Drosophila Mutants that Are Motile but Respond Poorly to All Stimuli Tested

These may have a defect in interaction with stimuli or in executive function.

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ABSTRACT

Adult *Drosophila melanogaster* fruit flies were placed into one end of a tube near to repellents (benzaldehyde and heat) and away from the other end containing attractants (light and a favored temperature). They escaped from the repellents and went to the attractants. Motile mutants that failed to do that were isolated. They did not respond to any external attractants tested or external repellents tested. In addition, they did not respond well to internal stimuli like hunger, thirst, and sleep. Some possibilities for explaining the deficiency: They may be defective in decision making that results from faulty interaction with the sensing mechanism, or they may be defective in executive function. The mutants failed at both 34°C and, in one case studied, at room temperature.

I. INTRODUCTION

The mechanism of behavior is summarized in Fig.1.

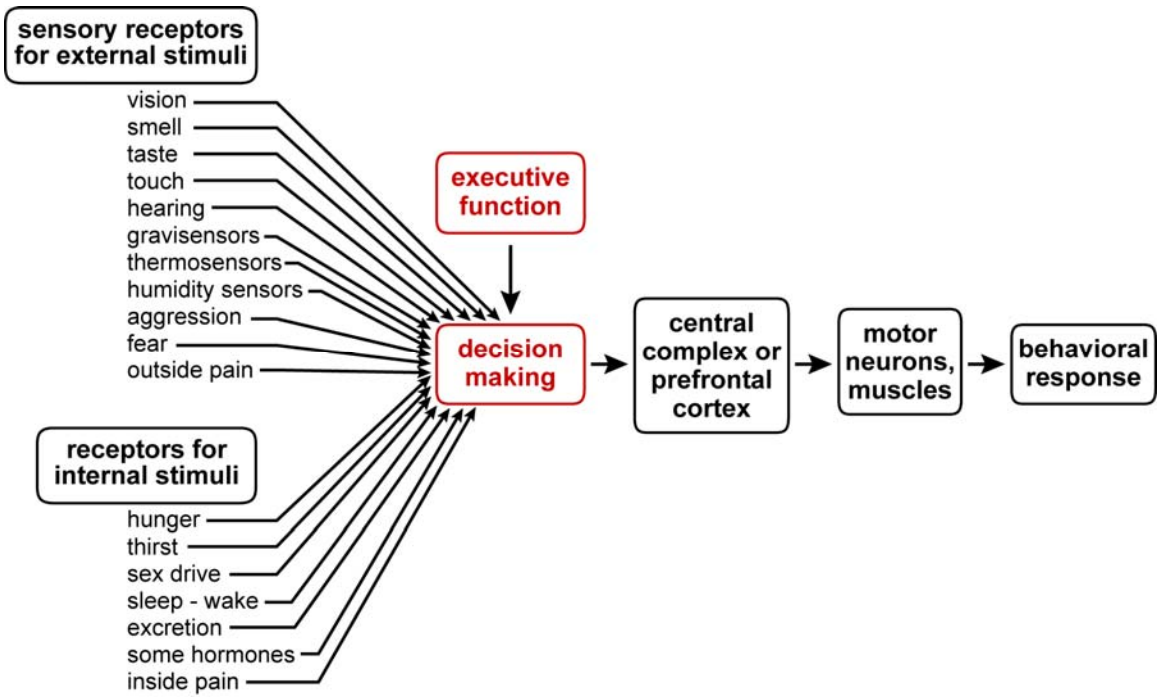

Fig 1. A scheme of how external and internal stimuli might yield a behavioral response. Hypothetical receptors act on decision making (in red). Decision making is controlled by executive function [1, 2] (in red). Information then goes on to the central complex in insects (S1A Fig) or the prefrontal cortex (S1B Fig) plus basal ganglia (S1C Fig) in vertebrates. This result is sent by means of motor neurons and muscles to generate a behavioral response. A variety of stimuli has been described [3-5].

To study the mechanism of behavior, one can isolate mutants and explore their properties. In this report we describe the isolation and study of *Drosophila* mutants that are motile and able to reproduce but they are poorly responsive to all stimuli tested, both external and internal stimuli. Some possibilities for explaining this are an abnormal relation between decision making and reception or an abnormal relation between decision making and executive function [1, 2]. See Discussion. A preliminary report of some of the results has been presented [3, 6].

II. RESULTS

A. RESPONSES TO EXTERNAL STIMULI

1. RESPONSE TO STIMULI USED TOGETHER

In a 34°C dark room flies were started near two repellents (0.1M benzaldehyde and 37°C) at one end of a tube, away from two attractants (light at 1000 lux and 27°C) at the other end (Fig 2). The parent responded by going away from the repellents and to the

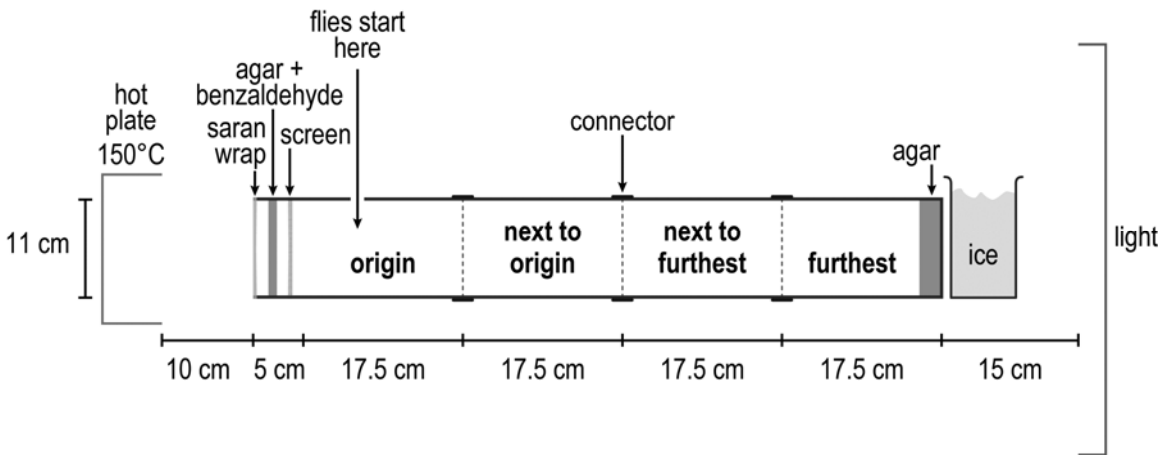

Fig 2. Apparatus for isolating and testing mutants in a 34°C room. At the left end were repulsive 0.1M benzaldehyde and repulsive 37°C (due to a hot plate at 150°C). At the right end were attractive light (1000 lux) and attractive 27°C (due to ice water). The middle was close to 34°C.

attractants (Fig 3A). Mutants that were not motile were rejected, only the motile mutants were studied. This consisted of five mutants, named 1 to 5. Fig 3B shows that such a mutant (Mutant 2) failed to respond when the four stimuli are together. S3 Fig shows that the other four mutants (Mutants 1, 3, 4, and 5) also failed to respond to the four stimuli together.

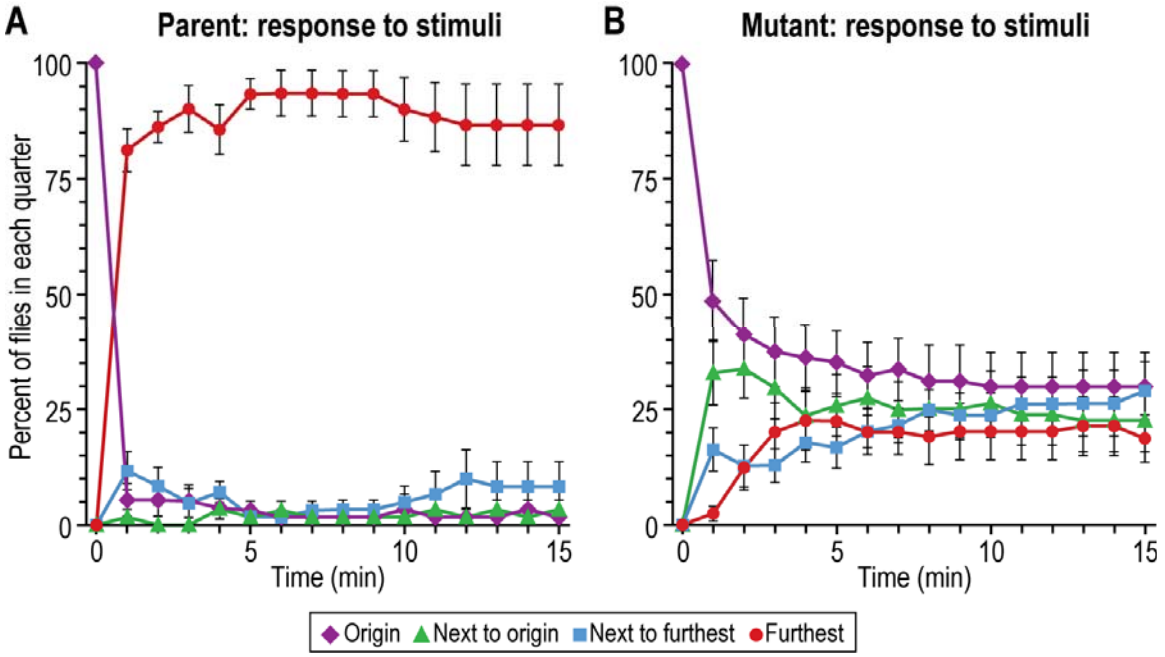

Fig 3. Response to stimuli used together. Repellents (0.1M benzaldehyde and high temperature (37°C) were at the origin end, attractants (light, 1000 lux, and a favored temperature (27°C) at the furthest end. (**A**) Parental response (n=7). (**B**) Mutant 2 (n=8). Flies were tested in a 34°C room with 10 to 20 flies used per trial. Data are mean±SEM.

2. RESPONSE TO INDIVIDUAL STIMULI

When only light was presented to flies that were derived from ones that had already experienced the four stimuli used together, the parent went to the light source (Fig 4A) while Mutant 1 did not (Fig 4B). Mutants 2, 3, 4, and 5 did not respond to only light either as shown in S4 Fig.

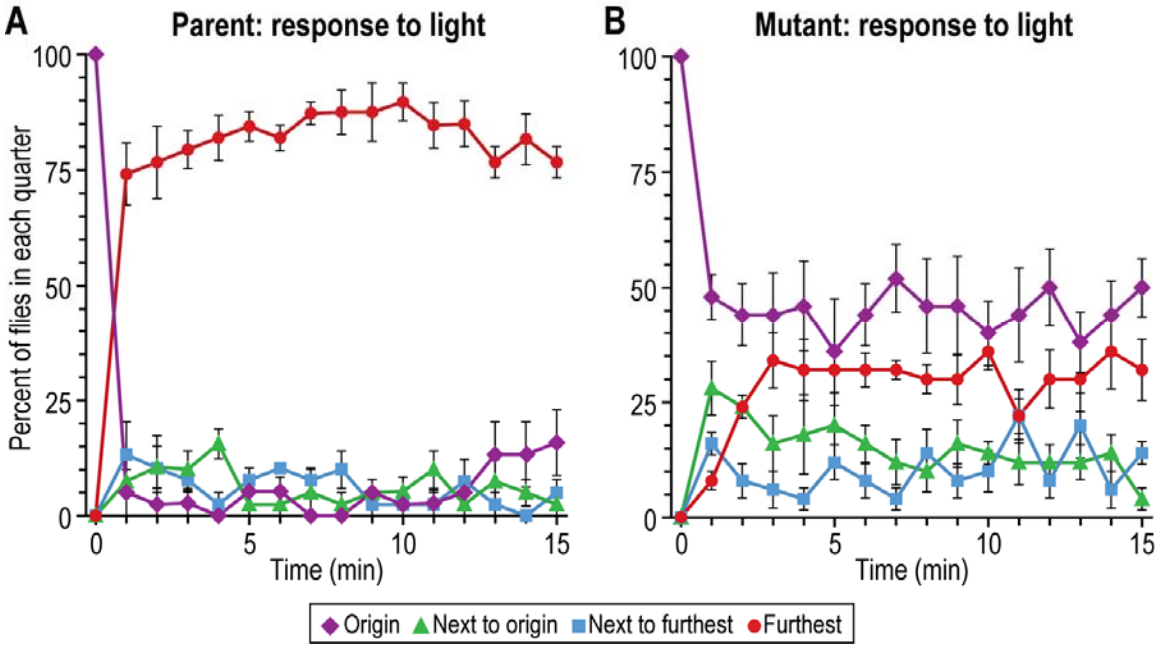

Fig 4. Response to light alone. Light (1000 lux) was placed at the right end as in Fig. 2. (**A**) Parental response (n=4). (**B**) Mutant 1 response (n=5). Flies were tested at 34°C with 10 to 20 flies used per replicate. Data are mean±SEM.

