

Drosophila as a new model organism for the neurobiology of aggression?

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Summary

We report here the effects of several neurobiological determinants on aggressive behaviour in the fruitfly *Drosophila melanogaster*. This study combines behavioural, transgenic, genetic and pharmacological techniques that are well established in the fruitfly, in the novel context of the neurobiology of aggression. We find that octopamine, dopamine and a region in the *Drosophila* brain called the mushroom bodies, all profoundly influence the expression of aggressive behaviour.

Serotonin had no effect. We conclude that *Drosophila*, with its advanced set of molecular tools and its behavioural richness, has the potential to develop into a new model organism for the study of the neurobiology of aggression.

Key words: *Drosophila melanogaster*, aggression, fighting behaviour, amine, mushroom body.

Introduction

Drosophila is the 'jack of all trades' in biology, but has not been studied in the context of the neurobiology of aggression. The fruitfly exhibits aggressive behaviour (Jacobs, 1960) and this behaviour is ethologically well characterized (Dow and von Schilcher, 1975; Jacobs, 1978; Lee and Hall, 2000; Skrzipek et al., 1979). The evolutionary relevance of this aggressive behaviour is also well established (Boake and Hoikkala, 1995; Boake and Konigsberg, 1998; Boake et al., 1998; Dow and von Schilcher, 1975; Hoffmann, 1988, 1989, 1994; Hoffmann and Cacoyianni, 1989; Ringo et al., 1983; Skrzipek et al., 1979; Zamudio et al., 1995). Finally, the ecological circumstances under which *Drosophila* exhibits territoriality and aggression have been examined in great detail (Hoffmann, 1987, 1988, 1989, 1994; Hoffmann and Cacoyianni, 1989, 1990). Under appropriate conditions, male flies try to occupy a food patch and defend it against other males, even in the laboratory. However, this aggressive behaviour in *Drosophila* has escaped the notice of most neurobiologists. Here we report the combination of ethological, ecological and evolutionary knowledge with molecular, genetic and pharmacological tools to manipulate the aggressive behaviour of *Drosophila melanogaster*.

To our knowledge, only two genetic factors have been reported to affect aggressive behaviour in *Drosophila*: the sex-determination hierarchy (SDH) and the β -alanine pathway. *fruitless* (*fru*) and *dissatisfaction* (*dsf*) mutants have been described as more aggressive than wild-type controls (Lee and Hall, 2000). Both genes are part of the SDH. Flies carrying mutant alleles of the *black* (*b*) gene appear less aggressive,

whereas *ebony* (*e*) mutants appear more aggressive (Jacobs, 1978). The enzymes encoded by the two genes regulate β -alanine levels (*b* flies have reduced and *e* flies elevated levels).

It is straightforward to expect genes of the SDH to affect sex-specific behaviours, but the pathways by which they modulate that behaviour are largely unknown. One possibility could be *via* the regulation of small neuroactive molecules (such as β -alanine and the biogenic amines) and their receptors. Biogenic amines play a key role in the regulation of aggressive behaviour, not only in vertebrates, but also in arthropods (e.g. Edwards and Kravitz, 1997; Heinrich et al., 1999, 2000; Huber et al., 1997a,b; Kravitz, 2000; Schneider et al., 1996; Stevenson et al., 2000). The biogenic amine system in flies is well described (see Monastirioti, 1999). Most serotonin and dopamine mutants in *Drosophila* are either lethal or affect both serotonin and dopamine, due to their shared pathways of synthesis (e.g. Johnson and Hirsh, 1990; Lundell and Hirsh, 1994; Shen et al., 1993; Shen and Hirsh, 1994). However, established protocols are commonly used to manipulate the levels of these amines individually in the adult fly (Neckameyer, 1998; Vaysse et al., 1988). Octopamine null mutants have been generated and characterized (Monastirioti et al., 1996). Interestingly, certain octopamine and dopamine receptors are preferentially expressed in a prominent neuropil in the *Drosophila* brain called the mushroom bodies (Han et al., 1996, 1998). Thus, all of the prerequisites for a systematic analysis of the neurobiological factors involved in the expression of aggressive behaviour are available: (1) a considerable body of knowledge about the behaviour and its

ecological context, (2) circumstantial evidence about possible neurobiological factors involved in regulating the behaviour, and (3) methods for manipulating these factors and for quantifying the behaviour.

