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Genetics of anesthetic response: autosomal mutations that render *Drosophila* resistant to halothane

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Abstract

Molecular mechanisms of anesthetic action are poorly understood. Genetic approaches to investigate mechanisms of anesthesia, although sparse and rather new, are turning out to be informative and add a new perspective. Before beginning a systematic investigation of anesthesia by this approach, it is necessary to have at hand a large collection of mutations in different loci that alter anesthetic response. We report here the isolation and characterization of six mutant autosomal lines that show a decreased sensitivity to the inhalation of anesthetic halothane. Two of these mutations, *Omar*^{82} and *Qajjem*^{211} are shown to map to separate loci on the third chromosome.

Keywords: Anesthesia; Genetics; Autosomal mutants; *Drosophila*; Inebriometer

1. Introduction

Mechanisms of general anesthesia are among one of the most intractable challenges in the behavioral sciences and pharmacology. While structure function studies have been successful to a great extent in delineating molecular mechanisms of action of many neuroactive compounds, they have been of dubious value in the case of general anesthetics. The reasons are not far to seek. A clear definition of the anesthetized state, let alone an understanding of the physiology, is lacking. The state of anesthesia is simply referred to as a reversible loss of consciousness defined strictly in terms of animal behavior. This state has been empirically known for over a hundred and fifty years and can be caused by diverse compounds ranging from nitrous oxide to isoflurane to halothane and even inert elemental gases like argon. The only property that binds these agents is hydrophobicity reflected by their oil/water partition coefficient. Meyer [10] and Overton [18] independently observed, around a hundred years ago, that the potency of general anesthetics had a direct correlation to their hydrophobicity. It was primarily due to this observation that for the greater part of the last century, it was believed that the site of action for volatile anesthetics (VAs) was the cell membrane. A particularly popular idea is that VAs dissolve in the lipids of membranes and fluidize them to cause by rather unknown mechanisms a shut down of neural activity. Virtually every avenue to prove this has been probed with no spectacular success. Biophysical methods have been the most challenged due to a clear demonstration that the action of VAs arises due to physical, as opposed to chemical, changes. Proteins, as direct targets of anesthetic action, are a relatively recent suggestion. VAs have been shown to bind many proteins ranging from hemoglobin to enzymes like luciferase [22]. Luciferase has also been shown to be competitively inhibited by VAs [20]. It is therefore conceivable that VAs bring about their effects by acting on membrane proteins or channels directly to disrupt neuronal function. Many molecules have thus far been identified, which function in an altered manner upon exposure to VAs. However, in most cases, it remains unclear if any of these molecules are potential receptors for VAs or if the alterations seen are a secondary effect. A common receptor for all VAs seems unlikely, because there is now evidence that questions the unitary target hypothesis for the action of anesthetics. It is possible that there may be multiple pathways for the action of VAs [14–17]. None of these hypotheses have yet been proven convincingly.

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A behavioral genetics approach, using appropriate model systems, is ideally suited for the study of a complex phenomenon like anesthesia. For one, behavioral phenotypes and the state of anesthesia are both defined at the level of the animal as a whole. Genetically altered animal models, if obtainable, will also afford delineation of the correlates of altered anesthetic response at cellular and molecular levels in an unprecedented fashion. Mice have been used to study sensitivities to general anesthetics and strains of mice with different sensitivities have been obtained [19]. However, the differences in these strains are at multiple loci, therefore, the genetics of these systems are complicated. *Drosophila* stocks obtained by selective breeding of sub-populations that were resistant to ether also suffered from the same lacunae [3,4]. A limited number of single gene mutations, responsible for changes in behavioral response to anesthetics, have been identified in the fruit fly *Drosophila melanogaster* [2,8], in the roundworm *Caenorhabditis elegans* [5,6,13–16], and even in the yeast *Saccharomyces cerevisiae*. In a remarkable example, yeast cells were shown to be sensitive to high concentrations of halothane, and a mutant resistant to growth inhibition by halothane was shown to be affected in a component of cellular transduction machinery [7]. In the case of the worm, a number of mutations known to cause constitutive uncoordinated behavior were shown to render it hypersensitive to the effects of VAs. Many interesting aspects of the phenomenon of anesthesia, particularly differential effects of anesthetics have emerged from a study of these mutants [13,14]. In *Drosophila*, Krishnan and Nash [8] had previously identified three loci in the fruit fly that render flies relatively resistant to the anesthetic effects of halothane. The behavioral response of these mutants to different anesthetics led them to question the unitary target hypothesis [11,16]. A mosaic analysis of this mutant behavior has implicated a specific part of the brain in the response of flies to anesthetics [12]. Thus, a genetic approach is proving itself potentially insightful.

