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GENERAL GENETICS

Novel Memory Mutants in *Drosophila*: Behavioral Characteristics of the P-Insertional Mutant *Ent2* P124

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Abstract—The *Drosophila* mutant P124, carrying insertion of P(*lacW*)vector in the second chromosome on the background of the *w* mutation in the X chromosome, was previously isolated as showing memory deficiency after courtship conditioning. Here we report additional features of the mutant phenotype: (1) abnormal adaptation to dark-light transition; (2) impaired perception by males of nuances of visual image of a female, specifically, of her fine movements such as preening and ovipositor extrusion; (3) subnormal performance index after odor-shock conditioning. These abnormalities are not related to a deficit of the eye screening pigments because they are also revealed on the background of the normal w^+ allele in the X chromosome. The visual and cognitive impairments are independent pleiotropic effects of the mutation. The mutation is caused by insertion of the P vector 12 nucleotides upstream of the transcription start of the gene *Ent2* (*equilibrative nucleoside transporter 2*). $Ent2^{P124}$ is the second mutant allele of this gene found in *Drosophila*. The genetic variability in *Ent2* locus should be considered as a factor of risk for development of mental retardation and disorders of visual perception.

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INTRODUCTION

The present study is performed by pupils of pupils of M. E. Lobashev, who have been working in the laboratory he had established, and is dedicated to his centenary.

Mikhail E. Lobashev is known not only as the scientist and the teacher who devoted himself to revival of genetics in our country and created the Department of Genetics in the Leningrad State University over again, but also as the founder of Laboratory of Lower Animals in the Pavlov Institute of Physiology and Pathology of Higher Nervous Activity of the Academy of Medical Sciences of the Soviet Union (now the Laboratory of Comparative Behavioral Genetics at the Pavlov Institute of Physiology, Russian Academy of Sciences). Being invited by the director of the institute, Academician Leon A. Orbeli, he headed the laboratory in 1950 and up to the end of his life stayed its informal leader, despite the fact that in 1957 he left the institute to become the head of Department of Genetics at the university. Owing to efforts of Lobashev, the genetic trend in physiological research received a power stimulus for further development in the institute, involving now already three genetic laboratories.

Drosophila, as a model genetic organism, has been successfully used to reveal molecular mechanisms of learning and memory [1–3]. Earlier, using the approach of forward genetics to this problem, we have isolated several P insertional mutants showing memory deficiency after courtship conditioning [4, 5], a form of classical conditioning [6, 7]. At present, one of these mutants, $nemy^{P153}$, has been characterized in most detail [8]. The mutation affects one of the splice variants of the gene *CG8776* coding for cytochrome b561 (carbon-monoxide oxygenase). In the present paper, we characterize another mutation from the same screening, P124. As it turned out, it affects the known gene *Ent2*—*equilibrative nucleoside transporter 2*.

MATERIALS AND METHODS

Drosophila Stocks

The outbred wild-type strain Canton-S (C-S) and the line P124, isolated earlier as showing memory deficiency after courtship conditioning [4], were used. The line P124 carried the insertion of P(*lacW*) vector in the second chromosome and mutation *white* (*w*) in the X chromosome. The P(*lacW*) vector (below referred to as the P vector) is a nonautonomous P element containing: (1) the marker *mini-white*$^+$ sequence making the eyes colored on the background of the *w* mutation in the X chromosome; (2) the reporter gene *lacZ* and (3) the gene of resistance to ampicillin, *ampR* [9]. For mutagenesis, transposition of P vector from the site of its initial localization in the X chromosome into one of the autosomes was induced by its combination for one generation with the source of transposase [10]. The

mutagenesis scheme [4] was analogous to that used earlier by other authors [11]. Prior to the experiments described, the initial mutant line P124 was equalized in genetic background with the C-S strain. First, ten cycles of crossing the *w* mutant line with C-S strain were performed. Each cycle consisted of crossing the w males with C-S females, further crossing the heterozygous *w*/+ females with C-S males and selection of white-eyed *w* males. Then males of this congenic *w*(C-S) line were used for tenfold crossing with *w*/*w*; P124/+ females, with selection of colored-eyed females in each generation. In addition, with use of balancer chromosomes and further analyzing crosses, P124w$^+$ line was created, which carried the P124 insertion in the second chromosome and the w^+ allele in the X chromosome derived from the C-S strain.

