

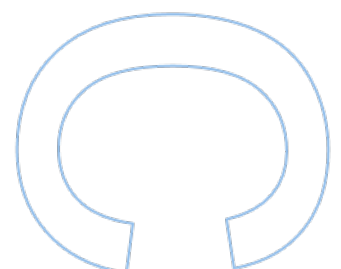
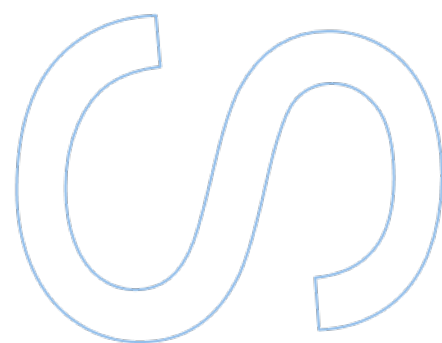
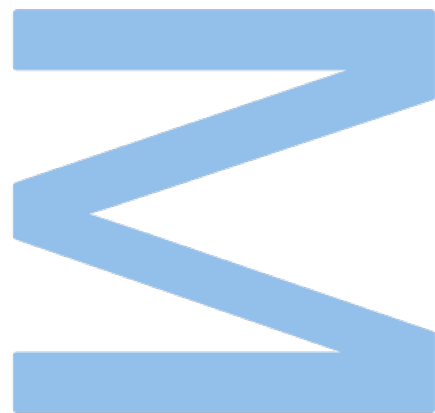
Curving Actin Rings: Anillin and IQGAP in the axonal periodic skeleton

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Master's Degree in Biochemistry

Faculty of Sciences of University of Porto (FCUP) and School of Medicine
and Biomedical Sciences (ICBAS)

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Resumo

O citoesqueleto, composto por microtúbulos, neurofilamentos e filamentos de actina, é crucial para manter a integridade estrutural dos neurónios, promovendo ao mesmo tempo o crescimento e transporte axonal. A actina desempenha um papel fundamental na morfologia e dinâmica celular, especialmente na polarização neuronal e no desenvolvimento de axónios e dendrites. No entanto, a organização da actina no interior dos axónios é um tema ainda por explorar. Avanços recentes em microscopia de super-resolução e estudos com axónios, revelaram uma organização sofisticada da actina, conhecida como o esqueleto periódico membranar (MPS). O MPS é formado por anéis de actina periodicamente distribuídos, com um espaçamento de aproximadamente 190nm, interligados por tetrâmeros de α II/ β II-spectrin. Inicialmente, acreditava-se que o MPS fornecia suporte mecânico aos axónios. O nosso grupo contribui para elucidar o papel do MPS na regulação do diâmetro do axónio através de aducina e miosina não muscular II (NMII). Como ainda há uma falta de compreensão sobre a biologia do MPS, pretendemos compreender completamente a sua biogénese e função. Atualmente, a nucleação da actina dentro dos anéis está a ser investigada, mas uma questão crítica ainda por resolver envolve os mecanismos de circularização da actina e os componentes bioquímicos implicados. Este trabalho visa investigar os papéis da anilina e IQGAP na determinação da circularização dos anéis de actina que constituem o MPS. Para isso, utilizamos uma abordagem genética, CRISPR-Cas9 e microscopia de super-resolução para explorar o envolvimento das mesmas na formação e manutenção do MPS em neurónios de hipocampo de rato. Os nossos resultados mostram que, enquanto a anilina parece não ser essencial para a formação e manutenção dos anéis de actina, a IQGAP é endogenamente mais expressa durante a manutenção do MPS e parece co-localizar com os anéis de actina. Quando mutada, observamos uma redução no diâmetro do axónio, o que não é verificado no controlo. Isto sugere que a proteína IQGAP1 pode desempenhar um papel crucial na circularização da actina durante a manutenção do MPS, em comparação com a formação inicial. As nossas descobertas contribuem para uma compreensão mais profunda da biologia celular neuronal.

Palavras-chave: esqueleto periódico membranar, anéis de actina, contratilidade do axónio, microscopia de super-resolução.

Abstract

The neuronal cytoskeleton, comprising microtubules, neurofilaments, and actin filaments, is critical for maintaining the structural integrity of neurons while promoting axonal growth and transport. Actin plays a pivotal role in cell morphology and dynamics, especially in neuronal polarization and axon/dendrite development. However, the organization of actin within axons has been less explored. Recent advances in super-resolution microscopy have unveiled an exquisite organization of actin within axons, known as the membrane periodic skeleton (MPS). The MPS consists of periodically distributed actin rings spaced approximately 190 nm apart, interconnected by tetramers of α II/ β II-spectrin. The MPS was initially considered to provide mechanical support to axons. Our group contributed to this field by elucidating the role of the MPS in regulating axon diameter via adducin and non-muscle myosin II. Despite these advances, the biology of the MPS remains incompletely understood. Our aim is to fully comprehend its biogenesis and function. Currently, actin nucleation within the actin rings is being investigated, but a critical unresolved question concerns the mechanisms of actin circularization and the biochemical components implicated. Our research focused on investigating the roles of Anillin and IQGAP in shaping the curved geometry of actin rings within the MPS. For that, we employed genetic approaches, such as Crispr-Cas9 and tau STED microscopy, to explore the involvement of these proteins in MPS formation and maintenance in primary hippocampal rat neurons. So far, our results show that while anillin appears to be non-essential for the formation and maintenance of the actin rings, IQGAP is endogenously upregulated during MPS maintenance and colocalizes with the actin rings. Once IQGAP is knocked-out, we find a reduction in axon diameter, which is not observed in the control. This suggests that IQGAP1 protein might play a key role in actin circularization during the MPS maintenance, rather than its initial formation. Our findings thus contribute to a deeper understanding of neuronal cell biology.

Keywords: membrane periodic skeleton, actin rings, axonal contractility, super-resolution microscopy.

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List of Abbreviations

ABP Actin binding protein

ADF Actin depolymerizing factor

AIS Axon Initial Segment

AnKG Ankyrin G

ANLN Anillin

ANS Autonomic Nervous System

APC Adenomatous polyposis coli

Arp2/3 Actin-related protein 2/3

ATP Adenosine triphosphate

Bp Base pair

Cas CRISPR-associated

CBh Chicken Beta-Actin Hybrid

CC Coiled-coil repeat region

Cdc42 Cell division control protein 42 homolog

CHD Calponin-homology domain

CLIP70 microtubule plus-end cytoplasmic linker protein 170

CMV Cytomegalovirus

CNS Central Nervous System

CRISPR Clustered regularly interspaced short palindromic repeats

CTRL Control

DAPI 4',6-diamidino-2-phenylindole

DIV Days *in vitro*

DMEM Dulbecco's Modified Eagle Medium

DNA Deoxyribonucleic acid

E-cadherin Epithelial cadherin

E. coli *Escherichia coli*

EB End-binding protein

eGFP Enhanced Green Fluorescent Protein

F-actin Filamentous actin

FBS Fetal Bovine Serum

G-actin Globular actin

GAP GTPase activating domain

GDP Guanosine diphosphate

GRD GTPase-activating protein-related domain

gRNA guide RNA

GTP Guanosine triphosphate

HBSS Hank's balanced salt solution

hCas9 human Cas9

hPGK human Phosphoglycerate Kinase

ICC Immunocytochemistry

IF Intermediate Filament

IQGAP IQ motif-containing GTPase-activating proteins

L-Glu L-Glutamine

LB Luria Broth

MAP Microtubule-associated protein

MBD Myosin binding domain

mDia2 Diaphanous-related formin 2

MT Microtubule

MTOC Microtubule Organizing Centre

N-cadherin Neuronal cadherin

NF Neurofilament

NMII Non-muscle myosin II

N-WASP Neuronal Wiskott-Aldrich syndrome

O/N Overnight

Opti-MEM Opti-Minimal Essential Medium

P/S Penicillin/Streptomycin

PAM Protospacer adjacent motif

PBS Phosphate-buffered saline

PFA Paraformaldehyde

PH Pleckstrin Homology

PLL Poly-L-Lysine

PNS Peripheral Nervous System

Rac1 Ras-related C3 botulinum toxin substrate 1

RBD Rho binding domain

RhoA Ras homology family member A

RhoG Ras homology family member G

RNA Ribonucleic acid

Rosa26 Reverse orientation splice acceptor 26

RT Room Temperature

SEM Standard Error of the Mean

sgRNA single-guide RNA

SH₃ Src-homology-3-binding

STED Stimulated Emission Depletion Microscopy

STORM Stochastic Optical Reconstruction Microscopy

TAE Tris-acetate-EDTA

TIPs+ Plus-end tracking protein

VASP Vasodilator-stimulated phosphoprotein

WW Proline-rich motif-binding region

γ -TURC Gama-tubulin ring complex

Introduction

1. The Nervous System

The nervous system is perhaps the most distinct and impressive part of the human body, capable of perceiving and regulating the body's response to internal and external stimuli (Sousa, Meyer et al. 2017, Martín-Durán and Hejnov 2021).

Topographically, this system is divided into two parts: the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS comprises the brain and spinal cord, which are surrounded by the bones of the axial skeleton. The PNS includes the cranial nerves, spinal nerves and the ganglia associated with these nerves (Figure 1) (Bazira 2021).

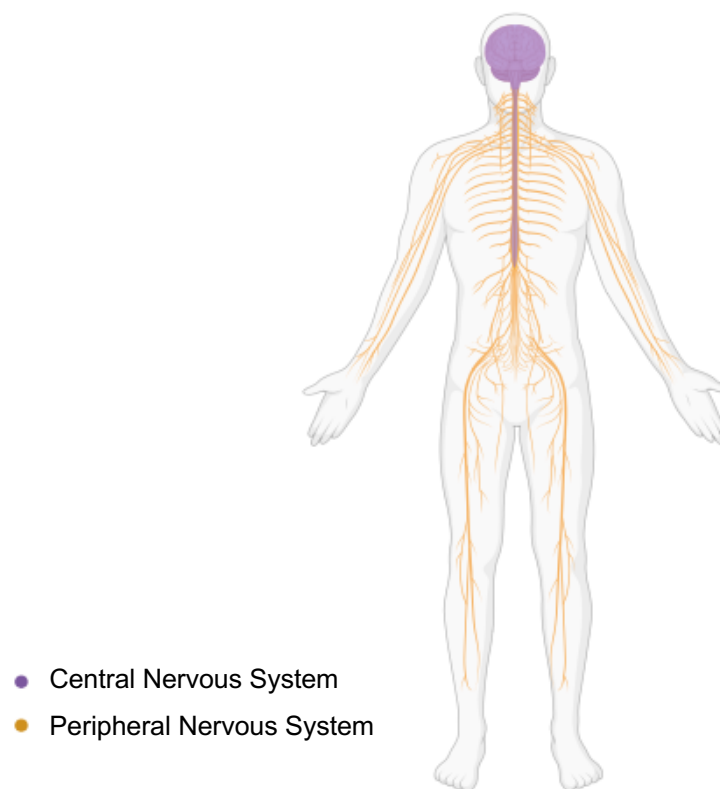


Figure 1 – Representation of the Nervous System. The CNS is presented in purple and it includes the brain and spinal cord. The PNS is presented in orange and includes the cranial, spinal nerves and the ganglia associated with these nerves. Made in BioRender.

From a microanatomical perspective, the nervous system consists mainly of two main types of cells: neuroglia (including oligodendrocytes in the CNS and Schwann cell in the PNS, astrocytes and microglia) and neurons. From a functional viewpoint, the

nervous system is highly interconnected, allowing for complex communication and control (Bazira 2021).

2. Basic Biology of Neurons

The neuron, a fundamental cellular unit also recognized as the building block of the nervous system, processes and propagates information between the nervous system and all body parts. Neurons are organized into complex neuronal networks and are responsible for the functional control of the entire organism (Fishell and Heintz 2013, Bazira 2021, Buentello, Garcia-Corral et al. 2024). Neurons communicate via a combination of chemical and electrical signals, allowing rapid conduction from one end of the cell to the other (Lovinger 2008, Azarfar, Calcini et al. 2018). This communication occurs at synapses, nanometric gaps between neurons that allow chemical transmission. The presynaptic neurons release neurotransmitters that are captured by receptors present in the postsynaptic neurons (Lovinger 2008, Azarfar, Calcini et al. 2018). Precise synapse development is important for accurate neuronal network activity and normal brain function (Kulkarni and Firestein 2012).

2.1. Main components

Both the size and shape of the neuron fluctuate depending on their functions. Despite this variability, most neurons, and as depicted in Figure 2, possess a cell body and two types of projections, dendrites and axons, discussed in more detail below, which are morphologically and functionally distinct (Kulkarni and Firestein 2012). Dendrites receive and integrate electrical synaptic inputs (signals) from thousands of neurons and communicate the information to other cells through axons. A single axon propagates signals by conducting impulses away from the cell body to adjacent neurons or to muscle or glandular cells in the form of an action potential (Lovinger 2008, Kevenaar and Hoogenraad 2015, Bazira 2021, Buentello, Garcia-Corral et al. 2024). These apparently simple structures interconnect not only with each other but also with other cell types to form complex networks that translate signals into cognitive functions (Bazira 2021, Buentello, Garcia-Corral et al. 2024).

2.1.1. Cell body

The cell body, also known as soma or perikaryon contains a bulky, spheroidal nucleus, with one, or more nucleoli, a nuclear membrane and a highly differentiated cytoplasm. The soma is characterized by an abundant rough endoplasmic reticulum, a well-developed Golgi apparatus and a considerable number of mitochondria and other organelles. These characteristics of the perikaryon indicate that this is the zone with the

greatest metabolic activity and protein synthesis within the neuron (Luengo-Sanchez, Bielza et al. 2015).

2.1.2. Dendrites

Dendrites present a complex morphology, sharing tree-like branching processes, designated dendritic arbor, which reflects their diverse functions as conduits for neural signals (Lolova, Lolov et al. 1997, Kulkarni and Firestein 2012, Tavosanis 2012, Lefebvre, Sanes et al. 2015). Additionally, they are essential for neural information processing and specialized brain functions and cognitive behaviors, such as social interaction, learning, and memory (Kulkarni and Firestein 2012, Lefebvre, Sanes et al. 2015). Minor alternation in dendrite size and morphology or defects in neuronal development, can impact connection and input processing and can contribute to several neurological and neurodevelopmental disorders (Lolova, Lolov et al. 1997, Kulkarni and Firestein 2012).

Dendritic morphology is shaped during development through various mechanisms, including branching, elongation, retraction and self-avoidance (Liao, Bird et al. 2023). Dendrite branching involves the reorganization of the neuronal cytoskeleton, with microtubules (MTs) and actin playing a crucial role in shaping the dendritic arbor. Far from being static, dendrites are highly dynamic during both development and adulthood, appearing to be capable of plastic changes (Kulkarni and Firestein 2012, Tavosanis 2012).

2.1.3. Axon

Axons are thin and extremely long structures, generally unbranched except at their ends (Kayasandik and Labate 2016, McMillan and Harris 2018). Structurally, axons consist of three main compartments: the axon initial segment (AIS), the shaft and the distal tip or axon terminals. During development, this tip is designated as the growth cone. After the connections are established, the growth cone is replaced by the presynaptic or axon terminals (Kamiya 2019, Costa and Sousa 2020).

Axons are responsible for generating and conducting action potential along their length (Safronov, Wolff et al. 2000). They conduct the presynaptic action potential from the cell body to the nerve terminals, allowing rapid conduction of electrical signals, from one region of the brain or body to another, travelling long distances (Debanne and Russier 2003, Griswold 2022). This propagation relies on the distribution of ionic conductance primarily driven by voltage-dependent sodium (Na^+) channels, which are crucial for the initiation and propagation of action potentials, while potassium (K^+) channels regulate this process (Debanne and Russier 2003, Kamiya 2019).

Axon Initial Segment (AIS)

The AIS is a short region located in the proximal region of the axon and adjacent to the soma. It occupies 20-60 μ m. The unique localization of the AIS in neurons is ideal for its major functions: to integrate synaptic inputs, thus generating the action potential, and to maintain neuron polarity (Jones and Svitkina 2016, Kamiya 2019). This structure exhibits structural plasticity, as it can alter its length and location relative to the soma in response to neuronal activity (Costa and Sousa 2020). Additionally, the AIS also acts as a diffusion barrier, restricting the movement of membrane proteins and lipids, acting as a filter and regulating selective intracellular cargo traffic between the soma and the axon (Kevenaar and Hoogenraad 2015, Kuijpers, van de Willige et al. 2016).

The AIS includes a wide and adaptable complex of proteins, including ankyrin G (AnkG), membrane proteins, scaffold proteins and cytoskeleton adaptors that respond dynamically to developmental and physiological cues (Jones and Svitkina 2016, Leterrier, Clerc et al. 2017). AnkG is responsible for the subsequent recruitment of most AIS-enriched proteins, and its depletion not only impairs the accumulation of other AIS components but also leads to a progressive loss of neuronal polarity (Leterrier, Clerc et al. 2017). The expression of Na⁺ channels is much higher in the AIS, to support reliable spike initiation at the AIS and safe spike propagation along the axon (Kamiya 2019). This region also contains high density of K⁺ channels (Gulledge and Bravo 2016). Some neurons also receive inputs either in the proximal portion of the AIS or along its distal region (Luengo-Sanchez, Bielza et al. 2015).

Axon Shaft

The axon shaft is the longest neuronal compartment and mediates the action potential propagation as well as long-distance transport of axonal proteins, lipids and organelles. It also includes the presynaptic mediators that are critical for presynaptic transmission (Prokop 2020, Ma, Deng et al. 2022). The diameter of the axon shaft remains constant, a feature believed to be crucial for effective action potential conduction (Ma, Deng et al. 2022). Along its length, the axon shaft typically forms varicosities or swellings with a bouton-like appearance. These boutons are distributed as a “pearl-chain structure” and each bouton contains numerous synaptic vesicles and multiple release sites. These enlarged structures significantly impact action potential propagation and synaptic transmission (Kamiya 2019, Ma, Deng et al. 2022).

Major determinants of membrane mechanics include lipid composition and cytoskeletal structure and these influence parameters such as axon shaft bending and

tension. These factors also control the localization of transmembrane proteins, including Na^+ and K^+ channels, essential for action potential firing tension (Griswold 2022).

The axon shaft contains specific arrangements of cytoskeleton elements: neurofilaments (NFs), MTs and actin filaments (Leterrier, Dubey et al. 2017). Very briefly, NFs play a crucial role in controlling axon diameter and thereby influencing axonal conduction (Kevenaar and Hoogenraad 2015). MTs provide a structural backbone for axonal transport, facilitating the trafficking of vesicles and organelles through motor proteins such as kinesins and dynein (Prokop 2020, Falconieri, Coppini et al. 2024). Actin filaments serve as a precursor to the formation of filopodia and lamellipodia and as an anchoring site for myosin motors and their cargoes (Arnold and Gallo 2014).

