Journal of Evolutionary Biochemistry and Physiology, Vol. 38, No. 6, 2002, pp. 706—733. Translated from Zhurnal Evolyutsionnoi Biokhimii i Fiziologii, Vol. 38, No. 6, 2002, pp. 557—577.

COMPARATIVE AND ONTOGENIC PHYSIOLOGY

Complex Study of *Drosophila* Mutants in the *agnostic* Locus: A Model for Connecting Chromosomal Architecture and Cognitive Functions[1]

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Received April 17, 2002

Abstract—After the devastation of genetics in our country academician Leon A. Orbeli has provided an opportunity for the elucidation of evolutionary conserved genes in control of the nervous system functioning, resulting behavior and conditioning. The progress in plant, worm, *Drosophila* and human genome projects and the bio-informatic analysis of genome sequences has revealed a high evolutionary conservation of gene structure and function. Studies in the *Drosophila* mutants have helped to unravel scaffold proteins which assemble supramolecular signal transduction complexes regulating neural functions and gene expression. It has turned evident that the chromosomal architecture predisposes to genomic disorders resulting from deletions, duplications, insertions and translocations and therefore, plays a role not only in evolution but also in generating human diseases with multiple manifestations including cognitive dysfunctions. A new approach—comparative genomics—has evolved which allows to identify a function of a newly isolated human disease gene on the basis of sequence homology to a known *Drosophila* gene having a number of well-studied mutant phenotypes. For this reason a *Drosophila* gene should be saturated with differently manifested mutations of diverse origin. Our complex behavioral and molecular-genetic study of spontaneous, induced and P-insertional mutations in the *Drosophila agnostic* locus and bio-informatic analyses of genomic sequences allowed to assign the locus to the *Drosophila* genomic scaffold AE003489 from the 11AB X-chromosomal region which contains the CG1848 gene coding for LIM-kinase 1. Mutations, insertions and deletions in the *agnostic* locus lead to: increased activity of Ca^{2+}/calmodulin-dependent PDE1, resistance to ether which inactivates synaptic transmission, impairments of the brain structures, learning and memory defects in conditioned courtship suppression paradigm, alterations in sound production and in structural-functional chromosomal organization. Therefore, the *agnostic* locus presents a model for a study of human Williams syndrome with multiple dysfunctions due to a contiguous deletion in the 7q11.23 spanning 17 genes, among them a gene for LIM-kinase 1 presumed to be responsible for cognitive defects. Williams syndrome is considered to be a most compelling model of human cognition, of human genome organization, and of evolution.

Key words: Drosophila, the agnostic locus, ether resistance, memory and learning, courtship sound production, neuroanatomy, Ca^{2+}/calmodulin-dependent phosphodiesterase, LIM-kinase, chromosomal architecture, Williams syndrome

[1] Authorized translation.

0022-0930/02/3806-0706$27.00 © 2002 MAIK "Nauka/Interperiodica"

INTRODUCTION

When speaking about the enormous contribution of L. Orbeli in the emerging of the modern evolutionary views one seldom mentions that he has also promoted the development of neurogenetics in our country, the field which has lead to a turning point in our notions about the evolutionary conservation of gene function and structure. In 1948, after the notorious Lysenko Session of the USSR Agricultural Academy, academician Orbeli has offered a laboratory in his Institute of Physiology and Pathology of Higher Nervous Activity of the Medical Academy of the USSR (now Pavlov Institute of Physiology) to professor M. Lobashev, the head of the devastated Department of Genetics of Leningrad State University. Michael Lobashev has presented his research program on the role of Pavlovian conditioning in mechanisms of environmental adaptation in animals with genotypic differences produced either by natural or artificial selection—different species of sturgeon fishes, hen brides and geographical races of the honey bee. Pretty soon the comparative studies of the research team of the lab (Anna K. Voskresenskaya, Nina G. Lopatina, Iraida A. Nikitina, Vladimir B. Savvateev and Valentina V. Ponomarenko) have demonstrated an astonishing fact. Though the vertebrates and insects separated in their evolutionary development at the ancestral stage preceding formation of the CNS, their mechanisms of conditioning have evolved in parallel and thereby, are similar. The mushroom bodies of the insect brain play the same functional role in conditioning as the certain parts of the mammalian brain [1–2]. This finding has justified the utility of the fruit fly *Drosophila melanogaster* for the studies of genetic determination of functional activity of the nervous system in behavioral performances including learning (conditioning). In the 1960s the advantages of this favorite genetic object have enabled a selection from natural populations of a vast collection of *Drosophila* strains differing in simple and easily scored neurophysiologic traits. For example, the spontaneous mutations affecting the reversible inactivation of synaptic transmission under ether anesthesia have been made homozygous by F3 of natural selection [3, 4].

The studies of the mode of inheritance of neurological, behavioral and genetic (meiotic recombination or crossing over) traits have allowed V. Ponomarenko to claim the principle of the gene homology according to which the basic functions of the nervous system in animals of different phylogenetic levels are controlled by structurally and functionally similar genes [4, 5]. Moreover, the concept of systemic regulation of genetic and cytogenetic processes has been proposed which implies that these processes in neural tissue are under backward regulation of the neuro-endocrine system executed in accordance with current needs of an organism, its individual experience and environmental demands [6].

In the middle of the 1970s behavioral geneticists have started to use genetic or mutational dissection [7]. This approach is based on the ability of *Drosophila* to give each 10 days a new and so numerous generation that rare mutants arising at a frequency of 10^{-4}–10^{-5} can be picked up in a progeny of parents treated with chemical mutagens. The strategy of mutational analysis implies that a gene and its putative product may be identified through a mutational change in gene *function*. Therefore, three main questions must be answered by a neurogenetic study: (1) how to find a gene? (2) how to find a function of a gene? (3) what relations do exist between behavior, neurochemistry and the brain structure? The last question should be answered irrespective of a starting point of a study which can date back to an induction of mutations disturbing (a) behavioral performance (a widely used approach with a long history [7]), (b) morphology of the brain structures (a very laborious and time-consuming approach made efficient in Heisenberg's lab [8]), (c) neurochemistry (a traditional approach in the prokaryote genetics, also used in our studies [9]).

However, in many cases the assigning function to a gene of interest following chemical mutagenesis, localization of a mutation in the *Drosophila* genome comprised of 4 pairs of the chromosomes, cloning and sequencing of a gene of interest lasted for decades. In the end of the 1980s a new approach of P-insertional mutagenesis has greatly reduced the time required to identify new mutations and analyze gene function. The essential nature of this approach is to use two separate P-elements to provide the two functions necessary for transposition. The first is a genetically marked P-element that is defective in production of transposase but contains the ends required for its own transposition. The second is a P-element with functional transposase activity but a much reduced likelihood for its own transposition. Transposition of the marked P-

element then is initiated by crossing flies that carry only the marked P-element to those that harbor only transposase and can be utilized to preferentially mutate the gene of interest. The marked P-element is a specially constructed vector which contains an easily scored eye-color marker to monitor the transpositions; a β-galactosidase fusion gene to reveal tissue specific expression of a host gene disrupted by P-element insertion and bacterial plasmid sequence for rapid cloning of a region near to insertion using "plasmid rescue" approach [10]. *In situ* hybridization to salivary gland polytene chromosomes can physically map a given P-element insertional mutation with an accuracy of 1–2 polytene bands. A massive gene disruption project (The Berkeley *Drosophila* Genome Project, BDGP) utilizing individual, genetically engineered P transposable elements to target open reading frames throughout the *Drosophila* genome [11], has helped to complete the total sequencing of its DNA in 2000 [12]. With the completion of the human [13], of the *Caenorhabditis elegans* [14] and of the small plant *Arabidopsis thaliana* [15] genomes the rapid development of the bioinformatics has allowed sequence database. On-line Internet searching [16], predicting the exon–intron structure of a putative gene, computer translation of a nucleotide sequence of a given gene into protein sequence of its putative product and large scale sequence similarity comparisons between different species. This has altered our estimate of the evolutionary relationship between vertebrate and invertebrate organisms and has lead to new surprising conclusions:

1. The genes of "low" and "high" organisms are more similar in structure and function than previously suspected. 75% of human genetic disease genes have clear homologs in *Drosophila*. The presenelin gene responsible for the early onset of human Alzheimer disease has been originally identified in the plant *A. thaliana* and nematode *C. elegans*. Therefore, it is not a quantitative comparison between the numbers of genes in different species, but rather the qualitative comparisons between the different modes of regulation of the expression of these genes that makes sense [14];

2. High structural and functional homology of genes between different species or high evolutionary conservation of the genes promotes the development of a new discipline named comparative genomics. For instance, the phenotypic manifestations of the *Drosophila* mutations in such genes help us to understand the putative function of a newly isolated human disease gene. Therefore, by utilising an array of genetic tools available to disrupt or misexpress the gene products, it is now feasible to perform large-scale genetic screens in *Drosophila* to saturate such genes with new phenotypes [17];

3. The eukaryotic genes are organized into chromosomes having modular structure: 5–10% of human genome is duplicated and duplicons flank many regions susceptible to human disorders. These disorders arise at a frequency of 10^{-3}, considerably exceeding the frequency of single gene mutations, from chromosomal rearrangements mediated by homologous recombination between different copies of a duplicon.. The resulting deletions, duplications, inversions and translocations of large chromosomal segments lead to dosage imbalance of multiple genes and thereby to human syndromes with multiple manifestations (cognitive defects, facial and body dysmorphisms, leukemia, etc.), termed as "genomic disorders" or "genomic diseases"[18].

Therefore, the foreseeing aspirations of academician Leon Orbeli to give neurobiologists genetic tools for deciphering genetic determinants of functional activity of the nervous system and such its manifestations as behavior and conditioning has proved to be highly justified. Also, the early conclusions of Michael Lobashev's genetic school about the parallel evolution of invertebrate and vertebrate organisms and gene homology—evolutionary conservation of genes, which control the basic properties of the nervous system—have been verified and confirmed. A fundamental challenge for a mammalian cell to reconcile its own vital needs with the demands imposed upon it by the organism it is a part of, formulated as a principle of the backward neuro-endocrine regulation of genetic and cytogenetic processes [6] has occupied one of the central places in modern neurobiology.

A good example is the development of our modern views about the organization of the second messenger systems—cyclic nucleotides, polyphosphoinositides and Ca^{2+}, which govern many cellular functions, including neuronal gene expression. These views have largely been formed due to the studies in *Drosophila* mutants. The first mutations picked up following chemical mutagenesis were screened for behavioral defects in vision (phototaxis and optomotor reflex) and learning/memory (olfactory electroshock condi-

tioning [7]). A detailed study of several learning/memory genes *dunce, rutabaga* and *amnesiac* helped to establish that behavioral defects resulted from the mutational changes in the genes for enzymes of the cAMP-messenger system [19, 20]; to identify four *dunce* homologs in rats and humans coding for cAMP-specific PDE which is inhibited by antidepressants [21]; and to gain a deeper insight into learning mechanisms not only in *Drosophila*, but also in mammals and humans [22, 23]. These processes are based on the plasticity which is a remarkable feature of the brain, allowing neuronal structure and function to accommodate to patterns of electrical activity. One component of these long-term changes is the activity-driven induction of new gene expression, which is required for both the long-lasting long-term potentiation of synaptic transmission associated with learning and memory, and the activity dependent survival events that help to shape and wire the brain during development. The molecular mechanisms, by which neuronal membrane depolarization and subsequent calcium influx into the cytoplasm lead to the induction of new gene transcription, involve activation of Ca^{2+}-binding transcriptional factor CREB, which in its turn recruits a transcriptional coactivator, the CREB binding protein (CBP). CBP promotes transcription through its recruitment of components of the RNA polymerase II transcription machinery and through its function as a histone acetyl transferase. CBP-catalyzed acetylation of lysine residues within histones helps to remodel chromatin structure into a form that is accessible to active transcription [24]. A multitude of cellular processes are controlled through Ca^{2+} signaling and, in turn, a multitude of external cellular signals induce or regulate Ca^{2+} signaling. Because so many systems respond to, or regulate, Ca^{2+} signaling, it is not surprising that dysfunctions of various aspects of Ca^{2+} signaling pathways underlie several important diseases [25]. When calcium signaling is stimulated in a cell, Ca^{2+} enters the cytoplasm from one of two general sources: it is released from intracellular stores, or it enters the cell across the plasma membrane. Both processes often occur either simultaneously or sequentially. Ca^{2+} entry can be signaled by a variety of processes, including direct activation by surface receptors, such as glutamate NMDA and AMPA receptors, and activation by a variety of second messengers. However, the most commonly observed mechanism of regulated Ca^{2+} entry in non-excitable cells is a process known as *capacitative* Ca^{2+} entry or *store-operated* Ca^{2+} entry. Among the proposed mechanisms of SOCE is a combination of the exocytosis and of conformational coupling models, presuming that communication between the ER and the plasma membrane involves direct multiple protein–protein interactions between components of different signaling systems and Ca^{2+}-channels which form a single supramolecular complex [25]. The remodeling of the actin cytoskeleton plays the crucial role both in the activation of Ca^{2} entry and in the exocytosis [26]. An emerging paradigm in eukaryotic signal transduction is that modular protein–protein recognition domains (PDZ domains) are used to wire the complex biochemical circuits that control cellular responses to external stimuli. This paradigm comes into being primarily due to the studies in the *Drosophila* mutants with defective vision [27]. One of the best-studied PDZ-containing proteins is the *Drosophila* protein INAD, which is composed almost entirely of five PDZ domains and has no direct catalytic function. INAD appears to serve as a scaffold for the G-protein-coupled phototransduction cascade in the fly eye. The PDZ domains in INAD interact with individual proteins in the signal transduction pathway, including G_q, $G\beta_e$, G_e, PLC-β, PKC and TRP (transient receptor potential Ca^{2+} channel). TRP, in its turn, interacts with calmodulin. This multiprotein complex mediates many sensory functions in *Drosophila*, including olfaction [28]. Seven mammalian and human homologs (TRPC1-7) of the *Drosophila* TRP [26] and mammalian PDZ-containing INADL protein [29] have been identified. PDZ domains were first identified as regions of sequence homology found in diverse signaling proteins. The name PDZ derives from the first three proteins in which these domains were identified: PSD-95 (a 95 kDa protein involved in signaling at the post-synaptic density which also contains NMDA-receptors), DLG (the *Drosophila melanogaster* Discs Large protein) and ZO-1 (the zonula occludens 1 protein involved in maintenance of epithelial polarity) [27]. Different signaling proteins can contain different number (from 1 to more than 5) of PDZ domains. These domains specifically recognize short C-terminal peptide motifs, but can also recognize internal sequences that structurally mimic a terminus. The ability of INAD to multimerize via PDZ oligomerization interactions may also help organize signal transduction cascades into large

