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The Structure and Dynamics of the Actin Cytoskeleton in the Axon Initial Segment

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Doctoral Dissertation

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Cover image: Egyptian hieroglyphic for ‘brain’, from the Edwin Smith Papyrus, originally 3000-2500 BCE

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Abstract

The axon initial segment (AIS) is the site of action potential initiation and plays an important role in maintaining neuronal polarity. Recent advances in super-resolution microscopy revealed the presence of an intricate membrane-associated periodic lattice in the AIS that contains sub-membranous actin rings periodically spaced ~190 nm and connected to a spectrin-ankyrin lattice. The precise function of these actin rings in unclear, as well as details of their structure and dynamics. The insensitivity of the AIS to actin-disrupting drugs led to the long-held view that actin is not a critical component of AIS structure. Here I show that the AIS contains a population of relatively stable, latrunculin-resistant actin filaments that are decorated by the tropomyosin isoform Tpm3.1. Disrupting these filaments through the perturbation of Tpm3.1 function led to the loss of accumulation of ankyrin G and other AIS markers, disruption of neuronal polarity, the loss of the clustering of voltage-gated sodium channels, and a rapid reduction in firing frequency. The findings I present in this thesis suggest that actin plays an important role in maintaining AIS structure and function, more important than previously appreciated.
Abbreviations

+TIP: Plus-end Tracking Protein
AIS: Axon Initial Segment
PAGFP: Photoactivatable Green Fluorescent Protein
SIM: Structured Illumination Microscopy
STORM: Stochastic Optical Reconstruction Microscopy
Tpm: Tropomyosin


Roman numerals are used in the text to refer to these publications.
1. Introduction

The ability of a neuron to process, transmit, and store information is the foundation of the nervous system. An intricate and brilliantly elaborate structure underlies and facilitates these functions in a neuron. As early as in the 19th century, neurons were described to possess a polar, compartmentalised structure (Deiters & Guillery, 2013). A typical neuron is divided into two domains; the somatodendritic domain and the axon. Shortly before the end of the 19th century, Ramon y Cajal postulated the law of ‘dynamic polarisation’, proposing the unidirectional flow of information where a neuron receives information at the somatodendritic end and sends its output through the axon.

The ‘initial segment of the axon’ was described in the mid-twentieth century as a specialised region at the proximal end of the axon where action potentials initiate (Eccles, 1964). The axon initial segment (AIS) is the stretch of the axon situated between the axon hillock and the myelin sheath, ranging between 20-60 μm in length. Concurrently, the development of electron microscopy allowed us to see the structure of this region in unprecedented detail, and identified the presence of specialised structural features—revealed hitherto for the first time: (i) a dense granular undercoating of the plasma membrane, (ii) scattered ribosome clusters, and (iii) microtubules bundled into one or more fascicles (Conradi, 1966; Palay, Sotelo, Peters, & Orkand, 1968). These unique features of the AIS earned it a name of its own, and sparked the interest of many for decades to come.

Today, we know that the AIS is implicated in numerous neurological and mental disorders (Hsu, Nilsson, & Laezza, 2014; Huang & Rasband, 2018). We know that the AIS plays a vital role in maintaining neuronal polarity (Leterrier, 2016). We also know that the initiation of action potentials is facilitated and modulated by ion channels in the AIS (Kole & Stuart, 2012). However, there is a lot we do not know. A substantial body of work carried out during the last five decades has revealed that the AIS contains a distinctive structural
complex that underlies its unique functions (Leterrier, 2018). In this current decade, the most remarkable addition to the list of things we know was perhaps the discovery of the unique structure of the actin cytoskeleton in the AIS, which organises into elegant, evenly-spaced, sub-membranous rings (Papandreou & Leterrier, 2018). Just as electron microscopy brought us face-to-face with the AIS for the first time, the development of super-resolution microscopy is allowing us to explore the structure of the AIS and its organisation in unprecedented detail. As a result, we now know that the actin cytoskeleton is an integral component of the AIS structural complex. However, we do not know the precise role actin plays in this structural complex. We do not know much about the properties of actin in the AIS. And we do not know how actin in the AIS is regulated. In my thesis work I examined the dynamic properties of the actin cytoskeleton in the AIS, and the role tropomyosin plays in its regulation.
2. Review of the Literature

In vertebrate neurons, the axon initial segment (AIS) is the stretch of axon lying proximal to the soma, between the axon hillock and the start of the myelin sheath. The AIS, therefore, marks the boundary between the axon and the somatodendritic domain. In fact, the AIS plays an important role in maintaining these two domains as two biochemically and functionally distinct entities (Brachet et al., 2010; Nakada et al., 2003; Song et al., 2009; Sun et al., 2014; Winckler, Forscher, & Mellman, 1999). In addition, the AIS is the site at which action potentials initiate, a process facilitated by the clustering of ion channels (Catterall, 1981; Eccles, 1964; Kole et al., 2008; Kole & Stuart, 2012; Wollner & Catterall, 1986). Unsurprisingly, defects in AIS structure or function are implicated in numerous neurological and mental disorders (Buffington & Rasband, 2011; Hsu et al., 2014; Huang & Rasband, 2018; Leterrier, 2016). In this chapter, I will briefly summarise the current literature on AIS function and structure, with emphasis on recent work on the AIS cytoskeleton.

2.1 AIS Function

The AIS is vital for proper neuronal function; the AIS serves as the site of action potential initiation and plays an important role in maintaining neuronal polarity.

2.1.1 Initiation of action potentials. The locus of origin of action potentials was hypothesised to be at the initial segment of the axon as early as in 1940 (Gesell, 1940), while an opposing theory attributed this job to the cell body (Fatt, 1957), sparking a lively debate. Araki and Otani (1955) observed that a spike was generated in the proximal non-myelinated segment of the axon before the soma upon both ortho- and antidromic stimulation, leading them to conclude that the threshold for firing was lower in that region. Similar observations and conclusions soon followed (Coombs, Curtis, & Eccles, 1957; Fuortes, Frank, & Becker, 1957), but the first direct evidence for the AIS being the site of action potential initiation came from recordings of the neuron of a crustacean stretch receptor (Edwards & Ottoson,
1958). By directly recording at several sites in the cell body and along the axon, the authors observed that the “impulse appears earliest in the initial part of the fibre” and concluded that “the impulses in the sensory neurone […] are initiated in the axon near to the cell body” (Edwards & Ottoson, 1958). Subsequent studies confirmed that the AIS is the site of action potential initiation in mammalian neurons as well (Meeks & Mennerick, 2007; Palmer & Stuart, 2006; Shu, Duque, Yu, Haider, & McCormick, 2007; Stuart & Hausser, 1994; Stuart, Schiller, & Sakmann, 1997). These findings raised an obvious and simple question: what makes the AIS the site of action of potential initiation? The answer, however, proved elusive.

