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Actin regulation in dendritic spines: from synaptic plasticity to animal behavior and human neurodevelopmental disorders

Iryna Hlushchenko

Minerva Foundation Institute for Medical Sciences
And
Division of Physiology and Neuroscience
Department of Biosciences
Faculty of Biological and Environmental Sciences
University of Helsinki
And
Neuroscience Center
And
Doctoral Program Brain and Mind

Academic dissertation

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Supervisor: Docent Pirta Hotulainen
Minerva Foundation Institute for Medical Research, Helsinki, Finland
University of Helsinki, Finland

Reviewers: Docent Miriam Nokia
University of Jyväskylä, Finland

and

Professor Marco Rust
Institute of Physiological Chemistry
Philipps-Universität Marburg, Germany

Opponent: Head of Research Group Marina Mikhaylova
University Medical Center Hamburg-Eppendorf, Germany

Custos: Professor Juha Voipio
Faculty of Biological and Environmental Sciences
University of Helsinki, Finland

Cover image: Olena Sokyrko

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1 The author wrote the parts of the review concerning plasticity and contributed to writing and editing of the whole manuscript along with the PH and MK.

2 The author designed the research, performed all the experiments and wrote the manuscript with PH.

3 The author contributed to behavioral phenotyping of mice and confocal imaging of mouse brain slices, as well as the analysis of ventricle wall cilia. The author contributed to writing and editing the manuscript along with the rest of the authors.

4 The author carried out the experiments and analyses for Figures 2, 6, 9, 11, 13; wrote the respective sections of the manuscript and contributed to final writing and editing of the manuscript along with the rest of authors. The author was also responsible for the final layout of the figures.
Abstract

This thesis explores the role of several actin-binding proteins in the regulation of brain physiology with a focus on dendritic spines. Dendritic spines are considered the plausible physical substrate for learning and memory, as their morphology allows for modulating incoming signals. Disruptions in spine density and morphology are also often associated with neuropsychiatric disorders. 

The two cellular processes representing neuronal learning are long-term potentiation (LTP) and long-term depression (LTD). Here, I show that the actin-severing protein gelsolin transiently relocates to dendritic spines upon LTD induction, but not LTP induction or spontaneous neuronal activity. It is plausible that the modest – but relatively long-lasting – LTD-induced elevation of Ca²⁺ concentration increases the affinity of gelsolin to F-actin, thus inducing the relocalization of gelsolin to dendritic spines.

Proper spine regulation is crucial for learning in live animals. MIM is an I-BAR containing membrane curving protein, shown to be involved in dendritic spine initiation and dendritic branching in Purkinje cells in the cerebellum. Behavioral analysis of MIM knock-out (KO) mice revealed defects in both learning and reverse-learning, alterations in anxiety levels and reduced dominant behavior, and confirmed the previously described deficiency in motor coordination and pre-pulse inhibition. Anatomically, we observed a decreased density of thin dendritic protrusions, enlarged brain ventricles and decreased cortical volume.

Genetic studies have pointed out that genes often disturbed in neuropsychiatric disorders encode synaptic actin regulators. We selected five genes encoding different actin-regulating proteins and induced ASD-associated de novo missense mutations in these proteins. These mutations induced changes in the localization of α-actinin-4, which localized less to dendritic spines, and for SWAP-70 and SrGAP3, which localized more to dendritic spines. Among the wild-type proteins studied, only α-actinin-4 expression caused a significant change in dendritic spine morphology by increasing mushroom spine density and decreasing thin spine density. We hypothesized that mutations associated with ASD shift dendritic spine morphology from mushroom to thin spines. An M554V mutation in α-actinin-4 (ACTN4) resulted in the expected shift in dendritic spine morphology by increasing the density of thin spines. In addition, we observed a trend toward higher thin spine density with mutations in myosin IXb and SWAP-70. Myosin IIb and myosin IXb expression increased the proportion of inhibitory synapses in spines. The expression of mutated myosin IIb (Y265C), SrGAP3 (E469K), and SWAP-70 (L544F) induced variable changes in inhibitory synapses.
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>ABP</td>
<td>Actin-binding protein</td>
</tr>
<tr>
<td>ADHD</td>
<td>Attention-deficit/hyperactivity disorder</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ASD</td>
<td>Autism spectrum disorder</td>
</tr>
<tr>
<td>AuD</td>
<td>Secondary auditory cortex, dorsal area</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>CA1</td>
<td>Cornu Ammonis 1</td>
</tr>
<tr>
<td>cLTD</td>
<td>Chemical long-term depression</td>
</tr>
<tr>
<td>cLTP</td>
<td>Chemical long-term potentiation</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DHPG</td>
<td>Dihydroxyphenylglycine</td>
</tr>
<tr>
<td>DIV</td>
<td>Days in vitro</td>
</tr>
<tr>
<td>DLEnt</td>
<td>Dorsolateral Entorhinal cortex</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E17</td>
<td>Embryonal day 17</td>
</tr>
<tr>
<td>E469K</td>
<td>Glutamic acid 469 to lysine</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HFS</td>
<td>High-frequency stimulation</td>
</tr>
<tr>
<td>HVC</td>
<td>High Vocal Center</td>
</tr>
<tr>
<td>ID</td>
<td>Intellectual disability</td>
</tr>
<tr>
<td>K1872R</td>
<td>Lysine 1872 to arginine</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>L544F</td>
<td>Leucine 544 to phenylalanine</td>
</tr>
<tr>
<td>LFS</td>
<td>Low-frequency stimulation</td>
</tr>
<tr>
<td>M554V</td>
<td>Methionine 554 to valine</td>
</tr>
<tr>
<td>mGluRs</td>
<td>Metabotropic glutamate receptors</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PSC</td>
<td>Pluripotent stem cells</td>
</tr>
<tr>
<td>PSD</td>
<td>Postsynaptic density</td>
</tr>
<tr>
<td>S1BF</td>
<td>Somatosensory 1, barrel field</td>
</tr>
<tr>
<td>SCZ</td>
<td>Seizophobia</td>
</tr>
<tr>
<td>SIM</td>
<td>Structural illumination microscopy</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>Y265C</td>
<td>Tyrosine 265 to cysteine</td>
</tr>
</tbody>
</table>
1 Introduction

Neurons are special cells with an intricate morphology. They typically have three distinct compartments: the soma, the axon, and the dendrites. There are nearly twenty types of neuronal cells that can be separated by their size, shape and features and even more by functionality. Precise neuronal morphology is based on the actin cytoskeleton, regulated by hundreds of actin-binding proteins and signaling cascades. A tightly-regulated actin meshwork supports such unique neuronal structures as the axon initial segment and the tiny dendritic spines, which hold synapses. The most remarkable thing about neurons, however, is their ability for information processing and retention of memories while at the same time being plastic. Therefore, the idea of a single ‘memory molecule’ has lingered in the research community for a couple of decades. During this time it became evident that the activity-induced changes in neuronal morphology, especially in dendritic spines, are governed by a combination of many different players. The cytoskeleton of dendritic spines is in constant recycling, as well as the structural proteins that keep it together. Nonetheless, the overall structure can persist for weeks, months and years throughout the whole life. In my Ph.D. study, I attempt to highlight the different ways in which the actin cytoskeleton is regulated in dendritic spines. I am looking into how actin-binding proteins can adjust the brain function starting from the level of a single cell and all the way through animal behavior to human neuropsychiatric disorders.

2 Background
2.1 Dendritic spines

2.1.1 Dendritic spine structure

The tiny protrusions on the excitatory neurons’ dendrites —dendritic spines— come in a variety of shapes. First described by Ramon y Cajal back in 1888, they are typically sized within the range of two micrometers. Although the morphologies of spines found in fixed tissue form a continuum of shapes (Arellano, 2007), they can be roughly divided into three groups based on the relationship between their main elements: the spherical head on the tip, which is usually between 0.1 and 1 μm in diameter, and the neck connecting the head to the dendrite, which is usually between 0.09 and 0.51 μm in diameter and between 0.01 and 2.21 μm in length (Harris and Stevens, 1989; Konur et al., 2003; Arellano, 2007). Currently, widely accepted classification labels spines with a thin neck compared to their larger head as ‘mushroom’, the slimmer spines with head size comparable to neck width are called ‘thin’, and the protrusions with a larger head and very short and wide neck are ‘stubby’ (Figure 1). As will be discussed further, these arbitrary categories also
reflect the developmental stage and functional properties of the spine, making them useful readouts in dendritic spine studies.

![Dendritic spine types based on morphology.](image)

**Figure 1.** Dendritic spine types based on morphology.

The most important element of the dendritic spine is the excitatory synapse, or, to be precise, the postsynaptic part, which holds the neurotransmitter receptors. Virtually all the excitatory postsynapses in pyramidal neurons are situated on the dendritic spine heads and the size of the postsynaptic density correlates with the volume of the spine head (Harris and Stevens, 1988, 1989; Arellano, 2007). Taking into account that dendritic spines are the consistent elements of the key computational neurons such as the pyramidal cells of the cortex and hippocampus, as well as the Purkinje cells in the cerebellum, it is fair to assume they must have a specific function in neural signal processing and computation.

