

Protein Phosphatase 1–Deficient Mutant *Drosophila* Is Affected in Habituation and Associative Learning

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The learning and memory of *Drosophila melanogaster* strains carrying the *Su-var(3)6⁰¹* mutation, which is known to affect the structural gene of a protein phosphatase 1 isoenzyme, PP1(87B), were studied in various behavioral paradigms. Three lines of *Drosophila* comprising the *Su-var(3)6⁰¹* mutation in different genetic backgrounds were shown to have diminished protein phosphatase 1 activity and behavioral anomalies. Associative olfactory learning and visual conditioning were impaired. Olfactory acuity for the odors used and response to electric shock were largely unchanged in the mutant lines. The motility and flight activity of the mutants were reduced. Habituation of the landing response, a nonassociative learning process, was more pronounced in heterozygotes of the mutants than in the wild-type control strains. Taken together with earlier data, the results indicate that protein phosphatase PP1(87B), while affecting several cellular processes, is also part of the biochemical machinery of various forms of neuromodulation in *Drosophila*.

[Key words: *Drosophila*, protein phosphatase 1, phosphatase-deficient mutant, landing response, habituation, associative learning, memory]

Several lines of evidence point to the mechanistic role of cAMP-dependent protein phosphorylation in the learning processes of the fruit fly *Drosophila melanogaster*. Mutants with abnormal cAMP metabolism have memory deficits: *dunce* is defective in a cAMP-specific phosphodiesterase (Byers et al., 1981; Chen et al., 1986; Davis and Davidson, 1986; Qui et al., 1991), whereas *rutabaga* lacks the calcium/calmodulin-activated component of adenylate cyclase (Dudai and Zvi, 1984; Livingstone et al., 1984; Levin et al., 1992). Feeding of phosphodiesterase inhibitors to wild-type flies mimics the learning/memory anomaly observed in these mutants (Folkers and Spatz, 1984; Asztalos et al., 1991).

Transgenic *Drosophila* expressing the heat-stable protein inhibitor of cAMP-dependent protein kinase (PKA) is impaired in learning (Drain et al., 1991). The findings that the calcium-activated neutral protease (calpain) is present in *Drosophila* (Pintér and Friedrich, 1988) and converts the regulatory subunit of PKA by limited proteolysis to a form showing less affinity to the catalytic subunit (Müller and Spatz, 1989) have led to a model in which the two signals of conditioning, cAMP and Ca²⁺, converge on PKA (Aszódi et al., 1991).

Since protein phosphorylation is a readily reversible modification, the enzymes catalyzing the removal of phosphate groups from protein Ser/Thr side chains, the protein phosphatases, are also expected to be part of the biochemical machinery of learning. Therefore, studies were initiated on *Drosophila* protein phosphatases, and both the type 1 and type 2 enzymes (cf. Cohen, 1989) were detected in the flies and were shown to be very similar to their mammalian homologs (Dombrádi et al., 1987a,b; Orgad et al., 1987). It was also demonstrated that protein phosphatase 1 (PP1) was the predominant active phosphatase in most *Drosophila* tissues, including larval brain (Dombrádi et al., 1987a). By exploiting the high homology with mammalian counterparts, four PP1 catalytic subunit genes were recognized and localized in the genome (Dombrádi et al., 1989). Mutants in one of these, gene PP1(87B), have diminished PP1 activity (Dombrádi et al., 1990a), and two alleles develop a mitotic delay in the third instar larvae (Axton et al., 1990). These mutants were originally isolated as lethals in the *ck19* complementation group (Gausz et al., 1981), and later it was shown that they were suppressors of position effect variegation (Reuter et al., 1987). One allele of this complementation group, *Su-var(3)6⁰¹* (Reuter et al., 1987), which carries a mutation in the PP1(87B) gene (Dombrádi et al., 1991), may reach adult age even in the homozygous state. This rendered it possible to study the behavioral effects of PP1(87B) deficiency in adult flies. It is shown below that both habituation of the landing response and associative learning are affected in these flies.

Materials and Methods

Drosophila strains. Canton-S and Berlin were used as wild-type strains. The position effect variegation suppressor mutant *Su-var(3)6⁰¹* was isolated by Reuter et al. (1987) from Canton-S carrying the inversion *In(1)w^{msh}* and was maintained in the following lines: line 51, *In(1)w^{msh};Su-var(3)6⁰¹*; line 34, *In(1)w^{msh};Su-var(3)6⁰¹ e ro*; line 106, *In(1)w^{msh};Su-var(3)6⁰¹ e red*; line 121, *In(1)w^{msh};cu Su-var(3)6⁰¹ e ro*. All four lines