As a first attempt to characterize the effects of various possible neurobiological factors that might regulate aggression, we report here the results of a competition experiment. Six male flies competed for a food patch and three mated females. The experimental males were manipulated in one of various ways: by a classical mutation affecting β -alanine levels, a P-element mutation affecting octopamine levels, or insertion of transgenes affecting synaptic output from the mushroom bodies, or by pharmacological treatment affecting serotonin or dopamine levels, and then tested for their aggressive behaviour.

Materials and methods

Flies

Animals were kept on standard cornmeal/molasses medium (for recipe, see Guo et al., 1996) at 25 °C and 60% humidity with a 16h:8h light:dark regime, except where noted. The females in all experiments were mated wild-type Canton S flies.

Mutants

Black¹ and *ebony¹* mutant strains from the laboratory's 18 °C stock collection (provided by S. Benzer in 1970) were kept at 25 °C for at least two generations. The M18 P-element octopamine mutant and control stocks (Monastirioti et al., 1996) were kept at 25 °C for two generations after arrival.

Transgenes

Sweeney et al. (1995) developed a method that constitutively blocks synaptic transmission by expressing the catalytic subunit of bacterial tetanus toxin (Cnt-E) in target neurons in the *Drosophila* brain using the P[GAL4] technique (Brand and Perrimon, 1993). Inspired by the preferential expression of certain dopamine and octopamine receptors in the mushroom bodies (Han et al., 1996, 1998), we used the Cnt-E transgene to block synaptic output from the mushroom bodies (Sweeney et al., 1995). Expression of another transgene, an inactive form of the tetanus toxin light chain (*imp-tntQ*), controlled for deleterious effects of protein overexpression (Sweeney et al., 1995). The P[GAL4] line mb247 (Schulz et al., 1996) served as a mushroom body-specific GAL4 driver (Zars et al., 2000) for both toxins. The trans-heterozygote offspring from the GAL4 driver strain and the two UAS_{GAL4} reporter strains (Cnt-E and *imp-tntQ*) entered the study.

Pharmacological treatments

Drosophila from the wild-type strain Berlin (*wtb*) were treated as described by Neckameyer (1998) and Vaysse et al. (1988). Briefly, the animals were fed a sucrose solution containing either 10 mg ml⁻¹ of the serotonin precursor 5HTP (5-hydroxy-tryptophan) or 10 mg ml⁻¹ of the serotonin

synthesis inhibitor pCPA (para-chlorophenylalanine) to manipulate serotonin levels. Effectiveness of the treatment was verified qualitatively with standard immunohistochemical techniques using rabbit serotonin antisera (data not shown; Buchner et al., 1986, 1988). Alternatively, the animals were treated with 1 mg ml⁻¹ of the dopamine precursor L-DOPA (L-3,4-dihydroxyphenylalanine) or 10 mg ml⁻¹ of the dopamine synthesis inhibitor 3IY (3-iodo-tyrosine) to manipulate dopamine levels. Effectiveness of the treatment was verified by observation of cuticle tanning. A dose of 10 mg ml⁻¹ L-DOPA was lethal, confirming unpublished data from Wendy Neckameyer (St Louis University School of Medicine).

Experimental groups

Using the different stocks described above, we arranged six different groups of 'low' versus 'high' males, such that the respective amine or the amount of synaptic output from the mushroom bodies was manipulated to produce relative high- and low-level subgroups.

(1) *Wild-type Berlin (wtb)*

Wild-type Berlin flies are randomly assigned to a 'high' or a 'low' group. No difference between the subgroups is expected (**negative control**).

(2) *Serotonin (5ht)*

(a) Wild-type Berlin with 10 mg ml⁻¹ 5HTP in sucrose solution. This treatment produces high levels of serotonin (5ht+).

(b) Wild-type Berlin with 10 mg ml⁻¹ pCPA in sucrose solution. This treatment produces low levels of serotonin (5ht-).

(3) *Octopamine (oa)*

(a) M18 P-element parental stock, from which the jump-out below was generated (red eyed). This strain has normal levels of octopamine (Monastirioti et al., 1996) and will be denoted the 'high' subgroup (oa+).

(b) M18 jump-out mutants. As tyramine-beta-hydroxylase (octopamine-producing enzyme) null mutants (white eyed), these flies have no detectable octopamine (Monastirioti et al., 1996) and will be denoted the 'low' subgroup (oa-).

(4) *Dopamine (da)*

(a) Wild-type Berlin with 1 mg ml⁻¹ L-DOPA in sucrose solution. This treatment produces high levels of dopamine (da+).