2.1.2 Dendritic spine function

Although quite a few functions were proposed for dendritic spines over the years, the ones that have the most supporting evidence so far are the biochemical isolation of the synapse and electrical modulation of the synaptic signal. These functions are strongly dependent on the precise spine morphology. It is quite straightforward to conclude that a spine neck with a diameter of a couple of hundred nanometers and a length of up to a couple of micrometers could limit the diffusion to or from the spine head. Indeed, it has been experimentally confirmed for a range of molecules: from large proteins to ions such as Ca\(^{2+}\) (Svoboda, Tank and Denk, 1996; Korkotian, Holcman and Segal, 2004; Noguchi et al., 2005; Tønnesen et al., 2014; Ramirez, Raghavachari and Lew, 2015). Moreover, the spine neck can also pose a diffusion barrier for membrane-bound molecules, such as neurotransmitter receptors (Adrian et al., 2017). The degree to which the neck can play its limiting role is often correlated to its diameter and length.
The electrical isolation of the signal, though not as obvious, was proposed as one of the spine functions since their discovery and attempts to model this in silico started in the seventies (Segev and Rall, 1988). With the advancement of experimental techniques, it has been shown that the amplitude of currents injected into the dendrite from a single spine increase with spine head diameter, but are also inversely proportional to spine neck length (Noguchi et al., 2005; Araya et al., 2006).

2.1.3 Dendritic spine development

Most dendritic spines form from pre-existing dendritic filopodia (Papa et al., 1995; Korobova and Svitkina, 2010). Filopodia arise from the dendritic shaft either randomly or in response to chemical stimulation (Portera-Cailliau, Pan and Yuste, 2003; Hamilton et al., 2012; Mattison et al., 2014). Although the detailed mechanisms of filopodia initiation are largely unknown, current evidence suggests that the development of a filopodium starts with bending of the dendritic membrane in the actin-rich regions of the dendritic shaft and continues with filopodium elongation (Korobova and Svitkina, 2010; Saarikangas et al., 2015). Filopodia are highly motile, and it is currently considered that this motility is needed to effectively probe the environment in the search for the optimal axon to form a synapse with (Wong and Wong, 2000; Dunaevsky and Mason, 2003). The current view is that after a synaptic connection is established the filopodium will start to form a distinct head, thus transitioning into a dendritic spine (Ziv and Smith, 1996). Simultaneously, the synaptic structure within the spine head will be formed and later stabilized.

In humans, spines form early during embryogenesis and their numbers rise steadily before and after birth. The spine and synapse density then peaks at 2-3 years old, which in rodents occurs at postnatal day 20-21. Later, during childhood and adolescence, spines are selectively eliminated to adult levels and plateau at the age of 12-18 years, which in rodents corresponds to postnatal day 35-49 (Huttenlocher, 1990; Bourgeois, Goldman-Rakic and Rakic, 1994; Semple et al., 2013; Mallya et al., 2017).

2.1.4 Spines in learning and animal behavior

What role do dendritic spines play in learning? As discussed before, almost all spines on principal neurons hold synapses. We have also noted that the synapse efficacy is tightly linked to the morphology of the dendritic spine it is situated on. Synapses and dendritic spines can thus mediate information processing in the brain, namely learning, forgetting and memory storage. The exciting idea that life experiences can be translated to physical changes in the brain has been gaining evidence for more than fifty years (Hubel and Wiesel, 1963; Le Vay, Wiesel and Hubel, 1980; Bailey and Kandel, 1993). Already during the seventies, scientists discovered that animals living in enriched environments have higher spine counts, while sensory deprived animals show a decreased number of spines in the corresponding sensory cortical areas (Globus et al., 1973; Freire, 1978). The
development of novel imaging techniques, such as two-photon microscopy, has enabled scientists to examine individual dendrites and spines over long periods of time in behaving animals (Lendvai et al., 2000; Hofer et al., 2009; Yang, Pan and Gan, 2009; Roberts et al., 2010). Notably, Yang et al. (2009) have observed how new spines form in the cortex of the mouse after learning and new sensory experiences, while also noticing facilitation in spine elimination. Despite constant structural rearrangement of synaptic connections, a fraction of the newly formed spines persisted through the observation period of 5 to 18.5 months during the experiment. Therefore, it was estimated that some spines can persist throughout the whole life of an animal (Yang, Pan and Gan, 2009). Others have also shown that spines formed during motor learning tasks are also preferentially stabilized during repeated learning (Xu et al., 2009). In 2010 Roberts et al. demonstrated that song learning in zebra finches increases dendritic spine turnover in the forebrain nucleus HVC, necessary for both learning and production of auditory information in songbirds. In addition, they observed the rapid spine stabilization and enlargement in vivo during learning when it was supplemented by tutoring from another bird (Roberts et al., 2010). Furthermore, if the remodeling of specific spines during learning is prevented it interferes with the learning process (Hayashi-Takagi et al., 2015). These experiments further backed the idea that dendritic spines are a plausible physical substrate for learning and lifelong memories.

2.1.5 Spine structural plasticity

In addition to the creation and elimination of dendritic spines, changes in existing spine morphology have gained interest along with functional plasticity studies (Harris, Jensen and Tsao, 1992; Lang et al., 2004). Dendritic spines are highly dynamic structures and even in the tissue, where space is limited by the extracellular matrix, their heads and necks are constantly morphing (Lendvai et al., 2000). However, there are specific, rapid and persistent changes induced by synaptic activity and accompanied by changes in the synapse strength.

Functionally, synaptic plasticity is a phenomenon of persistent change in the amplitude of synaptic response elicited by the same signal as a result of repeated synapse activation (Figure 2). Here I will discuss two types of long-term synaptic plasticity, which are associated with learning: long-term potentiation (LTP) and long-term depression (LTD).

2.1.5.1 Long-term potentiation

Long term potentiation—a persistent increase in the strength of the synapse—can be induced in glutamatergic synapses by trains of high-frequency activity (Bliss and Lomo, 1973). The postsynaptic events essential for the establishment of LTP include the depolarization of the postsynaptic membrane, NMDA receptor activation and short, significant increases in postsynaptic calcium concentration (Huganir and Nicoll, 2013). Single dendritic spines undergoing LTP exhibit a significant expansion
of the spine head along with shortening and widening of the spine neck (Figure 2) (Matsuzaki, Honkura, Graham C R Ellis-Davies, et al., 2004; Urban et al., 2011; Tønnesen et al., 2014). The potentiated synapses then increase in size and incorporate new receptors (Lu et al., 2001; Meyer, Bonhoeffer and Scheuss, 2014).

**Figure 2.** Structural plasticity of dendritic spines. High and low-frequency stimulation (HFS and LFS) lead to different functional and structural outcomes for dendritic spines and synapses. In LTP the existing spines enlarge and new spines may appear, which leads to an increase in the amplitudes of excitatory postsynaptic currents (EPSCs). On the other hand, in LTD the spines shrink and even retract and the amplitudes of EPSCs decrease.

### 2.1.5.2 Long-term depression

Long-term depression (LTD) is a form of synaptic plasticity crucial for developmental and experience-based network refinement. It is characterized by a decrease in the efficiency of a synaptic connection (Holtmaat and Svoboda, 2009; Chen, Lu and Zuo, 2014). Glutamatergic synapses in the hippocampal CA1 can undergo LTD after low-frequency stimulation (LFS) and this process requires postsynaptic NMDA receptor activation similar to LTP (Dudek and Bear, 1992; Mulkey and Malenka, 1992; Neveu and Zucker, 1996). However, the result is profoundly different for synapses and the spines, which shrink in size or even retract after induction of LTD (Figure 2) (Nägerl et al., 2004). Meanwhile, existing AMPA receptors leave the synapse (Carroll et al., 1999; Sanderson, Collingridge and Fitzjohn, 2011).

### 2.1.6 Disease-specific disruptions to dendritic spines

As discussed above, spines are important structural loci for excitatory synaptic transmission in the brain. Precise spine morphology and their density on the dendritic tree can regulate the signal processing by a neuron. Spines are also plastic, responsive to the environment and consequently easy to alter. It is not surprising that
disturbances in spine number, density or morphology have been observed in many neuropsychiatric disorders, especially the ones eliciting deficits in information processing (Penzes et al., 2011). For example, the brain of patients with autism spectrum disorder start to exhibit increased spine densities starting from childhood and the difference becomes pronounced during adolescence and then in the adult life (Hutslar and Zhang, 2010; Tang et al., 2014). On the other hand, in schizophrenia, the progressive loss of grey matter starting from adolescence and through young adult age is attributed to dendritic spine pruning and decreased connectivity between neurons (Garey et al., 1998; Glantz and Lewis, 2000). In Alzheimer’s disease the devastating changes occur later in life. However, the initial stages of the disease have been linked to synapse dysfunction and dendritic spine loss (DeKosky and Scheff, 1990; Knobloch and Mansuy, 2008). Although very different in onset time and symptoms, these diseases seem to have a common substrate –dendritic spines. Learning more about the regulation of dendritic spine structure would undoubtedly improve our understanding of these conditions and help to develop novel treatments to ease the lives of affected individuals.

2.2 Actin cytoskeleton

Neuronal cells have unbelievably intricate morphologies with several distinct compartments, and each neuron can look different depending on its type. Supporting these complicated structures are the cytoskeletal microfilaments composed of actin polymers and regulated by various actin-binding proteins. Actin is the most abundant protein in the eukaryotic cells and a remarkable one. Actin monomers have a molecular mass of 42 kDa (DOMINGUEZ and Holmes, 2011) and are called globular (or G-) actin. These monomers are able to assemble into filaments called filamentous (or F-) actin, which can then organize into a variety of 3D structures depending on which actin-binding proteins are involved in forming these structures.