One possible explanation for the lower threshold of firing at the AIS is an abundance of ion channels, particularly voltage-gated sodium ion channels (Dodge & Cooley, 1973; Moore, Stockbridge, & Westerfield, 1983). Modelling studies concurred (Mainen, Joerges, Huguenard, & Sejnowski, 1995; Rapp, Yarom, & Segev, 1996), and antibody staining did indeed show that the AIS contained a high density of voltage-gated sodium channels (Boiko et al., 2003; Inda, DeFelipe, & Munoz, 2006; Komada & Soriano, 2002; Kordeli, Lambert, & Bennett, 1995; Meeks & Mennerick, 2007; Van Wart, Trimmer, & Matthews, 2007; Wollner & Catterall, 1986; Zhou et al., 1998). However, patch-clamp recordings—where the electrical activity across a small area of membrane is recorded, revealing the properties of the ion channels present—from the AIS failed to show any difference in the density of sodium channels between the AIS membrane and that of the soma (Colbert & Johnston, 1996; Colbert & Pan, 2002). The controversy was eventually put to rest when Kole et al. (2008) discovered that the patch-clamp experiments failed because the membrane patches lost their sodium channels during the procedure, as the channels are too strongly attached to the actin cytoskeleton. Pharmacologically disrupting the actin cytoskeleton led to a 30-45-fold increase in the density of sodium channels detected (Kole et al., 2008). Interestingly, we know of no direct link between sodium channels and the actin cytoskeleton, although disrupting the actin
cytoskeleton leads to an increased mobility of ion channels in the AIS (Nakada et al., 2003). In fact, the clustering of sodium channels at the AIS is the result of binding to the AIS-specific adaptor protein ankyrin G through a motif in the II-III intracellular loop (Garrido et al., 2003; Gasser et al., 2012; Lemaillet, Walker, & Lambert, 2003; Srinivasan, Elmer, Davis, Bennett, & Angelides, 1988), and the accumulation of ankyrin G at the AIS is insensitive to actin-disrupting drugs (P. M. Jenkins, He, & Bennett, 2015).

The initiation of action potentials at the AIS is facilitated by the clustering of voltage-gated sodium channels, which results in a low threshold for firing. However, the morphology and molecular composition of the AIS also contribute to its role as the site of action potential initiation. The AIS constitutes a small electrical compartment, with a relatively smaller membrane capacitance requiring to be charged (B. D. Clark, Goldberg, & Rudy, 2009). The action potential current threshold is significantly lower at the AIS relative to the soma (Kole & Stuart, 2008), and the current generated is strong enough to depolarise the soma and induce back-propagation of action potentials, which is important for learning and other higher computational processes (Stuart, Spruston, Sakmann, & Hauser, 1997). Along with voltage-gated sodium channels—of which there are several isoforms—, the AIS also contains several types of calcium and potassium channels that may modulate action potentials and allow an extra layer of signal processing (Kole & Stuart, 2012). In addition, the length and position of the AIS affect neuronal excitability and change in response to different stimuli, providing an important mechanism of homeostasis and plasticity (Grubb et al., 2011; Yamada & Kuba, 2016). Together, these features facilitate—and regulate—the initiation and modulation of action potentials at the AIS, effectively making it the ‘output centre’ of the neuron.

2.1.2 Maintaining neuronal polarity. Axons differ from dendrites in their morphology: axons generally maintain a constant diameter while dendrites taper; axons are longer than dendrites, about a meter in length in some neurons; axons form branches at right
angles, dendrites generally form Y-branches; and axons lack dendritic spines, the bulbous protrusions on dendrites that house synapses (Glickstein, 2017). These distinct morphological features give a neuron its polar appearance, but the two neuronal compartments (the somatodendritic domain and the axon) are also functionally and biochemically distinct (Barnes & Polleux, 2009; Rasband, 2010; Takano, Xu, Funahashi, Namba, & Kaibuchi, 2015). Situated between these two compartments, the AIS has its own distinguishing features. The AIS acts as the border between the somatodendritic domain and the axon and presents a barrier that separates these two domains (Figure 1). In fact, the axons of neurons lacking the AIS progressively lose their axonal identity and acquire characteristics of dendrites (Hedstrom, Ogawa, & Rasband, 2008; Sobotzik et al., 2009). Dotti and Simons (1990) proposed that axonal and somatodendritic cargo are sorted at the Golgi apparatus and delivered to the axon or somatodendritic domain via microtubules, and that a diffusion barrier at the axon hillock hinders diffusion between the two compartments. We now know that the AIS—not the axon hillock—contributes to the maintenance of neuronal polarity by virtue of a membrane and cytoplasmic diffusion barrier, and an intracellular filter of vesicular cargo. The details of the mechanisms through which these processes operate, however, are not clearly understood.

Dotti and Simons (1990) suggested that there could be a ‘fence blocking lateral diffusion in the plasma membrane’ at the axon hillock, in their efforts to explain the polarised distribution of viral glycoproteins in cultured in hippocampal neurons. The authors then experimentally tested their hypothesis by observing the distribution of the membrane-anchored protein Thy-1 and fluorescent phospholipids and concluded that ‘there is a diffusion barrier at the axonal hillock/initial segment…’ (Dotti, Parton, & Simons, 1991; Kobayashi, Storrie, Simons, & Dotti, 1992). It was already known at the time that voltage-gated sodium channels have a reduced lateral mobility at the axon hillock (Angelides, Elmer, Loftus, &
Elson, 1988), and later studies demonstrated that the reduced lateral mobility at the AIS is dependent on an intact AIS (Nakada et al., 2003; Song et al., 2009), and an intact actin cytoskeleton (Nakada et al., 2003; Winckler et al., 1999). More recently, Albrecht et al. (2016) demonstrated that the diffusion of membrane-anchored GFP molecules was restricted between sub-membranous actin rings. Thus, sub-membranous actin in the rings in the AIS create an ‘obstacle course’ for membrane proteins and—probably—lipids. It is perhaps important to note that, while ion channels are immobilised at the AIS through directly binding to the AIS structural complex (as is the case for sodium channels, for example (Brachet et al., 2010)), the reduced lateral mobility of lipids and unbound proteins is generally non-selective. According to the model proposed by Kusumi et al. (2005), the cytoskeleton acts as a ‘fence’ and the abundant transmembrane proteins serve as ‘pickets’ that together hinder the diffusion of macromolecules in the membrane.

In addition to the membrane diffusion barrier, the AIS also restricts cytoplasmic diffusion in a seemingly non-selective but size-dependent manner (Song et al., 2009; Sun et al., 2014). The mechanism for this process, however, is not clear; it appears that the AIS acts as a sieve that is capable of blocking—or at least slowing down—large macromolecules (>70 kDa) (Leterrier & Dargent, 2014; Song et al., 2009). How large organelles overcome this intracellular filter is also unclear. However, one recent study suggests that the axonal cytoskeleton is an actomyosin network that can transiently stretch to accommodate organelle transport (Wang et al., 2018).

Finally, in addition to the membrane and the intracellular diffusion barriers, the AIS acts as a vesicle filter that can block the entry of vesicles carrying somatodendritic proteins into the axon (Al-Bassam, Xu, Wandless, & Arnold, 2012; Burack, Silverman, & Banker, 2000; Petersen, Kaech, & Banker, 2014; Song et al., 2009; Watanabe et al., 2012). The exact mechanism for this is debatable, but several studies showed that it is dependent on myosin
motors and an intact actin cytoskeleton (Balasanyan et al., 2017; Janssen et al., 2017; Lewis, Mao, & Arnold, 2011; Lewis, Mao, Svoboda, & Arnold, 2009; Watanabe et al., 2012). Upon entry into the AIS, vesicles carrying somatodendritic cargo halt at locations that contain a relatively higher concentration of actin filaments, and can sometimes reverse their direction (Balasanyan et al., 2017; Janssen et al., 2017; Watanabe et al., 2012). This inspired a model where kinesins carry vesicles containing somatodendritic cargo along microtubules into the AIS, only to be tethered and immobilised by the interaction of actin patches with myosin motors on these vesicles. Following this immobilisation, dynein motors may carry these vesicles along microtubules in the opposite direction (Leterrier, 2018; Leterrier & Dargent, 2014). However, Petersen et al. (2014) reported that the restriction of the entry of vesicles into the axon appears before the proper formation of an AIS, suggesting that the sorting mechanism lies upstream of the AIS. Furthermore, Farias et al. (2012) found that the polarised distribution of a number of transmembrane receptors in hippocampal neurons was dependent on the presence of a signal in their cytoplasmic domain that is recognised by the adaptor protein-1 complex. Therefore, it is probable that several mechanisms contribute to the regulation of the polarised distribution of vesicular cargo in neurons, and that these mechanisms may overlap.