Axon Terminals

As previously mentioned, the distal axonal tip initially features a growth cone during development, which originates presynaptic terminals or axon terminals once connections are established (Kalil and Dent 2014, Jones and Svitkina 2016, Costa and Sousa 2020). Growth cones, the motile tips of growing axons, are highly dynamic and polarized structures with protrusive activity that respond to extracellular signals or cues, thus guiding it towards a target region (Arnold and Gallo 2014, Kalil and Dent 2014, Nesler, Starke et al. 2016). In a typical growth cone, finger-like filopodia, composed of bundled actin filaments, extend from a lamellipodial region with loosely cross-linked actin webs (Ori-McKenney, Jan et al. 2012). Research on the dynamics of axon branching revealed three mechanisms of axon collateral branch initiation: formation of filopodia, lamellipodia and growth cone pausing (Armijo-Weingart and Gallo 2017). The growth cone is divided into three regions: the central domain, the transition zone and the peripheral domain (Costa and Sousa 2020). The central domain of the growth cone is rich in MTs and organelles, while the highly dynamic peripheral surrounding the growth cone is mainly composed of actin organized into lamellipodia and filopodia (Tahirovic and Bradke 2009, Stiess and Bradke 2011, Arnold and Gallo 2014).

Axon terminals branch into multiple axon terminals that form synapses, allowing neurons to integrate information from divergent regions of the nervous system. This extensive branching and formation of terminal arbors allow individual neurons to connect with multiple synaptic targets, establishing topographic specificity (Kalil and Dent 2014, Jones and Svitkina 2016, Costa and Sousa 2020). Synapses continue to remodel throughout development and during activity-dependent plasticity. As a consequence, changes in axon terminals can occur very rapidly. The journey of information initiation at a presynaptic terminal occurs through an action potential, ultimately culminating in the

transmission of chemical neurotransmitters to the postsynaptic cell. Within the presynaptic terminal, these neurotransmitters are carefully packaged into synaptic vesicles. When an action potential activates presynaptic voltage-gated calcium (Ca^{2+}) channels, the neurotransmitters are released via Ca^{2+} -triggered synaptic vesicle exocytosis into the synaptic cleft. In this synaptic cleft, they activate postsynaptic receptors, thus transmitting the information (Purves 2004, Südhof 2012, Squire 2013).

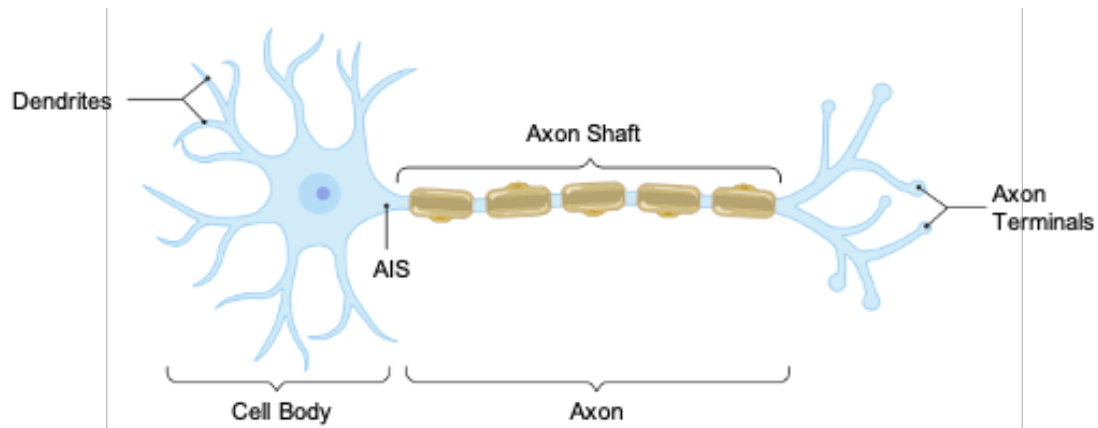


Figure 2 – Representation of the Neuron's main components. Neurons are composed by a cell body, dendrites and the axon. The axon is divided into the AIS, the shaft and axon terminals. Made in BioRender.

2.2. Neuronal Polarization

Neurons are structurally distinct from most other cells due to their unique capacity for polarization, which allows them to rearrange and assemble into neuronal circuits and propagate signals effectively (Bradke and Dotti 2000, Tahirovic and Bradke 2009). Depending on the species of origin and function, neurons can exhibit different polarities, they can be multipolar, unipolar or pseudounipolar and can function as motor neurons, interneurons or sensory neurons (Prokop 2020).

Neurons generally display both morphological and functional polarization. In the canonical multipolar neuron of the mammalian brain, neurons can be divided into distinct regions, a receptive and conductive apparatus (dendrites and soma), an emission apparatus (axon) and a distribution apparatus (axon terminals) (Luengo-Sanchez, Bielza et al. 2015). Neuronal polarization is essential for integrating and propagating information within the brain (Witte and Bradke 2008). This process is driven by the cytoskeleton, which determines cell polarity by forming the structural scaffold for establishing distinctive cell shape and organizes the polarized cell interior and intracellular transport (Stiess and Bradke 2011, Leite and Sousa 2016).

Studies using embryonic hippocampal neurons in culture, defined distinct developmental stages of polarization, summarized in Figure 3. After plating, neuronal development begins with a symmetrical cell containing a round soma that extends a motile lamellipodium with protrusive activity around the cell body, stably attaching to the substrate; this event is known as stage 1. Stage 2 can take up to 12 to 24 hours, during which the protrusion forms multiple short and cylindrical processes of roughly equal length, the neurites, transforming into multipolar cells. After approximately 1.5 days *in vitro* (DIV), these neurites, decorated with dynamic growth cones at their tips, exhibit characteristic alternations of extension and retraction, until one of them initiates a sudden and sustained growth. This neurite becomes the neuron's axon, marking a major polarity, classifying as stage 3. It is after this stage that AnkG begins to cluster in the proximal axonal region, indicative of early AIS assembly. During stage 4, the remaining neurites begin to slowly elongate and develop into highly branched dendrites at DIV4. By DIV7, or stage 5, functional polarization of axons and dendrites, including synaptic specializations and contacts are established. Neurite initiation and outgrowth depend on the local increase in actin dynamics in combination with microtubule stabilization (Kevenaar and Hoogenraad 2015, Jones and Svitkina 2016). Dendritic spines are formed after two weeks in culture, which is considered stage 6 (Tahirovic and Bradke 2009, Cáceres, Ye et al. 2012).

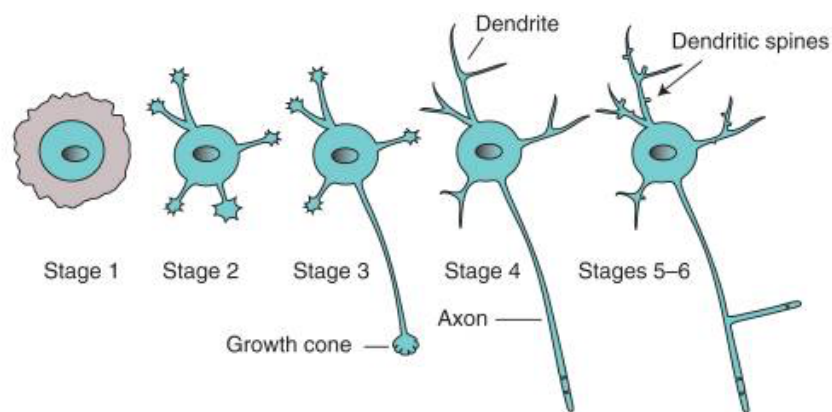


Figure 3 – Schematic representation of the neuronal polarization stages in rat embryonic hippocampal cultured neurons. Hippocampal neurons lose their round lamellipodia shape (stage 1) and become multipolar cells (stage 2). One neurite enlarges its growth cone and extends rapidly to become the axon (stage 3). The remaining shorter neurites will develop into dendrites (stage 4). This is followed by functional maturation and formation of dendritic spines and synapses (stages 5-6). Adapted from (Tahirovic and Bradke 2009).

The cytoskeleton both establishes and maintains neuron polarity. Not only, actin filaments and MTs rapidly convert molecular signals into structural changes modulating cell shape, but also, possess an intrinsic polarity, further explored (Tahirovic and Bradke

2009). Before morphological polarization occurs, the future axon shows enhanced growth cone dynamics and actin turnover. In contrast, future dendrites, which are not growing at that stage, have a static growth cone with a rigid actin cytoskeleton. Stable MTs in the axon may form a landmark to confer identity to the axon (Witte and Bradke 2008). Depletion of actin filaments from both morphologically and molecularly polarized cells results in the growth of multiple axons from pre-existing dendrites (Bradke and Dotti 2000). This polarized organization underlies many aspects of neuronal function and circuit formation. The loss of neuronal polarity may result in neurodevelopmental and neurodegenerative diseases (Eichel and Shen 2022).

3. The Neuronal Cytoskeleton

The extreme polarization of neurons, along with the plasticity they endure throughout their lifetime, creates a unique demand on the neuronal cytoskeleton. This cytoskeleton acts as a cellular shapeshifter, altering its morphology to accommodate the needs of the cell. As previously mentioned, it underlies the establishment of neuronal polarity, outgrowth and stabilization and integrity of the axon (Kevenaar and Hoogenraad 2015, Leterrier, Dubey et al. 2017, Gallo 2024).

In the initial stages of neuronal development and neurite outgrowth, the cytoskeleton is crucial for generating intracellular forces and mediates several signaling pathways (Kapitein and Hoogenraad 2015, Kevenaar and Hoogenraad 2015, Leterrier, Dubey et al. 2017). The cytoskeleton is also described as essential for the active transport of axonal proteins, vesicles, and organelles. This transport, driven by motor proteins, ensures that the correct proteins reach their proper location within the cell. Active transport is particularly important in axons due to their significant length (Kevenaar and Hoogenraad 2015).

The neuronal cytoskeleton includes three major constituents: NFs, MTs, and actin. Each performs a specialized role in the axon. These proteins are synthesized in the neuronal soma and transported along the axon (Leterrier, Dubey et al. 2017).

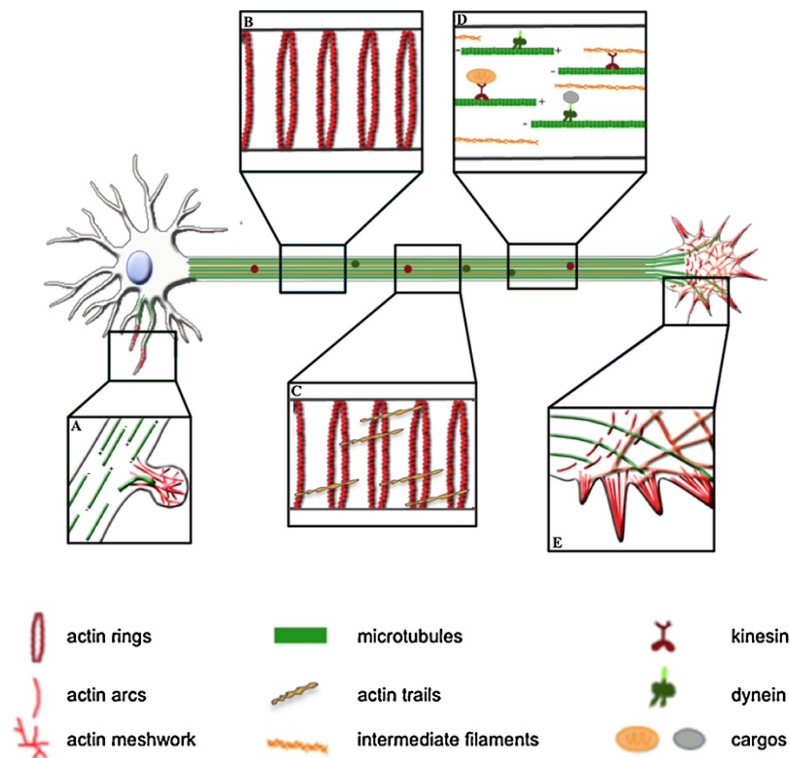


Figure 4 – Cytoskeleton organization of a developing neuron. Neurons have a tightly regulated cytoskeleton, which encloses three types of filaments, NFs, MTs and actin. **(A)** In vertebrate dendrites, MTs are mixed in orientation and actin filaments form patches within the dendritic spines **(B)** Actin rings, associated with adducin and spectrin, are found in both axons and dendrites **(C)** Actin trails are dynamic short filaments along the axon **(D)** In axons, MTs are polarized, with minus ends toward the cell body. Kinesin moves toward the plus end and dynein towards the minus end, while NFs form a structural mesh around MTs **(E)** At the axon's growing tip, actin forms lamellipodia and filopodia, with MTs extending into the growth cone, parallel to F-actin. Adapted from (Eira, Silva et al. 2016).

Both MTs and actin are very dynamic structures that continuously grow and shrink, facilitating the continuous remodeling of the cytoskeleton (Kevenaar and Hoogenraad 2015). The assembly, organization and remodeling of the actin and microtubule cytoskeleton are essential for different stages of neuronal development and for providing the structural basis of specialized axonal structures, such as axon branches, the AIS and presynaptic boutons (Kapitein and Hoogenraad 2015, Kevenaar and Hoogenraad 2015, Armijo-Weingart and Gallo 2017). Several developmental and neurological disorders, including Alzheimer and Parkinson's disease, involve defects in axonal transport, outgrowth and synapse functioning, which are often caused by disruptions of cytoskeleton-dependent processes (Kevenaar and Hoogenraad 2015).

3.1. Neurofilaments

NFs, also known as intermediate filaments (IFs), are heteropolymers measuring approximately 10nm in diameter and are highly stretch-resistant (Leterrier, Dubey et al. 2017, Prokop 2020). These filaments are particularly abundant in axons, whose primary

function is to provide mechanical support to the axon. They also play a crucial role in regulating axonal diameter and conductance (Kevenaar and Hoogenraad 2015). The loss of axonal NFs results in smaller caliber myelinated axons with slower conduction velocities (Costa, Pinto-Costa et al. 2018). Although NFs are abundant in neurons, their presence tends to be sparse in small axons. However, as axon diameter increases, the number of NFs rises considerably (Prokop 2020). Unlike actin and MTs, NFs are not polarized and do not require adenosine triphosphate (ATP) or guanosine triphosphate (GTP) for polymerization (Leterrier, Dubey et al. 2017).

3.2. Microtubules

MTs are present in all eukaryotic cells and are hollow and tubular polymers consisting of 13 protofilaments formed by alternating α and β -tubulin heterodimers (Kapitein and Hoogenraad 2015). These heterodimers bind GTP, which is hydrolyzed to guanosine diphosphate (GDP) after assembly. MTs are polarized structures, with a fast growing plus-end terminated by β -tubulin and slower growing minus-end (Kalil and Dent 2014, Kapitein and Hoogenraad 2015, Leterrier, Dubey et al. 2017). The plus end exhibits dynamic instability, involving cycles of polymerization and depolymerization, regulated by plus-end tracking proteins (+TIPS) like end-binding (EB) proteins. EB proteins autonomously track growing MT plus ends and interact with various others +TIPS through a conserved SxIP motif (Kapitein and Hoogenraad 2015). In axons, MTs are organized in stable parallel bundles with plus-end facing the axon tip, that run along the axons often accompanied by NFs (Kalil and Dent 2014, Kevenaar and Hoogenraad 2015, Prokop 2020). This unique organization is crucial for neuronal function, affecting the sorting of axonal and dendritic cargos (Kalil and Dent 2014, Kevenaar and Hoogenraad 2015).

MTs are typically nucleated by the gamma-tubulin ring complex (γ -TURC) at a microtubule-organizing center (MTOC), such as the centrosome, however, they can also be nucleated at other sites, such as the Golgi apparatus, though its role in axons is debated (Kapitein and Hoogenraad 2015, Leterrier, Dubey et al. 2017). Additionally, the severing of existing MTs by katanin and spastin contributes to axonal microtubule pool renewal (Leterrier, Dubey et al. 2017). These proteins promote collateral branch formation through different models: Spastin-driven local severing and Tau-regulated Katanin severing (Armijo-Weingart and Gallo 2017).

MTs provide structural support to axons and facilitate the transport of organelles and proteins to distal segments, essential for axon extension, branching, cell division and maintaining neuronal morphology. They serve as tracks for long-distance transport

of organelles and proteins to the distal part of the axon, offer mechanical support, and control local signaling events (Kapitein and Hoogenraad 2015, Leterrier, Dubey et al. 2017, Gallo 2024). MT defects can cause a wide range of nervous system abnormalities and neurodevelopmental disorders, linked to mutations in genes encoding for microtubule-associated proteins (MAPs) (e.g. Tau), MT severing proteins (e.g. spastin, katanin) and motor proteins (e.g. dynein, kinesin) (Kapitein and Hoogenraad 2015).

3.3. Actin

Actin is one of the most conserved and abundant proteins in eukaryotic cells, with three major isoforms (α, β, γ) that contribute to the dynamics and functions of actin filament-based structures in a neuron type-specific manner (Ladt, Ganguly et al. 2016, Gallo 2024). Actin exists both as soluble monomers (G-actin) as well as filaments (F-actin), in dynamic equilibrium, requiring ATP for nucleation and polymerization (Ladt, Ganguly et al. 2016, Leterrier, Dubey et al. 2017, Dubey, Jorgenson et al. 2018). Structurally, G-actin assembles into filaments with a helical twist (Gallo 2024). Actin filaments, approximately 8nm in diameter, are polarized and exhibit differential kinetics. They have a rapidly growing barbed end and a slower-growing pointed end. Monomers are added at the barbed end (plus tip), that is the primary site for polymerization, while on the opposite side, monomers dissociate at the pointed end (minus tip), main site for the depolymerization (Kalil and Dent 2014, Kevenaar and Hoogenraad 2015, Leterrier, Dubey et al. 2017, Gallo 2024). This polarization and the weak interaction between monomers allow actin filaments to rapidly shift between polymerization and depolymerization states and this dynamic nature is essential for various cellular functions (Kalil and Dent 2014). Within cells, filaments can be arranged in parallel (both barbed ends directed in the same direction) or anti-parallel (the barbed end of one filament aligns with the pointed end of another filament) (Gallo 2024).

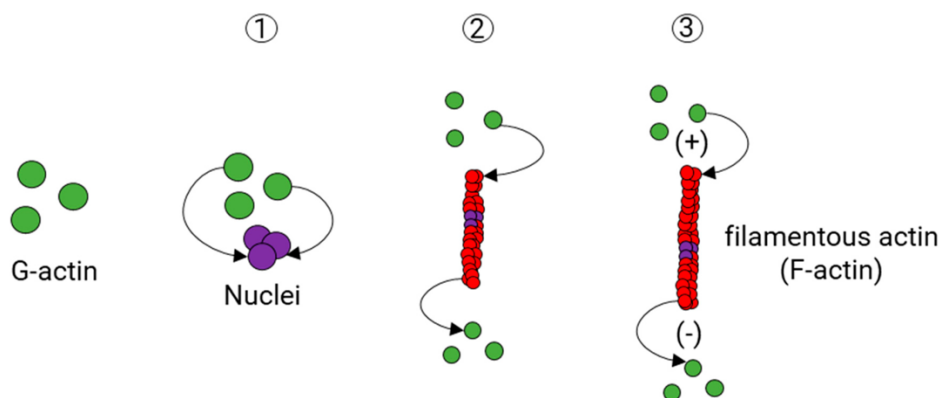


Figure 5 – Overview of F-actin formation. (1) G-actin associates and forms a nucleus suitable for elongation. (2) The proper formation of a nucleus elicits elongation of the filaments. (3) A steady state is achieved, actin treadmilling,

where new subunits of G-actin are added to the barbed end (+) and others are removed from the pointed end (-). G-actin, nuclei and F-actin are in green, purple and red respectively. Adapted from (Muñoz Lasso, Romá et al. 2020).