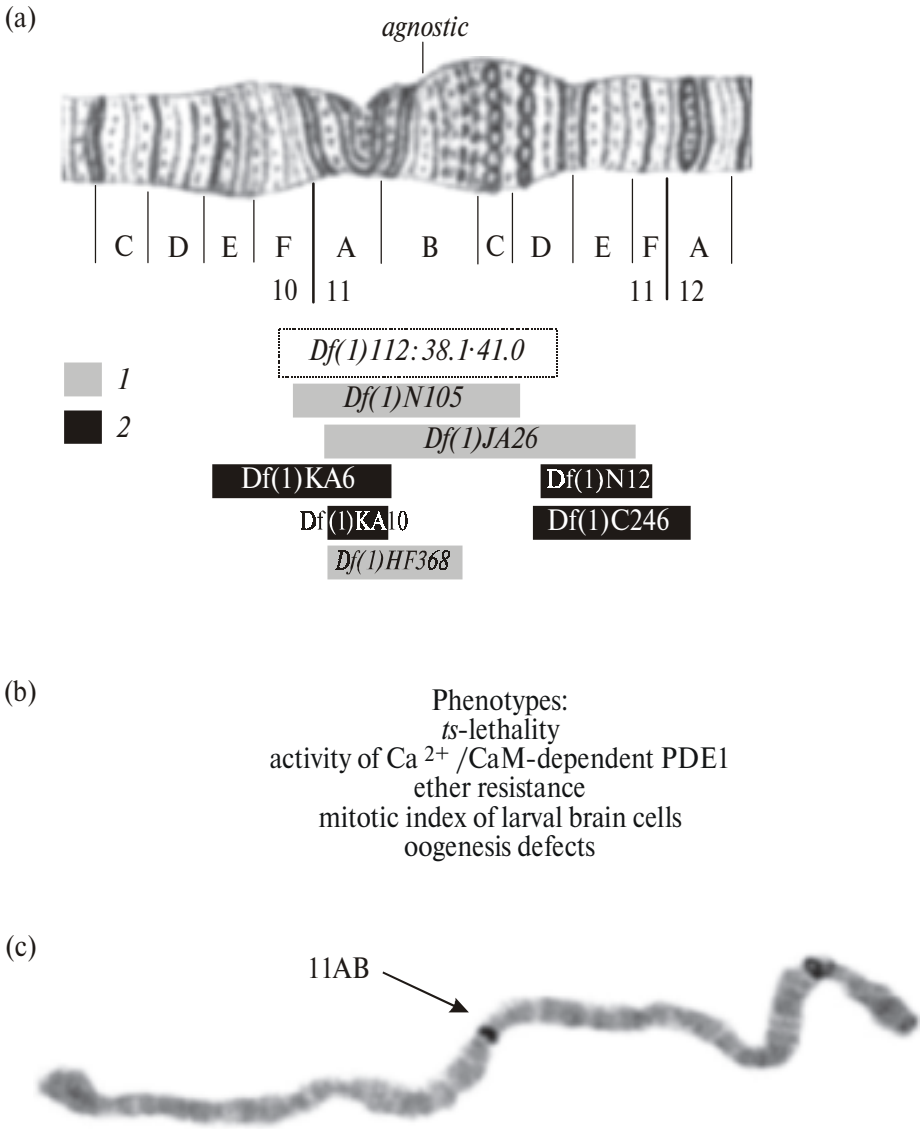


Fig. 1. Localization of the *agnostic* locus within the X-chromosome. (a) Deletion mapping. The length of rectangles (with the exception of microdeficiency *Df(1)112*, for which the limits of recombinational mapping are presented) indicates the X-chromosome regions spanned by deficiencies uncovering (*1*) and not uncovering (*2*) manifestations of mutant phenotypes (b) of the *agnostic* locus mutations; (c) *in situ* hybridization of P-element DNA to the polytene chromosomes of the P-element insertional mutant *P40*.

supramolecular complexes, possibly explaining the quantum bump phenomenon, in which at least a few hundred Ca^{2+} channels are activated in response to a single photon within 20 milliseconds after emptying the intracellular Ca^{2+}-storages. Although the modern view on the modular structure of signal transduction systems has mainly been derived from the studies in *Drosophila* mutants, a great majority of these studies have been carried out at cellular level. The prime goal of this report is to follow in a whole multicellular organism how a complex study of the *agnostic* locus based on the logics of genetic dissection, behavioral analysis, molecular cloning and bioinformatic analysis of nucleotide sequences helps to identify a certain defect in signal transduction systems and its contribution into behavioral formation. In other words, how the metabolism of second messengers, synaptic transmission, development of certain structures of the *Drosophila* brain, learning and memory in conditioned courtship suppression, sound production and chromosomal architecture are affected by spontaneous, chemically-induced and P-insertional mutations in the *agnostic* locus.

PHENOTYPES OF THE MUTANTS IN THE *AGNOSTIC* LOCUS

EMS-induced temperature-sensitive (ts) mutations. The *agnostic* locus was identified in a special screen designed for picking up ethanolmethylsulfonate (EMS)-induced mutations affecting the activities of the enzymes either of synthesis (adenylyl cyclase, AC), or of degradation of cAMP (phosphodiesterase, PDE). The importance of the enzymes of cAMP metabolism for normal functioning of an organism presumed that mutations in the genes coding for these enzymes could have lead to lethality and therefore, could have escaped their detection. Usually this obstacle might be overpassed while picking up temperature-sensitive (ts) mutations switched on when desired at a restrictive temperature (29°C for *Drosophila*). Our experimental design for screening the X-chromosome ts-mutations based on the lethality of its carriers during development at 29°C on selective media with inhibitors of AC, PDE and of calmodulin (CaM) lead to the isolation of several mutants [9, 30]. One of them appeared to be a strict ts-lethal irrespective of the inhibitors used. The mutant demonstrated an increased PDE activity, bright learning at 25°C and learning inability at 29°C. Therefore, it was named *agnostic* (agn^{ts3}). Exposure at 29°C lead to ts-lethality only when experienced before pupation, imago showed an increase in AC and PDE activities and alterations in behavioral performance after 30–40 min of temperature treatment [31]. The strict ts-lethality has served as a good tool both for the genetic and cytogenetic mapping of the mutation (I-38.9; 11AB), and for isolation of a small x-ray induced deficiency *Df(1)112*. This deficiency which lead to ts-lethality being opposed to agn^{ts3}, has been used to screen for the additional spontaneous and P-insertional mutations in the *agnostic* locus.

Spontaneous mutation. The strain *X1* was isolated during selection from natural population for a sensitivity to ether narcotization [3, 4]. The time of immobilization of an animal under ether anesthesia was presumed to reflect the mode of reversible inactivation of synaptic transmission, therefore any deviation from the wild type level (decrease or increase in a time period before complete immobilization) might indicate an altered function of synaptic proteins. The *X1* strain, having no morphological markers, has been preserving its resistance to ether anesthesia (long time preceding immobilization) without selection since 1966. Since the strain has been made homozygous by F3 of selection, it presumably carries a single gene mutation preexisted in natural population. A number of manifestations—an altered PDE activity [36, 43], impaired ability for olfactory electroshock conditioning, almost complete ts-lethality of homozygotes *X1/X1* and of the heterozygotes $X1/agn^{ts3}$, altered CaM content in *Df(1)112/X1* [38, 40] allowed to suggest that the spontaneous mutation *X1* might affect the *agnostic* gene. Moreover, the mutant strain *X1* has a low rate of recombination in the chromosome 2, which is drastically elevated by temperature exposures (29°C) *per se* and following transplantations of the *X1* gonads in normal organism. Also, the temperature treatments can increase the recombination rate in the X-chromosome region around the *agnostic* gene [40–42].

P-insertional mutations. P-insertional mutations *P40* и *P29* were obtained following mobilization of P-*lacW* element from the 2nd chromosome to the X-chromosome and screening for a decreased viability when heterozygous for *Df(1)112* at 29°C [44]. *In situ* hybridization to salivary gland polytene chromosomes gave map position of the both P-insertional mutations within the 11AB X-chromosome region of the *agnostic* locus. Combined data on recombinational and deletion mapping of the *agnostic* locus and on *in situ* hybridization are presented in Fig. 1.

These P-insertional mutations might have facilitated the cloning of the *agnostic* gene, provided that these mutations lead to alterations in the gene function similar to those caused by already studied mutations. Therefore, we have undertaken a study of the *P40* and *P29* phenotypes starting from the main one—the PDE activity.

ACTIVITY OF PHOSPHODIESTERASES OF CAMP

The relative rates of cAMP production and degradation are fundamental in shaping the intracellular cAMP signal. cAMP degrading phosphodiesterases (PDEs) are subjected to regulation by two of the main factors that also control adenylyl cyclases (Acs): Ca^{2+} and protein phosphorylation. PDEs and Acs also show similarities in their subcellular localization governed by scaffolding proteins organizing them in signaling modules for local control of cAMP content. Hence, systems where the rate of synthesis approximates that

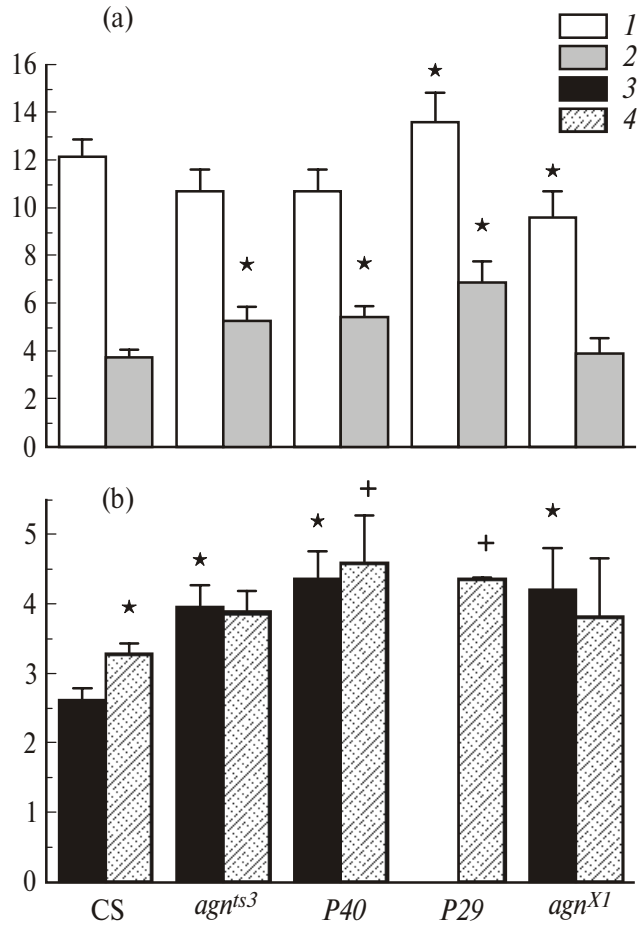


Fig. 2. Activity of phosphodiesterase (PDE) in normal, mutant, and heterozygous for *Df(1)112* flies (PDE; ordinate, nmol/cAMP/min/mg protein). The mean values of 5–10 determinations of the total PDE activity (*1*) and of the Ca^{2+}/CaM-dependent PDE1 activity (*2*) in males (a); and of the Ca^{2+}/CaM-dependent PDE1 activity in homozygous (females, (*3*)) and heterozygous for *Df(1)112* flies (*4*) (b). Other explanations are in the text and in [43]. *Asterisk* indicates differences from the wild type level, *cross*—from heterozygotes *CS/Df(1)112*, $p \le 0.05$ (Student's test).