2.2 AIS Structure

The AIS comprises a unique and highly stable structural complex that underlies its unique functions. Work over the span of several decades has gained us insight into the details of this structure, and technological advances continue to reveal even more. Interestingly, recent studies utilising super-resolution microscopy techniques speak of a significant resemblance between the ultra-structure of the cytoskeleton of the AIS and that of human red blood cells, in what constitutes a curious case of blatant plagiarism in evolution. In this
section I will briefly summarise the current literature regarding the different components that make up the core structure of the AIS and their place in the AIS structural complex.

2.2.1 Ankyrin G. In 1979, Vann Bennett and Peter J. Stenbuck isolated a protein from the membrane attachment site of human erythrocyte spectrin that they dubbed “ankyrin”, from the Greek word for anchor, ankyra (Bennett & Stenbuck, 1979). A few years later, Davis and Bennett (1984) reported the presence of what they described as a ‘closely related protein’ to the erythrocyte ankyrin in pig brain. This would come to be known as ankyrin B (B for broadly expressed), and erythrocyte ankyrin went by ankyrin R (R for restricted) (Bennett & Baines, 2001). Not long after discovering ankyrin B, while studying ankyrin in brains from ankyrin R knockout mice, the Bennett lab found a new ankyrin that was immunologically related to—but distinct from—ankyrin R (Kordeli & Bennett, 1991; Kordeli, Davis, Trapp, & Bennett, 1990). They then characterised the gene for this ankyrin and called it ankyrin G, owing to its ‘giant’ size; the full-length polypeptide has a mass of 480 kDa (Kordeli et al., 1995).

Davis and Bennett (1984) noted how “[B]rain ankyrin […] has the potential to interconnect microtubules and spectrin-associated actin filaments”. This is indeed one of the main functions of ankyrins in the axon, as we recently—nearly 30 years later—learnt (Leterrier, 2018). More specifically, ankyrin G has a central role in the AIS structural complex, where it acts as a hub linking structural components together and recruiting ion channels and adhesion molecules. In fact, the loss of ankyrin G leads to the loss of all AIS components (Hedstrom et al., 2008; S. M. Jenkins & Bennett, 2001; Zhou et al., 1998).

Ankyrins are modular proteins consisting of three conserved domains that are present in all family members and specialised domains that are specific to individual splice variants; an amino-terminal membrane-binding domain, a 62-kDa spectrin-binding domain, and a 12-kDa death domain are conserved in all ankyrins (Bennett & Baines, 2001). In the AIS, the
membrane-binding domain of ankyrin G is responsible for binding sodium (Garrido et al., 2003; Lemailliet al., 2003; Zhou et al., 1998) and potassium channels (Leterrier, 2018; Pan et al., 2006), as well as the L1 family adhesion molecules neurofascin-186 and NrCAM (Davis, Lambert, & Bennett, 1996). While the aptly named spectrin-binding domain of ankyrin G is responsible for binding βIV-spectrin (Leterrier, 2018), the C-terminal death domain has no known function in ankyrin G (Bennett & Baines, 2001). In addition to these conserved domains, ankyrin G contains a stretch of amino acids between the spectrin-binding and the death domains; both the 270 and the 480 kDa isoforms contain a stretch ~400-residues long that is rich in serine/threonine followed by a tail domain that is shorter in the 270 kDa isoform. Both isoforms also contain a tail region that anchors the dynein activator NdelI (Kuijpers et al., 2016). The full-length 480-kDa isoform, however, has a longer tail that contains several SxIP motifs, enabling it to bind microtubules through interactions with EB1 and EB3 proteins (Freal et al., 2016).

In the AIS, ankyrin G is arranged periodically at ~190 nm in the sub-membranous scaffold connecting spectrin tetramers (Figure 1). The membrane binding domain anchors various ion channels and cell adhesion molecules, while the tail domain (of the 480 kDa isoform) extends ~35 nm into the cytoplasm and binds microtubules, connecting them to the sub-membranous scaffold (Leterrier et al., 2015). Thus, ankyrin G serves as a hub connecting structural AIS components.

2.2.2 βIV-spectrin. “We suggest that this protein be called Spectrin since it is obtained from membrane ghosts [derived from “spectre”, meaning a visible disembodied spirit, a ghost (Merriam-Webster, 2019)].” These were the words used by Marchesi and Steers (1968), who were the first to isolate spectrin from erythrocytes about 50 years ago. Today we know that all metazoan cells express spectrin, where it may have several functions (Liu & Rasband, 2019). When Levine and Willard (1981) identified a similar protein in
neurons they named it ‘fodrin’ (from Greek fodros, lining). However, closer examination revealed that fodrin was merely the form of spectrin found in most cells, whereas erythrocytes and skeletal muscle cells contained unique variants (Glenney & Glenney, 1983). In the AIS, hetero-tetramers of αII- and βIV-spectrin are a component of the membrane-associated periodic scaffold (Leterrier et al., 2015; K. Xu, Zhong, & Zhuang, 2013; Zhong et al., 2014).

Spectrins are flexible molecules ~60-200 nm comprising two α/β antiparallel heterodimers assembled head to head to form a spectrin hetero-tetramer (Bennett & Baines, 2001; Brown et al., 2015). In the AIS, αII-spectrin comprises an N-terminal tetramerization motif, 21 spectrin repeats, an SH3 domain, a calmodulin-binding domain, and a calcium-binding EF domain, while βIV-spectrin contains two N-terminal actin-binding domains, 17 spectrin repeats, and a pleckstrin homology domain (Liu & Rasband, 2019). The so-called ‘specific domain’ of the tetramer binds ankyrin G through its ZU5 motif, as well as through a non-canonical phosphorylation-dependent site (P. M. Jenkins, Kim, et al., 2015; Kennedy, Warren, Forget, & Morrow, 1991). Super-resolution microscopy revealed that βIV-spectrin tetramers in the AIS bind ankyrin G and sub-membranous actin rings in the membrane-associated periodic scaffold, placing them ~190 nm apart (the length of the stretched βIV-spectrin tetramer) (Leterrier et al., 2015; K. Xu et al., 2013).

By binding the plasma membrane, the actin cytoskeleton, and ankyrin G, βIV-spectrin plays an important structural role in the membrane-associated periodic scaffold in the AIS. Interestingly, mice lacking or expressing a mutant form of βIV-spectrin do not altogether lack an AIS, but the accumulation of ankyrin G and sodium channels there is notably diminished (Komada & Soriano, 2002; Lacas-Gervais et al., 2004; Uemoto et al., 2007; Yang, Ogawa, Hedstrom, & Rasband, 2007). This suggests that βIV-spectrin is dispensable for AIS formation but is required for the maintenance of AIS structure. Perhaps unsurprisingly, mice
lacking βIV-spectrin or expressing mutant βIV-spectrin show ataxia, tremors, and auditory defects (Liu & Rasband, 2019). Recently, a study by Lorenzo et al. (2019) suggests that βII-spectrin (the prevalent spectrin in axons distal to the AIS) plays a role in axonal trafficking and organelle transport, facilitated by the association with several kinesins and dynactin. This novel function of βII-spectrin is in addition to its structural function in the membrane-associated periodic scaffold in the axon. Whether or not βIV-spectrin has a similar dual role, future studies will hopefully tell.

