The effect of the *inactive* mutation on longevity, sex, rhythm and resistance to *p*-Cresol in *Drosophila melanogaster*

K. M. C. O'DELL

Institute of Genetics, University of Glasgow, Church Street, Glasgow G11 5JS, Scotland, UK

Flies carrying the *inactive* (*iav*) mutation exhibit low locomotor activity and poor mating success, both of which are associated with a deficiency in the putative neurotransmitter, octopamine. Several other aspects of the *iav* mutant phenotype are described here. Male and female *iav* mutants show a small reduction in longevity but it is not clear whether this is a consequence of the *iav* mutation or their inactive phenotype. Young *iav* males show extended attractiveness to older courting males, which supports the notion that the *iav* gene has a role in post-eclosion maturation. The eclosion rhythm of *iav* mutants is normal, discounting the possibility of a role for octopamine in the maintenance of circadian rhythm. Flies carrying the *iav* mutation are highly susceptible to the octopamine analogue *p*-Cresol. Other phenotypically inactive flies show wild type levels of *p*-Cresol resistance. This is attributed to the deficiency of octopamine in *iav* mutants because low octopamine levels may be unable to out-compete the toxic effect of *p*-Cresol. Some inferences on the possible mode of action of the *iav* gene product are discussed.

Keywords: behaviour, courtship, *Drosophila*, longevity, octopamine, rhythm.

Introduction

Genetic and molecular approaches to the study of *Drosophila melanogaster* courtship may ultimately reveal underlying biochemical and developmental constraints associated with behaviour. Several mutations have now been isolated that alter specific aspects of *Drosophila* courtship behaviour (see Hall, 1982) but little is known about their mode of action. The *inactive* (*iav*) mutation, which was originally isolated by Kaplan (1977), provides one route by which we may understand the function of the putative *Drosophila* neurotransmitter, octopamine, during courtship and other behaviour.

The *iav* mutation and its allele *iav*² (formerly *hypoactive-B*¹, Homyk & Sheppard, 1977), were both isolated in flies showing locomotor-deficient phenotypes. Adult *iav* mutants show reductions in both the amount and speed of movement, exhibit poor reactivity and have several other abnormal locomotor-related phenotypes (O'Dell & Burnet, 1988).

The mating success of flies carrying the *iav* mutation is markedly reduced. Poor mating success of *iav* males is primarily the consequence of their inactive phenotype, which precludes the maintenance of close contact

during courtship. Homozygous *iav* and *iav*² females show reductions in mating propensity and extended courtship durations. However, given that *iav* females have normal cuticular hydrocarbon compositions and receive a high intensity of courtship from mature males, they can be considered as attractive but unresponsive (O'Dell *et al.*, 1989). Such behaviour is consistent with the aberrant perception and/or processing of incoming courtship information.

High performance liquid chromatography (HPLC) on flies carrying the *iav* mutation reveals that they have only 15 per cent of the wild type octopamine level but normal titres of dopamine and noradrenaline (O'Dell *et al.*, 1987). The precise role of octopamine in *D. melanogaster* is unclear, but it has been shown to have neurotransmitter, neuromodulator and neurohormone functions in several invertebrate species (David & Coulon, 1985). As yet it is unclear whether this deficit in octopamine is the cause or the consequence of the *iav* mutant phenotype, but it is consistent with the prediction of a neural focus for the *iav*² mutation by Homyk (1977).

It should be possible to use *iav* mutants to establish the role of octopamine in the *Drosophila* nervous system and its consequences for behaviour and

development. Livingstone & Tempel (1983) found a small octopamine deficit in rhythmless *period* mutants, and the effect of the *iav* mutation on circadian rhythm is described here. The effects of *p*-Cresol, a structural analogue of octopamine, on the survival of *iav* mutants is also described. *p*-Cresol is known to inhibit bovine dopamine β -hydroxylase (Goodhart *et al.*, 1987), the *Drosophila* equivalent of which is a candidate for the *iav* gene product.

A further consequence of the *iav* mutation appears to be a small developmental delay in the onset of some adult locomotor and sexual behaviours (O'Dell & Burnet, 1988; O'Dell *et al.*, 1989). This apparent delay may affect other developmentally regulated post-eclosion maturation events and the consequences of the *iav* mutation for the onset of male sexual behaviour and longevity are described.