Courtship Behavior Monitoring

Two approaches were used to register the courtship behavior: (1) visual observation of behavior of a courting male with simultaneous computer registration of male ethogram; (2) video recording of a courting pair with further computer registration and analysis of conjugate male and female ethograms. In both cases, the registration was performed for 300 s. The computer program fixed the sequence of the moments of initiation of behavioral elements performed by a male or a female. All registration and analyzing modules were written by N. Kamyshev using Delphi 7 Pro. The integral parameter of courtship intensity is the courtship index (CI), i.e., the percentage of time occupied by courtship including all its elements. Usually, it is a percentage of the total time of observing the male behavior. However, in this study we use also the instant CI calculated for the very restricted time interval prior or after a certain event.

Courtship Conditioning

In the context of this study, the courtship conditioning should be understood in its initial classic definition. After experience in courting the unreceptive fertilized female, a male reduces intensity of courtship of any subsequent female, virgin or fertilized [6]. It is considered to be a form of conditioning when after pairing of the courtship-stimulating stimuli, common to all females, with aversive stimuli, released only by fertilized females, the former become less attractive [7, 12]. One may test memory after courtship conditioning in two ways, by presenting to a male either a virgin female, lacking the aversive stimuli (test for memory retention), or another fertilized female (retraining test). In the latter test, good memory performance lasts longer [7]. In this study, only the retraining test was used.

Training and sham-training. Experimental males were collected for 1–2 h after eclosion from pupae without any anesthesia. For 5 days they were reared individually under 12 : 12 light-dark cycle. On the sixth day they were subject either to training or sham training under the two conditions: light and dark. For training, a male was placed into the Plexiglas experimental chamber (15 mm diameter, 5 mm high), and the wild-type C-S female, fertilized a day before, was added to the same chamber. A male was allowed to court a fertilized female for 30 min. For sham training, a male was placed into the chamber for the same time and under the same conditions, but without a female.

Performance index. Performance index (PI), also known as the learning index, was calculated by formula [13]:

$$PI = \frac{CI_N - CI_T}{CI_N} = 1 - \frac{CI_T}{CI_N},$$

where CI_N is the courtship index of naive males previously subject to sham training, and CI_T is the courtship index of males previously trained to court a fertilized female. PI is equal to 1 when training results in complete inhibition of courtship, and is equal to 0 when no courtship inhibition takes place (no learning).

Odor-Shock Conditioning

The odor-shock conditioning was performed as described earlier by other authors [14]. The odors of 3-octanol and 4-methylcyclohexanol were used. During training of a group of flies in a special apparatus, presentation of one odor was paired with electric shock, while presentation of the other odor was not. To test memory, the flies were allowed to choose between the two odors by moving into one of the opposite arms of the apparatus. In the two halves of each experiment the odor paired with electric shock and the unpaired odor alternated. Performance index (PI) was calculated for each half as a percent of flies choosing the unpaired odor minus the percent of flies choosing the odor paired with shock. The final PI resulted from averaging the two values. PI equal to zero corresponds to random distribution of flies. PI equal to 100% corresponds to the right choice of the unpaired odor by all flies. To estimate odor sensitivity, the odor avoidance was tested by allowing the flies to choose between the odor and the pure air. To estimate sensitivity to electric shock, the flies were allowed to choose between two electrified arms of the apparatus with only one of them connected to the electricity supply. In both cases, the avoidance index (AI) was calculated as a percent of flies avoiding the odor (or electric shock) minus the percent of flies making the opposite choice. AI equal to zero corresponds to random distribution of flies, AI equal to 100% corresponds to complete avoidance of the odor (or shock) by all flies.

