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SYMPOSIUM REVIEW

Imbalance of synaptic actin dynamics as a key to fragile X syndrome?

Kristin Michaelsen-Preusse[1], Jonas Feuge[1] and Martin Korte[1,2]

[1] *Zoological Institute, Division of Cellular Neurobiology, TU Braunschweig, Spielmannstr. 7, Braunschweig, 38106, Germany*
[2] *Helmholtz Centre for Infection Research, AG NIND, Inhoffenstr. 7, Braunschweig, 38124, Germany*

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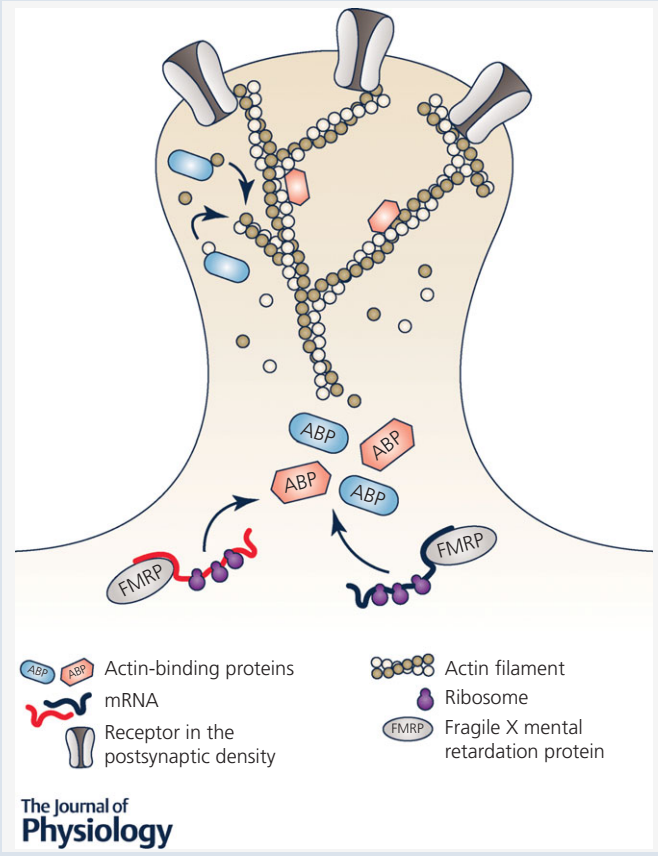

Martin Korte, Prof. Dr, is Head of the Zoological Institute and group leader of the Division of Cellular Neurobiology at the TU Braunschweig and co-affiliated to the Helmholtz-Center for Infectious Disease, AG Neuroinflammation and Neurodegeneration (AG NIND). He studies cellular mechanisms of learning and memory, the physiology of APP, neurotrophins and the functional role of NogoA. In addition he is interested in structural plasticity and the functional role of actin in processes of synaptic plasticity.

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Abstract Our experiences and memories define who we are, and evidence has accumulated that memory formation is dependent on functional and structural adaptations of synaptic structures in our brain. Especially dendritic spines, the postsynaptic compartments of synapses show a strong structure-to-function relationship and a high degree of structural plasticity. Although the molecular mechanisms are not completely understood, it is known that these modifications are highly dependent on the actin cytoskeleton, the major cytoskeletal component of the spine. Given the crucial involvement of actin in these mechanisms, dysregulations of spine actin dynamics (reflected by alterations in dendritic spine morphology) can be found in a variety of neurological disorders ranging from schizophrenia to several forms of autism spectrum disorders such as fragile X syndrome (FXS). FXS is caused by a single mutation leading to an inactivation of the X-linked fragile X mental retardation 1 gene and loss of its gene product, the RNA-binding protein fragile X mental retardation protein 1 (FMRP), which normally can be found both pre- and postsynaptically. FMRP is involved in mRNA transport as well as regulation of local translation at the synapse, and although hundreds of FMRP-target mRNAs could be identified only a very few interactions between FMRP and actin-regulating proteins have been reported and validated. In this review we give an overview of recent work by our lab and others providing evidence that dysregulated actin dynamics might indeed be at the very base of a deeper understanding of neurological disorders ranging from cognitive impairment to the autism spectrum.