Materials and methods

Fly strains

Unless otherwise stated, all comparisons are made between flies carrying the *iav*¹ allele of Kaplan (1977) and wild type. As described previously (O'Dell & Burnet, 1988), to avoid the effects of genetic background the *iav*¹ allele was crossed in to two wild type strains, Novosibirsk (N) and Sierra Leone (S), by means of multiply marked chromosomes and back crossing. These parental strains are designated *Niav*⁺, *Siav*⁺, *Niav*⁻ and *Siav*⁻, respectively.

To avoid the effects of inbreeding the F₁ individuals derived from a cross between appropriate wild type or *iav* parents are used, and for purposes of notation, the female parent is always designated first. Thus, a fly of the genotype *NSiav*⁺ is an F₁ wild type individual derived from a Novosibirsk mother and Sierra Leone father. If no difference is found in the behaviour and/or survival of the reciprocal crosses (NS and SN), then these scores are pooled for sex and genotype analyses.

The other strains used were not controlled for genetic background nor inbreeding. These are *inactive*² (*iav*², formerly *hypoactive-B*¹), *hypoactive-C* (*hypoC*) and *slow-receptor-potential*⁴ (*slrp*⁴, formerly *hypoactive-D*) all isolated by Homyk & Sheppard (1977) and exhibiting locomotor-deficient phenotypes; *retinal-degeneration-B*²³ (*rdgB*²³ formerly *hypoactive-F*, Homyk *et al.*, 1980); Oregon-R wild type; the selected low activity line La Mancha (van Dijken & Scharloo, 1979); *white*¹¹¹⁸ (*w*¹¹¹⁸, Hazelrigg *et al.*, 1984); *shibire*^{ts} (*shi*^{ts}, Grigliatti *et al.*, 1973); and two multiply marked lines constructed here in Glasgow, *w cr*⁶ *hypoC* and *iav m f*, the latter containing the original *iav* allele of

Kaplan (1977). Further information on these mutations is contained in Lindsley & Zimm (1992).

All flies were raised on standard oatmeal and molasses medium and maintained at 25 ± 0.5°C on a 12-h light:dark cycle. The sexes were separated under ether (longevity and courtship) or CO₂ (rhythm and *p*-Cresol survival) on the day of eclosion and maintained in groups of 10 in vials until required.

Longevity

Survival on standard medium was scored over a period of 15 weeks. Two hundred individuals of each sex (male and virgin female), genotype (wild type and *inactive*) and reciprocal cross (NS and SN) were divided on eclosion into groups of 10 and retained in vials seeded with live yeast. This gives a total of 1600 individual records of survival. Survival at 25°C was scored every day and freshly yeasted medium was provided on every second day.

Courtship of young males

All courtships were observed in circular mating cells as described previously (O'Dell *et al.*, 1989). The control (courting) flies were 3 days old *NSiav*⁺ wild type males. The target flies were single wild type (*SNiav*⁺) or *iav* (*SNiav*⁻) males at 2, 26 or 50 h old. These ages were chosen so that courtship could be observed at the same time each day on flies that can essentially be described as 0, 1 and 2 days old. Flies were kept singly in vials prior to their introduction to the mating chamber.

Preliminary analyses using *forked* mutant males (unpublished) confirm that courtship is exclusively expressed by the older (3 days old) male towards the younger male and that the reciprocal never occurs. Therefore, although the courting and target males are morphologically indistinguishable, we can be confident that the males expressing courtship are the 3-day-old *NSiav*⁺ wild type males.

Each morning, the behaviour of two control wild type courting males towards two target males, from each of the six classes, was observed. The order of the 12 trials was randomized and each trial was scored blind. The experiment was repeated on 10 mornings. The attractiveness of the target young males was measured using the sex appeal parameter (SAP) of Jallon & Hotta (1979). The SAP is calculated as the time the courting (older) male spends directing wing vibration towards the target fly (in this case the immature male) during the first 5 min of courtship. Each fly was used only once.

Circadian rhythm

The eclosion rhythm of two wild type (*NSiav⁺* and *SNiav⁺*) and two *iav* (*NSiav⁻* and *SNiav⁻*) strains was investigated using a protocol similar to that of Konopka & Benzer (1971). The 12/12 h light:dark acclimatized parental generation were collected as virgins, paired in the light and allowed to lay in yeasted bottles for 3 days. The parents were then discarded and the cultures placed at 25°C in constant darkness. At midnight on day 9–10, all the bottles were emptied under red light. Eclosing flies were collected every 3 h for the following 4 days. A total of 4133 wild type flies and 5425 *inactive* mutants eclosed during the 96-h observation period.