2.2.3 The cytoskeleton. The properties of the cytoskeleton in the AIS underlie and facilitate its specific functions. In this section I will summarise what we know about the structure of the cytoskeleton in the AIS and its regulation.

2.2.3.1 Microtubules. Microtubules form the backbone of the axon—including the AIS, as well as provide tracks for axonal transport (Conde & Caceres, 2009; Maday, Twelvetrees, Moughamian, & Holzbaur, 2014). Microtubules in the axon form tight bundles with uniform polarity, with their plus ends pointed distally (Baas & Lin, 2011). In the AIS, microtubules form fascicles—tightly-packed bundles of microtubules linked by thin bridges—that constitute a unique morphological feature used to identify the AIS in electron micrographs (Leterrier & Dargent, 2014; Palay et al., 1968). These unique features are partly the result of the action of several microtubule-associated proteins present in the AIS (Leterrier, 2018). Microtubules in the AIS are linked to the AIS structural complex through the interaction of ankyrin G with end-binding proteins EB1 and EB3 (Freal et al., 2016; Leterrier et al., 2011). EB proteins are microtubule plus-end tracking proteins (+TIPs) that typically accumulate at the ends of growing microtubules, regulating their dynamics and interactions (Akhmanova & Steinmetz, 2008). In the AIS, EB proteins are concentrated along the length of microtubules in the AIS (Nakata & Hirokawa, 2003), providing binding sites for ankyrin G tails that extend into the cytoplasm (Leterrier et al., 2015). Interference with this
interaction does not affect the formation of the AIS but causes a reduction in the concentration of other AIS components (Leterrier et al., 2011).

2.2.3.2 Actin. The role actin plays in axonal growth cones and branching points remained the focus of study of axonal actin for a long time (Papandreou & Leterrier, 2018), agreeing with the classical view of the role of actin in morphogenesis through the formation of highly dynamic structures that can exert or resist mechanical forces (Blanchoin, Boujemaa-Paterski, Sykes, & Plastino, 2014). In 1999, Winckler et al. (1999) reported that the molecular diffusion barrier at the AIS is dependent on an intact actin cytoskeleton. The mechanism for this process was not clear, and the discovery rekindled an interest in understanding the structure of the actin cytoskeleton in the AIS and its function. Nakada et al. (2003) proposed the ‘pickets and fences’ model, where actin filaments are part of a dense membrane mesh that also includes other proteins that together restrict diffusion in the AIS through steric hindrance. The actin cytoskeleton also proved to play a role in a cytoplasmic size-based diffusion barrier (Song et al., 2009). Furthermore, several studies implicated actin-based myosin motors in the selective filtering of cargo and diffusion barrier at the AIS (Al-Bassam et al., 2012; Lewis et al., 2011; Lewis et al., 2009), and described the role of patches of actin in the AIS in this process (Balasanyan et al., 2017; Janssen et al., 2017; Watanabe et al., 2012). Together, a strong case emerged for a role of actin in maintaining neuronal polarity by forming a diffusion barrier at the AIS (Leterrier & Dargent, 2014). Along these lines—until recently—the consensus viewed actin as important in maintaining neuronal polarity but plays no part in the core AIS structure. This view was supported by the fact that the mature AIS is insensitive to actin-disrupting drugs (Jones, Korobova, & Svitkina, 2014; Leterrier et al., 2015; Li et al., 2011; Qu, Hahn, Webb, Pearce, & Prokop, 2017; Sanchez-Ponce, DeFelipe, Garrido, & Munoz, 2011). However, proper AIS development requires an
intact actin cytoskeleton (X. Xu & Shrager, 2005), indicating that this consensus did not have the whole picture.

Indeed, the advent of super-resolution microscopy revealed that the actin cytoskeleton in the AIS is highly organised. The discovery of periodic rings of actin under the membrane in the axon (K. Xu et al., 2013; Zhong et al., 2014) urged a revisit of our view of the role of actin in the AIS; adducin-capped actin filaments form rings in the AIS that are oriented perpendicular to the direction of neurite propagation and connected via spectrin tetramers, which places them ~190 nm apart (Figure 1) (Barabas et al., 2017; D'Este, Kamin, Gottfert, El-Hady, & Hell, 2015; He et al., 2016; Leite et al., 2016; Leterrier et al., 2015; Qu et al., 2017; K. Xu et al., 2013; Zhong et al., 2014). This periodic structure of actin filaments in a spectrin-ankyrin lattice bears a striking resemblance to the membrane skeleton of red blood cells (Bennett & Baines, 2001; Leite et al., 2016; Papandreou & Leterrier, 2018). The precise function of actin rings in the AIS, and the regulation and dynamics of the actin filaments that make up these rings, are not clear. The capping of these filaments by adducin suggests that these filaments may be stable. However, actin rings persist in adducin knock-out mice (Leite et al., 2016). In addition, conflicting reports about the effect of actin-disrupting drugs on actin rings convolute our understanding of the dynamics of these filaments, and their importance for the membrane-associated periodic scaffold (Han, Zhou, Xia, & Zhuang, 2017; Leterrier et al., 2015; Qu et al., 2017; K. Xu et al., 2013; Zhong et al., 2014). Albrecht et al. (2016) reported that the sub-membranous actin rings constitute an obstacle that restricts lateral diffusion of membrane-bound GFP (GPI-GFP). This represented the first evidence for a role for actin rings in the membrane diffusion barrier in the AIS. It is probable that actin rings play a supportive structural role in the axon, resembling the hose of a vacuum cleaner (Papandreou & Leterrier, 2018). Moreover, defects in actin rings affect microtubule organisation in the AIS (Qu et al., 2017). Another recent report demonstrated that
Figure 1. The membrane-associated periodic scaffold of the axon initial segment. Top:
Schematic drawing of a neuron showing the typical features of the somatodendritic domain, the AIS, and the axon. Bottom: Schematic diagram showing details of the area within the dashed box. Actin filaments in the AIS form periodic sub-membranous rings connected by αII/βIV-spectrin tetramers and spaced 190 nm apart. Spectrin tetramers also bind ankyrin G which may be bound to microtubules through its tail domain. Actin filaments also form intracellular patches in the AIS.
phosphorylated myosin II light chains associate with actin rings in the AIS (Berger et al., 2018). Similarly, disruption of actin filaments, myosin II, or both leads to an increase in axonal diameter (Fan, Tofangchi, Kandel, Popescu, & Saif, 2017). An increase in axonal diameter was also reported in adducin knock-outs (Leite et al., 2016), suggesting that regulating axonal diameter is one of the functions of actin rings in the AIS. Future studies will undoubtedly reveal more about the precise function of actin rings in the AIS and their importance for the membrane-associated periodic skeleton.

