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BARD

FINAL REPORT

PROJECT NO. I-884-85

**Identification and Isolation of Genes that Code
for Formamidine Receptors and Formamidine
Sensitivity in Drosophila**

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1990

Standard BARD Cover Page for Scientific Reports

Date: Dec 29, 89

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BARD project No. I-884-85

Title

Identification and isolation of genes that code for
formamidine receptors and formamidine sensitivity in Drosophila

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Project's starting date: Oct 87

Type of Report: 1st Annual ☐ 2nd Annual ☐ Final ☒

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IDENTIFICATION AND ISOLATION OF GENES THAT CODE FOR FORMAMIDINE
RECEPTORS AND FORMAMIDINE SENSITIVITY IN *Drosophila*

Final Report
Submitted to
The United-States - Israel
Binational Agricultural Research and Development Fund (BARD)

by

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Rehovot

December 1989

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IDENTIFICATION AND ISOLATION OF GENES THAT CODE FOR FORMAMIDINE RECEPTORS AND FORMAMIDINE SENSITIVITY IN *Drosophila*

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December 1989

Abstract

The formamidines are a relatively novel and specific group of insect- and acarine- controlling agents. They exert both sub-lethal and lethal effects on sensitive species, and are highly effective in the control of some insects and acarines which have become resistant to conventional pesticides. The acute toxicity of formamidines for vertebrates is very low, though chronic exposure to high doses may result in long-term toxicity and possibly also, in some cases, in increasing the potential for malignancy. The mechanism(s) of action of formamidines differ(s) from that of common insecticides, such as organophosphates and carbamates which are cholinergic agents; it is generally assumed that the formamidines interact with aminergic transmission. The objective of this project was to contribute to the elucidation of the molecular and physiological mechanism of action of formamidines, and the genetic basis for formamide sensitivity. Specifically, we wished to characterize the mode of action of formamidines in the fruit-fly, *Drosophila melanogaster*, which is an excellent system for genetic manipulations, to identify genes that regulate sensitivity to formamidines in this organism, and to elucidate the role of the products of the relevant genes in mediating the physiological effects of formamidines.

Our research was conducted at the behavioural, physiological and molecular level. We found that formamidines, at conventional doses, are lethal to *Drosophila*, whereas at sub-lethal doses, they affect development, behaviour, and neuronal and muscular physiology. For example, formamidines delayed pupal eclosion, disrupted phototaxis and motor activity in the adult, impaired learning and memory, and led to abnormalities in the experience-dependent modification of the responsiveness of mechanosensory neurons. Molecular analysis revealed that formamidines interact with octopaminergic receptors that activate adenylate cyclase. The short-term effects of these toxins may be explained by an increase in the level of cAMP. The long-term effects, especially those on memory consolidation and on development, might be explained by an impairment in the activation of genes by cAMP. Indeed, we found that formamidines alter the synthesis of specific proteins in the nervous system of the fruit fly. We have also found that a mutation, *rutabaga*, that impairs memory and reduces the level of cAMP, confers relative resistance to formamidines. A partial molecular analysis of several transcripts in this X-linked locus was performed.

Introduction

The formamidines are a relatively novel and specific group of insect- and acarine-controlling agents. They exert both sub-lethal and lethal effects on sensitive species, and are highly effective in the control of some insects and acarines which have become resistant to conventional pesticides (Giles and Rothwell 1983, Hollingworth 1976, Lund et al. 1979, Matsumura and Beeman 1976). The acute toxicity of formamidines for vertebrates is very low, though chronic exposure to high doses may result in long-term toxicity and possibly also, in some cases, in increasing the potential for malignancy. The mechanism(s) of action of formamidines differ(s) from that of common insecticides, such as organophosphates and carbamates which are cholinergic agents; it is generally assumed that the formamidines interact with aminergic transmission (*ibid*). The objective of this project was to contribute to the elucidation of the molecular and physiological mechanism of action of formamidines, and the genetic basis for formamidine sensitivity. Specifically, we wished to characterize the mode of action of formamidines in the fruit-fly, *Drosophila melanogaster*, which is an excellent system for genetic manipulations, to identify genes that regulate sensitivity to formamidines in this organism, and to elucidate the role of the products of the relevant genes in mediating the physiological effects of formamidines. In accordance with these objectives, we have proceeded along the following lines of research:

1. We have analyzed, by a combination of pharmacological, neurochemical, and behavioral analysis, the effect of representative formamidines on the physiology and behavior of *Drosophila*, and on the short- and long-term function of second messenger cascades. This part of our research necessitated the development of experimental systems that permit the analysis of toxins on identified neurons that subserve identified behaviors in *Drosophila*, as well as the adaptation of molecular techniques for investigating neurochemical cascades in the fruit fly.
2. We have searched for mutations that affect formamidine sensitivity in *Drosophila*.
3. We have performed a molecular biological analysis of a chromosomal locus which increases resistance to formamidines.

Results.

1. The interaction of formamidines with *Drosophila* in vivo.

We have used three formamidines: Chlordimeform (N'-(4-chloro-2-methylphenyl)-N,N-dimethylformamidine-HCl) (CDMF); N,N-dimethyl-N₂-(2,4-dimethylphenyl)formamidine-HCl (DMPF); and Amitraz (2-methylbis(2,4-xylyliminomethyl)amine). CDMF (Fundal, Spanon, Galecron) and Amitraz (Tactic, Triatox) are in commercial use; all three compounds were generously donated by Schering, W. Berlin.

The three aforementioned toxins were tested by us extensively for their effects on pupal eclosion, adult viability under various feeding regimens, locomotion and phototaxis, muscle physiology, neural physiology, and learning in an olfactory classical conditioning

paradigm. Effects were found in all tests; most interestingly, we also found decrement in learning which could be dissociated from the sensory and motor defects. The results are detailed in the Appendices (Dudai et al. 1987; Corfas and Dudai 1990).

2. Characterization of the interaction of formamidines with *Drosophila* in vitro.

We have used several neurochemical tests to analyze the interaction of formamidines with *Drosophila* tissue in vitro. Among the tests that yielded significant results were binding of [³H]octopamine to membrane-associated binding-sites, activity of the enzyme adenylate cyclase in general and its stimulation by aminergic ligands in particular, protein phosphorylation, and protein synthesis. These assays were chosen because previous data indicated a possible action of formamidines on octopaminergic mechanisms (e.g., Beeman 1982; Hollingworth and Murdock 1980); from our studies we knew that octopamine activates very efficiently adenylate cyclase in *Drosophila* and is probably the major aminergic transmitter in this organism (Uzzan and Dudai 1982), and ample data indicate that the cAMP, generated by adenylate cyclase, can, in addition to activating protein kinase A and hence phosphorylating cellular proteins, also modulate gene expression (reviewed in Dudai 1987). We have verified and extended previously reported interactions of formamidines with the octopaminergic system. Our results indicate that at least part of the octopaminergic receptors mediate their effects via adenylate cyclase. Whereas CDMF and DMPF were antagonists of the octopaminergic receptor(s), Amitraz behaved as a partial agonist. CDMF and Amitraz also modulated the phosphorylation of several bands in SDS-PAGE electrophoretograms from *Drosophila*. Most interestingly, CDMF and DMPF had specific effects on the synthesis of specific proteins in the nervous system of *Drosophila*. This became evident from studies performed on larval brains preincubated with [³⁵S]methionine. The most marked effects were observed in polypeptides of MW of 76 and 53 kDa. The above results are presented in the Appendices (Dudai et al. 1987; Ofarim and Dudai 1988), and part are in preparation for publication (Buxbaum et al. 1990).