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Corresponding author M. Korte: TU Braunschweig, Zoological Institute, Division of Cellular Neurobiology, Braunschweig, Germany. Email: k.michaelsen@tu-bs.de

Abstract figure legend The role of the fragile X mental retardation protein (FMRP) in the regulation of actin in dendritic spines by direct interaction. mRNAs of actin-binding proteins can be targeted to dendrites as well as dendritic spines where they are locally translated upon need and crucially involved in processes of synaptic plasticity – a mechanism that might be dysregulated in fragile X syndrome.

Introduction

The immense computational power of the central nervous system depends on the formation of functional neuronal networks, which are further refined and adapted to environmental changes by processes of neuronal plasticity throughout the entire lifespan of an individual. Synapses are considered as the single processing units of these networks, and in the cortex the majority of excitatory synapses are located on spines, dendritic protrusions containing the postsynaptic compartment. Typically, dendritic spines show a variety of shapes and sizes even on a single neuron. Although spines are tiny in size, spine shape and function are fundamental for the network performance of our brain, a fact that we are just beginning to understand. Importantly, these parameters are significant indicators of proper neuronal function, as spines are constantly adapting their structural and functional characteristics to changes in neuronal activity (reviewed in Caroni *et al.* 2012). Although constant changes in spine morphology occurring within a time range of seconds to minutes (spine motility, short-term structural plasticity) are well described for more than 30 years, the underlying mechanisms remain rather elusive except for the principle involvement of the actin cytoskeleton (Fischer *et al.* 1998; Dunaevsky *et al.* 1999; Bonhoeffer & Yuste, 2002; Oertner & Matus, 2005; Michaelsen-Preusse *et al.* 2016). To date, motile spines have been described both *in vitro* and *in vivo* (Fischer *et al.* 1998; Dunaevsky *et al.* 1999; Berning *et al.* 2012). It is speculated that besides the obvious reason of facilitating contact formation between future synaptic partners, spine motility might in addition serve to orchestrate the positioning and reorganization of macromolecular assemblies in spine nanodomains or to modulate the engagement of the spine membrane with extracellular matrix components (Halpain, 2000; Frost *et al.* 2010; Koleske, 2013). Besides displaying basal motility (which might reflect subtle changes in baseline synaptic transmission), spines can exhibit more pronounced changes in shape in response to stronger alterations in synaptic efficacy, namely long-term potentiation (LTP) or long-term depression (LTD) (Engert & Bonhoeffer, 1999; Matsuzaki *et al.* 2004; Nagerl *et al.* 2004; for reviews see Bosch & Hayashi, 2012; Sala & Segal, 2014). These long-term structural plasticity processes can lead to substantial changes in spine volume and are considered as a key cellular correlate of learning and memory formation (as reviewed in Lamprecht & LeDoux, 2004; Caroni *et al.* 2012; Korte & Schmitz, 2016).

Structural plasticity and actin

These strong alterations in spine shape are directly associated with the dynamic actin cytoskeleton, which is highly enriched in dendritic spines (Fischer *et al.* 1998; Fukazawa *et al.* 2003; Chen *et al.* 2007; Honkura *et al.* 2008; Hotulainen *et al.* 2009; Bosch *et al.* 2014; Michaelsen-Preusse *et al.* 2016). In fact, up to 80% of actin filaments turn over in less than 2 min in the spine head (Star *et al.* 2002). Results from our lab show that actin filament turnover is modulated during LTP with a reduction of the turnover time during the initial phase possibly allowing spine head expansion and a significant increase after 1 h most likely important for the stabilization and maintenance of the new spine structure (Michaelsen-Preusse *et al.* 2016). Interestingly, we have recently shown that the actin-binding protein profilin2a (PFN2a) is crucially involved in this modulation, as activity-dependent spine head enlargement was completely abolished in PFN2a-deficient cells along with a significant general reduction in the F-actin turnover time, which showed no modulation during synaptic plasticity (Michaelsen-Preusse *et al.* 2016). An understanding of the detailed molecular machinery and identification of key molecules which control actin polymerization in space and time will help to reveal further details about spine function and might eventually also provide a better understanding of neurological disorders characterized by defects in spinogenesis and spine maintenance (Blanpied & Ehlers, 2004; Penzes *et al.* 2011). The strong structure-to-function relationship of dendritic spines (Holtmaat & Svoboda, 2009) and the fact that alterations in their morphology and density can be found in various neurological disorders such as autism spectrum disorder (ASD), schizophrenia and several forms of mental retardation in humans (reviewed in Fiala *et al.* 2002) opens the possibility that defects in the modulation of dynamic actin in spines might be a key neuropathological mechanism for the aetiology of these diseases.