Effect of *p*-Cresol on survival

The octopamine structural analogue *p*-Cresol (4-methylphenol) was obtained from Sigma. As described earlier, dilutions of *p*-Cresol into the standard medium were made to obtain food with a *p*-Cresol concentration of 1 per cent by volume or less. Females aged 5–7 days were introduced to vials containing food with a variety of dilutions of *p*-Cresol and control vials containing food with no added *p*-Cresol. Ten flies were introduced to each vial, and five vials were observed for each genotype for each *p*-Cresol dilution. Survival at 25°C was recorded every hour.

Results

Longevity

The effect of the *iav* mutation on longevity is shown in Fig. 1. The lifespan of *iav* males and females is significantly shorter than that of the equivalent wild type flies ($F=171.38$, d.f. = 1, $P<0.001$). The *iav* mutation decreases the lifespan by approximately 6 days under these conditions.

Interestingly, males show a shorter lifespan than females, the effect being somewhat greater than that observed between the two genotypes ($F=371.13$, d.f. = 1, $P<0.01$). Under these conditions females outlive males by approximately 12 days. There is also a small but significant interaction between genotype and sex ($F=5.52$, d.f. = 1, $P<0.05$), which is due to the greater difference in survival between wild type and *iav* females relative to males.

Courtship of young males

The results indicate that the attractiveness of wild type males decreases markedly with age, from an SAP of 0.223, soon after eclosion, to 0.023 after 26 h and

Fig. 1 Longevity of wild type and *inactive* males and females expressed as a percentage survival over age in days. Each point represents the data for 400 individuals. Mean longevity (and standard errors) in days are (■) wild type male 35.60 (0.50), (◆) wild type female 49.98 (0.72), (□) *iav* male 30.04 (0.52), (◇) *iav* female 41.28 (0.87).

0.002 after 50 h. The attractiveness of *iav* males also decreases with age over the same period from an SAP of 0.351 at 2 h, to 0.115 at 26 h and 0.053 at 50 h.

However, for each age examined the *iav* male is more attractive than its equivalent wild type male, the difference being most noticeable in the older (50 h) males. Here the *iav* males elicit about 25 times more wing vibration than their wild type counterpart. In fact the 50 h *iav* male elicits twice as much courtship as a 26 h wild type male.

An analysis of variance confirms the results alluded to above. As expected, attractiveness decreases with age ($F=92.11$, d.f. = 2, $P<0.01$) and wild type males are significantly less attractive than *iav* males ($F=29.12$, d.f. = 1, $P<0.01$). The observation that there is no significant interaction between age and genotype ($F=1.76$, d.f. = 2, $p=ns$), confirms there is a similar pattern of decreasing attractiveness with age for both wild type and *iav* males.

Circadian rhythm

The eclosion rhythms of wild type and *iav* mutant flies are presented in Fig. 2. As no differences were found between the rhythms of males and females nor between the genetic backgrounds of the parents, data were pooled for the two sexes and reciprocal crosses.

The results indicate that wild type flies show a 24-h eclosion rhythm. The peak 3-h eclosion rate is always between the hours of 09.00 and 12.00, immediately after perceived 'lights on'. The low point of eclosion is between 24.00 hours and 03.00, the middle of perceived 'night-time'.

Fig. 2 Eclosion rhythm of (a) wild type and (b) *inactive* flies. The bars represent the number of flies eclosing in each 3-h period starting at midnight on day 1 and continuing for 4 days.

The pattern of eclosion of *iav* mutants is similar to that of the wild type flies. Indeed the peaks and troughs of *iav* eclosion occur during precisely the same 3-h periods as wild type eclosion on each of the 4 days of observation. Clearly, *iav* mutants have a normal eclosion rhythm.

Effect of *p-Cresol* on survival

Preliminary trials showed that food medium supplemented with *p-Cresol* has a deleterious effect on the survival of both wild type and *iav* mutant females, as vials containing no *p-Cresol* show little or no mortality over the same observation period (data not shown). As revealed by Student's *t*-test, the survival rate of *iav* females is markedly reduced with respect to wild type females ($t=8.30$, d.f.=49, $P<0.001$). Indeed, the mean length of survival of *iav* females on 0.5 per cent *p-Cresol* is only 1.62 h compared to 4.46 h of wild type females.

Fig. 3 Survival of (□) wild type, (■) *iav*¹, (◇) *iav*², females and their hybrids (◆, *iav*⁺/*iav*¹; ●, *iav*¹/*iav*²) on medium containing 0.5% *p-Cresol*. $n=50$.

