Summary of Ph.D. thesis

ANALYSIS OF THE ROLE OF *DROSOPHILA* DAAM IN AXONAL GROWTH

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Introduction and aims

The connections between neurons are necessary for the formation of a functional nervous system. A major prerequisite of this process is the outgrowth and precise navigation of axons toward their target cells. The growing axons choose their specific path by sensing the environmental cues. In this process, the key structure is the growth cone, formed at the tip of the growing axon. During axonal growth, the finger-like filopodia and the veil-like lamellipodia located at peripheral region of the growth cone play crucial roles because their plasmamembrane is rich in receptors of the guidance cues. Depending on the nature of these signals (attractant and repellent), the growth cone changes its shape, and the axon grows toward a specific location. Growth cone dynamics is ensured by microtubules and actin filaments, the two main components of the cytoskeleton. The growth cone actin filaments organize into bundled structures in the filopodia, whereas in the lamellipodia they form a loosely interwoven network of filaments. Because axonal growth relies on the dynamic actin cytoskeletal rearrangements, it was expected that actin nucleator and polymerization factors play essential roles in the forming of neurites. Despite of that, at the beginning of our research no protein was clearly identified yet that would act as an assembly factor for the unbranched actin filaments of the growth cone. However, our preliminary studies revealed that the mRNA and the protein of a formin type of actin assembly factor, encoded by the *Drosophila* DAAM

gene (*dDAAM*), is highly enriched in the embryonic central nervous system (CNS), therefore dDAAM was a very good candidate for being the long sought-over axonal actin nucleation factor and we decided to investigate dDAAM function during neural differentiation.

Materials and methods

- tissue preparations:
    - embryo preparation:
        - fixing with methanol
        - „slow" fix
    - brain preparation
    - preparation of embryonic nerve cord cultures

- immunhistochemistry
- Western blot

Results and discussion

The first set of experiments we carried out was focused on the analysis of the embryonic CNS. To begin the functional analysis, we examined embryos homozygous for the null mutant *dDAAM*^{Ex68} allele, however, these zygotic null mutant embryos showed very subtle phenotypic differences as compared to wild

type. Because we knew that the maternal *dDAAM* mRNA is heavily loaded into the embryo, we thought that the lack of phenotypic effect is most easily explained by maternal effect. Therefore, next we generated maternal and zygotic mutant embryos to weaken the maternal effect. The CNS of most of these embryos clearly showed axonal growth defects without significantly altering neuron numbers. To prove that the phenotypic defects observed are indeed due to the impairment of dDAAM function, rescue experiments were carried out by overexpressing the full-length protein which was able to fully rescue the axonal phenotypes. Together, these results indicated that this formin type of actin assembly factor plays an important role during neuronal differentiation and axonal growth regulation.

To collect further evidences for our conclusion, we used an in vitro axonal regeneration test. During this experiment the constitutively active form of dDAAM enhanced the regeneration and growth of the axons exiting the ventral nerve cord, thus these results further confirmed that dDAAM has an important role in neurite formation. When the activated form of dDAAM was expressed in vivo within the CNS, we found that the constitutively active dDAAM has the ability to disrupt the normally highly stereotyped pattern of the embryonic axonal tracts. Comparing the phenotypes caused by two different activated forms, CDAAM and DADm-DAAM, our results suggested that the N-terminal region of the protein plays an important role in the regulation of the actin assembly activity provided by the C-terminal FH2 domain, and also plays a role

in the subcellular localization of the dDAAM protein, which is in good agreement with the findings for other DRF (Diaphanous related) formins.

To address the subcellular function of dDAAM, we generated primary neuronal cultures from *dDAAM*$^{mat/zig}$ mutant embryos and from embryos that overexpress the constitutively active form of dDAAM (DADm-DAAM). The results we collected indicated that in *dDAAM* mutants the axons grow to their normal length, however the number and the length of the filopodia is strongly decreased in the area of the growth cone. In contrast to this LOF data, cells that express the constitutively active form exhibited far more filopodia than their wild type counterparts. These results strongly suggested that the major subcellular function of dDAAM is to regulate filopodia formation in neuronal growth cones.

Once we have established that dDAAM has an essential role in embryonal neurite growth, we wanted to know whether this function is restricted to the embryonic stages or dDAAM is a more general regulator of axonal growth playing a role during the later stages of CNS development as well. By studying the larval, pupal and adult stages we revealed that *dDAAM* expression remains pan-neural during the whole course of CNS development. Because we noted that the dDAAM protein is heavily enriched in the developing mushroom body axons, we focused most of our subsequent studies on this particularly well known model system. Our results demonstrated that the decreased amount of dDAAM causes growth and guidance defects in the mushroom body, suggesting

that *dDAAM* is crucial for neurite outgrowth during the later stages of the neuronal development. Taking the embryonic, larval and adult phenotypic analysis together, dDAAM clearly appears to be a general regulator of neuronal differentiation.