Statistical Analysis

When analyzing the data concerning courtship conditioning, comparison of two PIs seems to be a complicated statistical task because it involves four rather than

two independent data samples. Because of this, the so-called randomization test, based on direct computing the probability of rejecting the null hypothesis, was used for this aim. The randomization test is a variety of the Monte-Carlo method [15]. The rationale for its usage were discussed earlier [7]. The necessary computer program was developed by N. Kamyshev. The test was also used to compare CIs.

To analyze the data based on video recording of courtship behavior, the paired *t* test for dependent samples and nonparametric Mann–Whitney test were used.

The data concerning the odor-shock conditioning were processed with *t* test for independent samples.

Localization of the P Vector Insertion Site in the P124 Line

To localize position of the inserted P vector in the second chromosome, the standard plasmid rescue technique was used [16]. The method is based on selective survival of only those bacterial cells, which carry plasmids with a part of P vector containing the gene of resistance to ampicillin flanked with a fragment of genomic DNA. The genomic DNA was isolated from the homozygous P124 inserts, cut with restriction endonuclease *Sac*II, ligated and electroporated into DH5a cells of *Escherichia coli.* The cells were seeded on the ampicillin-containing medium, and the resistant colonies were selected. The flanking fragment of genomic DNA was amplified using known sequence from the end of P vector as a primer, sequenced, and searched for in the annotated database of *Drosophila* genome (http://flybase.net/).

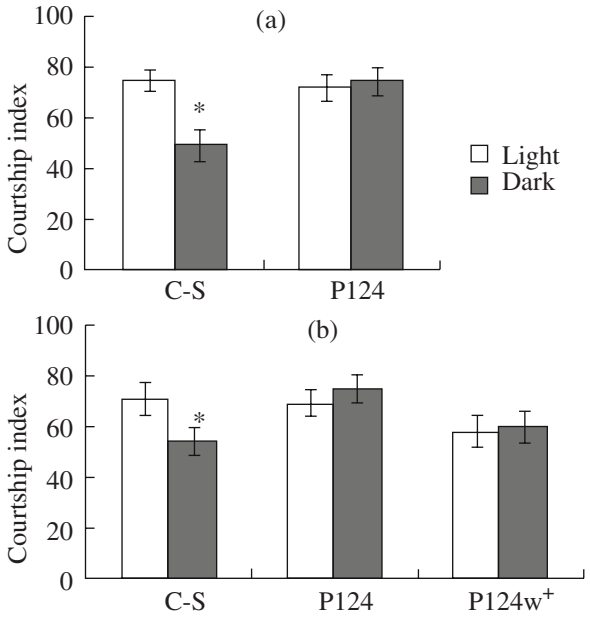

Fig. 1. Decrease in courtship intensity resulted from previous sojourn in the dark in the wild-type Canton-S (C-S) males and the absence of this reaction in the mutant P124 and P124w$^+$ males. Prior to the test on light, the sham training was performed: naive males were placed individually into experimental chambers for 30 minutes either under light or dark conditions. The experiments were carried out in 2002 (a) and in 2004 (b). Light, dark: conditions of sham training. Means with standard errors are shown. * Difference between the two conditions of sham training is significant (two-sided randomization test, $P < 0.05$). Visual registration.

RESULTS

Earlier it was shown that males of the line P124 carrying insertion of the P vector in the second chromosome revealed memory deficiency after courtship conditioning [4, 5]. However, this line also carries a *w* mutation in the X chromosome. The eye coloration is caused here by presence of the *mini-white*$^+$ sequence in the P vector with variable expression depending on the site of the P insertion. In P124 flies, color saturation of eyes does not reach the level of the wild type. To isolate the effects of possible visual defect caused by a deficit of eye screening pigments in P124 males, we created the line P124w$^+$ carrying the P insertion in the second chromosome and the normal w^+ allele in the X chromosome. As at that time we were very interested in investigating possible participation of visual stimuli in the courtship conditioning, we used the retraining test under light conditions to compare memory performance immediately after light or dark training in all three lines.