The fragile X syndrome

In light of this, fragile X syndrome (FXS) can be considered as a paradigmatic disease to study spine dysfunction. FXS is the most frequent inherited single-gene cause of ASD and mental retardation (Crawford *et al.* 2001) and is characterized by translational silencing of the fragile X mental retardation 1 (*fmr1*) gene. Caused by partial or complete loss of the *fmr1*-encoded fragile X mental retardation protein 1 (FMRP; Verkerk *et al.* 1991), patients suffering from FXS exhibit several neurological symptoms including hyperactivity, diversely severe forms of cognitive impairments, poor motor coordination and autistic behaviour (Beckel-Mitchener & Greenough, 2004). Surprisingly, despite having strong intellectual deficits, autopsy studies revealed that brains of FXS patients morphologically appear normal and only a subtle synaptic phenotype could be identified: a hyperabundance of long and thin dendritic spines (Rudelli *et al.* 1985; Hinton *et al.* 1991; Irwin *et al.* 2001).

To investigate the underlying cellular and genetic mechanism of FXS, *fmr1* knockout (*fmr1* KO) mice were generated (Bakker *et al.* 1994), which mirror some of the key behavioural phenotypes observed in humans, such as hyperactivity and increased anxiety. Most importantly, they also show deficits in cognitive function as learning in the Morris water maze is impaired (Kooy *et al.* 1996; D'Hooge *et al.* 1997). In line with this, there is evidence for an impairment in LTP both in the neocortex and in the hippocampus of *fmr1* KO animals (Desai *et al.* 2006; Lauterborn *et al.* 2007; Meredith *et al.* 2007; Wilson & Cox, 2007; Hu *et al.* 2008). Hence, these mice have been intensively used to study the pre- and postsynaptic function of FMRP. In some neurons FMRP has been shown to be present directly at the presynapse as well as in axon-restricted fragile X granules (FXGs), which can be observed especially during particularly plastic developmental stages (Christie *et al.* 2009; Akins *et al.* 2012). A presynaptic role has been suggested, as the absence of FMRP correlates with dysregulations in GABA release (Centonze *et al.* 2008; Kang *et al.* 2017), causing imbalances between inhibitory and excitatory neurotransmission. Additionally, several studies showed that FMRP is able to regulate presynaptic ion channel stability, trafficking, surface expression as well as sensitivity indicating a presynaptic role (Brown *et al.* 2010; Strumbos *et al.* 2010; Gross *et al.* 2011; Lee *et al.* 2011; Zhang *et al.* 2012; Ferron *et al.* 2014; Wang *et al.* 2014; Deng & Klyachko, 2016).

Nevertheless, most studies in *fmr1* KO mice focus on the postsynapse where investigations of neuronal morphology show that the immature spine profile of human patients can be mimicked. However, reports about the detailed changes in dendritic spine density and morphology are controversial (Braun & Segal, 2000; Nimchinsky *et al.* 2001; Galvez & Greenough, 2005; Antar *et al.* 2006; Grossman *et al.* 2006, 2010; Bilousova *et al.* 2009; Cruz-Martin *et al.* 2010; Pan *et al.* 2010; Levenga *et al.* 2011; Wijetunge *et al.* 2014). Whereas a large proportion of studies reported the classical immature spine phenotype in the hippocampus and neocortex (Nimchinsky *et al.* 2001; Antar *et al.* 2006; Grossman *et al.* 2006, 2010; Bilousova *et al.* 2009; Levenga *et al.* 2011), other studies analysing the same brain regions were not able to detect an impairment in spine maturation (Braun & Segal, 2000; Cruz-Martin *et al.* 2010) or detected only minimal alterations (Wijetunge *et al.* 2014). Similar controversies are reported for spine density analyses as well, especially in the hippocampus where a decrease (Braun & Segal,