A similar result was found for benzaldehyde alone: see parent being repelled in Fig 5A and the mutant, Mutant 1, not repelled in Fig 5B. S5 Fig shows the same for another mutant tested, Mutant 2.

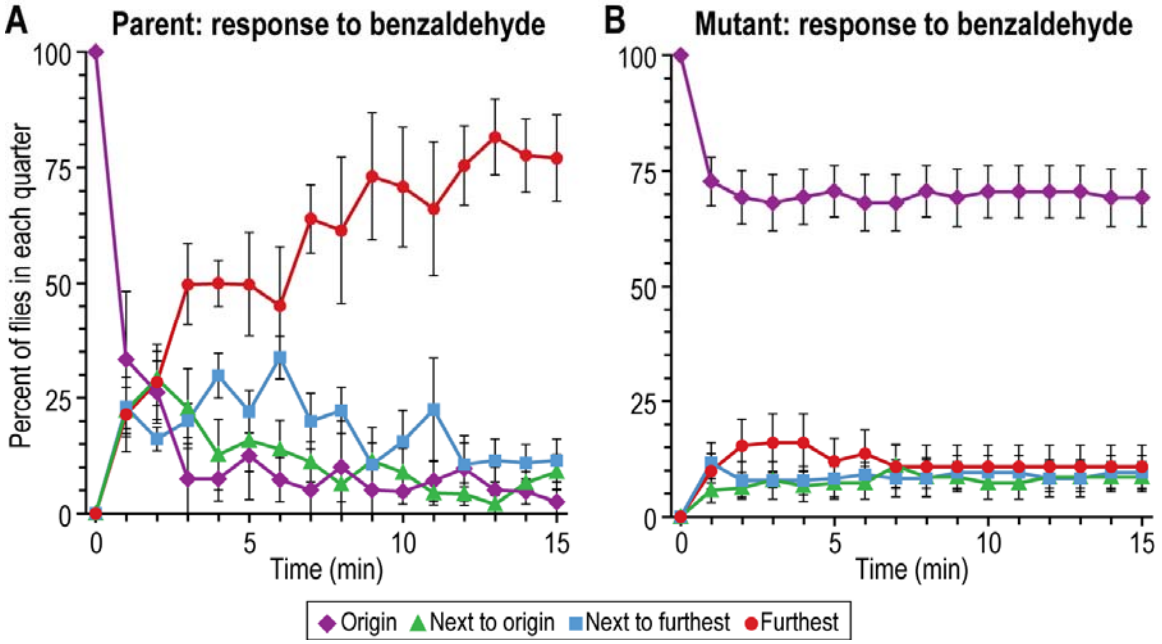

Fig 5. Response to benzaldehyde alone. Benzaldehyde (0.1M) was placed at the left end as in Fig 2. (**A**) Parental response (n=4). (**B**) Mutant 1 response (n=6). Flies were tested at 34°C with 10 to 20 flies used per trial. Data are mean±SEM. (The mutant largely remained near the benzaldehyde perhaps because its motility may have been inhibited by the benzaldehyde.)

Such was also found for heat alone: see results for parent (Fig 6A) and mutant (Fig 6B). S6 Fig shows the same for another mutant tested.

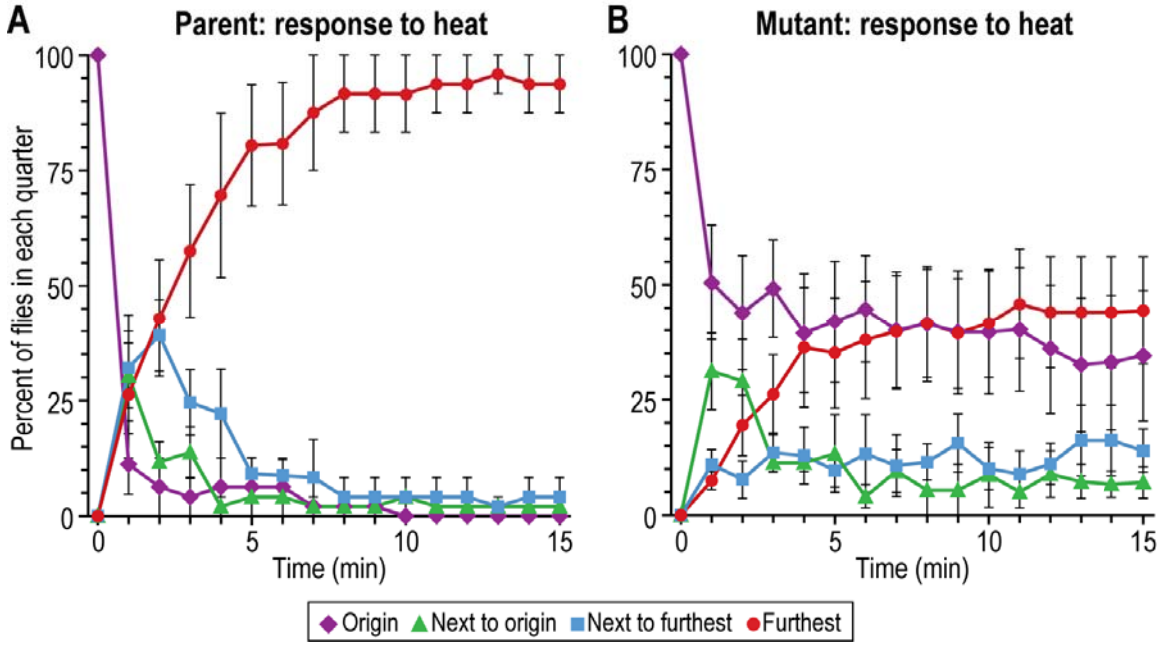

Fig 6. Response to heat gradient alone. The heat source was placed at the left end as in Fig. 2. (**A**) Parental response (n=4). (**B**) Mutant 1 response (n=5). Flies were tested at 34°C with 10 to 20 flies used per trial; the warm side measuring 37°C and the cool side at 27°C (as in Figure 2). Data are mean±SEM.

Thus the mutants were defective not only for the four stimuli used together but also for each stimulus used alone.

3. RESPONSE TO OTHER EXTERNAL STIMULI

These mutants were in addition tested with stimuli that were not among those four used to obtain the mutants.

In the case of the repellent quinine, flies were started in a 0.1M quinine half and then they had the opportunity to go into a non-quinine half (see ref. 4 for details of the method). The parent went into the non-quinine half (Fig 7A), but the mutant did not (Fig 7B). See S7 Fig for additional data.

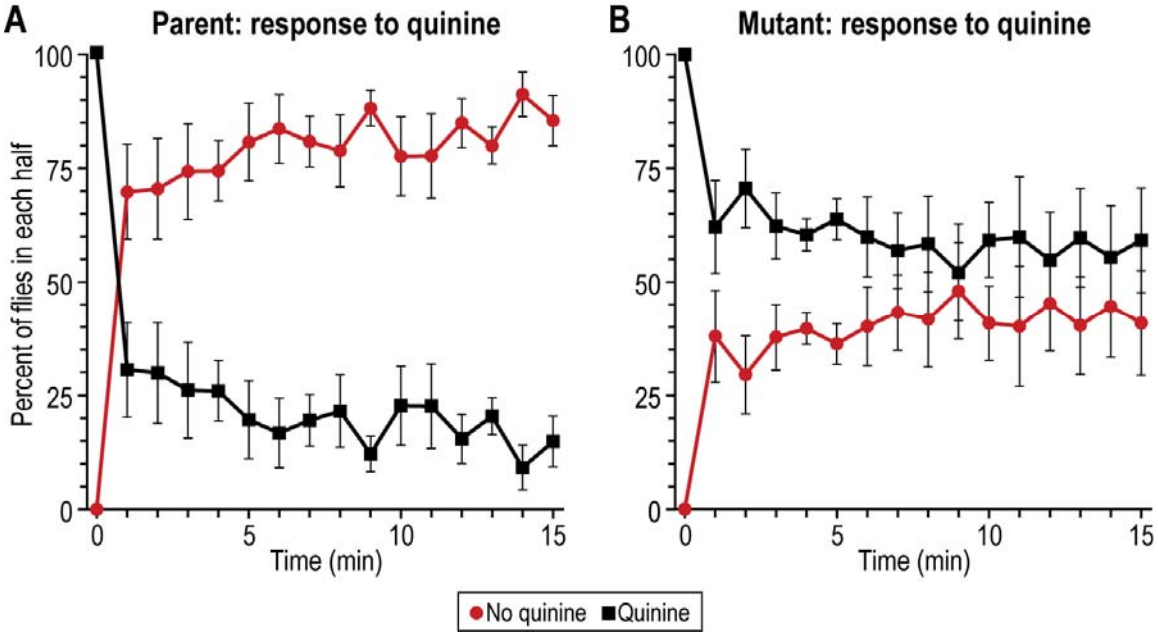

Fig 7. Response to quinine. (**A**) Parental response (n=5). (**B**) Mutant 2 response (n=5). Flies were tested at 34°C with 10 to 20 flies used per trial using 0.1M quinine. Data are mean±SEM.

These mutants were also tested for response to sucrose [5]. Compared to the wild-type, both Mutants 1 and 2 consumed significantly less sucrose, about 30 to 40% as much as the wild-type. See Fig 8 for details.

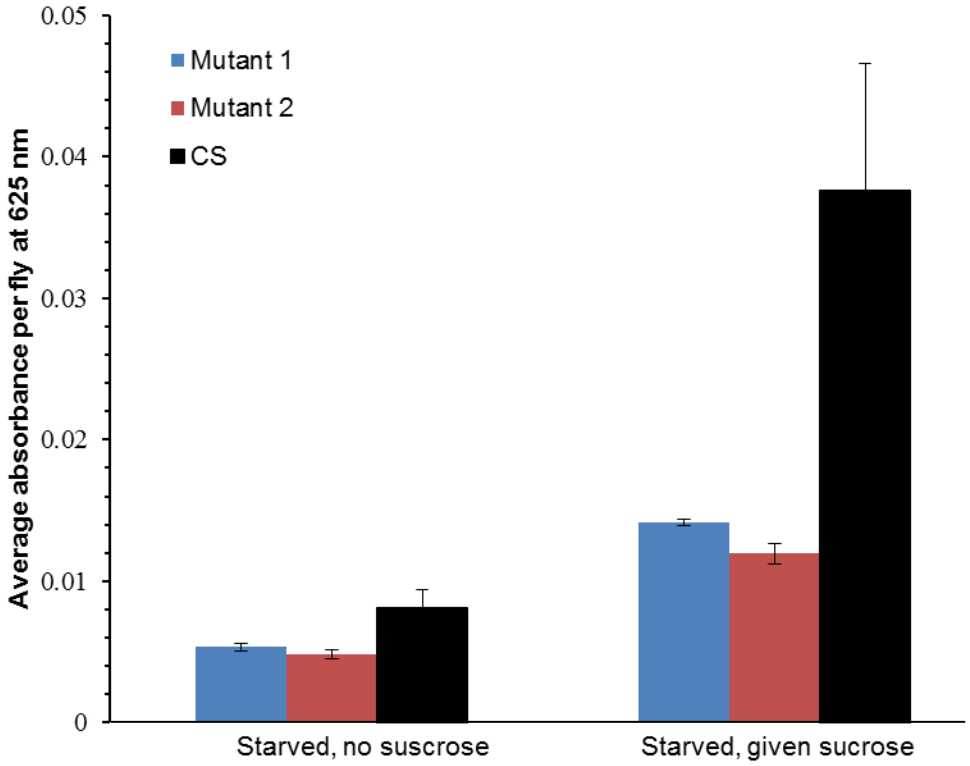

Fig 8. Response to sucrose. Between 15 and 20 flies were starved per trial on agar for 17 to 20 hours and then transferred to a tube containing agar and food dye FD & C Blue No. 1 solution for 5 minutes. These flies were homogenized and an absorbance was obtained. This was then compared to flies that were likewise starved on agar for 17 to 20 hours but were then transferred to a tube containing agar, food dye FD & C Blue No. 1, and 0.1M sucrose. No sucrose: Mutant 1 (n=3), Mutant 2 (n=3), and CS (n=3). With sucrose: Mutant 1 (n=2), Mutant 2 (n=2), and CS (n=3). Data are mean±SEM.

To test response to gravity, these flies were placed into a vertical tube and pounded down, then at every minute the flies in each third of the tube were counted (see ref. 4 for details of the method). The parent responded by climbing up (Fig 9A) while the mutant climbed up less (Fig 9B). See S9 Fig for additional data.