The fruit fly, *D. melanogaster*, can be anesthetized using VAs, and the concentrations and time scale of its behavioral response, seen as loss of postural control, are remarkably similar to humans. The mean alveolar concentration (MAC) or the concentration of anesthetic required to anesthetize 50% of the population of humans and the ED_{50} concentration in exposed air for 50% of flies to get anesthetized are virtually identical [11]. The fruit fly has been used previously to dissect complex behavior such as learning and memory, and we reasoned that the neural pathways of anesthetic action might similarly be amenable to genetic dissection. In order to have a comprehensive understanding of the processes that are affected by VAs, it is important to have a sufficiently large collection of mutants that are altered in their response to these anesthetics. Mutants previously isolated on the X chromosome show resistance to VAs at clinically relevant concentrations [8]. Autosomal loci have not yet been explored for such mutations because of difficulties involved in screening for recessive mutations on the autosomes. The only known locus so far identified is one for DDT resistance and is reported to affect the response of flies to halothane [2]. Here, we describe isolation and characterization of six dominant autosomal mutants that show resistance to halothane. We also report the recombination mapping of two of these loci, $Omar^{82}$ and $Qajjem^{211}$.

2. Materials and methods

Methane sulphonic acid ethyl ester (EMS) was purchased from Sigma (St. Louis, USA). 2 Bromo-2 chloro-1,1,1,trifloUroethane (halothane) was purchased from Imperial Chemical Industries India (Chennai, India).

2.1. Drosophila stocks and culture

D. melanogaster stocks were grown and maintained on medium that contained 83 g maize flour, 50 g dextrose, 25 g sucrose, 18 g agar, 15 g yeast tablet powder, 4 ml propionic acid, 0.6 ml *o*-phosphoric acid and 7 ml 10% methylhydroxy benzoate solution per liter of medium. The flies were reared at room temperature (22°C) in 200-ml bottles that contained 50 ml media. All experiments were done with flies that were 1–3 days old.

The Canton Special (CS) strain was used for mutagenesis and also as control flies. Third chromosome balancers used were either TM3, which had *Stubble* (*Sb*) as a marker, or TM6, which had *Tubby* (*Tb*) as a marker. The *rucuca* strain was used for mapping. All these stocks were from the TIFR stock collection.

2.2. Mutagenesis and screening

Ethylmethane sulphonate (EMS) mutagenesis was performed as described by Ashburner [1]. Batches of 100 males of the CS strain were starved for 6 h and then transferred to bottles for mutagenesis. Each of these bottles contained four circular Whatman filter discs soaked with 4 ml of a solution containing 0.75% EMS and 2% sucrose. The starved flies feed readily on this solution. The flies were allowed to feed for 12 h and then crossed en masse to virgin females, combined in equal numbers from a cluster of third chromosome deficiency stocks. This strategy would enable us to pick recessive mutants that were uncovered by any one of these deficiencies in the F1 generation. In addition, dominant mutations would also be recovered. F1 progeny were screened using the inebriometer assay. Flies that were not anesthetized after a 30-min exposure to 0.75% halothane in air were considered to be resistant to halothane and lines were set up with these flies. Approximately 200,000 F1 progeny were screened and 112 lines were set up with balancer flies. These lines were retested in subsequent generations and the lines that bred true were kept for further analysis.

2.3. Cantonization of lines

In order to isogenize the backgrounds of all the mutants, these flies were repeatedly out-crossed to the wild-type CS strain. The progeny that carried the dominant mutations were selected based on their anesthesia resistant phenotype in each generation. This procedure was repeated for seven generations to ensure that all other mutations, produced by EMS, did not contribute to the anesthesia resistance phenotype were lost from the fly's genome.

