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Genetic analysis of FMRF-amid related neuropeptides and their GPCR receptors in *Drosophila melanogaster*

Ph.D. thesis summary

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1. Introduction

Neuropeptides/neurohormones can be found in the whole animal kingdom. According to the definition of Burbach, neuropeptides are small peptides secreted under strict regulation by neurons of the central nervous system, which act on other neurons. Neuropeptides regulate the development of both the central (CNS) and peripheral nervous systems, reproduction, circadian rhythm, nutrition and behavior through their hormone activity.

One of the largest groups among neuropeptides are constituted by FMRF-amid related neuropeptides (FaRPs) which are named after the conserved sequence Phe-Met-Arg-Phe at their C-terminal end. FaRPs neuropeptides and their receptors comprise *Fmrf*, *Ms* and *Dsk* peptide, as well as *FR*, *MSR1* and *-2* and *Dsk-R1* and *-2* receptor genes. Fmrf is expressed in the central nervous system, heart and gut, and affects neuronal activity, behavior, stress-reaction, feeding and reproduction. Dsk has myoactive effect on the heart and crop. Ms affects visceral muscles and is named brain-gut peptide after its expression pattern in the brain and gut.

During the last decades FaRPs are identified biochemically, however, detailed functional and genetic studies are still to be performed. Since the genetic system of the fruitfly, *Drosophila melanogaster* is the best known among higher Eukaryotic organisms, we took advantage of this model animal to study the action of neuropeptides.

2. Specific aims

My goal was to study the roles of FaRP neuropeptides (Fmrf, Ms and Dsk peptides), as well as their receptors (FR, MsR1 and -2, Dsk-R1 and -2 receptors) in the stress response and other processes of life of *Drosophila melanogaster*.

To this purpose, I applied three different approaches of classic fly genetics and molecular biology: gene silencing by double stranded RNA molecules (RNAi), generation of intragenic deletions by P-element remobilization and ablation of specific peptiderg neurons.

1. In the **gene silencing experiments** I wanted to study the phenotypic effects of RNAi constructs for FaRP neuropeptides and their receptors available in the *Drosophila* stock centers.
2. As gene silencing frequently results in partial loss-of-function phenotypes corresponding to hypomorphic effcts, I **generated intragenic deletions** by remobilization of P-element insertions to confirm the RNAi phenotypes and rule out the possible "off-target" effects.
3. I also aimed to create **new Fmrf neuron-specific Gal4 drivers** suitable for studying the expression pattern of Fmrf and ablating Fmrf-producing neurons.
4. I planned to study the **effects of FaRP neuropeptides on the behavior** of adult flies which suffered gene silencing or carried intragenic deletions or ablation of specific Fmrf-producing neurons in a stress-induced behavior test.

3. Methods

1. Classic *Drosophila* genetics
2. Gene silencing through RNAi interference
3. Generation of mutants by P-element remobilization
4. RNA isolation and QRT-PCR
5. Testing chromosomal deletions by PCR technique
6. Recombinant DNA techniques
7. Immunofluorescent staining
8. Confocal laser scanning microscopy
9. Behavior tests
    a. Stress-induced locomotion
    b. Negative geotaxis tests

4. Summary of the results

4.1. Gene silencing through RNAi interference

Genes of FaRP neuropeptides and receptors were knocked down by induction of appropriate RNAi transgenes driven by the *Act5C-Gal4* driver. Silencing of *Fmrf, Ms, MsR1, MsR2,* and *DskR1* genes resulted in lethality, while silencing the other FaRP genes (*FR, Dsk,* and *DskR2*) did not affect the viability and fertility of the flies. The *Act5C-Gal4* driver ensures constitutive expression of the RNAi transgenes throughout the whole body in all the times. Because the neuropeptides are mainly expressed in the neurons of the central nervous system (CNS), we repeated the silencing experiment with the *elav-Gal4* pan-neuronal driver. As the *elav* promoter expresses the Gal4 protein exclusively in the neurons, the RNAi silencing is confined to the CNS. In this case all combinations were viable and fertile. By QRT-PCR we tested the efficiency of gene knock-down in these animals and found that in the case of certain receptor genes only mild reduction could be detected in the mRNA levels. From this we suppose that the cause of lethality in the case of *Act5C-Gal4*-induced ubiquitous knock down is the global silencing of the expression of FaRPs and/or the „off-target" effect of the RNAi construct, hitting genes that have their focus in other organs than the CNS.

4.2. Generating mutants by P-element remobilization

As an alternative approach, we remobilized P-transposons inserted in the *MsR1* or *Fmrf* genes to induce deletion mutants. We identified short deficiencies in both genes that either eliminate the whole coding region (*Fmrf*) or the transcription start point (*MsR1*). Surprisingly, these homozygous mutant

animals were viable and fertile, albeit the gene functions of *MsR1* or *Fmrf* were eliminated by the deletions in these flies. This is in clear conflict with the lethal phenotype showed by the silenced *MsR1-RNAi; Act5-Gal4* and *Fmrf-RNAi; Act5-Gal4* combinations, raising again the possibility of the "off-target" effects of the RNAi constructs. In the case of the *Fmrf* gene for example, the *Tango1* (*Transport and Golgi organization 1*) and *wit* (*wishful 71 thinking*) genes are possible off-targets. The *wit* gene is also known to take part in the regulation of the Fmrf expression in *Drosophila*. Further studies are needed to clarify this problem.