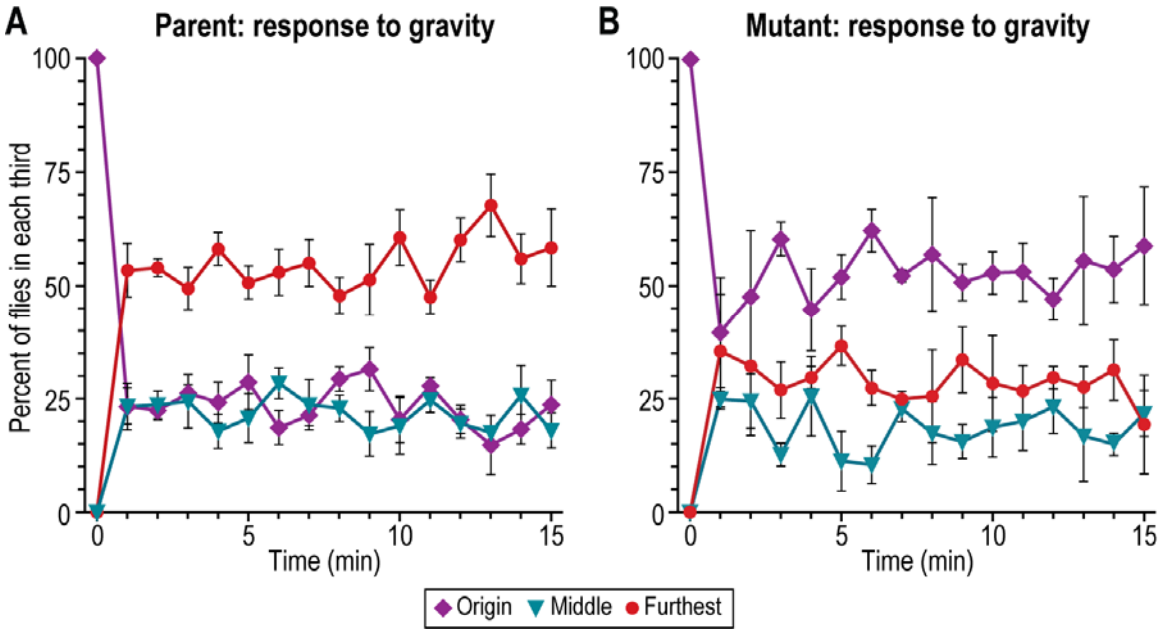

Fig 9. Response to gravity. (**A**) Parental response (n=5). (**B**) Mutant 2 response (n=4). Flies were tested at 34°C with 10 to 20 flies used per trial. Data are mean±SEM.

Thus these mutants, isolated by use of the four stimuli, were defective even for stimuli that were not present during their isolation.

4. MOVEMENT WITHOUT ANY ADDED STIMULI

In the absence of any stimulus added by the experimenters, the parent (Fig 10A) and the mutant (Fig 10B) moved similarly, indicating that motility alone is about the

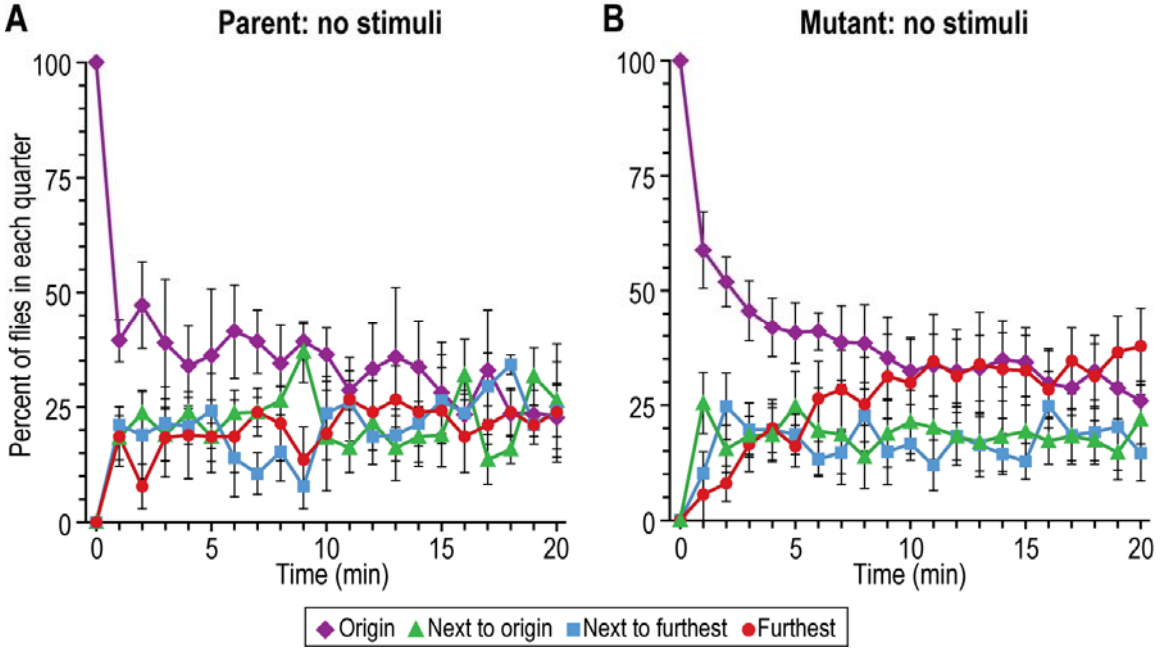

Fig 10. Response without added stimuli. (**A**) Parental response (n=4). (**B**) Mutant 1 response (n=6). Flies were tested at 34°C with 10 to 20 flies used per trial. Data are mean±SEM.

same in parent and mutant. This was found also for Mutant 4 (S10 Fig). Mutants 2, 3, and 5 showed a slightly slower motility (S10 Fig). These results tell that the mutants were motile.

5. EFFECT OF INCUBATION TEMPERATURE

All the work reported above was carried out in a 34°C room in order to allow, if necessary, isolation and study of conditional mutants, i.e. mutants defective at 34°C but not defective at room temperature. We found that at room temperature, when each stimulus was used together, there was substantial condensation along the interior of the tube, resulting in the flies becoming stuck when emptied into the test apparatus. Therefore we chose to measure response to light (1000 lux) at room temperature (21 to 23°C). The parent responded to light but all five of the mutants failed to respond to light or responded only 10% as well as the parent (S11 Fig), just as they did at 34°C. Thus the mutations are likely not conditional.

Being defective to all stimuli tested at the usual growth temperature, how could the mutants survive and grow? We don't know, but it seems that due to the crowded conditions and rich medium found in the growth tube at room temperature this defect is compensated for somehow.

B. RESPONSES TO INTERNAL STIMULI

1. HUNGER

Here we focus on hunger [4,5, 7-11]. To measure hunger we used an apparatus (Fig 11), inspired by and modified from an earlier design [12], described in Supplemental

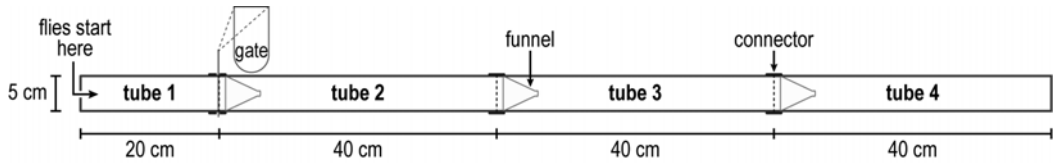

Fig 11. Apparatus for measuring hunger and for measuring thirst. For details see IV. METHODS, C. How to study response to internal stimuli. Tube 1 is called "origin". Flies were tested at room temperature (21-23°C).

Methods (Supplement: C. How to study response to internal stimuli, 1. Measuring hunger). Briefly, in a dark room at 21-23°C male flies – parent or mutants - were transferred into one end (tube 1) of a 5 x 140 cm apparatus containing throughout its length a 5 cm wide strip of wet paper to satisfy thirst but containing no food. Starvation

for food began once the flies were put in. Every 10 hours up to 40 hours the location of the flies was measured with light on for 30 seconds.

At 20 hours the parent had largely left the origin (tube 1) and had begun to accumulate at the end (tube 4) (Fig 12A, solid bars), while the mutant had moved

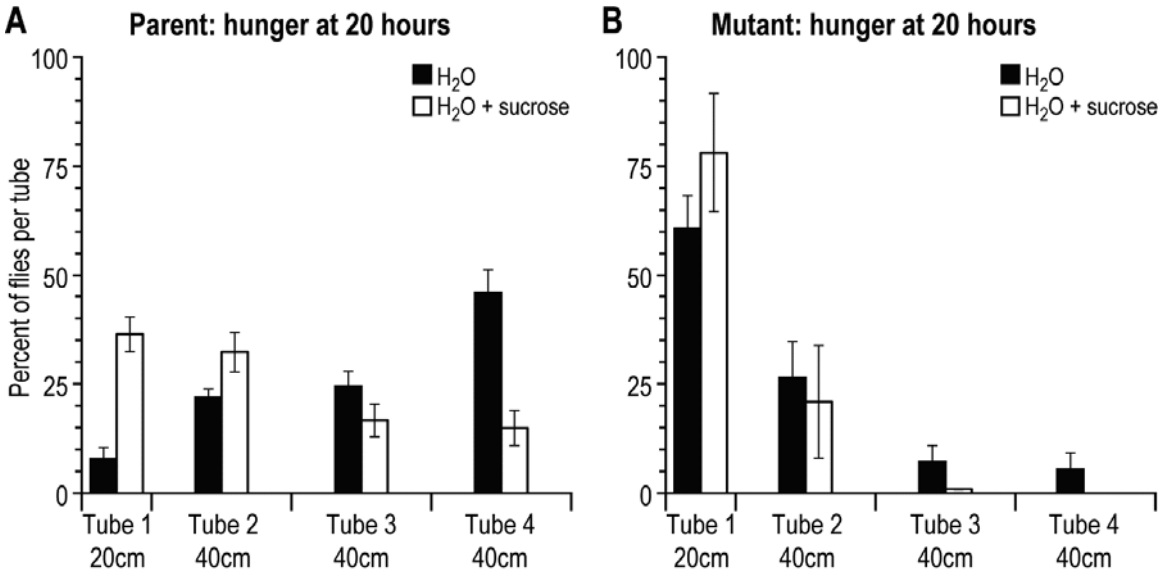

Fig 12. Movement of flies at 20 hours in search for food. Solid: water but no food (no sucrose). Open: water and food (0.1M sucrose). (**A**) Parental response with water only (n=5) and with water + sucrose (n=9). (**B**) Mutant 2 response with water only (n=5) and with water + sucrose (n=4). Data are mean±SEM. (See S13 Fig for Mutant 1.) Flies were tested at room temperature (21-23°C) with 40 to 60 flies used per trial.

towards the end very little (Fig 12B, solid bars). This is interpreted to mean that the parent is searching for food while the mutant is defective in searching for food. The flies at 40 hours had moved further than at 20 hours but this is not illustrated because by 40 hours the death of the flies, presumably due to starvation, had become a serious interference.

When food (0.1M sucrose) was added throughout the tube along with the wet strip of paper, the parent moved less far (rather than accumulating at the end) (Fig 12A, open bars), while the mutant remained mostly where placed (Fig 12B, open bars). Since sucrose inhibited the movement of the parent, it is supposed that movement without sucrose is due largely to hunger. From these results we conclude that the mutants are defective in hunger.

2. THIRST

To study thirst, flies were deprived of water by a procedure described in Supplemental Methods (Supplement: C. How to study response to internal stimuli, 2.

Measuring thirst). The procedure is the same as for hunger (Fig 11) except that water was omitted and solid sucrose was layered throughout.