2.2.1 Actin underlies the structure of the spine

Dendritic spines are extremely rich in filamentous actin. With the development of electron microscopy techniques, we learned that there are different subsets of actin filaments within dendritic spine compartments: the head of the spine is filled with an elaborate branched network of short actin filaments, whereas the neck contains also longer filaments which are better aligned (Landis and Reese, 1983; Korobova and Svitkina, 2010). This model of spine cytoskeleton structure was further refined with the development of super-resolution imaging techniques, which can reveal finer F-actin structures in mildly treated or even live neurons and further specify the stable and dynamic actin fractions (Frost et al., 2010; Urban et al., 2011). In addition to the long filaments in the spine neck and the branched filaments in the spine head, periodic actin ring structures were found in spine necks (Bär et al., 2016).
2.2.2 Actin regulation in spines

Most actin filaments are undergoing constant treadmilling: they polymerize at one end (barbed) and depolymerize at the other end (pointed). Using FRAP and photoactivation techniques, the fraction of dynamic actin filaments in the live dendritic spines was estimated to be around 85% (Star, Kwiatkowski and Murthy, 2002; Honkura et al., 2008; Frost et al., 2010). It would be fair to wonder how dendritic spines can maintain their structure while being largely composed of dynamic actin (Star, Kwiatkowski and Murthy, 2002). Even though we are far from completely unraveling this phenomenon, major progress has been achieved over the last few decades in identifying actin-binding proteins (ABPs), recently counted at 162, not including isoforms (dos Remedios et al., 2003; Lappalainen, 2016). Many of these actin regulators are present in neurons and can regulate dendritic spine structure. The better-characterized ones include the Arp2/3 complex (nucleates branched actin filaments), profilin (facilitates polymerization), coflin and ADF (depolymerize and sever actin filaments), CaMKIIb, α-actinin and drebrin (bundle and stabilize actin filaments).

2.2.3 Actin in synapse function and plasticity

Actin dynamics in dendritic spines can rapidly change in response to synaptic stimulation (Star, Kwiatkowski and Murthy, 2002; Okamoto et al., 2004). These changes are largely mediated by ABPs and can involve either recruitment of specific actin regulators to dendritic spines or their exodus from the spine heads (Bosch et al., 2014). During LTP formation, spines accumulate F-actin in two steps that are followed by the expansion of the PSD. Immediately after high-frequency stimulation (HFS), a large portion of the existing actin structure in the dendritic spine is disassembled by actin severing factors, then reorganized and expanded (Lang et al., 2004; Matsuzaki, Honkura, Graham C R Ellis-Davies, et al., 2004; Chen et al., 2015). In the second phase, the new structure stabilizes with the help of actin crosslinking proteins and disruption of these proteins’ function can prevent LTP stabilization (Rex et al., 2010; Bosch et al., 2014; Kim et al., 2015).

In LTD spines lose F-actin and decrease in size (Halpain, Hipolito and Saffar, 1998; Nagerl et al., 2004; Okamoto et al., 2004). Calcium-activated actin-severing proteins have been proposed as possible mediators of the synaptic signal to the actin cytoskeleton in LTD. However, strong experimental confirmation has only been achieved for a few of them. Namely, coflin activation through calcineurin has been shown to couple calcium influx to spine shrinkage (Pontrello et al., 2012). Gelsolin has often been proposed as a plausible player in LTD, but the experimental works exploring this idea have so far brought conflicting results (Star, Kwiatkowski and Murthy, 2002; Morishita, Marie and Malenka, 2005).
2.2.4 Actin-regulating proteins

2.2.4.1 Gelsolin

Gelsolin is a ubiquitously expressed actin-binding protein, which generally localizes to actin-rich structures (Li et al., 2012; Nag et al., 2013; Tanaka and Sobue, 1994). It is known as one of the most potent actin-severing factors and is activated by binding calcium ions (Yin and Stossel, 1979; Yin et al., 1981). However, the functions of gelsolin can also include capping actin filaments, sequestering actin monomers and, in vitro, nucleating actin filaments. Gelsolin has six homologous domains (G1-G6), each harboring a Ca\(^{2+}\) binding site. Upon elevation of calcium concentration gelsolin binds and severs F-actin (Forscher, 1989; Li et al., 2010).

![Image showing gelsolin's role in actin severing and capping](image)

**Figure 3.** Actin severing and capping by gelsolin.

Not much is known about the function of gelsolin in the nervous system and especially in the regulation of dendritic spines. In young neuronal cultures, gelsolin can regulate spine length in a calcium-dependent manner (Hu and Hsueh, 2014). Neurons cultured from mice lacking gelsolin show decreased depolymerization of actin in response to glutamate stimulation (Furukawa et al., 1997). Actin stabilization induced by LFS was disturbed in dendritic spines of neurons lacking gelsolin (Star, Kwiatkowski and Murthy, 2002). Functionally, however, LTD of postsynaptic currents was elicited normally in slices from gelsolin knockout mice (Morishita, Marie and Malenka, 2005).
2.2.4.2 MIM

First described in 2002, missing-in-metastasis (MIM/MTSS1) is an actinbinding protein highly expressed throughout the developing brain, and in Purkinje cells of the cerebellum in adult mice (Lee et al., 2002; Mattila et al., 2003). MIM possesses a G-actin binding WH2 motif and an inversed BAR (I-BAR) domain and can bind and curve PIP2-rich membranes (Mattila et al., 2007). In neurons, MIM initiated dendritic filopodia formation by curving dendritic membranes and subsequently facilitating Arp2/3-mediated actin polymerization (Saarikangas et al., 2015). MIM positively regulates dendritic spine density and negatively affects spine maturation. Mice that are deficient in MIM show disturbances in behavior and synaptic transmission (Saarikangas et al., 2015).

2.2.4.3 Alpha(α)-actinin-4

Alpha(α)-actinin-4 (ACTN4) monomers consist of the actin-binding domain and a CaM-domain separated by four spectrin repeats and are assembled into dimers in an anti-parallel fashion. This structure makes α-actinin well suited for crosslinking actin filaments into bundles and docking of proteins (Otey and Carpen, 2004). It has been shown in vitro that α-actinin-4 can form a ternary complex with the adhesion molecule densin-180 and CaMKIIα — both present in the PSD — and this complex formation is regulated by CaMKII phosphorylation (Walikonis et al., 2001).

In the mouse brain, α-actinin-4 is expressed in the hippocampus, cortex, and cerebellum. In cultured cortical neurons its expression plateaus at 14 DIV. In neurons, α-actinin-4 localizes at excitatory synapses along with group 1 metabotropic glutamate receptors (mGluRs) and supports the transition of dendritic spines from thin to mushroom. Depletion of α-actinin-4 disrupts the mGluR-dependent structural plasticity of dendritic spines (Kalinowska et al., 2015).

2.2.4.4 SWAP-70

Switch associated protein 70 (SWAP70) is a ubiquitously expressed protein that is involved in DNA recombination in the nucleus (Borggrefe et al., 1998). SWAP-70 regulates the actin cytoskeleton in the cytosol by bundling the filaments and associates with actin filament arrays generated behind actively protruding lamellipodia (Hilpelä et al., 2003; Chacón-Martínez et al., 2013). SWAP-70 has been shown to interact with coflin in vitro (Chacón-Martínez et al., 2013). Moreover, SWAP-70 possesses the pleckstrin homology domain which enables it to bind to PI(3,4)P₂ in the plasma membrane. Among the putative interaction partners for SWAP-70, myosin Ixb — another actin-binding protein — was identified in two-
hybrid screening (Hilpelä et al., 2003). SWAP-70 is also expressed in the brain (Hilpelä et al., 2003). However, its function in neurons has not been studied.

2.2.4.5 SrGAP3

Slit-Robo GTPase activating protein 3 (SrGAP3, Gene: SRGAP3, also called Mental disorder associated GAP protein (MEGAP) and WAVE-associated GAP (WRP)) is structurally similar to the other proteins in its SrGAP family. SrGAP1 and 2 possess an N-terminal F-BAR domain, a central GAP domain, and a C-terminal SH3 domain. SrGAP3 can inhibit cytoskeletal dynamics by inactivating Rac1 via its GAP domain, regulate actin-binding proteins through its SH3 domain and bind to membrane lipids via its F-BAR domain. In motile cells, SrGAP3 regulates lamellipodia dynamics and is involved in filopodia formation (Endris et al., 2011).

SrGAP3 is widely expressed throughout the murine brain (Bacon, Endris and Rappold, 2009). Mice lacking the SRGAP3 gene have enlarged brain ventricles and a reduced density of cilia on ventricle walls (Koschützke et al., 2015). They also show deficits in cognitive function, namely impaired learning and memory (Koschützke et al., 2015). In cultured neurons, SrGAP3 is required for dendritic spine development (Carlson et al., 2011). Furthermore, mutations in the SRGAP3 gene have been found in cases of severe mental retardation and ASD (Endris et al., 2011).

