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Neuronal polarity: establishing and maintaining the axon initial segment

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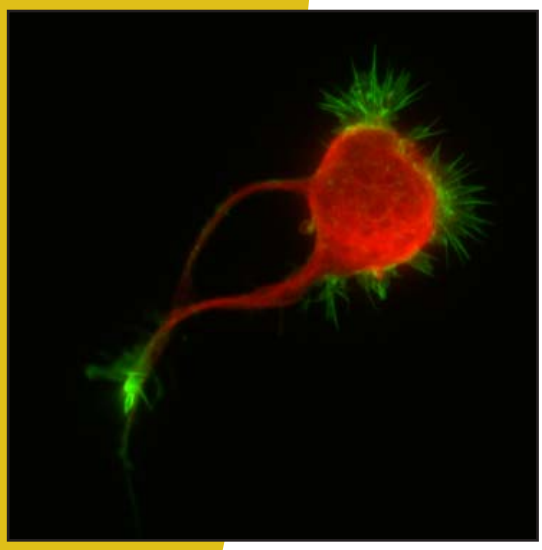

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Abstract

Neurons with their dendrites and axons have a highly polarized morphology. Axons are functionally different from dendrites, as they can generate an action potential, required for fast and consistent electric signal transfer. The axon initial segment (AIS) is a specialized region found at the beginning of the axon close to the cell body. This region is very important for the induction of the action potential. It has also been suggested to form a selective transport filter and diffusion barrier at the beginning of the axon. The AIS is therefore an important region required for maintaining neuron polarity. This thesis discusses briefly the current understanding of molecular mechanisms involved in neuron polarization, like NGF and TGF-β signalling. Both the appearance and the function of major AIS proteins, such as AnkG, NF-186, and voltage-gated sodium channels, are subsequently discussed more extensively in this thesis. These proteins play an important role in the three functions of the AIS, namely action potential generation, selective transport filter, and diffusion barrier. The question remains how the AIS is exactly build and how this relates to the induction of neuron polarization, a question which is examined in the discussion.

General introduction

The nervous system requires cells capable of electrical signalling as this is important for its functions such as memory, learning, and among others movement. The specialized cells that can fulfil this role are known as nerve cells, neuronal cells, or simply neurons. Neurons have a cell body (soma) and have very few till many tendrils which are called neurites. The morphology of neurons is highly polarized with mostly one or sometimes two axons and multiple dendrites. Axons can be very long, up to roughly a meter, and are able to send signals to recipient cells which can be another neuron or for instance a muscle cell. Dendrites are normally much shorter and receive signals from other neurons or have sensing abilities. Both dendrites and axons can branch extensively, creating many tendrils which will increase the input and output surface (to an average of 10^5 contacts per neuron) of which signals can be exchanged. Neuron contact, required for signal exchange between nerve cells, is established by synapses. The axon has a presynaptic terminal were neurotransmitters (chemical synapse) or sodium ions required for an membrane potential (electric synapse) are transferred via the synaptic cleft to the postsynaptic terminal (spine, Figure 1, stage 5) present at dendrites of receiving neurons or for instance receiving muscle cells. Together all neurons form an electric circuit that gives animals the ability to move, sense, learn, memorize, think, fear, anticipate etc. The most important function of neurons is sending and receiving electrical signals which have to travel fast and consistent from one end of the neuron to the other end in order to achieve a rapid communication (see Box 1). This electric signal is a change in voltage of the membrane, called a membrane potential. Neurons can respond to this change in membrane potential through several downstream actions, like for instance calcium influx that plays an important role in neuron signalling. Furthermore, neurons have the ability to boost the membrane potential, especially in the axon, so it can travel further and faster; this is called an action potential (see Box 1). The action potential originates at the beginning of the axon, also called the axon initial segment (AIS, see Box 1). However, before the AIS can generate the action potential, the AIS needs to be assembled, which is linked to neuron maturation into the highly polarized morphology. This thesis is aimed at reviewing the different processes and molecules known so far to be involved in neuron polarization and more specifically the role of the AIS in this process. First the molecular mechanisms involved in neuron polarization will be discussed followed by the role of the cytoskeleton in this polarization. The AIS composition and function will then be discussed in detail. Finally, in the discussion, we will try to couple neuron polarization to AIS assembly initiation although this has not been shown by experiments so far.

Box 1: Neuronal electric potential in a nut shell

Neurons generate a constant voltage across the cell membrane, called the membrane resting potential: negative at the inner side and positive at the outer side of the cell. Upon stimulation of the somatodendritic region ion channels in the postsynapse open, by for instance binding of a neurotransmitter, which result in a local influx of sodium ions. This influx of sodium ions depolarizes the membrane causing a local change in membrane resting potential (-60mV normally) towards a more positive charge. The electric signal that has been created is passively conducted along the membrane of the somatodendritic region, towards the axon hillock and AIS (Figure box 1A). During conductance the membrane potential decrease since the electric signal becomes less as it spreads along the membrane but also because it leaks out. This change in membrane potential that travels through the somatodendritic region after synapse stimulation is called a postsynaptic potential (PSP). Axons also require a fast electric signal transfer, however since axons can be much longer than dendrites this transfer must be more faithful and stronger. Therefore axons have a system that boots the electric signal to sustain the same membrane potential as it travels along the axon. This boosted electric signal in the axon is called an action potential. Many voltage-gated sodium channels at the AIS are required for this action potential, these channels open in response to a change in membrane potential. When the PSP is high enough, because of a significant stimulation, it will reach the threshold of the voltage-gated ion channels present at the AIS (Figure box 1B). When the threshold (-40mV) is reached, many of these ion channels at the AIS will open resulting in a large influx of sodium ions and a high peak in membrane potential (the action potential). The action potential has a sharp peak (called spike) with always the same change in voltage (amplitude). An increase in stimulation will result in an increase in spike frequency and not higher spike amplitude. To prevent leakage of the signal through the membrane, as the action potential propagates, the axon is covered with myelin (packed glial membranes) forming a sheet that works as an insulator. Furthermore, to maintain the action potential along the axon, there are gaps in the myelin sheet which are also enriched in voltage-gated ion channels. These gaps are called nodes of Ranvier and maintain the action potential by a new influx of sodium ions so the membrane potential can further propagate. This process is repeated until the membrane action potential reaches the presynaptic terminal where the depolarization of the membrane actives several output mechanisms.

Further reading

Extensive review on Action potential: (Bean, 2007)

Short review on PSP to action potential: (Giuliodori and Zuccolilli, 2004)

Nice review on action potential initiation at the AIS: (Clark et al., 2009)

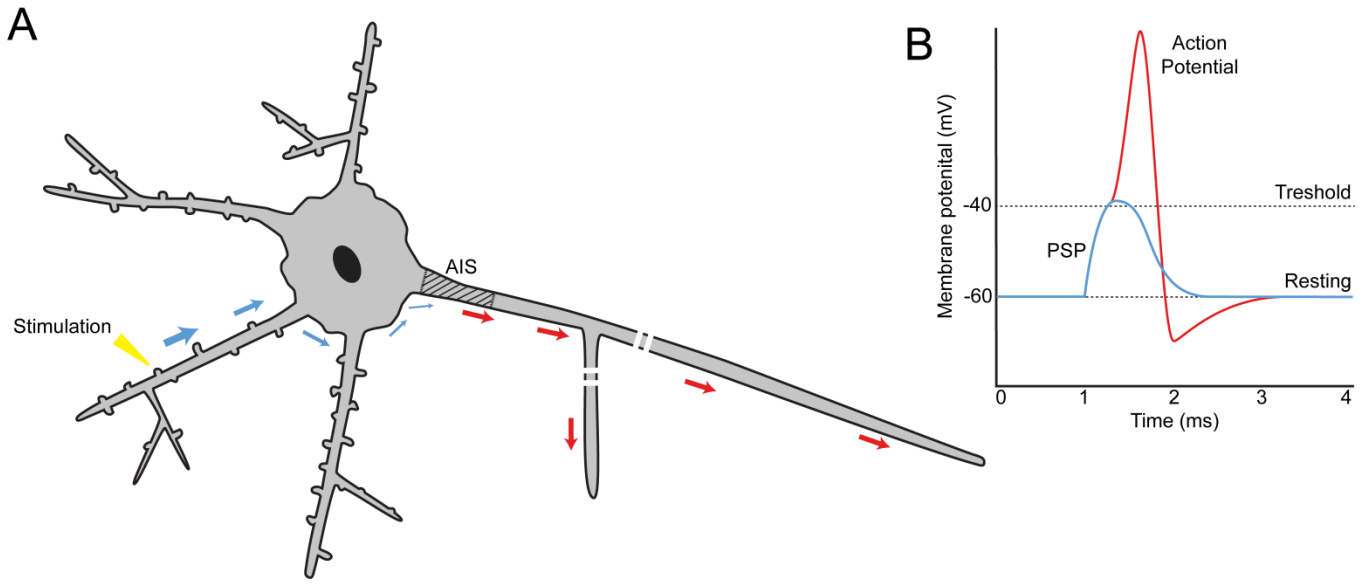

Figure box 1

A) The PSP (blue arrows) travels, after stimulation in the somatodendritic region, towards the AIS. The PSP decrease when it travels towards the AIS, indicated by the shrinking/decreased bleu arrows. An action potential (red arrows) is generated when a PSP above the action potential threshold reaches the AIS. This action potential then travels towards axon tips without losing any electric potential due to a boosting mechanism present in the nodes of Ranvier (not shown).

B) After neuron stimulation the resting membrane potential (-60mV) increases, creating an PSP (blue line). When the PSP is high enough, at the AIS, it will reach the action potential threshold resulting in the generation of an action potential (red line). The action potential recovers quickly back to the membrane resting potential after spiking. This allows the generation of a new spike when the PSP remains above the threshold (not shown).

Neuronal polarization: morphology

Neurons of the central nervous system (CNS) are derived from the neural tube (neuroectodermal tissue) during embryonic development. Nerve cell differentiation begins mostly with axon and dendrite outgrowth towards other neurons followed by establishing the synaptic connections. Neuron differentiation has been studied extensively *in vitro,* where undifferentiated neuron precursors were allowed to differentiate in culture. Dotti et al. were one of the first to describe the differentiation and polarity establishment of hippocampal neurons under culture conditions (Dotti et al., 1988). The hippocampus of 18 days (E18) old rat embryos were dissected and plated into special culture dishes and the change in morphology was followed over time using light microscopy. They discriminated five distinct stages of early neuron differentiation during several days of *in vitro* (DIV) culturing (Table 1 and Figure 1). Although the time of occurrence (in DIV) of the different stages can differ between labs and used culture conditions, the sequence of stages of hippocampal neuron differentiation remains the same. The ability of hippocampal neurons to grow and differentiate *in vitro* makes them a very good model system to study both neuron polarization and function.

An interesting step in neuron polarization is the determination of one neurite to become the axon. Once that is established, the remaining neurite become dendrites and develop accordingly. Subsequently, it is important to maintain this polarity in order to keep functioning as a neuron.

Molecular signals involved in neuron polarity

The different differentiation steps described in the introduction require several distinct signalling pathways leading to the breakage of the symmetric morphology. The four major signalling pathways involve lipids, GSK-3β, Par4/LKB1, Rho GTPases and more recently TGF β, which will all be discussed in the next paragraphs.

Lipid signalling

A generally important lipid signalling is the lipid Phosphatidylinositol (3,4,5)-trisphosphate ($PI(3,4,5)P_3$), which is formed after phosphorylation by phosphatidylinositol 3-kinase (PI3K) from $PI(4,5)P_2$. PI3K is in general activated by tyrosine receptor kinases (TrK) imbedded in the membrane which becomes active after extracellular substrate binding, which for nerve cells is the nerve growth factor (NGF). Interestingly, PI3K activity is required for axon formation since PI3K inhibition prevents axon formation whereas expression of a constitutively active PI3K protein results in multiple axon formation (Shi et al., 2003; Yoshimura et al., 2006). Likewise, expression of PTEN, which dephosphorylates $PI(3,4,5)P_3$ back to $PI(4,5)P_2$, also resulted in a loss of polarization (Shi et al., 2003). Both these results suggest that $PI(3,4,5)P_3$ is important for inducing and/or maintaining axon polarization in neurons.

Besides PI3K, also other lipid proteins, such as the plasma membrane ganglioside sialidase (PMGS) hydrolyses gangliosides generating GM1 ganglioside, which has a function in enhancing TrK activation after binding of NGF (Da Silva et al., 2005; Duchemin et al., 2002).