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were bred as heterozygous stocks with *TM3* balancer chromosome. Lines 34, 106, and 121 were derived from line 51 by appropriate recombinations, to remove possible unidentified mutations from the major part of the third chromosome. Thus, line 34 was generated by recombination between line 51 and strain *In(1)^{w^{msh}}*; *se ss e ro*, whereas in line 121 the third chromosome contained parts of chromosomes *cu kar ry* and *se ss e ro*, on either side of the *Su-var(3)^{6^{ol}}* mutation. In line 106, the original line 51 was recombined with a third chromosome carrying *e red* marker mutations. Strain *In(1)^{w^{msh}}*; *se ss e ro* was also used in this study as a "marker control." It follows from the above genotypes that all *Su-var(3)^{6^{ol}}* homozygotes and heterozygotes have normal red eyes, since this dominant mutation suppresses variegation of the *w⁺* gene, that is, the occasional lack of *w⁺* expression due to adjacent heterochromatin. White-eyed flies were only found in control strains *In(1)^{w^{msh}}* and *In(1)^{w^{msh}}*; *se ss e ro*, in which the red or brown (*se*) eye pigment was reduced to about 10%. The flies were grown on a standard medium of sugar, cornmeal, yeast, and agar that contained the mold inhibitor Nipagin. Breeding and experiments were carried out at 24°C and 60% humidity. Mutant flies were selected under nitrogen anesthesia, within less than 5 min, or cold anesthesia on the basis of morphological markers, and were kept on fresh standard cornmeal medium for at least 16 hr before the experiment.

For the landing response experiments, only red eyed imagos with persistent flight activity can be used. This condition would not be met with homozygous mutants or heterozygotes carrying the *TM3* balancer, since the latter comprises the dominant *Ser* mutation, which affects the wings. Therefore *TM3* was outcrossed with wild-type Berlin, a persistent flier: the progeny not carrying *TM3* from crosses between *In(1)^{w^{msh}}*; *Su-var(3)^{6^{ol}}*/*TM3* and wild-type Berlin were selected for the experiments. We used as control the progeny from crosses between Canton-S *In(1)^{w^{msh}}* (obtained from Dr. G. Reuter, Martin Luther University, Halle, Germany) and wild-type Berlin flies.

Olfactory associative learning was measured according to Tully and Quinn (1985) with some modifications as described earlier (Asztalos et al., 1991). The learning paradigm ran as follows. The flies were placed in the training tube, and odor-free air was sucked through at a rate of 40 liters/hr for 90 sec. Then flies were shocked with 90 V (AC) for 1 sec every 5 sec 12 times, and during this 60 sec period the A odorant, contained in a glass cup, was presented borne by the air stream as given above. Then the tube was flushed with odor-free air for 30 sec (interodor rest period). The B odor was then presented for 60 sec as above, but without electric shock, followed by another 30 sec of rest period. The flies were tested by transferring them after another 60 sec in the central sliding compartment to the choice point of the apparatus and were allowed to disperse in the two (A and B) testing tubes, with air-borne A and B odorants, respectively, for 120 sec. The flies in the tubes were anesthetized by cooling and counted. The experiment was then repeated with fresh flies and the two odors were interchanged in respect of association with electric shock. Instead of calculating the learning index *L* as done earlier (cf. Asztalos et al., 1991), the data were expressed in terms of a performance index, PI (Boynton and Tully, 1992). The PI also gives the fraction of flies avoiding the shock-associated odor corrected for the fraction of flies avoiding the shock-unassociated odor and averaged for the two half-experiments, but in this calculation the flies remaining in the central sliding compartment, that is, those not responding at all, are disregarded. The use of PI is warranted by the fact that *Su-var* flies have diminished motility, which results in an increase in the number of flies remaining in the central compartment. Inclusion of these flies in the calculations would have led to fallaciously low learning indices.

Odor avoidance was determined in Tully and Quinn's (1985) apparatus as described earlier (Asztalos et al., 1991) with the modification that odor intensity was adjusted not by varying the odor cup diameters, but rather by diluting the odorant with paraffin oil. The two odorants used were 4-methylcyclohexanol (MCH) and D,L-3-octanol (3-octanol), both purchased from Janssen Chimica (Beerse, Belgium). As with olfactory learning, flies remaining in the central compartment were not considered.

Electric shock avoidance was measured according to Drain et al. (1991) in Tully and Quinn's (1985) apparatus. Copper grids were inserted, as in the learning paradigm, into the tubes on either side of the choice point. During the test, one of the grids was electrified with 90 V AC. Flies were transferred to the choice point, where they distributed between the electrified and nonelectrified grids. After 2 min the flies were trapped in the tubes and counted. The procedure was repeated with

