Analysis of the role of the cytoskeleton regulatory protein dDAAM in synaptic development

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Ph.D. thesis summary

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INTRODUCTION

The building of complex neuronal circuits requires the formation of billions of synaptic connections in the brain. Elucidating synapse development and function is essential to understand cognitive processes such as learning, memory and perception. Over the last decades, a significant body of literature on the neuroscience has revealed that the dynamic cytoskeletal rearrangement is crucial for the stabilization and the remodeling of the synaptic connections. Despite the identification of numerous proteins that regulate actin and microtubule organization, the mechanisms of cytoskeletal control during synapse development have remained largely elusive. Our knowledge of the upstream cytoskeletal regulators is permanently growing, so we are starting to better understand how they function during synaptogenesis. However, much less is known about how the cytoskeletal effectors regulate the major elements of the cytoskeleton.

A few years ago, we began to investigate the role of a formin type of cytoskeleton regulatory protein, *Drosophila* DAAM, during neuronal development. By using different *Drosophila* neuronal model systems we have shown that the actin assembly activity of dDAAM is required for axonal growth and guidance. Later, we have provided several pieces of evidence that dDAAM is able to bind directly to the microtubules and plays a role in the coordination of actin/microtubule crosstalk. As an extension of these studies, here we investigated the role of dDAAM during NMJ development in *Drosophila*.

AIMS

Our goals were the followings:

1.) To investigate whether dDAAM, a cytoskeletal regulatory protein, is involved in synaptic development.

2.) To examine the role of dDAAM in cytoskeletal rearrangement processes during synaptic development.

3.) To investigate which signaling system has a connection with dDAAM by using genetic interaction studies during NMJ development.

4.) To investigate whether dDAAM plays a role in the formation of synaptic active zone.

5.) To examine whether lack of dDAAM can influence the efficiency of synaptic transmission by using electrophysiological methods.

METHODS

1. Recombinant DNA techniques
2. Fly genetics
3. Generation of a knock-in allele by using a gene conversation method
4. Generation of loss of function alleles by using a CRISPR/Cas9 system
5. Preparations of methanol fixed embryos
6. Preparations of dissected larvae
7. Immunofluorescent staining of embryos
8. Immunofluorescent staining of larval fillets
9. Western blot
10. Mass spectrometry analysis
11. Electron microscopy
12. Electrophysiology
13. STED (super-resolution stimulated emission depleted) microscopy

SUMMARY OF THE RESULTS

1. dDAAM is required for proper synaptic bouton formation

To address whether dDAAM might be involved in synaptogenesis, firstly we examined the role of dDAAM during embryonic NMJ development. We revealed that dDAAM is highly enriched at the motoraxon terminals and the lack of dDAAM causes defects in motorneuron growth and guidance in the embryos. These data suggest that the dDAAM protein might indeed be required for the early steps of synaptic development. We next analyzed the larval NMJs and showed that dDAAM is present on both the pre- and postsynaptic sides. Moreover, in the *dDAAM* mutant NMJs we observed a decreased synaptic bouton number and abnormal NMJ morphology with stretched inter-bouton regions, often designated as a bouton fusion phenotype. Although dDAAM is expressed in both synaptic compartments, further mutational analyses argue for a primarily presynaptic role.

2. dDAAM is required for organization of presynaptic microtubules

To gain deeper insights into presynaptic role of dDAAM in NMJ development, a set of experiments was carried out to analyze the underlying cytoskeleton in the motoraxon terminals. After large scale affinity purification and MS analysis, the Futsch protein which

is a widely used microtubule marker in the NMJ, appeared to be the most promising interaction partner of dDAAM. Previous findings have established that Futsch is able to promote microtubule stabilization and new bouton formation; therefore we tested whether lack of dDAAM affects presynaptic microtubule organization. In wild type NMJs the presynaptic microtubules are organized into a prominent bundle of core filaments that becomes gradually thinner in the distal region of the presynaptic motornerve terminal. By contrast, the *dDAAM* null mutation results in fragmentation of the core microtubule bundle and the microtubules are often absent from the terminal boutons. Although formins are best known for their ability to promote actin filament assembly, a mounting body of evidence supports that they are also involved in microtubule regulation; in particular, they have been implicated in microtubule stabilization in many cellular model systems. Hence, we tested which dDAAM activities are important for NMJ development. We carried out a series of rescue experiments with point mutant versions of the full-length dDAAM protein, in which we impaired actin interaction alone or actin and microtubule interactions together. The wild type and actin incompetent form of dDAAM rescued the *dDAAM* mutant NMJ phenotype while the actin and microtubule double mutant failed to restore the wild type NMJ morphology. In accordance with this, *dDAAM* mutant NMJ terminals do not appear to exhibit major alterations in presynaptic actin organization. These data suggest that the primary function of DAAM during NMJ development is presumably linked to its microtubule organizing role, whereas its actin filament assembly activity is likely to be dispensable for synaptic bouton formation.

3. The activated form of dDAAM provokes excessive synaptic bouton formation

To corroborate the presynaptic requirement of dDAAM for promoting bouton formation, we tested whether an increased activity of dDAAM affect synaptic bouton formation. We previously showed that removing the N-terminal part of dDAAM results in a constitutively active form, called C-DAAM. The presynaptically overexpressed C-DAAM caused an increase in bouton number at the NMJ terminals reinforcing further that this protein plays an instructive role in bouton formation.