Table 1: Staging differentiation steps *in vitro*

Stage	DIV	Differentiation step
1	0.25	Formation of membrane protrusions forming lamellipodia.
2	0.5	Formation and extension of multiple neurites which are not yet specified (cells remain un-polarized/symmetric).
3	1.5	Cells start to polarize and the fast growing neurite becomes the axon.
4	4	Complete polarization by specifying the remaining neurites into dendrites followed by growth and branching.
5	>7	Synapse formation and further neuron maturation.

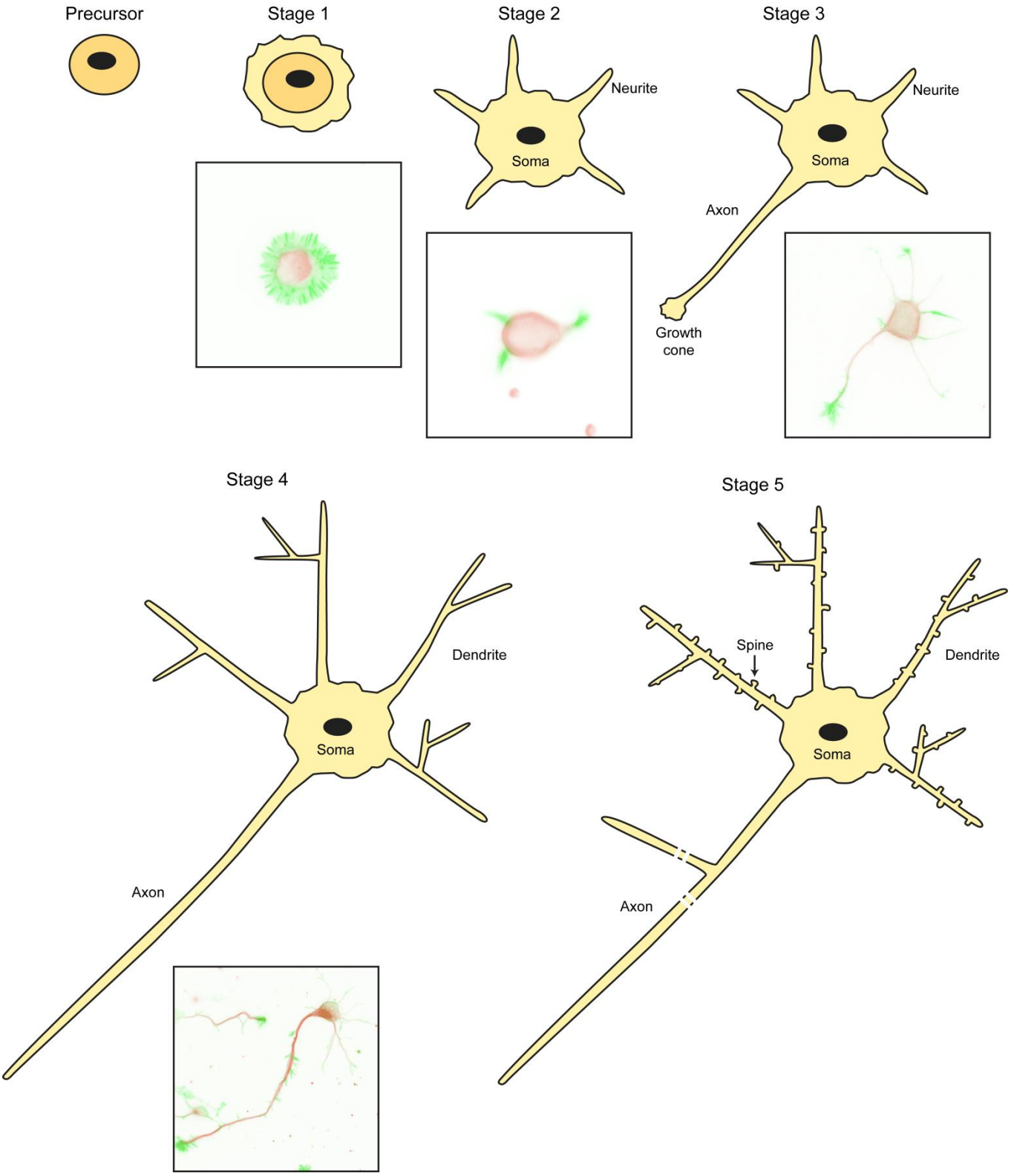

Figure 1

The different stages of hippocampal neuron development, as described in Table 1, are drawn in yellow. The drawings are accompanied with microscopic images of hippocampal neurons of these different stages, stained with actin (Phalloidin) in green and tubulin (Tuj) in red. The microscopic images were acquired by fixing neurons at different DIV stages in PFA and sucrose (4%/4%) followed by staining with mouse anti-Tuj overnight followed by Alexa568-conjugated anti-mouse and Alexa488-conjugated Phalloidin for 2h. Stained cells were visualized using fluorescent light microscopy (Olympus).

PMGS localizes at the neurite tip of the future axon in un-polarized neurons (Da Silva et al., 2005). Also, enhanced PMGS activity speeded up single axon formation, when in fact PMGS inhibition prevents axon formation. PMGS activity leads to the enhancement of TrK activation in the presence of NGF, which in its turn activates PI3K and its downstream targets (Da Silva et al., 2005). So in general these data suggest that local PMGS activity is important for enhancing axon specification and neuron polarization at a specific site after NGF stimulation.

GSK-3β signalling

After PI(3,4,5)P_3 has been formed at the plasma membrane it is important for the activation of downstream targets. One of them is the well described protein kinase Akt, also known as PKB, which after PI(3,4,5)P_3 binding, can be activated by several kinases. Also Akt phosphorylates many downstream targets and one of the important targets in neuron polarization is the glycogen synthase kinase 3β (GSK-3β). Akt phosphorylation of GSK-3β leads to its inhibition which has been linked to axon formation in neurons (Jiang et al., 2005; Yoshimura et al., 2005). Active GSK-3β phosphorylates and inhibits Collapsin response mediator protein-2 (CRMP-2) (Yoshimura et al., 2005), which results in multiple axon formation (Inagaki et al., 2001). Active CRMP-2 enhances axon elongation and branching possibly by promoting microtubule assembly (Arimura et al., 2004; Fukata et al., 2002), which will be discussed later. In short, these findings indicate that GSK-3β becomes inhibited upon TrK stimulation followed by PI(3,4,5)P_3 formation, this leads to the dephosphorylation and activation of CRMP-2 enhancing axon development.

LKB1 signalling

Liver kinase B1 (LKB1), homologue of the C. elegans *Par4*, has been implemented to be important in polarization of several asymmetric cell types. However, the role of LKB1 in neuron polarization remains largely unknown. Active LKB1 is known to specifically localize to the neurite which will become the axon during differentiation (Barnes et al., 2007). LKB1 function in the axon requires its phosphorylation by protein kinase A (PKA) and the binding by the pseudo kinase STE-20 Related Adaptor-α (STRADα). LKB1 can activate SAD-A/B kinases, by phosphorylation, in the future axon. These kinases can then, on their turn, phosphorylate and activate several microtubule associated proteins (MAPs) such as MAP, MAP4 and Tau (Barnes and Polleux, 2009). These MAPs enhance microtubule stabilization important for axon development and maintenance (discussed later in this thesis). Interestingly, cortical neurons lacking LKB1 are not able to develop an axon both *in vivo* and *in vitro* (Barnes et al., 2007). This suggests an important and essential role for LKB1 in axon development; however the upstream mechanisms by which LKB1 is activated in neurons are not yet clear.

Rho GTPases

Rho GTPases are a family of GTPases which bind and hydrolyse GTP to GDP. GTPases are active in their GTP bound form and can bind and activate downstream targets. Three important Rho GTPases that have been shown to play a role in neuron polarization are CDC42, Rac1 and RhoA. These Rho GTPases and their role in neuron polarization are extensively reviewed (Govek et al., 2005), here their role will be discussed only shortly.

Both CDC42 and Rac1 are activated after NGF stimulation, this activation is essential for neurite outgrowth (Govek et al., 2005; Nusser et al., 2002; Sarner et al., 2000). In fact, dominant negative mutants of both Rac1 and CDC42 results in cells unable to form neurites. Major downstream targets of both GTPases are the activation of the p21 activated kinases (PAKs), which are subsequently activated. Together with these kinases, CDC42 and Rac1 also regulate actin filament formation, elongation, and branching. This happens mostly through activation of cofilin, the Wiskott-Aldrich syndrome protein (WASP), and the Arp2/3 complex, which are important proteins that directly regulate actin dynamics required for neurite outgrowth. In addition, Rac1 also inactivates RhoA upon TrK binding by NGF and PMGS activity. This inactivation is caused by

PI3K activation by TrK followed by an increase in Rac1 activity which then inactivates RhoA (Da Silva et al., 2005; Nusser et al., 2002). This inactivation of RhoA indirectly enhances the stability of the actin cytoskeleton required for neurite outgrowth, since neurite outgrowth is lacking in neurons expressing a constitutively active mutant of RhoA (Da Silva et al., 2003).

TGF-β signalling

Besides the indications that NGF could be the extracellular factor inducing axon development, any hard evidence for a role of growth factors/external factors in the specification of the axon has been lacking. Recently, exogenous Tumor Growth Factor (TGF)-β has been shown to be an important growth factor in axon specification (Yi et al., 2010). The TGF-β receptor kinases are expressed in axons during both *in vitro* and *in vivo* development of neurons. Expression of constitutively active TGF-β receptors resulted in multiple axon formation whereas control cells formed long single axons. The TGF-β ligand is sufficient for axon specification, since axon development occurred only at the site of local TGF-β stimulation. This indicates that the ligand itself defines the site of axon development and not the site of TGF-β receptor expression. Neurons of animals lacking the neuronal TGF-β receptor did not form distinguishable axons, suggesting that indeed TGF-β signalling is required for axon development. Furthermore, an important downstream target of the TGF-β receptor in neurons, Par6, is activated by phosphorylation (Yi et al., 2010), which also has been shown to be involved in the polarization of epithelial cells (Ozdamar et al., 2005). Expression of a constitutively active Par6 phospho-mimic mutant in TGF-β knock-out neurons was found to rescue axon formation, so despite the absence of TGF-β signalling (Yi et al., 2010). In addition, expression of this Par6 mutant, which was like wild-type Par6 normally diffused throughout the cell, resulted in the expression in a single axon suggesting that there must also be some sort of repression mechanism present (in which phosphorylation plays a role). However, this is quite remarkable since a constitutively active TGF-β receptor lead to multiple axon formation. Therefore how these mechanisms actually work remain to be elucidated. Also, if Par6 is the only important downstream target in TGF-β induced neuron polarization remains unknown.

Interestingly, Par6 has a CDC42-Rac1 binding motif that directs the activity of these two proteins to regulate cytoskeleton reorganization (Joberty et al., 2000). Also, active Par6 inactivates RhoA by indirectly targeting it for protein degradation (Ozdamar et al., 2005). Both these mechanisms have previously been discussed to play an important role in actin cytoskeleton organization and axon formation. So it seems that TGF-β signalling could be the missing link in extracellular stimulation which activates a signalling cascade which eventually leads to a local reorganization of the cytoskeleton required for axon formation during neuron polarization.

Cytoskeleton dynamics in developing neurons

The (re)organization of the cytoskeleton is a major target of most of the previous discussed signalling pathways. The cytoskeleton consists of two major components, namely microtubules and actin filaments which differ both in composition and function.

Actin

Actin filaments (F-actin) are polymers of actin proteins which are polymerized from ATP bound actin (G-actin) with certain on and off rates (Pollard and Borisy, 2003; Pollard and Cooper, 2009). Actin filaments have two ends, the minus end also known as the slow growing end and the plus end which is also known as the fast growing end. The on and off rate differs between the minus and the plus end, generally the on rate is higher than the off rate at the plus end, whereas at the minus end the growing (on rate) is much slower. After polymerization, the GTP in the actin protein becomes hydrolysed resulting in GDP-actin. An ATP cap on the actin filament can be observed when the on rate is faster than the hydrolysis rate resulting in an enrichment of ATP-actin at the plus end. Moreover, actin filaments can be branched by the Arp2/3 complex which forms a scaffold on existing

filaments from which new actin can polymerize (Welch and Mullins, 2002). Also specific capping proteins can cap the actin filaments which will prevent them from shrinking and will act as actin filament stabilizer. Dense actin filament networks can generate enough force into the membrane to protrude the membrane outwards which is required for cell movement and cell shape (Pollard and Cooper, 2009; Ridley, 2011).