2.2.4.6 Myosin-IIb

Non-muscle myosin IIb (MYH10), or non-muscle myosin heavy chain IIb (NMMHCIIb) (referred to here as myosin IIb) is a myosin isoform expressed predominantly in neuronal tissue (Kawamoto and Adelstein, 1991). Myosin IIb structure includes two heavy chains and two pairs of regulatory light chains, which mediate the contractile activity of the molecule. Myosin IIb is a known regulator of spine morphology and development (Zhang, 2005; Ryu et al., 2006; Rex et al., 2010; Hodges et al., 2011). It is positioned at the base of the spine head where it crosslinks and stabilizes actin filaments (Korobova and Svitkina, 2010; Rubio et al., 2011; Koskinen et al., 2014). However, near the spine surface, the protein can exhibit the opposite function and enhance the dynamics of actin filaments through its ability to contract and break actin filaments (Koskinen et al., 2014). In recent years, MYH10 gene was associated with neuropsychiatric disorders such as ASD and schizophrenia (Fromer et al., 2014).

2.2.4.7 Myosin-IXb

Myosin IXb (Myo9b) is a single-headed molecular motor containing a Rho-specific GAP domain in the tail region, four binding sites for myosin light chains in its neck region and a C1 (Zn2+ binding) domain close to the RhoGAP domain (Wirth
et al., 1996). Human myosin IXb was previously mostly studied in cancer cell lines and macrophages, where it localizes to sites of actin polymerization and supports cell motility (van den Boom et al., 2007; Hanley et al., 2010). Myosin IXb is also expressed in the central nervous system, albeit to a lesser extent than myosin IXa. In cultured cortical neurons, it regulates growth and branching of dendrites by controlling RhoA activity (Long et al., 2013).

2.3 Neuropsychiatric disorders

At least one member in every fourth family worldwide is currently suffering from a mental or behavioral disorder (Murthy et al., 2001). This does not affect only the person diagnosed, but also the family members, who must adjust their lifestyle in a way that often prevents reaching their full potential. Neuropsychiatric disorders have been recognized as the biggest disease burden in Europe (Wittchen et al., 2011). Many neuropsychiatric diseases have a genetic background. Nevertheless, current treatments mostly focus on ameliorating symptoms, lack a personalized approach and in many cases do not really cure (McMahon and Insel, 2012).

Dendritic spine density and morphology is disturbed in a number of neuropsychiatric disorders and changes in synaptic function are now thought to lie at the core of these conditions. A new term has even been proposed—synaptopathy—to mark conditions with different symptoms but related to changes in the synapse.

2.3.1 Schizophrenia diagnosis and prevalence

Schizophrenia (SCZ) is a psychiatric disorder that interferes with a person’s ability to think clearly and manage emotions, and complicates the individual’s relationship with other people. Schizophrenia is typically diagnosed between early teens and twenties for men and between early twenties and thirties for women. The core symptoms of SCZ include hallucinations, delusions, lack of motivation and various cognitive impairments. The prevalence of schizophrenia is estimated in the range of 4 to 7 cases per 1 000 persons and generally does not differ between the sexes (Saha et al., 2005).

2.3.2 Studying the neurophysiology of schizophrenia in humans

The neuroanatomical changes in patients diagnosed with schizophrenia are rather well studied. Multimodal brain imaging studies point to disruptions in the structure of basal ganglia-thalamocortical circuits as a most common biomarker for schizophrenia (Zhao et al., 2019). Additionally, decreased volumes in cortical and other brain regions have been observed in schizophrenia, as well as a decrease in
dendritic spines density (Garey et al., 1998; Glantz and Lewis, 2000). Recent meta-analysis has revealed that although substantial heterogeneity is present in the evaluation of synaptic and spine densities in the schizophrenic brain, a significant decrease of postsynaptic elements is especially evident in the prefrontal cortex and cortical layer 3 (Berdenis van Berlekom et al., 2019). In addition, one of the most consistent brain abnormalities found in patients with schizophrenia is lateral ventricle enlargement (del Re et al., 2016).

2.3.3. Role of actin regulators in schizophrenia

Anatomical data point towards the idea that the pathology of schizophrenia is largely arising from synapse dysfunction. Genetic studies support this as well, showing particular enrichment of mutations in the genes coding for synaptic proteins (Fromer et al., 2014). Many of these synaptic proteins appear to be actin regulators required for the formation and maintenance of dendritic spines. Additionally, the expression of such actin regulators as the Rho GTPase cell division cycle 42 (Cdc42), kalirin and multiple subunits of Arp2/3 complex are altered in the prefrontal cortex of schizophrenia patients and animal models (Cahill et al., 2009; Datta et al., 2017; Hill et al., 2006).

2.3.4 Animal models of schizophrenia

Taking into consideration the core symptoms of schizophrenia, such as hallucinations or scattered thinking, it is evident that modeling this disorder in rodents is a complicated task. Indeed, most major symptoms are self-reported in humans and are not reliably relatable to mouse behavior. However, the behaviors relying on neural circuits affected in schizophrenia can be evaluated across species. Indeed, working memory and general cognitive abilities, as well as deficits in social interactions, can be tested in mice using standard behavioral assays (Powell and Miyakawa, 2006). Though the results of behavioral phenotyping of different knockout mouse models are heterogeneous, the common deficits they often share are shown in learning, social behavior and pre-pulse inhibition (Powell and Miyakawa, 2006).

2.3.5 Diagnosis and prevalence of ASD

Autism spectrum disorder (ASD) is neuropsychiatric condition usually diagnosed during the first two years of life, it is, therefore, often considered a developmental disorder. Affected individuals exhibit difficulties in social communication, restricted interests and repetitive behaviors. Their symptoms often hurt their ability to learn, work and in general function in various aspects of life. Autism is considered a ‘spectrum’ as the combinations and severity of symptoms can
vary greatly under the umbrella of an ASD diagnosis. ASD occurs in all racial, ethnic and socioeconomic groups. The estimated global prevalence of ASD was rated between 2.8 and 94 per 10 000 (Elsabbagh et al., 2012). The main symptoms of autism often coexist with other medical conditions, which include intellectual disability (ID), attention deficit hyperactivity disorder (ADHD), epilepsy, tics, anxiety, sleep disturbances and gastrointestinal problems (Gillberg, 2010).

2.3.6 Studying the neurophysiology of ASD in humans

Neuroanatomical changes in the ASD brain have been hard to pinpoint. The difficulty is largely related to the fact that ASD affects higher brain function and studying it with a top-down approach is limited to noninvasive methods and complicated by accompanying conditions. A few electroencephalography and functional brain imaging studies have suggested altered connectivity between brain regions in ASD patients, but a definitive neuroanatomical phenotype of ASD has not been found so far. Studies of post-mortem brain tissue of ASD-affected individuals have shown an increase in dendritic spine density (Hutsler and Zhang, 2010). Recently, it has become possible to obtain differentiated neurons from pluripotent stem cells (PSCs) of ASD patients and even grow brain organoids in culture dishes. This approach has been showing promising results in identifying physiological changes in the brain development of ASD patients (Wang, 2018).

2.3.7 Genetics of ASD

The last couple of decades have seen tremendous improvements in genetic research, which aided studies on neuropsychiatric disorders, including autism. They started as epidemiological studies of families and twins and have shown that ASD is a highly heritable condition with the concordance rate for monozygotic twins reaching 90% (Steffenburg et al., 1989). Even though these epidemiological studies have largely clarified the genetic contribution of ASD, they could not inform us of the genes involved or the frequency and number of their variants. To uncover the physiological changes in ASD a bottom-up approach using methods of quantitative molecular genetics has shown much promise: starting from identifying genes affected in ASD we can predict and test, in vitro, how the physiology is altered upon the perturbation of the functions of these genes.

Up to 60% of ASD cases can be explained by common variants, and it was only in the last couple of years that genetic studies finally reached the power required to detect some of them (Grove et al., 2019). In the future, we will likely see more results from similar studies. However, identifying single rare and de novo penetrant variants has hitherto been most useful. The cases where major symptoms are caused by the presence of one genetic alteration make up 10-25% of ASD cases. Knowing the products of these crucial genes is valuable for uncovering the altered physiology of ASD on the cellular level. Large whole-exome sequencing studies have pointed out that genes altered in ASD are connected to the regulation of synaptic function,
actin cytoskeleton regulation, protein synthesis and degradation, chromatin remodeling and transcription.

2.3.8 Role of actin regulators in ASD

Many genes can be affected in ASD. In this study, we focus on those related to actin regulation. As I discussed before, dysregulation of neuronal actin can cause an alteration in dendritic spine morphology and density, therefore changing synaptic transmission efficacy. At dendritic spines and synaptic sites actin is regulated by a number of proteins. More than 30 genes encoding actin regulators were identified so far to be associated with ASD. Products of these genes can control actin polymerization and depolymerization, crosslinking or linking to other proteins, synaptic scaffolds or trans-synaptic adhesion molecules, and activate or inhibit actin regulation pathways (Joensuu, Lanoue and Hotulainen, 2017).