The first effect of P124 insertion, which was found, had no relation to conditioning but concerned behavior of the control naive (having no previous experience of courtship) males subjected to sham training (see Materials and Methods). In males of the wild-type strain C-S, the dark sham training led to significant decrease in courtship intensity in comparison to the light sham training (Fig. 1). Thus, for normal males after their sojourn in the dark, some time is required to adapt to conditions of the light test. Males that were not subject to previous visual deprivation court females more intensely because of a stronger stimulation by a visual image of a female. Males of all P insertional lines isolated by us as showing memory deficiency after courtship conditioning (*nemy*^{P153}, P171, P95) behaved similarly to the wild type (data not shown) with the exception of P124 males showing no difference in CI after sham training in the dark and on the light (Fig. 1). This fact points to impairment of some physiological process related to light adaptation, suggesting a visual defect in the mutant. The abnormality is due to the insertion of P vector, and not to a deficit of eye screening pigments, because it also exists in P124w$^+$ males.

In contrast to earlier data [5], obtained with the P124 line just after making it congenic with the wild-type strain C-S, in the series of experiments made in 2002 and 2004, the performance index of P124 males in the immediate retraining test after light training was significantly above zero and did not differ from PI of C-S

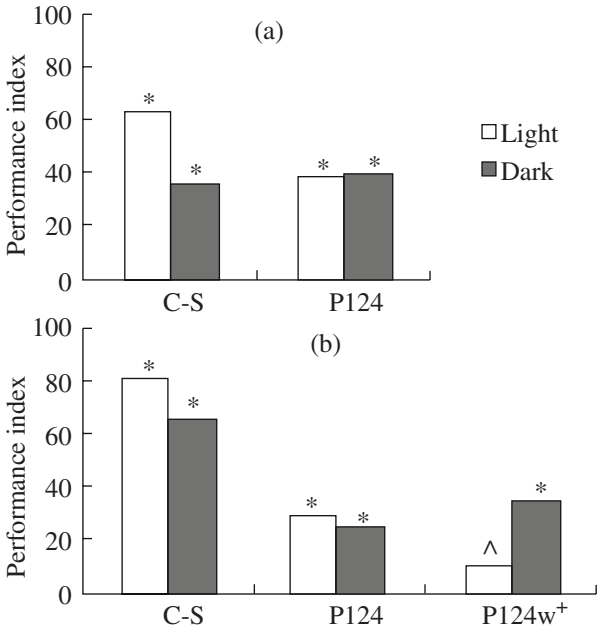

Fig. 2. Memory performance in the immediate retraining test after courtship conditioning in the wild-type males C-S and in the mutant males P124 and P124w$^+$. The experiments were carried out in 2002 (a) and in 2004 (b). Light, dark: conditions of training. * Performance index (PI) is significantly above zero (one-sided randomization test, $P < 0.05$). ^ PI significantly differs from that of C-S strain (two-sided randomization test, $P < 0.05$). Visual registration.

males (Fig. 2). That seems to be a result of selection with time of the genes modifying the mutational defect towards the norm. This phenomenon is widely known [17]. Replacement of the X chromosome, carrying the *w* mutation, by an X chromosome, carrying the w^+ allele, and related to this process renewal of genotypic environment resulted in that in the same test P124w$^+$ males showed PI, which did not significantly differ from zero and significantly differed from PI of C-S males (Fig. 2b). Firstly, this evidences in favor of P insertion, and not a deficit of eye screening pigments, as a cause of memory deficiency in P124 males. Secondly, this supports the supposition that memory deficiency in the mutant males is influenced by genes-modifiers.

Changes of the instant courtship index in naive males resulted from execution of certain actions by a female

Female's action	Genotype of male	
	C-S	P124w$^+$
Ovipositor extrusion coupled with locomotion	–	0
Rest in the "face-to-face" attitude	++++	0
Forelegs preening directed to a male	+++	0

Notes: The instant (measured for 3 s) courtship index was estimated prior to and after the execution of a given action by a female. Comparison of the two CIs was performed by paired *t* test ($P < 0.05$). – significant decrease of CI up to 10%; +++ significant increase of CI up to 30%; ++++ significant increase of CI by more than 30%; 0, nonsignificant. Analysis of video records.