(b) Wild-type Berlin with 10 mg ml⁻¹ 3-iodo-tyrosine in sucrose solution. This treatment produces low levels of dopamine (da-).

(5) *β -alanine (b/e)*

(a) *ebony* mutants with high β -alanine levels (*e*).

(b) *black* mutants with low β -alanine levels (*b*). This group serves as the **positive control**, as it is known that *e* flies are more aggressive than *b* flies (Jacobs, 1978).

Table 1. *Experimental time-course*

| | Day | | | | | | | | | | |
|--------------|-----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| Put in vials | 5ht | oa | wtb | mb | da | b/e | | | | | |
| | wtb | da | b/e | 5ht | oa | mb | | | | | |
| Mark | | | | | 5ht | oa | wtb | mb | da | b/e | |
| | | | | | wtb | da | b/e | 5ht | oa | mb | |
| Record | | | | | | 5ht | oa | wtb | mb | da | b/e |
| | | | | | | wtb | da | b/e | 5ht | oa | mb |

Two groups were treated in separate vials but in parallel each experimental day. Each group was treated in two replicates, starting with different flies on different days.

For abbreviations see Materials and methods.

(6) *Mushroom bodies (mb)*

(a) Offspring of P[GAL4] line mb247 with the UAS-IMP-tntQ line. This strain has normal levels of synaptic output from the mushroom bodies and will be referred to as the 'high' subgroup (mb+).

(b) Offspring of P[GAL4] line mb247 with the UAS-Cnt-E line. This strain has no synaptic output from the mushroom bodies and will be called the 'low' subgroup (mb-).

Thus, we arranged four experimental groups and two control groups. For each group, the two subgroups ('high' and 'low') compete against each other in one recording chamber. Each group was tested twice with different animals.

Recording chambers

Aggression was studied in cylindrical cages similar to those used by Hoffmann (1987), i.e. 100 mm Petri dishes, top and bottom separated by a 40 mm high spacer (i.e. a cylindrical chamber of 100 mm diameter and 40 mm height). The bottom of the chamber was filled with 2% agar to moisturize the chamber. Flies were introduced by gentle aspiration through a small hole in the spacer. A food patch (10 mm diameter, 12 mm high) was positioned in the centre of the chamber, containing a mixture of minced 2% agar, apple juice, syrup and a live yeast suspension (after Reif, 1998), filled to the level of the rim of the containing vial. The chamber was placed in a Styrofoam box (used to ship biochemical reagents on dry ice; outer measurements: 275×275 mm, height, 250 mm; inner measurements: 215×215 mm, height, 125 mm) to standardize lighting conditions and to shield the chambers from movements by the experimenters. Two Styrofoam boxes with one chamber each were arranged underneath video cameras, focused on the food patch in a darkened room at 25 °C. Ring-shaped neon-lights (Osram L32W21C, power supply Philips BRC406) on top of the boxes provided homogenous illumination throughout the experiment.

Experimental time course

The stocks were treated completely in parallel (see Table 1). A 5% sucrose solution (in *Drosophila* ringer) with or without added treatment was pipetted onto 5 pieces of filter paper

snugly fitting in cylindrical (12×40 mm) vials before transferring newly eclosed (0–24 h) male flies into the vials. The flies were transferred into new vials with new solution and new filter paper on a daily basis for 5 days. Each group was treated in two replicates, starting with new flies on different days (see Table 1). On the fifth day, 4–6 flies per subgroup were briefly immobilised on a cold plate and marked with one small dot on the thorax in either green or white acrylic paint. At 08.00 h (1 h after lights-on) on the sixth day, the animals of the two groups treated in parallel were transferred into the recording chambers (three mated, but otherwise untreated, Canton S females, and six males, three from each paired subgroup) and placed underneath the video cameras under identical conditions to those used during the recording time, except that the video recorders (VCRs) were turned off. Continuing the parallel treatment of two groups per day, two video set-ups were used simultaneously ('left' and 'right'). After an acclimatisation period of 2 h, the VCRs were set to record. For each group, we recorded 4 h of fly behaviour, once in each location (yielding the two replicates for each group), resulting in 12 video tapes (see Table 2). Data from both replicates were pooled. Since each group was measured twice with six (3+3) experimental animals (males) for each recording, the total number of observed males was 6 animals×2 replicates×6 groups=72. Recording of the experiments was randomised across days.