Actin is an important player in neuron polarization. The actin cytoskeleton is already required for lamellipodia formation (Ridley, 2011), which is one of the initial steps of hippocampal neuron development [see stage 1, Figure 1 (Dotti et al., 1988)] Another important role of the actin cytoskeleton in neuron development is the establishment of small lamellipodia at the tip of the growing axon, also known as the growth cone. The growth cone also requires filopodia for invasion and axon guidance, these filopodia are thin finger like protrusion made by parallel F-actin bundles (Ridley, 2011). The actin cytoskeleton of the axon growth cone is very dynamic resulting in continuous actin induced membrane protrusions, but it also allows microtubule induced protrusion at the growth cone required for the elongation and growth of the axon (Bradke and Dotti, 1999). In contrast, the neurites that will become dendrites, and are not growing during initial axon elongation, have a stable actin cytoskeleton in their growth cone. This stabilization does not only prevent actin induced membrane protrusion but it is also believed to prevent microtubules from inducing membrane protrusions (Forscher and Smith, 1988; Tahirovic and Bradke, 2009). Inducing destabilisation of actin in one early neurite induces this neurite to transform into an axon (Bradke and Dotti, 1999, 2000). This suggests that a stable actin cytoskeleton is required for dendrite fate whereas a dynamic actin cytoskeleton is needed for axon formation.

The actin cytoskeleton has its specific group of motor proteins, called myosins, which walk along actin filaments thereby transporting different kinds of cargo. This is especially important in both pre-synapse and post-synapse (spine) formation and maintenance, by transporting necessary proteins into these region where the actin cytoskeleton is predominant (Kapitein and Hoogenraad, 2011). Finally, it is believed that the actin cytoskeleton also plays a very important role in the formation of a dense cytoskeleton network at the site of the AIS. This dense network is required for linking and fixing AIS proteins to the membrane and the function of the AIS as a diffusion barrier. The actin requirement of the AIS will be discussed later in this review.

Microtubules

Microtubules (MT) are cylindrical polymers of α and β tubulin dimers which have, like actin, a minus and a plus end (Desai and Mitchison, 1997). They polymerize from GTP bound α and β tubulin dimers that are incorporated mostly at the plus or growing end of the microtubule. Like actin, tubulin is hydrolysed after polymerization into GDP bound tubulin. Tubulin in this GDP bound state tends to bend, resulting in the collapse of a microtubule filament. However this can be prevented by a continuous polymerization of GTP tubulin dimers, resulting in a GTP cap that is much more stable. Furthermore microtubule stabilization can also be enhanced by certain microtubule stabilizing factors such as MAP2 and Tau, which will both be discussed shortly later. Microtubules usually originate from a γ-tubulin ring complex that can be found at the minus end. These microtubule polymerization initiation complexes are concentrated at the centrosomes, large protein organelles from which most microtubules originate (Desai and Mitchison, 1997).

Microtubules play an important role in, axon extension and transport and are also thought to be involved in neuron polarization initiation and maintenance. Microtubule nucleation is important for axon extension as the elongating microtubule filaments generate a pushing force towards the membrane in the axon growth cone resulting in protrusions. The microtubule elongation in axons is promoted by, among others, CRMP2 which is believed to bind tubulin dimers and transports them to the growing plus-end (Arimura et al., 2004; Kimura et al., 2005). CRMP2 is responsible for axon extension and branching by promoting microtubule elongation, since a mutated form of CRMP2 has a dominant

negative effect on axon formation (Fukata et al., 2002; Inagaki et al., 2001).

The centrosome as microtubule organizer has an important role in neuron development and axon formation [reviewed by: (Kuijpers and Hoogenraad, 2011)]. In general, although controversial, the centrosome is thought to be required for microtubule assembly and neuron polarization. Interfering with centrosome activity in the neocortex affects axon formation and outgrowth, and therefore neuron polarization (de Anda et al., 2010). However, centrosome independent axon extensions have been observed in developing hippocampal neurons (Stiess et al., 2010).

Interestingly, microtubule organization differs between axons and dendrites since axon microtubules have a uniform orientation (Figure 2) whereas the MTs in dendrites do have a mixed orientation (Baas et al., 1988; Burton, 1988). Generally, all the microtubules found in the axon have their minus end orientated towards the cell body and their plus and growing end towards the axon tip. In dendrites the microtubules have a mixed orientation, meaning that minus and plus ends can be found orientated towards both the cell body and the dendrite tips, approximately in a 50-50 ratio, depending on the position in the dendrite. It is believed that this different microtubule organization plays a role in transport regulation in both the axon and dendrites. The current model is that axon microtubule transport requires only plus end directed motors, kinesins, to reach the end of a axon, whereas microtubule transport in dendrites requires also minus end directed motors – like dynein – to transport vesicles to dendrite tips (Kapitein and Hoogenraad, 2011; Kapitein et al., 2010).

Important for microtubules are MAPs, which are a wide-range of proteins with different functions (Conde and Caceres, 2009; Mandelkow and Mandelkow, 1995). Many of them have a role in microtubule dynamics, like enhancing polymerization of tubulin dimers, stabilization of microtubule filaments, or even induction of microtubule collapse. Two major MAPs found in neurons are MAP2 and Tau which are mainly associated with either dendritic or axon microtubules respectively (Conde and Caceres, 2009). Knock down of MAP2 together with MAP1B, which have synergistic functions, results in neuron abnormalities such as lack of microtubule bundling and neurite outgrowth altering predominantly dendritic morphogenesis (Teng et al., 2001). Tau localizes very early to a specific neurite on the onset of axon formation and is therefore suggested to be important in neuron polarization (Kempf et al., 1996). And although the knock-down of Tau does not have a severe impact on axon morphogenesis (Harada et al., 1994), it does have an effect on microtubule stability and organization at certain axon regions. Like MAP2, Tau could have a synergistic partner that can take over its function in axon formation. Although, several discoveries concerning MAP2 and Tau have been made over ten years ago, the details of MAP2 and Tau function in neuron polarization remain unknown.

Microtubules can also be modified by post-translational modifications. Three important microtubule modifications in neurons are detyrosination, acetylation, and polyglutamylation. Detyrosination involves the mechanism in which the C-terminal tyrosine of α-tubulin is removed. This affects the binding of certain proteins and also reduces microtubule disassembly (Fukushima et al., 2009; Ikegami and Setou, 2010; Wloga and Gaertig, 2010). Furthermore, detyrosinated microtubules are believed to be more stable than tyrosinated microtubules.

Acetylation of α-tubulin lysine 40 is commonly found and is associated with stable microtubules (Fukushima et al., 2009; Hammond et al., 2008). Axons have been shown to have a higher acetylated/tyrosinated MT ratio than dendrites (Hammond et al., 2010; Witte et al., 2008). There is some evidence that tubulin acetylation is required for kinesin-1 transport (Reed et al., 2006), however the exact function of acetylated microtubules in neuronal polarization remains to be elucidated further.

The third modification common for neuronal microtubules is polyglutamylation, which is the polymerization of a chain of glutamates on a single or multiple C-terminal glutamic acid residues of tubulin (Fukushima et al., 2009; Ikegami and Setou, 2010). Addition of multiple glutamates results in more negatively charged

microtubules since the charge of glutamate is negative. It is strongly believed that this change in charge affects the binding of MAPs and kinesins. This has been shown in more detail for KIF1A, which distribution in neurons is distorted when the polyglutamylation mechanisms is impaired (Ikegami et al., 2007).

Finally, differences in microtubule stability have also been implicated in neuronal polarization, especially axon formation. Microtubules appear to be more stable in future axon neurites and also in axons after polarization since they are more detyrosinated compared to microtubule dendrites (Witte et al., 2008). Cell treatment with low doses of the microtubule stabilizing drug taxol resulted in the formation of multiple axons expressing several axon markers including Tau (Witte et al., 2008). These results suggest that indeed enhanced microtubule stability can be linked to axons, however this is predominantly based on microtubule modification measurements. Recent research showed that the microtubule turnover does not differ between axons and dendrites suggesting there is no major difference in microtubule dynamics between axons and dendrites (Hammond et al., 2010). Therefore linking enhanced microtubule stabilization to axon formation and maintenance remains controversial.

Axon initial segment

The AIS is a specialised region which is characterized by a dense network of both cytoskeleton proteins, membrane proteins, and scaffold proteins which link on their turn the membrane proteins to the cytoskeleton. The AIS is believed to have unique properties and a special function in neuron function since it is the site of action potential initiation, mainly because the AIS clusters ion channels which are required for this process. The fact that the unique region such as the AIS is only found at the beginning of the axon and not in dendrites also implies an important role of the AIS in the establishment and maintenance of neuronal polarity. After extensive research initial evidence for this hypothesis was actually shown by the existence of a diffusion barrier at the site of the AIS between the somatodendritic region and the axon region (Kobayashi et al., 1992; Winckler et al., 1999). This diffusion barrier suggests that the axon and dendrites are physically different and therefore polarized. A couple of years ago also further evidence showed that the AIS can function as a selective transport filter (Song et al., 2009), which strengthened the believe that the AIS plays an important role in neuron polarity. Several proteins are important for the AIS main function of diffusion barrier, selective transport filter, and action potential initiation. Both these proteins and functions will be discussed more extensively.

It should be noted that many of these proteins can also be found in the nodes of Ranvier, which have a role in propagating the action potential. In fact, an intriguing phylogenetic based study strongly suggests that the nodes of Ranvier are evolutionary derivatives of the AIS (Hill et al., 2008). This study showed that during evolution a clustering mechanism of voltage-gated sodium channels evolved early in the chordate lineage. The primitive chordate Lamprey does have AIS like structures, however this organism lacks myelinated neurons and therefore nodes of Ranvier are not present. Jawed vertebrates, on the other hand, do have myelinated neurons and nodes of Ranvier. Since Lampreys evolved earlier in evolution than jawed vertebrates, and the fact that the node of Ranvier are very much like the AIS, it is likely that the nodes of Ranvier are derived from the AIS during evolution (Hill et al., 2008).

The fact that the AIS is very conserved in vertrebrates implies an essential role in neuronal functioning. However, although extensive research has been done concerning the AIS, there are still many uncertainties to be elucidated. In the upcoming chapters the composition and functions of the AIS will be extensively described. Also in the last chapter the initiation of AIS assembly, although largely unknown, will be discussed together with the future perspectives.

AIS proteins

To fully understand AIS formation and function we first have to understand all the proteins and their function making up the AIS (see supplemental table). Therefore this section will focus on the most important proteins known so far that can be found in a functional AIS (Figure 2).

Ankyrin G

Ankyrin G (AnkG or ANK3) is a 480kDa cytoskeleton scaffolding protein involved in organizing membrane domains and therefore maintaining cell polarity in a large subset of cells, mainly those of the CNS. AnkG has been found to specifically localize to the AIS and nodes of Ranvier (Kordeli et al., 1995). AnkG is (so far found) the first protein localizing at the AIS and is therefore believed to be of important function in AIS assembly (Boiko et al., 2007). There are also different ankyrins, namely ankyrin B (AnkB) which is also found in neurons and ankyrin R (AnkR) which is mostly found in erythrocytes. A high degree of homology between the different ankyrins can be found, however AnkG only localizes to the AIS and nodes of Ranvier whereas AnkB localizes throughout the axon, which is likely due to differences in domains between AnkB and AnkG (Bennett and Lambert, 1999; Zhang and Bennett, 1998). The ankyrin transcripts are subjected to alternative splicing creating different isoforms. The AnkG 480kDa is the major isoform and is found at the AIS together with the 270kDa isoform, due to the presence of a serine-rich domain in both isoforms (Zhang and Bennett, 1998).

One of the main functions of AnkG first described is the clustering of voltage-gated sodium channels at the AIS, required for action potential firing (Zhou et al., 1998). Local knockout of ankyrin G in the mouse cerebellum showed a loss of Na^+ channels at the AIS followed by a lack of faithful action potential initiation. Besides its requirement for action potential firing, ankyrin G has also been described to be essential for maintenance of both the AIS and neuronal polarity (Hedstrom et al., 2008). Hedstrom et al. showed that shRNA AnkG positive cells (AnkG knockdown) have a loss of βIV-spectrin, NrCAM, NF-186, and Nav staining at the AIS. In addition, AnkG knockdown resulted in the localization of dendritic markers like MAP2 and KCC2 to what used to be the axon. Even spines expressing postsynaptic densities were formed in these former axons (Hedstrom et al., 2008) indicating the former axon has become a dendrite. Additional research showed that the role of AnkG in maintaining neuronal polarity is also true *in vivo* (Sobotzik et al., 2009). Purkinje cells lacking AnkG in mice developed spine like protrusions in the axon which stained positive for several postsynaptic proteins like the metabolic glutamate receptor mGluR1. Finally, AnkG was also found as the initiator of ion channel immobilization (at 4 DIV) at the AIS resulting in a diffusion barrier (at 10 DIV) between the soma and axon membrane domains (Brachet et al., 2010). Expression of AnkG in neuroblastoma cells resulted in a decrease in diffusion of an exogenously expressed Kv-Nav chimera, this diffusion was increased when the AnkG binding motif was mutated. In developing hippocampal neurons the diffusion restriction of the Kv-Nav chimera was reversed by CK2 inhibition, suggesting that CK2 phosphorylation is required for AnkG binding. In mature neurons AnkG is also still required for ion channel immobilization and faithful maintenance of the diffusion barrier (Brachet et al., 2010). Only one paper describes the phenotype of AnkG knock-out mice: mice lacking AnkG in the cerebellum walk uncoordinatedly, have reduced locomotion, and progressive ataxia (Zhou et al., 1998) most likely due to malfunctioning Purkinje cells.