In neurons, F-actin is also important for providing scaffolding at synapses and remodeling synaptic structures during neuronal plasticity, explaining why actin is particularly enriched in growth cones (Kevenaar and Hoogenraad 2015, Ladt, Ganguly et al. 2016, Leite and Sousa 2016). The regulation of actin dynamics is crucial for axon guidance and branching (Kalil and Dent 2014).

Recent advancements in super-resolution microscopy have revealed previously unknown actin structures along the axon shaft. These include dynamic actin pools that continuously polymerize and depolymerize, termed actin trails, the periodic actin rings that circumferentially wrap underneath the plasma membrane and the actin hotspots (Leterrier, Dubey et al. 2017). These structures contribute to the organization and function of the axon (Ladt, Ganguly et al. 2016, Dubey, Jorgenson et al. 2018).

Numerous actin-binding proteins (ABPs) control actin dynamics by sequestering G-actin, nucleating new filaments (formins and Arp2/3), capping ends (adducin), severing filaments (coronin and cofilin), bundling or stabilizing F-actin (anillin) and crosslinking with actin (IQGAP) (Brandt and Grosse 2007, Piekny and Maddox 2010, Zhang and Maddox 2010, Tuan and Lee 2020, Palani, Ghosh et al. 2021). The actin-related protein 2/3 (Arp2/3) complex is a key ABP that nucleates branched network, essential for the formation of axonal filopodia and collateral branches. Specifically, Arp2/3 binds to an existing “mother” filament and nucleates a new filament with its pointed end embedded within the complex, resulting in a branched organization of actin filaments. In contrast, formins nucleate actin filaments *de novo*, playing a critical role in neurite initiation and filopodia formation (Arnold and Gallo 2014, Kalil and Dent 2014, Gallo 2024). The Ena/Vasodilator-stimulated phosphoprotein (Ena/VASP) proteins, binds to the barbed end of actin filaments, enhancing their elongation by antagonizing capping proteins that would otherwise inhibit this process, modulating morphology and behavior of these structures, while actin depolymerizing factor/cofilin (ADF/cofilin) increases actin depolymerization and severs filaments to increase actin turnover, promoting new filament assembly. Additionally, myosin II is an actin-binding mechanochemical enzyme that plays a fundamental role in regulating cellular contractility and is a prevalent binding partner for actin filaments within cells (Kalil and Dent 2014, Armijo-Weingart and Gallo 2017, Gallo 2024).

Actin plays critical roles in shaping and maintaining cell morphology and supporting various cellular functions, including cell motility, division, signaling and intracellular transport. In neurons, actin is essential for establishing neuronal polarity, growing neurites and stabilizing synaptic structures, as previously discussed (Xu, Zhong et al. 2013, Ladts, Ganguly et al. 2016, Leterrier, Dubey et al. 2017, Dubey, Jorgenson et al. 2018).

3.3.1. Anillin

Anillin (ANLN) is a conserved protein with a multi-domain structure, involved in cytoskeleton dynamics, especially during cytokinesis. It acts as a scaffold protein, binding F-actin, non-muscle myosin II (NMII), septins, plasma membrane lipids, as well as regulatory players, including active RhoA. Mutations or depletion of ANLN result in disorganized contractile rings (Zhang and Maddox 2010, Rehaian and Maddox 2015, Tian, Diao et al. 2015). ANLN includes a myosin-binding domain (MBD) and an actin-binding domain (ABD) at the N-terminal, a Rho-binding domain (RBD) and a Pleckstrin Homology (PH) domain at the C-terminal (Tuan and Lee 2020, Lebedev, Chan et al. 2023).

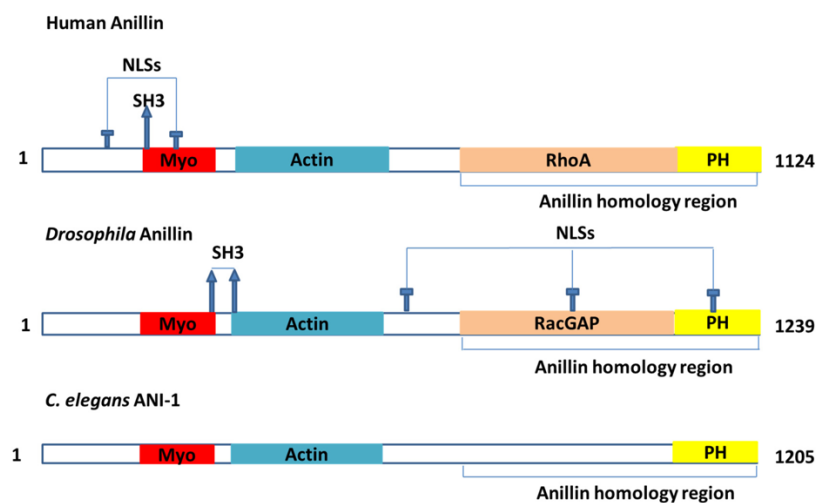


Figure 6 – Structure of ANLN. In its structure, ANLN includes several domains, being the majority conserved between species; Myo, myosin-II-binding domain; Actin, actin-binding domain; NLS nuclear localization signal; SH₃ Src-homology-3-binding consensus sequences. PH, Pleckstrin homology domain; RhoA, region that interacts with the GTPase RhoA; RacGAP, region that interacts with the RacGAP50C component of the centralspindlin complex. Adapted from (Tuan and Lee 2020).

ANLN binds and bundles F-actin specifically during cell division, enhancing actomyosin contractility and promoting F-actin polymerization by stabilizing diaphanous-related formin 2 (mDia2) in the active form, after binding RhoA, thus promoting the continuous polymerization of actin. This process is crucial for the efficient formation and

constriction of the contractile ring, leading to successful cytokinesis (Piekny and Maddox 2010, Zhang and Maddox 2010, Tuan and Lee 2020).

Human ANLN mediates actin ring formation and contractility *in vitro* independently of myosin by generating contractile forces through actin filament depolymerization (Kučera, Siahaan et al. 2021). ANLN enhances contractility by maximizing overlap lengths between bundled actin filaments, which is further increased by actin disassembly. When actin filaments form a ring, this contractility leads to ring constriction. As a non-motor actin crosslinking and scaffolding protein, ANLN efficiently converts actin filaments depolymerizing into sliding-driven contraction of actin bundles (Kučera, Siahaan et al. 2021, Lebedev, Chan et al. 2023).

ANLN is highly expressed in adult neuronal tissues, indicating potential roles in post-mitotic neurons and central nervous system development (Rehain and Maddox 2015). Recent studies show that ANLN is recruited to the leading-edge during cell migration and neurite growth, through a physical association mediated via RhoG. The actin-binding domain of ANLN is sufficient to stabilize F-actin by antagonizing the F-actin severing activity of cofilin (Tian, Diao et al. 2015).

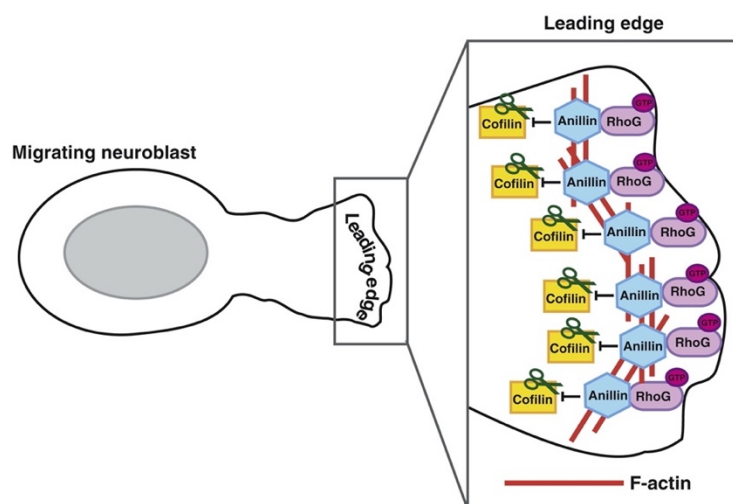


Figure 7 – Anillin stabilizes F-actin at the leading edge of migrating neuroblasts. A member of the Rho family of small GTPases, RhoG, recruits anillin to the leading edge of migrating neuroblasts. There, ANLN binds active RhoG and stabilizes F-actin. Stabilization of actin is achieved by ANLN's ability to inhibit both actin monomer dissociation and the F-actin severing activity of cofilin. Adapted from (Rehain and Maddox 2015).

3.3.2. IQGAP

The IQGAP (IQ motif-containing GTPase-activating proteins) family of proteins, which includes IQGAP1, IQGAP2 and IQGAP3, is a group of scaffolding proteins involved in a wide range of cellular processes, particularly in the regulation of actin dynamics and

cytoskeleton organization (Brandt and Grosse 2007, Le Clainche, Schlaepfer et al. 2007, White, Erdemir et al. 2012, Nakamura, Hui et al. 2023, Thines, Roushar et al. 2023). These proteins share a similar multidomain structure that includes an N-terminal calponin-homology domain (CHD), a coiled-coil repeat region (CC), a proline-rich motif-binding region (WW), an IQ motif-containing domain, a GTPase-activating protein (GAP)-related domain (GRD) and a RasGAP domain in the C-terminal (White, Erdemir et al. 2012, Nakamura, Hui et al. 2023, Thines, Roushar et al. 2023). These domains allow IQGAP proteins to interact with various binding partners and modulate key cellular functions. All three isoforms share some common binding partners, including actin (binds to the CHD), the Ca^{2+} -binding protein calmodulin (to the IQ motifs), the small Rho GTPases Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division control protein 42 homolog (Cdc42) (that bind to the GRD) (Le Clainche, Schlaepfer et al. 2007, White, Erdemir et al. 2012, Thines, Roushar et al. 2023).

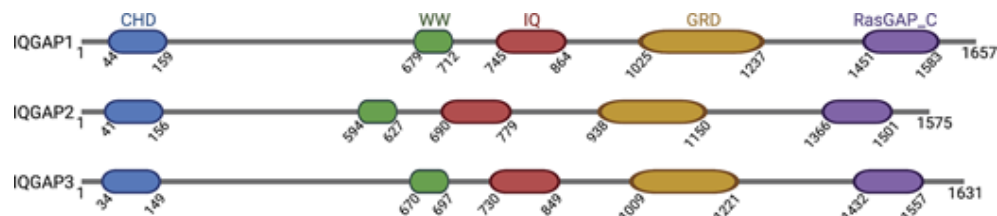


Figure 8 – Model of the isoforms of human IQGAPs. The three isoforms (IQGAP1, IQGAP2 and IQGAP3) share a similar multidomain composition, each containing a calponin homology domain (CHD), WW domain, IQ domain containing several IQ motifs, GTPase-activating protein (GAP)-related domain (GRD) and a RasGAP domain. Adapted from (Thines, Roushar et al. 2023).

IQGAP2 and IQGAP3 share 62% and 59% of homology with IQGAP1, respectively. Although exhibiting some degree of homology in their structure, they differ in many aspects, such as function, tissue distribution, and subcellular localization. IQGAP1 is the most well-characterized member of this family and is ubiquitously expressed, whereas IQGAP2 and IQGAP3 have more restricted expression patterns, being predominantly expressed in the liver and brain, respectively (White, Erdemir et al. 2012, Hedman, Smith et al. 2015, Thines, Roushar et al. 2023).

One of the primary roles of IQGAP proteins is the regulation of actin cytoskeleton dynamics. IQGAP1 binds directly to actin filaments through its CHD, promoting the crosslinking of these filaments. This interaction is crucial for maintaining the stability and organization of the actin cytoskeleton. Notably, when IQGAP1 is tethered to lipid membranes, it can induce the bending of individual actin filaments into curved geometries, forming ring-like structures. This ability to bend actin filaments distinguishes

IQGAP1 from other actin-binding proteins, which typically form rings by bundling multiple filaments together, such as anillin (Brandt and Grosse 2007, Palani, Ghosh et al. 2021).

In addition to its role in actin dynamics, IQGAP1 also regulates microtubule dynamics by interacting with the microtubule plus-end cytoplasmic linker protein 170 (CLIP-170). The IQGAP1/CLIP-170 complex mediates the transient capture of MTs at the cortical regions and regulates dendritic arbor growth in both cortical and hippocampal neurons. Another mechanism by which IQGAP1 modulates MTs is via adenomatous polyposis coli (APC). IQGAP1/APC are necessary for the immobilization of CLIP-170 at leading edges, suggesting that IQGAP1 and APC work together to stabilize the plus end of MTs (White, Erdemir et al. 2012).

IQGAP1 also plays a significant role in neurite outgrowth, synaptic plasticity and dendritic spine formation (Hedman, Smith et al. 2015). In hippocampal neurons, a reduction of this protein leads to decreased spine density and impairs both synaptic and memory formations. IQGAP1 achieves this by interacting with neuronal cadherin (N-cadherin) and components of actin polymerization machinery (Wang, Watanabe et al. 2007, White, Erdemir et al. 2012, Jausoro, Mestres et al. 2013).

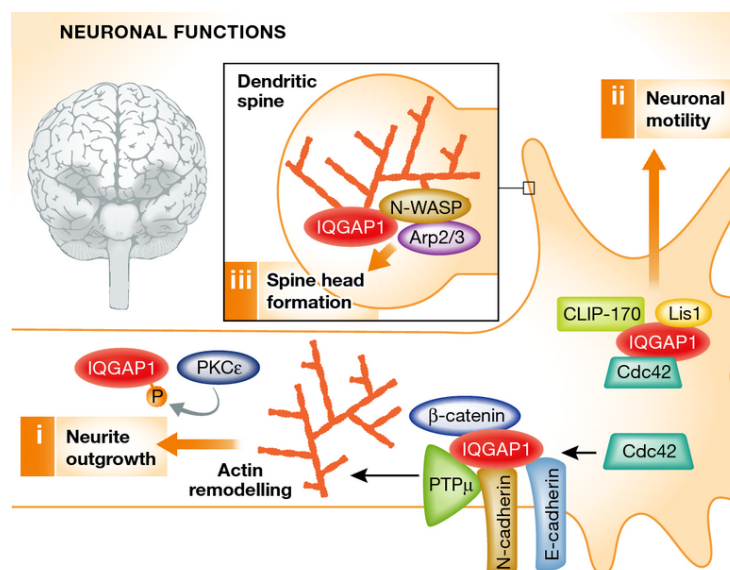


Figure 9 – The role of IQGAP1 in neurons. (i) PTPμ, IQGAP1, N-cadherin, E-cadherin and β-catenin form a complex in ganglion cells. Cdc42 promotes the interaction of IQGAP1 with PTPμ to stimulate actin remodelling and neurite outgrowth (ii) IQGAP1 forms a complex with active Cdc42, Lis1 and CLIP-170 that appears necessary for cerebellar neuronal motility (iii) In hippocampal neurons IQGAP1/N-WASP and Arp2/3 complex promotes dendritic spine head formation. Adapted from (Hedman, Smith et al. 2015).

IQGAP1 stimulates branched actin filament assembly through its interaction with neuronal Wiskott-Aldrich syndrome (N-WASP), which then activates the Arp2/3 complex

to promote actin polymerization and branching, an essential process for the formation of lamellipodia at the leading edge of migrating cells (White, Erdemir et al. 2012, Thines, Roushar et al. 2023). The CHD domain of IQGAP1 binds actin filaments with high affinity, bending them into a high curvature in an anti-clockwise manner. IQGAP1 recruits and activates N-WASP in a Cdc42-like manner, relieving the autoinhibited fold of N-WASP which then leads to the activation of the Arp2/3 complex. The C-terminal of IQGAP1 activates N-WASP, while the N-terminal domain can inhibit this activation, suggesting an autoinhibited conformation. Phosphorylation of a serine residue may relieve this autoinhibition, allowing N-WASP to interact with Arp2/3 to promote actin filament formation. As actin filaments are inherently helical, some bound proteins can change the pitch of actin and induce twisting or bending forces to give rise to high-order actin assemblies such as rings (Le Clainche, Schlaepfer et al. 2007, Nakamura, Hui et al. 2023, Thines, Roushar et al. 2023).

IQGAP3, while sharing many functional similarities with IQGAP1, such as binding to actin filaments and influencing neurite outgrowth, has unique roles as well. This isoform is specifically located in the contractile ring during cytokinesis and interacts with ANLN, a key protein of this process. The localization of IQGAP3 to the contractile ring is dependent on ANLN, and together they ensure proper cytokinesis (Adachi, Kawasaki et al. 2014, Hedman, Smith et al. 2015). The suppression of IQGAP1 also has similar inhibitory effects on cytokinesis and simultaneous suppression of both IQGAP1 and IQGAP3 induces severe effects (Brandt and Grosse 2007). IQGAP2, although less studied, it is also involved in cytoskeleton regulation (Adachi, Kawasaki et al. 2014).

In summary, the IQGAP family of proteins plays a critical role in the regulation of the actin and microtubule cytoskeleton, influencing various cellular processes such as cell migration, cytokines, dendrite formation, synaptic plasticity, memory formation and neurite outgrowth (Hedman, Smith et al. 2015).

4. The Membrane Periodic Skeleton (MPS)

Upon the development of super-resolution microscopy, Stochastic Optical Reconstruction Microscopy (STORM) and Stimulated Emission Depletion Microscopy (STED) were used to study the organization of the axonal actin and spectrin cytoskeleton in hippocampal neurons, both *in vitro* and *in vivo* (Arnold and Gallo 2014, Leterrier, Dubey et al. 2017). Surprisingly, what was once thought to be a patchy distribution of actin along the axon shaft turned out to be a periodic cytoskeleton assembly designated of membrane periodic skeleton (MPS). The MPS includes actin organized as rings, periodically interspaced by approximately 190-200nm and connected by tetramers of

α II/ β II-spectrin (Xu, Zhong et al. 2013, Arnold and Gallo 2014, Leterrier, Dubey et al. 2017, Dubey, Jorgenson et al. 2018).