2.3.9 Animal models of ASD

Animal models, though not perfect, provide a tool for investigating the role of genetic mutations and environmental factors in the behavioral changes associated with ASD. ASD is a uniquely human disorder, therefore it has been challenging to define behavioral assays for rodents that would be relevant to diagnostic symptoms of autism. Nevertheless, the number of behavioral tests assessing sociability, repetitive behaviors and accompanying conditions such as seizure and sleep disorders are currently successfully used in evaluating animal models for ASD (Silverman et al., 2010). The most frequently used rodent models for studying ASD are created by generating targeted mutations corresponding to the ones found in ASD-affected individuals in the mouse genome. The promising mouse models for ASD are the Fmr1 knockout mouse, which mimics the silencing in FMR1 gene observed in Fragile X syndrome, and the Shank3-deficient mouse, which mimics the insufficiency in the Shank3 gene associated with autism. Both animal models show behavioral symptoms analogous to humans such as social deficits and repetitive behaviors (Bozдачи et al., 2010; Dolan et al., 2013; Duffney et al., 2015). These models allowed a deeper look at the underlying neuronal function disruptions in ASD and a potential way to reverse the functional deficiencies. Fmr1 KO mice do exhibit an increased dendritic spine density similarly to humans, likely mediated through mGluR 1- and 5-associated plasticity (Comery et al., 1997; Bhakar, Dölen and Bear, 2012). Shank3-deficient mice show disrupted glutamatergic synaptic function and actin regulation (Bozдачи et al., 2010; Duffney et al., 2015). Additionally, a few inbred mouse lines exhibit robust deficits in social behaviors relevant to ASD. These models are considered a better representation of idiopathic ASD, as there are no known genes responsible for the ASD-like symptoms in these cases (Silverman et al., 2010).
2.3.10 Can core ASD symptoms be treated in animal models?

Currently, ASD is treated by behavioral therapy and pharmacological solutions exist only for alleviating associated symptoms such as aggression, self-injury, and temper tantrums, rather than addressing the core symptoms of ASD. Mouse models of ASD have been used by scientists in pharmacological companies as well as in universities to test new drugs and therapies for ASD. From genetic studies we can uncover potential drug targets or if the disruption of single genes can lead to behavioral symptoms. New approaches in gene manipulations have opened the possibility of restoring affected genes, and animal studies have provided a demonstration that some of the symptoms can be rescued with gene therapy even in adults (Mei et al., 2016). However, a deeper understanding of the pathways affected by disrupted genes can provide hints about ways to restore the damaged function without resorting to gene editing. A good example would be results obtained in studies on SHANK3-deficient mice, where fixing the disrupted actin regulation through the administration of a cofilin inhibitor could compensate for the social deficits and repetitive behaviors (Duffney et al., 2015). Similar success was achieved in Fragile X model mice, where the rescue of dendritic spines by manipulating actin dynamics was able to partly restore neurological and behavioral symptoms (Dolan et al., 2013). Even though these therapy strategies have not yet been tested in the clinic, they provide proof that there are many approaches that can be effective. It is worthwhile to keep in mind though, that as ASD is a developmental disorder the effectiveness of a treatment would depend on the developmental stage at which it is administered. Nevertheless, a deeper understanding of physiological changes in ASD holds promise of finding ways to discover new treatments.
3 Aims of the study

Actin is a building block of neuronal cell structures and is regulated by hundreds of actin-binding proteins. My work aims to show the importance of actin regulation specifically at dendritic spines for neuronal function spanning from the level of a single neuron to animal behavior and human neuropsychiatric disorders.

1. The ability of dendritic spine cytoskeleton structure to rearrange in accordance with functional changes in synapses underlie memory formation. The first aim of my study is to summarize and critically evaluate the current knowledge on actin regulation in dendritic spines during synaptic plasticity. Furthermore, I aim to show how the actin-binding protein gelsolin is involved in actin disassembly in long-term depression (I, II).

2. Actin regulation has been shown to be important for synaptic function and, thus, underlies behavior. The second aim of this work is to show how a deficiency in the actin-binding protein MIM can affect rodent brain physiology and ultimately animal behavior (III).

3. A number of genetic studies have pointed out that genes often disturbed in neuropsychiatric disorders such as schizophrenia and ASD encode synaptic actin regulators. The third aim of this study is to investigate how single-point mutations in genes encoding for five actin regulators (α-actinin-4, SWAP-70, SrGAP3, myosin Ixb, and myosin IIb) can affect the protein and its function in neuronal dendritic spines and inhibitory synapses (IV).
4 Materials and Methods

For the large part of the experimental work presented in this thesis, I have used primary hippocampal neuronal cell cultures obtained from Wistar rat embryos at E17. In addition, mice were used in study III. All animals were handled in accordance with Finnish laws and ethics under the EU Directive 2010/63/EU (licenses: ESAVI-4943-04.10.07-2016 and GMO 3/S/12). The detailed descriptions of the materials and methods used can be found in the original publications. All the methods used in the thesis work are summarized in Table 1.

Table 1. Summary of experimental methods.

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<th>Method</th>
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<td>Live cell confocal imaging</td>
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5 Results

5.1 Plasticity-induced actin remodeling in dendritic spines (Study I, II)

5.1.1 Refining the current view on actin remodeling in LTP and LTD

The first aim of my study was to summarize the currently available knowledge on actin cytoskeleton remodeling during spine maturation and long-term synaptic plasticity events. Initially, the growth of dendritic spine heads during development and plasticity was exclusively attributed to increased actin polymerization. In my review of current research papers exploring actin dynamics, I highlighted and summarized the emerging view that the actin structures in spines are disassembled and rebuilt during long-term potentiation to suit the increase in synapse size and number of receptors. I also noted that a big number of actin regulators are involved in this process at different times and the whole process can be divided into three main phases. Furthermore, I noted that the long-term decrease in spine size and the accompanying actin remodeling have got little attention from the scientific community in recent years compared to LTP. So far, the detailed mechanisms of spine size decrease and elimination are not known and only a few actin-binding proteins were studied in LTD. Several studies have proposed gelsolin as a likely player in spine remodeling during LTD. However, only a couple of studies have attempted to clarify its role in plasticity.

5.1.2 Gelsolin accumulates transiently in dendritic spines upon chemical LTD induction

In the second study, I aimed to clarify how gelsolin is involved in plasticity. In his Ph.D. thesis work, Zhao (2003) showed that gelsolin can accumulate in dendritic spines of cultured neurons after stimulation with glutamate. I started with testing whether gelsolin would respond similarly to chemical LTD induction mediated by NMDA receptors. To achieve that, I treated days in vitro (DIV) 18-21 neurons expressing gelsolin-GFP along with mCherry with a solution containing 50 μM NMDA and 1 μM glycine for 4 minutes. I acquired one z-stack of images of the same dendritic segment each minute for 10 minutes before NMDA application, 3 minutes during application and 30-40 minutes after the washout of NMDA. This experiment showed that already during NMDA application, diffusely distributed gelsolin started to accumulate in dendritic spines. The accumulation peaked at 8 minutes after starting the NMDA application and sustained significantly above the baseline for an average of 7 minutes (timepoints 7-13 min). Simultaneously followed mCherry distribution did not change.
To further supplement this finding, I repeated the same experiments while following changes in distribution of F-actin (using Lifeact-RFP), GluR1 subunits of AMPA receptors and the calcium indicator GCaMP6f. I saw that the onset of the accumulation of gelsolin in dendritic spines also correlated with a 20% decrease in Lifeact-RFP signal in dendritic spines. The decrease in F-actin accumulation in spines was statistically significant 10 minutes after starting NMDA application and remained so until the end of recording (40 minutes). This experiment also showed that the signal obtained from surface GluR1 in dendritic spines started to decrease upon the application of NMDA and continued to fall in a linear fashion throughout the duration of recording (35 min) reaching as low as 40% of the baseline value. Imaging with GCaMP6f further revealed a fast elevation in Ca$^{2+}$ concentration in dendritic shafts and spines, which lasted throughout the NMDA application.

Taken together, these experiments showed that the induction of NMDAR-mediated cLTD results in an immediate increase in Ca$^{2+}$ concentration followed by a slower decrease in cell surface expression of GluR1, re-localization of gelsolin from dendritic shafts to spines and loss of F-actin from dendritic spines.

5.1.3 Gelsolin is enriched in spines upon cLTD but not cLTP induction

Both LTD and LTP require elevation in Ca$^{2+}$ concentration. I compared the changes in gelsolin localization upon cLTD and cLTP induction by analyzing gelsolin distribution in fixed cells. I first induced chemical LTD or LTP (Lu et al., 2001) in DIV 21 hippocampal neurons, and then fixed them immediately or 30 min after chemical stimulation. As expected, gelsolin enrichment in spines was observed immediately after cLTD induction, however, induction of cLTP did not affect gelsolin distribution. Like in live experiments, gelsolin enrichment was transient, and I did not detect it 30 minutes after the washout.

During LTP, the elevation of calcium is typically shorter. It is possible that we missed the short redistribution of gelsolin by fixing at only two timepoints. Therefore, we performed a live cLTP experiment where we followed gelsolin-mCherry and GCaMP6f. Although Ca$^{2+}$ levels were elevated briefly during the stimulation, we did not detect any redistribution of gelsolin. We also followed gelsolin and Ca$^{2+}$ during spontaneous activity, where Ca$^{2+}$ levels rise in single dendritic spines for 2-3 seconds. No gelsolin redistribution was observed under these condition. We neither detected changes in gelsolin localization in mGluRs-dependent LTD, induced by a 7-minute application of dihydroxyphenylglycine (DHPG). These results show that NMDARs-dependent cLTD, but not cLTP, spontaneous activity or mGluRs-dependent LTD, induce a transient accumulation of gelsolin in spines.
5.1.4 Gelsolin co-localizes with actin filaments but not with synaptic structures

The binding of Ca$^{2+}$ by gelsolin can open its structure and increase its affinity to actin filaments. As F-actin is enriched in dendritic spines and hardly visible in the dendritic shaft, binding to actin would then be a plausible mechanism for recruiting gelsolin to spines upon NMDA treatment. Thus, I followed GFP-gelsolin and Utrophin-mCherry in DIV 21 neurons using structural illumination super-resolution microscopy (SIM) to see whether gelsolin would colocalize with F-actin during the translocation. Although SIM does not allow the quantification of changes in fluorescent intensity, we can estimate the amount of gelsolin co-localizing with F-actin based on the reconstructed structure of dendrite and spines. Upon LTD induction, gelsolin distribution was transiently more similar to that of F-actin. Next, I tested whether gelsolin co-localizes with synaptic markers in spines and whether the enhanced binding to the receptor area could explain the accumulation of gelsolin in spines upon NMDA treatment. SIM imaging revealed that gelsolin is located close to synapses, visualized by anti-PSD-95 antibody staining. Although gelsolin was located close to synaptic structures, it seldom co-localized with synaptic markers and this localization was not changed by NMDA treatment.