Fig. 4 Survival of females on medium containing 0.5% *p-Cresol*. $n=50$. (□) *hypoC*, (◆) *iav m f*, (■) Oregon-R, (◇) *rdgB*²³, (○) *shi*^{1s}, (●) *slrp*⁴, (▲) La Mancha, (△) *w ct*⁶ *hypoC*, (×) *w*¹¹¹⁸.

Females carrying the *iav*² allele show a susceptibility to *p-Cresol* that is indistinguishable from that of *iav* females (Fig. 3). Exposure to *p-Cresol* is similarly deleterious to the survival of F₁ hybrid *iav*/*iav*² females. F₁ hybrid *iav*⁺/*iav* females show wild type like resistance to *p-Cresol*.

The data in Fig. 4 confirm that the deleterious effect of *p-Cresol* is specific to flies carrying a mutation in the *iav* gene. It shows that flies with a locomotor-deficient phenotype, superficially similar to that of *iav*, are indistinguishable from wild type flies in their response to *p-Cresol*. This is irrespective of their relative inactivity being associated with a single mutation (*hypoC*, *rdgB*²³, *slrp*⁴ and *w*¹¹¹⁸), several mutations (*w ct*⁶ *hypoC*) or resulting from an extended period of selection (La Mancha). The multiply marked strain (*iav m f*), derived from the original *iav* mutation, is similar in its susceptibility to *p-Cresol* to the isogenized *iav* line.

Discussion

Longevity

It is a prediction of the 'Rate of Living' hypothesis of Pearl (1928) that activity should be inversely related to age at death. Trout & Kaplan (1970) demonstrated just such an effect in the relatively short-lived but hyperactive *Drosophila* mutant *Shaker*. The problem in measuring the expression of longevity in mutant lines of *D. melanogaster* is the marked sensitivity of the phenotype to the external environment, particularly crowding, and effects of genetic background (Lamb, 1978). However, in these trials these problems have largely been eliminated.

The observation that wild type flies have a longer life span than the hypoactive *iav* mutants clearly runs contrary to the 'Rate of Living' hypothesis. However, the extended longevity of females relative to males fits the observations of several authors (Allemand *et al.*, 1973; Maynard Smith, 1958). Why this should be so is unclear.

Although the 'Rate of Living' hypothesis has been the subject of some recent criticism (Le Bourg, 1987), the observation that phenotypically inactive flies have a shorter lifespan than wild type individuals need not necessarily be taken as evidence against the hypothesis. The apparent reduction in *iav* mutant longevity may be a consequence of an, as yet, unknown function of the *iav* gene. Given that the genotypic difference in longevity is greater between wild type and *iav* females than between males, it may also indicate some female-specific function of the *iav* gene. Alternatively, the small decrease in *iav* mutant longevity may reflect some aspect of their inactive behavioural phenotype that contributes to an age-independent increase in their mortality.

Courtship of young males

Observations on the effect of ageing on locomotor activity and female sexual receptivity suggest there is a short delay in the onset of mature adult behaviour in young *iav* adults (O'Dell & Burnet, 1988; O'Dell *et al.*, 1989). The delay in the onset of female receptivity may be associated with the late occurrence of 'switch on', that is the point at which young virgin females change from an unreceptive to receptive state (Manning, 1967). However, no delay in maturation has yet been demonstrated in any aspect of the courtship behaviour of *iav* males.

Detailed analyses of *Drosophila* courtship have revealed the unexplained phenomenon of male/male courtship. Males that are only a few hours old stimulate older males to court them (Cook & Cook, 1975). This

occurs in all *D. melanogaster* strains so far investigated (McRobert & Tompkins, 1983) and is due to the production of female-like sex pheromones by the young males (Tompkins *et al.*, 1980; Jallon, 1984). However, this attractiveness falls rapidly and 2-day-old wild type males elicit little or no courtship from older males (Jallon & Hotta, 1979).

If the *iav* mutation has a consequence for some or all aspects of maturation in newly eclosed adults, then we might expect young *iav* males to exhibit extended attractiveness. This appears to be the case as *iav* males are still highly attractive 2 days after eclosion, whereas wild type males are not.

These results show that *iav* males are more attractive than wild type males at all three ages investigated. This may be due in part to their inactive phenotype, which largely precludes their escaping the attention of older courting males. Similar observations have been made in courtships involving virgin *iav* females, which attract a high frequency of wing vibration from courting males. This is thought to be a consequence of their relative inactivity, which precludes their decamping and other avoidance and escape behaviours (O'Dell *et al.*, 1989). However, the observation that the relative attractiveness of *iav* males to wild type increases over time, clearly demonstrates extended attractiveness.