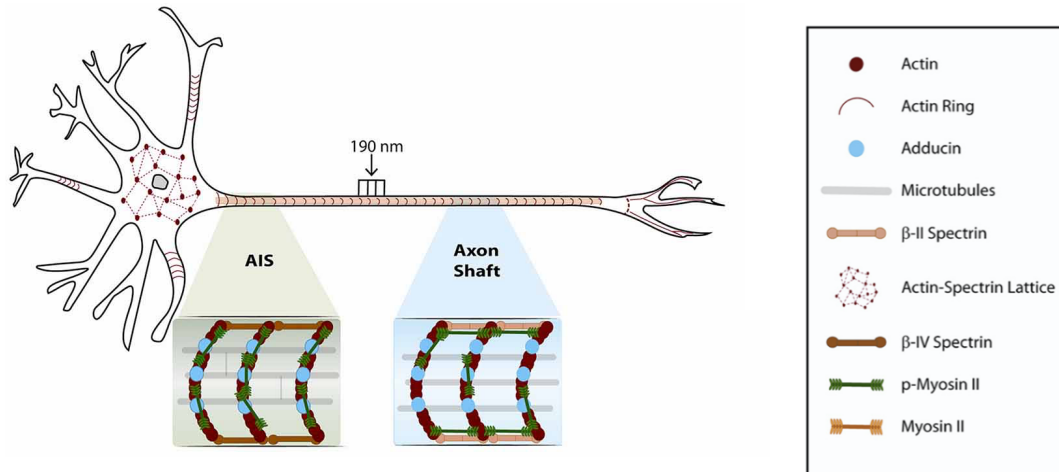


Figure 10 – Schematic representation of the MPS in neurons and its associated proteins. The MPS is composed of actin rings evenly spaced by spectrin, periodically every 190nm throughout the entire length of the axon and in a small fraction, also in dendrites. The actin rings are composed by filaments of actin capped by adducin. Adapted from (Costa, Pinto-Costa et al. 2018).

The MPS emerges early during axon development in cultured hippocampal neurons and propagates from proximal regions to distal axonal ends and in a small fraction of dendrites, it is present in every neuron type inspected so far, either belonging to the CNS or PNS (Xu, Zhong et al. 2013, Lukinavičius, Reymond et al. 2014, Zhong, Gutierrez et al. 2014, D'Este, Kamin et al. 2015, D'Este, Kamin et al. 2016, He, Zhou et al. 2016, Leite, Sampaio et al. 2016, Costa and Sousa 2021). Unmyelinated axons in the CNS and myelinated sciatic nerve axons contain this periodic lattice (D'Este, Kamin et al. 2016, Leterrier, Dubey et al. 2017). The MPS is not exclusive to neurons, it is also present in different glial cell types, including astrocytes, microglia, NG2 glia, Schwann cells and oligodendrocytes (Zhong, Gutierrez et al. 2014, D'Este, Kamin et al. 2016, Hauser, Yan et al. 2018). This unique arrangement is observed in axons across species, from invertebrates (*C. elegans* and *D. melanogaster*) to vertebrates (chicken, mice, rats and humans), supporting its importance for neuronal function (Xu, Zhong et al. 2013, Arnold and Gallo 2014, Lukinavičius, Reymond et al. 2014, Zhong, Gutierrez et al. 2014, D'Este, Kamin et al. 2016, He, Zhou et al. 2016, Leite and Sousa 2016, Leterrier, Dubey et al. 2017, Dubey, Jorgenson et al. 2018, Costa and Sousa 2021, Gallo 2024).

While the MPS exhibits a unique ultrastructure and arrangement, it shares some structural components with the erythrocyte membrane-cortical cytoskeleton (EMCC)

(Unsain, Stefani et al. 2018, Wang, Simon et al. 2019). Spectrins, are found as heterotetramers composed of two antiparallel α/β dimers, organized in a long rod shape, (Arnold and Gallo 2014, Leite and Sousa 2016, Costa and Sousa 2021, Gallo 2024). Spectrin subunit composition differ according to the region of the axon. Spectrin tetramers formed by $\alpha II/\beta IV$ -spectrin are found in the AIS, whereas the axon shaft is enriched in $\alpha II/\beta II$ -spectrin (Leterrier, Dubey et al. 2017, Costa and Sousa 2021). In the dendrites, $\alpha II/\beta III$ -spectrin predominates (Stankewich, Gwynn et al. 2010). This protein is crucial for axons, providing structural support to prevent axons from breaking in response to naturally occurring forces (Hammarlund, Jorgensen et al. 2007, Gallo 2024). The spacing of actin/ βII spectrin network in erythrocytes is approximately 80nm, suggesting a relaxed lattice network in these cells, unlike in axons, where it may be under tensile stress (Dubey, Jorgenson et al. 2018).

Adducin is an ABP that has three main functions in regulating actin dynamics: it promotes the bundling of actin filaments, caps the barbed ends of actin filaments, thus inhibiting the incorporation of new monomers, and crosslinks with spectrin tetramers. Previous findings have shown that adducin and NMII colocalize with the actin rings and they can regulate the diameter of the actin rings (Xu, Zhong et al. 2013, Leite, Sampaio et al. 2016, Leite and Sousa 2016, Costa, Sousa et al. 2020, Gallo 2024).

Besides actin, spectrin, ion channels, adducin, and NMII, recent proteomic and mass spectrometry studies, identified the presence of more than 400 possible components of the MPS, such as motor proteins, cell adhesion molecules, signalling molecules. Initially, it was believed that the MPS primarily provided mechanical stability to axons (Xu, Zhong et al. 2013, Leite, Sampaio et al. 2016, Costa and Sousa 2020, Wang, Li et al. 2020, Zhou, Han et al. 2020). However, in line with the high number of components associated with the MPS, it has become clear that the MPS plays a crucial role in various aspects of neuronal biology. These include serving as an inter- and intra-signaling platform, regulating axon diameter, which affects axonal transport efficiency and electrophysiological properties, participating in the assembly and function of the AIS and controlling the stability of axonal MTs (Leite and Sousa 2016, Costa, Sousa et al. 2020).

The disassembly of the MPS has also been identified as an early factor in axon degeneration (Costa and Sousa 2021, Macarrón-Palacios, Hubrich et al. 2024). The implication of the MPS in so many functions of the neuron strongly supports its relevance for neuronal biology. Although the advances of super-resolution microscopy highly contributed to the development of this field, there are still questions that are not clear.

The process of actin nucleation and polymerization into rings and the components involved are some of them. Due to the observation that adducin, an ABP capping the actin, is also periodically distributed within the rings, the first model proposes that actin is organized as short filaments. In contrast, in 2019, platinum replica electron microscopy (PREM) and super-resolution studies have shown that actin rings are organized as two long intertwined filaments. This braided actin arrangement was suggested to provide more stability to the structure, as it is expected to be stiffer than a ring made of short actin filaments (Vassilopoulos, Gibaud et al. 2019).

As previously stated, recent studies using *in vitro* assays have found that proteins including IQGAP and ANLN can influence the bend or curvature of actin filaments (Nakamura, Hui et al. 2023). Despite these insights, the mechanism of nucleation, assembly, and maintenance of the actin rings and the components involved in the circularization of the rings within the MPS have not yet been identified.

Objectives

To provide answers to some of these questions, our main aim is to unravel the mechanisms underlying MPS biogenesis, maintenance and function, focusing mainly on the role of anillin and IQGAP in the circularization of the axonal actin rings. The following objectives were delineated:

- i. Determine the endogenous levels of these proteins in both the formation (DIV5) and maintenance (DIV14) of the MPS;
- ii. Develop and validate a CRISPR-Cas9 strategy to knockout ANLN and IQGAP;
- iii. Clarify the role of ANLN and IQGAP in the actin circularization for the actin rings within the MPS.

Together, this knowledge will provide the basis for studies on the circularization of the axonal actin rings.

Methods

1. Animals

The animals were handled according to both the National Decreto-lei no. 113-2013 and the European Union Directive 2010/63/EU regulations. All animals were bred and housed within the i3S animal facility and maintained under a 12-hour light/dark cycle, fed with hard pellets and distilled water, *ad libitum*. Environmental conditions were maintained at 20 to 24°C while the humidity level was carefully maintained at 45-65%.

2. Primary Neuron Cultures

2.1. Coatings

Round coverslips (1.5H 13mm; Marienfeld, #0117530) were coated with 50mg/mL poly-L-lysine hydrobromide (PLL) (Sigma-Aldrich, #P2636-100MG) in 24-well plates (VWR Corning, #734-1606). The incubation with PLL was preceded by a plasma cleaning process using a Tergeo Plasma Cleaner (Tergeo Plasma Cleaner, Pie Scientific (TG100) to remove any residues and organic contaminants, thus enhancing the surface cleanliness and promoting optimal adherence for subsequent experimental procedures. This process involved exposing the coverslips to oxygen plasma for 1.5 minutes at 75W under vacuum conditions. Afterwards, the plate was incubated with 500 μ L of PLL/well for 1 hour at 37°C in a 5% CO₂ incubator, and finally, the coverslips were washed two times with 1xPhosphate-buffered saline (PBS).

2.2. Hippocampal Neuron Cultures

Wistar female rats, pregnant at embryonic day 18 (E18) were euthanized using a CO₂ chamber, following the guidelines recommended by the animal facility. The embryos were carefully removed to extract the hippocampi. Rat hippocampal neuron cultures were performed, following an adapted version of the Kaech and Banker protocol (Kaech and Banker 2006). The dissected hippocampi were collected in Hank's balanced salt solution (HBSS) (Sigma-Aldrich, #H9394-500mL) in ice, to ensure optical preservation conditions. In a cell incubator, at 37°C and 5% CO₂, after washing three times with HBSS, the hippocampi were digested 15 minutes in 0.06% trypsin from porcine pancreas solution (Sigma-Aldrich, #T4799-5G) in HBSS. Subsequently, 10% Fetal Bovine Serum (FBS) (Sigma Aldrich, #F9665-500mL) in HBSS, was added to stop the trypsin activity. Neurobasal medium (Thermo Fisher Scientific, #21103-049 500mL) was added to the hippocampi and was mechanically dissociated. The resulting solution was filtered through a 70 μ m cell strainer (Corning, #352350), to ensure the removal of any remaining

undissociated tissue. Cells were counted in a Neubauer Chamber (VWR International, #631-0696), followed by a 1:1 dilution with 0.4% trypan blue (Sigma-Aldrich, #T8154).

Neurons were cultured in Neurobasal medium supplemented with 2% B27 (Thermo Fisher Scientific, #17504044), 1% P/S (penicillin/streptomycin) (Alfagene, #15140-122) and 2mM L-glutamine (Thermo Fisher Scientific, #25030024). Cells were plated onto the pre-coated coverslips in 24 well-plates (VWR Corning, #734-1606). Different densities were plated accordingly to the specific experimental objectives and requirements. To analyze endogenous levels of the proteins and to perform transient transfections, 10,000 and 50,000 cells were plated, respectively. Throughout the experiments, cells were maintained at 37°C under a 5% CO₂ atmosphere.

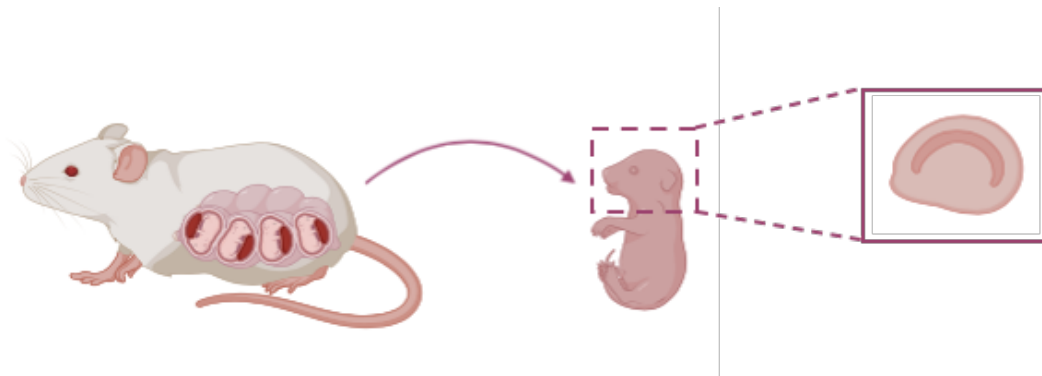


Figure 11 – Representation of the steps involved in the dissection of hippocampal neurons. Pregnant female rats are euthanized at E18, the embryos are carefully removed, and the hippocampi are extracted. Made in BioRender.

3. Cell Line Culture

To validate the CRISPR-Cas 9 approaches, RAT-2 (ATCC, #CRL-1764), a rat fibroblast cell line, was used. The cells were suspended in complete medium, consisting of Dulbecco's Modified Eagle Medium (DMEM) High glucose (Alfagene, #10569010), supplemented with 10% FBS (Sigma Aldrich, #F9665-500mL) and 1% P/S (Alfagene, #15140-122) within a T25 flask (Corning, #430639). The cell line was maintained under optimal conditions throughout the experiment. Cells were routinely monitored for viability and confluence. Based on the experimental requirements, the cell culture was either split into different flasks to ensure consistent growth and prevent over-confluence or cryopreserved for future use.

4. Immunocytochemistry (ICC)

Upon dissection, as previously described, hippocampal neurons were plated at either 10,000 cells/well for the analyzes of the endogenous levels of the proteins or 50,000 cells/well for the transfections in a 24-well plate containing properly PLL-coated glass coverslips, respectively.

In all conditions, after removing the complete media, cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) (Electron Microscopy Sciences #15713-1L) in PBS (pH of 7.4) for 15 minutes at room temperature (RT). Upon washing, cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, #T9284-100ML) in PBS for 3 minutes at RT, following an incubation with 200mM ammonium chloride (NH₄Cl) (VWR Merck, #1.01145.0500) prepared in water for also 3 minutes at RT to quench the autofluorescence. For blocking purposes, the cells were treated with 10% FBS in PBS and incubated 1 hour at RT. The coverslips were carefully removed from the wells and transferred to a humid chamber. The primary antibodies (Table 1), were diluted in blocking buffer and 50μL was added to each coverslip, incubating at 4°C overnight (O/N). Then, coverslips were washed 3x with PBS and secondary antibodies (Table 2) were added for 1 hour at RT, after being diluted with the blocking buffer.

To analyze the actin cytoskeleton, a solution of phalloidin (Abberior STAR 635, #ST635) was added, after the secondary incubation, to each coverslip for 30 minutes at 37°C. Coverslips were mounted using either a mounting media with 4',6-diamidino-2-phenylindole (DAPI) (Ibidi, #50011), or a 90% glycerol mounting media for STED samples. The process was completed by sealing the coverslips with clear nail polish.

Table 1 – Primary antibodies used for ICC. Host, dilution and source of each antibody.

Primary Antibody	Host	Dilution	Source
<i>βIII</i> –tubulin	Mouse	1:1000	Promega, #G712A
<i>βIII</i> –tubulin	Guinea pig	1:1000	SYSY, #302304
Anti-IQGAP1	Mouse	1:50	Santa Cruz, #376021
Anti-IQGAP1	Mouse	1:50	BD Transduction Laboratories, # 610611
Phospho-Myosin Light Chain 2	Rabbit	1:50	Cell Signaling Technology/ #3674

Anti β II –spectrin	Mouse	1:100	BD Transduction Laboratories, # 612562
Anti-Anillin	Rabbit	1:50	GeneTex, #GTX107742

Table 2 – Secondary antibodies used for ICC. Host, dilution and source of each antibody.

Secondary Antibody	Host	Dilution	Source
Anti-Rabbit 488	Donkey	1:200	Jackson ImmunoResearch, #711545152
Anti-Rabbit 580	Goat	1:200	Abberior STAR, #41367
Anti-Rabbit 635	Goat	1:200	Abberior STAR, #53399
Anti-Mouse 580	Goat	1:200	Abberior STAR, #52403
Anti-Mouse 635	Goat	1:200	Abberior STAR, #40734
Anti-Guinea Pig 488	Donkey	1:200	Jackson ImmunoResearch, #706545148

Table 3 – Probe-conjugated dyes used for ICC. Host, dilution and source of each conjugated dye.

Conjugated dyes	Host	Dilution	Source
Anti-Rabbit Rhodamine-conjugated phalloidin 635	Goat	1:50	Abberior STAR, #ST635

5. CRISPR-Cas9

CRISPR-Cas9 gene editing was employed to target and modify ANLN and IQGAP in RAT-2 cell line and hippocampal neurons. We used Geneious software to analyze potential guide RNA targeting distinct exons in ANLN and IQGAP. The target sequences, described in Table 4, displayed high on-target activity and low off-target scores and were used to synthesize dual and single gRNAs. The gRNAs were designed to bind to the ANLN and IQGAP specifically, and the Cas9 nuclease was utilized to induce a double-strand break at this location, only in the presence of a specific sequence on the non-

targeted DNA strand, known as the protospacer adjacent motif (PAM). The gRNA and Cas9 were delivered into cells using a plasmid-based system, one for gRNA (dual and single for each gene) and the other one for Cas9, as described below.

Following lipofectamine transfection, cells were selected with puromycin and expanded. Successful editing was confirmed by PCR or DNA genomic sequencing analysis. When a single guide RNA (sgRNA) is used, small insertions or deletions (indels) are expected to be introduced at the target site, which can be only detected through genomic DNA sequencing. When a dual guide is used, and if both guides are properly working, a larger fragment of DNA should be deleted, which will be detected by PCR and agarose gel electrophoresis (Ma, Zhang et al. 2014).

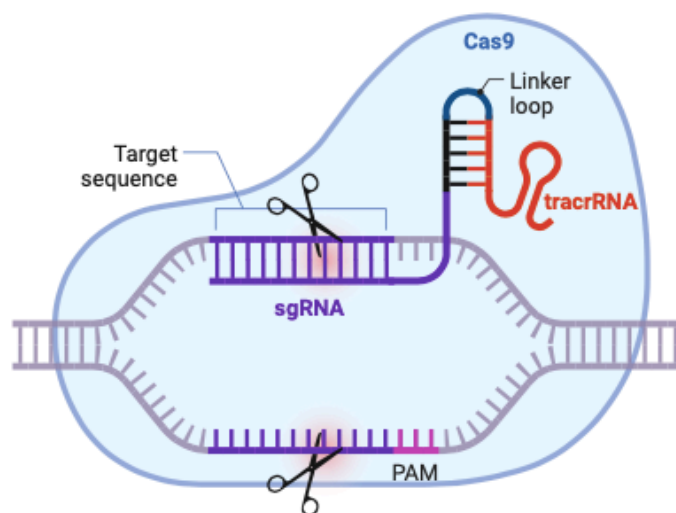


Figure 12 – CRISPR-Cas9 process. Made in BioRender.

5.1. Plasmids used in ANLN and IQGAP CRISPR- gene editing

Cas9 Vector:

The vector VB220503-1078vkb (pRP[Exp]-Puro-CBh-hCas9), from VectorBuilder, is a 8908 base pairs (bp) size mammalian expression vector. hCas9 is expressed under the chicken beta-actin hybrid (CBh), followed by puromycin resistance under the cytomegalovirus (CMV) promoter (Figure 13).

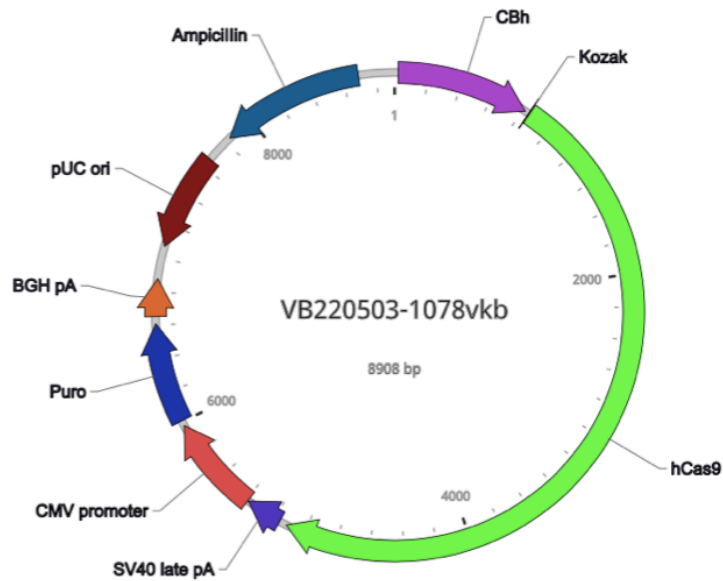


Figure 13 – VB220503-1090vtr plasmid construct used for Cas9.