4.3. Creating new Fmrf-Gal4 drivers

Previously, Benveniste and Taghert described three DNA sequences in the 5' upstream and intronic regions regulating the expression of the *Fmrf* gene. When these DNA fragments were cloned into a vector upstream of the *β-galactosidase* gene, the staining pattern reproduced specific parts of the Fmrf expression pattern in the CNS. We chose three of these regulatory sequences (*RS8, RS11* and *RS17*), amplified by PCR and inserted them upstream to the coding sequence of the *Gal4* reporter gene in the pBPGUw vector. By embryo-injecting the purified plasmid DNAs of the new clones (RS8-Gal4, RS11-Gal4 and RS17-Gal4) we created three transgenic *Drosophila* lines carrying each one of these constructs at the *attP2* "landing platform" on the third chromosome. We crossed the flies carrying each construct to *UAS-GFP*-carrying flies and determined the *Gal4*-driven expression pattern of the GFP in the offspring. These new drivers can be used in future experiments to target any UAS-dependent expression to these neurons. Upon the GFP expression, we detected new Fmrf-positive neurons in the larval brain complex and found ectopic expression in the midgut's enteroendocrine cells and the imaginal discs.

By crossing the *RS8- Gal4, RS11-Gal4, RS17-Gal4* or *Fmrf-Gal4* driver males to *UAS-rpr* transgenic females, we created offspring where the *rpr* (*reaper*) gene was expressed in the Fmrf-positive neurons. This in turn induced apoptotic death and hence the selective ablation of these neural cells. The offspring in which the *UAS-rpr* was driven by the *RS11- Gal4, RS17-Gal4* or *Fmrf-Gal4* drivers, showed normal development, and normal-looking as well as viable adults eclosed from the pupae. However, the majority of the animals in which the *UAS-rpr* was driven by the *RS8-Gal4* construct, died as pharate adults. Moreover, in approximately 20% of the flies eclosed, the wings were distorted, the movement of the animals was uncoordinated and within 2–3 days after eclosion they died. The distorted wings of the animals carrying the *UAS-rpr; RS8-Gal4* combination could be the consequence of the *RS8-Gal4*-specific expression pattern we observed with *UAS-GFP* in the wing disc.

4.4. Behavior tests

4.4.1. Effects of knocking-down the FaRP peptides and receptors on the stress-induced behavior

Myotropic and behavioral effects of most FaRP peptides have already been suggested by previous studies. Therefore, we used the Repetitive Startle-induced Hyperactivity (ReSH) test to examine the behavior of animals silenced for FaRPs peptides and/or their specific receptors in different combinations. During the test, we examined the locomotor activity of flies by measuring the mean velocity of movement (MVM) after stress (repeated air-puffs). In most of the combinations tested, silencing the FARPs and/or their receptors resulted in significant decrease of the MVM. We detected the weakest stress-induced reaction in two combinations. In the first one, both MS-receptor genes (*MsR1* and *MsR2*), while in the second one, *Dsk* and its receptor were parallelly

knocked-down. Interestingly, we could observe small differences between the stress-induced reactions of different combinations of RNAi constructs silencing the same genes. Moreover, some of these differences could be detected sex-specifically. This latter phenomenon most probably could be attributed to the different position effects of the chromosomal regions surrounding the randomly inserted, different RNAi constructs of the 'GD' series we used. Taken together, we can conclude that silencing of the FaRP neuropeptides and/or their receptor genes - in the tested combinations - affects the locomotor activity and stress-induced reaction of the silenced animals.

4.4.2. Effects of Fmrf-specific cell ablation on stress-induced behavior

In the next series of experiments, we tested the effect on the stress-induced behavior of ablating Fmrf-producing cells by the expression of the *UAS-reaper* apoptosis-inducing gene construct driven by the *RS8-*, *RS11-*, *RS-17-* and *Fmrf-Gal4* drivers. Similar to the RNAi-treated animals, ablating the different patterns of the Fmrf peptide-producing neurons resulted in different behavioral effects in the males and females. In the case of *RS8-Gal4* and *RS17-Gal4*, the female flies showed significantly reduced movement activity as compared to the controls. In the case of *RS8-Gal4*, the males did not show any significant difference in their stress response from the controls. Similarly, the *RS17*-induced ablation caused a weaker, albeit significant effect in the males than in the females. In the case of the *RS11-* and *Fmrf-Gal4* drivers, there was no difference in the behavior of males and females. The ineffectiveness of the *Fmrf-Gal4*-induced ablation came as a surprise because this driver induced the apoptotic death of all the Fmrf-producing neurons, so in this case one could expect the strongest effect. A possible explanation for this contradiction may be

to suppose that, in the original intact Fmrf expression pattern, the active cells are in some kind of "functional balance" with each other. If the entire expression pattern is removed, the missing functions would equalize each other's effect so that the system would remain in balance. However, when the ablation removes some specific cells/part of the expression pattern, the balance is upset yielding the observed stress-induced movement activity phenotypes.

In negative geotaxis test we examined the *RS8-Gal4* driver carrying females that showed the strongest phenotype in the stress-induced locomotion test and did not observe significant differences compared to control animals. This result suggests that negative geotaxis and stress-induced locomotion are regulated by different mechanisms.

5. Summary

Taken all together, our results show that decreasing the production of the Fmrf-related neuropeptides and/or their receptors as well as ablating the peptiderg neurons by apoptosis have a serious negative effect on the stress-induced locomotor activity of the flies.

6. Publications

Publication used for the thesis:

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IF: 2,674

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Cumulative IF: 12.178