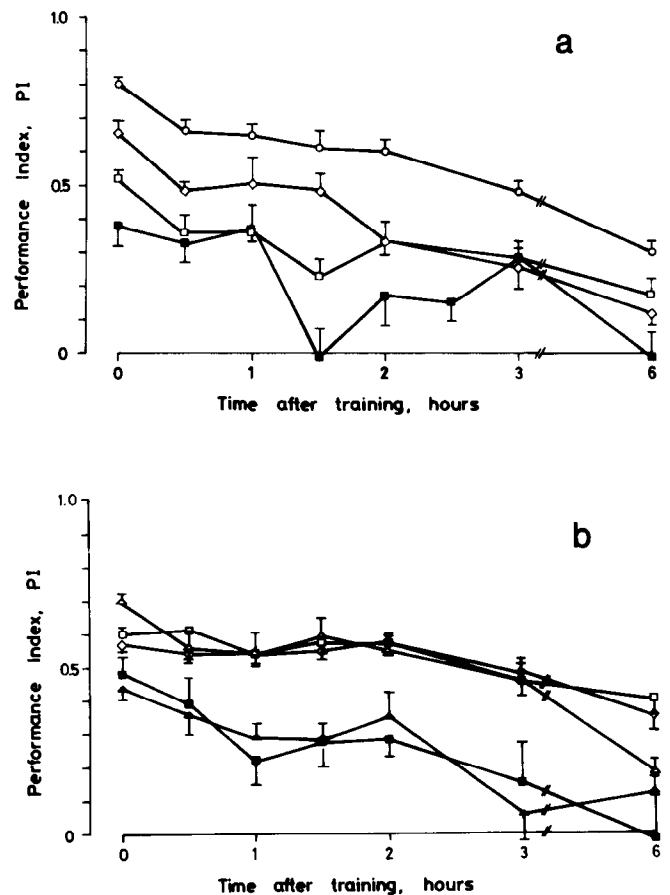


Figure 1. Time course of memory decay of wild-type and mutant flies in the olfactory learning paradigm. PI is indicated as a function of time after training. The data are mean \pm SEM of 5–15 measurements. *a*: \circ , wild-type Canton-S; \diamond , *In(1)^{w^{msh}}*; \square , line 51 heterozygote; \blacksquare , line 51 homozygote. *b*: \diamond , *In(1)^{w^{msh}}*; *se ss e ro*, "marker control"; \triangle and \square , heterozygotes of lines 34 and 121, respectively; \blacktriangle and \blacksquare , homozygotes of lines 34 and 121, respectively.

fresh flies and with the other grid electrified to exclude any side bias. After this a PI was calculated as with olfactory learning, averaged for the two half-experiments.

In all above population behavioral experiments, wild-type and control strains as well as *Su-var* heterozygotes were tested with 80–120 flies at a time, whereas *Su-var* homozygotes were tested with about 15 flies at a time, because of the reduced viability of homozygotes (5–10% in the fly populations). Control experiments with Canton-S flies showed that the results were not influenced by this difference in the number of flies. In the shock-odor learning paradigm, the following PI values were obtained (in separate experiments, but corresponding to the Canton-S curve in Fig. 1) with 100 and 15 flies, respectively: 0 time, 0.76 ± 0.02 ($n = 6$) and 0.78 ± 0.06 ($n = 5$); 1 hr, 0.71 ± 0.04 ($n = 4$) and 0.70 ± 0.09 ($n = 4$); 3 hr, 0.56 ± 0.04 ($n = 4$) and 0.59 ± 0.04 ($n = 5$). A two-way analysis of variance (ANOVA) yielded an insignificant effect of number [$F(1,20) = 0.05$, $p > 0.10$]. This result is in accord with Byers' (1980) measurement (see also Tully, 1984) showing that the learning index is the same whether a group of flies is tested in one run or flies are tested individually, one each in a run, and the data summated. Flies of both sexes were used.

Habituation of the landing response. The paradigm used is described in Wittekind (1987), Wittekind and Spatz (1988), and Rees and Spatz (1989). At variance with the original design landing was elicited by the progressive movement of two black vertical stripes as described by Waldvogel and Fischbach (1991). Within the binocular field of vision of the fly, the screen carries a black mask, such that the return of the black stripes to the starting position cannot be seen. The landing activity and the degree of habituation of the response depend *inter alia* on stripe velocity and on the contrast of the gray stripe against the background. In the experiments reported here, the stripe velocity was 180 degrees/

Table 1. Specific activity of protein phosphatase 1 in *Su-var(3)6⁰¹* flies

Strain/line	Specific activity mU/mg protein (n)
Canton-S	6.60 ± 0.70 (10)
<i>In(1)w^{m4h}</i>	5.39 ± 0.86 (10)
<i>In(1)w^{m4h}; se ss e ro</i>	6.76 ± 1.61 (25)
<i>Su-var(3)6⁰¹</i>	
Heterozygotes	
Line 51	3.89 ± 0.41 (8)
Line 34	3.83 ± 0.54 (5)
Line 121	3.88 ± 0.67 (5)
Homozygotes	
Line 51	2.39 ± 0.52 (7)
Line 34	1.89 ± 0.37 (5)
Line 121	2.54 ± 0.11 (4)

Heterozygotes carried the *TM3* balancer chromosome. The means ± SD of n measurements are given.

sec with respect to the fly's viewing field. The contrast was adjusted to reach appropriate response levels. In the test, the landing stimulus is repeated 125 times at 2 sec intervals.