βIV-spectrin

βIV-spectrin belongs to the spectrin family, a family believed to form a molecular scaffold between the actin cytoskeleton and the cell membrane and its proteins. βIV-spectrin was originally described by Berghs et al. who reported that βIV-spectrin was most abundant in the brain and the islets of Langerhans (Berghs et al., 2000). The expression of this protein in neurons was mainly seen in the nodes of Ranvier and the AIS where it co-localizes with AnkG. The localization of βIV-spectrin to the AIS requires AnkG but precedes sodium channel

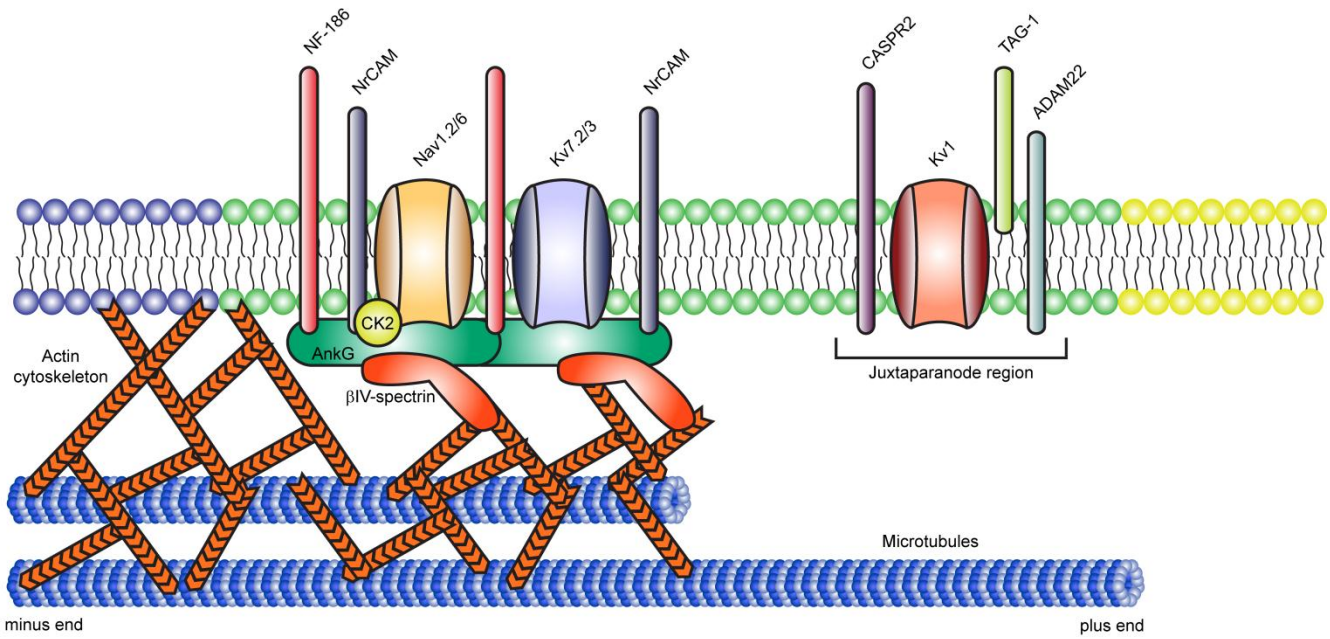

Figure 2

Membrane region of the AIS (green phospholipids), flanking both the soma region (blue phospholipids), and the axon (yellow phospholipids). Embedded in the membrane are the different ion-channels and several neuronal adhesion molecules. Most of them are believed to be coupled to the cytoskeleton via AnkG and βIV-spectrin. In addition CK2 is also shown, which regulates the Nav-AnkG interaction by phosphorylation. The microtubules in the axon are uniformly orientated with the minus end towards the soma. The juxtaparanode, which is considered as part of the AIS in myelinated axons, is also shown.

and neurofascin-186 localization (Jenkins and Bennett, 2001). βIV-spectrin has a C-terminal actin binding domain followed by 17 spectrin repeats. It has been shown that βIV-spectrin binds AnkG through its 15th spectrin repeat, and that this interaction is essential for βIV-spectrin to localize to the AIS both *in vitro* and *in vivo* (Yang et al., 2007). Interestingly, experiments done with mice lacking βIV-spectrin showed that adult neurons lack localization of the major AIS components at the AIS, including AnkG (Komada and Soriano, 2002). In addition, mice lacking a functional βIV-spectrin protein have impaired AIS and nodes of Ranvier morphology and molecular organization (Lacas-Gervais et al., 2004; Yang et al., 2004). This suggests that βIV-spectrin is essential for the stability of the AIS and nodes of Ranvier possibly through its interaction with the actin cytoskeleton. However, AIS formation and AnkG localization in βIV-spectrin mutant mice is not affected *in vivo* in developing neurons, suggesting that βIV-spectrin is not required for AIS assembly but presumably for AIS maintenance (Yang et al., 2007).

Neurofascin-186

Neurofascin (NFASC) is a cell adhesion molecule expressed in neurons and was discovered in the mid-nineties, among other cell adhesion molecules to have ankyrin binding abilities (Davis and Bennett, 1993, 1994; Davis et al., 1993). An abundant neurofascin isoform is Neurofascin-186 (NF186) as its molecular weight is 186kDa (uniprot NFASC isoform 1). NF186 is mainly localized in the AIS and nodes of Ranvier where it co-localizes with AnkG (Davis et al., 1996). In Purkinje cells, NF186 forms a subcellular gradient along the AIS-soma axis, with a high signal at the AIS and dorsal soma which decreases towards the ventral soma (Ango et al., 2004). This NF186 localization to the AIS is absent in cerebellum specific AnkG knock-out mice, indicating that it requires AnkG (Ango et al., 2004; Jenkins and Bennett, 2001). These results emphasises the main function of AnkG in keeping all the proteins at the AIS(Hedstrom et al., 2008).

RNAi knock-down of NF186 does not affect the localization of AnkG, βIV-spectrin, or sodium channels to the AIS whereas knock-down of AnkG does (Hedstrom et al., 2007). Nonetheless, NF186 knock-down inhibits the

formation of a brevican based extracellular matrix which is enriched at the AIS and nodes of Ranvier. The exact function of this matrix is unknown, but it is suggested that it may stabilize NF186 and its interaction partners. Recently it has been shown that developing neurons of NF186 knock-out mice are able to develop an AIS, although NrCam cannot localize to the AIS in these cells (Zonta et al., 2011). In addition, culturing of these mature NF186 deficient neurons resulted in the disintegration of the AIS after several weeks. This was confirmed *in vivo* using a conditional NF186 knock-out, showing a loss of the AIS in mature neurons after the conditional deletion (Zonta et al., 2011). These results suggest that although NF186 is not important for AIS assembly, it is important for AIS maintenance.

NrCAM

The neuronal cell adhesion molecule (NrCAM) protein is, like NF186, a cell adhesion molecule expressed in neurons and having ankyrin binding activity (Davis and Bennett, 1993, 1994; Davis et al., 1993). Antibody stainings showed that NrCAM localizes to both the AIS and the nodes of Ranvier (Davis et al., 1996; Hedstrom et al., 2007). This localization is abolished in NF186 knock-out mice, suggesting that NF186 is required for NrCAM function at the AIS, possibly through the brevican matrix (Zonta et al., 2011).

NrCAM null mice have delayed clustering of sodium channels and AnkG during node of Ranvier development (Custer et al., 2003). Although delayed, the nodes are still able to develop properly, suggesting a redundant clustering mechanism independent of NrCAM. The same evolutionary origin of nodes and the AIS suggests that NrCAM could have a similar role in clustering proteins in the AIS as it has in nodes, however this is still left to investigate. Mice lacking NrCAM have abnormalities in axonal guidance (Heyden et al., 2008), which could be due to impaired cell-cell or cell-extracellular matrix contact. Although not shown in these mice, NrCam is maybe required for extracellular influences on the AIS and an interesting and presumably clinically relevant protein to investigate.

Other neuronal cell adhesion molecules

Several other neuronal cell adhesion molecules that can localize to the AIS, are the proteins CASPR2, TAG-1, and ADAM22. Although not well described, they are all found at the AIS, at the juxtaparanode region to be more precise. This region can be found in myelinated nerve cells at the beginning of the myelin sheet, which can be found shortly after the AIS. Furthermore the juxtaparanode is also part of the node of Ranvier in these neurons. CASPR2 has been shown to be required for maintaining potassium channels at the juxtaparanode region (Poliak et al., 2003). To do so, TAG-1 has to be linked to the membrane via its GPI linker in this region. ADAM22 was also found to be associated with potassium channels (Kv1) at the juxtaparanode region (Ogawa et al., 2010). However, ADAM22 knock-out did not results in a loss of potassium channels, whereas it did result in the loss of some scaffolding proteins found at the juxtaparanode region. Besides the potassium channel clustering at the AIS, the exact importance of these proteins in fulfilling the role of the axonal initial segments remains largely unknown.

Sodium channels

Voltage-gated sodium (Nav) channels are highly enriched in the AIS (Wollner and Catterall, 1986). This highly dense Nav channel network is required in the AIS to fulfil its role in action potential initiation (Kole et al., 2008). Nav channels are transmembrane proteins that form a pore or channel in the cell membrane trough which specifically sodium ions (Na^+) can pass. These channels open in response to a change in voltage, allowing Na^+ ions into the cell causing depolarization of the cell membrane, the initial step of the action potential (see also Box 1).

Two distinct isoforms of Nav channels have been found in the AIS, namely Nav1.2 and Nav1.6 (Boiko et al., 2003). The Nav1.2 isoform is the single isoform in developing neurons where it is, during later stages of neuron development, accompanied by Nav1.6 at the AIS. Nav1.2 is mainly found at the proximal regions and Nav1.6 at distal regions of the AIS, where they have both been shown to fulfil different functions (Hu et al., 2009). Ankyrin G

appears to be essential for NaV channel localization to the AIS, as the NaV channels are abolished in the absence of AnkG (Hedstrom et al., 2008; Jenkins and Bennett, 2001; Zhou et al., 1998). In addition, an 9 amino acid specific AnkG binding motif has been found in Nav channels which bind AnkG at the AIS (Garrido et al., 2003; Lemaillet et al., 2003). Addition of this AnkG binding motif to somatodendritic localized potassium channels is sufficient to target this channel to the AIS. The high degree of homology between AnkG and other ankyrins like AnkB, which is found throughout the axon, suggests that these other ankyrins can also target for instance Nav1.6 channels elsewhere in the axon. Therefore it is believed that additional mechanisms are required for Nav – AnkG binding, enabling AIS specific localization of the Navs at the AIS.

Potassium and calcium channels

Voltage-gated potassium (Kv) channels are, like Nav channels, also important in action potential firing (Kole et al., 2007). However, they are mainly involved in stabilizing the resting potential after depolarization by Na^+ ions and thereby limiting repetitive firing. Upon depolarization these Kv channels open, allowing K^+ to flow out of the cell and thereby counteracting the Na^+ induced depolarization of the membrane.

The two main Kv channels found at the AIS are Kv7.2 (KCNQ2) and Kv7.3 (KCNQ3). They have both been reported to co-localize with ankyrin G and Nav channels at the initial segment and nodes of Ranvier (Devaux et al., 2004). Both Kv channels contain an AnkG binding motif similar to that of Nav channels, which is not only sufficient but also required for the AnkG interaction (Pan et al., 2006). In addition, a lack of Kv concentration at the AIS could be observed in AnkG knock-out mice. This shows again the importance of ankyrin G in localization of AIS components and AIS function. Finally, Kv1 has also been reported to be present at the distal (juxtaparanode region) AIS (Kole et al., 2007; Van Wart et al., 2007). It is believed that this different distribution of Kv1 compared to Kv7.x may have an important function controlling action potential firing (Rasband, 2010). The scaffold post synaptic density 93 (PSD-93) was recently also reported to localize to the AIS and required for Kv1 clustering, as a PSD-93 knock-down abolishes Kv1 AIS localization (Ogawa et al., 2008). However, the exact mechanism of PSD-93 induced clustering of specific protein to the AIS remains unclear.