2.3 The erythrocyte membrane skeleton

As already mentioned, the composition of the membrane-associated periodic skeleton—or what we so far know about it, anyway—is vividly reminiscent of that of the membrane skeleton of mammalian erythrocytes; periodically arranged actin filaments in a spectrin-ankyrin lattice (Bennett & Baines, 2001; Leite et al., 2016; Papandreou & Leterrier, 2018). In fact, the mammalian erythrocyte membrane contributed significantly to our understanding of the role of actin in membrane skeletons, earning it the title of the “Rosetta Stone for […] membrane-cytoskeleton structure” (Fowler, 2013). Nonetheless, there are important differences between these two structures that are not only related to their functions or their respective contexts, but also in their composition and arrangement. For one, the main components of the spectrin-ankyrin lattice—namely, spectrin and ankyrin—exist as different isoforms in the AIS and erythrocytes (Bennett & Baines, 2001; Liu & Rasband, 2019). The 2D quasi-hexagonal geometry of the erythrocyte membrane skeleton (Fowler, 2013) also contrasts with the current model of the architecture of the AIS membrane-associated periodic skeleton (Papandreou & Leterrier, 2018). In addition, several proteins that play a role in the structure of the erythrocyte membrane skeleton have not been detected in the AIS, at least not yet: dematin contributes to the association of actin filaments with the membrane and promotes spectrin binding (Koshino, Mohandas, & Takakuwa, 2012; Takakuwa, 2000);
tropomodulin-1 caps the pointed ends of actin filaments in the erythrocyte membrane skeleton and regulates the length of the filament (Fowler, 1987; Weber, Pennise, Babcock, & Fowler, 1994); and, finally, actin filaments in the erythrocyte membrane skeleton are decorated by tropomyosins, which regulate actin dynamics and its interactions with actin-binding proteins (Fowler & Bennett, 1984a). Indeed, tropomyosin is predicted to play a role in the AIS membrane-associated periodic scaffold (Leite et al., 2016; K. Xu et al., 2013). However, to the best of my knowledge, the study presented in this dissertation is the first to directly examine the role of tropomyosin in the AIS. In the next section, I will briefly summarise the available literature on tropomyosins and their potential role in the AIS.

2.3.1 Tropomyosin. Originally isolated from myofibrils and predicted to be a precursor to myosin (hence the name, tropomyosin) (Bailey, 1948), tropomyosin (Tpm) comprises a sizable family of actin-binding proteins that are found in different isoforms in muscle and non-muscle cells (for an excellent review of different tropomyosin isoforms and the similarities and differences between them, refer to Schevzov, Whittaker, Fath, Lin, and Gunning (2011)). In mammals, 4 genes code for tropomyosin, expressing up to 40 different isoforms (P. Gunning, O’Neill, & Hardeman, 2008). All Tpm isoforms polymerise head to tail to form a double-stranded α-helical coiled-coil along the major groove of actin filaments (Geeves, Hitchcock-DeGregori, & Gunning, 2015). Tpm stabilises actin filaments and regulates their interactions with other actin-binding proteins in an isoform-specific manner, which allows for a great diversity of actin structures and dynamics (Vindin & Gunning, 2013). Fowler and Bennett (1984a) reported that human erythrocyte membranes contain two isoforms of Tpm in a quantity that is enough to decorate all actin filaments in the erythrocyte membrane. Years later, these two isoforms were identified as isoforms Tpm1.9 (Sung et al., 2000) and Tpm3.1 (Sung & Lin, 1994), products of the TPM1 and TPM3 genes, respectively.
Neurons express several Tpm isoforms that regulate the neuronal actin cytoskeleton in specific compartments or neuronal structures at different developmental stages (Figure 2), but details of the role of tropomyosin in the regulation of the neuronal actin cytoskeleton are lacking (Curthoys, Gunning, & Fath, 2011; Gray, Kostyukova, & Fath, 2017; Schevzov, Curthoys, Gunning, & Fath, 2012). Indeed, working out these details can be a daunting task, owing to the large number of Tpm isoforms and the similarities among them (Schevzov et al., 2011). However, using a combination of virtually all Tpm antibodies available, Schevzov, Vrhovski, et al. (2005) examined the tissue-specific Tpm isoform expression in the mouse. The authors demonstrated that the mouse brain expresses Tpm3.1, but not Tpm1.9 (Schevzov, Vrhovski, et al., 2005). Thus, of the two Tpm isoforms found in the erythrocyte membrane skeleton, only Tpm3.1 is expressed in the brain.

Tpm3.1 binds actin filaments with high affinity (Gateva et al., 2017). The decoration of actin filaments by Tpm3.1 lowers the rate of depolymerisation (J. R. Stehn et al., 2013). Tpm3.1 enhances the phosphorylation of cofilin (Bryce et al., 2003), which inhibits the severing of filaments and negates the effect of cofilin on depolymerisation at the pointed end (Broschat, 1990). Tpm3.1 also recruits tropomodulin to the pointed ends of actin filaments (Sung & Lin, 1994), which further reduces the rate of depolymerisation (Weber et al., 1994; Yamashiro et al., 2014). Furthermore, Tpm3.1 enhances the recruitment and activation of myosin II (Bryce et al., 2003; Gateva et al., 2017). Tpm3.1 also inhibits Arp2/3-mediated branching of actin filaments (Kis-Bicskei, Vig, Nyitrai, Bugyi, & Talian, 2013). Thus, Tpm3.1 generally stabilises actin filaments and regulated its interactions with other actin-binding proteins. But does it have any role in neurons? In 1995, Hannan, Schevzov, Gunning, Jeffrey, and Weinberger (1995) reported that Tpm3.1 mRNA localises to the axons of differentiating neurons, both in vitro and in vivo. The authors described the “segregation of [Tpm3.1]” as “the earliest known marker of neuronal polarity”, and reported that Tpm3.1
associated with a subset of actin filaments involved in the initiation and maintenance of axonal outgrowth. (Hannan et al., 1995). The localisation of Tpm3.1 to developing axons was further confirmed by Weinberger et al. (1996). It is not clear what Tpm3.1 does in the developing axons, but studies have implicated Tpm3.1 in regulating the pool of F-actin in growth cones (Schervoz et al., 2008), the motility of growth cones (Fath et al., 2010), and in neurite branching (Schervoz, Bryce, et al., 2005). The role of Tpm3.1 in mature neurons, however, is not clear (Vindin & Gunning, 2013).

<table>
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<tr>
<th>TPM1</th>
<th>1a 2a 2b 1b 3 4 5 6a 6b 7 8 9a 9b 9c 9d</th>
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<tr>
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<td>growth cones in developing neurons</td>
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<tr>
<td>Tpm1.9</td>
<td>growth cones in developing neurons</td>
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<tr>
<td>Tpm1.10</td>
<td>presynaptic compartment</td>
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<td>cell body</td>
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<td>Tpm1.12</td>
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<tr>
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<tbody>
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<td>developing axon</td>
</tr>
<tr>
<td>Tpm3.2</td>
<td>developing axon</td>
</tr>
<tr>
<td>Tpm3.3</td>
<td>cell body</td>
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<tr>
<td>Tpm3.4</td>
<td>cell body</td>
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<td>cell body</td>
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<tbody>
<tr>
<td>Tpm4.2</td>
<td>cell body, axon, postsynaptic compartment</td>
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**Figure 2. Neuronal tropomyosin isoforms.** Tropomyosin isoforms expressed in neurons, their exon composition, and known localisation.
3. Aims

The unique features of the AIS are a function of the unique underlying structure. Recent advances in imaging techniques have revealed substantial detail about the architecture of the AIS structural complex. Actin filaments present a highly-organised arrangement in sub-membranous rings in the membrane-associated periodic scaffold, but the dynamics and regulation of these filaments, as well as their role AIS structure, is not well understood. This thesis investigates the dynamics of actin filaments in the AIS (I, II), their regulation (II), and their significance for AIS structure and function (II).
4. Materials and Methods

To conduct the experiments for the work presented in this thesis, I used primary cultures of rat hippocampal neurons in conjunction with an array of molecular biological and imaging techniques. The details of the materials and methods are described in the respective manuscripts. I harvested hippocampal neurons from rat embryos at E16/17 (I, II) and cultured dissociated neurons under optimal growth conditions. I used pharmacological (I, II) and genetic agents (II) to manipulate neurons for individual experiments, and a variety of fluorescent proteins and immunocytochemistry techniques to label proteins of interest in both fixed (I, II) and live cells (II). For imaging, I used several fluorescence microscopy techniques including widefield and confocal microscopy (I, II), live-cell imaging (II), structured illumination microscopy (SIM) (I, II), and stochastic optical reconstruction microscopy (STORM) (II). Details of analyses are described in the respective publications.
5. Results

In this chapter, I will summarise the results presented in this thesis.