4. *dDAAM* interacts genetically with *wg* and *Ank2*

We next aimed to decipher which signaling pathways control the activity of dDAAM. Our genetic interaction experiments suggested that dDAAM works together with Wg and Ank2 during NMJ development. Interestingly, dDAAM has previously been linked to Wnt/Frizzled signaling in other cellular contexts. Furthermore, prior studies have revealed that presynaptic Wg signaling regulates the synaptic microtubule cytoskeleton in conjunction with Ank2. Thus, these data support the notion that dDAAM acts in concert with Wg and Ank2 during synapse development. It has previously been shown that Ank2 is likely to be a downstream factor of Wg signaling in NMJ development. Consequently, we attempted to determine the place of dDAAM in this hierarchy by epistasis analysis. In accordance with previous studies, *Ank2* null mutant NMJs displayed reduced bouton numbers with bouton fusions and strong microtubule accumulations in the presynaptic

terminal. By contrast, in the double homozygous *dDAAM; Ank2* animals the absence of dDAAM was able to suppress the microtubule aggregation phenotype of *Ank2* and only a weak central microtubule bundle formed, and boutons no longer exhibited a fused appearance. These data regarding microtubule organization suggest that dDAAM is epistatic to Ank2, which implies a downstream regulatory position as a microtubule stabilization factor. The NMJ morphology and microtubule organization of *dDAAM; Ank2* double mutants are not identical to any of the single mutants suggesting a more complex interaction between dDAAM and Ank2 than a simple linear pathway. In addition to Ank2, the microtubule associated Futsch protein has also been shown to be a downstream effector protein of Wg signaling in synaptic growth. Similarly to Ank2, we examined *futsch/dDAAM* double mutant NMJ terminals to determine the place of dDAAM in this divergent canonical Wg signaling pathway. The double mutant NMJs strongly resembled those of *dDAAM* single mutants, with significantly reduced bouton numbers, the presence of bouton fusions, and fragmented microtubule organization. Hence, *dDAAM* is clearly epistatic to *futsch*, and these results argue for dDAAM acting either downstream of Futsch or in a parallel pathway.

5. dDAAM has a modulatory function in synaptic transmission associated with its Brp-dependent recruitment to the active zone scaffold

Using two different anti-dDAAM antibodies we revealed that besides its cortical accumulation, dDAAM is strongly expressed at the presynaptic terminal, forming a spot-

like pattern. The application of STED microscopy allowed us to determine that dDAAM is enriched in a ring-like pattern in the immediate vicinity of the C-terminus of Brp, the distal part of active zone complex. Furthermore, we have shown that the presence of Brp is required for dDAAM localization at the active zone, but not vice versa. Moreover, we have proposed several lines of evidence that lack of dDAAM affects active zone formation and function. Although the lack of dDAAM has no major effect on active zone organization, active zones were slightly bigger in dDAAM mutant synapses. Finally, electrophysiological experiments were carried out to investigate whether *dDAAM* could influence the efficiency of synaptic transmission due to its prominent localization close to the sites of neurotransmitter release. Although spontaneous release was not altered, we observed a significant reduction in evoked excitatory NMJ currents. Collectively, these data suggest that dDAAM is strongly associated with the active zone scaffold, and its absence impairs synaptic vesicle release.

SUMMARY

Our work described that dDAAM, a formin type of cytoskeleton regulatory protein, is able to promote the new synaptic bouton formation and appears to have a modulatory role during the formation and functioning of the active zones.

LIST OF PUBLICATIONS

Publications supporting the dissertation:

Migh E, Gotz T, Foldi I, Szikora S, Gombos R, Darula Z, Medzihradszky KF, Maleth J, Hegyi P, Sigrist S, Mihaly J Microtubule organization in presynaptic boutons relies on the formin DAAM. DEVELOPMENT 145:(6) Paper dev158519. 13 p. (2018) IF: 5.843 MTMT: 3343168

Additional publications:

A T Vig, I Földi, S Szikora, E Migh, R Gombos, M Á Tóth, T Huber, R Pintér, G C Talián, J Mihály, B Bugyi The activities of the c-terminal regions of the formin protein disheveled-associated activator of morphogenesis (daam) in actin dynamics JOURNAL OF BIOLOGICAL CHEMISTRY 292:(33) pp. 13566-13583. (2017) IF: 4.125 MTMT: 3242535

Szikora S, Foldi I, Toth K, Migh E, Vig A, Bugyi B, Maleth J, Hegyi P, Kaltenecker P, Sanchez-Soriano N, Mihaly J The formin DAAM is required for coordination of the actin and microtubule cytoskeleton in axonal growth cones. JOURNAL OF CELL SCIENCE 130:(15) pp. 2506-2519. (2017) IF: 4.431 MTMT: 3240757

M Á Tóth, A K Majoros, A T Vig, E Migh, M Nyitrai, J Mihály, B Bugyi Biochemical Activities of the Wiskott-Aldrich Syndrome Homology Region 2 Domains of Sarcomere Length Short.: WH2 domains in sarcomeric actin regulation JOURNAL OF BIOLOGICAL CHEMISTRY 291: pp. 667-680. (2016) IF: 4.125 MTMT: 2974845

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Migh E, Foldi I, Molnar I, Szikora S Developmental signaling pathways in human cancer Selected Topics from Contemporary Experimental Biology, Volume 2. 288 p. Szeged: MTA Szegedi Biológiai Központ, (2015) pp. 171-188. IF: - MTMT: 3003062

Molnár I, Migh E, Szikora S, Kalmár T, Végh A G, Deák F, Barkó S, Bugyi B, Orfanos Z, Kovács J, Juhász G, Váró G, Nyitrai M, Sparrow J, Mihály J DAAM is required for thin filament formation and sarcomerogenesis during muscle development in Drosophila PLOS GENETICS 10:(2) Paper e1004166. 15 p. (2014) IF: 7.528 MTMT: 2506301

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