In P124w$^+$ males, PI significantly exceeded zero after dark training and did not differ from zero after training on light (Fig. 2b). Thus, P124 insertion causes more severe memory deficiency after light training than after dark training. This might mean that light training includes vision-dependent processes impaired by P124 mutation and that just these processes are influenced by genes-modifiers, which are accumulated in P124 line leading to absence of difference between PIs after light and dark training (Fig. 2).

In a special series of experiments, which included video recording of a courting pair and further analysis of conjugate male and female ethograms, it was found that naive wild-type males responded to certain actions of a female, such as the "face-to-face" attitude and the forelegs preening directed to a male, by intensification of courtship. These female actions, which, entirely or partly, were perceived by a male through vision, provoked no response from the mutant naive males (table). The ovipositor extrusion by a moving female provoked a moderate decrease of courtship intensity in the pursued her wild-type male, but did not affect behavior of the mutant males (table).

From the very beginning of training, the wild-type males decreased the intensity of courtship after the attempt to copulate (Fig. 3). However, significant reduction in the instant CI took place only when a female extruded her ovipositor during the attempted copulation. At the end of training and in the immediate test with another fertilized female, a drop in courtship intensity after attempted copulation became more pronounced, and significant difference was observed between cases when a female extruded her ovipositor and when she did not. The mutant males, at the beginning of training, did not decrease the intensity of their courtship after attempted copulation (Fig. 3). This response had been developed only after training and was observed in the retraining test, only if during the attempted copulation a female extruded her ovipositor. On the other hand, ovipositor extrusion during the attempted copulation led to essential extension of the attempt duration in the mutant males (Fig. 4). In the wild-type males, this response was absent. Thus, ovipositor extrusion during the attempted copulation produces through stimuli of different modality two effects on male's will to court: the inhibitory (manifested as a decrease in courtship intensity after termination of the attempted copulation) and the stimulatory (manifested as prolongation of the attempted copulation). It seems that in the wild-type males, both effects are present, but the inhibitory effect predominates from the very beginning and strengthens at the end and after training (Fig. 3). In the mutant males, the inhibitory effect is absent (Fig. 3)

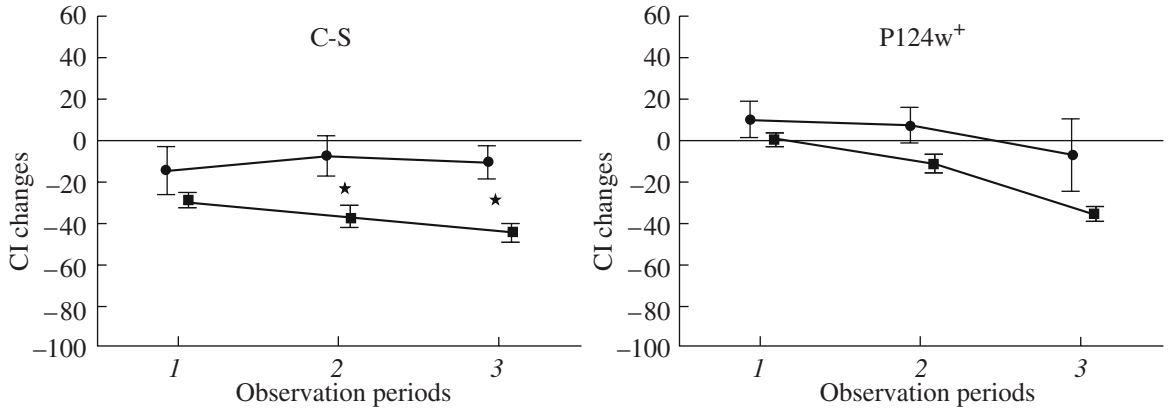

Fig. 3. Effect of ovipositor extrusion on change in courtship intensity resulted from attempt to copulate in the wild-type and mutant males. Abscissa, successive periods of observation: (*1*) the first 5 minutes of 30-min experience in courting a fertilized female (training); (*2*) the last 5 minutes of training; (*3*) the immediate test with another fertilized female. Ordinate, change of the instant (measured for 3 s) courtship index (CI) resulted from execution by a male of attempted copulation (AC): CI after AC minus CI prior to AC. Means with standard errors are shown. * Significant effect of the ovipositor extrusion (two-sided Mann–Whitney test, $P < 0.05$). Analysis of video records. ● The female did not extrude ovipositor during the copulation attempt; ■ the female extruded ovipositor during the copulation attempt.