Visual associative learning. The conditioning apparatus developed by Menne and Spatz (1977) was described in detail by Bicker and Reichert (1978). The conditioning procedure consisted of a training phase and a test. The training phase was subdivided into several 1 min training cycles, in which two lights, one of them coupled with shaking, an aversive stimulus, were presented sequentially, each for 30 sec. One population (Pop I) experienced shaking together with violet light (DAL interference filter, $\lambda_{\max} = 406$ nm, 110 mW/m²) and a resting period together with green light (IL interference filter, $\lambda_{\max} = 507$ nm, 8 mW/m²). During the test, the two light stimuli were presented simultaneously, each in one half of the container. At the beginning of the test, the flies were tapped into one end of the container by tilting it to one side. Immediately thereafter the container was brought back into the horizontal position. The flies were given 2 min to distribute themselves between the two lights and then their distribution was photographed. Then the container was tilted to the opposite side and brought back into the horizontal position, and the distribution of the flies again was photographed after 2 min.

A second population (Pop II) underwent an identical procedure except that these flies experienced the aversive stimulus, shaking, together with the green light and the resting period together with the violet light.

The distribution index (P_{406}) is the fraction of the flies found to be in the violet illuminated half of the container in the test. The conditioning index (Δ) is defined as the difference between the distribution indices of the two sets of populations [$\Delta = P_{406}(\text{Pop II}) - P_{406}(\text{Pop I})$].

By comparing two sets of populations that had undergone the same conditioning procedure except for the coupling of aversive and conditioned stimuli, a direct measure of associative learning is derived, because this way pseudoconditioning effects cancel out against each other.

Measurement of protein phosphatase 1 specific activity. PP1 activity was measured in the homogenate of adult flies on the basis of radioactivity released from ³²P-labeled phosphorylase *a* in the absence and presence of inhibitor-2 protein (Dombrádi et al., 1990a). Protein concentration was measured according to Read and Northcote (1981).

Results

Protein phosphatase 1 levels in *Su-var(3)6⁰¹*

Table 1 shows the specific activity values for PP1 in adult flies of Canton-S, *In(1)w^{m4h}* (derived from Canton-S by an inversion in the X-chromosome and used for EMS mutagenesis to produce *Su-var(3)6⁰¹*), the "marker control" *In(1)w^{m4h}; se ss e ro*, and three *Su-var(3)6⁰¹* lines. It is seen that whereas there are no significant differences in PP1 specific activity among strains Canton-S, *In(1)w^{m4h}* and the "marker control" *In(1)w^{m4h}; se ss e ro*, the heterozygotes and homozygotes exhibit progressively and significantly diminishing values. The residual activity in the homozygotes probably stems from PP1s other than PP1(87B),

which are known to exist in *Drosophila* (Cohen and Dombrádi, 1989; Dombrádi et al., 1990b), although the PP1(87B) carrying the *Su-var(3)6⁰¹* mutation may not be completely inactive (see Discussion).

Olfactory learning of *Su-var(3)6⁰¹* flies

The memory decay curves, that is, PI versus time after training, for Canton-S, *In(1)w^{m4h}*, *In(1)w^{m4h}; se ss e ro*, and three *Su-var(3)6⁰¹* lines are shown in Figure 1. The zero time PI values ("acquisition") decrease in the order Canton-S, *In(1)w^{m4h}*, line 51 heterozygote, line 51 homozygote (Fig. 1a). The curve for *In(1)w^{m4h}* runs otherwise nearly parallel with that of Canton-S. Line 51 heterozygote and homozygote fall below *In(1)w^{m4h}* only in the first 2 and 3 hr, respectively. Line 51 homozygote has a decay curve with a minimum around 1.5 hr. The two-way ANOVA of these curves yielded the following results: *In(1)w^{m4h}* versus line 51 heterozygote, highly significant strain effect [$F(1,71) = 27.39, p < 0.001$], highly significant time effect [$F(6,71) = 26.01, p < 0.001$], and highly significant strain × time interaction effect [$F(6,71) = 4.38, p < 0.001$]; *In(1)w^{m4h}* versus line 51 homozygote, highly significant strain effect [$F(1,85) = 35.88, p < 0.001$], highly significant time effect [$F(6,85) = 11.34, p < 0.001$], and significant strain × time interaction effect [$F(6,85) = 2.78, p < 0.025$]. In Figure 1b the heterozygotes of lines 121 and 34 can hardly be distinguished from *In(1)w^{m4h}; se ss e ro* ("marker control," a reference strain more adequate than Canton-S), whereas their homozygotes run much lower with a slight minimum around 1 hr. The two-way ANOVA for "marker control" versus mutant strains yielded the following results: line 34 heterozygote, no significant strain effect [$F(1,82) = 0.66, p > 0.1$], highly significant time effect [$F(6,82) = 21.65, p < 0.001$], and a significant strain × time interaction effect [$F(6,82) = 3.72, p < 0.005$]; line 121 heterozygote, no significant strain effect [$F(1,73) = 0.82, p > 0.1$], a highly significant time effect [$F(6,73) = 8.84, p < 0.001$], and insignificant strain × time interaction effect [$F(6,73) = 0.52, p > 0.1$]; line 34 homozygote, a highly significant strain effect [$F(1,90) = 73.86, p < 0.001$], a highly significant time effect [$F(6,90) = 9.79, p < 0.001$], and no significant strain × time interaction effect [$F(6,90) = 1.51, p > 0.1$]; and line 121 homozygote, a highly significant strain effect [$F(1,65) = 54.73, p < 0.001$], a highly significant time effect [$F(6,65) = 5.53, p < 0.001$], and no significant strain × time interaction effect [$F(6,65) = 0.87, p > 0.1$]. These data clearly indicate that performance in the olfactory learning test is impaired in *Su-var(3)6⁰¹* flies. The rapid initial decrease in performance found so far with most "memory" mutants (Tully and Quinn, 1985), with the exception of *latheo^{P1}* (Boynton and Tully, in press), however, cannot be seen here.