By 30 hours the parent had moved out, presumably to search for water since addition of water inhibited this (Fig 13A). The mutant moved out less well than the

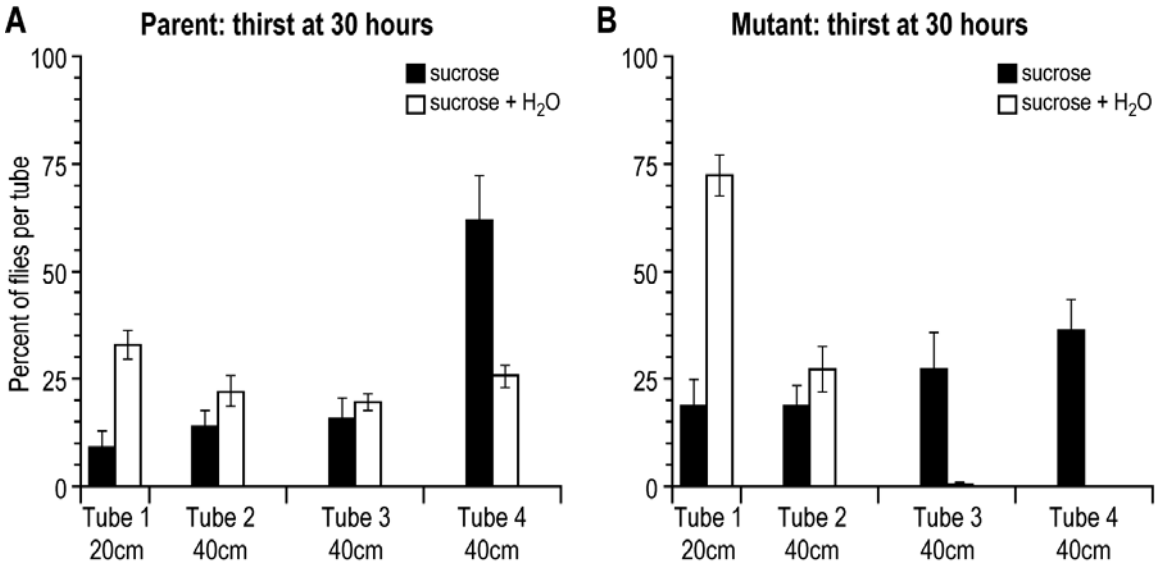

Fig 13. Movement of flies at 30 hours in search for water. Solid: food (sucrose crystals) but no water. Open: food (0.1M sucrose) and water. (**A**) Parental response with sucrose only (n=5) and with water + sucrose (n=6). (**B**) Mutant 1 response with sucrose only (n=3) and with water + sucrose (n=3). Flies were tested at room temperature (21-23°C) with 40 to 60 flies used per trial. Data are mean±SEM. (See S13 Fig for Mutant 2 data.)

parent (Fig 13B), so we conclude that the mutants are defective in thirst. Already by 30 hours dying, presumably from thirst, had become a problem; by 40 hours this had become severe, so those results are not illustrated.

3. SLEEP-WAKE

The parent and mutants isolated here were studied for sleep and wake according to the procedure of Pfeiffenberger et al. [13]. The parent was different from the mutants (Fig 14). The parent showed greatest activity at the start and end of the day but not in the

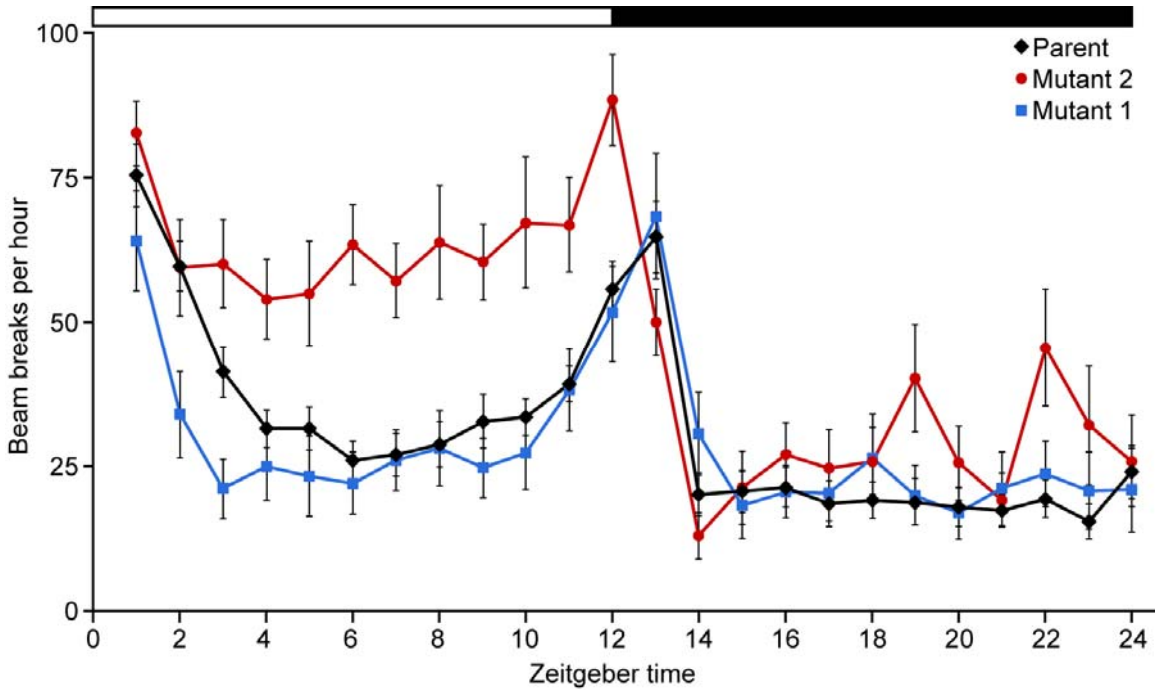

Fig 14. Circadian response. Individual flies are placed into a tube (5 x 20 mm) with an infrared light beam intersecting at the middle of the tube. Mutant 1 (n=24), Mutant 2 (n=24), and parental response (n=24) are recorded over a 24 hour period at 22° C. Data are mean±SEM. (S14 Fig shows smaller differences between parent and three other mutants.)

middle of the day. Mutant 2 showed high activity throughout the day. Mutant 1 was less active than the parent at the start of the day.

C. MAPPING OF THE MUTANTS

We used recombination and deficiency kits [14-16]. Mutants isolated were mated to attached-X females. When presented with the four stimuli (light, favorable and unfavorable temperature, and benzaldehyde) together, attached-X females behaved wild-type (S15 Fig), indicating that both Mutants 1 and 2 are X-linked. The mutants were then crossed to an X chromosome bearing *y cho cv v f*.

Mutant 1 was found to be between *v* and *f*, approximately in position 12 or 13. Of the deficiencies that can be found for this region, we found that *Df(1)BSC705* uncovered Mutant 1. We then tested two deficiencies that overlap *Df(1)BSC705*, *Df(1)BSC628* and *Df(1)ED7229*. *Df(1)BSC705* uncovered Mutant 1 but *Df(1)ED7229* did not. Therefore the mutation must lie within *Df(1)BSC705* but to the left of *Df(1)BSC628*. Of the genes that were found within this region, 12E3 to 12E5, and that we tested, all complimented. This would suggest that the gene may lie in a small gap between the deficiencies.

Mutant 2 was found to be between *cv* and *v*, approximately in position 8 or 9. Of the deficiencies tested in this region, *Df(1)BSC539* uncovered Mutant 2. Two deficiencies

that overlap with *Df(1)BSC539*, *Df(1)BSC754* and *Df(1)ED7005*, did not uncover Mutant 2 and the mutation must then be in the *Df(1)BSC539* segment to the left of *Df(1)BSC754*. Of the genes that were found within this region and that we tested, CG1791 failed to compliment, so we conclude that Mutant 2 lies in the *CG1791* gene, which has been shown to be related to fibrinogen [17, 18]. The *CG1791* gene has 3 transcripts: RA, RB, and RC [19] but we do not yet know how *CG1791* is connected to decision making or executive function.

This new information can be used to go on to learn what the next steps are. Ultimately all the steps between sensory reception and response can be studied in mutants in order to define the entire sequence.

D. RELATION TO REPRODUCTION

Although the mutants reported here are defective in the responses to all external stimuli tested and all internal stimuli tested, nevertheless they are able to reproduce, so it must be that the mechanism for mating is still active in these mutants. In a normal fly, mating uses many systems: hearing, vision, smell, taste, and contact; mating can, however, take place without some of these [20-23], but presumably contact is still required since the male must touch the female in mating. Contact may well be intact in the mutants isolated here and perhaps presence of the flies in crowded tubes might make some of the other requirements for mating not necessary.

In our previous work on isolating mutants such as these by G. Tang, V. Toniolo, Y. H. Ng, L. Vang, and J. Adler [24], the mutants isolated failed to reproduce. Possibly they lacked mating due to lacking this contact. The procedure for isolating mutants was different from the procedure here: instead of using the four stimuli together (as described in Fig 2), the stimuli were used one at a time.

III. DISCUSSION

Here we describe the isolation and some properties of *Drosophila* mutants that are motile but yet they each fail in response to all external attractants and repellents tested (Figs 3-9) and also they are deficient in response to internal stimuli tested (Figs 12-14). Thus, although the mutants are motile, they have

1. decreased responsiveness to light
2. decreased responsiveness to repulsive chemicals (like benzaldehyde)
3. decreased responsiveness to heat and to favorable temperature
4. decreased responsiveness to bitter tastants (like quinine)
5. decreased responsiveness to sweet tastants (like sucrose)
6. decreased responsiveness to gravity
7. decreased responsiveness to hunger
8. decreased responsiveness to thirst
9. abnormality in some sleep

Because all of these different behaviors are defective in each of these mutants (Figs. 3-9, 12-14), it seems reasonable to say that there is a central place that is responsible. This might possibly be due to these:

Decision making involves interaction with stimuli. The simple sensing-response relationships long assumed to underlie behaviors is actually more complex [25]. When there are no stimuli added there is a resting state, called "default" or "intrinsic", which yet allows many behaviors to occur, and when stimuli are presented there is an activated state, called "reflexive" or "responsive" [26-28]. Our mutants could be defective in making this relation between resting state and activated state so that responses to all stimuli are poor. Presumably some factor is needed for this coming together of the resting state and the activated state, and that factor may be deficient in the mutants.

What is defective may be the interaction between decision making and executive function [1, 2]. "Executive functions are the high-level cognitive processes that facilitate new ways of behaving…The operation of executive processes accompanies a very wide range of behaviours…Similar progress has been made on work in animals" [1].

A defect in The Boss is yet another possibility. The Boss is a hypothetical identity that controls each organism's behavior, metabolism, reproduction, and other functions [6]. It may be that executive function responds to the The Boss and that the mutants isolated here are defective in that response. The Boss hypothetically controls DNA synthesis plus RNA synthesis plus protein synthesis, and through that it controls behavior (Figure 10 of "A search for The Boss", submitted 2016).

Isolating a larger number of mutants than just the five studied here may result in finding that all of these above mechanisms might be involved. Also, it is possible that locomoter mutants need to be considered, which was done in some of the previous work [33, 41, 42].

The place where the consequences of action of the present mutants appears is not known. Possibilities are: 1) Outside of known bodies but rather in the surrounding fibers [29, p. 540]. 2) In the mushroom bodies [29, pp. 523, 548; 30], but this is not likely because when we tested mutants in the mushroom bodies they had a normal response (see S16 Fig). 3) In the central complex [29 pp. 521, 31]:

In the case of insects, known parts of the central complex consist of the protocerebral bridge, the fan-shaped body, the ellipsoid body, and noduli (S1A Fig) and the central complex is involved in bringing about a behavioral response [32-36]. In the case of vertebrates, it is the prefrontal cortex [37] (S1B Fig) plus the basal ganglia [38] (S1C Fig) that determines a behavioral response.

Some 30 different mutants of *D. melanogaster* have been isolated that have defects in various parts of the central complex [33, 39-45]. These mutants have been kindly given to us by Roland Strauss, Burkhard Poeck, and Douglas Armstrong. Now we

have shown here (S17 Fig) that several of these mutants can block the parental response away from benzaldehyde plus heat and towards light plus a favored temperature that is shown in Figure 3A; these several mutants are *cbd* ^{ks96}, *nob*^{ks49}, and c*cb*^{ks145} [33] and *EB1* and *62y* [45]. This result may suggest that the mutants we isolated and report here cause defects in the central complex.

Neusser, Triphan, Mrontz, Poeck, and Strauss found that *D. melanogaster* mutants, unlike their parent, failed to remember where they were located just before they were moved [46], see the diagram of this by Tomchik and Davis [47] (S2 Fig). The Strauss et al. work suggested to them that this is related to the prefrontal cortex of humans [46]. Our own work on decision making [48] also describes mutants of *D. melanogaster* that appear to lack steps like those handled by the prefrontal cortex of humans and other vertebrates. It would seem that the insect brain and the brain of higher-order animals share many qualities.

According to Straussfeld and Hirth [49], there is behavioral homology between the response by insects and the response by vertebrates: see Figure 2 of ref. 49 (S1D Fig) and they propose that this homology originated in very early times. Studies by Arendt's group indicate that the responses by insects and the by vertebrates are descended from a common ancient ancestor that represents an early stage of evolution of the nervous system and muscles [50].

Altogether, the mutants that we isolated and reported here may well have bearing for both insects and vertebrates.

RELATION TO CHARGE SYNDROME AND DEAF-BLIND CONDITION

The *Drosophila* mutants isolated in this report seem related to a rare human genetic condition that occurs in roughly 1 per 10,000 live births [51], called CHARGE syndrome, reviewed recently [52]. "Children with CHARGE syndrome are truly 'multi-sensory impaired', having difficulties not only in vision and hearing but also with the senses that perceive balance, touch, temperature, pain, pressure, and smell, as well as problems with breathing and swallowing, eating and drinking, digestion, and temperature control" [53]. This syndrome was described independently in 1979 by Hall [54] and Hittner et al. [55]. The term CHARGE was then coined for the disease to cover its various symptoms [56], although not all symptoms are always present. A human gene, *CHD7*, is known to be associated with some 65% of 379 published cases of human CHARGE syndrome [57-60].