of breakdown will show dramatic changes of cAMP levels upon relatively small changes of cAMP production or breakdown. The hydrolysis of cAMP in mammals is achieved by more than 10 PDE subfamilies. One of the mostly wide distributed gene subfamily is Ca^{2+}/CaM-regulated PDE1 which is switched on upon an agonist stimulation of a cell and is capable of degrading both cAMP and cGMP [45]. In *Drosophila*, in addition to PDE1, two other forms of PDEs have been identified: cGMP-hydrolyzing PDE3 and cAMP-hydrolyzing PDE2, which corresponds to mammalian PDE4 and is encoded by the *dunce* gene [46, 47]. The both forms regulate basal cellular content of cyclic nucleotides and are thermolabile. Therefore, a short preheating of crude *Drosophila* homogenate at 55°C, abolishing PDE3 and PDE2, leads to predominant determination of PDE1 activity. The first isolated *agnostic* gene mutant agn^{ts3}, besides an increase in AC activity, also demonstrated a significant increase in PDE activity proportional to the elevation of incubation temperature from 22 to 37°C. CaM isolated from the mutant was capable of very potent *in vitro* activation of PDE1 isolated from normal flies, either in presence or absence of Ca^{2+}, and this activation was eliminated by CaM inhibitors [33, 37–39]. This allowed to assume that a product of the *agnostic* gene was capable of increasing CaM activation potency towards both AC and PDE. Since the activation potency of CaM is commonly measured as alterations in PDE1 activity, we have determined it, as well as the total PDE activity, in the *agnostic* gene mutants and in normal Canton-S (CS) flies. The obtained results are presented in Fig. 2. As can be seen, all mutants demonstrate characteristic increase in PDE1 activity over the wild type level, though *P29* and agn^{X1} show their own peculiarities. Whereas the *P29* mutant males show an increase both in total PDE and PDE1 activities, in agn^{X1} mutant the PDE1 activity is increased in females, but not in males, and their total PDE activity is even reduced. The levels of PDE1 activity in mutant females, i.e. when any of the *agnostic* gene mutations is homozygous, significantly exceeds that of normal females CS/CS and is reproduced in each case when flies carry the *Df(1)112*-bearing X-chromosome and the mutant X-chromosome. PDE1 activity in *Df(1)112/P40* and *Df(1)112/P40* is significantly higher ($p < 0.05$), than in *Df(1)112*/CS. It is noteworthy that the activity of this Ca^{2+}/CaM-regulated form of PDE in *Df(1)112/CS* exceeds that of CS/CS females, but do not differ from CS/Y males (Fig. 2). Thus, any disturbance of normal structure of the X-chromosome within the region of the *agnostic* locus due to either deficiency *Df(1)112,* or P-element insertions (*P40* and *P29*) leads to an increase in activity of Ca^{2+}/CaM-regulated PDE1. This fact indicates that probably the PDE1 activity might be a marker of complicated interrelations in multimolecular complex of signal transduction, when physical disruptions in the gene integrity lead to altered interactions inside this complex. Moreover, the effect of the deficiency and P-insertions indicates that a specific chromosome

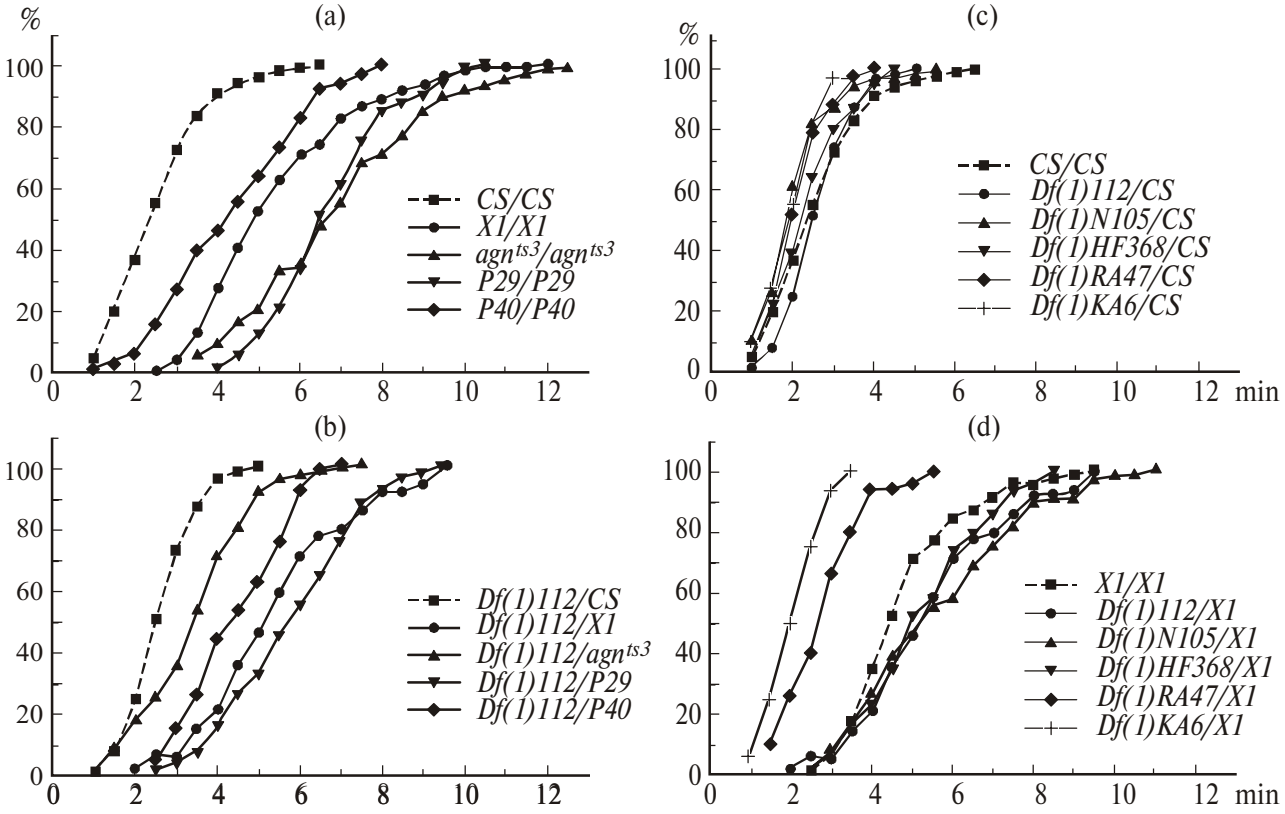


Fig. 3. Dynamics of ether anesthesia of normal and mutant flies (a), of the flies heterozygous for *Df(1)112* (b), and of the flies heterozygous for deletions overlapping within the region of the *agnostic* locus (c, d). Cumulative curves for data of 5–16 experiments, in each of which the time (min) was recorded of complete immobilization of 10 flies subjected to 0.1% concentration of vapors produced by 0.02 ml of ethyl ether in a Plexiglas vial (see in detail in [4]). Description of genotypes of the tested flies is given on plots (a)–(d) and in the text. *Abscissa*: time (min), *ordinate*: the number of immobilized flies (%).

architecture may be present around the *agnostic* locus. The peculiarities of the *P29* and agn^{X1} manifestations also have promoted the necessity of a study of intragenic allelic interactions provided that all aforementioned mutations indeed belong to the same locus, i.e., are allelic.

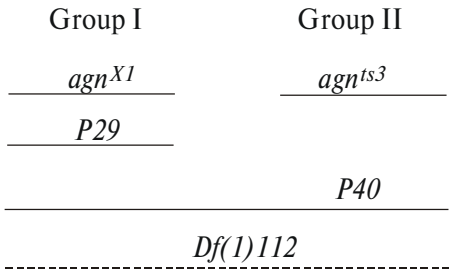
ALLELIC INTERRELATIONS IN THE *AGNOSTIC* LOCUS AND THEIR EFFECTS ON SYNAPTIC FUNCTIONS REVEALED UNDER ETHER ANESTHESIA

A special approach in genetic analysis aimed to establish whether different mutations belong to the same gene, is known as functional test for allelism. For this, different mutants are crossed to each over in all possible combinations, and the progeny from each cross is analyzed whether it shows wild type (if mutations affects different genes) or mutant (if mutations affect the same gene) manifestations of an easily scored genetic trait. To obtain the evidence that the aforementioned mutations indeed belong to the *agnostic* gene, we could not make use of either biochemical and behavioral tests because of their complexity, neither we could use ts-lethality because of its ambiguity: for instance, *Df(1)112*/CS is not ts-lethal. Therefore, we have used such a trait, as "a time of immobilization under ether anesthesia," which has proven to be convenient both for genetic analysis and for a study of possible mutational effects on synaptic functions. At the same time, it is possible to reveal allelic interactions within a gene and to construct a map of intragenic allelic complementation, when all mutations under study are allelic and recessive. A recessive manifestation of a trait might be stated when the scores of the heterozygotes *CS/mut,* or *Df/mut* (if a deficiency

Table 1. Functional test for allelism. using the trait "the mean time of 50% immobilization under anesthesia with 0.1% ether vapors" in progeny from inter-strain crosses

	Male parents				
	agn^{ts3}	*X1*	*P40*	*P29*	*CS*
Female parents: *agn^{ts3}*	**6.1 ± 0.25** (13)				
X1	3.1 ± 0.12 (4)	**5.3 ± 0.36** (16)			3.3 ± 0.18 (7)
P40	**4.1 ± 0.33** (7)	**4.3 ± 0.40** (9)	**3.8 ± 0.29** (14)		
P29	2.8 ± 0.26 (6)	**5.5 ± 0.38** (9)	**4.9 ± 0.54** (7)	**6.6 ± 0.29** (8)	
CS	3.3 ± 0.12 (9)	3.1 ± 0.10 (20)	2.6 ± 0.08 (10)	2.5 ± 0.15 (7)	2.7 ± 0.27 (14)
Df(1)112	**3.6 ± 0.16** (10)	**4.8 ± 0.17** (20)	**5.1 ± 0.38** (8)	**6.3 ± 0.45** (8)	2.4 ± 0.08 (11)

Map of intragenic allelic complementation in the *agnostic* locus



Note: The numbers in parentheses – the number of experiments; differences from the wild type level of the Canton-S (CS) strain ($p \leq 0.05$, Student's test) are in bold font.

does not uncover a given gene) have the wild type level. As shown in Fig. 3a, not only the *agn^{X1}*, as it has been known earlier, but also the other mutations, when homozygous, lead to ether resistance, i.e., to an increase in the time of immobilization. The same is characteristic for heterozygotes *Df(1)112/mut* (Fig. 3b). The dynamics of ether anesthesia in the heterozygotes *Df(1)112/agn^{ts3}* is similar to that of males (the single X-chromosome), but not of females (the two X-chromosomes, the data are not shown), thus indicating an absence of the gene dosage compensation. The deficiencies usually used for the deletion mapping of the *agnostic* gene [48] do not affect the manifestation of the trait when opposed against the X-chromosome from the wild type CS strain (Fig. 3c) and only those, which uncover the gene when opposed to the X-chromosome from the *agn^{X1}* (Fig. 3d), show the characteristic ether resistance. For the convenience of the genetic analysis we have chosen the trait "a mean time of 50% immobilization" at a given ether concentration. As shown in Table 1, the trait is, indeed, recessive, since the scores in the heterozygotes CS/mut are similar to those in the homozygotes CS/CS. Statistically significant differences from the wild type level which are characteristic for the mutants and for the crosses between them (Table 1, shown in bold), allow to claim that the mutations are allelic and therefore, to construct a map of intragenic allelic complementation. This map indicates that there are two groups of complementation, *X1* and *P29* belonging to one group, *agn^{ts3}* and *P40*, having the most extended mutational impairment in the gene, to the other. *Df(1)112* has a similarly extended impairment, but probably indicates the absence of dosage compensation in the *agn^{ts3}* (transcription in the sex chromosomes in *Drosophila* is regulated in such a way as to equal the amounts of gene products, i.e., the gene dosage, one X-chromosome in males and two in females).