After having clearly demonstrated that the formin *dDAAM* is a critical element of axon growth, we wanted to know whether dDAAM is the only formin which involved in the regulation of this process. To address this question we reduced the level of other formin proteins by using RNAi tools in a neuronal specific manner. By analyzing the mushroom bodies we discovered that out of the six formin genes, only the *dDAAM* and *dia* mutants exhibit a neuronal phenotype. With respect of the Dia protein we revealed that it is enriched in the newly borned axons of the mushroom body, which is very similar to our findings about dDAAM. Despite of that similarity in the expression pattern, the two mutants are characterized by different phenotypic effects. Contrasting to *dDAAM*, in the case of *dia* we didn't find any positive effect to the neurite outgrowth, instead we noticed that cytokinesis is disrupted in the Kenyon cells of the mushroom body. As a consequence, most of the cells became multi-nuclear that prevents the formation of functional neurons. The only observation that hinted towards dia having an effect in the axonal growth regulation, was that the β-axons of the mushroom body failed to terminate at the midline of the brain and overextended into the opposite hemisphere. This observation implied that if dia has a role in the axonal grow, it works as a negative regulator. This is

in good agreement with another set of data we obtained by studying *RhoA*. The dominant negative form of the small GTPase RhoA, that has previously been shown to be a negative regulator of neurite growth, caused very similar cell division phenotypes as the lack of *dia*, therefore RhoA is the likely activator of Dia in the mushroom body both acting as an inhibitor of axonal growth. Together our data suggest that dDAAM is the only formin type of actin assembly factor which plays an essential role in neurite formation.

As typical for the members of DRF family, it is thought that dDAAM activation requires the binding of an activated Rho GTPase to the GBD domain (GTPases binding domain) of the protein. Given that the *Drosophila* genome contains several small GTPases of the Rho family, we were curious to know which one is responsible for the activation of dDAAM in the CNS. With the examination of the phenotypes of five Rho GTPases, combined with genetic interaction tests, we demonstrated that the major activators of dDAAM during neuronal development are the Rac GTPases.

By using genetic interaction assays, we identified further interaction partners, these include Profilin and Enabled that we think to cooperate with dDAAM during all stages of neuronal growth. This interaction was further supported by co-immunoprecipitation experiments, and it is also consistent with the immunostaining experiments showing that dDAAM at least partially colocalizes with these protein in the region of the growth cone, in particular within the filopodia.

According to recent findings, the PCP genes play a crucial role during the growth of the mushroom body neurites. Our genetic interaction experiments suggested that the dDAAM protein works together with the PCP proteins during the regulation of axonal growth and guidance in the mushroom body. Based on these data, we propose that dDAAM acts in the Wnt5 signaling pathway. In this pathway the binding of the Wnt5 guidance cue leads to the activation of the Fz receptor that in turn leads to the activation of Dsh while cooperating with the Stbm/Pk/Fmi proteins. Subsequently, the Dsh protein activates dDAAM by binding to its DAD domain that promotes local actin assembly. The directed actin elongation leads to the formation of new filopodia, which drives axon growth towards the direction of the guidance cue. At present, it is not clear whether Wnt5 is the only guidance cue that regulates the activity of dDAAM or other navigation factors also play a role, the investigation of this question awaits future experiments.

Taken all our data together, we uncovered a whole novel signaling system from an axon guidance cue to the direct regulator of the actin cytoskeleton that plays an essential role during directed axonal growth regulation. These studies not only deepened our knowledge about neuronal growth but we also learnt a lot about the in vivo functions of the DAAM formin subfamily.

List of publications directly related to the subject of the thesis:

Matusek T, Gombos R, Szecsenyi A, Sanchez-Soriano N, Czibula A, Pataki C, Gedai A, Prokop A, Rasko I, Mihaly J (2008) Formin Proteins of the DAAM Subfamily Play a Role during Axon Growth. *J Neurosci*. 28:(49) pp. 13310-13319. **IF: 7.452**

List of publications not related to the subject of the thesis:

Barkó, Sz., Bugyi, B., Carlier, M.F., Gombos, R., Matusek, T., Mihály, J., and M. Nyitrai. (2010) Characterization of the Biochemical Properties and Biological Function of the Formin Homology Domains of *Drosophila* DAAM. *J. Biol. Chem*., 285(17)**,** 13154–13169. **IF: 5.329**

Goncalves-Pimentel C, Gombos R, Mihaly J, Sanchez-Soriano N, Prokop A. (2011) Dissecting Regulatory Networks of Filopodia Formation in a Drosophila Growth Cone Model., *PLoS One* 6(3), pp. 18340-18348 **IF: 4.411**