Behavioural scoring

Only male–male interactions were counted. Mated females lose their receptivity to male advances and the males cease courting quickly, refraining from courting for a number of hours (courtship conditioning; e.g. Greenspan and Ferveur, 2000). Little courtship behaviour was thus observed after the acclimatisation period.

Behavioural scoring was done blind, before the colour codes on the flies' thoraces were decoded into 'high' and 'low'. An interaction between two males was classified as either aggressive or non-aggressive as defined by Hoffmann (1987). Briefly, we classified encounters that contained the previously described boxing, head-butting, lunging, wrestling, tussling, charging and chasing behaviours (Dow and von Schilcher,

Table 2. *Colour codes and recording dates*

| Day | Number | Left | Number | Right |
|-----|--------|---------------------------|--------|---------------------------|
| 6 | 1 | 5ht+, green / 5ht-, white | 2 | wtb |
| 7 | 3 | oa+, green / oa-, white | 4 | da+, green / da-, white |
| 8 | 5 | wtb | 6 | e, green / b, white |
| 9 | 7 | mb-, green / mb+, white | 8 | 5ht+, green / 5ht-, white |
| 10 | 9 | da+, green / da-, white | 10 | oa+, green / oa-, white |
| 11 | 11 | e, green / b, white | 12 | mb-, green / mb+, white |

Each group was measured twice, once under each camera with different flies. Each of the 12 experiments was saved on individually numbered, 4 h video tapes. This table was used to break the code after the behavioural scoring had been done blindly.

For abbreviations see Materials and methods.

1975; Hoffmann, 1987, 1988, 1989, 1994; Hoffmann and Cacoyianni, 1989, 1990; Jacobs, 1978; Skrzipek et al., 1979) as aggressive. Encounters that only contained approach, leg contact, wing vibration or wing flapping were classified as non-aggressive. If the encounter was classified as aggressive, it was straightforward to discern the aggressor as one animal attacking and/or chasing the other. Non-aggressive encounters could usually not be classified directionally. Thus, with three 'high' and three 'low' animals in the recording chamber, any interaction between them falls into seven categories, listed below:

- (1) High attacks, high aggressive encounter (1ag)
- (2) High attacks, low aggressive encounter (2ag)
- (3) High/high, non-aggressive encounter (3nonag)
- (4) High/low, non-aggressive encounter (4nonag)
- (5) Low/low, non-aggressive encounter (5nonag)
- (6) Low attacks, high aggressive encounter (6ag)
- (7) Low attacks, low aggressive encounter (7ag)

This design thus yielded seven values, one for each of the respective interaction categories, giving each of the six groups a characteristic aggression profile (Fig. 1A).

Data analysis

A log-linear analysis (delta=0.005, criterion for convergence=0.0005, maximum iterations 500) was performed over the 6×7 table of observed behavioural frequencies to determine the effect of the treatments on the distribution of behavioural classes. To normalize for the total number of encounters, two derived parameters were computed from the raw data. The first is the likelihood that an individual of one subgroup will attack during an encounter (attack probability, P_A). It is calculated as the fraction of all encounters in that group involving a 'high' (or 'low', respectively) animal, where such an animal was the aggressor:

$$P_A = \frac{\text{Number of 'subgroup' attacking encounters}}{\text{Number of encounters with 'subgroup' participation}}, \quad (1)$$

i.e.:

$$P_{A,\text{high}} = \frac{1ag + 2ag}{1ag + 2ag + 3nonag + 4nonag + 6ag} \quad (2)$$

and

$$P_{A,\text{high}} = \frac{6ag + 7ag}{2ag + 4nonag + 5nonag + 6ag + 7ag}. \quad (3)$$

Thus, P_A describes the probability that a given individual will act aggressively against any other individual it encounters. The second derived parameter assesses the representation of each subgroup in the total number of encounters (encounter probability, P_E). It is calculated analogously to the first parameter as the fraction of all encounters in a group, where an animal of a specific subgroup (i.e. 'high' or 'low') participated:

$$P_E = \frac{\text{Number of encounters with 'subgroup' participation}}{\text{Total number of encounters in the group}}, \quad (4)$$

i.e.:

$$P_{E,\text{high}} = \frac{1ag + 2ag + 3nonag + 4nonag + 6ag}{1ag + 2ag + 3nonag + 4nonag + 5nonag + 6ag + 7ag} \quad (5)$$

and

$$P_{E,\text{low}} = \frac{2ag + 4nonag + 5nonag + 6ag + 7ag}{1ag + 2ag + 3nonag + 4nonag + 5nonag + 6ag + 7ag}. \quad (6)$$

Thus, P_E describes the probability that an individual of one subgroup will be a participant in an encounter.