Olfactory acuity, electric shock avoidance, and motility of *Su-var(3)6⁰¹* flies

We tested whether the sensory modalities involved in the above learning paradigm were affected in the mutant flies. The olfactory acuity of all three *Su-var(3)6⁰¹* lines was measured in terms of a PI of the avoidance of the two odorants used, MCH and 3-octanol. Only the data for line 51 are shown (Fig. 2); lines 121 and 34 gave more or less similar results. The two-way ANOVA gave the following values (the curve for each strain is compared to the appropriate curve for Canton-S). For MCH, line 51 heterozygote, no significant strain effect [$F(1,62) = 0.54, p > 0.1$]; line 51 homozygote, no significant strain effect [$F(1,54) = 2.80, p > 0.1$]; line 34 heterozygote, no significant strain effect [$F(1,54) = 0.03, p > 0.1$]; line 34 homozygote, a significant

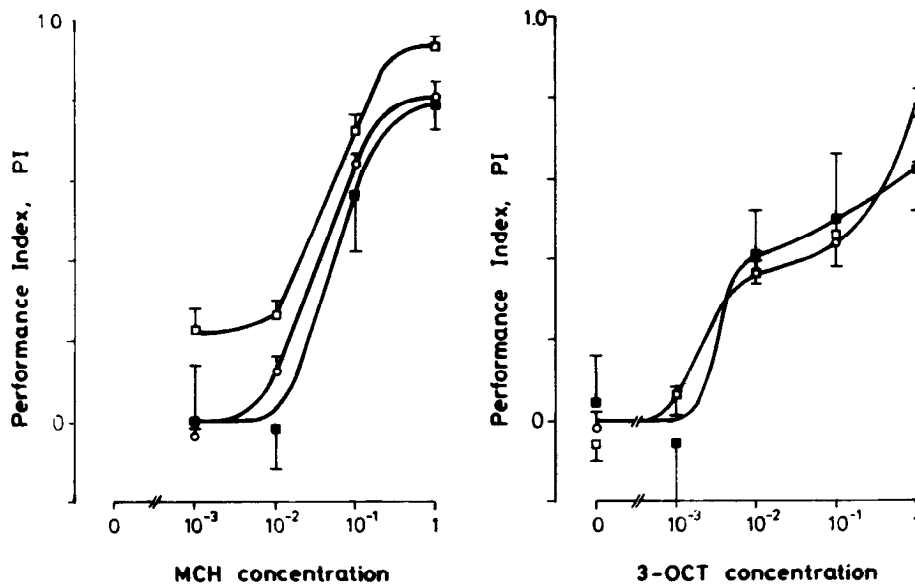


Figure 2. Odor avoidance of Canton-S and *Su-var(3)6⁰¹* line 51 heterozygous and homozygous flies. Odor concentration of 1 is undiluted odorant. The data are mean \pm SEM of 6–12 measurements. *Left*, MCH avoidance; *right*, 3-octanol (3-OCT) avoidance. O, wild-type Canton-S; \square , line 51 heterozygote; \blacksquare , line 51 homozygote.