Vector containing guide RNA to the Rosa26 locus (CTRL):

Guide RNAs targeting the Rosa26 locus has been used as a control in CRISPR experiments mainly because as it does not contain coding sequences, gene editing does not cause unintended effects. The vector VB230127-1090vtr (pRP[gRNA]-EGFP-U6>{guide RNA control_ROSA26}), from VectorBuilder, is a mammalian expression vector with 3914 bp. It has one gRNA with the sequence GCAGATCACGAGGGAAGAAG, under the control of the Human U6 small nuclear promoter. This vector contains enhanced green fluorescent protein (EGFP), allowing the visualization of transfected cells, under the promoter human phosphoglycerate Kinase (hPGK), as represented in Figure 14.

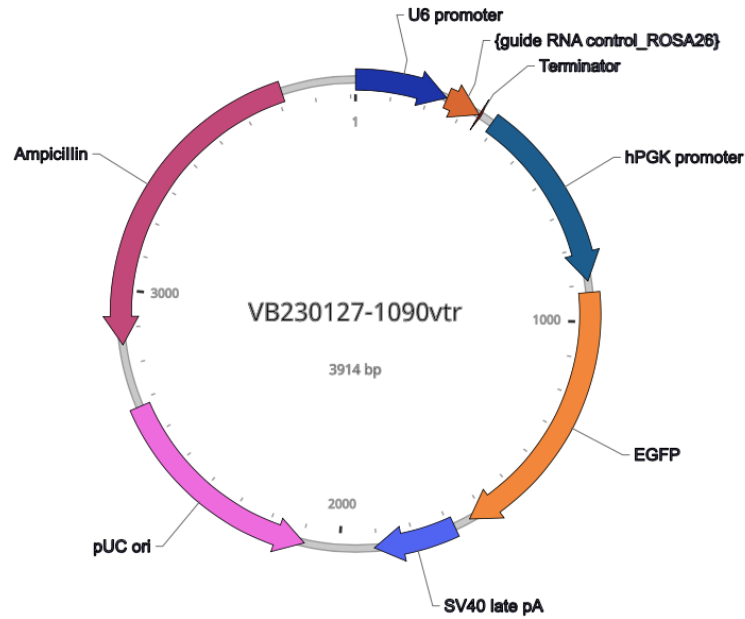


Figure 14 – VB230127-1090vtr plasmid construct used for Rosa26 (CTRL).

Vector containing ANLN dual gRNA:

The vector VB221115-1124hkw (pRP[2gRNA]-EGFP-U6>{g1ANLN}-U6>{g2ANLN}) is a mammalian dual gRNA expression vector with 4274 bp. It has two guides: gRNA1 (g1ANLN) with the sequence AGTGGTTCTTGAATGCTGCC and a gRNA2 (g2ANLN) with the sequence CTGGAGTTGTTAGCCGCCAT, targeting sequences in exon 2 of ANLN. Both gRNAs are expressed under the control of the Human U6 small nuclear promoter. This vector contains EGFP, under the promoter hPGK, as depicted in Figure 15.

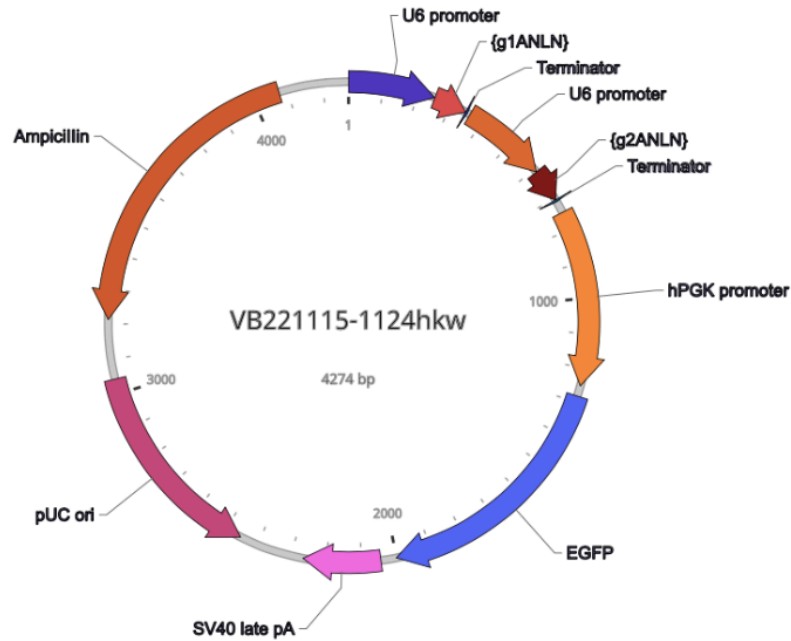


Figure 15 – VB221115-1124hkw plasmid construct used for ANLN dual-gRNA.

Vector containing ANLN single gRNA:

The vector VB240213-139jgz (pRP[gRNA]-EGFP-U6>{RngRNAANLN_B_2982}) is a mammalian sgRNA with 3914 bp. It has one gRNA with the sequence TTGTTCTGACAAAATCGAAC, targeting the sequence in exon 2 of ANLN. The gRNA is expressed under the control of the Human U6 small nuclear promoter. This vector contains EGFP, under the promotor hPGK, as indicated in Figure 16.

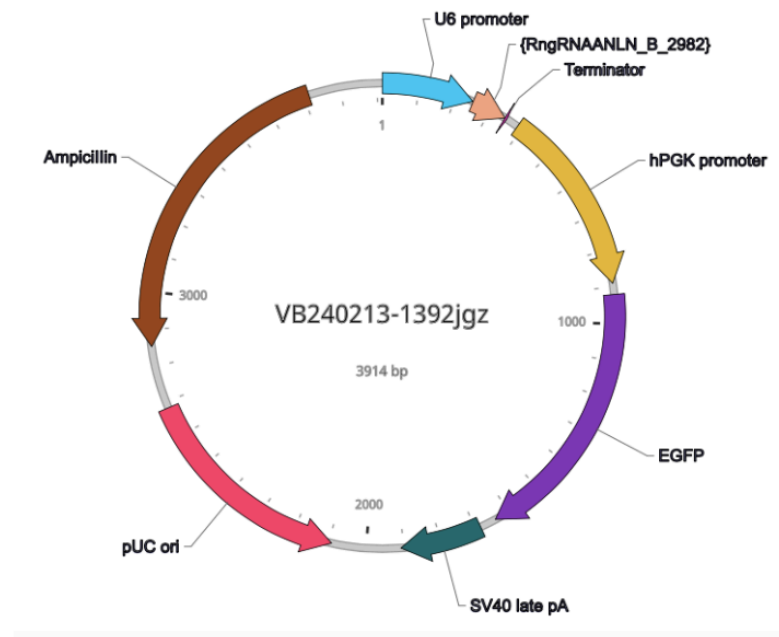


Figure 16 – VB240213-1392jgz plasmid construct used for ANLN sgRNA.

Vector containing IQGAP1 dual gRNA:

The vector VB221115-1127cds (pRP[2gRNA]-EGFP-U6>{g1IQGAP1}-U6>{g2IQGAP1}) is a mammalian dual-gRNA expression vector with 4274 bp. It has two guides: g1IQGAP1 with the sequence CCCCTAGGCTCTCTAAAGTAAAA and a g2IQGAP1 with the sequence GCTCTGGTCCTTCGAAAGAGCGG, targeting sequences in exon 2 of IQGAP1, under the control of the Human U6 small nuclear promoter. This vector contains EGFP under the promoter hPGK (Figure 17).



Figure 17 – VB221115-1127cds plasmid construct used for IQGAP1 dual-gRNA.

Vector containing IQGAP1 single gRNA:

The vector VB240229-1273vzu (pRP[gRNA]-EGFP-U6>{RN_gRNA_IQGAP1_B_guide23}) is a mammalian sgRNA expression vector designed for CRISPR-Cas9 with 3914 bp. It has one gRNA with the sequence GGTGTGGTCCGGCCGCACTA, targeting the sequence in exon 2 of IQGAP1. The gRNA is expressed under the control of the Human U6 small nuclear promoter. This vector contains EGFP, under the promoter hPGK, as represented in Figure 18.

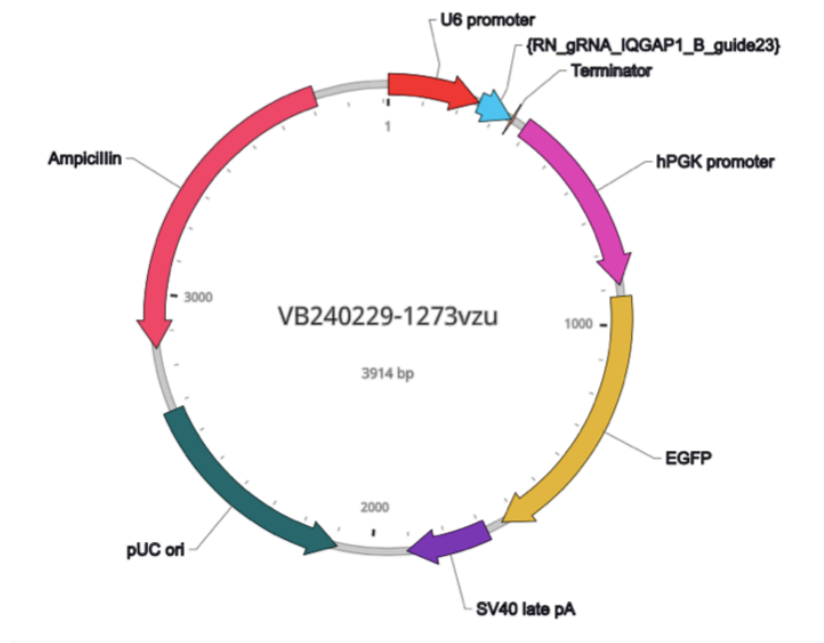


Figure 18 – VB240229-1273vzu plasmid construct used for IQGAP1 sgRNA.

Vector containing IQGAP123 dual gRNA:

For the three isoforms of IQGAP, only the dual guide approach was used. The vector VB221115-1129uvj (pRP[2gRNA]-EGFP-U6>mlqgap1[grna#462]-U6>mlqgap3[grna#102]) is a mammalian dual gRNA vector with 4274 bp. It has two guides: mlQGAP1 and an mlQGAP3. The mlQGAP1 recognizes the sequence CAGAGGAGATGGATGAGAGG in exon 2 of IQGAP1 and IQGAP2 while the mlQGAP3 recognizes the sequence CCGGAATGGAGTGCTTCTGG in exon3 of IQGAP3. Both gRNAs are expressed under the control of the Human U6 small nuclear promoter. This vector contains EGFP under the promoter hPGK, as depicted in Figure 19.

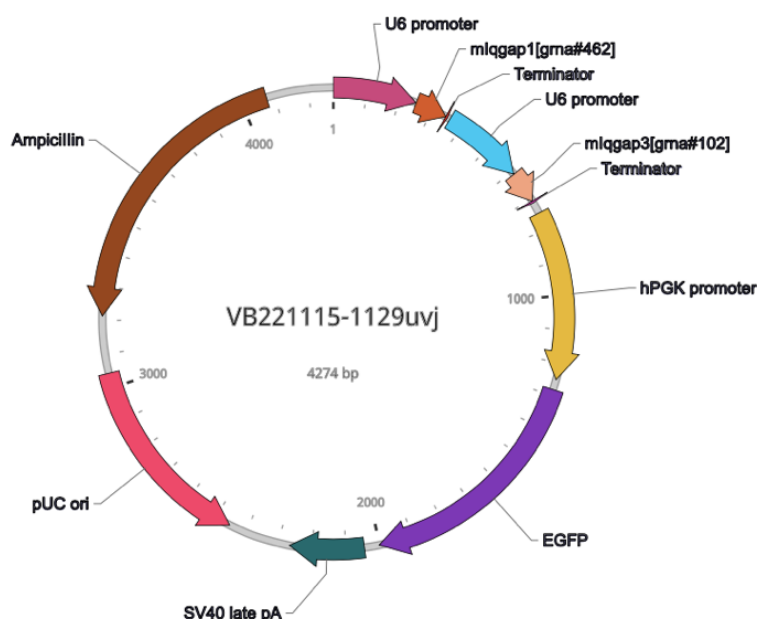


Figure 19 – VB221115-1129uvj plasmid construct used for IQGAP1 dual-gRNA.

5.2. Transformation and Plasmid Production

All plasmid production was done using NZYStar Competent cells (NZYTech, #MB00501). After thawing the bacteria, 100-200ng of plasmid was added to 45µL of cells and incubated in ice for 30 minutes. After this incubation, the cells were heat-pulsed in a water bath at 45°C for 45 seconds. Cells were again incubated in ice for 2 minutes. To the volume of cells, 100µL of Luria Broth (LB) (NZYTech, #MB02803) was added and incubated at 37°C for 1 hour with shaking (200-250rpm). The transformed cells were then plated in a Petri dish containing agar (NZYTech, #MB11801) mixed with LB plus and the resistance antibiotic of the given plasmid, in our case, ampicillin (Sigma-Aldrich, #A9518-5G), and incubated at 37°C O/N.

One colony was then isolated and added to 5mL of LB supplemented with ampicillin and left to grow at 37°C O/N with continuous shaking. The subsequent day, the culture was transferred to a larger Erlenmeyer flask, containing 250mL of LB supplemented with ampicillin and left to grow at 37°C O/N with continuous shaking. For plasmid DNA isolation, the NZYMaxiPrep kit (NZYTech, #MB05102) was used and the provided protocol was followed. The cell pellet was resuspended in 24mL of buffer M1 containing RNase A, followed by the gentle addition of 24mL of Buffer M2 (SDS solution) and incubation at room temperature (RT) for 5 minutes to achieve cell lysis. Next, 24mL of pre-cooled Buffer M3 (potassium acetate) was added, mixed, and centrifuged at

$\geq 20,000g$ for 30 minutes at 4°C to neutralize the lysis. The lysate was applied to an equilibrated NZYTech Plasmid Maxi Column, washed with 32mL of buffer MW, and the plasmid DNA was eluted with 15mL of buffer ME. The DNA was precipitated with 10.5mL isopropanol, washed with 5mL of 70% ethanol, air-dried, and finally dissolved in 100-150 μ L of endotoxin-free water. DNA concentration was measured using a Nanodrop.

6. Cell Transfection

6.1. RAT-2 cell line Transfection

In the day prior to the transfection, RAT-2 cells were plated. For this, the complete medium was removed from the T75 and washed with 1xPBS. Then, cells were suspended in Trypsin 0,05% EDTA 1x (Alfagene, #25300062) and incubated for 5 min at 37°C. After the cells detached from the T75, DMEM complete medium was added. A 1:1 dilution with 0.4% trypan blue and cells were counted using a Neubauer chamber. A cell density of 50,000 cells/well was plated in the 24 well-plate and stored at 37°C under a 5% CO₂ atmosphere.

In the day of the transfection, the media of each well was removed and 250 μ L of DMEM High Glucose was added. Transfections were done using the Invitrogen™ Lipofectamine™ 2000 Transfection Reagent (Invitrogen, #11668030). The lipofectamine (2 μ L of lipofectamine in 100 μ L of Opti-MEM per well) and the DNA (0.5 μ g of DNA in 100 μ L of Opti-Minimal Essential Medium (Opti-MEM) 1x (Invitrogen, #31985070) were prepared in different eppendorfs. The DNA mixture was added to lipofectamine mixture and incubated for 15 minutes at RT. The conditions were: 1) lipofectamine, ANLN guide vector and Cas9 vector, 2) lipofectamine, IQGAP1 guide vector and Cas9 vector and 3) lipofectamine, IQGAP123 vector and Cas9 vector. To each well, 100 μ L of the final mixture was added and cells were incubated at 37°C in a 5% CO₂ incubator for 2 hours. At the end of the incubation time, the medium was removed and replaced by the RAT-2 complete media, previously heated at 37°C.

6.2. Primary Hippocampal Neuron Transfection

Hippocampal neurons underwent transfection at DIV3-DIV7 and DIV10-DIV14, to effectively evaluate the effects of knocking-out ANLN, IQGAP1 and IQGAP123, during formation and maintenance of the MPS, respectively. As previously stated, on the culture day, a cell density of 50,000 cells/well was plated for transfection. At DIV3 and DIV10, the medium of each well was removed and 250 μ L of neurobasal medium was added.

Transfections were once again done using the Invitrogen™ Lipofectamine™ 2000 Transfection Reagent. The lipofectamine (2 μ L of lipofectamine in 100 μ L of Opti-MEM

per well) and the DNA (0.5 μ g of DNA in 100 μ L of Opti-MEM 1x) (Invitrogen, #31985070) were prepared in different eppendorfs. The DNA mixture was added to lipofectamine mixture and incubated for 15 minutes at RT. To each well, 100 μ L of the final mixture was added and cells were incubated at 37°C in a 5% CO₂ incubator for 1 hour. At the end of the incubation time, the medium was removed and replaced by the hippocampal neuron's complete media, previously heated at 37°C.

7. Cell Line Sorting

After transfection, 2 μ L/mL puromycin (Merck-Millipore, #540222) was added to the RAT-2 cells until they are prepared for sorting. For that, the media from the 24-well plates was removed, and each well is washed with 1xPBS; 200 μ L 0,05% trypsin-EDTA 1x was added to each well and incubated at 37°C in a 5% CO₂ incubator for 5 minutes. After the incubation period, the same volume of 10% FBS in PBS was added. The cells were transferred to a 15mL falcon and centrifuged for 5 minutes at 1000rpms. Subsequent of discarding the supernatant, the cell pellet underwent a PBS wash and cells are centrifuged again in the same conditions. After removing the supernatant, cells followed a 1:1 dilution with 0.4% trypan blue and were counted in a Neubauer chamber. The cells are then resuspended in the quantity of medium needed to reach a final concentration of 10x10⁶/ml and filtered to a FACS tube (Corning, #352052) with a 70 μ m cell strainer. Cell Sorting was performed by the Flow Cytometry Unit at i3S, with a FACS Aria Fusion (BD FACSAria™ Fusion Cell Sorter). After sorting the EGFP positive cells, they were transferred to a 6-well plate and later on to a T25 with RAT-2 complete medium.