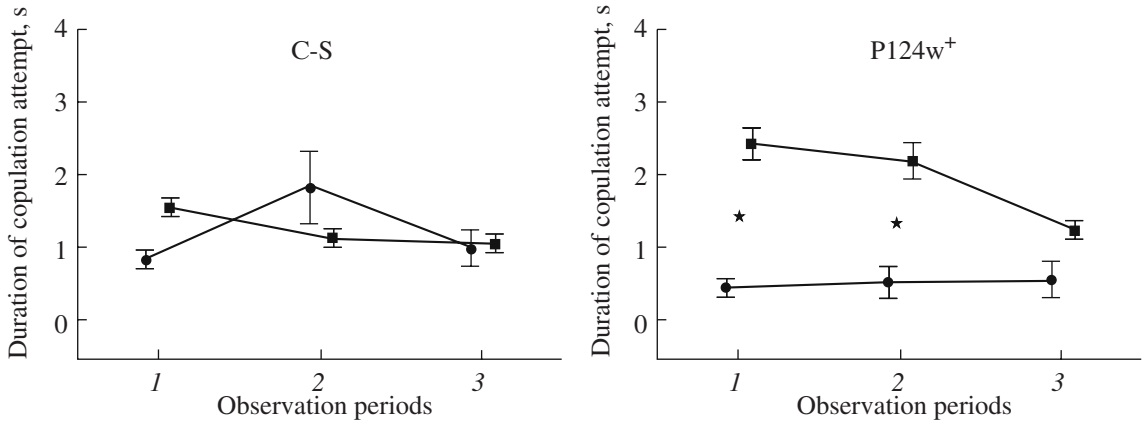

Fig. 4. Effect of ovipositor extrusion on duration of the attempted copulation in normal and mutant males. Abscissa, successive periods of observation: (*1*) the first 5 minutes of 30-min experience in courting a fertilized female (training); (*2*) the last 5 minutes of training; (*3*) the immediate test with another fertilized female. Means with standard errors are shown. * Significant effect of the ovipositor extrusion (two-sided Mann–Whitney test, $P < 0.05$). Analysis of video records. ● The female did not extrude ovipositor during the copulation attempt; ■ the female extruded ovipositor during the copulation attempt.

that allows the stimulatory effect to become fully apparent at the beginning of training with its further slackening after training (Fig. 4).

The ability to learn and remember in flies of P124 line was also examined using the odor-shock conditioning. From the moment of training completion and for five next hours, the mutants demonstrated the performance index significantly decreased by approximately 20% in comparison to the wild type (Fig. 5a). At the same time, neither the sensitivity to the odors used (Fig. 5b) nor the sensitivity to the electric shock (Fig. 5c) were altered in the mutants.

Cloning the fragment of genomic DNA, flanking the P vector insertion in P124 line, showed the exact localization of the P insertion: 12 nucleotides upstream of the transcription start of the gene *Ent2*, coding for a protein, equilibrative nucleoside transporter 2.

DISCUSSION

Insertion of the P vector into the second chromosome causes memory deficiency after courtship conditioning in males of P124 line [4, 5]. In the retraining test under conditions of training used, memory lasts for at least 8 h in the wild-type males [7]. In the mutant males, memory deficit was apparent immediately after training [5]. In the memory retention test, memory performance did not significantly differ from zero already 0.5 h after training completion in the mutant males as opposed to