What implies this increased ether resistance of the *agnostic* mutants? Although general volatile anesthetics have been introduced into clinical practice over 150 years ago, the mechanisms of general anesthesia in the central nervous system and the sites of its action are at the beginning of molecular examination. As a result of research during the past several decades, especially in *Drosophila* and nematode, a group of ligand-gated ion channels have emerged as plausible targets for general anesthetics, including ether. This group includes GABA, glycine, AMPA, kainite and NMDA receptors. Molecular biology techniques have greatly accelerated attempts to classify ligand-gated ion channel sensitivity to general anesthetics, and have identified the sites of receptor subunits critical for anesthetic and alcohol modulation using chimeric (recombinant) and *in vitro* mutated receptors [49, 50]. When alleles of several loci that encode or regulate subunits of ion channels were compared with control stocks in *Drosophila*, it was shown that a given ion channel mutation often affected the response to one anesthetic but not another [51]. By screening existing mutants of the nematode *C. elegans*, it was found that a mutation in the neuronal syntaxin gene dominantly conferred resistance to volatile anesthetics. By contrast, other mutations in synaptic Ca^{2+}-binding proteins syntaxin and in the syntaxin-binding proteins synaptobrevin and SNAP-25 produced hypersensitivity. Both the resistant and hypersensitive mutations decreased synaptic transmission [52]. Components of cAMP system and, therefore, multimolecular complexes of signal transduction, which regulate SOCE (see Introduction), are also the molecular targets both for anesthetics and alcohols [53]. Another site of anesthetic action is the remodeling of the actin cytoskeleton. Clinically effective doses of chloroform and ether have been shown to block actin-based motility not only in dendritic spines, but also in fibroblasts, indicating that their action is independent of neuron-specific components and thus identifying the actin cytoskeleton as a general cellular target of anesthetic action [54]. The sensitivity of spine motility to general anesthetics lends further support to the idea that rapid morphological changes at central synapses contribute both to brain development and short-term memory and learning [54]. Therefore, it is likely that both the brain structure and learning/memory might be affected in the *agnostic* mutants.

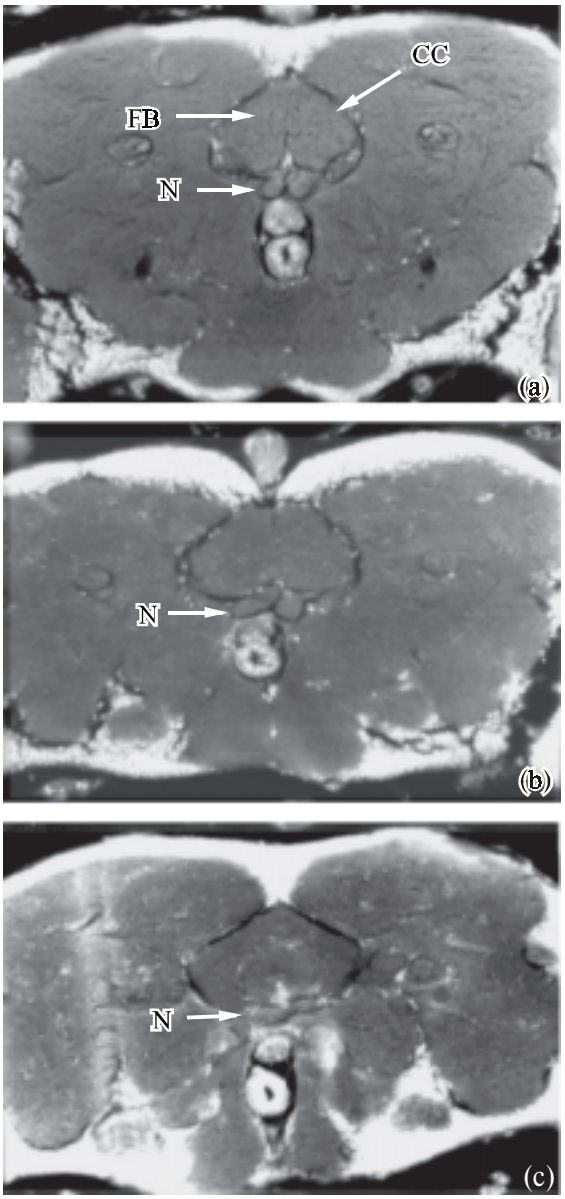


Fig. 4. Structure of the central complex of the brain of the wild type Canton-S flies (a) and of the mutants *agn* ^{X1} (b) and *P29* (c). Photos of frontal autofluorescent paraffin sections (7 μm) of the Drosophila brain: *dark part*—neuropil formed by the neural fiber extensions of peripherally located nerve cells (*light part*). *CC*—The central complex of the brain; *FB*—the fan-shaped body; *N*—noduli. For details, see [8]. The deranged noduli in the mutants are demonstrated.

THE STRUCTURE OF THE BRAIN IN THE *AGNOSTIC* MUTANTS

One of intensively and for a long time studied sites of action of cAMP/CaM-dependent phoshphorylation is the regulation of cell cycle and modification of

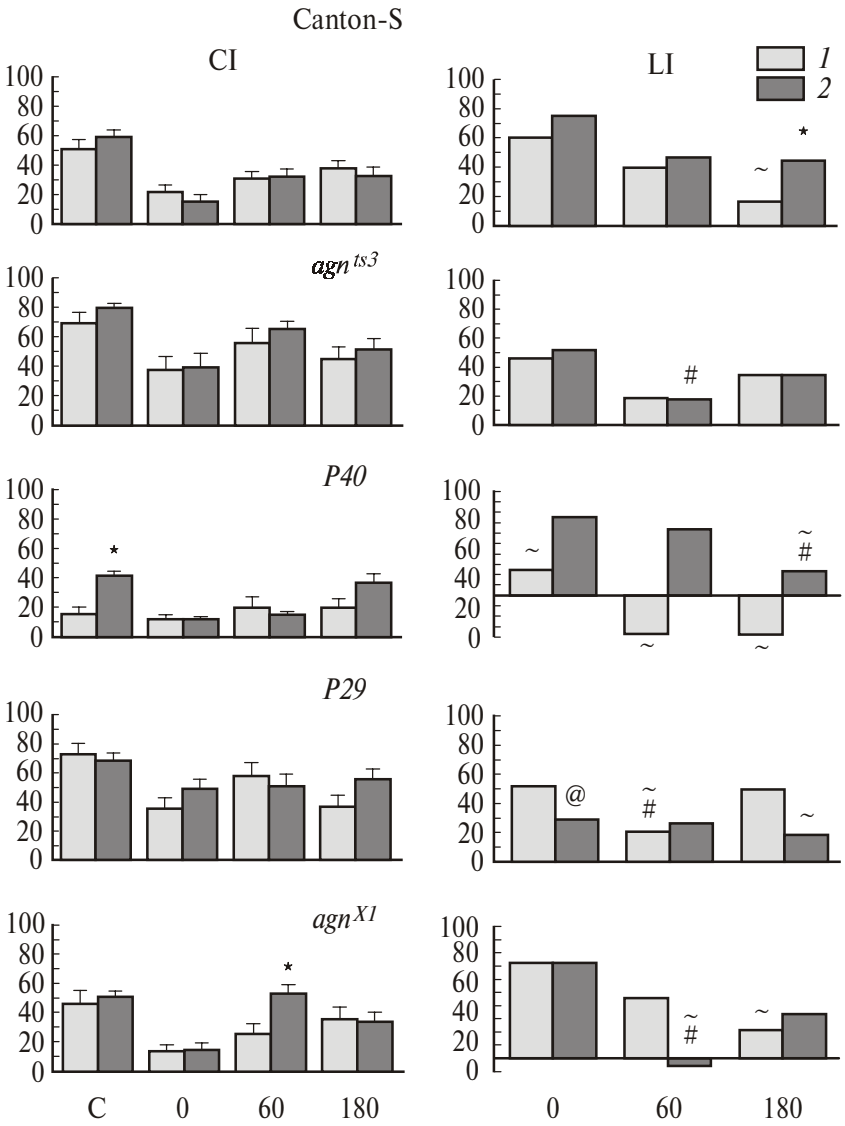


Fig. 5. Dynamics of conditioned courtship suppression in the wild type strain Canton-S and the *agnostic* locus mutants. *Abscissa*: time after training (min), *ordinate*: CI—Courtship Index, LI—Learning Index. Indices in the test with a virgin female (*1*), in the test with a fertilized female—(*2*). The sample size for each point is 20 males. Comparison with randomization test (one-sided), $\alpha_R < 0.05$ (see in detail [58, 59]): *—differences between two tests; #—LI in a given delayed test is lower than LI in the test immediately after training; @—LI differences from the corresponding scores in the wild type strain Canton-S; ~—the absence of differences from 0.

the actin cytoskeleton in neuronal growth cones. Changes in the duration of different stages of cell cycle during development of the brain might affect the formation of its structures. Alterations in the neuritis outgrowth and in synaptogenesis lead to defective morphology of certain regions of the brain involved in learning and memory formation under neurodegenerative disorders [55]. As it has been shown earlier, both the agn^{ts3}/agn^{ts3} and $Df(1)112/agn^{ts3}$ demonstrate a 3-fold increase in mitotic index in the cells of the larval brain, which can be brought down to the wild type level by CaM inhibitor triftazin [56]. The defective structure of the central complex of the adult brain (deranged noduli) has been first documented in the agn^{X1} mutant [57]. The present histological inspection of the brain structure in the *agnostic* mutants has revealed that the mutations from the same group of complementation, agn^{X1} and *P29*, but not of the other one, agn^{ts3} and *P40*, lead to a similar defect in morphology of the noduli of the central complex in the adult brain (Fig. 4).

LEARNING AND MEMORY IN CONDITIONED COURTSHIP SUPPRESSION PARADIGM

It is well documented that the synaptic pathology, as well as morphologic defects in the brain structures lead to impairments of learning and memory. Our previous study on the olfactory conditioning in spontaneous and EMS-induced *agnostic* mutants demonstrated that learning ability was drastically diminished in agn^{X1} and, depending on temperature regime of testing ts-mutants, could be either increased or decreased [40]. However, olfactory conditioning based on the electro-shock negative reward associated with an artificial odor is not pertinent to biology of *Drosophila*. At present, the conditioned courtship suppression paradigm (CCSP) based on biologically important stimuli and more appropriate for the analysis of learning and memory, is widely used [58]. This paradigm is highly suitable for testing small samples of rare genotypes (transgenic *Drosophila* strains) and for mutational screens [59]. Therefore, we have used CCSP for simultaneous testing of the previously studied *agnostic* mutants and the newly isolated P-insertions. Male courtship is triggered by a courtship-stimulating pheromone (aphrodisiac), which is emitted by both virgin and mated females. Fertilized females additionally produce the courtship-suppressing pheromone (antiaphrodisiac) which they emit in response to male courtship. Courtship suppression in males which have previous experience in courting a mated female, is the result of counterconditioning of an attractive unconditioned stimulus after its pairing with an aversive unconditioned stimulus, the antiaphrodisiac. In the commonly used retention test with a virgin female lacking the antiaphrodisiac, males retain a diminished level of courtship for about 2–3 h after training. In contrast, in the retraining test with a mated female, memory lasts for at least 8 h [58].

The comparison of learning ability and memory retention in the *agnostic* mutants in two test with virgin or fertilized Canton-S females is presented in Fig. 5, in which the left part demonstrates the courtship levels (courtship indices, CI), the right part shows learning indices (LI). The learning indices are computed to enable comparisons to be made irrespective of genotype- or test-dependent fluctuations in the courtship levels of naive males. The agn^{ts3} mutants demonstrate similar performance in the both tests: their memory retention is drastically diminished by the 1st h after training, as evidenced by an increased CI and decreased LI (Fig. 5). Behavioral performance of *P40* is rather atypical—their CI in the test with a virgin female is significantly lower than in the test with a fertilized female, and this is reflected in LIs. In the first case the *P40* mutant demonstrates neither learning, nor memory retention (LI does not differ from 0), in the second case the mutant has a memory retention defect 3 h after training (LI_{M3} does not differ from 0). The *P29* mutants, despite high CIs in the both tests, have defective learning and diminished memory retention immediately, 1 h and 3h after training, especially in the test with a fertilized female.

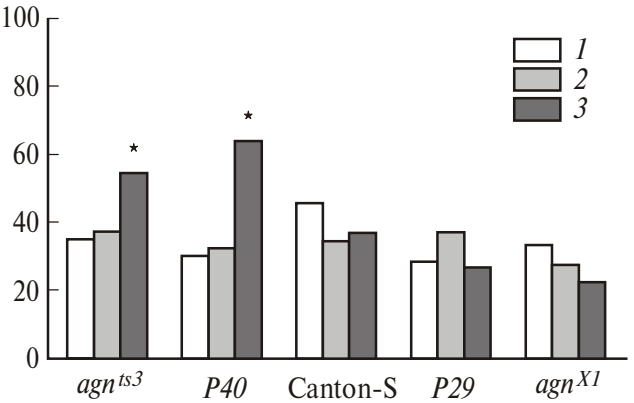


Fig. 6. Memory retention in the conditioned courtship suppression paradigm in 3 h after training (*abscissa*) at different temperature regimes in the wild type strain Canton-S and in the *agnostic* locus mutants. *Ordinate*: Learning Index. (*1*)—training, rest, and test at 22°C; (*2*)—training at 29°C, rest, and test at 22°C; (*3*)—training, rest, and test at 29°C. The sample size for each point—20 males. *Asterisks*—LI differences from the corresponding scores at 22°C, comparison with randomization test (one-sided), $\alpha_R < 0.05$.