5.2 The knockout of MIM changes brain structure and behavior of mice (Study III)

MIM is an actin-regulating protein that initiates new dendritic spines by locally curving the membrane of the dendrite. MIM is highly expressed during brain development (Mattila et al., 2003; Saarikangas et al., 2015). Previously, mice deficient in MIM (MIM -/- or MIM KO as referred to further) showed lower spine densities in Purkinje cells in vivo as well as in vitro in cultured hippocampal pyramidal neurons. MIM KO mice also exhibited defects in motor coordination and functional properties of Purkinje cells (Saarikangas et al., 2015). To achieve a comprehensive picture of brain physiology and the behavioral phenotype of MIM knockout mice we subjected them to a broad array of behavioral tests and histological analysis. First, we confirmed MIM expression in different brain areas by Western blotting and immunohistochemistry. Similar to previous studies (Saarikangas et al., 2015), MIM was highly expressed in the cerebellum and less so in the cortex and hippocampus.

5.2.1 MIM KO mice show deficiencies in learning and motor coordination

The first cohort of mice was examined when they were young (16±2 weeks old) and aged (42±2 weeks old). The young group had 12 males and 12 females in
the control group (MIM ++), and 10 males and 8 females in the KO group. In the older group, we tested the same controls and 9 males and 6 females in the KO group (3 KO mice died before reaching the age of 42 weeks). As learning is believed to be partially dependent on the generation of new spines, we focused on examining the spatial memory of MIM KO mice. Young MIM KO mice showed no significant spatial memory decline in the Morris water maze as compared to their wild-type littermates. When re-tested at an older age, however, they showed a significant deficiency in learning and reversed learning and older MIM KO mice were also performing worse in the probe trial. Similar results were also seen when spatial learning was assessed in older mice with Barnes test. We also accounted for possible health problems that might have affected the performance of the MIM KO mice in these learning tasks. Their deficiency was not related to muscular weakness (the measured velocity was not different between the genotypes) or vision impairment (no difference in finding the visible platform in the water maze).

In addition to the spatial memory test we carried out other behavioral tests to achieve a comprehensive view of the role of MIM in mouse behavior. We observed reduced anxiety-like behaviors in MIM KO mice (tested in open field, elevated zero maze and light/dark box test), and impaired motor coordination and strength (tested in rota-rod, vertical grid, grip strength and footprint tests). The possible deficits in the social interactions were tested in the tube dominance test, which reliably measures dominant behavior in male rodents as a percentage of wins in the total number of matches. In this test, MIM KO male mice showed less dominant behavior compared to wild-types, winning fewer matches at both young age and when re-tested at 42 weeks old.

The observed behavioral phenotype with impairment in cognitive function, decreased anxiety levels and defects in motor coordination are typically present in mouse models of schizophrenia. The histological changes in these models typically include disrupted dendritic spine density and morphology, increased ventricle volume and decreased cortical volume. Therefore, we next examined these parameters in MIM KO mice.

5.2.2 MIM KO show reduced densities of thin and stubby spines in the hippocampus

We hypothesized that the spatial learning deficits were a result of a deficiency in MIM-dependent regulation in the initiation of dendritic spines. Therefore, we transduced a GFP expression-inducing lentiviral vector in CA1 pyramidal neurons of the mice used in the behavioral tests at P378. After two weeks of expression, the mice were sacrificed and their brains fixed and sliced. The slices were then additionally stained with DAPI and anti-GFP-antibodies to reveal the nuclei and amplify the GFP signal. The dendrites of CA1 pyramidal neurons revealed by GFP signal were imaged with a confocal microscope and spine density and morphology were analyzed based on 3D reconstructions using the NeuronStudio software. According to our analysis, total spine density did not change in MIM KO mice compared to controls. However, the densities of thin and stubby spines were reduced.
5.2.3 Brain ventricles are enlarged in MIM KO mice

We next examined ventricle size in the same mice that were used in the behavioral tests. After the tests were completed, the mice were sacrificed and their brains were fixed and sliced. The slices were further stained with DAPI to visualize the cell nuclei. We compared total brain area and ventricle area in MIM KO slices at different Bregma (0.0, -0.5, -1.5, -2.0, -3.0) to wild-type mice slices. Total brain area was similar for MIM KO and wild-type mice. However, the area of ventricles in all Bregma was significantly larger in MIM KO mice slices than in WT slices.

We, therefore, generated a separate group of MIM KO and WT mice for a longitudinal in vivo magnetic resonance imaging (MRI) study which allowed us to follow water-content changes in brain parenchyma as well as ventricular volume increase over time. As expected, we observed the increase in the volume of ventricles, which was already evident at postnatal week 12 and progressing throughout the 2 months of the study. Additionally, we observed a sex-related phenotype within the MIM KO group: 2 out of 3 male transgenic mice developed a hydrocephalic condition and were not used in subsequent analysis. The last analyzed MIM KO male mouse showed increased ventricular volume compared to female MIM KO and leakage of cerebrospinal fluid (CSF) from the ventricles.

To examine the CSF circulation more closely, at the end of the longitudinal study the left lateral ventricle of each mouse was injected with a solution of Evans Blue (EB) tracer dye and sacrificed after 2 hours. The EB distribution in the brains of MIM KO mice was atypical, with an accumulation of the tracer in the ventricles and periventricular areas, suggesting a deficiency in fluid dynamics. Hydrocephalic mice showed a diffuse bilateral EB signal in the caudal brain areas, indicating that the tracer could freely distribute from the ventricles in the parenchyma.

5.2.4 Cilia are not altered in the ventricle or aqueduct walls of MIM KO mice

MIM KO mice showed a deficiency in fluid dynamics and cases of hydrocephalus. Genetic animal models of hydrocephalus show increased ventricular volumes that are related to ciliary defects affecting the CSF circulating system (Kim et al., 2012). It was also previously demonstrated that MIM regulates ciliogenesis in mouse skin dermal cells (Bershteyn et al., 2010). Therefore, increased ventricle volume of MIM KO mice might be a result of cilia defects on the ventricle walls and cerebral aqueduct. Thus, we measured cilia density and length both in the lateral ventricle walls and the cerebral aqueduct. Cilia were visualized in brain slices by acetylated-tubulin staining and the ventricle walls were imaged using a confocal microscope. However, we found no significant differences in any of the parameters studied.
5.2.5 Cortical thickness is reduced in MIM KO mice

One of the underlying reasons for increased ventricle size could be the loss of brain tissue, which is also found in schizophrenia models. Therefore, we examined the cortical volumes of MIM KO mice as compared to their control littermates using MRI. This study revealed a significant thinning in MIM KO mice compared to wild-type animals in all the areas studied, namely in somatosensory 1, barrel field (S1BF), secondary auditory cortex, dorsal area (AuD), dorsolateral entorhinal cortex (DLEnt) and striatum. These results suggest that the cortical thinning is related to the bilateral ventricular expansion.

5.3 ASD-associated mutations in actin regulators affect dendritic spines (Study IV)

A large portion of mutations associated with neurodevelopmental disorders such as ASD is found in genes encoding synaptic actin regulators. However, it is hard to estimate without experimental testing whether these mutations affect the function of the resulting protein. Therefore, I chose five genes with ASD-associated missense mutations and studied how they change the function of the encoded protein in neuronal dendritic spines and synapses. I relied on the lists of genes and mutations associated with different neuropsychiatric diseases published by Fromer et al. (2014) and focused on the genes encoding known actin-binding proteins that are expressed in the brain: ACTN4, MYO9B, SWAP70, MYH10, and SRGAP3. The mutations identified in these genes are all unique, one-allele de novo mutations leading to mixed expression of wild-type and mutated proteins.

To study the possible effects of the selected mutations on protein function in synapse regulation I overexpressed either the wild-type or the mutated protein in cultured rat hippocampal neurons at DIV15. I compared those two groups between each other and to control neurons expressing GFP. I further compared the localization of the protein in neurons as well as overexpression effects on dendritic spine density and morphology and on inhibitory synapses.