Voltage-gated calcium (Ca^{2+}) channels are responsible for muscle contraction and a calcium influx leading to, among other output mechanisms, presynaptic vesicle release both in response to an action potential. These channels are thought to be normally not concentrated at the AIS. However, recently they have been discovered at the AIS of dorsal cochlear nucleus interneurons (Bender and Trussell, 2009). They have been described to influence action potential timing, as inhibition of these channels causes a delay in the action potential. The significance of Ca^{2+} channels at the AIS remains unclear, also the targeting and localization towards the AIS of these channels is not determined.

Casein kinase 2

It has been discussed that Nav channels have a specific ankryin binding motif, however it remains unclear whether this motif is sufficient to target Nav channels to the AIS by AnkG. Recently a protein kinase has been found which is believed to regulate the Nav-AnkG interaction. This kinase, casein kinase 2 (CK2) is a protein kinase that consists of four subunits; alpha, alpha', and two beta. CK2 expression is most abundant at the AIS and the node of Ranvier both *in vitro* and *in vivo* (Brechet et al., 2008). An *in vitro* phosphorylation assay showed that the ankyrin binding motif of Nav1.2 is phosphorylated by active CK2. This phosphorylation is essential for Nav channel localization to the AIS, since the use of a CK2 inhibitor abolishes the sodium channel accumulation at the AIS. A second assay showed that the binding of the Nav ankyrin binding motif to ankyrin increased with a 1,000 fold upon CK2 phosphorylation of the flanking serines (Brechet et al., 2008). This suggests that the CK2 phosphorylation indeed enhances the ankyrin – Nav channel interaction at the AIS and

nodes. However, the enhanced binding was both true for ankyrin G and ankyrin B, which share a high degree of homology (Brechet et al., 2008). The specific localization of CK2 to the AIS and nodes could explain why Nav channels are only bound to AnkG at these spots and not throughout the axon at spots where ankyrin B, but no CK2, can be found. In addition, knock-down of AnkG has been shown to impair CK2 concentration at the AIS, but also CK2 inhibition diminishes AnkG and Nav channels at the AIS (Sanchez-Ponce et al., 2011). This suggests that both AnkG and CK2 are required for each other's localization. The results could be explained by an impaired stability of AnkG to the AIS: it cannot longer bind to Nav channels and is therefore easier dissociated from the AIS. Furthermore, CK2 has been discovered to associate with microtubules (Serrano et al., 1989). This could be another important role in AIS development since microtubule organization and stabilization is important in axon formation (Baas et al., 1988; Burton, 1988; Witte et al., 2008).

EB1 and EB3

Recently two additional proteins have been described at the site of the axon initial segment, namely EB3 and EB1 (Leterrier et al., 2011; Nakata and Hirokawa, 2003). These are microtubule end-binding (EB) proteins that belong to the well described group of microtubule plus-end tracking proteins (+TIPS) which have a role in regulating microtubule dynamics and interactions [reviewed by: (Akhmanova and Steinmetz, 2008, 2010)]. EB3 forms a stable fraction at the AIS, where it co-localizes with AnkG (Leterrier et al., 2011). Pull-down experiments showed a positive interaction of EB3 and EB1 with AnkG, but not with AnkB (only shown for EB3). Experiments done with different mutants indicate that the EB3 interaction with AnkG requires the amino acid residues between positions 245-259, which is part of the EB3 hydrophobic pocket. Furthermore, knock-down of AnkG resulted in an increased localization of EB3 and EB1 to the somatodendritic region, which could be caused by a translocation of EB3 and EB1 from the AIS. Finally, both a knock-down of EB3 and EB1 resulted in a partial loss of AnkG from the AIS (Leterrier et al., 2011). This could suggest that AnkG is localized to the AIS via EB3 and EB1 to microtubules. However, a loss of EB3 and EB1 could also affect microtubule dynamics and therefore AnkG localization to the AIS, which can be considered as an indirect effect of EB3/1 loss. Although the study described here gives us some more insights about AnkG localization, many experiments are left before we completely understand AIS assembly and AnkG targeting to this segment.

AIS vs. nodes of Ranvier

Both the AIS and nodes of Ranvier play an essential role in action potential initiation and propagation. Also the molecular proteins are almost identical as they both contain AnkG, βIV-spectrin, NF186, NrCAM, sodium channels etc. However, the assembly of both regions has been reported to be different. The nodes of Ranvier require the binding of myelinated Schwan cells to the axon (Eshed et al., 2005), whereas the AIS does not require them for assembly (Ogawa and Rasband, 2008). This suggests that signals from these Schwan cells are important for node but not AIS assembly.

Interestingly, it has been shown that NF186 is required for node but not for AIS formation, since knock down of NF186 does not affect the AIS, but does leas to loss of AnkG, Nav channels and NrCAM localization to the nodes of Ranvier (Dzhashiashvili et al., 2007; Hedstrom et al., 2007). Moreover, it was also found that the intracellular domain of NF186 is sufficient for AIS localization, whereas the extracellular domain of NF186 is sufficient for its node localization (Dzhashiashvili et al., 2007). However, even though NF186 can localize to nodes without AnkG, mutants of NF186 lacking the AnkG binding domain showed that AnkG binding by NF186 is required for node assembly. Overall this study indicates that NF186 is essential for node formation initiation, independent of AnkG, and this is possibly through interaction with the myelinating Schwan cell. Although AnkG is not required for node initiation, it is required for NF186 maintenance at nodes and recruitment of other components

after binding of NF186. At the AIS, on the other hand, AnkG initiates AIS formation independent of NF186. So in short it is believed that nodes formation is initiated extracellular by NF186 whereas AIS formation is initiated intracellular by AnkG. However, the exact mechanisms of NF186 recruitment to nodes and AnkG to the AIS remain largely unknown.

Roles of the AIS

Diffusion barrier

A distinct difference in membrane proteins can be observed between axons and the somatodendritic region, which are important for their different functions. Normally many membrane proteins can diffuse more or less freely within the membrane. However, in neurons this diffusion has to be blocked at the beginning of the axon to maintain neuronal polarity. Otherwise specific dendritic membrane proteins, receptors for instance, can diffuse to the axon or the other way around, which will impair neuronal function.

In 1992 Kobayashi et al. fused liposomes of fluorescent phospholipids specifically into the axonal membrane of polarized neurons (Kobayashi et al., 1992). The fluorescence was followed over time but no diffusion of the lipids could be detected in the somatodendritic region. However, FRAP (fluorescence recovery after photobleaching) experiments showed that the lipids within the axon could still diffuse freely. This suggested that there is some sort of barrier present, preventing free diffusion of membrane proteins between the axon and somatodentritic region. Additional experiments also showed that the observed diffusion barrier was present in both the inner and outer leaflet of the membrane (Kobayashi et al., 1992). The study, described here, showed evidence of a diffusion barrier existing at the AIS, preventing lipids to diffuse freely from the polarized axon towards the cell body and the other way around. Interestingly, a scientific correspondence pointed out that because of the length of the axon the fluorescent lipids would simply not reach the cell body by diffusion in the time span used by Kobayashi et al. (Futerman et al., 1993). Because of this argumentation the existence of a diffusion barrier was still an uncertainty by then. More evidence was published several years later that (also) membrane proteins were not able to pass a diffusion barrier at the AIS (Winckler et al., 1999). Using optical tweezers it was shown that membrane proteins could not be dragged physically along the AIS. Moreover, after disrupting the actin filaments using the drug latrunculin-B this impaired mobility along the AIS was abolished (Winckler et al., 1999). This does not only suggest that the function of the diffusion barrier is true for membrane protein but also that this barrier is dependent on actin filaments. The need of actin filaments is not very surprising as it is believed that proteins of the AIS are coupled by scaffolds to the actin, and thereby inducing its immobility.

A delicate study showed that all membrane proteins anchored (indirectly) to the cytoskeleton, at the site of the AIS, are themselves part of the diffusion barrier by forming a dense network preventing diffusion (Nakada et al., 2003). By using the phospholipid L-α-dioleoylphosphatidylethanolamine (DOPE) coupled to the fluorophore Cy3, they showed that at the side of the AIS the diffusion was significantly impaired compared to other adult neuron regions. Interestingly, they showed that the binding of AnkG and Nav channel to the AIS, a measurement for AIS development, coexisted with the decrease diffusion observed at this site. This suggested that the localization of AIS components to the axon is important for the function of a diffusion barrier. In addition, a further decrease in diffusion is observed when the F-actin stabilizing drug Jasplakinolide is used. In contrast, again an increase in diffusion was measured when F-actin depolymerisation was induced (Nakada et al., 2003). These data conforms the already described involvement of the actin cytoskeleton in anchoring and stabilizing the membrane proteins, important for maintenance of the diffusion barrier. A model was proposed by Nakada et al. where membrane proteins were anchored in a very dense network and thereby function as “rows of pickets” preventing diffusion of both membrane lipids and proteins (Nakada et al., 2003). Although the studies described here did give us

further insights about the existence of a diffusion barrier at the AIS, the exact mechanism remains to be unravelled.

Selective transport filter

A second proposed role of the AIS, which remains largely unknown, is its function as a selective filter for active transport into the axon. So far two elegant studies describe evidence of the existence of such a filter. The first study showed a difference in microtubule based selective transport between the axon and the dendrite, without showing any evidence regarding the involvement of the AIS (Burack et al., 2000). Using GFP tagged chimeras of both transferring receptor (TfR) and neuron-glia cell adhesion molecule (NgCAM) a selective transport of these proteins into dendrites or axons was observed. TfR is a dendritic specific protein which was in this study only observed in the dendrites. Also transport by (mainly) exocytotic carrier vesicles containing TfR was almost only found into the dendrite and not the axon, suggesting that TfR containing vesicles are prohibited from axon entry. On the other hand, GFP-NgCAM chimera distribution was found throughout the neuron, so in both axon and dendrite. Even vesicle transport of NgCAM was observed into both axon and dendrite (Burack et al., 2000). So NgCAM vesicles were not blocked from axon entry, neither were they from entering the dendrite. These data could suggest that there may be some sort of filter present at the beginning of the axon, which is not found at dendrites, that allows selective transport entry and blocks others. Having said that, as already discussed a difference in microtubule organization and motor transport is observed between dendrites and axons which is also believed to play an important role in selective transport (Kapitein and Hoogenraad, 2011), and could explain the observed phenotype described by Burack et al.

The second study showed the first real evidence for a selective transport filter at the AIS (Song et al., 2009). Two different sizes of the dextran sugars (normally equally distributed throughout the cytoplasm) was injected into the soma at different stages of developing neurons. After 3 DIV (presumably stage 2-3 neurons), both 10 and 70kDa diffused into the axon within minutes after injection. However, after 5 DIV the 10kDa dextran could still enter the axon whereas the 70kDa dextran could not pass the site of the AIS. Interestingly the prohibited cytoplasmic diffusion of 70kDa dextran pass the AIS was dependent on F-actin but not on microtubules, which was shown by using either actin or microtubule disrupting, or even microtubule stabilizing drugs. The filtering at the AIS was also abolished when AnkG expression was inhibited using siRNA (Song et al., 2009). This data suggests that the AIS do not only form a passive membrane diffusion barrier but also a cytoplasmic diffusion barrier, which depends on the actin and not the microtubule cytoskeleton.

To study the regulation of active transport into the axon additional experiments were carried out. The dendritic targeting membrane protein NR2B is exclusively transported to the somatodendritic region, and not in the axon, after 5 DIV. This selective transport into dendrites depended on microtubules whereas the blockage from axon entry depended on actin. Furthermore, after 7 DIV, transport of VAMP2, an important presynaptic protein, was impaired but not abolished into the axon by the AIS, as shown by FRAP experiments. However, complete VAMP2 transport was restored after F-actin was disrupted (Song et al., 2009). The described data suggest that AIS development coexists with the formation of a selective transport filter at the AIS site, regulating entry of active transport into the axon. To further test the observed selective active transport into axons both the kinesins KIF17 and KIF5B and their role in this observed phenotype were investigated. Normally KIF5B drives transport of VAMP2 vesicles into the axon whereas KIF17 drives transport of NR2B vesicles into the somatodendritic region and not the axon. Interestingly, a KIF17 mutant, who cannot carry any vesicles but is still motile, is able to pass the AIS and enter the axon. In addition, KIF5B normally also transports GluR2 vesicles to dendritic regions and when carrying this load, KIF5B cannot enter the axon (Song et al., 2009). This suggests that not the motor itself but the KIF-cargo complex as a whole is responsible for its own selective transport. Maybe this complex

is recognized and filtered by the AIS allowing its entrance or not.

Both studies described here contribute to the growing evidence and acceptance of the existence of both a cytoplasmic diffusion barrier and active transport filter at the site of the AIS. However, many experiments remain to be done before we understand the mechanisms of transport filtering at the axon initial segment.