2000), no alterations (Pfeiffer & Huber, 2007; de Vrij *et al.* 2008; Levenga *et al.* 2011) or an increase in spine density (Antar *et al.* 2006; Grossman *et al.* 2006; Swanger *et al.* 2011) was described. In part, this controversy might be due to different labelling methods as, for instance, it is not clear if there is a specific subpopulation of cells labelled by Golgi–Cox impregnation and if so whether this population might be affected by the loss of FMRP. Despite the wealth of data, it is therefore still under debate if postsynaptic alterations in FXS are persistent or transient, which brain regions are affected most and whether some neuronal subpopulations might be specifically impaired (also reviewed extensively in He & Portera-Cailliau, 2013). In addition, super-resolution microscopy techniques will help in the future to reveal details of the structural phenotype at synapses which might be very subtle in some brain regions or in specific neuronal subpopulations (Wijetunge *et al.* 2014) but could be much more pronounced in other areas or following different experiences. In this respect it has been shown that rearing in an enriched environment either led to the enhancement of the immature spine profile or ameliorated it (Restivo *et al.* 2005; Lauterborn *et al.* 2015).

Given the fact that FMRP has both pre- and postsynaptic functions, it seems to be especially important in future studies to view the pre- and postsynapse as entities which at best should be studied together in order to obtain more conclusive results. We recently made the effort to analyse pre- as well as postsynaptic specializations at the same time, which allowed us to uncover a novel role of FMRP in restricting development of one of the most powerful synapses in the central nervous system, the mossy fibre synapse (Scharkowski *et al.* 2017). It is composed of presynaptic large mossy fibre terminals (LMTs) of granule cells connecting with postsynaptic thorny excrescences (TEs) on proximal dendrites of CA3 pyramidal neurons in the stratum lucidum. Each TE consists of multiple postsynaptic densities, rendering this synapse especially strong (Amaral & Dent, 1981), and together with their location close to the soma of CA3 neurons, these synapses are able to drive the firing of CA3 neurons just by activation of a few TEs (reviewed in Evstratova & Toth, 2014). Accordingly, the mossy fibre synapse can be described as a 'detonator' or 'teacher' directing the storage of information in the CA3 network (Urban *et al.* 2001). This allows single granule cells to precisely time the activity of CA3 pyramidal neurons and thereby provide the necessary depolarization needed for Hebbian plasticity at the associational/commissural inputs in the stratum radiatum and stratum oriens (see also Henze *et al.* 2000). Mutations in a different gene (*grik2*, coding for the kainate receptor subunit GluK2) are also associated with ASD and intellectual disability and have been shown to influence LMT-TE development (Lanore *et al.* 2012). Moreover, a key role of the mossy fibre synapse in mediating homeostatic synaptic plasticity was reported in mature hippocampal neurons (Lee *et al.* 2013). Behavioural phenotypes appearing in autism spectrum disorders, as hyperactivity and hypersensitivity, especially point towards an impaired homeostatic synaptic plasticity, which therefore renders the mossy fibre synapse a key structure for investigating the cellular basis of ASD.