5.1 Distribution and dynamics of actin filaments in the AIS

Actin filaments form different structures in axons, including in the AIS (Papandreou & Leterrier, 2018). Actin rings in the membrane-associated periodic scaffold are largely believed to play a structurally supportive role (Leterrier, 2016), with some evidence for a role in the AIS membrane diffusion barrier (Albrecht et al., 2016). On the other hand, several studies suggest that actin patches in the AIS are important for the filtering of somatodendritic cargo (Balasanyan et al., 2017; Janssen et al., 2017; Watanabe et al., 2012), a process that is sensitive to actin-disrupting drugs like latrunculin (Song et al., 2009; Winckler et al., 1999). I was able to detect both actin patches (II) and sub-membranous actin rings (I, II) in the AIS and examined their dynamics by observing their sensitivity to latrunculin (I, II) or by directly monitoring their rate of depolymerization (II). To achieve this, I used primary cultures of rat hippocampal neurons grown on glass coverslips. To detect sub-membranous actin rings (I,II) or actin patches in the AIS (II), I fixed mature cultures and used fluorescence-tagged phalloidin to label F-actin. To study the dynamics of the actin cytoskeleton in the AIS, I treated cultures using the actin-disrupting drug latrunculin under different conditions (I). In addition, I used photoactivatable GFP-actin to monitor the rate of depolymerisation of actin filaments in the AIS (II).

5.1.1 Actin filaments in AIS actin rings are resistant to latrunculin. The AIS is insensitive to actin-disrupting drugs (Jones et al., 2014; Sanchez-Ponce et al., 2011; Song et al., 2009). Whether this is because the actin cytoskeleton is not required for maintaining AIS structure, or because actin filaments in the AIS are resistant to these drugs is unclear. In addition, studies examining the effect of actin-disrupting drugs on the AIS actin cytoskeleton used different treatment conditions. Unsurprisingly, there is no consensus regarding the effect
of these drugs on actin in the AIS. To examine the effect of actin-disrupting drugs on the AIS actin cytoskeleton I used two commonly used drugs, namely latrunculin A and B, under different treatment conditions (I). Both latrunculin A and B disrupt the actin cytoskeleton by sequestering actin monomers leading to the inhibition of polymerisation and increased depolymerisation. I found both drugs to be effective in visibly reducing the overall levels of F-actin in the neurons treated (I, Figure 1). However, sub-membranous actin rings in the AIS persisted under all treatment conditions, and their periodicity remained intact (I, Figure 2). The findings of this study suggest that actin filaments constituting sub-membranous rings in the AIS may have a relatively low rate of depolymerisation. As a result, the sequestering of monomers by latrunculin is not enough to destroy these filaments and abolish actin rings.

5.1.2 AIS actin patches have a low rate of depolymerisation. To examine the dynamics of actin patches in the AIS, we used photoactivatable GFP-actin (PAGFP-actin) to monitor their rate of depolymerisation (II, Figure 2). Actin patches in the AIS are resistant to strong detergent extraction (Watanabe et al., 2012), which may indicate an increased stability. In line with this, we found that AIS actin patches have a lower rate of depolymerisation compared to regions in the AIS outside the patch and in comparable dendrites (II, Figure 2). By monitoring the rate of fluorescence decay after photoactivation, I was able to observe the rate at which PAGFP-actin monomers—initially immobilised by incorporation into an actin filament—dissociate from filaments through depolymerisation. This enabled me to examine the dynamics of actin filaments in actin patches in the AIS compared to regions in the AIS outside the patches or in dendrites.

5.2 Tropomyosin Tpm3.1 in the AIS

Actin filaments in the erythrocyte membrane skeleton are decorated by two isoforms of tropomyosin (Fowler & Bennett, 1984b), namely Tpm1.9 (Sung et al., 2000) and Tpm3.1 (Sung & Lin, 1994). While the structure of the AIS cytoskeleton is in many ways similar to
that of mammalian erythrocytes, Tpm3.1 is the only isoform of the two expressed in the mouse brain (Schevzov, Vrbovskí, et al., 2005). In my second study (II), I investigated the role of tropomyosin Tpm3.1 in the AIS.

5.2.1 Tpm3.1 decorates actin filaments in AIS actin rings and patches. I used a combination of immunocytochemistry and microscopy techniques to investigate the distribution of Tpm3.1 in the AIS in primary cultures of rat hippocampal neurons. For this purpose, we used an antibody specific to exon 9d of the TPM3 gene that is capable of specifically recognising isoforms Tpm3.1 and Tpm3.2 (Schevzov et al., 2011). To confirm that the immunofluorescence pattern in the AIS is due to Tpm3.1, we examined the distribution of exogenous YFP-labelled Tpm3.1 and Tpm3.2 after brief expression. YFP-Tpm3.1, but not YFP-Tpm3.2, showed a non-uniform distribution in the AIS, with patches of higher concentrations similar to actin patches. In contrast, YFP-Tpm3.2 was diffuse and faint in the AIS and showed a more prominent localisation to dendritic spines (II, Supplementary Figure 3). Tpm3.1 presented a periodic distribution of ~190 nm in the AIS, corresponding AIS actin rings (II, Figure 1), and co-localised with AIS actin patches (II, Figure 3). Together, these data suggest that Tpm3.1 is present in the AIS where it decorates actin filaments in sub-membranous actin rings and AIS actin patches.

5.2.2 Tpm3.1 is required for the maintenance of AIS structure and function. I used both pharmacological and genetic manipulations to perturb Tpm3.1 function in primary cultures of rat hippocampal neurons. For pharmacological inhibition, we used two distinct small-molecule inhibitors of Tpm3.1, namely TR100 and Anisina (Rynkiewicz, Stehn, Gunning, & Lehman, 2017; J. Stehn et al., 2016; J. R. Stehn et al., 2013). Neither of these inhibitors interfere with the binding of Tpm3.1 with actin filaments. Instead, the presence of these inhibitors negates the effects of Tpm3.1 on the dynamics of the filament and the regulation of their interactions with different binding partners (Bonello et al., 2016; Currier et
al., 2017; Jancz et al., 2019). Acute inhibition of Tpm3.1 using these small-molecule inhibitors led to the loss of the accumulation of ankyrin G at the AIS in a dose- and time-dependent manner (II, Figure 4). The inhibition of Tpm3.1 also led to the loss of the accumulation of other AIS markers, namely TRIM46, EB1, and neurofascin-186 (II, Supplementary Figure 7). In addition, Tpm3.1 inhibition led to the disruption of the periodicity of actin rings in the AIS (II, Figure 9). Similarly, genetic manipulation of Tpm3.1 in our primary cultures using shRNA to deplete Tpm3.1 (II, Supplementary Figure 11) or in cells isolated from Tpm3 conditional knockout mice (II, Figure 5) revealed that the loss of Tpm3.1 led to a reduction in the accumulation of ankyrin G at the AIS.