3. The effect of formamidines on the activity of an identified sensory neuron that subserves a modifiable behavior.

The aforementioned results pinpointed at least a major target of formamide action to the octopaminergic system in *Drosophila*. However, these results were obtained from experiments performed on the intact organism, or the whole nervous system, or homogenates. It is of great importance to test the effect of formamidines on the physiology of identified neurons in *Drosophila*. Such a cellular approach complements the genetics and should provide valuable data on the molecular effects of mutations (see below). Appropriate experimental systems of this kind were missing, and we set out to develop such a system. Our newly developed experimental approach is based on a mechanosensory system that subserves a cleaning reflex in the fruit fly. The system is described in detail in Corfas and Dudai 1989, 1990a,b (Appendices). In brief, we have found that formamidines affect the physiology of the sensory neuron that mediates the reflex in a similar manner to octopamine. The effect is best seen when testing the effect of repetitive mechanical stimulation on a sensory neuron (i.e., sensory fatigue). Low concentrations of formamidines accelerate this experience-dependent modification in neuronal responsiveness. This provides further evidence for the octopaminergic nature of formamide action in *Drosophila*.

4. The search for mutations that affect formamide sensitivity in *Drosophila*.

We have searched for mutations that affect formamidine sensitivity in *Drosophila*. This was done by two methods: a. mutagenesis and screening for sensitivity to formamidine (administered by feeding) among newly generated mutants; b. screening for formamidine sensitivity among existing mutants that were isolated due to other morphological, physiological, developmental or behavioral abnormalities.

We have screened EMS-mutagenized lines generated by methods that should lead to isolation of X-linked or autosomal-linked mutations. About 5,000 independent lines were screened altogether. The results of these screens were initially encouraging. We have isolated ca. 40 putative mutations, mostly on the X, that increased the sensitivity of adult flies to CDMF administered in sucrose solutions. However, in all the lines so isolated, the sensitivity returned to normal after a maximum of 8-12 generations of breeding isogenic lines. This disappointing outcome of a very tedious experimental procedure, repeated thrice, could be explained either by very rapid accumulation of genetic modifiers, or by dominance which was not appropriately preserved in our genetic breeding schemes. However, the results of our screening of existing mutations were much more rewarding. We established that two mutations, *dunce* (*dnc*) and *rutabaga* (*rut*), both X-linked, EMS-induced mutations that were initially isolated as learning mutants (reviewed in Dudai, 1988), affect the sensitivity of the flies to formamidines. *dnc* is now known to increase cAMP and to be lesioned in a structural gene for cAMP-phosphodiesterase (reviewed as above). It is more sensitive to formamidines than the wild-type, CS, from which it was isolated. *rut* is suspected to be lesioned in the cAMP-generating system (*ibid.*) and confers resistance to formamidines. At that stage, since the results with *rut* were striking and the molecular nature of the locus unknown, we have decided to pursue the molecular basis for the formamidine resistance in *rut* by molecular biological means.

5. Molecular biology of the *rut* chromosomal locus, that confers increased resistance to formamidines.

The results with the cAMP mutants were the first neurogenetic indication that formamidines indeed interact with the cAMP cascade. The search for the molecular nature of the *rut* locus was therefore deemed of primary importance in this project. We have previously mapped *rut*, on the basis of its behavioral and biochemical phenotypes, to a small segment of the X chromosome, spanning polytene bands 12F5-13A1. This region could be estimated to contain approximately 150 kb DNA. In order to identify the gene product(s) that are related to formamidine actions, we have decided to clone the region. This is not an easy task, since we had no nucleic acid or protein probes for the relevant genes or their products, respectively. We have jumped into 12F-5 by using a clone of *heldup* (17C1; obtained from Dr. G. Rubin, Berkeley) and a chromosomal rearrangement, In(1)P363. Following identification of a junction fragment, we have started walking towards 13A1. In the last year, the chromosomal walk was completed, leading to isolation of 16 successive clones spanning a distance of approximately 150 kb (see Appendices for additional details of the following results; also Orgad and Dudai 1990). *In situ* hybridization to polytene chromosome indeed proved that the clones were in the appropriate loci. At a later stage, a complementary walk from the 13A1 locus, using a DNA clone 1129.1 obtained from Dr. Barry Ganetzky (Madison, Wisconsin), indeed proved that the walk was completed when an overlapping clone from the two opposing walks was isolated.