Although a central connection for hippocampal network function, this synapse had not been studied in the FXS mouse model before. Somewhat surprisingly, we found TEs to be premature during development in *fmr1* KO mice with their number and size being increased both *in vitro* and *in vivo* (Fig. 1; Scharkowski *et al.* 2017). In contrast to this, presynaptic LMTs were decreased in size leading to a significant change in the LMT/TE area ratio in *fmr1* KO animals compared to WT mice. In parallel, single TEs contained more clusters of the actin-binding protein synaptopodin, which is accumulated in the spine apparatus thereby indicating enhanced synapse maturation. In line with this premature phenotype, we found that the structure of TEs was hyperstabilized during development as short-term structural plasticity was significantly decreased together with a reduction in actin polymerization rates. This phenotype could be rescued by overexpression of the actin binding protein profilin 1 (PFN1), a target of FMRP with reduced expression levels in the FXS mouse model (Scharkowski *et al.* 2017).

In addition to the structural alterations described above, we were also able to detect functional changes in the absence of FMRP as well. We used expression of Super Ecliptic pHluorin (SEP)–glutamate receptor subunit 1 (GluR1) (Kopec *et al.* 2007), which specifically labels surface AMPA receptors to quantify the amount of GluR1-containing AMPA receptors at TEs of WT and *fmr1* KO neurons (Scharkowski *et al.* 2017). The intensity of SEP–GluR1 clusters was analysed under baseline conditions and following increased activity. Interestingly, already under baseline conditions the SEP–GluR1 signal was stronger in *fmr1* KO TEs compared to WT neurons, indicating an increased content of surface AMPA receptors. Synaptic activation via KCl led to a significant increase in the SEP–GluR1 signal both in WT neurons and in *fmr1* KO cells compared to baseline conditions, but this increase was more pronounced in the absence of FMRP. In contrast to the premature phenotype of TEs, CA3 dendrites located in the stratum radiatum displayed the well documented immature spine profile with an overabundance of thin spines in *fmr1* KO neurons (Fig. 1). It was indeed an intriguing finding that two different synapse types found at the same neuron are affected differentially, notably even in opposite directions, in the FXS mouse model, most likely with detrimental outcome for information processing in CA3 neurons.

Imbalance of the actin cytoskeleton in FXS

The dysregulation of the actin cytoskeleton as observed in TEs in the FXS mouse model might emerge more and more as a central element in mediating aberrant spine phenotypes in the course of FXS and probably even in ASD in general (Chen *et al.* 2010). The FMRP target PFN1 with reduced levels in *fmr1* KO neurons can be seen as an example where expression of recombinant PFN1 rescued actin dynamics in TEs described above as well as the immature spine phenotype of regular spines (Michaelsen-Preusse *et al.* 2016; Scharkowski *et al.* 2017). This is in line with other studies, which also hypothesized that the rescue of spine phenotypes by manipulation of the actin cytoskeleton might represent a treatment to ameliorate ASD-like behavioural symptoms. It was shown that a potent small molecule inhibitor of group I p21-activated kinases (PAKs) reversed dendritic spine phenotypes in *fmr1* KO mice. Moreover, this PAK inhibitor (FRAX486) rescued seizures and behavioural abnormalities such as hyperactivity and repetitive movements (Dolan *et al.* 2013). Also Bongmba and colleagues (2011) showed that pharmacological manipulation of overactive Rac1 partially reversed altered long-term plasticity. Additionally, increased Rac1 activity could be also blocked by increased training time in a fear-conditioning paradigm thereby preventing cognitive deficits (Martinez & Tejada-Simon, 2017). Therefore, the regulation of Rac1 may indeed provide a functional