Action potential initiation

Already in the fifties the first evidence arose of the AIS being the site of action potential initiation. Several studies have been published during that time all suggesting that the site of action potential initiation can be somewhere found at the beginning of the axon including, soma, axon hillock and AIS [shortly reviewed by: (Clark et al., 2009)]. Edwards et al. were one of the first, among others, describing a study suggesting that the site of action potential initiation could be found at the axon initial segment (Edwards and Ottoson, 1958). Measurements using micro-electrodes on lobster stretch receptor neurons showed that upon receptor activation the first electrical pulse occurred at the beginning of the axon but away from the soma, where the AIS can be found. Also, after activation, the pulse travelled from the AIS backward/in reverse (antidromically) towards the somatodentritic region and forwards (orthodromically) towards the axon end (Edwards and Ottoson, 1958). Both results suggest that therefore the initial segment is very likely to be the site of action potential initiation. Several decades later in the nineties these observations were confirmed by several research groups. Using multiple whole cell recording experiments researchers were able to show that indeed the AIS is the initiator of action potential since the potential propagated towards axon synapses and back into dendrites (Colbert and Johnston, 1996; Stuart and Sakmann, 1994). Computational modelling indicated that a high density of sodium channels at the AIS is required for action potential initiation (Mainen et al., 1995). As discussed previously sodium channels are very abundant at the AIS, which could be the reason why especially the AIS is important for action potential initiation.

Perhaps the most significant study on why the AIS is so important for action potential initiation, was published in 2008. Using patch-clamp recordings no difference in sodium peak current between the AIS and the soma was observed, suggesting also no difference in sodium channel abundance between these regions (Kole et al., 2008). However, immunohistochemistry experiments show that there are significantly more sodium channels present at the AIS compared to the soma. They suggest that the fixation of sodium channels to the cytoskeleton at the AIS prevents them from being pulled into the path-clamp pipette and are therefore not measured. Indeed F-actin destruction by the drug cytochalasin B increased the sodium peak current, which was restored when the actin stabilizer drug phalloidin was also added. Furthermore they also used fluorescent sodium dyes during action potential to show that the sodium influx at that time is the highest at the site of the AIS. Finally, computational simulation showed that the AIS requires a sodium channel density of at least 50 times more than in the somatodendritic region (2,500 versus 50 pS μm^{-2}) (Kole et al., 2008). Results from this study strongly indicate that a high density sodium network at the AIS is required for faithful action potential initiation and that this network is dependent on the actin cytoskeleton for anchoring.

After an action potential is initiated at the AIS, a quick repolarization of the membrane is required before a new potential can be initiated. Therefore also potassium channels are required at the AIS to restore the membrane potential back to resting. Inhibition of potassium channels leads to an increase in action potential peak duration (Kole et al., 2007; Shu et al., 2007). A longer peak after potassium channel inhibition does also lead to an increase in action potential response, measured in presynaptic activity (Kole et al., 2007). This indicates that not only sodium channels but also potassium channels at the AIS are important for faithful action potential firing and its response.

Without the three main functions of the AIS mentioned here the axon is not unique anymore compared to dendrites which will eventually

impair neuron function. Therefore it could be argued that these three function themselves, especially the diffusion barrier and selective transport filter, are important for neuron polarity maintenance since the loss of these functions will result in a loss of axon function. The question remains how the AIS formation is initiated and how this relates to the initiation of neuron polarity. Is the axon defined first by a different mechanisms followed by AIS formation, or does AIS formation in an unpolarized neurite define the axon? These and others question remains largely to be elucidated.

Discussion

Although the exact molecular mechanisms of neuron polarization are not completely unravelled, several mechanisms are known that are believed to play a predominant role on cytoskeleton reorganization. However, how exactly axon development is initiated remains largely undiscovered. Also at what stage a neurite has been determined to become the axon and which signals are required at this stage is not known at the moment. The stage at which AIS formation is initiated is also unidentified, it is even possible that AIS formation on itself determines the axon. We know that AnkG localization is important for AIS development; its localization is the first known event, up to now, in AIS formation. However, it remains unclear what mechanisms recruit AnkG to the AIS or whether other unknown proteins localize to the AIS first. All these mechanisms remain to be elucidated before we completely understand all the features of neuron polarization including axon development.

It has been indicated that microtubule growth and organization are possibly the key stones in axon formation. Fast growing microtubules in a uniform orientation are associated with a growing axon during the first stages of neuron polarization. This uniform orientation could be the foundation of axon associated processes and domains like the AIS. Interestingly, it has been shown that the neurites of stage 1-3 neurons that will become dendrites also have uniform oriented microtubules (minus end towards cell body), until they start to develop as a dendrite in stage 4 (Baas et al., 1989). So if indeed the uniform microtubule orientation is important for axon development then it could be argued that predominant axon domains, such as the AIS, are likely to develop during or after dendrite formation unless secondary signals are also involved. This latter is more likely the case since in most cellular mechanisms several signals are intertwined to regulate and initiate different processes. But the question is then, what are these secondary signals? Or does the uniform orientation of microtubules not play a role at all in AIS formation? To answer these questions we first have to be sure what the sequential events are in AIS development and which action can be considered as the initiation step in AIS formation. As mentioned, it remains unclear what recruits AnkG to the site that will become the AIS. Possibly this unknown mechanism is the key in AIS initiation. To find these mechanisms, many experiments have to be done like RNAi screening to see which knock-downs impair AIS formation during development. However this could be very challenging in *in vitro* developing hippocampal neurons, therefore other techniques could be more suitable. Recent findings suggest, again, that the missing link in AIS development can possibly be found in the microtubule network. Both the +TIPs EB3 and EB1 have been linked to the AIS, as well as GTP capped microtubules (Leterrier et al., 2011; Nakata and Hirokawa, 2003; Nakata et al., 2011). This suggests that the microtubules plus ends plays a role in the AIS, possibly in AIS assembly. Although it is likely that the microtubules plus ends are not the key to AIS initiation, it is tempting to look for AIS initiators in the pool of proteins that can bind microtubules. When candidates are found, it would be interesting to express these in neurons or organisms that are believed to not have an AIS to see whether this protein is sufficient to initiate AIS formation.

Another very interesting question arises when discussing the exact function of the AIS. For a long time it was believed that the major function of the AIS is the generation of an action potential. However, more evidence arises that the AIS also has other important functions, like the maintenance of polarity by the diffusion

barrier and selective transport filter. So the question is: what defines the AIS? C. elegans for instance do not express voltage-gated sodium channels, therefore people accept that they do not have an AIS. However, they do have polarized neurons and it is for this reason that it is still possible that they have an AIS that functions as a diffusion barrier or selective transport filter. On the other hand people believe that dorsal root ganglia neurons also do not have an AIS because they are differently polarized, however they do generate an action potential. These findings question the exact functions of the AIS and opens the debate on what the AIS exactly is.

Furthermore, additional research is required in the plasticity and dynamics of the AIS. If we could completely unravel the plasticity of the AIS then this would give us more insights in the ability of the AIS to form again after injury. Recent publications showed that the size and location of the AIS depends on the excitation (Grundemann and Hausser, 2010). Constant neuron stimulation alters the exact location of the AIS, which alters the action potential threshold, in order to fine-tune the response (Grubb and Burrone, 2010). In addition, a deprivation of neuron stimulation regulates the voltage-gated Na^+ channels distribution in the AIS, thereby increasing the AIS size (Kuba et al., 2010). This actually suggests that the AIS is not a static domain in the axon but that it can also relocate or expand. FRAP experiments on AnkG and for instance Nav channels could give us more insights of the stability of these proteins in the AIS. These results should show us if the proteins form a immobile fraction meaning that the AIS is a very static region, or if they are mobile which would mean that the AIS is actually dynamic. If the AIS is indeed more dynamic after development, based on its ability to expand and relocate, then it could possibly also form elsewhere, which would mean that a dendrite could replace an injured axon. Interestingly, upon axonal damage in spinal cord injury the AIS is disintegrated by a calcium influx which results in the activation of calcium dependent proteases that cleaves the proteins holding the AIS together, like AnkG (Schafer et al., 2009). This would result in a loss of the AIS and therefore also in a loss of axon function. Several papers show that axons are able to regenerate after injury (Fenrich and Rose, 2009; Hellal et al., 2011), even axon like formation from dendrites has been observed (Gomis-Ruth et al., 2008). However, new AIS formation at these sites has never been shown. Overcoming AIS degradation and inducing axon formation after spinal cord injury has many therapeutic potential for patients suffering with these injuries.

Future perspectives

First it is would be important to find the missing links in AIS initiation: is AnkG alone really sufficient for AIS formation and if not which other proteins are required. Furthermore, at which stage of neuron polarization does AIS formation exactly occur and which signalling pathways are involved in its induction. Hopefully, in the future we will have an idea of the exact sequence of events required for complete neuron polarization including axon and AIS formation. This does not only help us to better understand neuronal functioning and therefore memory, learning etc, but it could also help us in treating patients with neurological diseases like Alzheimer or spinal cord injuries. When more data will be reported during time on the AIS and its function the exact definition can be more easily discussed in the field.

Database entries

UniProt: http://www.uniprot.org/

ANK3 | βIV-spectrin | NFASC | NrCAM | CASPR2 | TAG-1 | ADAM22 | Nav1.2 | Nav1.6 | KCNQ2 | KCNQ3 | CK2 alpha, alpha', and beta | EB3 | EB1

References

Akhmanova, A., and Steinmetz, M.O. (2008). Tracking the ends: a dynamic protein network controls the fate of microtubule tips. Nat Rev Mol Cell Biol *9*, 309-322.

Akhmanova, A., and Steinmetz, M.O. (2010). Microtubule +TIPs at a glance. J Cell Sci *123*, 3415-3419.

Ango, F., di Cristo, G., Higashiyama, H., Bennett, V., Wu, P., and Huang, Z.J. (2004).

Ankyrin-based subcellular gradient of neurofascin, an immunoglobulin family protein, directs GABAergic innervation at purkinje axon initial segment. Cell *119*, 257-272.

Arimura, N., Menager, C., Fukata, Y., and Kaibuchi, K. (2004). Role of CRMP-2 in neuronal polarity. J Neurobiol *58*, 34-47.

Baas, P.W., Black, M.M., and Banker, G.A. (1989). Changes in microtubule polarity orientation during the development of hippocampal neurons in culture. J Cell Biol *109*, 3085-3094.

Baas, P.W., Deitch, J.S., Black, M.M., and Banker, G.A. (1988). Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and nonuniformity in the dendrite. Proc Natl Acad Sci U S A *85*, 8335-8339.

Barnes, A.P., Lilley, B.N., Pan, Y.A., Plummer, L.J., Powell, A.W., Raines, A.N., Sanes, J.R., and Polleux, F. (2007). LKB1 and SAD kinases define a pathway required for the polarization of cortical neurons. Cell *129*, 549-563.

Barnes, A.P., and Polleux, F. (2009). Establishment of axon-dendrite polarity in developing neurons. Annu Rev Neurosci *32*, 347-381.

Bean, B.P. (2007). The action potential in mammalian central neurons. Nat Rev Neurosci *8*, 451-465.

Bender, K.J., and Trussell, L.O. (2009). Axon initial segment Ca2+ channels influence action potential generation and timing. Neuron *61*, 259-271.

Bennett, V., and Lambert, S. (1999). Physiological roles of axonal ankyrins in survival of premyelinated axons and localization of voltage-gated sodium channels. J Neurocytol *28*, 303-318.

Berghs, S., Aggujaro, D., Dirkx, R., Jr., Maksimova, E., Stabach, P., Hermel, J.M., Zhang, J.P., Philbrick, W., Slepnev, V., Ort, T., *et al.* (2000). betaIV spectrin, a new spectrin localized at axon initial segments and nodes of ranvier in the central and peripheral nervous system. J Cell Biol *151*, 985-1002.

Boiko, T., Vakulenko, M., Ewers, H., Yap, C.C., Norden, C., and Winckler, B. (2007). Ankyrin-dependent and -independent mechanisms orchestrate axonal compartmentalization of L1 family members neurofascin and L1/neuron-glia cell adhesion molecule. J Neurosci *27*, 590-603.

Boiko, T., Van Wart, A., Caldwell, J.H., Levinson, S.R., Trimmer, J.S., and Matthews, G. (2003). Functional specialization of the axon initial segment by isoform-specific sodium channel targeting. J Neurosci *23*, 2306-2313.

Brachet, A., Leterrier, C., Irondelle, M., Fache, M.P., Racine, V., Sibarita, J.B., Choquet, D., and Dargent, B. (2010). Ankyrin G restricts ion channel diffusion at the axonal initial segment before the establishment of the diffusion barrier. J Cell Biol *191*, 383-395.