The agn^{X1} mutants demonstrate significant difference between CIs in the both tests only 1 h after training, thus indicating severe memory retention defect in the test with fertilized female (Fig. 5). In the test with virgin female, the mutants show constant decline in memory retention, so that 3 h after training their LI_{MB} does not differ from 0.

Temperature dependency of learning and memory retention in the *agnostic* mutants has been characterized in the test with a fertilized female when either training alone, or training, 3 h period before the test (rest) and the test have been carried out at 29°C. The both temperature exposures do not affect LIs in wild type, *P29* and agn^{X1}. On the contrary, agn^{ts3} and *P40* demonstrate significant increase in 3 h memory, when the whole experiment has been carried out at 29°C (Fig. 6).

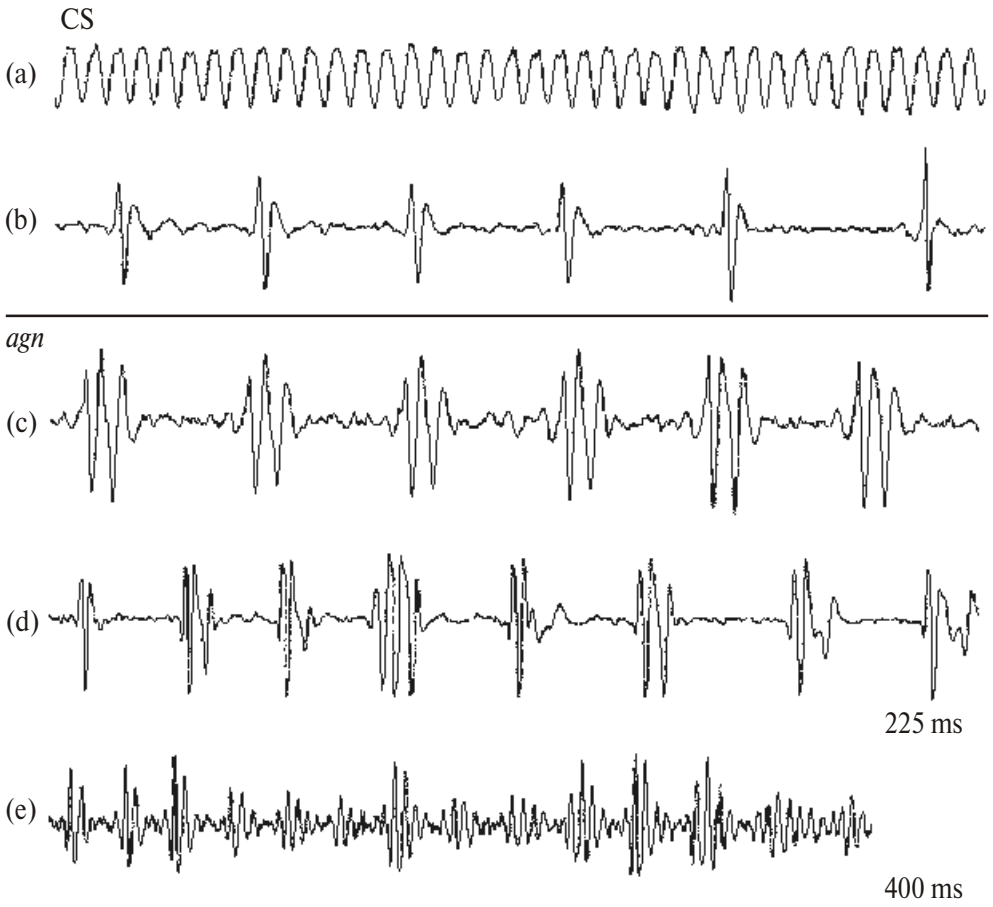


Fig. 7. Courtship sound signals of the wild type (CS) and mutant males. (a) The sine, (b) the pulse songs of wild type flies; (c)–(e) the periodically appearing distorted sound pulses in the mutant song. Oscillogram duration (ms) is indicated by numbers on the right; for (a)–(d) it is equal.

Thus, a certain learning and memory display in CCSP is also characteristic of a given mutation, and temperature dependency can be revealed in the mutants from the same group of complementation, agn^{ts3} and *P40*. Different performance of the mutants *P40*, *P29* and agn^{X1} in the both tests might indicate possible defects in motivation, thus promoting a detailed study of their courtship.

ACOUSTIC BEHAVIOR AND SOUND PRODUCTION IN THE *AGNOSTIC* LOCUS MUTANTS

Courtship behavior of *Drosophila* males is inborn and consists of several successive acts, finally resulting in copulation. Singing is its important component which can serve as a reliable objective indicator of courtship intensity, i.e., of male motivation level and of the state of animal effectors, sensory and nervous centers. That is why the bioacoustic method is invaluable for reveling of different pathologies and for the screening of various pharmacological agents for their action on the nerve-muscle system and motivational centers.

Communicative sound signals produced by wild type males while courting females have been earlier described in detail [60]. The males of all 4 *agnostic* mutant strains emit during courtship the same two types of sound signals (the pulse and the sine songs) as the wild type flies (Figs. 7a, 7b). However, if the sine song of the mutants practically does not differ from that of CS, their pulse song, especially in agn^{ts3} and *P29*, has much more unstable pulses both in form and amplitude. The so-called "distorted" pulses much more often appear in their pulse trains (Figs. 7c, 7d). Sometimes they generate the so-called "mixed" song, consisting of the sine modulated with the repetition rate of pulses in the pulse song (Fig. 7e).

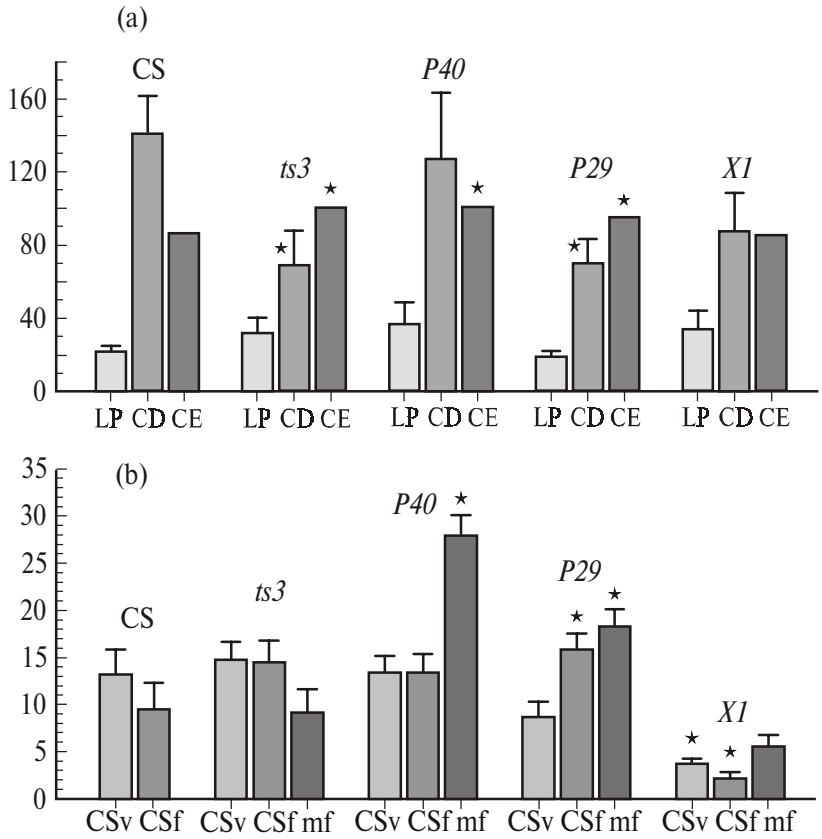


Fig. 8. Courtship characteristics in males of the wild type (CS) and of mutant strains. (a): LP—latent period, CD—duration and CE—efficiency of the male courting virgin wild type females. *Ordinate*: seconds (for LP and CD) and percent (for CE). The mean values with standard errors are presented for samples not less than 20 pairs. Courtship efficiency was evaluated by the percent of pairs copulated during 10 min of the test. (b): Singing index, i.e. the percent of the test time spent in singing (5 min—for fertilized females; till copulation or till the end of the 5-min test—for virgin females), of male courting virgin (CSv) and fertilized (CSf) wild type females and fertilized females of their own strain (mf). *Asterisks* above the columns—statistically significant difference from CS ($p < 0.05$, Student test). The methods of sound recording and testing were described earlier [60].

At the same time, the courtship efficiency of males from all mutant strains in contacts with virgin CS females is at least not lower than in wild type males (Fig. 8a). Although the mean latency of courtship onset, which shows how quickly the males detect and identify a female by her chemical signals, is a bit longer in three mutant strains (agn^{ts3}, *P40* and agn^{X1}) than in wild type flies, these differences appears to be statistically insignificant. Courtship duration before copulation in mutants is even shorter than in CS males, and the percent of pairs copulated during 10 min test is larger in agn^{ts3}, *P29* and *P40*. Thus, the *agnostic* mutations do not lower the courtship efficiency.

Singing index (the percent of time spent in singing) which reflects the level of male motivation, in a different way in each mutant depends on courtship object (Fig. 8b). In CS it goes down by lowering the proportion of the pulse song when a virgin female is replaced by a fertilized. In agn^{ts3} males the singing index is the same in cases of courting both virgin and fertilized CS females, and when agn^{ts3} males are courting a fertilized mutant female of their own strain their singing index goes down, again due to diminished proportion of the pulse song, i.e., mutant females appear to be less attractive for them than the wild type females. On the contrary, the own females are much more attractive for *P29*, *P40* and agn^{X1} males: singing index while courting them is much higher than while courting CS females. For *P29* males fertilized females (CS and own) are more attractive than virgin CS females which stimulate them much weaker to generate the pulse and especially the sine signals. Singing index of agn^{X1} males is lower than in the other *agnostic* mutants and wild type flies irrespec-

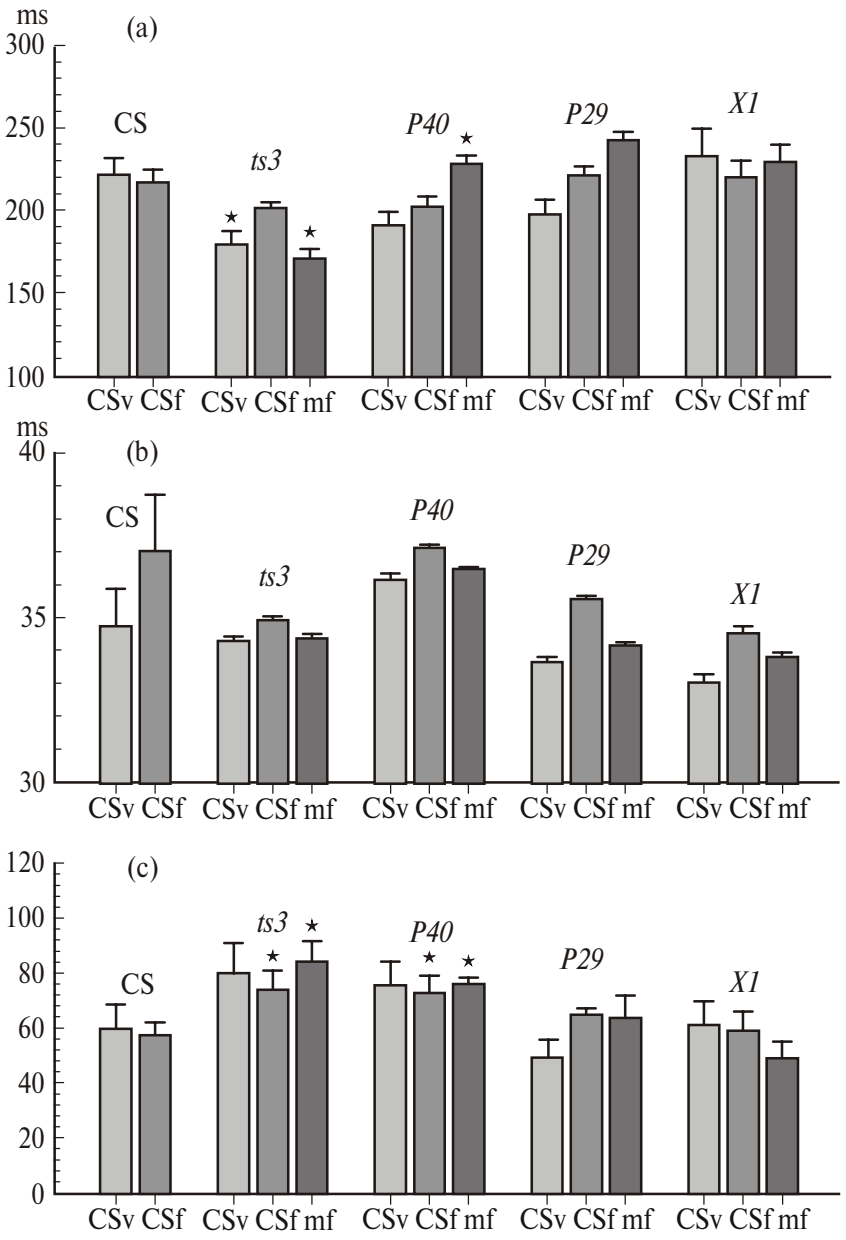


Fig. 9. Characteristics of the male pulse song emitted while courting virgin (CSv) and fertilized (CSf) wild type females and fertilized females of their own strain (mf). (a) The mean train duration of the pulse song; (b) the mean interpulse interval; (c) the IPI variance. The mean values with standard errors for the total sample of 10 males are presented. *Asterisks* above the columns—statistically significant difference from CS ($p < 0.05$, Mann–Whitney U test for (a), Student test—for (b)). In (b) all the differences are statistically significant due to the large sample size (> 1000 of measurements).

tive of courtship object. Thus, here we face a clear allelic polymorphism of phenotypic manifestations in respect of the male motivation level, which may be connected to differences in the rate and extent of impairment of sensory systems.