While P_A can be said to describe the level of aggression of a certain subgroup, P_E can be perceived as a control measure for the overall number of interactions in that subgroup, as influenced by, for example, general activity, visual acuity, etc. After the data transformation, the resulting probabilities were tested against random distribution using χ^2 tests.

Results

We performed two 4 h experiments with four experimental and two control groups in each experiment. In all, 48 h of video tape were analysed containing 9881 encounters (an average of 3.4 encounters min⁻¹ or 137.2 encounters male⁻¹). The two 4 h experiments were pooled for each group, yielding one 7-score aggression profile for each group (Fig. 1A). A log-linear analysis over the six groups and the seven behavioural classes yields a $P < 0.0001$ (Pearson $\chi^2 = 6479.426$, d.f. = 30), suggesting the various treatments were effective in changing the proportions of the different classes of encounters in each group.

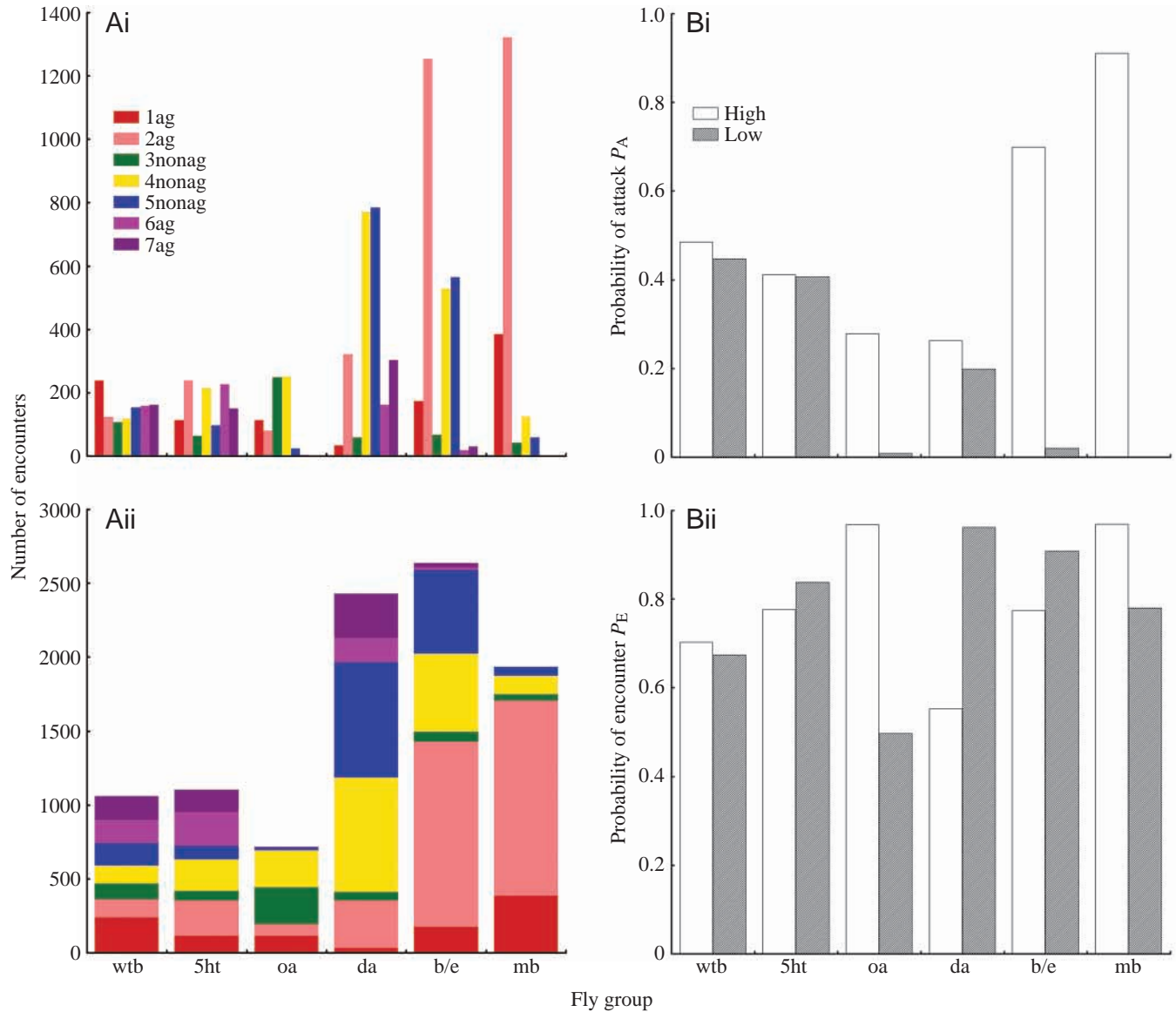


Fig. 1. Raw and derived data from all six groups. (A) Raw behavioural scores. Two different graphs depict the same data in order to facilitate the interpretation of the complex data structure obtained from our experiments. (Ai) Multiple bars graph, (Aii) single bar graph. *1ag*, high attacks, high aggressive encounter; *2ag*, high attacks, low aggressive encounter; *3nonag*, high/high attacks, non-aggressive encounter; *4nonag*, high/low attacks, non-aggressive encounter; *5nonag*, low/low attacks, non-aggressive encounter; *6ag*, low attacks, high aggressive encounter; *7ag*, low attacks, low aggressive encounter. (B) Derived probabilities. (Bi) The probability of attacking P_A . For each subgroup (*high*, *low*) the fraction of encounters where a member of that subgroup was the aggressor is calculated from the total number of subgroup encounters. (Bii) The probability of an encounter P_E . For each subgroup (*high*, *low*) the fraction of encounters (irrespective of classification) in which a member of that subgroup participated is calculated from the total number of encounters. Wtb, wild-type Berlin; 5ht, serotonin; oa, octopamine; da, dopamine; b/e, β -alanine; mb, mushroom bodies. See Materials and methods for details of behavioural classification and fly groups.