5.3.1 α-actinin-4 point mutation M554V alters protein localization and dendritic spine phenotype

The missense mutation A to G in the ACTN4 gene leads to an amino acid substitution [methionine (M) 554 to valine (V)] in the spectrin repeat-3 of the α-actinin-4 protein. Such change may interfere with the formation of α-actinin homodimers required for efficient actin filament cross-linking activity. I showed that wild-type α-actinin-4 was highly enriched in dendritic spines at a ratio of 6.64 ± 0.50 compared to diffuse GFP with a ratio of 0.98 ± 0.01 (Table 2). M554V-α-actinin-4 also localized to dendritic spines —albeit to a much lesser extent than wild-type α-
actinin-4 at a ratio of 4.10 ± 0.29 (Table 2). The overexpression of wild-type α-actinin-4 resulted in a more mature dendritic spine phenotype. With wild-type overexpression, the density of mushroom spines increased and that of thin spines decreased, while mutated α-actinin-4 failed to induce such change when compared to control cells expressing mCherry (Table 2). I analyzed the distributions of width-to-length ratios of spines and saw that in cells overexpressing wild-type or mutant α-actinin-4 more spines possessed a mature morphology (i.e., wide head, short neck) compared to controls. This suggests that changes in spine widths and lengths occur proportionally in the same spines. The mutation in α-actinin-4 enhanced the effect of wild-type protein in decreasing inhibitory synapse density but left other parameters of inhibitory synapse undisturbed. Based on the results obtained, the M554V mutation leads to a loss-of-function or reduced-function effect in α-actinin-4 in the regulation of dendritic spines.

<table>
<thead>
<tr>
<th>Comparison of wild-type (wt) protein phenotypes to control phenotype and mutant expression phenotype to the wild-type phenotype</th>
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<tbody>
<tr>
<td>α-actinin-4</td>
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<td>Localized to spines (ratio spine/shaft)</td>
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<td>Ctrl vs. wt</td>
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<td>Ctrl vs. mut</td>
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<tr>
<td>wt vs. mut</td>
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<tr>
<td>Total spine density (spines/µm)</td>
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<td>Ctrl vs. wt</td>
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<td>Ctrl vs. mut</td>
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<td>wt vs. mut</td>
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<tr>
<td>Thin spine density (spines/µm)</td>
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<td>Ctrl vs. wt</td>
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<td>Ctrl vs. mut</td>
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<td>wt vs. mut</td>
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<tr>
<td>Mushroom spine density (spines/µm)</td>
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<td>Ctrl vs. wt</td>
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<td>Ctrl vs. mut</td>
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<td>wt vs. mut</td>
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<tr>
<td>Total spine head size (µm)</td>
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<td>Ctrl vs. wt</td>
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<td>Ctrl vs. mut</td>
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<td>Inhibitory synapse density (synapses/µm)</td>
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<td>Inhibitory synapse size (µm)</td>
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<td>wt vs. mut</td>
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<tr>
<td>The proportion of inhibitory synapses in spine versus shaft</td>
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<td>Ctrl vs. mut</td>
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<td>wt vs. mut</td>
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Table 2. Result summary table. Values obtained from wild-type (ctrl vs wt) or mutated protein (ctrl vs mut) expressing cells are compared to control cells, and values obtained from cells expressing wild-type proteins are compared to values of cells expressing the mutant protein (wt vs mut). A statistically significant (p<0.05) increase is highlighted in green, decrease in blue.
5.3.2 Myosin IIb mutation Y265C changes inhibitory synapse size

A de novo missense mutation T to C in the MYH10 gene leads to the substituiton of tyrosine-265 (Y265) to cysteine (C) in the myosin IIb protein. Based on the location of this changed amino acid in the protein structure we hypothesized that the mutation affects the stability of the myosin IIb motor domain. Analysis of the protein’s localization and spines only showed a slight difference in spine morphology with mutant-expressing neurons having a larger proportion of wide-head spines and a smaller proportion of long spines. However, larger effects were seen in inhibitory synapses. In neurons overexpressing myosin IIb-Y265C, inhibitory synapses were smaller compared to wild-type and fewer of them resided on spines (Table 2).

5.3.3 Myosin IXb increases the proportion of inhibitory synapses in spines

An amino acid change [lysine (K) 1872 to arginine (R)] in the RhoGAP domain of myosin IXb is a result of the de novo missense mutation A to G in the MYO9B gene (Iossifov et al., 2014). The position of the changed amino acid in the protein structure suggests that this mutation is unlikely to bring a significant functional change. Not surprisingly, I observed that both wild-type and mutated myosin IXb were similarly enriched in dendritic spines and showed no significant differences in spine phenotype. Inhibitory synapse size and density were not changed either. However, the proportion of inhibitory synapses residing in spines was higher in both wild-type and mutated myosin IXb overexpression (Table 2).

5.3.4 SWAP-70 mutant L544F expression enhances localization to spines and reduces spine width and inhibitory synapse density

An amino acid change of leucine (L) 544 to phenylalanine (F) is a result of the de novo missense mutation A to T in the SWAP70 gene. This mutation is situated in the domain of SWAP70 important for actin binding. Therefore, such a change might affect the protein’s actin-binding ability. SWAP-70 was not previously studied in neurons. I found that wild-type SWAP-70 is only slightly more enriched in spines compared to dendrites (Table 2). In contrast, changing leucine (L) 544 of SWAP-70 to phenylalanine (F) significantly increased the localization of the protein to spines (Table 2).

Spine phenotype was also changed by the overexpression of SWAP-70, with an increased density of spines. Especially the number of thin spines was increased. The L544F mutation enhanced the effects induced by wild-type SWAP-70. Mutant SWAP-70 expression also made spines thinner on average and decreased the density of inhibitory synapses (Table 2). Based on these results, it seems that an ASD-associated mutation in the SWAP70 gene enhances the effects of SWAP-70 in spines.
It is plausible that this is mainly achieved through an enhanced spine localization of the protein.

5.3.5 SrGAP3 mutant E469K expression increases the proportion of inhibitory synapses in spines

A de novo C to T missense variant in the SRGAP3 gene was identified by Sanders et al. (2012). This leads to glutamic acid-469 being changed to lysine (E469K) in the SrGAP3 protein. I found that this mutation in SrGAP3 increased the protein’s localization to dendritic spines (Table 2). Neurons overexpressing SrGAP3-E469K also had a significant increase in the density of spines and especially thin spines (Table 2). Furthermore, wild-type SrGAP3 expression decreased the size of inhibitory synapses, whereas the expression of E469K SrGAP3 increased the proportion of inhibitory synapses located on spines (Table 2).
6 Discussion

Synaptic plasticity has gained a lot of attention since its discovery. Nevertheless, the detailed mechanisms of actin regulation during the process are still far from clear, especially when it comes to LTD. A number of actin regulators were shown to be involved in spine remodeling during long-term potentiation. Some of these proteins are recruited to spines in the early phases of LTP establishment and some in the later phases, and the time when the protein is recruited to the spine can provide hints about their role in the process. In this thesis work, I showed that gelsolin transiently relocates to dendritic spines upon cLTD induction, but not by cLTP induction. These results further support the earlier evidence that gelsolin is involved in LTD (Furukawa et al., 1997; Star, Kwiatkowski and Murthy, 2002). As gelsolin is activated by Ca$^{2+}$-binding (Kwiatkowski, Janmey and Yin, 1989), it is plausible that calcium activation is a crucial step in gelsolin translocation. Both LTD and LTP require calcium elevation as a trigger mechanism to launch the plasticity processes. However, according to our results, gelsolin only reacts to the LTD stimulus. One possible explanation of this phenomena is that the prolonged elevation in calcium level seen in LTD is needed to fully activate gelsolin and increase its affinity to F-actin. All six domains of gelsolin have Ca$^{2+}$-binding sites and it is plausible that gelsolin structure opens step-wise depending on the number of Ca$^{2+}$ ions bound, so to open it completely and to achieve full affinity for F-actin the increase in Ca$^{2+}$ needs to be long enough.

It has been estimated that during LTD the increase in calcium level would last for 1 minute (in our setup it lasted the whole 4 minutes of chemical stimulation), and for LTP induction, the elevation would last for only a few seconds (Yang, Tang and Zucker, 1999). Dendritic spines are rich in F-actin and therefore activated gelsolin is able to relocate to the dendritic protrusions. Gelsolin did not localize with synaptic structures but was found around them, so its function may be severing the F-actin found around the postsynaptic density.

As we overexpressed gelsolin for most of our experiments in this study, we checked how the overexpression itself influences spine morphology and dynamics. Under normal conditions, we only found minor changes in spine morphology. This, together with previous studies, suggests that gelsolin only exhibits its function in the presence of high Ca$^{2+}$ concentrations (Hu and Hsueh, 2014).

Deficiency in actin regulation can have devastating consequences for overall brain physiology and, as a result, the behavior of the animal. Our study has provided yet another example, showing a number of behavioral and physiological deficiencies in MIM-deficient mice. We have demonstrated for the first time that MIM KO mice have deficits in learning and reversed-learning. MIM KO mice also show lower anxiety levels and reduced dominant behavior. Additionally, we have confirmed the behavioral deficits reported earlier (Saarikangas et al., 2015).

Further, we analyzed the few plausible underlying physical causes for these changes. The previous study has shown a decrease in total spine density in Purkinje cells, where MIM is highly expressed (Saarikangas et al., 2015). Here we assessed spine morphology and density in pyramidal cells of the hippocampal CA1 area of the
56-week old mice. However, the total density of dendritic spines in the hippocampus was unchanged in the transgenic animals. Such discrepancy can be explained by the different MIM expression levels in the cerebellum and hippocampus. Moreover, additional spine-initiation factors might be expressed in the hippocampus and cortex, compensating for the MIM deficiency. At the same time, thin spine density was decreased in the hippocampal neurons of the MIM KO mice, similar to earlier in vitro data (Saarikangas et al., 2015). Thin spines are highly dynamic and are considered to be converted to stable spines with stronger synapses while learning (Matsuzaki, Honkura, Graham C. R. Ellis-Davies, et al., 2004). Therefore, it is possible that depletion in this pool of spines available for conversion can contribute to the impairment in learning we see in MIM KO. On the other hand, the magnitude of the decrease in thin spine density was small, and considering that enlarged ventricles can result in similar problems in learning (McMullen, Baidwan and McCarthy, 2012), it would be unfair to conclude that the learning impairment is a result of thin spine deficit. It is possible that increased stability of spines throughout the life of MIM KO mice interferes with learning and especially reversed-learning. However, this idea would require further confirmation.