CHARGE syndrome is now being studied also in mice [61], zebrafish [62], and *Drosophila* [63]. A number of genes have been identified that are closely related to the human gene. In *Drosophila*, kismet *(kis*, the *Drosophila* homolog of human *CHD7*) is located in chromosome 2 [64]. In our own work on *Drosophila* (this report) the mutants described are located in the X chromosome, so the research here might deal with a different, additional gene for CHARGE syndrome, or else it deals with a different function altogether.

While in CHARGE syndrome the deaf-blind symptoms occur already at birth, in a different inherited disease, Usher syndrome, the deaf syndrome occurs at birth but the blind syndrome occurs years later [65]. That defect is known to be caused by an abnormal autosomal gene as well as a number of still unidentified genes [66].

IV. METHODS

See Supplemental Methods for details. A. Isolation of mutants. B. How to study response to external stimuli. C. How to study response to internal stimuli.

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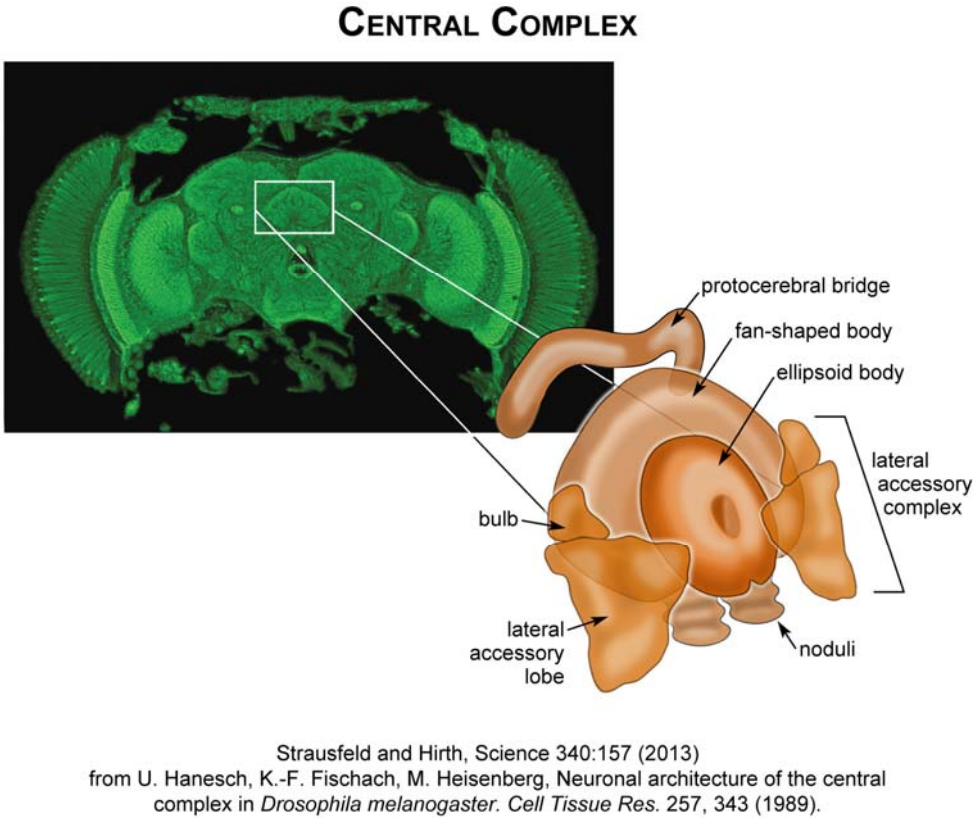
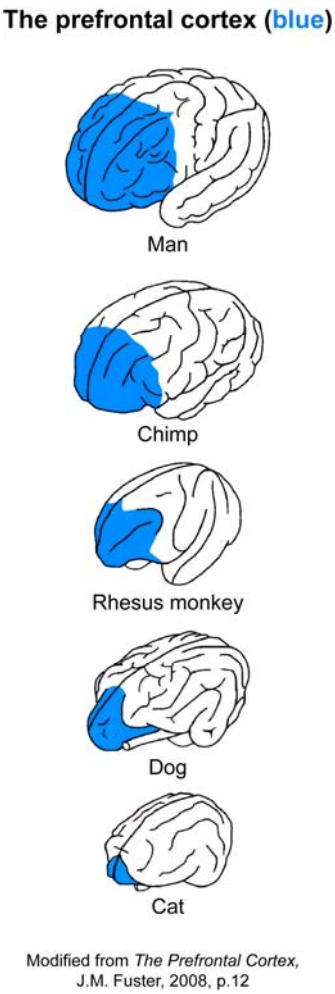
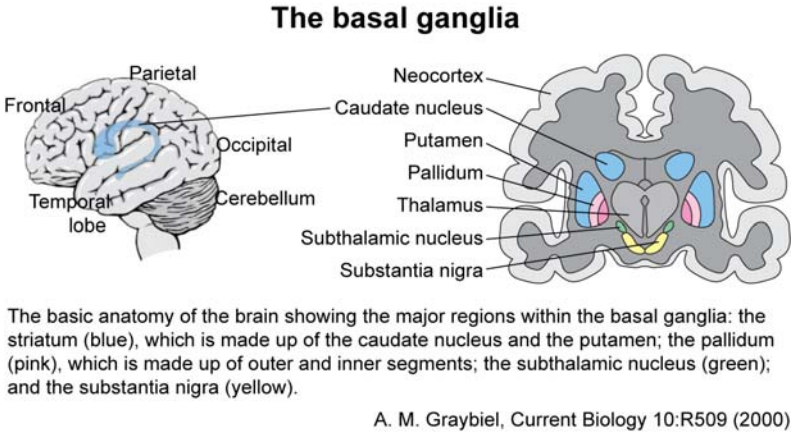
SUPPLEMENTAL FIGURES



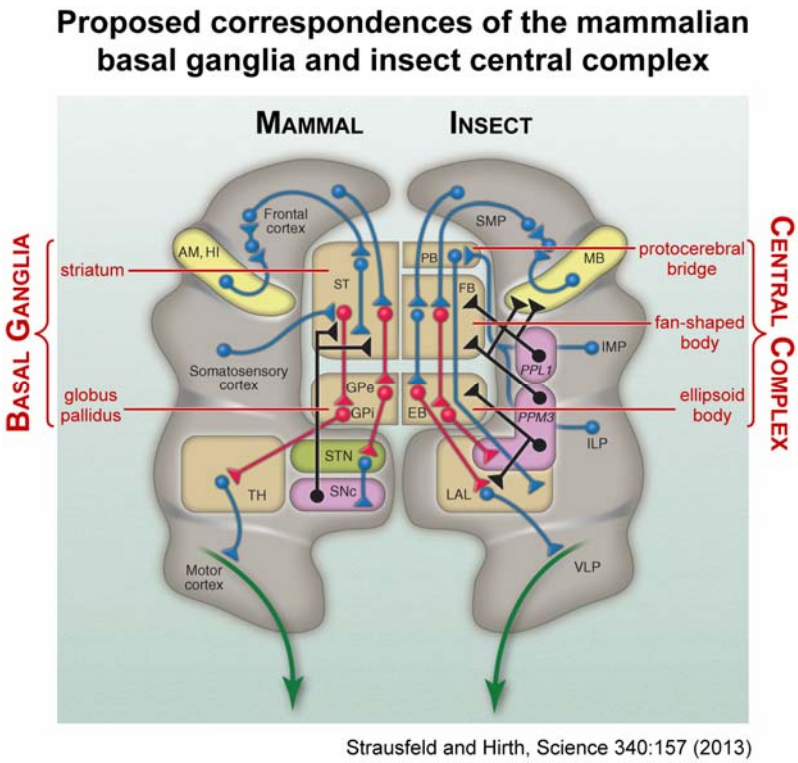
S1A Fig. Central complex of the *Drosophila* brain.



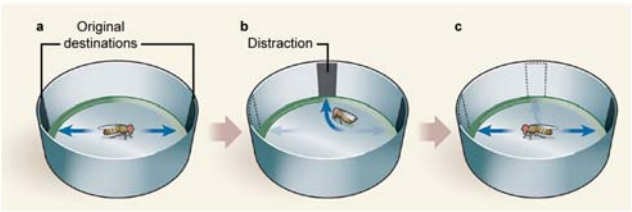
S1B Fig. The prefrontal cortex of various mammalian species.



S1C Fig. The basal ganglia of the human brain.



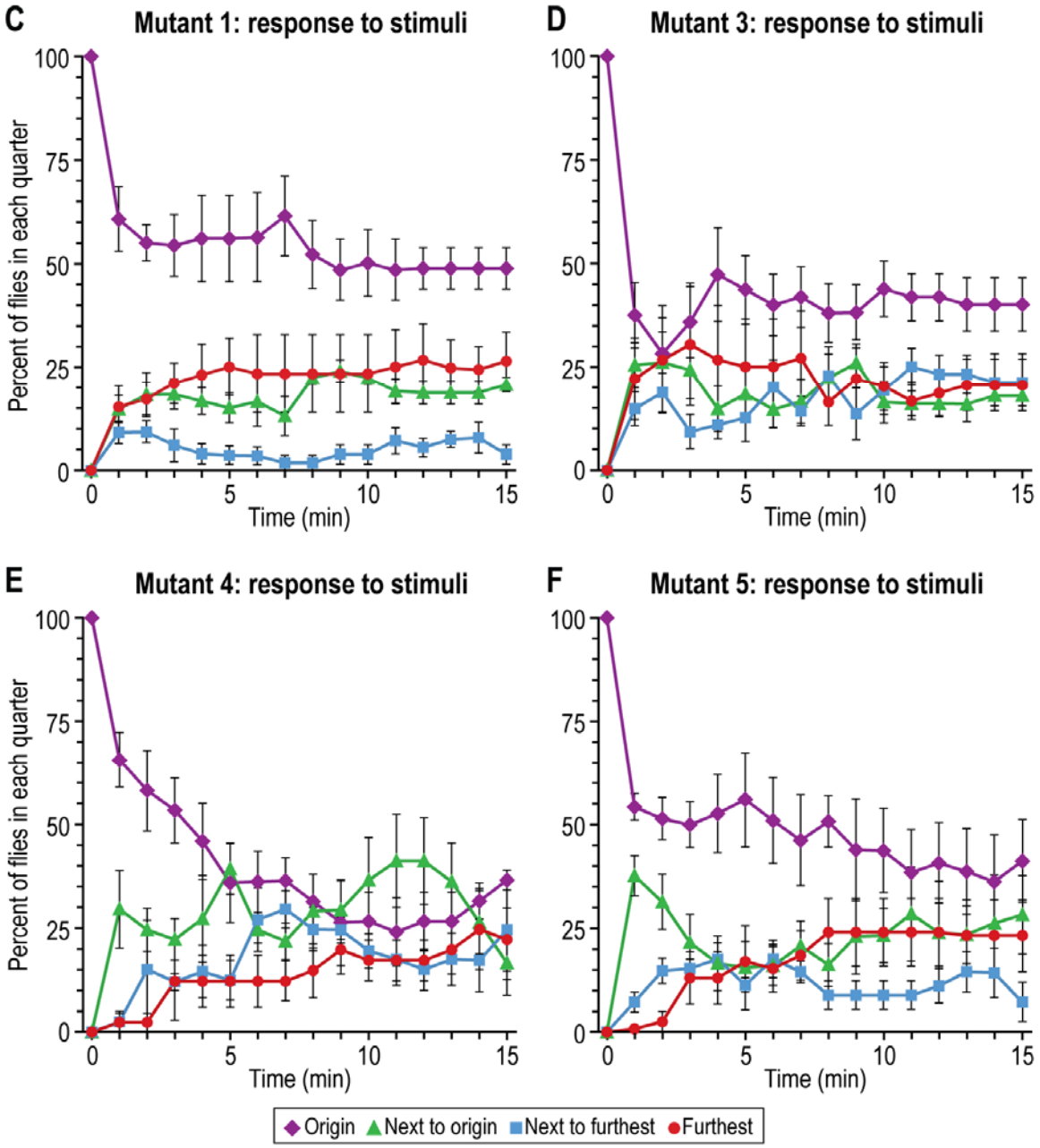
S1D Fig. Comparison of the mammalian brain and the insect brain.