The raw data (Fig. 1A), reveal that the two control groups behaved according to our expectations. The wtb negative control shows a uniform distribution of aggressive encounters, whereas the β -alanine positive control is skewed towards the mutants with high levels of β -alanine (Fig. 1Ai).

The clearest effects among experimental groups were obtained from the octopamine mutants and the mb group. Both octopamine null mutants (*oa*⁻) and animals with inhibited mushroom bodies (*mb*⁻) are virtually non-aggressive

(Fig. 1A). In Fig. 1Aii, the octopamine group seems similar to the wild-type control except for the missing values for *6ag* and *7ag*. However, while the *oa*⁺ animals appear to show a wild-type level of aggression, the *mb*⁺ animals show elevated levels of aggression compared to all other groups (Fig. 1A).

It also appears that our serotonin treatment had little effect on aggression (Fig. 1A).

The dopamine treatment appears to be somewhat effective in decreasing the number of aggressive encounters in animals with

Table 3. χ^2 -Statistics for derived probabilities

| Fly group | Probability of attack P_A | | Probability of encounter P_E | |
|------------------|------------------------------|----------------|--------------------------------|----------------|
| | χ^2 (Yates; d.f., 1) | P (Yates) | χ^2 (Yates; d.f., 1) | P (Yates) |
| Wild-type Berlin | 0.01 | =0.92 | 1.85 | =0.17 |
| Serotonin | 0.31 | =0.58 | 13.11 | <0.0003 |
| Octopamine | 92.33 | <0.0001 | 403.71 | <0.0001 |
| Dopamine | 36.62 | <0.0001 | 1109.17 | <0.0001 |
| β -alanine | 2080.64 | <0.0001 | 177.13 | <0.0001 |
| Mushroom bodies | 3061.61 | <0.0001 | 315.84 | <0.0001 |

high levels of dopamine, while the animals with low levels of dopamine seem to have numbers of aggressive encounters similar to, if not slightly higher than, the wild-type controls. Obviously, the number of non-aggressive encounters in the dopamine-treated animals is strongly elevated (Fig. 1A). Interestingly, the two subgroups show inverted profiles for intra- and inter-subgroup aggression (i.e. 1ag/2ag and 6ag/7ag).

The total number of encounters also varies considerably between the different treatments (Fig. 1Aii).