One can judge about the action of a given mutation on motor centers, nervous centers and their coordination basing on the state of preservation of the pulse song parameters. According to our data, the mean train duration of the pulse song in agn^{ts3} males is shorter (especially in cases of courting CS virgin and own fertilized females), and in *P29* males is longer (while courting own females) than in wild type flies (Fig. 9a). In *P40* and *P29* mutants the shortest trains are emit-

ted by males courting the virgin CS females. Thus, in respect of this parameter of the pulse song the effects of the *agnostic* locus mutations are mutation-specific.

The main parameter of the pulse song determining its recognition by females—the mean interpulse interval (IPI)—is similar in all mutant strains. The differences, statistically significant only because of large samples size, are just few milliseconds (Fig. 9b). It is typical that in all the strains including the wild type the IPI are longer (i.e., the pulse rate is lower) while courting the fertilized CS females than when the males are courting virgin CS or fertilized own mutant females. The shortest IPIs are characteristic for agn^{X1}, the longest—for *P40*.

The IPI variance determines the working stability of the pulse song pacemaker. In mutants *P29* and agn^{X1} it is the same as in wild type flies, whereas in *P40* and agn^{ts3} it is for sure higher,—an indication of a certain distortion of the working stability of the pulse generator in animals of these strains (Fig. 9c). To test the temperature sensitivity, the mutants agn^{ts3} and *P40* have been subjected to temperature treatment at 29°C for the same time as in CCSP experiments. It appears that the mutants *P40* are practically insensitive to temperature treatment,—neither characteristics of their courtship nor the main parameters of the courtship sound signals change after such treatment. In agn^{ts3} males the temperature treatment causes a slight increase of courtship latency and double increase of courtship duration before copulation with virgin females. At the same time, there are no changes either in courtship intensity, or in courtship success. Apart from this, agn^{ts3} males having the same singing index produce the sine song much more often than the pulse song (respective indices were 8.2 and 6.2%). The relation between these two signals under normal conditions is inverse. Finally, the train duration in the pulse song of this mutant after the temperature treatment decreases for 25% (in *P40* males only for 11%). Thus, these two mutants differ slightly in their reaction to temperature treatment. The fact that in both cases the singing index remains unchanged is the important evidence that the sharp improvement of memory retention under similar conditions of CCSP experiments (Fig. 6) results from temperature action not on courtship characteristics, but rather on the central mechanisms of memory formation.

Thus, all four mutants have their own phenotypic manifestations both at motivation level and in the parameters of the emitted courtship sound signals. This evidences a complex functional organization of the *agnostic* gene. The *agnostic* mutants have temperature-sensitive phenotypes, and in *Drosophila* many ts-mutations are at the same time the ion channels mutations which also affect the sound production parameters [61–63].

Do the central complex defects affect learning and sound production? According to our data, parameters of sound production, but not of learning, are very similar in *P29* and agn^{X1} mutants having defects in this brain structure and belonging to the same complementation group. However, the study of the both forms of behavior has been carried out in males without using *Df(1)112* which uncovers the *agnostic* gene, because its localization in the X-chromosome has allowed to obtain only heterozygous females. At the same time, the effect of *Df(1)112* which is independent from the effects of the mutations and is manifested as an increase in Ca^{2+}/CaM-regulated PDE1 activity has required a more detailed study of the chromosome architecture around the gene.

CHROMOSOME ARCHITECTURE

We have already mentioned that signal transduction systems can regulate gene transcription activity via chromatin remodeling and thereby via modulation of the spatial 3-dimensional organization of homologous and non-homologous chromosomes in the nuclei. The latter is based on the ectopic contacts formed by a certain region with different regions of homology in the same or different chromosomes of a nucleus. There are no mitotic divisions following chromosome replication in the nuclei of larval salivary glands of *Drosophila*, and 4 chromosomes represented by maternally and paternally derived homologs become polytene, i.e., formed by numerous chromatin threads. They can be visualized under light microscope as arms stretched out from the common chromocenter: one for the X-chromosome, 2L and 2R for the chromosome 2, 3L and 3R for the chromosome 3, and one small arm for the chromosome 4. The banding pattern, individual for each chromosome arm, allows to assign a unique code, which identifies each band within one of 102 numbered chromosome divisions, and therefore, gives enormous advantages for cytogenetic analysis. This has helped to reveal all

Table 2. Frequency of ectopic contacts in the left arm of the chromosome 2 (2L)

Growth conditions	CS/CS	*Df(1)112/w*	*agnts3/ agnts3*	*P40/P40*	*P29/P29*	*Df(1)112/P29*	*agnX1/agnX1*
I experimental series							
Normal media	46.9 ± 3.18		70.0 ± 2.56*				59.4 ± 3.88*
II experimental series							
Normal media	91.3 ± 1.32	95.0 ± 1.98*	96.2 ± 1.31*	96.9 ± 3.03*	96.7 ± 1.45*	96.7 ± 2.28*	
Media supplemented with triftazin	97.9 ± 1.03*		98.1 ± 0.94				

*—Difference from CS, $p < 0.05$.

the numerous ectopic contacts (EC) in *Drosophila* genome [64]. As it has been shown earlier, the agn^{ts3} mutation increases the frequency of ectopic contacts (FEC) around the region of its localization (11A) and in other regions of the X-chromosome (10A, 10B, 11E), as well as in 2L (33AB, 36 AB, 38E) [65]. Also, the chromosomes of the mutant are more tightly packed or condensed. ECs have been demonstrated to form at the stages of embryo and larvae II when contrast temperature treatments (37° or 15°C) lead to an increase in FEC of the wild type chromosomes to the agn^{ts3} mutant level [66]. In the present study we have demonstrated an increased FEC in all *agnostic* mutants and also in *Df(1)112/w* (Table 2). This is similar to an increased PDE1 activity observed in the heterozygotes when the normal X-chromosome is opposed with the X-chromosome which carry *Df(1)112*. Addition of CaM inhibitor triftazin to fly media (0.2%, 50 μl/ml), when flies are allowed to develop on such a media from an egg, does not affect FEC in agn^{ts3}, but increases it in the wild type strain CS.

Chromatin architecture has functional significance not only in *Drosophila*, but in humans as well. In *Drosophila* impairments of olfactory conditioning and some other behavioral performances are brought about by mutations in the *Suvar(3)6(01)* gene which control mitotic behavior of chromosomes, chromatin condensation and activity of proteinphosphatase 1 [67–69]. The region of the localization of the *agnostic* locus 11AB resides in intercalary heterochromatin [64, 67] and is the long known hot spot for rearrangement breakpoints, ectopic contacts, underreplication and increased recombination. These properties of the region allow to use it as a marker of intercalary heterochromatin and a test-system in the analysis of different cytogenetic phenomena in *Drosophila* [70]. Many sporadic human syndromes, such as Williams, Angeliman and Prader–Willi syndromes, originate as a result of a hemizygous deletion of a number of genes leading to mental retardation and defects in different types of memory [18]. If specific chromosome architecture predisposes to recombination and thereby to chromosome rearrangements leading to human disorders, can it also underlie the aforementioned impairments caused by P-element insertions and deletions in the *agnostic* locus? The answer to this question might be given by the cloning of the genomic region and its molecular-genetic characterization. The detailed data will be presented in a special paper, here we shall focus on the crucial points of our molecular-genetic studies of the *agnostic* locus.

CLONING AND MOLECULAR-GENETIC STUDY OF THE GENOMIC REGION CONTAINING THE *AGNOSTIC* LOCUS

In the beginning of the 1990s a new approach, which facilitates gene cloning while correlating genetic and cytogenetic maps and molecular-genetic structure of each polytene chromosomes division in one integrated physical map, has been developed [71]. For this, one chromosome division after another has been microdissected from a single polytene chromosome, microamplified and cloned in the Lorist 6 cosmid vector. Ordered overlapping cosmid clones

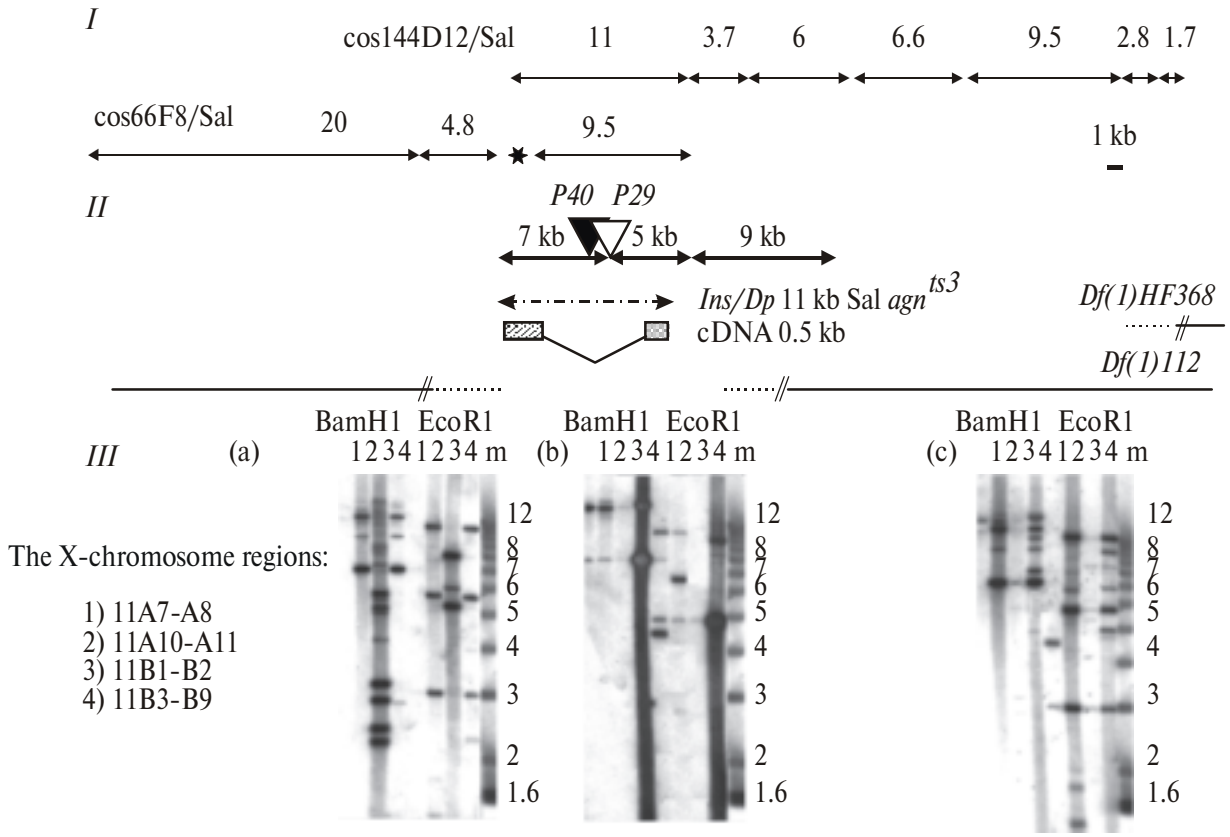


Fig. 10. Genomic structure of the X-chromosome region around the *agnostic* locus. (*I*): Alignment of cosmids cos144D12 and cos66F8 [71], spanned by deficiencies *Df(1)HF368* and *Df(1)112*, with indication of the order and the size (1 kb = one thousand base pairs) of isolated Sal1 fragments used as probes in Southern-blot analysis of the genomic DNA from the region ((*II*), (*III*)). (*II*): Arrangement of 7, 5, and 9 kb EcoRI fragments sub-cloned from cos144D12 and cos66F8 and localized by *in situ* hybridization to the X-chromosome region removed by narrow *Df(1)112*. Indicated are: the sites of P-element insertions (*black* and *white triangles*); of insertion/duplication of 11 kb Sal1-fragment in the agn^{ts3} mutant (*shaded* and *dotted line*); 0.5 kb cDNA—the only positive clone detected at screening of the Drosophila imago head cDNA library in the λEXLX(⁻) vector using genomic fragments flanking the P-element insertion *P40* as probes (*shaded rectangles*—exons, *broken line*—introns). Translated BLAST analysis of the cDNA [16] reveals homology between the region delimited by amino acid residues 2 and 46 and terminal amino acids of the Drosophila CG15221 gene that has yeast and mammalian homologs ITRB2 (integrin β2) and SV2 (mammalian synaptic Ca^{2+}-protein) [97]. *Dotted line* indicates the location of *Df(1)112* breakpoints and of proximal breakpoint of *Df(1)HF368*. (*III*): Southern-blot analysis of the X-chromosome genomic regions contained in P1-phages (numbered from 1 to 4) using as [α-dCTP–P^{32}] labeled probes distal 20 kb SalI fragment from cos66F8 (a); internal 5 kb EcoR1fragment (b); proximal to the centromere 9.5 kb SalI fragment from cos144D12 (c).