Furthermore, the inhibition of Tpm3.1 negatively affected the functions of the AIS in maintaining neuronal polarity and the initiation of action potentials. Tpm3.1 inhibition led to the redistribution of the somatodendritic marker GluA1 into the axon (II, Figure 6). The inhibition of Tpm3.1 also led to the loss of the clustering of voltage-gated sodium channels (II, Figure 7), and a rapid reduction in firing frequency (II, Figure 8).

Taken together, our results indicate that the tropomyosin isoform Tpm3.1 is required for maintaining the structure and function of the AIS.
6. Discussion

The data I present in the studies that constitute this thesis suggest that the AIS contains a population of latrunculin-resistant actin filaments, and that this resistance is likely due to their low rate of depolymerisation. These filaments are decorated by the tropomyosin isoform Tpm3.1 which shows a periodic distribution similar to that of the actin rings of the membrane-associated periodic scaffold and co-localises with actin patches in the AIS. The perturbation of Tpm3.1 led to a partial loss of the uniformity of actin rings in the AIS, indicating they may be required for their maintenance. Furthermore, the perturbation of Tpm3.1 led to the loss of the AIS vesicle filter, a significant reduction in the accumulation of ankyrin G and other AIS markers, and a rapid reduction in firing frequency.

The formation of the AIS requires an intact actin cytoskeleton (X. Xu & Shrager, 2005), but actin-disrupting drugs seem to have no effect on the structure or function of the mature AIS, only affecting the integrity of the AIS vesicle filter (Jones et al., 2014; Leterrier et al., 2015; Sanchez-Ponce et al., 2011; Song et al., 2009; Winckler et al., 1999). This has generally led to the conclusion that the actin cytoskeleton is a non-critical component of the AIS. In addition, several studies examined the effect of different actin-disrupting drugs on actin rings in the membrane-associated periodic scaffold, employing different treatment conditions (Han et al., 2017; Leterrier et al., 2015; Qu et al., 2017; K. Xu et al., 2013; Zhong et al., 2014). The result of this inconsistency is that these studies have described actin rings as resistant, partially resistant, or not resistant to actin-disrupting drugs. I tried to address this by using two of the most commonly used actin-disrupting drugs in a variety of concentrations and treatment durations. I then analysed the effects of these treatments on the neuron as a whole and on the actin rings in the membrane-associated periodic scaffold and found that actin rings persisted under all treatment conditions despite the overall loss of F-actin in the neuron.
To complicate things further, the super-resolution microscopy techniques used to visualise actin rings tend to be sensitive to the quality of the signal coming from the sample. While a healthy wild-type neuron may provide enough binding sites for F-actin probes that allow an abundance of high-quality fluorescence, treatment with actin-disrupting drugs may compromise the quality of the signal. Thus, the imaging settings that work for untreated neurons may fail to resolve actin rings in treated neurons. For this reason, I used structural illumination microscopy (SIM) to resolve actin rings, which proved to be the super-resolution microscopy technique most tolerant for relatively poor signal from the sample. I also optimised the imaging protocol for increased sensitivity, which allowed me to visualise actin rings in neurons that overall had a significantly lower intensity of fluorescence of the actin probe compared to wild-types.

Both latrunculin A and B disrupt the actin cytoskeleton by sequestering actin monomers—thus inhibiting actin polymerisation—and by severing and depolymerising ATP-actin filaments (Fujiwara, Zweifel, Courtemanche, & Pollard, 2018). Unable to polymerise, dynamic filaments continue to depolymerise in the presence of latrunculin and may be totally lost. However, filaments with a low rate of depolymerization—or that do not depolymerise at all—remain unaffected. Actin filaments in AIS actin rings are capped by adducin at their barbed ends, which inhibits polymerisation (Leite et al., 2016; K. Xu et al., 2013). The fact that these filaments endure under normal conditions without continuous polymerisation suggests they may be relatively stable, which allows them to persist in the presence of latrunculin and the inhibition of polymerisation. Interestingly, these actin rings are not lost in neurons lacking adducin (Leite et al., 2016), which strongly suggests that other factors may be contributing to their stability. Two such factors contribute to the stability of actin filaments in the erythrocyte membrane skeleton by lowering the rate of depolymerisation: tropomyosin and tropomodulin (the only known pointed-end cap) (Fowler, 2013).
While several studies have investigated the role of different tropomyosin isoforms in neurons and their development (Curthoys et al., 2011), the role of tropomyosin in the AIS has not been examined. This is possibly in part a result of the prevailing view that actin is not a critical component of AIS structure and function, apart from its role in vesicle filtering and the diffusion barrier. This advent of super-resolution microscopy and the discovery of actin rings in the AIS challenged this view and sparked interest in the AIS actin cytoskeleton.

Actin filaments form periodic rings in the membrane-associated periodic skeleton in the AIS, as well as intracellular patches (Papandreou & Leterrier, 2018). I was able to show that actin filaments in the AIS actin patches have a relatively low rate of depolymerisation. Together with resistance of actin rings to latrunculin, it is evident that the AIS contains a population of relatively stable actin filaments with a low rate of depolymerisation. The regulation of the actin cytoskeleton and its dynamics is largely a function of actin-binding proteins and the interplay between them. A potential role for tropomyosin in regulating the actin cytoskeleton in the AIS was discussed but never examined (Leite et al., 2016; K. Xu et al., 2013).

Although the capping of the barbed ends of actin filaments in actin rings by adducin may confer stability to these filaments, the mild effects of knocking out adducin on AIS structure and its actin cytoskeleton indicate that other factors may also be involved.

The periodic distribution of the tropomyosin isoform Tpm3.1 and its co-localisation with AIS actin patches suggests it is one of the factors responsible for stabilising actin filaments in the AIS and lowering their rate of depolymerisation. Tpm3.1 typically strongly binds actin filaments and lowers their rate of depolymerisation (Gateva et al., 2017; J. R. Stehn et al., 2013). Equally importantly, Tpm3.1 regulates the interaction of actin filaments with other binding partners. Of particular interest in this context is the ability of Tpm3.1 to recruit tropomodulin to the pointed ends, which it in fact does in the erythrocyte membrane skeleton (Sung & Lin, 1994). The capping of pointed ends by tropomodulin can further lower
the rate of depolymerisation (Weber et al., 1994; Yamashiro et al., 2014). Also noteworthy is
that Tpm3.1 regulates the interactions of actin filaments with motor proteins (P. W. Gunning,
Hardeman, Lappalainen, & Mulvihill, 2015). Tpm3.1 recruits myosin II (Bryce et al., 2003;
Gateva et al., 2017), which has recently been shown by several labs to play a role in AIS
structure (Berger et al., 2018; Evans, Tufo, Dumitrescu, & Grubb, 2017; Fan et al., 2017;
Wang et al., 2018). Thus, Tpm3.1 is well-poised for a role in maintaining AIS structure. The
effects of Tpm3.1 perturbation on AIS structure and function indicate this is indeed the case.