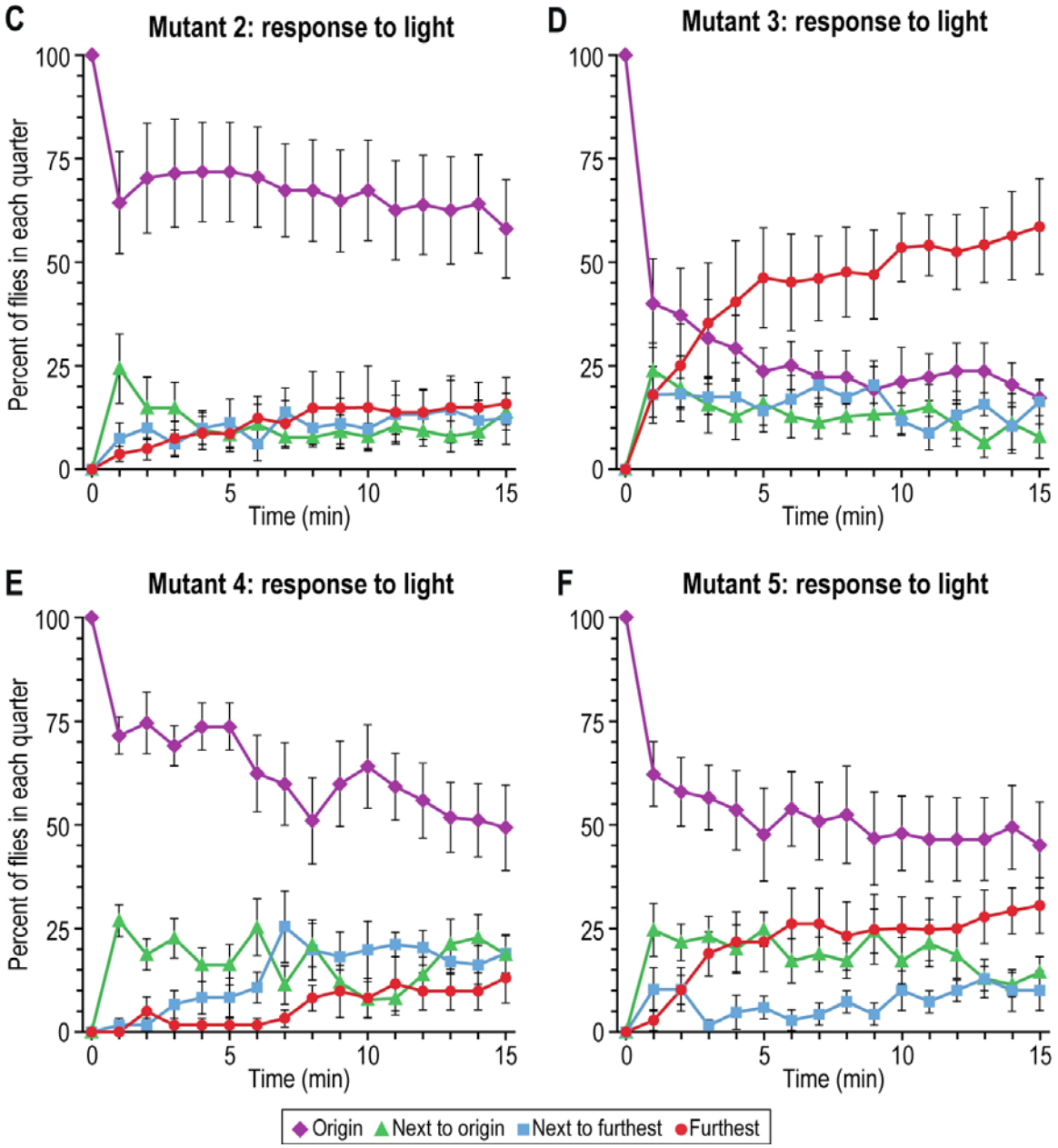
a, A fly will walk back and forth between two opposing black stripes in a circular arena even if, midway, the target stripe disappears. Neuser et al.[1] find that if (**b**) a fly is distracted midway by a new black stripe to its side, the insect still remembers the position of the original stripe, and (**c**) when the distracter stripe subsequently disappears, resumes walking along its original course. The authors find that this behaviour depends on ring neurons of the ellipsoid body in the fly brain.
Tomchick & Davis, Nature 453 (2008) p. 1193

[1]Neuser, Triphan, Mronz, Poeck & Strauss, Nature 453 (2008) p.1244-1247

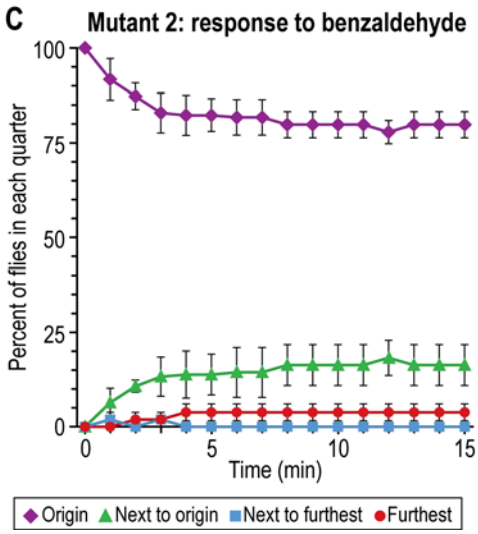
S2 Fig. Assay used to show that a fly remembers its original location.



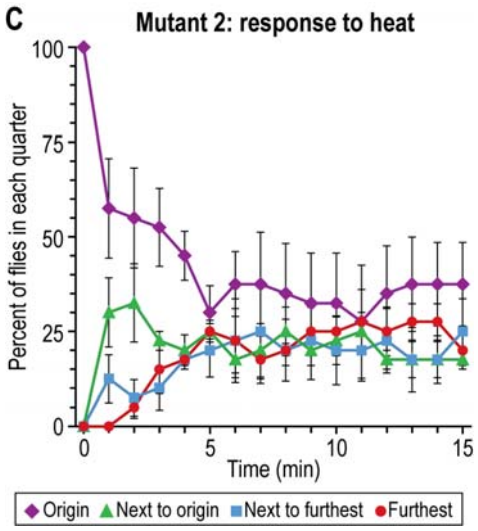
S3 Fig. Response to four stimuli together. Repellents (0.1M benzaldehyde and high temperature (37°C)) were at the origin end, attractants (light and a favored temperature (27°C)) at the furthest end. (**C**) Mutant 1 response (n=5). (**D**) Mutant 3 response (n=5). (**E**) Mutant 4 response (n=5). (**F**) Mutant 5 response (n=4). Flies were tested in a 34°C room with 10 to 20 flies used per trial. Data are mean±SEM.



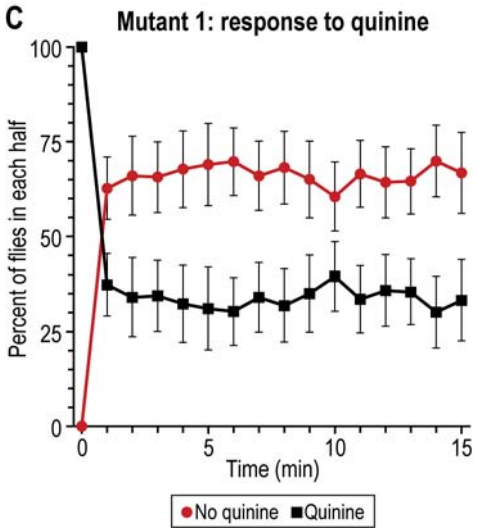
S4 Fig. Response to light alone. (**C**) Mutant 2 response (n=8). (**D**) Mutant 3 response (n=7). (**E**) Mutant 4 response (n=6). (*F*) Mutant 5 response (n=8). Flies were tested at 34°C with 10 to 20 flies used per trial. Data are mean±SEM.



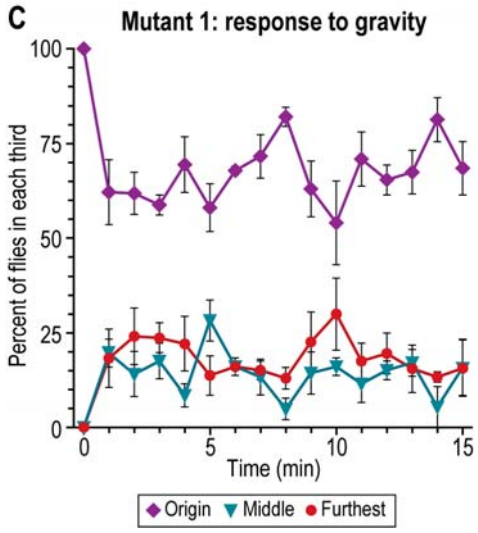
S5 Fig. Response to benzaldehyde alone. (**C**) Mutant 2 response (n=4). Flies were tested at 34°C with 10 to 20 flies used per trial. Data are mean±SEM.



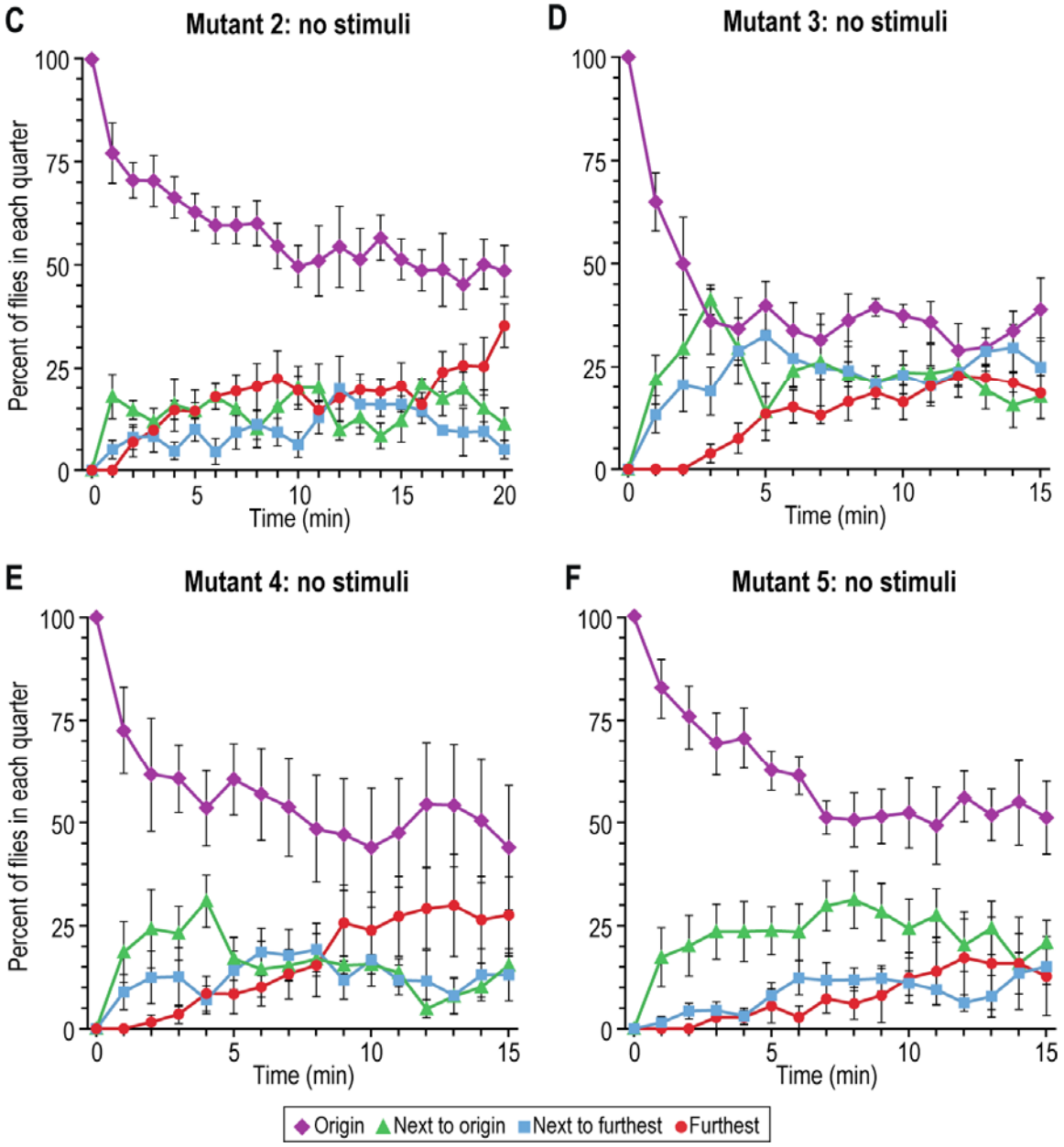
S6 Fig. Response to heat gradient alone. (**C**) Mutant 2 response (n=4). Flies were tested at 34°C with 10 to 20 flies used per trial; the warm side measured 37°C and the cool side 27°C. Data are mean±SEM.