Learning and motor defects could also be connected to the enlarged brain ventricles, which appeared to be the most prominent physiological change in MIM KO mice. We have addressed the possible cause for this condition by analyzing the cilia on the ventricle walls. As the cilia density and lengths were not changed, the underlying cause of the ventricular enlargement is still unclear. The altered clearance of Evans Blue in MIM KO mice may indicate a lymphatic defect as a potential cause for the enlarged brain ventricles. Therefore, our best explanations for the change in ventricle volume so far would be the decreased cortical volume we detected in the MRI study, deficiency in fluid dynamics and a possibly impaired lymphatic clearance of CSF.

The behavioral and physiological changes we observed in MIM KO mice are similar to the ones found in mouse models for schizophrenia (Powell and Miyakawa, 2006), the disorder characterized in humans by hallucinations, social withdrawal and cognitive decline. Additionally, one of the consistent abnormalities in schizophrenia patients is lateral ventricle enlargement (del Re et al., 2016). Even though schizophrenia has a strong genetic background, there has been no single gene responsible for the condition found in humans, but rather many genes were associated with this disorder, such as ones involved in neurogenesis, neuronal migration, dendrite maturation and synaptogenesis (Fromer et al., 2014). In the case of knock-out mouse models for schizophrenia we are usually discussing the role of one gene on cell function, and in the case of MIM KO mice, the exact reason for the observed phenotype is hard to pinpoint. There are generally two possibilities: either the changes in specific cell types drive the resulting phenotype, or the increased ventricle volume leads to brain tissue damage. In the latter case, the gene in question might be crucial only for the function of one cell type which would lead to all the observed secondary effects. To clarify this, one should knock-out the studied gene in specific cell populations.

Many cytoskeletal proteins that are known regulators of dendritic spines have been found to be affected in ASD (Joensuu, Lanoue and Hotulainen, 2017). Nevertheless, changes in protein functionality upon gene disruption with respect to
spines and synapses are largely unknown. The definite common physiological phenotype for ASD has not been found yet, but studies have pointed out spine and synapse disruptions. Therefore studying changes in spine and synapse phenotypes can be a helpful tool in identifying the disruptive potential of mutations. Additionally, the proper localization of the protein is an important marker of its functionality. Therefore, we included this parameter in our analysis. The selection of these mentioned parameters is also supported by the fact that these are quite easy to analyze, so such an approach can later be implemented in high throughput screenings for disruptive mutations.

All the proteins studied here were enriched in dendritic spines, suggesting that they can have a role in the regulation of dendritic spine number or morphology. Localization analysis confirmed earlier published results for α-actinin-4 (Kalinowska et al., 2015), myosin IIb (Korobova and Svitkina, 2010; Rubio et al., 2011), and SrGAP3 (Carlson et al., 2011). For three out of five proteins studied, the ASD-associated mutation changed their localization. For α-actinin-4 it reduced the localization to dendritic spines, and for SWAP-70 and SrGAP3 increased spine localization. These results demonstrate that a single amino acid change can affect the subcellular localization of proteins and a mutation can either reduce (loss-of-function) or induce (gain-of-function) a specific localization.

As for dendritic spine phenotypes, they have been variable for autism-linked proteins studied before. Based on the results of spine analysis, together with published results, we conclude that wild-type α-actinin-4 (Kalinowska et al., 2015), myosin IIb (Rex et al., 2010; Hodges et al., 2011), and SrGAP3 (Carlson et al., 2011) have a clear function in regulating dendritic spine density and morphology (summarized in Table 2). The α-actinin-4-M554V mutation resulted in a shift of spine morphology from mushroom towards thin. This change also resembles the one observed upon the overexpression of ASD-associated Shank3 mutants (Durand et al., 2012). In addition to significant changes with the ACTN4 mutation, we saw a trend towards more thin spines with mutations in myosin Ixb and SWAP-70 when compared to wild-type proteins. Taken together, although most of the changes were mild and the differences between wild-type and mutated proteins were not always significant, we observed a trend toward an increased proportion of thin spines.

It has been proposed that thin spines are involved in learning whereas mature mushroom spines are needed for long-term memory storage (Kasai et al., 2003). Thus, it is probable that the most frequently observed effect of the mutations—the increased density of thin spines—also affects neuronal function and behavior and this idea is supported by studies on the Fragile-X syndrome (FXS) and Shank1 ASD-mouse models (Huber et al., 2002; Hung et al., 2008).

The excitatory/inhibitory imbalance has been proposed as one of the physiological problems underlying ASD. Thus, we analyzed the size, density, and localization of inhibitory synapses. Myosin IIb and myosin Ixb increased the proportion of inhibitory synapses in spines, whereas SrGAP3 decreased the size of inhibitory synapses. The detailed molecular mechanisms for such regulation are still unknown. Currently, it has been proposed that the positions of inhibitory synapses on dendritic shafts can determine the hotspots where both new spines and new inhibitory synapses are more likely to be formed (Chen et al., 2012; Isshiki et al., 2014). It has also been noted that inhibitory synapses, when positioned in dendritic
spines, do stabilize them. Therefore, the loss of inhibitory synapses from spines could indicate an increased spine turnover rate, precisely what was found earlier in two ASD mouse models (Ishiki et al., 2014). We found that the expression of mutated myosin IIb (Y265C) reduced, but mutated SrGAP3 (E469K) increased, the proportion of inhibitory synapses in spines (Table 2). In addition, α-actinin-4, myosin IXb, and SWAP-70 mutations showed a trend toward a reduced proportion of inhibitory synapses in spine heads. Furthermore, the expression of myosin IIb-Y265C decreased inhibitory synapse size and SWAP-70-L544F expression decreased the density of inhibitory synapses.

It is important to note that our study is limited to the synaptic function of the proteins mentioned. However, they might be involved in other aspects of CNS development and function such as dendrite growth or glial function. Functional defects of glial cells are implicated in ASD (Petrelli, Pucci and Bezzi, 2016) and, in fact, all genes studied here are expressed in higher levels in human astrocytes than in neurons (Zhang et al., 2014). With overexpression analysis in cultured neurons, we can only determine whether a mutation changes the protein’s function. Nonetheless, one must remember that it might not be enough to assume the mutation will contribute to the development of ASD. Comprehensive studies on knock-in animal models would be beneficial in this regard, albeit time-consuming and expensive to carry out. Therefore, pre-screening of mutations is necessary to pre-select mutations for detailed studies. Furthermore, our screening-type experiments give a broader view of the impact of de novo missense mutations and which cellular parameters should and could be used as readouts for protein functionality. From these five genes, only the α-actinin-4 mutation showed a substantial effect on dendritic spines and should be taken to further animal studies. The effects of all the other mutations were relatively mild, but it is possible that under suitable circumstances, possibly enhanced by other mutations or risk-variants, these mutations can contribute to the development of autism.
7 Conclusions

This thesis project brought together and explored various aspects of dendritic spine regulation via actin-binding proteins. In recent years we have seen a tremendous increase in our understanding of how structure and function are tightly interconnected in the brain. Regulation of dendritic spines and synapses is the one important example. By showing that an actin-regulating protein, such as gelsolin can discern between two types of long-term synaptic plasticity, I demonstrated how the underlying spine structure can be regulated differently during these plasticity events.

The study of MIM knockout mice further establishes this idea of structure-function synergy. MIM is a spine-initiating factor important for dendritic spine development, and this study demonstrates how devastating the absence of this one structural regulator can be for brain function. Our transgenic mice had impairments in various aspects of behavior, especially higher brain functions such as learning and social interaction.

If the function and structure are so intertwined, could we use structural readouts as a diagnostic tool? In the last part of this thesis, I examined how single-point mutations in actin-binding proteins that are associated with ASD could change dendritic spine and synaptic structures. This study demonstrated that dendritic spines are very often affected by the change in the functionality of structural regulators. This knowledge is very important in the case of neurodevelopmental disorders, such as autism spectrum, where no apparent alterations in overall brain physiology are present, but we see clear behavioral changes. Dendritic spines are fine-tuned to support the functionality of synapses and, therefore, disruption of this fine-tuning process can lead to major behavioral alterations.

So could we save the function by restoring the structure? Some studies have already addressed this question, and it appears that at least in some cases it is possible. However, to do so in the most efficient way, we should gain more insight into the ways the structural components of the neuron control its function and how various compensatory mechanisms come into play. Currently, there are more than 30 variants for actin-binding proteins associated with ASD and we continue to find new ones. This area of research is very new but promising and it would greatly benefit if we could develop high-throughput assays to read-out structural changes in the neurons in an efficient way, and focus in detail on more promising variants.
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Helsinki, 2019

Iryna Hlushchenko
References


Kim, I. H. *et al.* (2012) ‘Disruption of wave-associated Rac GTPase-activating protein (Wrp) leads to abnormal adult neural progenitor migration


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