strain effect [$F(1,66) = 5.40, p < 0.025$]; line 121 heterozygote and homozygote, no significant strain effect [$F(1,59) = 0.64, p > 0.1$, and $F(1,69) = 1.64, p > 0.1$, respectively]. For 3-octanol: line 51 heterozygote and homozygote, no significant strain effect [$F(1,55) = 0.29, p > 0.1$, and $F(1,68) = 0.59, p > 0.1$, respectively]; line 34 heterozygote and homozygote, no significant strain effect [$F(1,58) = 0.07, p > 0.1$, and $F(1,50) = 0.34, p > 0.1$, respectively]; line 121 heterozygote, a highly significant strain effect [$F(1,63) = 8.00, p < 0.001$]; and line 121 homozygote, no significant strain effect [$F(1,72) = 1.2, p > 0.1$]. On the whole, the *Su-var(3)6⁰¹* mutation affected slightly, if at all, the olfactory acuity of flies. The only significant changes were found with the MCH avoidance of line 34 homozygotes, where avoidance decreased by about 50%, and with the 3-octanol avoidance of line 121 heterozygotes, where avoidance slightly increased. These differences in olfactory performance are highly unlikely to underlie the anomalies observed in olfactory learning, all the more so since before each olfactory learning experiment the 3-octanol versus MCH odor choice value was adjusted to 0.5 at an avoidance PI for MCH of about 0.7–0.8 (except line 34 homozygote, where it was about 0.4) (see Asztalos et al., 1991). While with MCH all avoidance curves conformed to a single sigmoidal, with 3-octanol the curves were more complex with all three mutant lines, as well as with the controls. We do not know the reason of this anomaly. It may reflect two distinct receptor populations for 3-octanol or the odorant may have a general, unspecific membrane effect at high concentrations (Siddiqi, 1991).

The other stimulus in the olfactory learning paradigm is electric shock. Table 2 shows the shock avoidance of the various flies. In comparison with Canton-S, the PI values of *Su-var* lines differ insignificantly ($p > 0.05$, Student's *t* test) with the exception of line 34 homozygote, where the PI is significantly decreased ($p = 0.034$, Student's *t* test). This suggests that their sensing and responding to this stimulus are hardly impaired. On the other hand, the motility of *Su-var(3)6⁰¹* flies is diminished, which is clearly seen with the homozygotes (Table 2).

Visual learning

Only heterozygotes from crosses between *Su-var(3)6⁰¹* strain (line 51 and line 121) with wild-type Berlin could be tested in

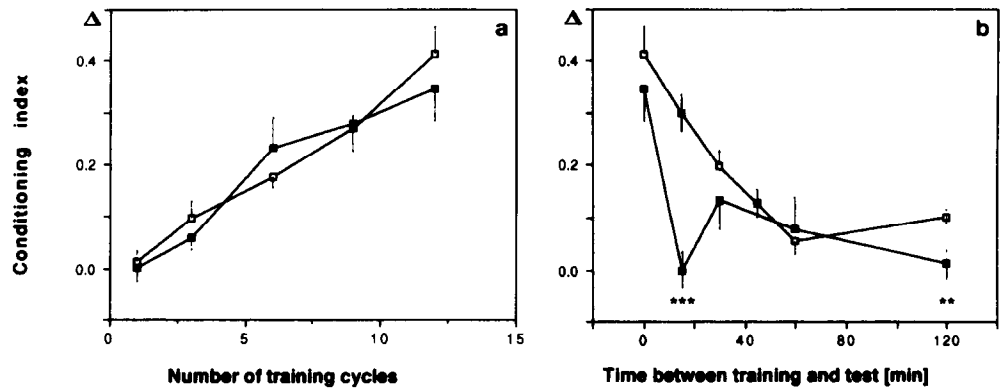
visual learning. In acquisition experiments (Folkers and Spatz, 1981), no significant differences of the conditioning indices were observed in comparison to the controls resulting from crosses between the *In(1)w^{mdh}* background strain and wild-type Berlin (Fig. 3a). Homozygotes for the *Su-var(3)6⁰¹* mutation were too sluggish to be evaluated in this paradigm. In contrast, differences are seen in the memory curves (Fig. 3b). In these experiments, flies were conditioned in 12 training cycles and tested at a given time after training. Each population was tested only once. During the interval between training and test, the flies were left undisturbed in white light (Folkers and Spatz, 1981). In contrast to the control strain, after 2 hr the conditioning index for the heterozygous *Su-var(3)6⁰¹* strain is not significantly different from zero. The most striking difference, however, is found 15 min after training: there is a significant drop with the heterozygote followed by a slight recovery at 30 min. The two-way ANOVA yielded a highly significant strain effect [$F(1,150) = 12.48, p = 0.005$], a highly significant time effect [$F(4,150) = 14.12, p <$

Table 2. Electric shock avoidance and motility of *Su-var(3)6⁰¹* flies

Strain/line	Shock avoidance PI \times 100 (n)	Motility % (n)
Canton-S	71 \pm 4 (5)	90 \pm 1 (36)
<i>In(1)w^{mdh}</i>	63 \pm 7 (3)	91 \pm 1 (38)
<i>In(1)w^{mdh}; se ss e ro</i>	70 \pm 3 (4)	79 \pm 1 (40)
<i>Su-var(3)6⁰¹</i>		
Heterozygotes		
Line 51	71 \pm 4 (4)	92 \pm 1 (38)
Line 34	69 \pm 4 (4)	91 \pm 1 (34)
Line 121	54 \pm 6 (4)	80 \pm 1 (42)
Homozygotes		
Line 51	67 \pm 12 (4)	88 \pm 1 (38)
Line 34	44 \pm 8 (4)	79 \pm 2 (40)
Line 121	67 \pm 15 (4)	69 \pm 2 (43)

Electric shock avoidance is expressed as PI \times 100. Motility is expressed as the percentage of flies that left, in either direction, the central compartment in the olfactory learning test. Heterozygotes carried the *TM3* balancer chromosome. The means \pm SEM of *n* measurements are given.