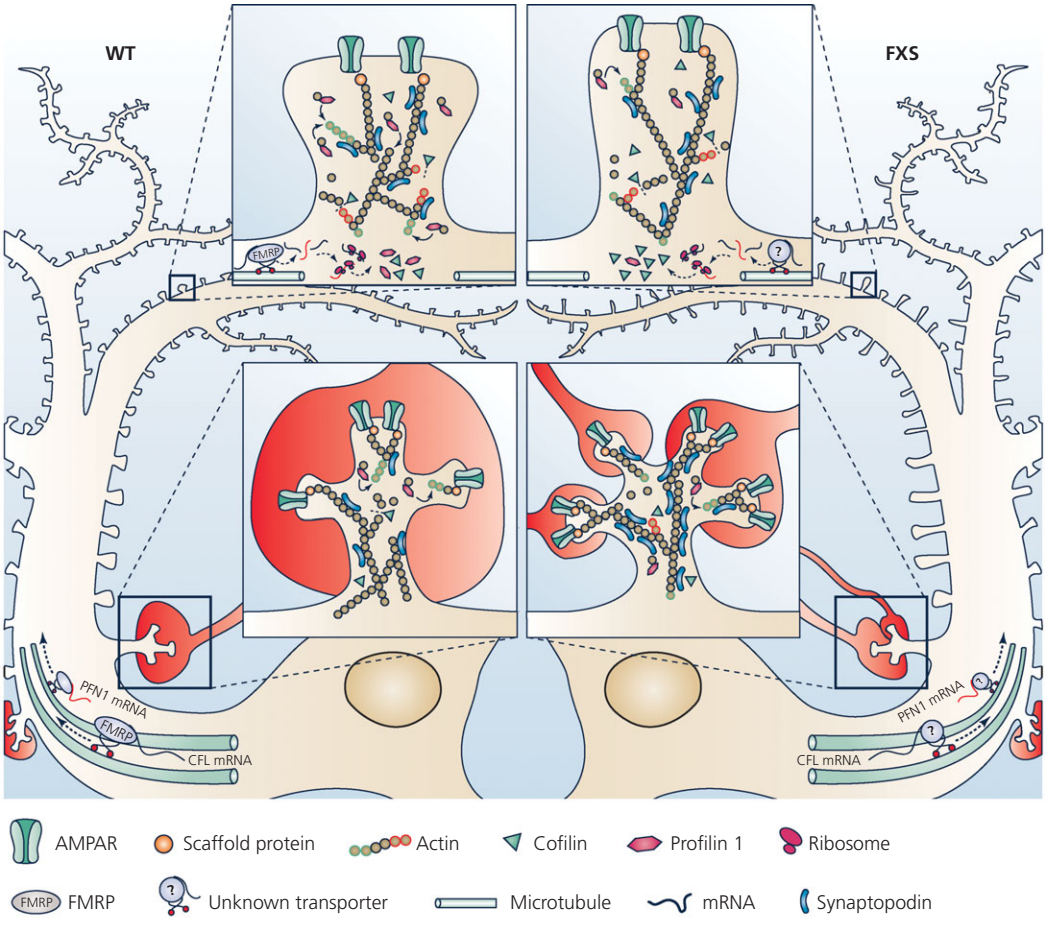

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Figure 1. Two opposite synaptic phenotypes shown on a single FXS CA3 neuron
In comparison to WT CA3 neurons (left side), FXS neurons (right side) show a hyperabundance of long and thin dendritic spines in the stratum radiatum (right upper tile). Given the fact that FMRP has an important function in mRNA transport and the regulation of local translation, our work suggests that this phenotype might be based on altered actin dynamics, caused by a dysregulation of actin-binding proteins (ABPs) (right upper tile, here shown for the ABPs profilin 1 (PFN1) and cofilin 1 (CFL)). Surprisingly, we found mossy fibre synapses on CA3 neurons in the stratum lucidum (lower tiles) to be structurally altered as well, having increased numbers of thorny excrescences (TE), containing more surface AMPA receptors as well as more clusters of the ABP synaptopodin.

link between alterations in neuronal morphology, deficits in synaptic plasticity and impaired cognition in FXS. As mentioned above, dysregulation of actin-binding proteins and the accompanying spine phenotypes might be central not only to FXS but to ASD. Support of this comes from the Shank3 autism mouse model where pharmacological blocking of overactive cofilin 1 prevented autism-like social deficits and repetitive behaviours, as well as significantly diminished NMDA receptor synaptic function and synaptic distribution in the prefrontal cortex (Duffney *et al.* 2015; for reviews see Yan *et al.* 2016; Joensuu *et al.* 2018). Normalization of excessive activity in pathways modulating actin polymerization to normalize cortical actin dynamics might indeed offer a potential therapeutic strategy to ameliorate cognitive and synaptic defects in autism. In this respect it is important to note that the expression of many actin regulators is often brain specific thereby reducing potential detrimental side effects. The identification of actin-interacting proteins with distinct transcriptional activity in different brain regions and in the periphery would help to reveal potential therapeutic targets. Manipulation of these actin regulators (for instance of human PAK3, which is involved in X-linked mental retardation; Allen *et al.* 1998) could enable the specific normalization of actin dynamics at glutamatergic synapses (see also Yan *et al.* 2016).