The inhibition of Tpm3.1 in our cultures for the duration of our experiments reduced
the uniformity of actin ring periodicity in the AIS, compared to untreated controls. Actin
rings nonetheless presented visible periodicity and autocorrelation analysis showed a
prominent lag at 200 nm. Interestingly, the accumulation of ankyrin G and other AIS markers
was significantly reduced after the same treatment. While the mechanism behind this loss of
ankyrin G accumulation is yet to be elucidated, the discrepancy between the effect of Tpm3.1
inhibition on ankyrin G accumulation and actin ring periodicity suggests that the former is
not the result of the loss of actin rings or a complete collapse of AIS structure. Owing to the
central role of ankyrin G in recruiting various AIS components and maintaining AIS structure
(Hedstrom et al., 2008; S. M. Jenkins & Bennett, 2001; Zhou et al., 1998), the loss of AIS
markers and AIS function upon Tpm3.1 inhibition could be viewed as a consequence of the
loss of ankyrin G. One possible explanation is that the inhibition of Tpm3.1 disrupts the
interactions of actin rings with a critical binding partner(s) that is required for keeping the
entire lattice intact. I know of no direct link between actin and ankyrin G, in the AIS or
elsewhere. The spacing in the lattice also makes such a direct interaction unlikely. Could
there be a protein that acts as an adaptor linking actin rings to ankyrin? An obvious candidate
for fulfilling such a role is βIV-spectrin, and it is imperative to examine the effect of Tpm3.1
inhibition on its distribution. In addition, recent studies on the role of myosin II in the AIS
(Berger et al., 2018; Evans et al., 2017; Fan et al., 2017; Wang et al., 2018) could provide important clues; it is plausible that the effects of Tpm3.1 inhibition are at least partly the result of the loss of myosin II recruitment to the membrane-associated periodic scaffold, although this needs to be tested. Future work will hopefully provide answers to these questions.

The sensitivity of the selective filtering of somatodendritic cargo at the AIS to latrunculin (Song et al., 2009; Winckler et al., 1999) suggested a role for actin in the process. This was further demonstrated by discovering that the process involves myosin motors (Al-Bassam et al., 2012; Janssen et al., 2017; Lewis et al., 2011; Lewis et al., 2009) and the presence of patches of F-actin in the AIS where somatodendritic cargo occasionally halt after they enter the AIS (Balasanyan et al., 2017; Janssen et al., 2017; Watanabe et al., 2012). Actin patches appear to contribute to the sorting of vesicles by tethering and immobilising somatodendritic cargo, triggering a chain of events that culminates in the recruitment of the minus-end microtubule motor dynein, which carries the cargo along the unipolar microtubules toward the soma (Leterrier, 2018). The tethering of cargo to actin patches is mediated by myosin motors, particularly myosin V (Balasanyan et al., 2017; Janssen et al., 2017; Lewis et al., 2009; Watanabe et al., 2012). Tropomyosins regulate the interaction of actin filaments with myosin motors by recruiting specific isoforms and inhibiting others (P. W. Gunning et al., 2015). In yeast, tropomyosin enhances the interaction of actin filaments with class V myosin, and increases its duty ratio (Hodges et al., 2012). Interestingly, on the other hand, Myo1b—a myosin motor involved in organelle transport—does not bind Tpm3.1-decorated actin filaments (Kee et al., 2015; McIntosh, Holzbaur, & Ostap, 2015; Tang & Ostap, 2001). Whether this is how bulky organelles overcome the selective filtering of cargo at the AIS remains to be tested, however. In my experiments, Tpm3.1 inhibition did not abolish actin patches in the AIS, but led to the entry of somatodendritic cargo (the
somatodendritic marker GluA1) into the axon. Thus, it is plausible that the inhibition of Tpm3.1 disrupted the interaction of myosin motors with actin patches, which interfered with the tethering and immobilisation of the somatodendritic cargo (GluA1). However, details of the precise manner through which tropomyosins—particularly Tpm3.1—regulate the interaction of actin filaments with myosin motors may hold valuable answers that help us better understand the mechanism of vesicle sorting at the AIS.

I used two structurally distinct small-molecule Tpm3.1 inhibitors, namely TR100 (Bonello et al., 2016; Kee et al., 2018; Kee et al., 2015; J. R. Stehn et al., 2013) and Anisina (also known as ATM-3507) (Currier et al., 2017; Janco et al., 2019; J. Stehn et al., 2016). Originally developed as anti-cancer compounds, both these inhibitors operate not by interfering with the binding of Tpm3.1 with actin filaments but by negating the effects of Tpm3.1 on actin dynamics the interaction with other proteins (Bonello et al., 2016; Janco et al., 2019). Importantly, the effects of TR100 on glucose re-uptake were absent in Tpm3.1 knockout cells, demonstrating its specificity (Kee et al., 2018; Kee et al., 2015). In my experiments, the effects of both TR100 and Anisina were also dose- and time-dependent. For both TR100 and Anisina, the presence of saturating amounts of the calpain inhibitor MDL28170 had no effect on the extent of loss of ankyrin G under any treatment condition. This is significant as the loss of ankyrin G accumulation may happen as a result of calpain-mediated proteolysis after oxidative stress (K. Clark, Sword, & Dupree, 2017) or neuronal injury (Schafer et al., 2009).Unlike our experiments with Tpm3.1 inhibition, the disruption of the AIS upon neuronal injury could be rescued by applying the calpain inhibitor MDL28170, however (Schafer et al., 2009). Throughout my work I used both inhibitors interchangeably. This is partly due to the fact that Anisina was only available to me at a later stage during my work. In addition, both inhibitors showed highly similar effects and share the same mode of action, despite differences in their chemical structure.
The effects of TR100 and Anisina were demonstrably more penetrant than genetic manipulations of Tpm3.1 levels. This is not surprising as the genetic manipulations we employed (shRNA-mediated knock down and Cre-dependent conditional knock out) only reduced—but did not completely deplete—the amount of Tpm3.1. Acute inhibition using small-molecule inhibitors is thus inherently more rapid and absolute. The large number of Tpm isoforms also convolutes genetic approaches to manipulate the levels of specific isoforms. The target sequence of our shRNA construct lies in exon 9d of the *TPM3* gene, which is shared by two Tpm3 isoforms: Tpm3.1 and Tpm3.2. The reduction of Tpm3.1 levels in neurons expressing our shRNA construct, calculated based on the relative intensity of Tpm3.1/2 immunofluorescence, was approximately 25%. The corresponding reduction in the accumulation of ankyrin G was approximately 23%. Although milder than the effects of small molecule-mediated inhibition, the sensitivity of ankyrin G accumulation to the levels of Tpm3.1 highlights its importance for maintaining AIS structure. Importantly, the effects of Tpm3.1 knockdown were readily rescued by the exogenous expression of human Tpm3.1, which differs in sequence in the target region. Similarly, for culture preparations from Tpm3 conditional knockout mice, we only introduced Cre after the neurons were dissociated and plated on glass coverslips (at embryonic day 16). Consequently, the neurons may have maintained residual amounts of Tpm3.1 that explain the milder effect compared to acute inhibition.

Finally, our functional recordings showed that the application of Anisina had a rapid effect on firing frequency. While the effect of Anisina on the accumulation of ankyrin G was readily detectable after incubating neurons for two hours in media containing 5 μM Anisina, a clear reduction in firing frequency was evident less than fifteen minutes after the introduction of a patching solution containing 2.5 μM Anisina (likely leading to an effectively lower intracellular concentration of Anisina). This clearly highlights the
advantage of using functional assays in terms of sensitivity. I hope future work will expand the use of functional assays to benefit from this.
7. Conclusions

In my thesis work I show that the AIS contains a population of relatively stable actin filaments, and that these filaments are critical for maintaining AIS structure and function. It is the stability of these filaments that has led to them being overlooked for decades of research on the AIS, leading to a common view of actin as dispensable for AIS structure. Here I show that relatively stable actin filaments form sub-membranous rings and actin patches in the AIS, and reveal the function of the tropomyosin isoform Tpm3.1 in regulating these filaments and its importance for maintaining AIS structure and function.

The AIS is critical for neuronal function, and defects in the AIS are implicated in numerous neurological and mental disorders. Understanding the structure of the AIS and its regulation will undoubtedly contribute to a better understanding of these disorders. Furthermore, the well-defined population of actin filaments described in this thesis may serve as a valuable drug target.
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References


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