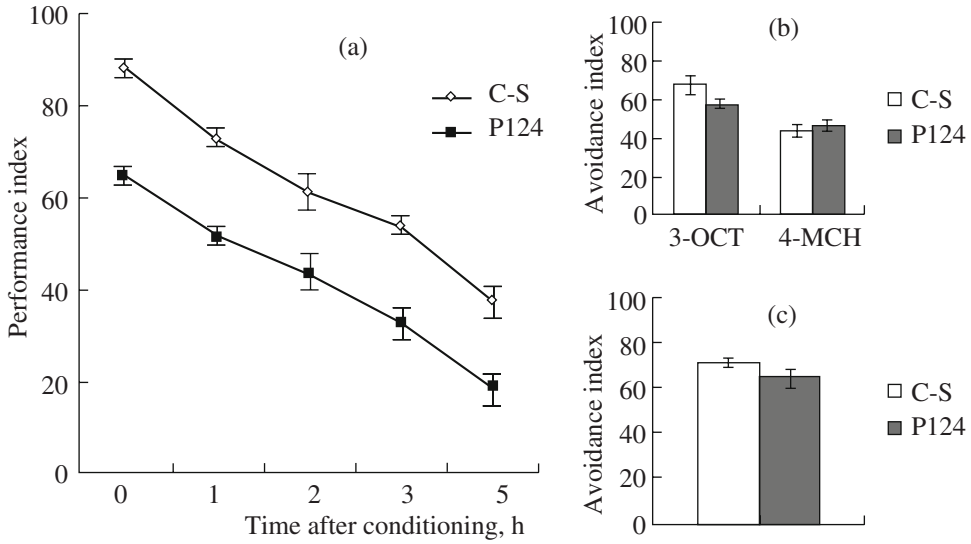

Fig. 5. Memory deficiency in P124 flies after odor-shock conditioning. (a) Time course of performance index. At each time point the difference between C-S and P124 flies is significant (*t* test for independent samples, $P < 0.05$). Odor avoidance: 3-OCT, 3-octanol; 4-MCH, 4-methylcyclohexanol. (c) Avoidance of electric shock. In (b) and (c), the difference between C-S and P124 flies is insignificant (*t* test for independent samples, $P > 0.05$).

3 h in the wild type [5]. In the present study, we found additional manifestations of the mutant phenotype in P124 flies and showed that neither memory deficiency after courtship conditioning nor these additional manifestations were related to deficit of eye screening pigments because they were also present in P124w$^+$ flies.

In contrast to the wild type, the mutant males did not decrease the intensity of their courtship after previous sojourn in the dark (Fig. 1) that pointed to the impairment of some physiological process involved in the adaptation to light. In *Drosophila*, the light adaptation includes translocation of several molecular components, participating in phototransduction, from the rhabdomeres, a special region of photoreceptors where the phototransduction takes place, to the cell bodies of photoreceptors [18]. There are two stages of light-dependent translocation of the phototransduction components, which are based on different molecular mechanisms. The first one goes on during 5 minutes, the second requires more than 6 hours [18]. One may suggest that P124 mutation impairs one of the mechanisms participating in these translocation processes, especially those related to the fast stage including the reverse direction, from light to dark. On the other hand, the base mechanisms of compartmentalization of the phototransduction components inside the photoreceptor cells may be affected. Each of these possible abnormalities should to be accompanied by, at least, moderate visual defect.

The presence of visual defect in the mutants is supported by the fact that P124 insertion produces more severe memory deficiency after courtship conditioning on light than after training in the dark (Fig. 2b). The visual defect in the mutants is also evidenced by the absence in the mutant males of response to such female's actions as the attitude "face-to-face" and the forelegs preening directed to a male (table).

While examining the details of the male–female interaction during various stages of courtship conditioning, additional specific features of behavior in the mutant males were found. Particularly, the wild-type and the mutant males differed in their response to the ovipositor extrusion by a female during the attempted copulation (Figs. 3, 4). It was suggested that there are two effects of the ovipositor extrusion, mediated by stimuli of different modality, on courtship of the wild-type males: the courtship-stimulating one and the inhibitory one, with predominance of the latter. In the mutant males, the inhibitory effect was absent (Fig. 3) that allowed to visualize the stimulatory effect (Fig. 4). The dual effect of the ovipositor extrusion was demonstrated also by other authors [19]: the partial extrusion stimulated courtship of the wild-type males, while the complete extrusion inhibited it. It was also observed that presence of a quickly evaporating drop at the end of the ovipositor strongly stimulated male courtship [19]. Taking into account all these facts, one may suggest that the stimulatory effect of the ovipositor extrusion is mediated by the chemosensory perception, while the inhibitory one by visual perception of a fully extruded ovipositor. It is the latter effect that is absent in P124 males, which according to the data presented above have a visual defect. It should be noted that severe visual deficiency leads to a great decline in courtship index [20] that is not the case of P124 mutants. That is why the most correct conclusion will be that P124 mutants are not able to perceive the