With significant effects of our treatments on the distribution of the behaviours within each group, we can process the data in order to determine the effect of our treatments on the propensity of the animals to become aggressive. The fraction of all encounters involving a 'subgroup' animal, where such an animal was the aggressor, is calculated (Fig. 1Bi; P_A , see Materials and methods). The P_A value allows us to estimate the effects of the treatments on aggression. χ^2 tests can be computed on P_A values to test the null hypothesis that our treatments had no effect on the probability of the fly being aggressive. Table 3 summarizes the χ^2 results for all six groups. The statistics confirm the effects already visible in the raw data (Fig. 1A): the two control groups (wtb and b/e) were consistent with our expectations. The obvious effect of octopamine null mutants being completely non-aggressive is corroborated by our statistical analysis, as are the extreme effects of expressing active and inactive tetanus toxin, respectively, in the flies' mushroom bodies (Fig. 1Bi). The serotonin treatment had no significant effect on the probability of the flies becoming aggressive during an encounter, despite the fact that we could verify the effectiveness of the treatment immunohistochemically (data not shown). The group in which the dopamine levels were manipulated shows a moderate, but statistically reliable, effect of high dopamine levels leading to a higher probability to attack in an encounter.

Despite the fact that most of our treatments have a record of influencing aggression in other animals, the possibility exists that the different treatments may have altered the number of aggressive encounters indirectly by altering the total number of encounters, through other factors such as general activity, visual acuity, etc. The distribution of encounters over the subgroups, P_E , should reveal such candidate variables. For instance, if the treatment rendered the animals of one subgroup inactive, the P_E of that subgroup should be smaller than the P_E of the other subgroup. If the obtained aggression scores were but a reflection

of asymmetric P_E values, they should follow the pattern of P_E asymmetry. Fig. 1Bii depicts the distribution of encounters over the two subgroups, independently of encounter classification. Again, χ^2 statistics were performed and summarized in Table 2. All treatments led to a significant asymmetry in P_E between subgroups, with the exception of the negative wtb controls. However, the pattern of asymmetry does not seem to match the pattern of asymmetry in the level of aggression (see Discussion).

Discussion

Most importantly for this first study of the effects of various treatments on *Drosophila* aggression, the animals in the control groups behaved exactly as expected: no differences were detected among the subgroups of the wtb negative control, and previously published higher aggression levels in the *ebony* (high β -alanine) than in the *black* (low β -alanine) flies (Jacobs, 1978) could be reproduced. These findings corroborate our pilot studies in which we repeatedly observed the same pattern (A. Baier, B. Wittek and B. Brembs, unpublished data).

Octopamine null mutants exhibit strongly reduced aggression, as do flies with low levels of synaptic output from their mushroom bodies. Interestingly, certain types of octopamine and dopamine receptors are preferentially expressed in the mushroom bodies of wild-type flies (Han et al., 1996, 1998). It is tempting to interpret this phenocopy of the octopamine mutants as resulting from Kenyon cells being the major regulators of octopamine- (and/or dopamine-) mediated aggression. Recently, temperature sensitive *shibire^{ts1}* constructs have been developed to conditionally block synaptic transmission (e.g. Dubnau et al., 2001; Kitamoto, 2001; McGuire et al., 2001; Waddell et al., 2000). Unfortunately, at the time of our experiments, the *shibire^{ts1}* constructs were not yet available. Future experiments definitely should include *shibire^{ts1}* constructs in order to replicate our mb- results, examine the high levels of aggression in the mb+ flies and look for other brain areas involved in aggression. Replication of our results using the *shibire^{ts1}* constructs would also eliminate the possible explanation that the expression of tetanus toxin anywhere in the fly's brain abolishes aggressive behaviour and solve the problem of UAS promoter leakiness. The octopamine result is conspicuous in another respect: it is consistent with studies in crickets, where depletion of octopamine and dopamine decreases aggressiveness (Stevenson et al., 2000), but contrasts with studies in crustaceans, where high octopamine levels tend to bias behaviour towards submissiveness (Antonsen and Paul, 1997; Heinrich et al., 2000; Huber et al., 1997a).

The high aggression observed in the mb+ animals is difficult to interpret. In principle, the inactive toxin should not have any effect on the secretion of neurotransmitter at the synapse. More likely is an insertion effect of the P-element containing the *impntQ* transgene. In that case it would be extremely interesting to characterize the genetic environment within which the P-element lies in order to find the gene responsible for such aggressiveness. One may argue that high aggressiveness by flies of one subgroup may produce low aggression in the respective

other subgroup. In the case of the mb group, this is unlikely, because there still should be at least some aggression between mb- animals, even if mb+ animals attacked every other male they encountered. Moreover, mb- animals seemed unaffected by the repeated attacks from mb+ males and kept coming back to the patch soon after an mb+ male chased it off (the reason for the high 2ag value in Fig. 1). However, mb- animals were never observed to be the aggressor. It thus seems more likely that the high frequency of attacks by mb+ males is due to a combination of high levels of aggression due to insertion effects of the imp-tntQ transgene and returning mb- males repeatedly eliciting aggressive behaviours in the mb+ males.