Local translation of actin-binding proteins in FXS

The altered spine morphology phenotype in FXS can be linked to a direct role of FMRP in activity-dependent mRNA transport, docking and local translation (reviewed in Bassell & Warren, 2008; Dictenberg *et al.* 2008; Kao *et al.* 2010). Several studies showed that the RNA-binding protein localizes together with actively translating polyribosomes in cultured neuronal and non-neuronal cells as well as in isolated brain synaptoneurosomes (Feng *et al.* 1997; Khandjian *et al.* 2004; Stefani *et al.* 2004) and translational dysregulation has recently been suggested to be a major factor in ASD (Santini *et al.* 2013). Surprisingly, although hundreds of putative FMRP-associated mRNAs were identified (Darnell *et al.* 2011; Ascano *et al.* 2012), little is known about the direct correlation between FMRP and actin-binding proteins and only a very few direct interactions of actin-binding protein mRNAs and FMRP have been validated (Reeve *et al.* 2005; Michaelsen-Preusse *et al.* 2016). The example of MAP1B as a cytoskeletal regulatory protein described as an FMRP target indicates that there might be indeed highly conserved target mRNAs – ranging from *Drosophila* to mammals – which could be key molecules responsible for the most prominent phenotypes of the disease (Darnell *et al.* 2001; Zhang *et al.* 2001). However, studies on the interaction of FMRP with the mRNAs of actin regulators showed controversial results. Whereas Ascano and colleagues (2012) identified prominent candidates as profilin, cofilin, cortactin and members of the Arp2/3 complex and also work form our own lab showed an interaction of the mRNAs of PFN1 and cofilin 1 with FMRP (Michaelsen-Preusse *et al.* 2016 and unpublished data), others did not detect an interaction with these mRNAs (Darnell *et al.* 2011). Interestingly, our results show that profilin 1 and cofilin 1 levels are altered in opposite directions in *fmr1* KO animals further indicating potentially different FMRP-dependent regulatory mechanisms. FMRP has been in general described as a negative regulator of translation for many of its targets; however, it was also shown that the protein can stabilize target mRNAs leading to a decreased expression in the absence of FMRP as is the case for PSD95 (Zalfa *et al.* 2007).

Conclusion

In summary, a variety of neurodevelopmental diseases such as schizophrenia, cognitive impairment and ASD share a common phenotype: alterations in dendritic spine morphology and density across different regions throughout the brain. It has to be emphasized that the reasons for impaired spine structure and function could be both pre- and postsynaptic. Therefore, future studies should concentrate more on a characterization of the synapse as an entity to get a deeper insight into dysregulated synaptogenesis and synaptic plasticity. The example of our own work where we found the postsynaptic site (TEs on CA3 pyramidal neurons) to be premature and the presynaptic site to be immature (LMTs of dentate granule cells) emphasizes that the phenotypes can differ extensively which will influence the way potential treatment strategies could be designed. It becomes clear that the mechanisms mediating disease phenotypes as for instance the role of FMRP for both presynaptic and postsynaptic development and function in FXS are highly complex reducing the likelihood to find a single treatment. However, whether behavioural and cognitive impairments originate from presynaptic defects, e.g. by dysregulations in ion channel composition and transmitter release or a postsynaptic defect in spinogenesis, spine maturation and spine plasticity, eventually, pathways converge on the modulation of postsynaptic actin dynamics. Thus, a deeper understanding of the spine actin cytoskeleton could provide versatile future tools and potential treatment strategies for such diverse disorders as cognitive impairment, schizophrenia and ASD.

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Additional information

Competing interests

The authors declare no competing financial interests.

Author contributions

All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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