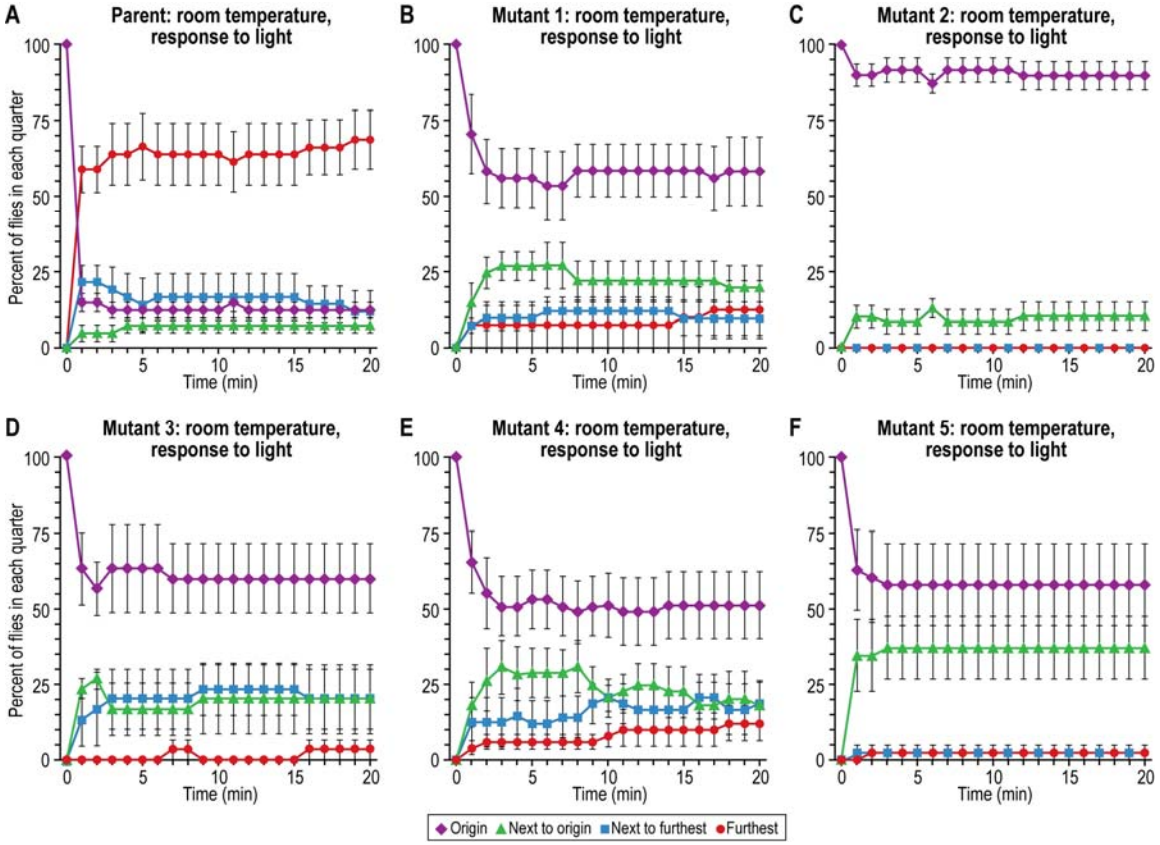
S7 Fig. Response to quinine. (**C**) Mutant 1 response (n=10). Flies were tested at 34°C with 10 to 20 flies used per trial. Data are mean±SEM.



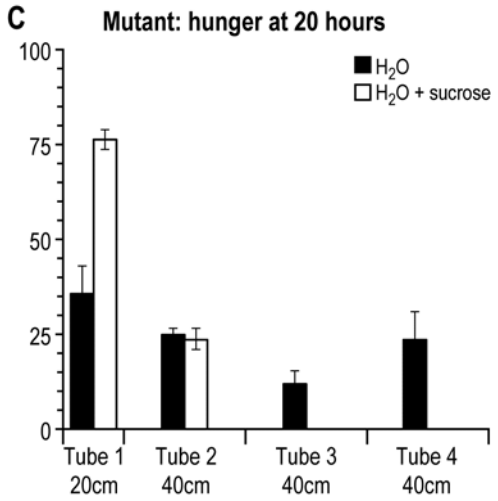
S9 Fig. Response to gravity. (**C**) Mutant 1 response (n=4). Flies were tested at 34°C with 10 to 20 flies used per trial. Data are mean±SEM.



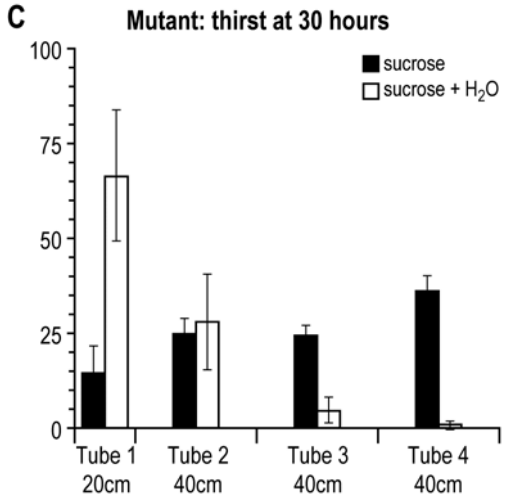
S10 Fig. Response without added stimuli. (**C**) Mutant 2 response (n=6). (**D**) Mutant 3 response (n=5). (**E**) Mutant 4 response (n=6). (**F**) Mutant 5 response (n=6). Flies were tested at 34°C with 10 to 20 flies used per trial. Data are mean±SEM.



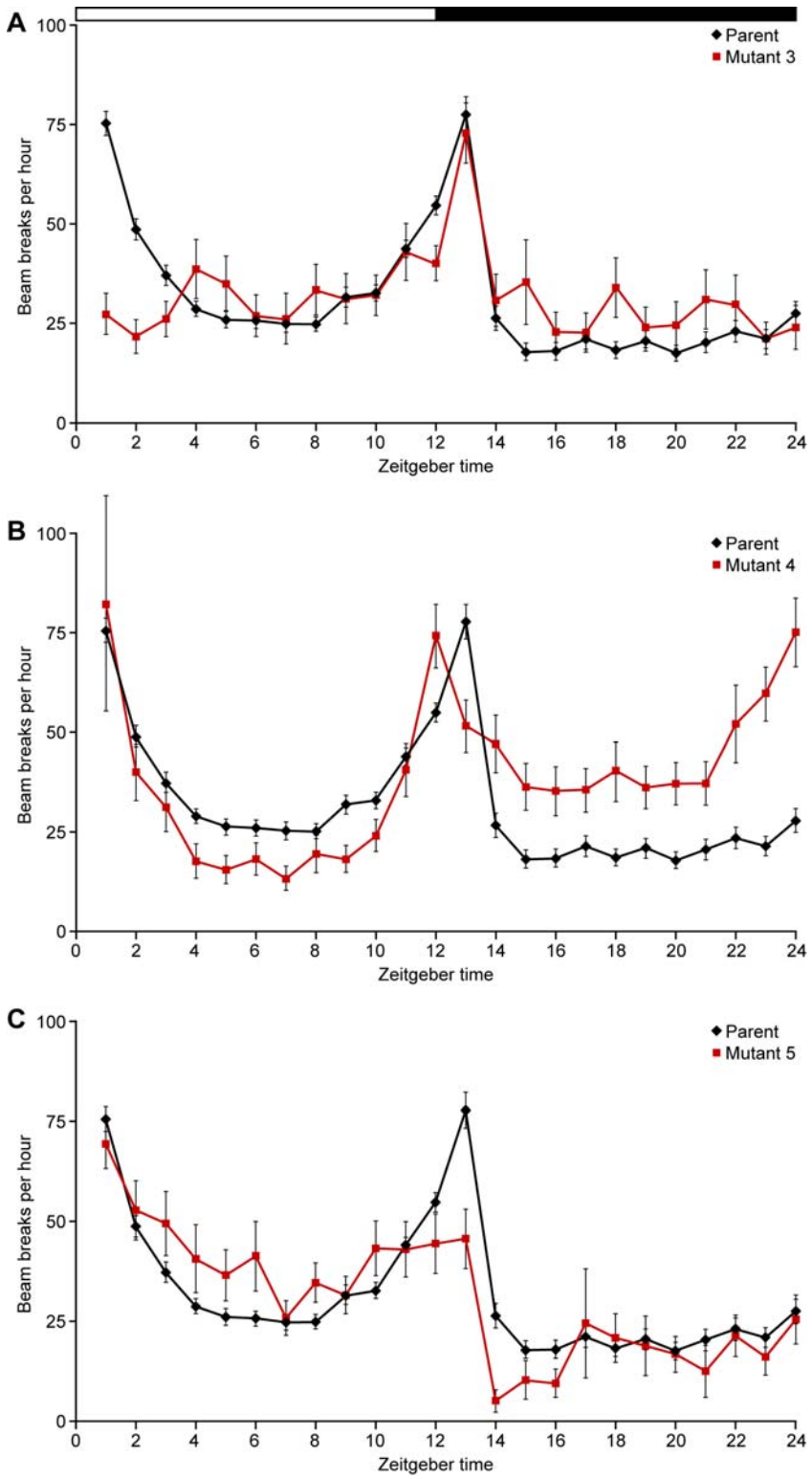
S11 Fig. Response to light at room temperature. (**A**) Parental response (n=4). (**B**) Mutant 1 response (n=4). (**C**) Mutant 2 response (n=6). (**D**) Mutant 3 response (n=3). (**E**) Mutant 4 respnose (n=5). (**F**) Mutant 5 response (n=4). Approximately 10 to 20 flies were used per trial. Data are mean±SEM.



S12 Fig. Movement of flies at 20 hours in search for food. Solid: water but no food (no sucrose). Open: water and food (sucrose). (**C**) Mutant 1 response with water only (n=4) and with water+sucrose (n=4). Flies were tested at 22º C with 40 to 60 flies used per trial. Data are mean±SEM.

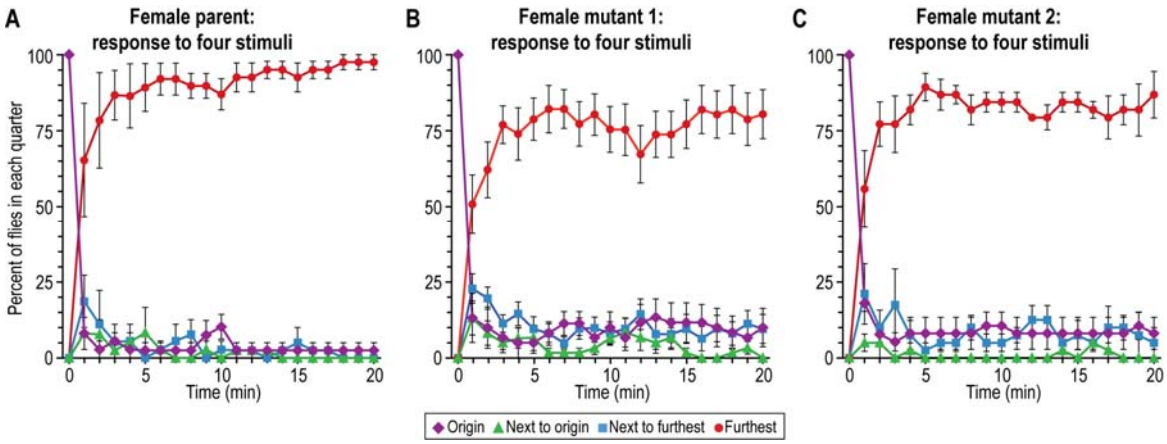


S13 Fig. Movement of flies at 30 hours in search for water. Solid: food but no water. Open: food and water. (**C**) Mutant 1 response with sucrose only (n=5) and with water+sucrose (n=4). Flies were tested at 22º C with 40 to 60 flies used per trial. Data are mean±SEM.