Northern blot analysis identified three transcriptional units within the cloned segment of 150 kb DNA (Fig. 1). The first was hybridized to the 6 kb fragment of clone a₁, and is not likely to be related to the formamidine sensitivity phenotype, because we found that the breakpoint within this fragment does not lead to the phenotype. The second transcript hybridized to the 4 kb fragment of clone a₂, and the third is a region of 11 kb that spans clone e₇ and has 6 transcripts. This limited number of transcriptional units made it feasible to proceed by isolation of cDNA clones corresponding to the relevant genomic restriction fragments. Several positive cDNA clones were identified from both whole-body and head-specific libraries, part of which were prepared at the laboratory of our US collaborator, Professor Seymour Benzer at Caltech. These clones were analyzed and compared to known polypeptides. In several of them sequences which might be interpreted as consensus sequences of ATP binding sites were revealed, as might be expected if one concludes that the *rut* locus codes for polypeptides that regulate cAMP production. The clones seem to represent polypeptides unknown to date. Interestingly, a cDNA clone isolated by the genomic 5kb from a head-specific library, displayed substantial homology to the *Notch* protein from *Drosophila*, known to regulate neural development. Additional data are presented in the Appendices.

Conclusions.

The aforementioned project carried with the generous support of BARD led to several conclusion:

1. Formamidines have clear-cut effects on *Drosophila*. These effects are lethal at high doses of the toxin. Lethality probably results from neural toxicity (see below). However, at low doses, formamidines exert interesting behavioral effects. They include disruption of experience-dependent modification of mechanosensory receptors, and of learning ability. Since experience is necessary for successful mating in *Drosophila*, formamidine may be considered as agents for confusing insects and disrupting their mating. In addition, formamidine delay development, including hatching.

2. Formamidines clearly interact with octopaminergic transmission in *Drosophila*. At least a major part of this interaction is based on binding to an octopamine receptor that activates adenylate cyclase. This interaction with the cAMP cascade results in the short run in disruption of experience-dependent modification of sensory response (see above). In the long run, it leads to alteration in protein synthesis. The long-term effect of formamidines may explain their developmental effects.

3. A chromosomal locus has been identified in *Drosophila* that confers resistance to formamidines. This locus codes for a polypeptide that regulates cAMP production, but also for several transcripts whose role is not yet understood. Sequences of these proteins have been characterized.

In general, our research cast light on behavioral, physiological and molecular aspects of formamidine action in a dipteran. It supports and extends the notion that aminergic systems are the major locus of formamidine toxicity. Our data are the first genetic evidence for the interaction of formamidine with the cAMP cascade in behavior and development. We did not succeed in obtaining new stable mutagenized lines that confer resistance to formamidine, though many putative mutated chromosomes displayed initial resistance. We cannot explain this finding. We did succeed in identification of formamidine resistance in

an existing locus. This paves the way for further molecular biological analysis of the locus. We expect to pursue this goal in the future.

Our research during the period of this grant culminated in several publications, which are attached herewith. The detailed data on the effect of formamidines on protein phosphorylation and protein synthesis, and the detailed analysis of the *rut* locus, are currently in preparation and the manuscripts will be submitted to BARD upon completion.

We wish to express our deep gratitude to BARD for supporting our research during the period of this grant.

Collaboration.

The US collaborating laboratory was actively engaged in two facets of this project: a. Development of the behavioral assays. b. Molecular biological analysis of the *rut* locus. Several genomic as well as cDNA libraries from *Drosophila*, including from *Drosophila* CNS, were prepared in Caltech and used by us. The strategy of the molecular biological analysis was learned from the Caltech laboratory. In addition, we exchanged data and ideas with respect to this project on a regular basis. I have visited Dr. Benzer's laboratory during the project, to coordinate research. I have lectured on our mutual project in Caltech during this visit. This work was also presented at several international forums, including the International Meeting on Catecholamines, the American Society for Neurochemistry in Chicago, and two Annual Meetings of the American Society for Neuroscience.

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