Figure 3. Visual conditioning of wild-type and mutant flies. *a*, Learning. The conditioning index Δ was measured as a function of the number of training cycles. *b*, Memory. The conditioning index Δ was measured as a function of time between training and test. The error bars denote the SD of the mean of eight parallel experiments. The asterisks denote levels of significance (***, $p < 0.001$; **, $p < 0.01$) of the difference between Berlin/*In(1)w^{m4h}* (□) and Berlin/*Su-var(3)6⁰¹* (■), line 51 or 121.



0.0001], and a significant strain \times time interaction effect [$F(4,150) = 3.85, p = 0.052$].

Habituation of the landing response

For the habituation of the landing response, only heterozygotes produced by crosses with the persistent flier wild-type Berlin could be used (see Materials and Methods). Homozygous mutants had only poor flight activity. Figure 4 demonstrates that the heterozygotes of line 51, line 34, line 106, and line 121 habituate faster than the control strain or wild-type Berlin. Under the stimulus conditions used, the time between the start of the stimulus and the onset of the landing response, a parameter characteristic of this paradigm (Wittekind and Spatz, 1988; Rees

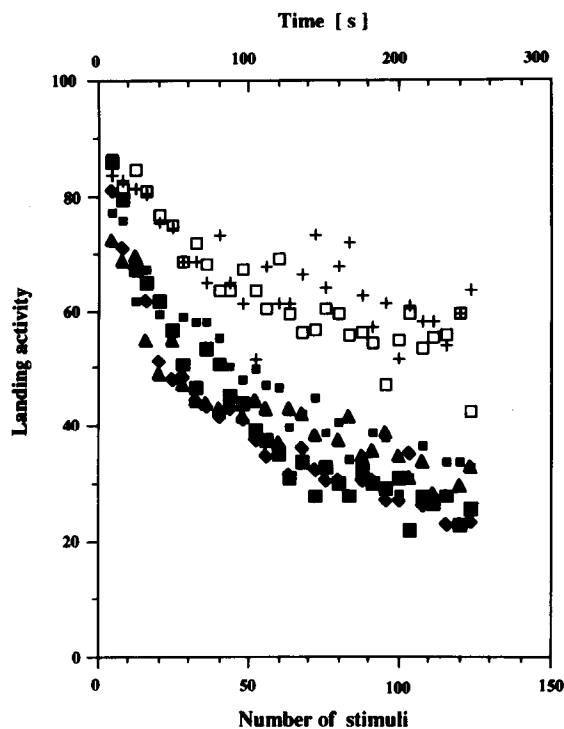


Figure 4. Habituation of the landing response in wild-type and *Su-var(3)6⁰¹* heterozygous flies. The landing activity is plotted as a function of the number of stimuli presented in 2 min intervals. +, wild-type Berlin ($n = 21$); □, *In(1)w^{m4h}*/Berlin ($n = 38$); ■, line 34/Berlin ($n = 44$); ▲, line 106/Berlin ($n = 52$); ◆, line 121/Berlin ($n = 52$). Approximately equal numbers of male and female flies were tested. n = number of flies tested; evaluation made as described by Wittekind and Spatz (1988).

and Spatz, 1989), did not differ in the heterozygous strains and the control strains. Furthermore, there was no significant change, as compared with the controls, in the time between the onset and completion of the response (data not shown; see Wittekind and Spatz, 1988).

Discussion

Drosophila melanogaster lines carrying the *Su-var(3)6⁰¹* mutation in different genetic backgrounds exhibit anomalies in associative and nonassociative learning paradigms. As expected from recent work (Dombrádi et al., 1991) showing that *Su-var(3)6⁰¹* is a mutation in the structural gene of a PP1 enzyme, PP1(87B), all examined lines had diminished PP1 activities in a dose-dependent manner. It is assumed that the behavioral deficits are due to this enzyme deficiency. Nevertheless, it cannot be excluded that there is an unidentified mutation, in addition to *Su-var(3)6⁰¹* between loci *cu* and *e*, that underlies the abnormal behaviors, since this region of the third chromosome has not been replaced in any of the lines. Narrowing this region by deficiency mapping was obviated by the fact that the relevant available deletion strains [*Df(3R)e⁰⁷⁹* and *Df(3R)TE45*] are lethal or give very few progeny over *Su-var(3)6⁰¹*, suggesting that *Su-var(3)6⁰¹* is a hypomorph rather than amorph. The learning anomalies could not have originated from the *ro* and *e* markers, which had been shown to affect olfactory learning (Dudai, 1977), since our "marker control," *In(1)w^{m4h}; se ss e ro*, performed nearly as well as wild-type Canton-S and significantly differed from *Su-var(3)6⁰¹* homozygotes.