8. Genomic DNA Sequencing

8.1. Genomic DNA Extraction

When cells reached a confluency of 70-80% after sorting, RAT-2 EGFP-positive transfected cells with either the control or the experimental conditions detailed above, were prepared for genomic DNA extraction. The media from the T25 was removed and each T25 was washed 2x with PBS, 500 μ L; 0,05% trypsin EDTA 1x was added and incubated at 37°C in a 5% CO₂ incubator for 5 minutes. After the incubation period, the same volume of 10% FBS in PBS was added. The cells were transferred to a 15mL falcon and centrifuged for 5 minutes at 1000rpms. Supernatant was discarded and the cell pellet underwent a PBS wash and it was transferred to an eppendorf, where another round of centrifugation was performed in the same conditions. The supernatant was once again discarded. After the PBS removal, 75 μ L of 25mM NaOH/0.2mM EDTA were added. Samples were heated for 3 to 5 minutes at 98°C in a heating block. After this

incubation time, samples were cooled down until reaching RT. Finally, 75 μ L of 40mM Tris HCl with a pH of 5.5, was added. Samples were stored at -20°C until required.

8.2. Genomic DNA PCR and Analysis

Using the previously extracted genomic DNA, a PCR mix was prepared. For each sample, 6.25 μ L of the Supreme NZYTaQ II 2x Green Master Mix (NZYTech, #MB36002) was mixed 1 μ L of primer forward (FWD), 1 μ L of primer reverse (RVS) (primer stock at 10uM), and 1 μ L of the genomic DNA. MilliQ water was calculated and added to reach a final volume of 12.5 μ L. Samples were then placed in a thermocycler (VWR, #732-1359) and the amplification protocol included an initial denaturation and enzyme activation at 95°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds and a final extension step at 72°C for 5 minutes. All primers were designed using the Primer3Plus interface (Table 4).

Table 4 – List of primers used for PCR and respective sequences.

Approach	Primer	Sequence
Dual guide	ANLN (FWD)	5' GTCTTTCTCTACCTTATATTCTGA 3'
	ANLN (RVS)	5' TGGGAGTCAAACCTCAGGTGTC 3'
	IQGAP1 (FWD)	5' AGAGAATGTCGGGTCCCCTG 3'
	IQGAP1 (RVS)	5' GTTGGAGGCTGGTCGTTGAG 3'
Single guide	ANLN (FWD)	5' GGCTGTGAAAACCTGCAGAGG 3'
	ANLN (RVS)	5' TAGATTTGTGGCCTGCCTGG 3'
	IQGAP1 (FWD)	5' GCGTCTTCAAGGTCTCTGCT 3'
	IQGAP1 (RVS)	5' CAGGAGGACTGACTGTGCAG 3'
	IQGAP123	IQGAP1 (FWD)
		5' CCCTGGTCAACAAGTGCTCT 3'
	IQGAP123	IQGAP1 (RVS)
		5' AGTGTTCCCTAACTGCCGAGC 3'
	IQGAP123	IQGAP2 (FWD)
		5' GAGGAGAGGGGGAGAGGATG 3'
		IQGAP2 (RVS)
		5' GACACCACACTCACCTAGGC 3'
	IQGAP123	IQGAP3 (FWD)
		5' TGTGGCAGTTTGGGGAAAGT 3'
	IQGAP123	IQGAP3 (RVS)
		5' ATGTCCAAGACAACCAGGGC 3'

A 1% agarose gel was prepared using 2g of agarose (Lonza, #50004) and 200mL of 1xTris-acetate-EDTA (TAE) (stock TAE buffer at 50x) (BioRad, #161-0773). The

mixture was microwaved for approximately 3 minutes, until the agarose was completely dissolved. Afterwards, it was cooled down at RT and 8 μ L of the GreenSafe Premium (NZYTech, #MB13201) a DNA-binding dye, was added, and the mixture was poured into a casting tray with the appropriate well comb. The gel was solidified at RT for about 20 minutes. Once the gel solidified, the comb was gently removed to create the wells and the gel was placed into an electrophoresis chamber or gel box and covered with 1xTAE.

Carefully, the NZYDNA Ladder III (NZYTech, #MB04401) and the samples were loaded into the wells. The gel ran for about 1h and 15 minutes at 300mA and 130V. After the run was complete, the gel was removed and placed on the Molecular Imager Gel Doc XR+ System (BioRad, #1708195EDU). The resulting bands were visualized and photographed by the Image Lab Software version 5.21.

8.3. Purification of PCR products

To purify the PCR products, the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, #D4045) was used and the manufacturer's protocol was followed. After analyzing the bands on the Image Lab connected to the Gel Doc, the wanted band size was excised from the gel using a scalpel and stored in an eppendorf. Afterwards, each agarose gel slice was weighted and 300 μ L of ADB buffer per 100mg agarose gel slice was added. The tubes with the agarose were placed in a heating block at 55°C for 5-10 minutes. When the gel slice appeared to be fully dissolved, the sample was mixed through inversion. The melted agarose solution was transferred to a Zymo-Spin Column in a collection tube, centrifuged for 1 minute and the flow-through discarded. Then, 200 μ L of DNA Wash Buffer was added to each column and centrifuged for 30 seconds and the flow-through was discarded. This step was repeated. Lastly, the column was placed in a microtube and 10 μ L of DNA elution buffer or endotoxin-free H₂O was added directly onto the center of the column and subjected to a 1-minute centrifugation cycle to yield the final eluate.

8.4. Sequencing of the PCR amplicon

DNA Sequencing was performed by the Genomics Platform at i3S, employing the Sanger Sequencing Reaction method using the Applied Biosystems 3500 Genetic Analyzer (S/N 29113-030). The results were analyzed using the SnapGene version 2.2.2. Software.

9. Andor BC43 Confocal Imaging

Confocal imaging was performed on an Andor BC43, a Benchtop Spinning Disk Confocal Microscope (OXFORD Instruments, United Kingdom), using the following timepoints:

DIV5 and DIV14 for the endogenous studies and DIV7 and DIV14 for the transfected cells in hippocampal neurons. The hippocampal neurons endogenous levels were imaged with a 100x glycerol objective while transfected images were obtained using a 40x dry objective.

10. STED microscopy

STED imaging was performed on an inverted Leica Stellaris STED 3, using the following timepoints: hippocampal neuron cultures at DIV5 and DIV14 for the studies on endogenous expression and at DIV7 and DIV14 for the transfected hippocampal neurons. Hippocampal neurons were imaged at the AIS and the shaft, approximately at a distance of 80-100 μ m from the cell body, with a 93x glycerol objective (HC PL APO 93x NA 1.4 STED glycerol objective) from Leica Microsystems using confocal and STED modes. The 2D vortex STED images with lateral resolution enhancement were recorded with 20nm pixel size in xy and dwell times in typically 600ns. The STED far-red channel (Abberior STAR 635P) was recorded with 633nm excitation using the pulsed white light laser with 80MHz repetition rate and STED depletion was performed with a synchronized pulsed 775nm depletion laser. The detection bandpass was set to 650 to 750nm and the pinhole was set to 0.93UA. The following acquisition settings were applied: 16x line averaging and detector gating on a Hybrid Detector (HyD, Leica Microsystems) of 0.3ns to 6ns. The second STED channel (Abberior STAR580) was recorded in line sequential mode with 561nm excitation and 775nm depletion using a detection window from 580 to 620nm. All other settings remained constant.

11. Statistical Analysis

The data is presented as the mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed by GraphPad Prism 10.2.3. for Windows (GraphPad Software, California, USA). Data comparison between groups was performed using One-Way ANOVA followed by Student's t-test. Statistical significance was determined by a p-value inferior to 0.05.

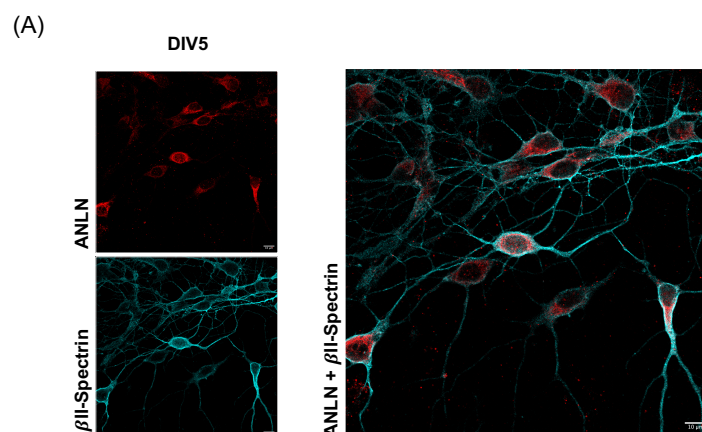
Results

1. ANLN is not relevant for axonal actin ring formation and maintenance

1.1. Endogenous levels of ANLN in hippocampal neurons

As previously mentioned, the main goal of this study is to investigate the process of the actin circularization of the actin rings within the MPS. Recent studies have highlighted that ANLN can affect the bending and the curvature of actin filaments (Kučera, Siahaan et al. 2021, Lebedev, Chan et al. 2023). Therefore, the role of ANLN in the circularization of the actin rings in the context of the MPS is of particular interest. To assess the involvement of ANLN in this process, we first examined the endogenous levels of this protein in hippocampal neurons at two developmental stages: DIV5 and DIV14. These time points were selected because at DIV5 the basic structure of the actin rings is already visible, whereas at DIV14, the structure is mature, making these two stages relevant for comparison.

As an initial approach, we analyzed ANLN expression exclusively at DIV5, where we observed a clear and distinct staining of the protein localized primarily to the neuronal cell body (Figure 20A). This prompted further analysis to compare expression at DIV14, to assess potential changes during neuronal maturation. Hippocampal neurons were immunostained for βIII -tubulin, a neuron-specific protein commonly used as a marker, βII -spectrin, which is enriched in the axon, and ANLN. As shown in Figure 20A, ANLN is predominantly located in the neuronal cell body, with very faint expression observed in the axon. Quantification of endogenous ANLN levels, normalized to βIII -tubulin intensity, demonstrated no statistically difference in ANLN expression between DIV5 and DIV14 for the cell body and the axon (Figure 20B, C).



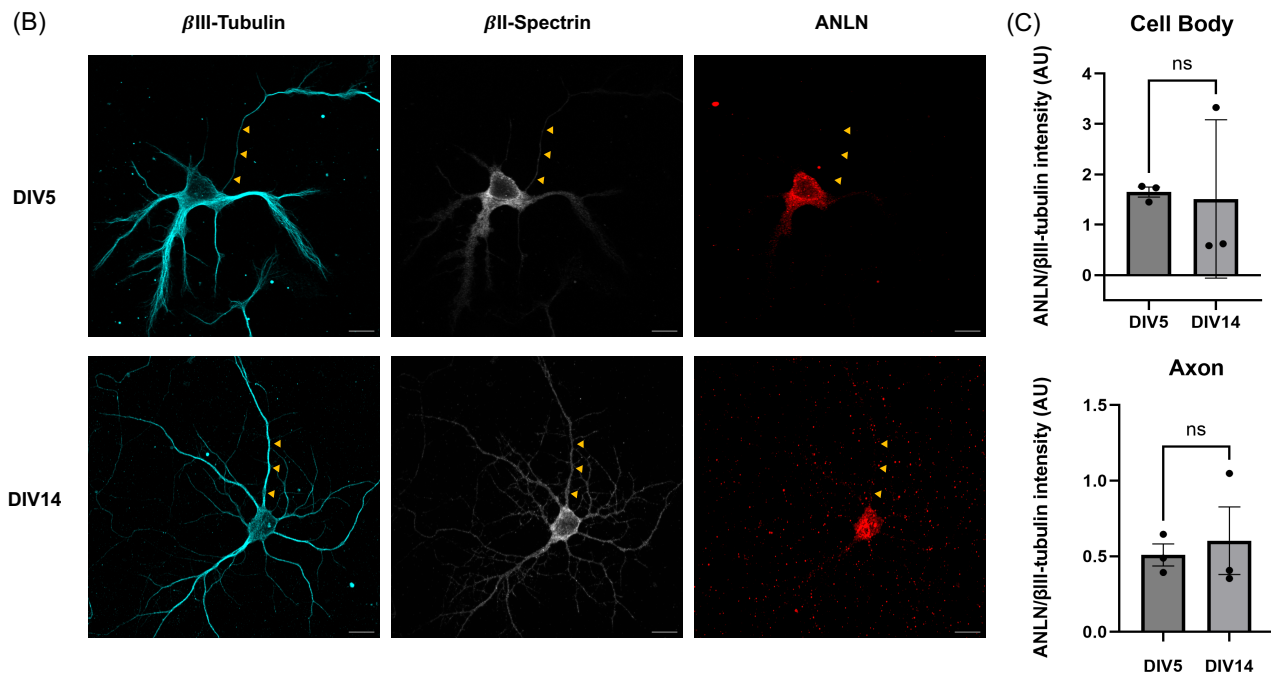


Figure 20 – ANLN is predominately located in the cell body. (A) Representative images of embryonic hippocampal neurons at DIV5 stained with β II-spectrin and ANLN (B) Representative images of embryonic hippocampal neurons at DIV5 and DIV14, stained with β III-tubulin, β II-spectrin and ANLN. The axon is indicated with an orange arrow. (C) Representative quantifications of the cell body and the axon analyzed in GraphPad. Data represent mean $\pm$ SEM (n=3). Statistical significance determined by Student's t-test: ns non-significant *p<0.05.

Following this initial analysis, the next step was to resolve ANLN with higher resolution using STED microscopy and determine whether using this alternative type of higher resolution microscopy ANLN might be seen in the axon, specifically colocalizing with actin rings. However, due to the low levels of ANLN detected in the axon at both DIV5 and DIV14, we were unable to detect the protein in this compartment. This result is consistent with our earlier observations, which showed faint ANLN staining in the axon at both time points, further suggesting that its expression in this region is minimal (Figure 20).

Although endogenous levels of ANLN were very faint in the axon, since the quantity of protein needed to perform a function is not known, we continue to investigate the role of this protein in this context.

1.2. CRISPR-Cas9 efficiently depleted ANLN expression

1.2.1. RAT-2 cell line

After evaluating the endogenous levels of ANLN, we intended to determine the importance of this protein for the actin ring formation and maintenance within the MPS.

To achieve this, we used CRISPR-Cas9 technology to genetically deplete ANLN and assess its impact on the actin ring organization.

To evaluate the efficiency of the dual guide RNA designed to target exon 2 of the ANLN gene, RAT-2 cells were transfected with the dual gRNA, each targeting different sequences. If both guides were active, we would expect the formation of a 257bp product (Figure 21). We used a guide targeting the Rosa26 as control (CTRL). However, we were unable to detect this expected product, as shown in Figure 21, even after testing different annealing temperatures (53°C; 55°C and 58°C), suggesting that this approach may not be optimal.

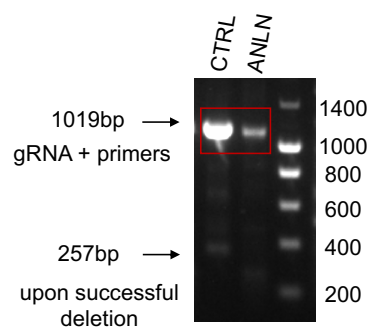


Figure 21 – Validation of the CRISPR-Cas9 genetic manipulation visualized through an agarose gel indicated that the dual guide approach is not effective. Representative agarose gel showing amplicons for the CTRL and ANLN (1019bp). Upon successful deletion a 257bp fragment would be expected.

Since the initial dual gRNA strategy was not successful, we tested a sgRNA approach to target the exon 2 of ANLN gene. Transfections were performed in the RAT-2 cell line, using a guide RNA targeting the Rosa26 locus as the CTRL. To enrich for successfully transfected cells, we performed FACS sorting of EGFP expressing cells, followed by selection of puromycin-resistant cells. This allowed us to isolate the population that had undergone successful CRISPR-Cas9 editing.

As previously mentioned, if this strategy works as expected, small indels should be introduced at the target site. Since these indels cannot be detected by agarose gel electrophoresis, genomic DNA sequencing is necessary. Initially and as expected, we observed no detectable differences between the CTRL and ANLN-targeted cells when analyzing PCR products by agarose gel electrophoresis (Figure 22A). However, subsequent sequencing of the targeted region revealed the presence of indels in the ANLN-targeted cells, that were not detected in the CTRL (Figure 22B). This indicates that the sgRNA successfully induced CRISPR-mediated editing at the target site in ANLN.

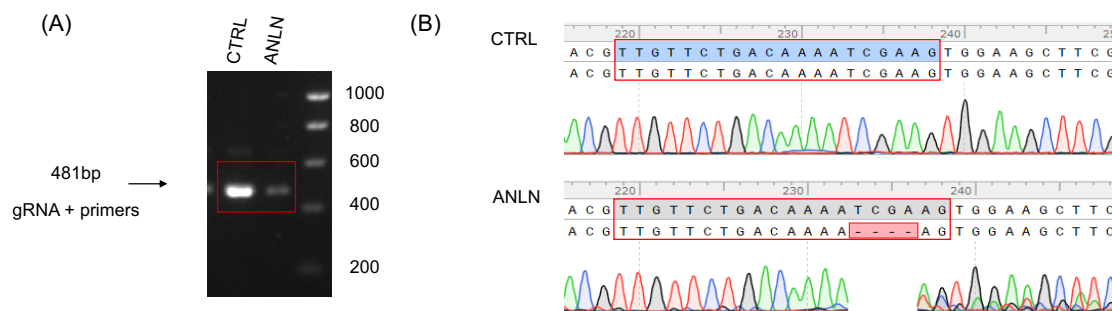


Figure 22 – Validation of the CRISPR-Cas9 genetic manipulation through genomic DNA sequencing indicates the single guide approach is effective. (A) Representative agarose gel showing amplicons for the CTRL and ANLN (481bp) **(B)** Sanger sequencing confirmed the presence of indels in the ANLN transfected cells when compared to the control (bottom electropherogram).

Following these results, we proceeded with the analysis of ANLN depletion and its impact on actin ring formation and maintenance in hippocampal neurons.

1.2.2. Hippocampal primary neurons

To validate the efficiency of ANLN depletion in hippocampal neurons following CRISPR-Cas9 transfection, immunofluorescence staining was performed. Neurons were transfected with the sgRNA targeting exon 2 of the ANLN gene at DIV3 and DIV10 and fixed at DIV7 and DIV14, respectively, to analyze the formation and maintenance of the MPS. Immunofluorescence analysis revealed a significant reduction in ANLN staining in neurons transfected with the CRISPR-Cas9 constructs compared to the control group (non-transfected cells on the same coverslip) (Figure 23A).

As previously described, ANLN was predominantly located in the neuronal cell body, with weak staining observed in the axon. Consequently, quantifications were performed on the cell body rather than the axon (Figure 23B). This reduction in ANLN expression validates the efficiency of the CRISPR-Cas9 system in hippocampal neurons, providing a solid foundation for further analysis of its impact on actin ring formation and organization.

