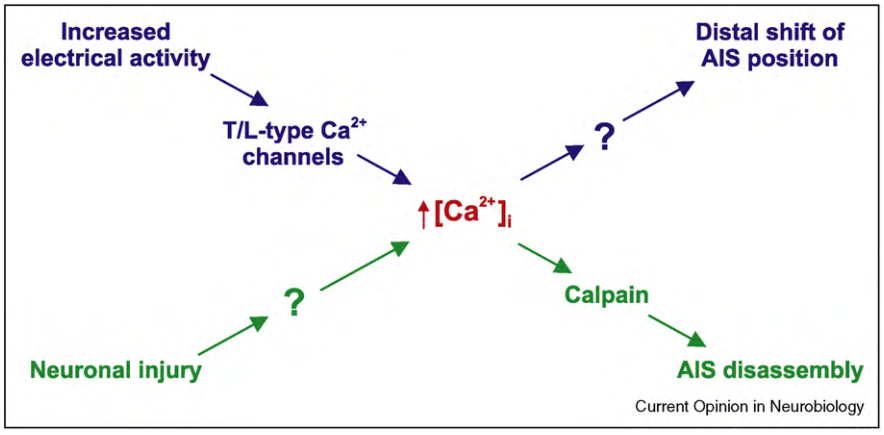
Once the AIS has formed with its specialised sub-domains, how static a structure is it? The few experiments that have examined protein dynamics at the AIS suggest that under normal conditions its components are actually rather stable. After its initial development, the specific knockdown of several AIS component proteins, including NF186, β-IV-spectrin and Na_v channels, showed that they had a long half-life of at least two weeks. Similar findings were also observed using repetitive live labelling of extracellular NF186 [48]. Both approaches indicate that AIS proteins undergo very slow baseline turnover. However, experiments looking at the mobility of $K_v2.1$ channels within the AIS found it to be quite dynamic. Using fluorescence recovery after photobleaching (FRAP), mixing of $K_v2.1$ channels at the AIS was shown to occur with a time-constant of around 11 s [52]. This mixing appears to be at odds with the existence of restricted sub-domains within the AIS, and raises interesting questions about the mobility of transmembrane proteins and possible crosstalk between different AIS sub-compartments.

AIS composition can certainly change over longer timescales. During postnatal development, as discussed above, the AISs of retinal ganglion cells switch their subunit composition from being dominated by $Na_v1.2$ to $Na_v1.6$ [22,23], and both AIS number and AIS length decrease significantly with development in monkey prefrontal cortex [53]. Chronic diseases are associated with AIS abnormalities, too: a decrease in AnkG-labelled AISs is seen in prefrontal cortex of people with schizophrenia [54], whilst the number of AIS-targeting GABAergic synapses is reduced in the cortex following long-term epilepsy [55,56].

Recent evidence has also revealed much more rapid and dramatic changes at the AIS as a response to neuronal injury [57••]. Following ischaemia *in vivo* or oxygen glucose deprivation (OGD) in culture, neurons rapidly degrade the AIS protein scaffold, producing a huge decrease in AIS component proteins after only ~2 hours. This response is independent of cell death or axon degeneration, is dependent upon the calcium-activated degradation enzyme calpain, and, as might be expected from the data discussed above, results in former axonal processes taking on dendritic characteristics [57••]. Importantly, since AIS degradation appears to be irreversible, it could be prevented both *in vivo* and *in vitro* injury models by pharmacological calpain inhibition [57••]. Although this could represent a promising new avenue for drug treatment of brain injury, if AIS degradation is a drastic attempt by dying neurons to escape the consequences of pathological over-excitation, such treatment could do more harm than good.

Dynamic changes at the AIS are certainly not restricted to conditions of neuronal injury, however, nor to mechanisms of scaffold disassembly. Recent work has shown that long-term changes in electrical activity can result in significant changes in AIS *location*. The precise position of the AIS within the axon is known to vary significantly both across [58] and within [59] different cell types, and modelling studies have shown that this variation can produce significant differences in neuronal excitability [59,60] and sensory response properties [59]. The mechanisms determining AIS location within the proximal axon, however, were entirely unknown. Recent findings have shown that chronic two day depolarisation of mature cultured hippocampal neurons produces a significant distal shift in the position of the AIS, moving the molecular complex up to 17 μm further away from the soma [61••]. This relocation is reversible upon return to control conditions, depends on calcium entry through T-type and/or L-type channels, and, under conditions of chronic stimulation using the light-gated channel channelrhodopsin-2, requires specific bursting patterns of electrical activity. Activity-dependent AIS relocation also seems linked to changes in neuronal excitability, as predicted [59]: cells with more distal AISs have higher current thresholds for action potential initiation [61••].

Figure 2



AIS plasticity requires elevated intracellular calcium. Are any other pathway components common?

Conclusions

Recent studies are beginning to give us a new view of the AIS. Rather than being a uniform, static structure involved solely in action potential initiation, the AIS is actually a diverse, dynamic neuronal compartment that also plays a key role in axon–dendrite partitioning. Future studies should focus on the molecular mechanisms that first establish AnkG localisation in the proximal axon — this event is utterly vital to AIS formation, and may turn out to be intimately linked to processes of axon–dendrite specification. Information on the development, maintenance and potential plasticity of AIS subdivision would also be extremely welcome. How exactly does the structure become functionally divided? And can these subdivisions change according to recent neuronal activity? Finally, plasticity at the AIS is a fascinating new area to explore, especially the similarities and differences between injury-induced AIS degradation [57••] and activity-dependent AIS relocation [61••] (Figure 2), and the possible relationships of both phenomena to modification of local ion channel densities (and/or distributions [41]. It may turn out that multiple features of the AIS can be modified in parallel to produce exquisite fine-tuning of neuronal excitability.

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