The enzyme-deficient flies showed impaired olfactory associative learning without any major deficit in olfactory acuity and sensitivity to electric shock. The memory decay curves of homozygotes differed significantly from those of heterozygous and control flies: they had a low initial PI value and a local minimum around 1–1.5 hr. Such a trough has also been observed, though to a lesser extent, with both wild-type and various memory mutant flies (Spatz, 1985; Tully and Quinn, 1985), and has been interpreted by assuming different, overlapping memory phases (Tully et al., 1990; Tully, 1991).

Another feature of the olfactory learning of *Su-var(3)6⁰¹* homozygotes is that there is no rapid initial memory decay observed with the *dunce*, *rutabaga*, and *amnesiac* memory mutants of *Drosophila* (Tully and Quinn, 1985). From our current incomplete knowledge of the biochemical reactions underlying olfactory learning in *Drosophila*, we may assume, by analogy with *Aplysia* (Braha et al., 1990) and *Hermisenda* (Alkon and Nelson, 1990), that short-term memory resides in the phosphorylation of some protein(s), possibly related to K^+ channels.

The decay of this short-term memory should then involve dephosphorylation, as indicated in the case of *Aplysia* (Endo et al., 1991). We do not know, however, how the lack of PP1(87B) in *Drosophila* brings about the observed time course of memory decay.

In the visual learning paradigm, *Su-var(3)6⁰¹* homozygotes could not be evaluated owing to their decreased motility. Impairment in motility was also observed in olfactory learning, but it did not interfere with the measurement, because of the lesser demand for movement in the olfactory paradigm. In visual learning, the heterozygotes did not differ from the control strain in acquisition, but failed to show a conditioned response 15 min after training. This phenomenon is reminiscent of a rapid initial decrease of the conditioning index seen in many but not all of our experiments on wild-types (see Folkers and Spatz, 1981). The phosphatase mutant may offer a chance to study whether this is an indication of an early phase of memory consolidation (Matthies, 1989).

It may look puzzling that the data on visual learning do not coincide with those on olfactory learning. It should be recalled, however, that several other differences have been observed between paradigms based on these two sensory modalities (Folkers, 1982; Folkers and Spatz, 1984). Most strikingly, the mutant *mbd* (*mushroom body deranged*) is normal in visual learning while it is severely impaired in olfactory learning (Heisenberg et al., 1985), which suggests that these two types of learning may be associated with different brain regions in the fly. Furthermore, it should be borne in mind that enzyme levels, here the significant differences in PP1 activities between *Su-var(3)6⁰¹* heterozygotes and homozygotes, cannot be directly correlated with behavioral parameters. At what degree partial enzyme deficiency becomes critical depends on the type of learning and the paradigm used.

Habituation of the landing response was found to be faster in *Su-var(3)6⁰¹* heterozygotes than in wild-type controls. Wittekind and Spatz (1988) have shown that muscle fatigue contributes little if at all to the response decrement seen during repetitive stimulus presentation. A direct indicator, recorded automatically for every response, is the time between the onset and completion of the landing reaction. This time is constant over 125 stimulus presentations. In these experiments, no difference was detected in this response time between the heterozygous *Su-var(3)6⁰¹* and the control strains. It therefore seems unlikely that different degrees of muscle fatigue can explain the differences in the response decrement, even if a contribution by fatigue cannot be totally excluded.

It would be far-fetched to envisage tentative molecular mechanisms for the opposing effects of the *Su-var(3)6⁰¹* mutation on associative learning and habituation. At our current level of understanding, even the data on habituation in *Drosophila* cannot be assembled in a coherent picture. The habituation of proboscis extension reflex, triggered by a gustatory stimulus, is attenuated in *dunce*, *rutabaga* and *amnesiac* (Duerr and Quinn, 1982). In contrast, the same mutants habituate faster in the landing response, but interference with cAMP metabolism by feeding wild-type flies phosphodiesterase inhibitors has an inverse effect: habituation is almost completely prevented (Wittekind and Spatz, 1988). Habituation of the cleaning reflex, elicited by tactile stimulation, is observed in *rutabaga*, but its duration is much shorter than in the wild-type (Corfas and Dudai, 1989).

The above apparently puzzling set of findings warns us that

the construction of unifying molecular models for associative learning mechanisms and habituation mechanisms is premature, if ever attainable. Each sensory modality poses individual problems, and the details of central connectivity changes are unknown. An inherent difficulty in the study of these mechanisms is that they involve enzymes and other proteins, or their different isoforms, that take part in several cellular processes. Thus, the *Su-var(3)6⁰¹* flies, apart from the learning anomalies, have decreased motility and flight activity, moderate viability, strong suppression of position effect variegation, and possibly other so far undetected phenotypic traits. This inevitable pleiotropy (see, e.g., Kulkarni et al., 1988) does not invalidate the message that the enzyme PP1(87B) is involved in the biochemical machinery of associative learning and habituation in *Drosophila*.

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