nuances of female visual image, including her fine movements (preening, ovipositor extrusion, etc.).

The fact that P124 mutants show memory deficiency also after the odor-shock conditioning (Fig. 5), the learning type, which does not depend on vision, tells us that the visual defect discussed above is not the cause of memory deficiency. In other words, these are the two independent manifestations of pleiotropic gene action. Just after the training completion and for five next hours the mutants reveal surprisingly constant decrease in CI by 20% from the wild type (Fig. 5). Thus, one may suggest that the mutation does not affect the memory storage but rather impairs memory formation (learning acquisition) or memory retrieval.

Cloning of a fragment of genomic DNA, flanking the P vector, showed that the P vector was inserted into the regulatory region of the *Ent2* (*CG31911*) gene, 12 nucleotides upstream its transcription start. Accordingly, it is suggested to name this mutant allele as $Ent2^{P124}$. The gene consists of one exon, has one transcript and one polypeptide product, ENT2 (equilibrative nucleoside transporter 2) [21]. Earlier, only one mutant allele of the gene, *Ent2,* $Ent2^{GS2163A}$ having the extended wings as a visible phenotype, was described [22]. In that case, the P insertion occurred six nucleotides upstream the transcription start.

Some properties of protein ENT2 in *Drosophila* were described quite recently [23]; however, they did not concern the effect of mutations in this locus on behavior. It is known that ENT2 facilitates diffusion of purine and pyrimidine nucleosides, as well as nucleobases [24]. The nucleosides play the key role in physiology of the eukaryotes functioning as signal molecules, the neuromodulators and participating in cardiovascular activity [23]. Adenosine, the purine nucleoside and neuromodulator, exerts wide effects on the central and peripheral neurons through G-proteins-associated adenosine receptors of various classes [24]. Through changes in the adenosine level, the nucleoside transporters in the CNS influence such physiological processes as sleep and arousal, formation of addictions, reinforcement, nociception [24]. Participation of ENT2 in the mechanisms of einforcement corresponds to the supposition that $Ent2^{P124}$ mutation impairs memory formation (the definitive level of acquisition) rather than memory storage. ENT2 is localized mainly in the basolateral membranes of the polarized epithelial cells [24]. Thus, it is not surprising that one of the mutation manifestations concerns the function of photoreceptors, the specialized type of epithelial cells, whose plasmatic membrane is subdivided to the apical, containing the light-sensitive rhabdomeres, and the basolateral parts [18]. The role of adenosine, regulated by ENT2, in the control of circadian rhythms and adaptation to light conditions is shown for mammalian retina [25].

Thus, in the present study the following effects of mutation in *Drosophila Ent2* locus were revealed: (1) abnormal adaptation to light conditions; (2) impaired perception by males of nuances of female visual image; (3) independent from the two above, impairment of ability to conditioning. The fact that all these defects, particularly the learning ability decreased by 20%, are rather moderate shows that the mutation affects not a key, but a secondary molecular component participating in these processes. Nevertheless, the present results show that hereditary variability in *Ent2* locus should be considered as a factor of risk in pathogenesis of visual disorders and mental retardation. In industrially advanced countries, 1–3% of the population suffer from mental retardation. This problem cannot be resolved by finding only the genes playing a key role in the learning mechanisms (whose mutations lead, naturally, to complete idiotism), but requires the wide search of other, less significant, genes. The essential help here may come from studying the model genetic organism, *Drosophila* [26].

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