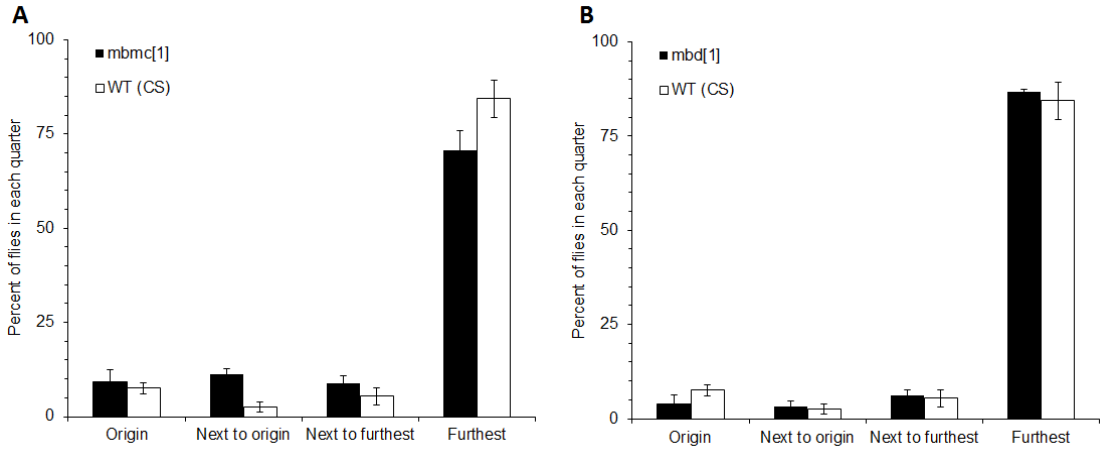


S14 Fig. Circadian response. Individual flies are placed into a tube (5 x 20 mm) with an infrared light beam intersecting at the middle of the tube. (**A**) Mutant 3 response (n=62), (**B**) Mutant 4 response (n=63), (**C**) Mutant 5 response (n=58), and parental fly response

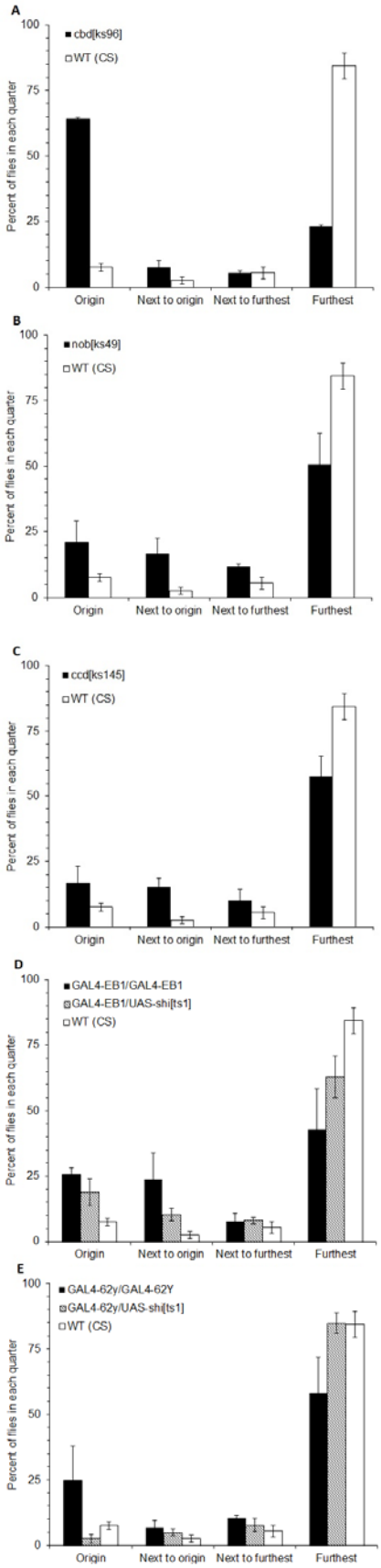
(n=156). Flies were tested at 22º C. Graphs were obtained from traces of the original graphs as the original data were lost. Data are mean±SEM.



S15 Fig. Response of attached-X females from their respective mutant mate to four stimuli together. Repellents (0.1M benzaldehyde and high temperature (37°C)) were at the origin end, attractants (light and a favored temperature (27°C)) at the furthest end. (**A**) Female of parent response (n=4). (**B**) Female of Mutant 1 response (n=6). (**C**) Female of Mutant 2 response (n=4). Flies were tested at 34°C with 10 to 20 flies used per trial. Data are mean±SEM.



S16 Fig. Response of mutant strains with malformations of the mushroom bodies. Repellents (0.1M benzaldehyde and high temperature (37°C)) were at the origin end, attractants (light and a favored temperature (27°C)) at the furthest end. (**A**) Mutant *mbmc*^{1} response (n=3) and (**B**) mutant *mbd*^{1} response (n=3) compared to wild-type CS (n=7).). Flies were tested at 34°C with 10 to 20 flies used per trial. Data are mean±SEM. See [30] for more details.



S17 Fig. Response of GAL4 driver lines and mutant strains with malformations of the central complex. Repellents (0.1M benzaldehyde and high temperature (37°C)) were at the origin end, attractants (light and a favored temperature (27°C)) at the furthest end. (**A**) Response of *cdb*^{ks96} (n=3), (**B**) response of *nob*^{ks49} (n=6), (**C**) response of *ccd*^{ks145} (n=4), (**D**) response of *GAL4-EB1*/+ (n=3) and *GAL4-EB1/UAS-shi*^{ts1} (n=10), and (**E**) response of *GAL4-62y/+* (n=3) and *GAL4-62y/UAS-shi*^{ts1} (n=4) compared to wild-type CS (n=7). Flies were tested at 34°C with 10 to 20 flies used per trial. Data are mean±SEM. See [33, 45] for more details.

SUPPLEMENTAL METHODS

A. ISOLATION OF MUTANTS

D. melanogaster (strain Canton-S) and attached-X flies were provided by Robert Kreber.

Flies were maintained on standard cornmeal-molasses agar medium at room temperature (21-23°C) in a room that was light for 12 hours and dark for 12 hours. Male flies were mutagenized overnight with 25mM ethyl methane sulfonate in 1% sucrose on Kimwipe paper (Kimberly-Clark, Roswell, GA). Then they were mated with virgin attached-X (*y w f* C(1)DX) females. At about six days the adult flies were removed. At around two weeks the F1 adults appeared and they were studied here.

At 2 to 5 days some 5300 of these F1 males plus females were screened about 500 at a time by a procedure shown in Fig 2. From 4-liter graduated cylinders (10.8 cm x 58 cm Nalgane polymethylpentene, Fisher, 08-572-5J, Hanover Park, IL) four 17.5 cm sections were cut (the end with the spout was not used) and labeled sections 1, 2, 3, and 4. (In Fig 2 sections 1, 2, 3, and 4 are called “origin”, “next to origin”, “next to furthest”, and “furthest”, respectively.) At room temperature the right end of section 4 was filled with 40 ml of 1.5% agar for control of the temperature-gradient described below. A 2-cm diameter hole was made in the middle of section 1 to be the point of entry for the flies. Then these four pieces were put into a 34°C room, where the experiments were to be carried out. Sections 1, 2, 3, and 4 were each fixed together with a transparent connector (a 15x20 cm piece cut from a transparency film for copiers, 3M Company, St. Paul, MN). A 45 cm fluorescent light (15 watts, cool white, lamp VTU15RTCPCO used without plastic cover, obtained from American Fluorescent Corp., Waukegan, IL or local hardware store such as Mennards, Madison, WI) was placed perpendicularly 15 cm from the right end of section 4 to produce a light gradient (stimulus 1). A temperature gradient (stimulus 2) was generated by placing a 2 L beaker of ice 1 cm from the right end of section 4 and by placing a hotplate (Barnstead Thermolyne, 1900, Dubuque, IA) on its side at a setting of 150°C 10 cm away from the left end of section 1; this was ready to use in about 20 minutes, when the temperature at the cool end was about 29° and at the warm end 38° (but not greater, so as to avoid a block in motility), as determined with a digital thermometer (63-1032, RadioShack, Fort Worth, TX) inside each end of the tube. At room temperature 0.1M benzaldehyde in 5 ml of melted 1.5% agar was pipetted onto the interior walls of a 5 cm-long cap (cut from a 17.5 cm section, see above) while rotating the cap until the agar hardened (cooler temperature may be needed). One end of the cap was then closed with Saran Wrap (S.C. Johnson Inc., Racine, WI). White tulle fabric or white organza fabric (Gifts International Inc., Ontario, CA, or local fabric store such as Hancock Fabrics, Madison, WI) was folded in half and taped to the other end of the benzaldehyde cap to provide a screen between section 1 and the cap in order to prevent the flies from making direct contact with this toxic benzaldehyde solution. Then the benzaldehyde cap was brought into the 34°C room and was affixed to the left end of

section 1 with white label-tape (Fisher, 15-938). The benzaldehyde was allowed to diffuse for 30 minutes to provide a gradient of it (stimulus 4). (If a single stimulus had been used instead of four stimuli together, the resulting mutants would more likely be defective in a gene for sensing that single stimulus instead of in a gene for the final pathway.)

A previous attempt to produce such mutants (2005-2007) by Grace Tang, Victor Toniolo, Yi Hang Ng, Lar Vang, and Julius Adler used an alternative yet similar procedure [23], but it failed. After initial mutagenization of males, about 33000 *Drosophila melanogaster* were tested, around 1500 males plus females at a time. Selected were those that failed in response to light, then those were tested in response to benzaldehyde, then those were tested in response to heat. After these three tests, finally 29 mutants resulted but they did not reproduce. Then we started over with the present procedure, which puts all the stimuli together for the isolation of mutants.

B. HOW TO STUDY RESPONSE TO EXTERNAL STIMULI

Flies were removed from their food vials into empty vials. The flies (about 500 flies for isolation of mutants, about 10 in later experiments) were then placed at 34°C in the dark for 30 minutes. They were added through the hole in section 1 by use of a funnel, and then the fluorescent lamp was turned on, which marked the beginning of the assay. Every minute for 20 minutes the number of flies in each quarter of the tube was counted, unless the number of flies was too large to easily count, in which case estimates were made. In cases where the flies were getting killed by the heat and/or benzaldehyde, the assays were run for a shorter time.

Parental flies went almost entirely to the attractant end (compartment 4). For isolation of mutants, those flies that failed to go to the attractant end (the ones in compartments 1, 2, and 3) were saved, placed in food vials, and retested in the same way one to two days later. Then each of those males that again failed was mated with virgin females, and the next generation (F2) of males was tested in the same way. This procedure yielded five mutants, called 1 to 5. Only males were used in all of the experiments presented in this article. (If a single stimulus had been used instead of four stimuli together, the resulting mutants would more likely be defective in a gene for sensing that single stimulus instead if in a gene for the final pathway.)

When light alone is used, the setup is the same as in the isolation of mutants but with only the light source used.

When heat alone is used, the setup is the same as in the isolation of mutants but with only the heat source used and with a light source placed parallel to the entire length of the tube.

When no stimuli was used, the setup is the same as in the isolation of mutants but with no stimuli present except for a parallel light source placed along the entire length of the tube.

See ref. 4 on testing for quinine and gravity. Each trial used about 10 male flies.

See ref. 5 on testing for sucrose. Each trial used about 10 male flies.

C. HOW TO STUDY RESPONSE TO INTERNAL STIMULI

1. Measuring hunger

The apparatus for measuring hunger consisted of four tubes as shown in Fig 10. Tube 1 is 20 cm long and 5 cm wide, tubes 2, 3, and 4 are 40 cm long and 5 cm wide (cut from clear acrylic tubes 6 feet long and 2 inches wide, TAP Plastics info@tapplastics.com). Each of the four tubes was drilled with four rows of 1 mm holes every 2.5 cm to provide exchange of air (possibly these holes are not needed when only 50 flies are used). Each of the four tubes was saturated with water by adding a strip of Kimwipe (15 in x 17 in, Kimberly-Clark) 5 cm wide and 20 or 40 cm long to which 5 or 10 ml of distilled water was added, and then the excess water was poured off. Between each of the four tubes was placed a funnel (45 mm wide, Kimble Kimax 28950-45) with its stem cut off (see Fig 10), to direct flies from one tube into the next and to minimize movement backward. Then as connector to connect the tubes, transparent packaging tape (5 cm width, SureTech Brands, Avon, OH) was placed all the way around between tubes 2 and 3 and between 3 and 4; between 1 and 2 it was placed over only the bottom half because the gate is in the way elsewhere. Then a gate (see Fig 10) (cut from transparency film for copiers, 3M, St. Paul, MN) was inserted between 1 and 2 (used only during the loading of the flies, then removed). The two open ends of the apparatus were capped with an acrylic cap we made, though Saran wrap (S. C. Johnson & Sons, Racine, WI) would be perhaps as effective.

The apparatus was put into a dark room at 21 to 23°C. About 50 male flies per trial were placed from their growth medium into tube 1. After about 5 minutes to allow the flies to become quiescent, the gate was removed and replaced by putting transparent packaging tape over the top half. The apparatus was then covered with black garbage bags to ensure darkness. Then the number of flies in each tube was counted at 0, 10, 20, 30, and 40 hours by having lights on for 30 seconds.

The effect of adding water plus sucrose was studied as above except that 0.1M sucrose was poured onto the strip of Kimwipe, 5 ml for tube 1 and 10 ml for tubes 2, 3, and 4, and then the excess was poured off at 10 sec.

2. Measuring thirst

The same apparatus (Fig 10) and the same method was used for studying thirst as for measuring hunger except there was no water added and sucrose crystals were placed on the Kimwipe, 250 mg in tube 1 and 500 mg in each of tubes 2, 3, and 4.