have been assembled into contigs separately for each chromosome division. We have used cosmids from the 11AB region originated by the aforementioned approach for *in situ* hybridization to polytene chromosomes when the X-chromosome has been represented by one normal homolog and one carrying *Df(1)112*. If a given cosmid has been derived from a region deleted in *Df(1)112*, hybridization signal may be observed only in the structurally normal, but not in the deleted homolog. In that way we have picked up 6 cosmids, 5 similar and one overlapping, but oppositely orientated, each containing a genomic insert about 40 kb in size. As a result, the resriction map of the 80 kb genomic region has been constructed. Figure 10 presents the sizes and restriction map position of cosmid fragments obtained after SalI digestion. These fragments have been used as probes for Southern-blot analysis of genomic DNA isolated from the *agnostic* mutants and flies which carry *Df(1)112* and *Df(1)HF368*, uncovering the *agnostic* locus. This has enabled to find the break points of short *Df(1)112* and a proximal break point of contiguous *Df(1)HF368* and

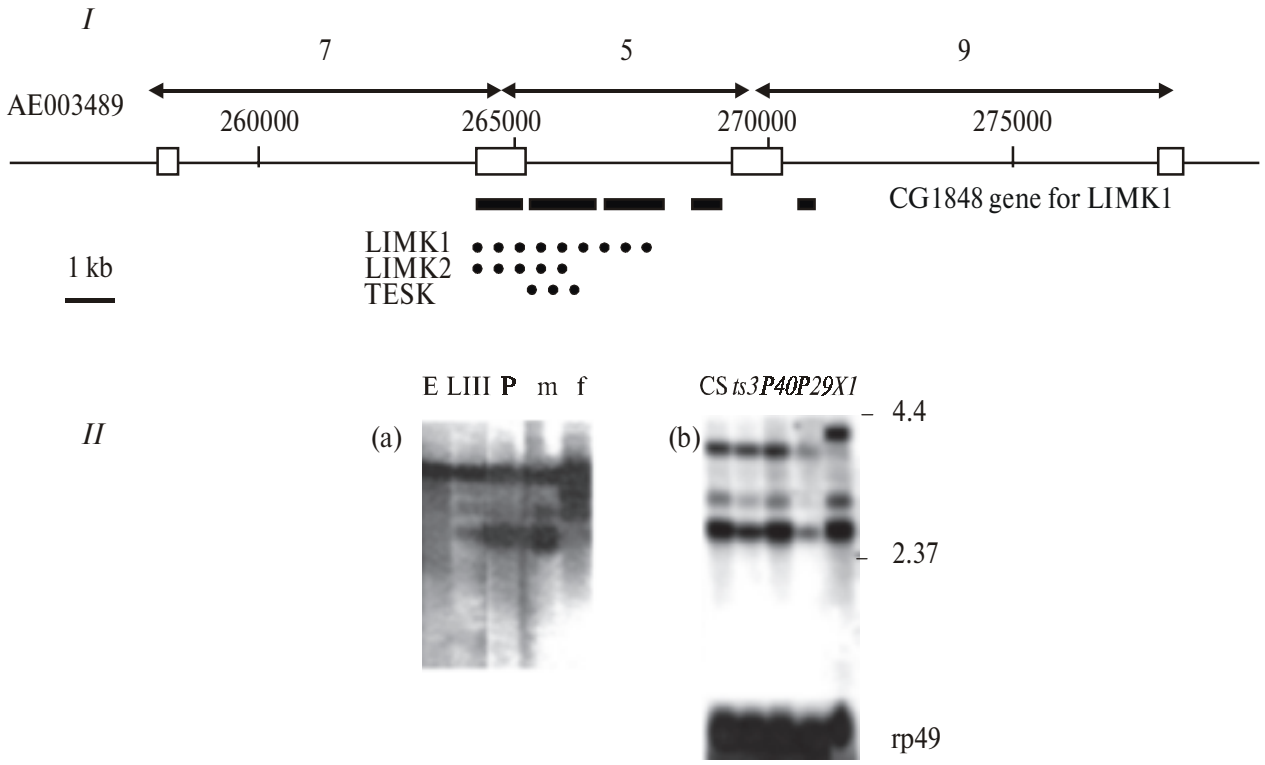


Fig. 11. Assignment of the 7, 5, and 9 kb EcoR1 fragments to the published Drosophila genomic scaffold AE003489 from the X-chromosome 11AB region containing the CG1848 gene for LIMK1. (*I*): Location of the sequenced ends (*black arrows*) of the genomic 7, 5 and 9 kb EcoR1 fragments within the published sequence [12, 16, 97] of the Drosophila genomic scaffold AE003489 (*open rectangles*). Numbers indicate the nucleotide positions in the genomic scaffold AE003489, *dark rectangles*—exons of the Drosophila CG1848 gene for LIMK1, *black circles*—alignment of amino acid sequences of the CG1848 gene product and homologous LIMK2 and TESK from different species. (*II*): Northern-blot analysis of transcripts detected by [α-dCTP–P^{32}]-labeled 5 kb EcoR1 fragment in RNA isolated from different Drosophila developmental stages (a) and from the *agnostic* locus mutants—male larvae III (b). (a) E—embryo; LIII—larvae from stage III; P—pupae; m—adult males; f—adult females; (b) figures at the right side indicate the transcript size; rp49—control for the uniformity of RNA loading monitored by reprobing the blots with rp49 cDNA.

to demonstrate that the cosmids overlap in the genomic region which predisposes to high frequency of breaks and EcoRI-fragment-length polymorphism. *In situ* hybridization to polytene chromosomes has revealed that three such adjacent sub-cloned EcoRI-fragments—7, 5 and 9 kb large—are contained in the genomic region removed by *Df(1)112,* a part of the 9 kb EcoRI fragment extending beyond the deficiency. Both the *P29* and *P40* P-elements are inserted into this polymorphic region. Southern blot analysis of genomic DNA isolated from mutants and probed with 5 kb EcoRI fragment detects a microdeletion of the fragment in *P40* mutants and an additional 11 kb SalI-fragment in agn^{ts3} resulting from either an insertion or a duplication. Almost all genomic region (80 kb) contained in the cosmids is represented by contigous repeats. This has been demonstrated while using the EcoRI-fragments and plasmid rescue fragments of both P-element insertions to probe Southern-blots of genomic DNA of P1-phage vectors which can carry large, 120 kb-long regions, dissected from polytene chromosomes [72]. Since the P1-phage vectors used contain the X-chromosome genomic material from 11A7 to 11B9, this enables to aassign each cosmid fragment to a certain polytene chromosome division. Presumably, the EcoRI-fragments reside in the genomic region 11B1-2–11B3-B9 which contains at least a part of the *agnostic* locus affected by the mutations *P29*, *P40* and agn^{ts3} (Fig. 10). Therefore, the ends of the 7, 5 and 9 kb EcoRI fragments have been sequenced and resulting 0.4–0.8 kb sequences compared to the published *Drosophila* genome sequences [12]. Results of bio-informatics analysis assign 7, 5, and 9 kb EcoRI-fragments to the X-chromosome

11AB region of the genomic scaffold AE003489 (Fig. 11). This region, removed by *Df(1)112*, contains the CG1848 gene coding for LIM-kinase 1 (LIMK1). Its homologs have been identified in different species including humans. About 40 LIM-proteins have been identified in eukaryotes. These proteins contain evolutionary conserved cysteine-rich LIM-domain having two "zinc fingers." Alternative splicing produces homologous protein kinases—LIMK1, LIMK2 and TESK (testis specific kinase). Our bio-informatics analysis reveals homology of the *agnostic* locus, predominantly of the 5 kb EcoRI-fragment, to these three protein kinases from different vertebrate species. The alignment of these protein kinases is shown in Fig. 11. LIMK1 is a component of signal transduction cascades activated by integrin signaling to Rho-family small GTPases. LIMK1 efficiently phosphorylates cofilin at Ser-3 site responsible for its actin-depolymerizing activity, and therefore, is crucial for the remodeling of the actin cytoskeleton. LIMK1 has characteristic structural features composed of two LIM domains and a PDZ domain at the N-terminus and a protein kinase domain for serine-threonine kinases at C-terminus. In humans, chromosomal localization of LIMK1 is assigned to 7q11.23 and its hemisizygotic deletion is linked to impairments of visuospatial constructive cognition in patients with Williams syndrome which is also associated with other multiple manifestations. The deletion spans 150 kb and includes about 17 genes. Large repeats containing genes and pseudogenes flank the deletion breakpoints and mutation mechanism commonly appears to be unequal meiotic recombination [73, 74]. Figure 11 demonstrates the results of Northern-analysis when 5 kb EcoRI-fragment, containing the largest part of the genomic sequence for LIMK1, is used to probe RNA isolated from different developmental stages of *Drosophila* (Fig. 11a) and from the *agnostic* mutants (Fig. 11b). This probe detects three transcripts—one large (3.7 kb), which is present at all developmental stages, and two smaller transcripts which appear at the larval stage III and increase in size in adult females. The *agnostic* mutants (larvae III, males) demonstrate different expression patterns of these three transcripts. Functional significance of this observation is still obscure. At the same time, human 3.3 kb mRNA for LIMK1 from 7q11.23 is expressed in all tissues, but predominantly in the brain. Human LIMK2 (22q12) is represented by two isoforms—3.7 and 1.6 kb, the small isoform being expressed only in skeletal muscles [75]. The *Drosophila* gene for LIMK1 (CG1848) spans about 6.7 kb and has 6 exons. Two isoforms—short and long—are derived by alternative splicing [76]. However, there are no data on developmental and tissue expression patterns of the gene. At the same time, in vertebrates as demonstrated in the rat, LIMK1 is most intensively expressed within neurons and is concentrated at presynaptic terminals of neuromuscular synapses after 2nd week after birth and coincides with synapses maturation [77]. Different LIM–kinase isoforms with deletions of either LIM- or PDZ domains show tissue- and stage-specific expression patterns, in particular, in male germ cells they are involved in spermatogenesis, especially in meiotic and/or postmeiotic processes [78]. It is noteworthy that genomic sequence adjacent to the *P40* insertion, shows homology both to human genomic sequence from 7q11 and to the *Drosophila* sisA gene [79]. This gene participates in the complex of sex determination and gene dosage compensation. It should be reminded that the *agnostic* locus does not show gene dosage compensation [40]. However, genomic sequences close to P-element insertions *P29* and *P40*, derived by plasmid rescue, show homology to intron (non-coding) part of the *Drosophila inaF* gene (the data are not shown). This gene codes for INAF protein regulating TRP Ca^{2+}channels, but its reported localization (10C2-E3) is somewhat distant from that of the region studied [80].

DISCUSSION

Pleiotropy, which is exemplified by differently manifested mutations at the same locus, is a common feature of behavioral genes [81]. This is also true for the *agnostic* locus, and numerous phenotypic manifestations of mutations at the locus are caused by two reasons: by complex organization of biochemical cascades in which participates the product of the locus, and by specific chromosome architecture of the region of its genomic localization. Recently, the advances of the human genome project and the completion of total genome sequences for many species, have enabled investigators to view genetic information in the context of the entire genome. As a result, it is recognized that the mechanisms for some genetic diseases are best understood at a genomic level. The evolution of the mammalian genome has resulted in the dupli-

cation of genes, gene segments and in origination of repeat gene clusters (direct and inverted repeats). This genome architecture provides substrates for homologous recombination between nonsyntenic regions of chromosomes. Such events can result in DNA rearrangements that cause disease, in particular, human syndromes with multiple manifestations, termed as genomic disorders. Characterization of various genomic disorders showed that they can be classified into three groups. In the first well-characterized group, homologous recombination between the small duplicons generates an imbalance of a dosage-sensitive gene, leading to a disease phenotype. The other two groups of genomic disorders involve rearrangement of a large segment of the chromosome, yet the flanking duplicons are structurally simple in the second group, whereas the third group of disorders involves complex duplicons [18]. Among the simple duplicons mediated genomic disorders of the second group are peripheral neuropathies, hemophilia A and neurofibromatosis Type1. Genomic disorders of the third group arise from chromosome rearrangements involving complex duplicons**.** Among such disorders are deletion Williams syndrome (WS) 7q11.23, deletion–duplication syndrome 17p11.2, DiGeorge and velocardiofacial syndromes (VCFS) 22q11, etc. The rearranged DNA segments usually span several megabases and are flanked by large duplicons that often contain several genes and/or pseudogenes. Multiple copies are present both at the common breakpoint regions and elsewhere within the same subchromosomal region. As the duplicons are large in size, have high homology, and multiple copies, thus leading to genome instability, the mapping and cloning of regions within these duplicons has proven to be difficult [82–84]. However, the architecture of such regions may result in predisposition not only to deletions, but may also cause translocations and inversions: the inversion is hemizygous in 27% atypical affected individuals who show a subset of the WS phenotypic spectrum but do not carry the typical deletion. In 33% families with a proband carrying the WS deletion, the inversion was exclusively observed in the parent transmitting the disease-related chromosome [83, 85].