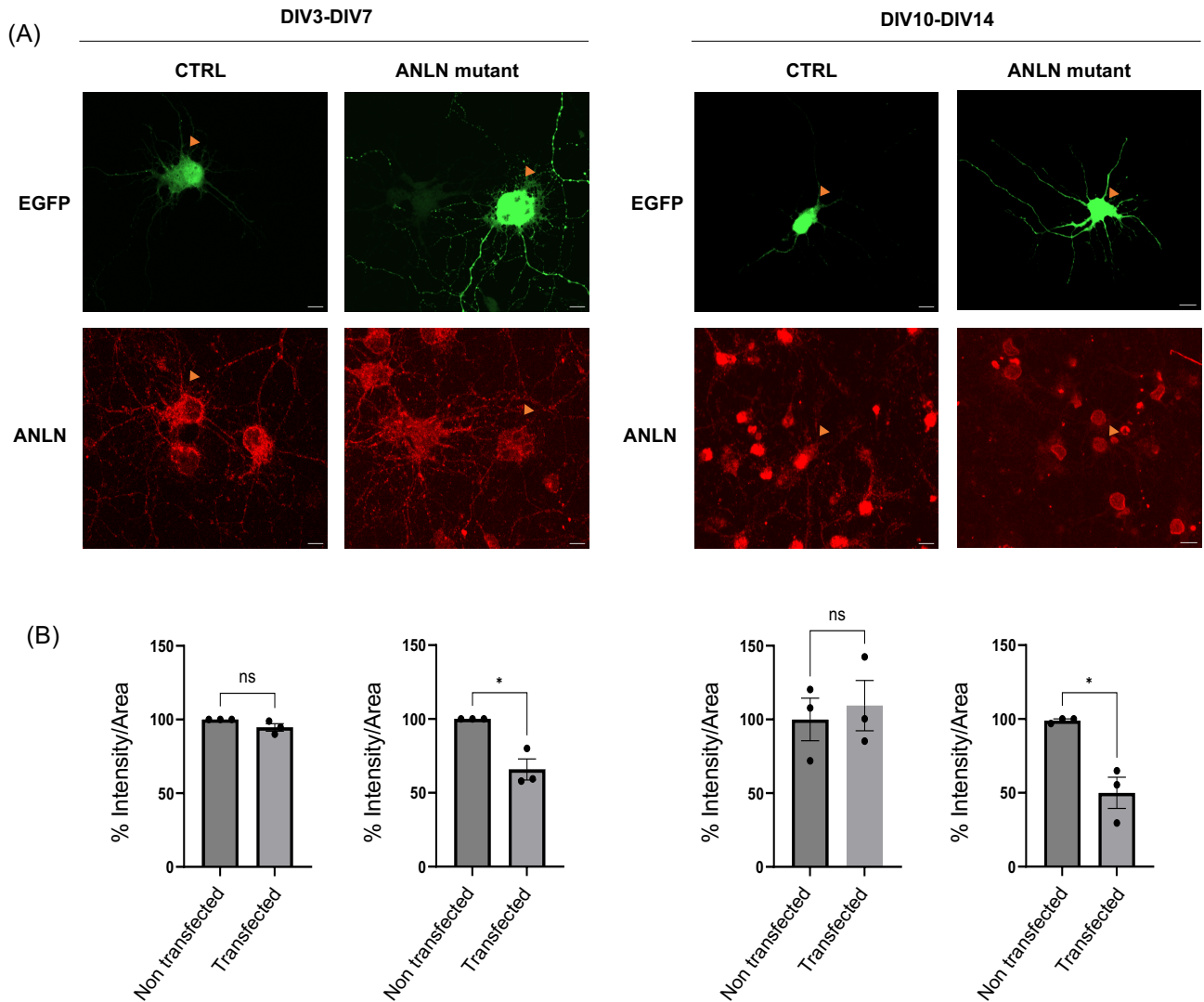


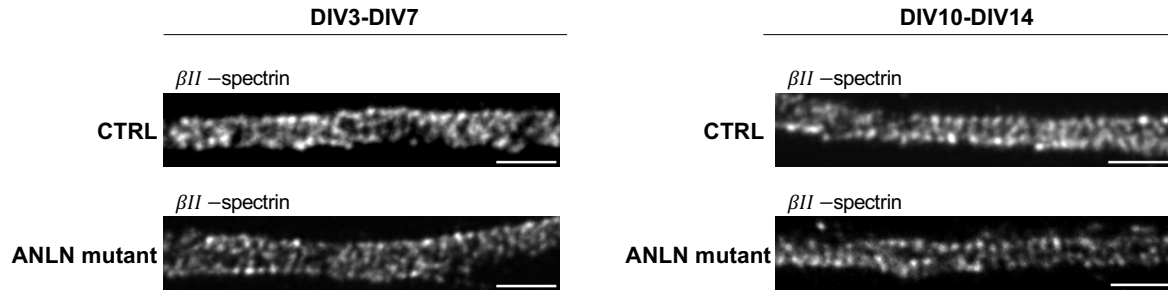
Figure 23 – Validation of the ANLN Crispr-Cas-9 in hippocampal neurons through immunofluorescence indicates that this strategy is effective. (A) Representative images of hippocampal neurons transfected at DIV3 (left panel) and DIV10 (right panel) and fixed at DIV7 (left panel) and DIV14 (right panel), respectively, with the single gRNA and Cas9. CTRL neurons showed no significant alterations in ANLN expression levels between non-transfected and transfected neurons. In contrast, ANLN-transfected (EGFP +) neurons exhibited reduced expression ANLN expression levels compared to non-transfected (EGFP -) neurons **(B)** Representative quantifications of expression levels in the cell body analyzed in GraphPad. Data represent mean $\pm$ SEM (n=3). Statistical significance determined by Student's t-test: ns non-significant *p<0.05.

1.3. ANLN does not affect the organization of the actin rings within the MPS both during its formation and maintenance

Following the successful depletion of ANLN using CRISPR-Cas9 technology, we investigated the effect of mutant ANLN on actin ring formation and organization within hippocampal neurons. Hippocampal neurons transfected with the sgRNA targeting exon

2 of the ANLN gene were analyzed for alterations in the structure and periodicity of actin rings using STED microscopy (Figure 24A).

(A)



(B)

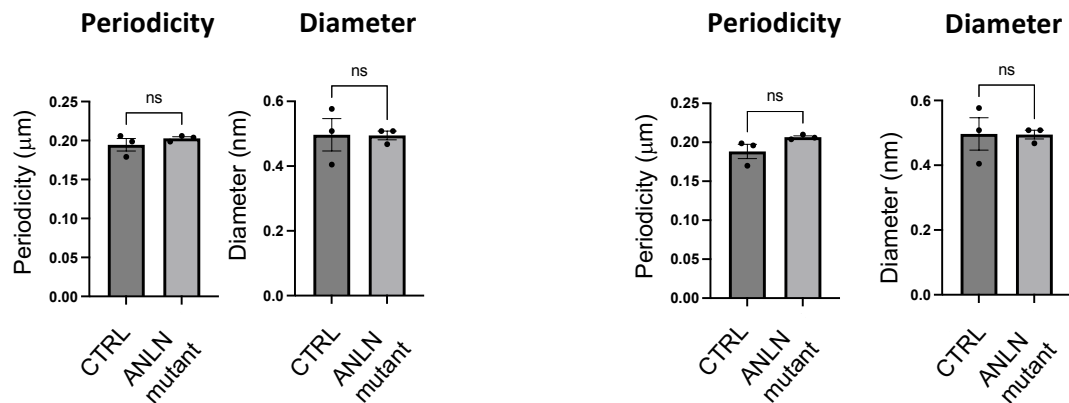


Figure 24 – ANLN does not affect the organization of the actin rings within the MPS either in its formation and maintenance. (A) Tau-STED representative images of embryonic hippocampal neurons transfected with the sgRNA approach at formation and maintenance, stained with β II-spectrin and ANLN. Transfected neurons showed no significant alterations in the organization of the rings (B) Representative quantifications of expression levels analyzed in GraphPad. Data represent mean $\pm$ SEM (n=3). Statistical significance determined by Student's t-test: ns non-significant *p<0.05.

Our analysis revealed that the depletion of ANLN did not result in significant alterations in the overall organization of the actin rings within MPS. The periodicity (190nm) and diameter (450nm) of the actin rings in mutant neurons were comparable to those observed in the control neurons, which were either non-transfected or transfected with Rosa26 (Figure 24B). These results suggest that ANLN is not essential for the formation or maintenance of the actin rings in hippocampal neurons.

These findings suggest that while ANLN is present in hippocampal neurons, it probably does not play a critical role in the formation or maintenance of the actin rings within the MPS.

2. IQGAP is involved in the circularization of the actin rings within the MPS only when the structure is mature

2.1. Endogenous levels of IQGAP1 in hippocampal neurons

We next analyzed the role in MPS biology of IQGAP1, a protein that when tethered to the lipid membrane through its CHD domain can bend actin filaments forming ring-like structures, to further investigate its potential involvement in MPS organization (Brandt and Grosse 2007, Palani, Ghosh et al. 2021).

As we did for ANLN, we measured the endogenous levels of IQGAP1 in hippocampal neurons at two developmental stages: DIV5 and DIV14, due to the reasons stated previously. Hippocampal neurons were immunostained for β III-tubulin, a neuron-specific protein, 2x phospho myosin light chain (2x phospho MLC), a marker for the AIS and the axon, and finally IQGAP1 (Figure 25A). Our results revealed that, unlike ANLN, the IQGAP1 is well expressed in the axon and upon quantification, an increase of expression from DIV5 to DIV14 is observed (Figure 25B).

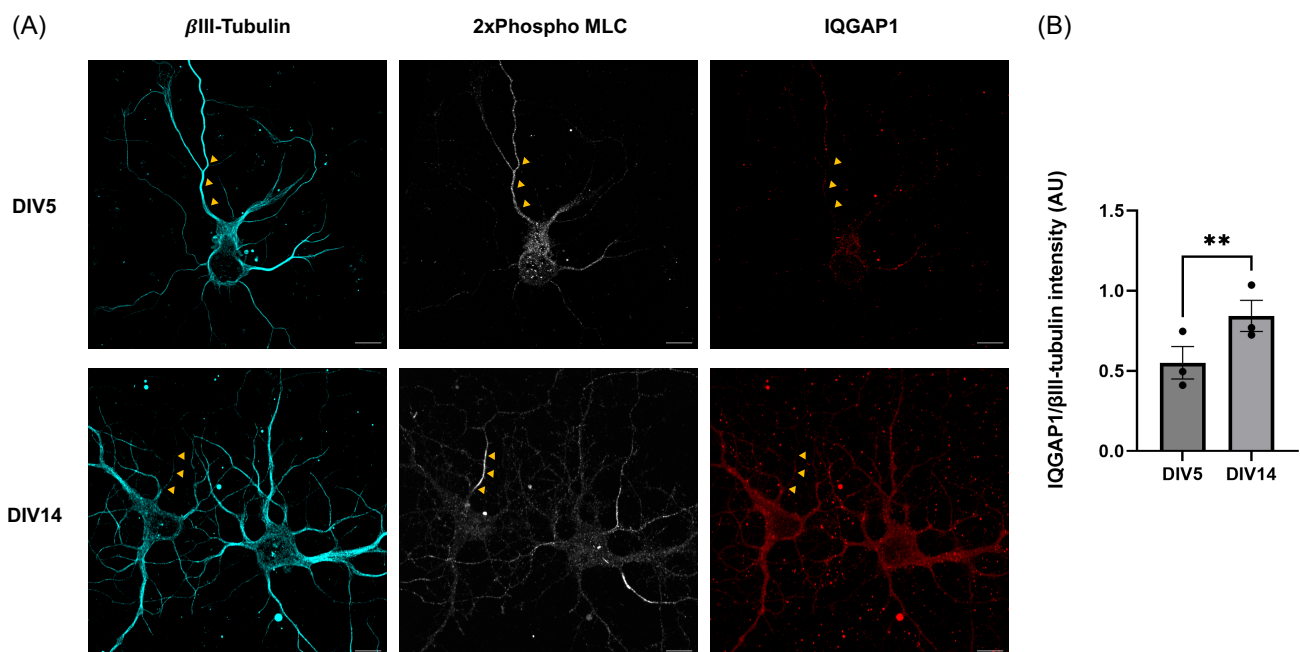


Figure 25 – IQGAP1 is endogenously expressed in hippocampal neurons and its expression increases at DIV14. (A) Representative images of embryonic hippocampal neurons at DIV5 and DIV14 stained with β III-tubulin, 2xPhospho MLC and IQGAP1. The axon is indicated with an orange arrow (B) Representative quantifications of the axon, analyzed in GraphPad. Data represent mean $\pm$ SEM (n=3). Statistical significance determined by Student's t-test: ns non-significant *p<0.05.

After assessing the endogenous levels of IQGAP1 and observing that this protein is present in the axon at both DIV5 and DIV14, we next determined whether IQGAP1 colocalized with the actin rings. To investigate this, an immunofluorescence was performed with phalloidin to stain for actin and IQGAP1. Both time points were observed using a STED microscope.

As expected, STED revealed the characteristic periodic actin rings at both DIVs and the increased expression of IQGAP1 at DIV14. Notably, we could detect some degree of colocalization between actin and IQGAP1 at DIV14, suggesting that IQGAP1 may play a role in maintaining the structure of the rings during this later stage of neuronal development (Figure 26). However, further analysis is required to confirm the extent of this colocalization and to fully elucidate the functional role of IQGAP1 in the maintenance of the actin rings.

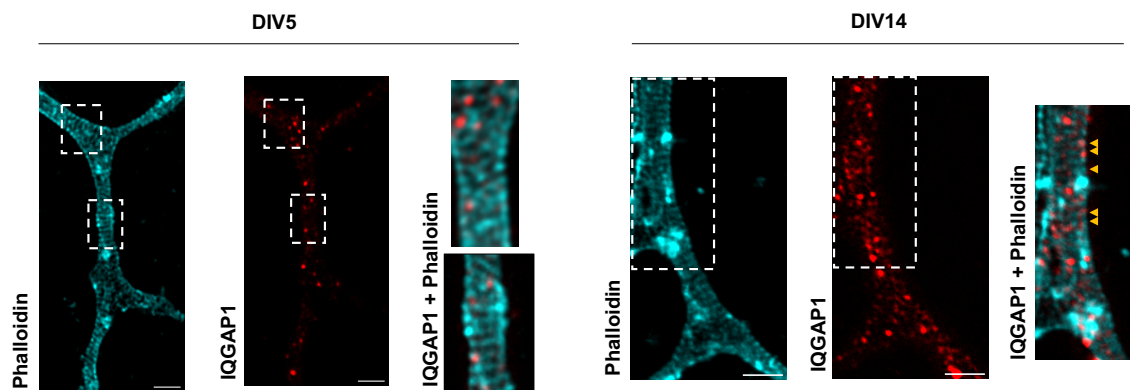


Figure 26 – IQGAP1 colocalizes with the actin rings in hippocampal neurons. Representative images of embryonic hippocampal neurons at DIV5 and DIV14 stained with Phalloidin (cyan) to visualize actin filaments and IQGAP1 (red). The insets display merged channels at both time points. Orange arrows indicate examples of the colocalization with the actin rings.

2.2. CRISPR-Cas9 efficiently depleted IQGAP expression

2.2.1. RAT-2 cell line

After evaluating the endogenous levels of IQGAP, we intended to determine its importance for actin ring formation and maintenance within the MPS. To achieve this, we used CRISPR-Cas9 technology to genetically deplete IQGAP and all its isoforms, IQGAP1, IQGAP2, and IQGAP3 and assess its impact on the actin ring organization.

To evaluate the efficiency of the dual guide RNA designed to target exon 2 of the IQGAP1 gene, we followed the same methodology as we did for ANLN. Briefly, RAT-2 cells were transfected with the dual gRNA, each targeting different sequences within exon 2. We used a guide RNA targeting the Rosa26 locus as the CTRL. We were unable

to detect the 214bp expected product in IQGAP1-depleted conditions, as shown in Figure 27, even after testing different annealing temperatures (55°C, 58°C, 60°C and 63°C), suggesting that this approach did not successfully target exon 2.

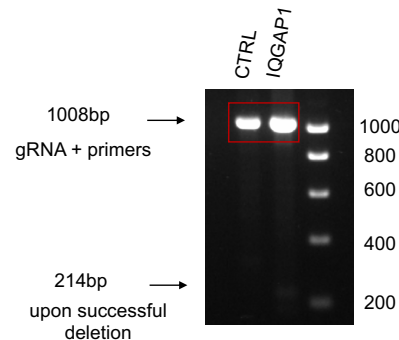


Figure 27 – Validation of the CRISPR-Cas9 genetic manipulation visualized through an agarose gel indicated that the dual guide approach is not effective. Representative agarose gel showing amplicons for the CTRL and IQGAP1 (1008bp). Upon successful deletion a 214bp fragment would be expected.

Alternatively, a single guide approach was tested for IQGAP1. As previously mentioned, if this strategy works as expected, small indels would be introduced at the target site, exon 2, which can only be detected by genomic DNA sequencing. For this particular construct, we could not detect the presence of indels in the genomic DNA. For this reason, this strategy for this isoform of IQGAP was not pursued further.

Simultaneously, we used a construct designed to target all IQGAP isoforms. This construct targets an identical sequence in exon 2 of both IQGAP1 and IQGAP2, as well as a sequence in exon 3 specific to IQGAP3. Consequently, this vector, although being a dual guide vector, functions as a single guide vector. As such, we did not expect a large fragment of DNA to be deleted but rather the introduction of small indels at the target sites. Transfections were performed in RAT-2 cells, with a guide RNA targeting the Rosa26 locus used as the CTRL. As shown in Figure 28A, no differences in the bands were observed in the gel between the CTRL and the IQGAPs, as expected, and all isoforms displayed the anticipated band sizes: 508 bp for IQGAP1, 592 bp for IQGAP2, and 501 bp for IQGAP3. However, upon analyzing the electropherograms of the genomic DNA, we observed no differences in the CTRL, whereas the IQGAP targeted cells exhibited indels in all three isoforms: IQGAP1, IQGAP2, and IQGAP3 (Figure 28B).