Bradke, F., and Dotti, C.G. (1999). The role of local actin instability in axon formation. Science *283*, 1931-1934.

Bradke, F., and Dotti, C.G. (2000). Differentiated neurons retain the capacity to generate axons from dendrites. Curr Biol *10*, 1467-1470.

Brechet, A., Fache, M.P., Brachet, A., Ferracci, G., Baude, A., Irondelle, M., Pereira, S., Leterrier, C., and Dargent, B. (2008). Protein kinase CK2 contributes to the organization of sodium channels in axonal membranes by regulating their interactions with ankyrin G. J Cell Biol *183*, 1101-1114.

Burack, M.A., Silverman, M.A., and Banker, G. (2000). The role of selective transport in neuronal protein sorting. Neuron *26*, 465-472.

Burton, P.R. (1988). Dendrites of mitral cell neurons contain microtubules of opposite polarity. Brain Res *473*, 107-115.

Clark, B.D., Goldberg, E.M., and Rudy, B. (2009). Electrogenic tuning of the axon initial segment. Neuroscientist *15*, 651-668.

Colbert, C.M., and Johnston, D. (1996). Axonal action-potential initiation and Na+ channel densities in the soma and axon initial segment of subicular pyramidal neurons. J Neurosci *16*, 6676-6686.

Conde, C., and Caceres, A. (2009). Microtubule assembly, organization and dynamics in axons and dendrites. Nat Rev Neurosci *10*, 319-332.

Custer, A.W., Kazarinova-Noyes, K., Sakurai, T., Xu, X., Simon, W., Grumet, M., and Shrager, P. (2003). The role of the ankyrin-binding protein NrCAM in node of Ranvier formation. J Neurosci *23*, 10032-10039.

Da Silva, J.S., Hasegawa, T., Miyagi, T., Dotti, C.G., and Abad-Rodriguez, J. (2005). Asymmetric membrane ganglioside sialidase activity specifies axonal fate. Nat Neurosci *8*, 606-615.

Da Silva, J.S., Medina, M., Zuliani, C., Di Nardo, A., Witke, W., and Dotti, C.G. (2003). RhoA/ROCK regulation of neuritogenesis via profilin IIa-mediated control of actin stability. J Cell Biol *162*, 1267-1279.

Davis, J.Q., and Bennett, V. (1993). Ankyrin-binding activity of nervous system cell adhesion molecules expressed in adult brain. J Cell Sci Suppl *17*, 109-117.

Davis, J.Q., and Bennett, V. (1994). Ankyrin binding activity shared by the neurofascin/L1/NrCAM family of nervous system cell adhesion molecules. J Biol Chem *269*, 27163-27166.

Davis, J.Q., Lambert, S., and Bennett, V. (1996). Molecular composition of the node of Ranvier: identification of ankyrin-binding cell adhesion molecules neurofascin (mucin+/third FNIII domain-) and NrCAM at nodal axon segments. J Cell Biol *135*, 1355-1367.

Davis, J.Q., McLaughlin, T., and Bennett, V. (1993). Ankyrin-binding proteins related to nervous system cell adhesion molecules: candidates to provide transmembrane and intercellular connections in adult brain. J Cell Biol *121*, 121-133.

de Anda, F.C., Meletis, K., Ge, X., Rei, D., and Tsai, L.H. (2010). Centrosome motility is essential for initial axon formation in the neocortex. J Neurosci *30*, 10391-10406.

Desai, A., and Mitchison, T.J. (1997). Microtubule polymerization dynamics. Annu Rev Cell Dev Biol *13*, 83-117.

Devaux, J.J., Kleopa, K.A., Cooper, E.C., and Scherer, S.S. (2004). KCNQ2 is a nodal K+ channel. J Neurosci *24*, 1236-1244.

Dotti, C.G., Sullivan, C.A., and Banker, G.A. (1988). The establishment of polarity by hippocampal neurons in culture. J Neurosci *8*, 1454-1468.

Duchemin, A.M., Ren, Q., Mo, L., Neff, N.H., and Hadjiconstantinou, M. (2002). GM1 ganglioside induces phosphorylation and activation of Trk and Erk in brain. J Neurochem *81*, 696-707.

Dzhashiashvili, Y., Zhang, Y., Galinska, J., Lam, I., Grumet, M., and Salzer, J.L. (2007). Nodes of Ranvier and axon initial segments are ankyrin G-dependent domains that assemble by distinct mechanisms. J Cell Biol *177*, 857-870.

Edwards, C., and Ottoson, D. (1958). The site of impulse initiation in a nerve cell of a crustacean stretch receptor. J Physiol *143*, 138-148.

Eshed, Y., Feinberg, K., Poliak, S., Sabanay, H., Sarig-Nadir, O., Spiegel, I., Bermingham, J.R., Jr., and Peles, E. (2005). Gliomedin mediates Schwann cell-axon interaction and the molecular assembly of the nodes of Ranvier. Neuron *47*, 215-229.

Fenrich, K.K., and Rose, P.K. (2009). Spinal interneuron axons spontaneously regenerate after spinal cord injury in the adult feline. J Neurosci *29*, 12145-12158.

Forscher, P., and Smith, S.J. (1988). Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. J Cell Biol *107*, 1505-1516.

Fukata, Y., Itoh, T.J., Kimura, T., Menager, C., Nishimura, T., Shiromizu, T., Watanabe, H., Inagaki, N., Iwamatsu, A., Hotani, H.*, et al.* (2002). CRMP-2 binds to tubulin heterodimers to promote microtubule assembly. Nat Cell Biol *4*, 583-591.

Fukushima, N., Furuta, D., Hidaka, Y., Moriyama, R., and Tsujiuchi, T. (2009). Post-translational modifications of tubulin in the nervous system. J Neurochem *109*, 683-693.

Futerman, A.H., Khanin, R., and Segel, L.A. (1993). Lipid diffusion in neurons. Nature *362*, 119.

Garrido, J.J., Giraud, P., Carlier, E., Fernandes, F., Moussif, A., Fache, M.P., Debanne, D., and Dargent, B. (2003). A targeting motif involved in sodium channel clustering at the axonal initial segment. Science *300*, 2091-2094.

Giuliodori, M.J., and Zuccolilli, G. (2004). Postsynaptic potential summation and action potential initiation: function following form. Adv Physiol Educ *28*, 79-80.

Gomis-Ruth, S., Wierenga, C.J., and Bradke, F. (2008). Plasticity of polarization: changing dendrites into axons in neurons integrated in neuronal circuits. Curr Biol *18*, 992-1000.

Govek, E.E., Newey, S.E., and Van Aelst, L. (2005). The role of the Rho GTPases in neuronal development. Genes Dev *19*, 1-49.

Grubb, M.S., and Burrone, J. (2010). Activity-dependent relocation of the axon initial segment fine-tunes neuronal excitability. Nature *465*, 1070-1074.

Grundemann, J., and Hausser, M. (2010). Neuroscience: A plastic axonal hotspot. Nature *465*, 1022-1023.

Hammond, J.W., Cai, D., and Verhey, K.J. (2008). Tubulin modifications and their cellular functions. Curr Opin Cell Biol *20*, 71-76.

Hammond, J.W., Huang, C.F., Kaech, S., Jacobson, C., Banker, G., and Verhey, K.J. (2010). Posttranslational modifications of tubulin and the polarized transport of kinesin-1 in neurons. Mol Biol Cell *21*, 572-583.

Harada, A., Oguchi, K., Okabe, S., Kuno, J., Terada, S., Ohshima, T., Sato-Yoshitake, R., Takei, Y., Noda, T., and Hirokawa, N. (1994). Altered microtubule organization in small-calibre axons of mice lacking tau protein. Nature *369*, 488-491.

Hedstrom, K.L., Ogawa, Y., and Rasband, M.N. (2008). AnkyrinG is required for maintenance of the axon initial segment and neuronal polarity. J Cell Biol *183*, 635-640.

Hedstrom, K.L., Xu, X., Ogawa, Y., Frischknecht, R., Seidenbecher, C.I., Shrager, P., and Rasband, M.N. (2007). Neurofascin assembles a specialized extracellular matrix at the axon initial segment. J Cell Biol *178*, 875-886.

Hellal, F., Hurtado, A., Ruschel, J., Flynn, K.C., Laskowski, C.J., Umlauf, M., Kapitein, L.C., Strikis, D., Lemmon, V., Bixby, J.*, et al.* (2011). Microtubule stabilization reduces scarring and causes axon regeneration after spinal cord injury. Science *331*, 928-931.

Heyden, A., Angenstein, F., Sallaz, M., Seidenbecher, C., and Montag, D. (2008). Abnormal axonal guidance and brain anatomy in mouse mutants for the cell recognition molecules close homolog of L1 and NgCAM-related cell adhesion molecule. Neuroscience *155*, 221-233.

Hill, A.S., Nishino, A., Nakajo, K., Zhang, G., Fineman, J.R., Selzer, M.E., Okamura, Y., and Cooper, E.C. (2008). Ion channel clustering at the axon initial segment and node of Ranvier evolved sequentially in early chordates. PLoS Genet *4*, e1000317.

Hu, W., Tian, C., Li, T., Yang, M., Hou, H., and Shu, Y. (2009). Distinct contributions of Na(v)1.6 and Na(v)1.2 in action potential initiation and backpropagation. Nat Neurosci *12*, 996-1002.

Ikegami, K., Heier, R.L., Taruishi, M., Takagi, H., Mukai, M., Shimma, S., Taira, S., Hatanaka, K., Morone, N., Yao, I.*, et al.* (2007). Loss of alpha-tubulin polyglutamylation in ROSA22 mice is associated with abnormal targeting of KIF1A and modulated synaptic function. Proc Natl Acad Sci U S A *104*, 3213-3218.

Ikegami, K., and Setou, M. (2010). Unique post-translational modifications in specialized microtubule architecture. Cell Struct Funct *35*, 15-22.

Inagaki, N., Chihara, K., Arimura, N., Menager, C., Kawano, Y., Matsuo, N., Nishimura, T., Amano, M., and Kaibuchi, K. (2001). CRMP-2 induces axons in cultured hippocampal neurons. Nat Neurosci *4*, 781-782.

Jenkins, S.M., and Bennett, V. (2001). Ankyrin-G coordinates assembly of the spectrin-based membrane skeleton, voltage-gated sodium channels, and L1 CAMs at Purkinje neuron initial segments. J Cell Biol *155*, 739-746.

Jiang, H., Guo, W., Liang, X., and Rao, Y. (2005). Both the establishment and the maintenance of neuronal polarity require active mechanisms: critical roles of GSK-3beta and its upstream regulators. Cell *120*, 123-135.

Joberty, G., Petersen, C., Gao, L., and Macara, I.G. (2000). The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. Nat Cell Biol *2*, 531-539.

Kapitein, L.C., and Hoogenraad, C.C. (2011). Which way to go? Cytoskeletal organization and polarized transport in neurons. Mol Cell Neurosci *46*, 9-20.

Kapitein, L.C., Schlager, M.A., Kuijpers, M., Wulf, P.S., van Spronsen, M., MacKintosh, F.C., and Hoogenraad, C.C. (2010). Mixed microtubules steer dynein-driven cargo transport into dendrites. Curr Biol *20*, 290-299.

Kempf, M., Clement, A., Faissner, A., Lee, G., and Brandt, R. (1996). Tau binds to the distal axon early in development of polarity in a microtubule- and microfilament-dependent manner. J Neurosci *16*, 5583-5592.

Kimura, T., Watanabe, H., Iwamatsu, A., and Kaibuchi, K. (2005). Tubulin and CRMP-2 complex is transported via Kinesin-1. J Neurochem *93*, 1371-1382.

Kobayashi, T., Storrie, B., Simons, K., and Dotti, C.G. (1992). A functional barrier to movement of lipids in polarized neurons. Nature *359*, 647-650.

Kole, M.H., Ilschner, S.U., Kampa, B.M., Williams, S.R., Ruben, P.C., and Stuart, G.J. (2008). Action potential generation requires a

high sodium channel density in the axon initial segment. Nat Neurosci *11*, 178-186.

Kole, M.H., Letzkus, J.J., and Stuart, G.J. (2007). Axon initial segment Kv1 channels control axonal action potential waveform and synaptic efficacy. Neuron *55*, 633-647.

Komada, M., and Soriano, P. (2002). [Beta]IV-spectrin regulates sodium channel clustering through ankyrin-G at axon initial segments and nodes of Ranvier. J Cell Biol *156*, 337-348.

Kordeli, E., Lambert, S., and Bennett, V. (1995). AnkyrinG. A new ankyrin gene with neural-specific isoforms localized at the axonal initial segment and node of Ranvier. J Biol Chem *270*, 2352-2359.