Williams syndrome is considered as a most compelling model of human cognition, of human genome organization, and of evolution [83]. WS is also associated with such multiple manifestations as distinctive facial appearance, cardiac abnormalities, infantile hypercalcemia, and growth and developmental retardation presumably due to hemizygosity at the gene loci for elastin (ELN), synaptic Ca^{2+}-protein syntaxin (STX1A) and LIMK1. LIMK1 hemizygosity may contribute to characteristic uneven cognitive-linguistic profile together with mild to severe mental retardation (low IQ), extraordinary musical talent alongside with severe impairments of visuospatial constructive cognition [73, 74]. In each patient hemizygosity of different length arises as a result of 7q11.23 chromosome rearrangements involving hot spots of chromosomal breaks. Complex study of each WS patient helps to generate an integrated physical, genetic and transcriptional map of "cognitive impairments" in the locus and flanking regions.

The *agnostic* locus may be viewed as a model for WS with such an exception that this locus containing *Drosophila* gene for LIMK1 is located in highly repetitive and unstable region not of the autosomes, as in humans, but of the X-chromosome. Therefore, deletion hemizygosity at the locus leads to lethality. At the same time, when the deleted X-chromosome is opposed against the normal X-chromosome, the flies of this genotype can manifest many of mutant phenotypes. Of course, molecular nature of the *agnostic* mutations needs a more detailed study, but the data obtained present a working model for future experiments. It seems plausible that the ts-mutation agn^{ts3} is not a single-gene point mutation, but rather a rearrangement mutation that results from an increase in recombination in 11A, which has been documented for EMS effects [86]. This might lead to either an insertion or duplication detected as 11 kb SalI-fragment. Therefore, it is not surprising that standard genetic mapping procedures reveal 3-fold map expansion around the agn^{ts3} mutation, usually considered to be indicative of a duplication [39]. Presumably, the agn^{X1} mutation has the same recombination origin: the genomic region which contains the *agnostic* locus is a part of Kossikov duplication showing homology between 11A and 12D, an also between 11B and 12E [87]. The effects of the both mutations on the recombination in the region [40–42] have allowed to develop a model for unequal recombination in alternating repeats [40]. P-insertional mutations *P40* and *P29* uncovered by the *agnostic* locus deletion *Df(1)112* and detected on Southern-blots probed with 5 kb EcoRI fragment which contains LIMK1 gene, may result

from inversions or translocations known to be produced by P-element insertions. Since the plasmid rescue genomic material of *P29* and *P40* shows homology to intron (non-coding) part of the *Drosophila inaF* gene, the mutations may result, for instance, from recombination between the normal *inaF* gene and an *inaF* pseudogene, still unidentified due to complexity of the 11AB X-chromosome region. Since the gene product, INAF protein, is a regulator of TRP-channels, and therefore, is involved in complexes of signal transduction formed due to protein–protein interactions of structurally similar regions, this similarity at nucleotide level can predispose recombinational generation of chromosome rearrangements.

The finding of the modular organization of signal transduction systems exemplified by such a close interaction of its components in phototransduction in *Drosophila* that the *inaF* mutants more closely resemble *trp* in electrophysiological phenotypes than any other mutant identified to date [80], has lead to identification of 7 mammalian homologs of TRP channels. Intensive study of TRP functioning in different species uncovers their role in ever-growing number of processes, including olfaction, pain perception and male aggression [88]. It is assumed that still not fully understood phenomenon of SOCE implies the following mechanisms. Ca^{2+} release from internal stores and the consequent influx from external space are important steps of Ca^{2+} signaling that follows the stimulation of phospholipase C. Ca^{2+} release is triggered by the binding of inositol 1,4,5-trisphosphate (IP_3) to IP_3 receptors (IP_3Rs), and the depletion of Ca^{2+} from the stores in turn activates Ca^{2+} influx via numerous Ca^{2+} channels, in particular TRPs. Presumably, their heteromultimeric complexes form a pore for Ca^{2+} entry during SOCE. *Drosophila* TRP and TRPL (TRP-like isolated in a screen for calmodulin-binding proteins) channels, which participate in such complexes, are regulated by CaM. Two CaM-binding sites have been localized on the C terminus of TRPL, one of which overlaps with the IP_3R binding domain. TRP channels are activated by a Trp-binding peptide from IP_3R that displaces CaM from CaM/IP_3R common binding site, and liberated CaM becomes capable of activating other Ca^{2+}/CaM proteins. This could be registered using an assay for PDE1 activity, a standard procedure for monitoring CaM-activating functions. This mechanism has been studied in detail while analyzing functions of human Trp3 channel [89]. A surprising variety of ion channels found in a wide range of species from *Homo* to *Paramecium* use CaM as their constitutive or dissociable Ca^{2+}-sensing subunits. Our understanding of CaM chemistry and its relation to enzymes has been instructive in channel research, yet the intense study of CaM regulation of ion channels has also revealed unexpected CaM chemistry. The findings on CaM channel interactions have indicated the existence of secondary interaction sites in addition to the primary CaM-binding peptides and the functional differences between the N- and C-lobes of CaM [90]. At the same time, normal functioning of ion channels, including NMDA and AMPA receptors, requires simultaneous interaction both with CaM and with the actin cytoskeleton [91], which is mediated by PDZ domains of postsynaptic density [92]. In the light of these findings the results of the study on the *agnostic* locus mutants acquires a new meaning. The main manifestation of the *agnostic* mutations, the increased activity of Ca^{2+}/CaM-dependent PDE1, may result from CaM displacement by IP_3R from CaM/IP_3R common binding site of TRP and TRPL-channels of the mutants, thus indicating that these Ca^{2+}-channels may be constantly active. The actin cytoskeleton plays an inhibitory role in SOCE preventing coupling of endoplasmic reticulum and of plasma membrane. Remodeling of the actin cytoskeleton, which requires 40 min [26], depends in its turn on LIMK1 activity. Disruption of the second LIM domain or the PDZ domain or deletion of the entire amino terminus leads to an increase in LIMK1 activity and, thereby, to an increased phosphorylation of cofilin and increased actin aggregation [93]. Since the mutant phenotypes are either reproduced or mimicked when the *Df(1)112*-bearing X-chromosome is in a heterozygote with the mutant or normal X-chromosome, correspondingly, it can be concluded that all *agnostic* mutations affecting LIMK1 activity, alter the actin polymerization [54]. It is believed that LIMK1, LIMK2 and TESK phosphorylate the actin-depolimerizing protein cofilin but being differently regulated, control different functions of the actin cytoskeleton [94, 95]. Among these functions is the neurites outgrowth and the forming of the structures of the brain during its development. The fact that only two *agnostic* locus mutants—*P29* and agn^{X1} demonstrates defects in the noduli of the central complex of the brain, is probably explained by differential regulation of these closely

related protein kinases by Rho family small GTPases: LIMK1 is activated by Rac and LIMK2 is activated by Rho and Cdc42 [94]. In their turn, Rac, Rho and Cdc42 are down-stream effectors of integrin signaling, regulating all three LIM-kinases [95]. It has been demonstrated that *Drosophila* mutations affecting integrin signaling are involved in the control of learning and memory [96]. Therefore, it is not surprising that each of the *agnostic* locus mutants has a characteristic defect in learning and memory, which is most drastically manifested in the *P40* mutant. According to our unpublished data, cDNA derived from the genomic region of the *P40* insertion contains a part of the *Drosophila* gene CG15221, which is homologous to mammalian beta2-integrins and synaptic Ca^{2+}-regulating protein SV2B [97, preliminary data]. It is worth reminding that a gene for synaptic Ca^{2+}-protein syntaxin functionally related to SV2B and involved in regulation of exocytosis, falls within WS deletion [98]. Also, the mutations of the nematode syntaxin gene affect the ether resistance [52]. Time duration of the actin cytoskeleton remodeling during SOCE can probably explain why manifestations of the *agnostic* locus mutation need 40 min at 29°C for pronounced development and why temperature treatments can lead in some mutants to memory improvements. WS patients demonstrate uneven cognitive-linguistic profile and mild to severe mental retardation alongside with severe impairments in visuospatial constructive cognition. Such visuospatial defect, originally documented for agn^{X1} [57], is characteristic for each of the *agnostic* mutants and their heterozygotes with *Df(1)112* and is very severe in *P40* mutants (R. Strauss, personal communication). The influence of the *agnostic* locus mutations on the parameters of sound production is in line with the observation that mutations affecting ion-channel function in *D. melanogaster* are often associated with temperature sensitivity, paralysis, and altered love song. Among these mutations are *Shaker*, $para^{ts1}$, nap^{ts1}, *comatose*, *cacophony (cac)* [61, 62]. *cac* is a mutation in the gene for α1 subunit of voltage-sensitive Ca^{2+}-channels (Dmca1A) which are hetero-oligomeric assemblies of α1, α2, δ, β- and γ-subunits. Channel diversity is generated by multiple genes, alternative splicing of transcripts from a given gene, and perhaps by combinatorial assembly of variant isoforms of the subunits. The *cac* locus was mapped cytogenetically using inversions and deletions to the X chromosomal region 11A2 and the Dmca1A transcript spans distal breakpoint associated with *Df(1)HF368* [63]. This deletion have been also used for mapping the *agnostic* locus which appears to be located quite near to the opposite, proximal breakpoint of *Df(1)HF368*.

It is believed that LIMK1 may play an important role in the cell cycle progression through regulation of the actin cytoskeletal rearrangements [99]. Recent work has begun to provide evidence for important roles for actin in a number of nuclear processes, ranging from chromatin packaging at apoptosis to dynamic chromatin remodeling and its spatial organization in the nuclei. Particular chromatin structures may nucleate domains that are permissive or restrictive of transcription, to which active or inactive loci could be recruited, thus forming a structural basis of epigenetic phenomena, such as the inheritance of a "cellular memory" of gene expression status [100, 101]. This might result in more tight chromosome packaging in the *agnostic* locus mutants and their increased frequency of ectopic contacts in certain genome regions. It is noteworthy that such regions of the X-chromosome, for example 10B, harbor genes which contain PDZ domains mediating multiple protein–protein interactions. These genes might be located in the regions of ectopic contacts implicated in chromatin remodeling for rapid and efficient transcriptional regulation. For example, *Disc large* gene, first shown to contain PDZ domain, maps to 10B7-8, the *dishevelled* gene involved in the control of Wnt-frizzeled signal cascades maps to 10B4-5 [97]. Among the Williams syndrome deletion hemizygous genes is the homolog of the *Drosophila* gene *frizzeled* and the WBSCR11 gene for a transcriptional factor involved in chromatin remodeling in eukaryotes [102].

To summarize, the *agnostic* locus mutants manifest multiple phenotypes depending on a level of a given study—biochemical, morphological, behavioral and cytogenetical. The complex of these manifestations results from genetic alterations in the genomic locus containing the *Drosophila* gene for LIMK1 in agreement with multiple cellular functions documented for LIMK1-dependent regulation of the actin cytoskeleton reorganizations. Naturally, each of these multiple functions has been elucidated using modern approaches and artificial experimental systems at cellular level. These observations await their confirmation at the level of an organism [103] and this might be enabled by the *agnostic* locus mutants. Moreover, the

agnostic locus mutants may be considered as an unique model for Williams syndrome, i.e., for coupling genomic structure and cognition [83].

ACKNOWLEDGMENTS

The work was supported by a contract with the Russian Federation Ministry of Industry and Sciences "Genetics of development, biological protection, and behavior," project no. 43.073.1.1.2507 (E.V. Savvateeva-Popova, N.G. Kamyshev), Volkswagen–Stiftung, Germany (E.V. Savvateeva-Popova), and the Russian Foundation for Basic Research (no. 01-04-48847, E.V. Savvateeva-Popova; no. 01-04-49574, A.V. Popov; no. 02-04-48502, N.G. Kamyshev).

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