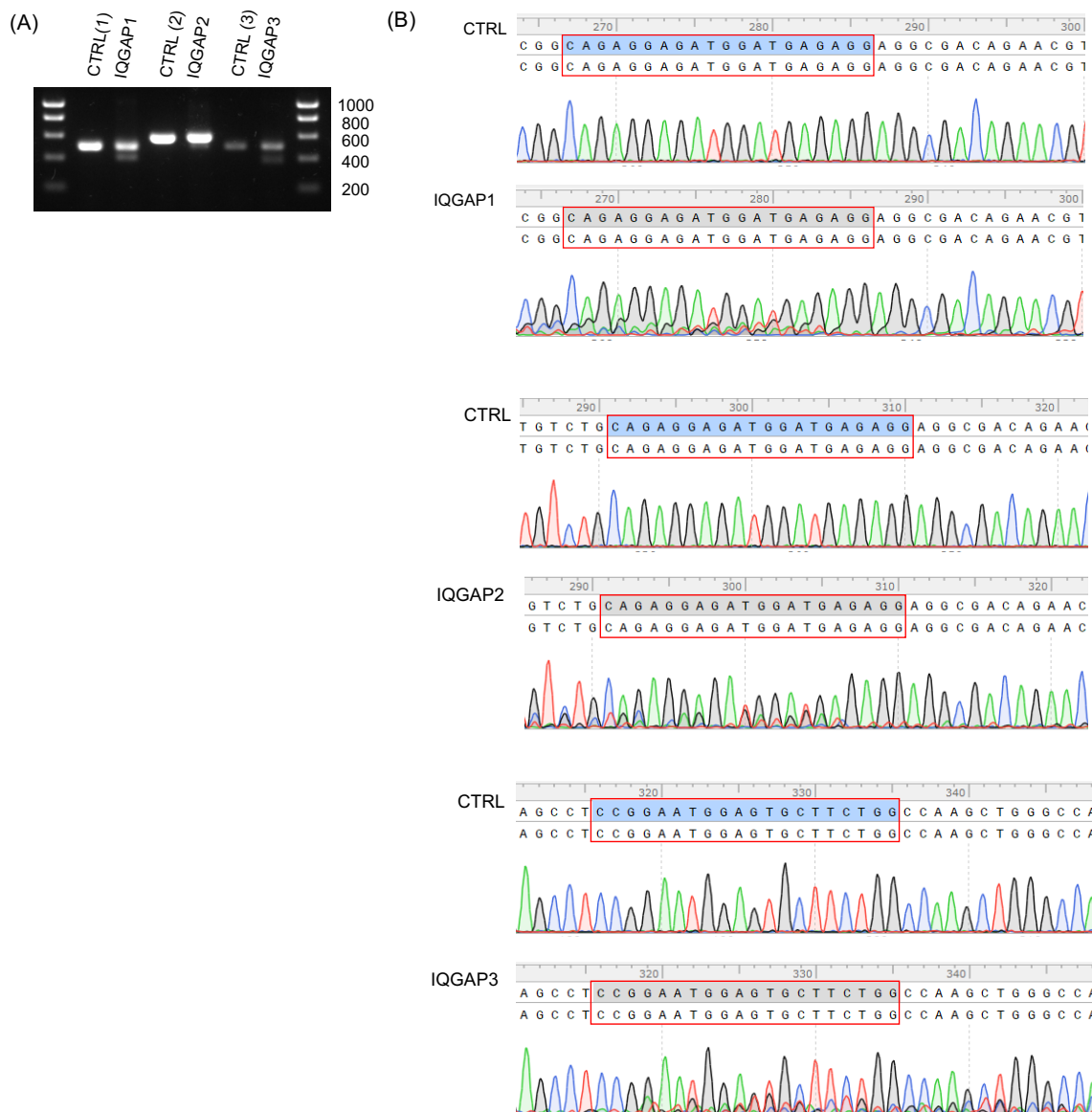


Figure 28 – Validation of the CRISPR-Cas9 genetic manipulation through genomic DNA sequencing indicates the dual guide approach is effective against all IQGAP isoforms. (A) Representative agarose gel showing amplicons for the CTRL and IQGAP1 (508bp), IQGAP2 (592 bp) and IQGAP3 (501 bp) **(B)** Sanger sequencing confirmed the presence of indels in the IQGAP1,2,3-transfected cells compared to the CTRL.

2.2.2. Hippocampal primary neurons

To assess the efficiency of IQGAP1, IQGAP2, and IQGAP3 depletion in hippocampal neurons following CRISPR-Cas9 transfection, immunofluorescence staining was performed. Neurons were transfected with vectors containing two single-guide gRNAs, one targeting IQGAP1 and IQGAP2 and a separate one for IQGAP3, at DIV3 and DIV10 and fixes at DIV7 and DIV14, respectively, to analyze the effect of IQGAPs in the

formation and maintenance of the MPS. Immunofluorescence analysis of IQGAP1 revealed a significant reduction in IQGAP1 signal in neurons transfected with the CRISPR-Cas9 constructs compared to non-transfected controls on the same coverslip, specifically at DIV14 (Figure 29A). Quantification of the fluorescence intensity in the axonal compartment confirmed the depletion of IQGAP1 expression only at DIV14, validating the efficiency of the CRISPR-Cas9 system in hippocampal neurons (Figure 29B). These findings support the use of this system to investigate the role of IQGAP proteins in actin ring organization, specifically during the period of maintenance of the structure.

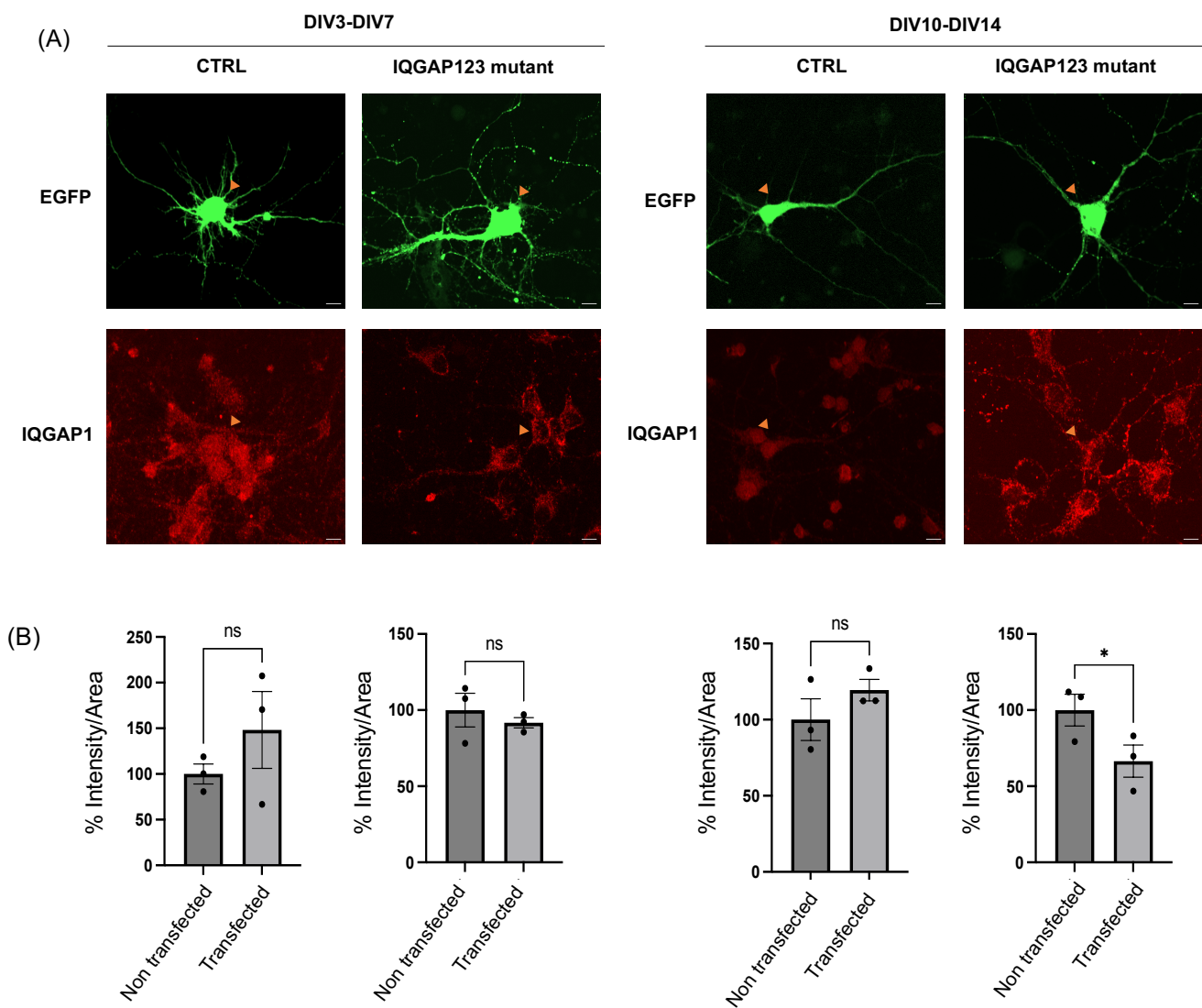


Figure 29 – Validation of the IQGAP1,2,3 Crispr-Cas-9 in hippocampal neurons through immunofluorescence indicates that this strategy is effective. (A) Representative images of hippocampal neurons transfected at DIV3 (left panel) and DIV10 (right panel), and fixed at DIV7 (left panel) and DIV14 (right panel), respectively, with the dual gRNA for all IQGAP isoforms: 1,2 and 3 and Cas9 showed a reduction in the levels of expression only in the

maintenance of the MPS (DIV10-DIV14) **(B)** Representative quantifications of expression levels analyzed in GraphPad. Data represent mean $\pm$ SEM (n=3). Statistical significance determined by Student's t-test: ns non-significant *p<0.05.

2.3. IQGAP1 affect the organization of the actin rings within the MPS in its maintenance

Following the successful depletion of IQGAP1 using CRISPR-Cas9 technology, we investigated the effect of mutant IQGAP on actin ring formation and organization within hippocampal neurons. Hippocampal neurons transfected with the sgRNA targeting exon 2 of IQGAP1 and IQGAP2 and exon 3 of IQGAP3 were analyzed using STED microscopy for alterations in the structure and periodicity of actin rings (Figure 30A).

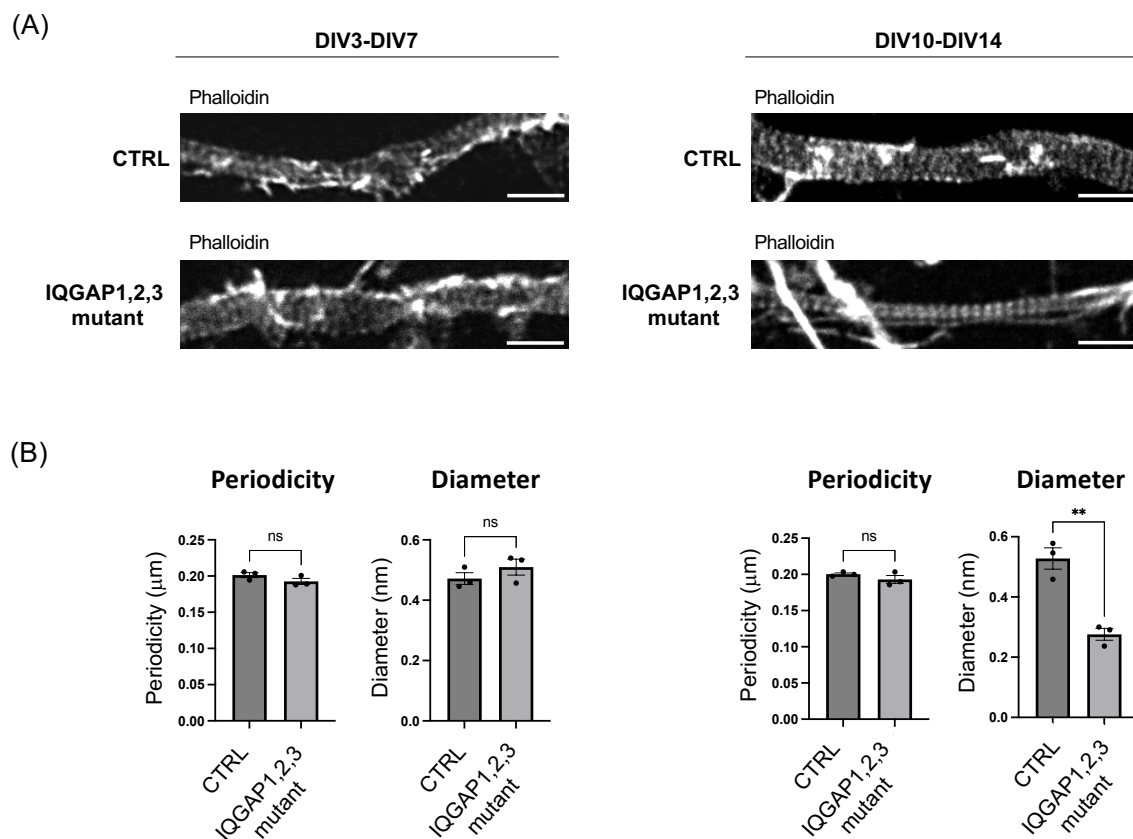


Figure 30 – IQGAP1 is involved in the maintenance of the axonal actin rings within the MPS. (A) Tau-STED representative images of embryonic hippocampal neurons transfected with the dual gRNA approach for all the IQGAP isoforms at formation and maintenance, stained with Phalloidin and IQGAP1. IQGAP1,2,3-transfected neurons showed a reduction in the diameter of the actin rings **(B)** Representative quantifications of expression levels analyzed in GraphPad. Data represent mean $\pm$ SEM (n=3). Statistical significance determined by Student's t-test: ns non-significant *p<0.05.

Our analysis revealed that the indels in IQGAP did not significantly alter the overall organization of the actin rings within the MPS for both time points since its presence and periodicity (190 nm) are maintained. Of note, given that at the initial

timepoints used to study MPS formation, no decrease in the levels of IQGAPs were found, the absence of effect was expected. However, in the maintenance stage (DIV10-DIV14), transfected neurons showed a strong reduction in actin ring diameter (approximately 250 nm) that was not detected in the non-transfected nor in the CTRL axons (approximately 450-500 nm) (Figure 30B). These results support the relevant role of IQGAP in the maintenance of the actin rings within the MPS.

Discussion

The MPS is a highly intricate structure within neuronal cells, composed of actin rings spaced by spectrin tetramers along the axon. It serves critical roles beyond structural support, including signal transduction, AIS filtering, and regulation of axonal diameter (Costa and Sousa 2021). Although several questions have been addressed with the super-resolution tools, there are still many unknowns related with the precise molecular mechanisms governing its formation and maintenance.

Previous work demonstrated that knocking out α -adducin in neurons resulted in a significant increase in the diameter of actin rings, implying its involvement in maintaining axonal diameter (Leite and Sousa 2016). Similarly, NMII, another ABP responsible for actin contraction and relaxation, was shown to regulate axonal diameter, affecting both signal propagation velocity and the transport of large cellular cargoes (Costa and Sousa 2020, Wang, Li et al. 2020). These studies highlight the complex interplay between actin-binding proteins and MPS integrity, particularly concerning axonal diameter.

To further investigate the molecular regulation of the MPS, we focused on the potential roles of ANLN and IQGAP1—both actin-binding proteins known to modulate actin filament dynamics in other cellular contexts, such as the cytokinesis. In this context, ANLN was described to bind and bundle F-actin during cell division, enhancing actomyosin contractility and promoting actin polymerization by stabilizing mDia2 in its active form through RhoA binding. This is crucial for the formation and constriction of the contractile ring, essential for successful cytokinesis (Piekny and Maddox 2010, Zhang and Maddox 2010, Tuan and Lee 2020).

Furthermore, *in vitro*, human ANLN mediated actin ring formation and contractility independently of myosin by generating contractile forces through actin filament depolymerization. ANLN enhances contractility by maximizing the overlap between bundled actin filaments, further driven by actin disassembly, leading to ring constriction. As a non-motor actin crosslinking protein, ANLN efficiently converts actin depolymerization into contraction of the actin bundles (Kučera, Siahaan et al. 2021, Lebedev, Chan et al. 2023).

Due to the impact of ANLN in the circularization of the cytokinetic ring, we wondered if this protein would also be implicated in the circularization of the actin rings within the MPS. Our experiments aimed to test this hypothesis at two critical time points: DIV3/5 (formation) and DIV10/14 (maintenance) of the actin rings, in the first time point

we can detect complete actin rings, while at DIV10/14 neurons are mature in a super-resolution microscope.

Despite the established role of ANLN in actin-based contractile processes, our data revealed minimal endogenous ANLN expression in axons at both DIV5 and DIV14. Furthermore, CRISPR-Cas9-mediated depletion of ANLN did not result in any significant alterations in the formation (DIV3-DIV7) and maintenance (DIV10-DIV14) of organization of the actin rings. These findings suggest that ANLN is not required to organize actin rings within the MPS. This lack of involvement might indicate functional redundancy within neurons, where other actin-regulatory proteins compensate for the absence of ANLN, such as IQGAP family of proteins.

The IQGAP, a three-isoform family of proteins, has been described to regulate actin dynamics and cytoskeleton organization. Within its structure, IQGAP contains a CHD, which allows it to bind actin filaments and bend them into curved geometries, forming ring-like structures (Brandt and Grosse 2007, Palani, Ghosh et al. 2021). Based on this, we aimed to investigate whether IQGAP is involved in the circularization of actin rings. To explore this, we employed the same experimental approach as we did for ANLN.

Our findings revealed an increase in the endogenous levels of IQGAP1, the ubiquitously expressed isoform of the IQGAP family, at DIV14. Additionally, we observed co-localization of IQGAP1 with the actin rings at this later time point, suggesting that this protein plays a role in the maintenance or function of these structures, particularly during the later stages of development. Supporting this, when all the isoforms of IQGAP1,2 and 3 were mutated using a CRISPR-Cas9 approach, we observed the presence of highly periodic (190 nm) actin rings, but with a striking reduction in their diameter (from 450 nm to 250 nm, approximately).

These results highlight the involvement of IQGAP1 in the curving and stabilising actin rings in more mature neurons. The mechanism by which IQGAP participates in curving the actin rings within the MPS is still unknown but we could speculate the presence of additional actin nucleators, such as Arp2/3 and formins. IQGAP1's ability to interact with actin-nucleating complexes, such as N-WASP and the Arp2/3 complex, suggests that it may coordinate branched actin filament assembly to support MPS architecture (Le Clainche, Schlaepfer et al. 2007). As previously mentioned, the C-terminal of IQGAP1 activates N-WASP, leading to the activation of the Arp2/3 complex, which is essential for actin branching. The N-terminal of IQGAP1, however, inhibits this activation, suggesting a potential autoinhibitory mechanism that may be released under

specific conditions (Brandt and Grosse 2007). This dynamic regulation could be critical for the fine-tuning of actin ring size and the maintenance of MPS structure during axonal development. Moreover, we could not exclude the possible participation of formins in these processes. Although IQGAP1 regulates actin dynamics through the Rho GTPases family, including Cdc42 and Rac1, which participate in formin-mediated pathways, a link between these two proteins is yet to be established.

Conclusion

In conclusion, our findings highlight the differential roles of ANLN and IQGAP1 in the organization of actin rings in hippocampal neurons. ANLN appears dispensable for the formation and circularization of actin rings, indicating that its well-characterized function in actin ring formation during cytokinesis does not extend to the MPS of neurons. Conversely, IQGAPs play a crucial role in maintaining the structural integrity of actin rings, particularly during the later stages of neuronal development. The increased expression of IQGAP1 at DIV14 and its colocalization with actin rings suggests that IQGAP1 is necessary for curving, stabilizing and regulating the maturation of these structures.

These results suggest that IQGAP1 may interact with specific actin nucleators, such as Arp2/3 and potentially formins, to regulate actin dynamics within neurons. IQGAP1's ability to modulate both branched actin assembly and actin filament stabilization likely supports the circularization and maintenance of actin rings. Such interactions could play a role in fine-tuning the architecture of the cytoskeleton, which is critical for axonal diameter, neuronal signalling, and the transport of intracellular cargo.

Further research is required to delineate the molecular pathways through which IQGAP influences actin nucleation and stabilization. Investigating how IQGAP1, 2, and 3 interact with other actin-binding proteins and signalling molecules could provide valuable insights into the regulatory networks that maintain cytoskeletal integrity in neurons. Understanding these mechanisms is particularly important in neurodegenerative diseases, where disruptions in cytoskeletal organization are often implicated in disease progression.

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