Kuba, H., Oichi, Y., and Ohmori, H. (2010). Presynaptic activity regulates Na(+) channel distribution at the axon initial segment. Nature *465*, 1075-1078.

Kuijpers, M., and Hoogenraad, C.C. (2011). Centrosomes, microtubules and neuronal development. Mol Cell Neurosci.

Lacas-Gervais, S., Guo, J., Strenzke, N., Scarfone, E., Kolpe, M., Jahkel, M., De Camilli, P., Moser, T., Rasband, M.N., and Solimena, M. (2004). BetaIVSigma1 spectrin stabilizes the nodes of Ranvier and axon initial segments. J Cell Biol *166*, 983-990.

Lemaillet, G., Walker, B., and Lambert, S. (2003). Identification of a conserved ankyrin-binding motif in the family of sodium channel alpha subunits. J Biol Chem *278*, 27333-27339.

Leterrier, C., Vacher, H., Fache, M.P., d'Ortoli, S.A., Castets, F., Autillo-Touati, A., and Dargent, B. (2011). End-binding proteins EB3 and EB1 link microtubules to ankyrin G in the axon initial segment. Proc Natl Acad Sci U S A *108*, 8826-8831.

Mainen, Z.F., Joerges, J., Huguenard, J.R., and Sejnowski, T.J. (1995). A model of spike initiation in neocortical pyramidal neurons. Neuron *15*, 1427-1439.

Mandelkow, E., and Mandelkow, E.M. (1995). Microtubules and microtubule-associated proteins. Curr Opin Cell Biol *7*, 72-81.

Nakada, C., Ritchie, K., Oba, Y., Nakamura, M., Hotta, Y., Iino, R., Kasai, R.S., Yamaguchi, K., Fujiwara, T., and Kusumi, A. (2003). Accumulation of anchored proteins forms membrane diffusion barriers during neuronal polarization. Nat Cell Biol *5*, 626-632.

Nakata, T., and Hirokawa, N. (2003). Microtubules provide directional cues for polarized axonal transport through interaction with kinesin motor head. J Cell Biol *162*, 1045-1055.

Nakata, T., Niwa, S., Okada, Y., Perez, F., and Hirokawa, N. (2011). Preferential binding of a kinesin-1 motor to GTP-tubulin-rich microtubules underlies polarized vesicle transport. J Cell Biol *194*, 245-255.

Nusser, N., Gosmanova, E., Zheng, Y., and Tigyi, G. (2002). Nerve growth factor signals through TrkA, phosphatidylinositol 3-kinase, and Rac1 to inactivate RhoA during the initiation of neuronal differentiation of PC12 cells. J Biol Chem *277*, 35840-35846.

Ogawa, Y., Horresh, I., Trimmer, J.S., Bredt, D.S., Peles, E., and Rasband, M.N. (2008). Postsynaptic density-93 clusters Kv1 channels at axon initial segments independently of Caspr2. J Neurosci *28*, 5731-5739.

Ogawa, Y., Oses-Prieto, J., Kim, M.Y., Horresh, I., Peles, E., Burlingame, A.L., Trimmer, J.S., Meijer, D., and Rasband, M.N. (2010). ADAM22, a Kv1 channel-interacting protein, recruits membrane-associated guanylate kinases to juxtaparanodes of myelinated axons. J Neurosci *30*, 1038-1048.

Ogawa, Y., and Rasband, M.N. (2008). The functional organization and assembly of the axon initial segment. Curr Opin Neurobiol *18*, 307-313.

Ozdamar, B., Bose, R., Barrios-Rodiles, M., Wang, H.R., Zhang, Y., and Wrana, J.L. (2005). Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. Science *307*, 1603-1609.

Pan, Z., Kao, T., Horvath, Z., Lemos, J., Sul, J.Y., Cranstoun, S.D., Bennett, V., Scherer, S.S., and Cooper, E.C. (2006). A common ankyrin-G-based mechanism retains KCNQ and NaV channels at electrically active domains of the axon. J Neurosci *26*, 2599-2613.

Poliak, S., Salomon, D., Elhanany, H., Sabanay, H., Kiernan, B., Pevny, L., Stewart, C.L., Xu, X., Chiu, S.Y., Shrager, P.*, et al.* (2003). Juxtaparanodal clustering of Shaker-like K+ channels in myelinated axons depends on Caspr2 and TAG-1. J Cell Biol *162*, 1149-1160.

Pollard, T.D., and Borisy, G.G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. Cell *112*, 453-465.

Pollard, T.D., and Cooper, J.A. (2009). Actin, a central player in cell shape and movement. Science *326*, 1208-1212.

Rasband, M.N. (2010). The axon initial segment and the maintenance of neuronal polarity. Nat Rev Neurosci *11*, 552-562.

Reed, N.A., Cai, D., Blasius, T.L., Jih, G.T., Meyhofer, E., Gaertig, J., and Verhey, K.J. (2006). Microtubule acetylation promotes kinesin-1 binding and transport. Curr Biol *16*, 2166-2172.

Ridley, A.J. (2011). Life at the leading edge. Cell *145*, 1012-1022.

Sanchez-Ponce, D., Munoz, A., and Garrido, J.J. (2011). Casein kinase 2 and microtubules control axon initial segment formation. Mol Cell Neurosci *46*, 222-234.

Sarner, S., Kozma, R., Ahmed, S., and Lim, L. (2000). Phosphatidylinositol 3-kinase, Cdc42, and Rac1 act downstream of Ras in integrin-dependent neurite outgrowth in N1E-115 neuroblastoma cells. Mol Cell Biol *20*, 158-172.

Schafer, D.P., Jha, S., Liu, F., Akella, T., McCullough, L.D., and Rasband, M.N. (2009). Disruption of the axon initial segment cytoskeleton is a new mechanism for neuronal injury. J Neurosci *29*, 13242-13254.

Serrano, L., Hernandez, M.A., Diaz-Nido, J., and Avila, J. (1989). Association of casein kinase II with microtubules. Exp Cell Res *181*, 263-272.

Shi, S.H., Jan, L.Y., and Jan, Y.N. (2003). Hippocampal neuronal polarity specified by spatially localized mPar3/mPar6 and PI 3-kinase activity. Cell *112*, 63-75.

Shu, Y., Yu, Y., Yang, J., and McCormick, D.A. (2007). Selective control of cortical axonal spikes by a slowly inactivating K+ current. Proc Natl Acad Sci U S A *104*, 11453-11458.

Sobotzik, J.M., Sie, J.M., Politi, C., Del Turco, D., Bennett, V., Deller, T., and Schultz, C. (2009). AnkyrinG is required to maintain axo-dendritic polarity in vivo. Proc Natl Acad Sci U S A *106*, 17564-17569.

Song, A.H., Wang, D., Chen, G., Li, Y., Luo, J., Duan, S., and Poo, M.M. (2009). A selective filter for cytoplasmic transport at the axon initial segment. Cell *136*, 1148-1160.

Stiess, M., Maghelli, N., Kapitein, L.C., Gomis-Ruth, S., Wilsch-Brauninger, M., Hoogenraad, C.C., Tolic-Norrelykke, I.M., and Bradke, F. (2010). Axon extension occurs independently of centrosomal microtubule nucleation. Science *327*, 704-707.

Stuart, G.J., and Sakmann, B. (1994). Active propagation of somatic action potentials into neocortical pyramidal cell dendrites. Nature *367*, 69-72.

Tahirovic, S., and Bradke, F. (2009). Neuronal polarity. Cold Spring Harb Perspect Biol *1*, a001644.

Teng, J., Takei, Y., Harada, A., Nakata, T., Chen, J., and Hirokawa, N. (2001). Synergistic effects of MAP2 and MAP1B knockout in neuronal migration, dendritic outgrowth, and microtubule organization. J Cell Biol *155*, 65-76.

Van Wart, A., Trimmer, J.S., and Matthews, G. (2007). Polarized distribution of ion channels within microdomains of the axon initial segment. J Comp Neurol *500*, 339-352.

Welch, M.D., and Mullins, R.D. (2002). Cellular control of actin nucleation. Annu Rev Cell Dev Biol *18*, 247-288.

Winckler, B., Forscher, P., and Mellman, I. (1999). A diffusion barrier maintains distribution of membrane proteins in polarized neurons. Nature *397*, 698-701.

Witte, H., Neukirchen, D., and Bradke, F. (2008). Microtubule stabilization specifies initial neuronal polarization. J Cell Biol *180*, 619-632.

Wloga, D., and Gaertig, J. (2010). Post-translational modifications of microtubules. J Cell Sci *123*, 3447-3455.

Wollner, D.A., and Catterall, W.A. (1986). Localization of sodium channels in axon hillocks and initial segments of retinal ganglion cells. Proc Natl Acad Sci U S A *83*, 8424-8428.

Yang, Y., Lacas-Gervais, S., Morest, D.K., Solimena, M., and Rasband, M.N. (2004). BetaIV spectrins are essential for membrane stability and the molecular organization of nodes of Ranvier. J Neurosci *24*, 7230-7240.

Yang, Y., Ogawa, Y., Hedstrom, K.L., and Rasband, M.N. (2007). betaIV spectrin is recruited to axon initial segments and nodes of Ranvier by ankyrinG. J Cell Biol *176*, 509-519.

Yi, J.J., Barnes, A.P., Hand, R., Polleux, F., and Ehlers, M.D. (2010). TGF-beta signaling specifies axons during brain development. Cell *142*, 144-157.

Yoshimura, T., Arimura, N., Kawano, Y., Kawabata, S., Wang, S., and Kaibuchi, K. (2006). Ras regulates neuronal polarity via the

PI3-kinase/Akt/GSK-3beta/CRMP-2 pathway. Biochem Biophys Res Commun *340*, 62-68.

Yoshimura, T., Kawano, Y., Arimura, N., Kawabata, S., Kikuchi, A., and Kaibuchi, K. (2005). GSK-3beta regulates phosphorylation of CRMP-2 and neuronal polarity. Cell *120*, 137-149.

Zhang, X., and Bennett, V. (1998). Restriction of 480/270-kD ankyrin G to axon proximal segments requires multiple ankyrin G-specific domains. J Cell Biol *142*, 1571-1581.

Zhou, D., Lambert, S., Malen, P.L., Carpenter, S., Boland, L.M., and Bennett, V. (1998). AnkyrinG is required for clustering of voltage-gated Na channels at axon initial segments and for normal action potential firing. J Cell Biol *143*, 1295-1304.

Zonta, B., Desmazieres, A., Rinaldi, A., Tait, S., Sherman, D.L., Nolan, M.F., and Brophy, P.J. (2011). A critical role for Neurofascin in regulating action potential initiation through maintenance of the axon initial segment. Neuron *69*, 945-956.

Supplemental table: AIS proteins

General AIS region			
Protein	**Description**	**Function in the AIS**	**Key references**
AnkG	Cytoskeletal adaptor (Ankyrin G)	Linking membrane proteins of the AIS to the cytoskeleton, preventing diffusion.	(Brachet et al., 2010; Hedstrom et al., 2008; Kordeli et al., 1995; Zhou et al., 1998)
βIV-Spectrin	Spectrin beta chain, brain 3 of the spectrin family.	Essential for the stability of the AIS, possibly by linking important components to the actin cytoskeleton.	(Berghs et al., 2000; Yang et al., 2007)
Nfasc186 (NF-186)	Cell adhesion molecule (Neurofascin-186)	Anchoring key elements of the AIS (like AnkG) to the membrane.	(Davis et al., 1996; Hedstrom et al., 2007)
NrCam	Neuronal cell adhesion molecule	Exact function of NrCam at the AIS remains unknown.	(Davis and Bennett, 1994; Davis et al., 1993)
Nav1	Sodium (Na^+) channel	Axonal action potential (AP) initiation.	(Boiko et al., 2003; Kole et al., 2008)
Kv7 (KCNQ2/3)	Potassium (K^+) channel	Controlling the axonal AP threshold.	(Devaux et al., 2004; Pan et al., 2006)
CK2	Protein kinase (Casein kinase 2)	Phosphorylates ankyrin-binding motif of Nav1, regulating the Nav1 – AnkG interaction.	(Brechet et al., 2008)
EB3 and EB1	Microtubule plus-end binding proteins	Links microtubules to AnkG.	(Leterrier et al., 2011; Nakata and Hirokawa, 2003)
Juxtaparanode region			
Protein	**Description**	**Function in the AIS**	**Key references**
CASPR, TAG-1, and ADAM22	Neuronal cell adhesion molecules	Associated with potassium channels, and believed to be important for the maintenance of these channels at the juxtaparanode.	(Ogawa et al., 2010; Poliak et al., 2003)
Kv1	Potassium (K^+) channel	Enhancing axonal AP fidelity.	(Kole et al., 2007; Van Wart et al., 2007)