Males carrying the *fruitless* (*fru*, Tompkins *et al.*, 1980; Gailey & Hall, 1989) or *apterous* (*ap*, Jallon & Hotta, 1979) mutations are attractive to mature males throughout their lives. In both cases this is associated with the permanent presence of the immature male pheromone coat. In fact solvent cuticular extracts of old *fru* males are sufficient to stimulate other mature males to display courtship behaviour (Tompkins *et al.*, 1980). The presence of extended attractiveness in young *iav* males suggests there may be some delay in the switch of the *iav* male pheromonal coat from the immature to mature state. The pheromone coat of 4-day-old *iav* males is indistinguishable from that of wild type (O'Dell *et al.*, 1989) but the nature of the pheromone coat of the attractive 2-day-old *iav* males used here is unknown. However, the observation that young *iav* males exhibit extended attractiveness supports the hypothesis that the *iav* mutation delays post-eclosional maturation.

Circadian rhythm

According to Livingstone & Tempel (1983), the circadian rhythm mutant *period* (Konopka & Benzer, 1971) has a small deficit in octopamine. If octopamine is the cause rather than a consequence of the aberrant circadian rhythm then one might reasonably expect *iav* mutants, which have only 15 per cent wild type octopa-

mine (O'Dell *et al.*, 1987), to exhibit a rhythmless phenotype. However, wild type and *iav* mutant eclosion rhythms are indistinguishable, and given that *iav* flies also show a normal locomotor rhythm (B. Kyriacou, personal communication), it is unlikely that octopamine has any causative role in the expression or maintenance of normal circadian rhythm. The small deficit in octopamine in *per* mutants is therefore unlikely to have a causative role in the expression of their aberrant rhythm phenotype.

Effect of *p*-Cresol on survival

The observation that *iav* mutants have only 15 per cent of the wild type levels of octopamine but normal dopamine titres (O'Dell *et al.*, 1987), suggests that the *iav* gene product may be involved in the synthesis of octopamine from tyrosine, presumably via tyramine (see review by Wright, 1987). A candidate gene product for the *iav* gene would therefore be tyramine β -hydroxylase (TBH), the enzyme that converts tyramine into octopamine.

In invertebrates, TBH is thought to play a role analogous to that of dopamine β -hydroxylase (DBH) in mammals (see Fig. 5). Indeed the TBH and DBH enzymes are thought to share evolutionary history, an observation that is supported by the characterization of lobster TBH by Wallace (1976). As a consequence of this similarity in DBH and TBH function and ancestry, it may be possible to use inhibitors of vertebrate DBH to investigate the alleged role of TBH in *iav* mutants.

Work on mechanism-based inactivation of the bovine DBH enzyme has revealed two inhibitors of enzyme activity, *p*-Cresol (Goodhart *et al.*, 1987) and

β -ethynyltyramine (DeWolf *et al.*, 1989). *p*-Cresol is a simple structural analogue of dopamine which lacks a basic side chain nitrogen and binds DBH in a mechanism that is indistinguishable from normal dopamine substrate binding (Goodhart *et al.*, 1987). If *p*-Cresol binds to and inactivates *Drosophila* TBH in a manner analogous to that for bovine DBH, then flies deficient for TBH may find it more toxic than do wild type flies.

All the lines that show susceptibility to *p*-Cresol have a mutation in the *iav* gene (*iav*, *iav*² and *iav m f*). Heterozygous *iav*⁺/*iav* females show wild type levels of resistance to *p*-Cresol, confirming the recessive nature of the *iav* allele. Given that other phenotypically inactive lines, such as La Mancha and *w*¹¹¹⁸, show wild type survival rates, the mode of action of *p*-Cresol must be a consequence of mutations in the *iav* gene rather than a consequence of their inactive phenotype.

These data support both the notion that an octopamine deficit is associated with the *iav* phenotype and the possibility that TBH is the *iav* gene product. Alternatively, *p*-Cresol may mediate its effect by mimicking and out-competing the action of octopamine. In *iav* mutants, the relative scarcity of octopamine would allow *p*-Cresol to mediate its toxic effect at lower concentrations. It therefore seems likely that the behavioural abnormalities associated with the *iav* mutation are a consequence, rather than a cause of a deficit in octopamine.

Acknowledgements

This work was made possible by the award of a Wolfson Special Grant to David Sherratt FRS. The author would also like to thank Barrie Burnet, David Roberts, Bambos Kyriacou, Jane Davies and Marshall Stark whose help and advice is gratefully acknowledged.

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Fig. 5 Reactions catalysed by dopamine β -hydroxylase (DBH) and tyramine β -hydroxylase (TBH).

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