Our serotonin treatment has no significant effect on aggression, despite the fact that we could verify the effectiveness of the treatment immunohistochemically (data not shown). Also, Vaysse et al. (1988) observed effects on learning and memory after identical treatment, indicating that this pharmacological manipulation of serotonin levels in principle can have behavioural effects. Moreover, we observed a noticeable increase in activity in the 5ht- flies, a subjective impression that is corroborated by the significantly increased P_E of this subgroup (Fig. 1Bii). Nevertheless, the possibility remains that the observed difference in serotonin immunoreactivity was not high enough to generate significant differences in aggression, although it was high enough to affect other behaviours. The lack of serotonergic effect on aggression was also repeatedly observed in our pilot studies (A. Baier, B. Wittek and B. Brembs, unpublished data). Lee and Hall (2001) have reported that the pattern of serotonergic cells in the *Drosophila* brain is unaltered in the more aggressive fru mutants, confirming the idea that serotonin is not crucial for regulation of aggressive behaviour in the fly. The serotonin results presented here are also consistent with data in crickets, where serotonin depletion appears to have no effect (Stevenson et al., 2000); they contrast with data in crustaceans, where injections of serotonin increase the level of aggressive behaviour (Edwards and Kravitz, 1997; Huber et al., 1997a,b; Kravitz, 2000). Our serotonin data thus parallel our octopamine data in conforming with insect data but contrasting with observations in crustaceans. Perhaps aminergic control of aggression functions fundamentally differently in those two arthropod groups?

Our dopamine treatment had complex effects. The absolute number of non-aggressive encounters appears elevated compared to the wild-type controls (Fig. 1A), reducing overall aggression probabilities (Fig. 1Bi; P_A). Also, while the raw data indicate higher aggression scores in the animals with low dopamine (Fig. 1Ai), the P_A is higher in animals with high dopamine levels (Fig. 1Bi). Taking the number of encounters that each subgroup experiences (Fig. 1Bii, P_E) into account, it seems as if the higher raw scores for the 'low' dopamine animals is generated by the higher P_E in this subgroup. Once that factor is accounted for (Fig. 1Bi), the perceived difference between raw and derived data disappears.

A general point of concern is possible side effects of our treatments. Both *e* and *b* flies exhibit varying degrees of visual impairment (A. Baier, B. Wittek and B. Brembs, unpublished

data; Heisenberg, 1971, 1972; Hovemann et al., 1998; Jacobs, 1978), with *e* flies showing more severe defects than *b* flies (A. Baier, B. Wittek and B. Brembs, unpublished data; Jacobs, 1978). Without screening pigments (i.e. *white*⁻), the M18 octopamine jump-out mutants are expected to have severely impaired vision compared with the control strain still carrying the P-element. Also, the extent by which the treatments may affect general activity is largely unknown (but see Martin et al., 1998). One may assume that a subgroup's P_E should reflect overall activity. Not surprisingly, the more visually impaired *e* and *oa*- flies have lower P_E values than the *b* and *oa*+ subgroups, respectively (Fig. 1Bii). However, the probability to attack seems entirely unaffected by this measure of general activity, as the relationships are reversed. Moreover, both the dopamine and the mushroom body groups show a higher probability to attack in the respective 'high' subgroup (Fig. 1Bi), but their P_E values are inverted with respect to their P_A values (Fig. 1Bii). Thus, while both vision and general activity may influence aggression, those factors seem to have only marginal effects compared to the determinants studied here.

Of course, this study is only a beginning. We did not examine encounter duration, behavioural composition or opponent identity/recognition, let alone investigate potential mechanisms as to how the identified factors might exert their effects. However, our method successfully reproduced published data (the *e/b* group) and yielded new insights into the neurobiological determinants of aggression in *Drosophila melanogaster*. Serotonin appears to have no effect, while dopamine, octopamine and the mushroom bodies could be linked to the promotion of aggressive behaviour. We hope that our work will inspire others to exploit *Drosophila*'s numerous technical advantages for studying the neurobiology of aggression.

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