2.4. Behavioral assay

All assays were done in an inebriometer. The inebriometer was a modified version of the one developed by Weber to study the effects of alcohol on *Drosophila* [21]. The inebriometer was constructed and assays performed on it as described previously [17]. It consists of a 1.2-m-tall glass column of about 7.6 cm diameter and 16 hemiconical nylon baffles placed inside the column. A constant concentration of anesthetic is maintained within the column. Flies are loaded at the top of the column and initially hold on to the baffles at the top of the column. As the flies get anesthetized, they lose postural control and tumble down the column. If they have lost complete postural control, they fall right through the column and are collected in a vial placed below the column. However, if the flies have not lost their postural control completely, they can reorient themselves during their fall and can rest on baffles at subsequently lower levels. The flies that are "eluted" out of the column are collected in vials at the bottom of the column at 2-min intervals. Flies that are more easily anesthetized loose their postural control sooner and fall through the column faster and are collected in the fractions collected at earlier time points.

Anesthetic was delivered into the inebriometer using an Ohmeda Floutec 3 model hospital kettle (Yorkshire, England) and compressed air. A flow rate of 6 l/min was used during the experiments. The concentration of halothane was maintained at 0.75% for the screen and at 0.5% for all other experiments. Concentration of halothane was checked using a Riken model 18 gas indicator. Before loading the flies, the inebriometer was equilibrated with halothane for a period of 15 min. Approximately 90–100 flies were loaded onto the column during each run and anesthetized flies that fell through the column were collected every 2 min for a total of 30 min. Flies that remained in the column, after 30 min were eluted by flushing the column with CO_2. A Response Index was calculated as the number of flies eluted at a given time point divided by the total number of flies loaded. A mean elution time (MET) was calculated as:

$$\mathrm{MET} = \frac{\sum n_t t}{\sum n}$$

where n_t is the number of flies anesthetized in time t, and $\sum n$ is the total number of flies. The CO_2 fraction was considered as the 32nd-minute fraction while calculating the MET for each line. Three to eight runs per line were done, and an average MET was calculated for each line.

2.5. Recombination mapping

Mutant flies were crossed to a strain that contained multiple recessive markers spread over the third chromosome. The markers present in this stock were *rughoid* (*ru* — 0.0), *hairy* (*h* — 26.5), *thread* (*th* — 43.2), *scarlet* (*st* — 44.0), *curled* (*cu* — 50.0), *stripe* (*sr* — 62.0), *ebony* (*e* — 70.7) and *claret* (*ca* — 100.7). The female progeny that contained one copy of the mutation and one multiply marked third chromosome was crossed back to the marker strain and the progeny were scored to determine the linkage between the mutation, that caused anesthesia resistance, and the various markers. A Kruskal–Wallis test was performed to estimate the rank order of each recombinant class. Random lines were set up with all classes of recombinants to confirm their halothane resistance phenotype.

2.6. Statistical analysis

An ANOVA was performed using the METs or Response Indices from each run, and then the Fisher PLSD test was done to evaluate statistically significant differences between mutant and wild-type METs. To estimate the rank order of various recombinant classes during the mapping of *Omar* and *Qajjem*, the Kruskal–Wallis test was used. All statistical analyses were done using the Statview (Abacus Concepts, USA) statistics package for Macintosh.

3. Results

Flies elute out of the inebriometer as a function of the time of exposure to a given concentration of anesthetic. In the case of the wild-type CS strain, in an assay using 0.5% halothane, flies begin to fall within the first few minutes and 90% or more of the flies are eluted from the column within 30 min. Mutations in *Drosophila* can dramatically alter its response to VA in the inebriometer assay. It had been earlier shown that mutations at the *Shaker* (*Sh*) locus make the fly hypersensitive to anesthetics in the tail-flick assay [9]. As a part of our standardization procedure we checked the response of Sh^{KS133} flies in the inebriometer. Fig. 1 illustrates the three types of responses that can be observed using the inebriometer. CS flies show a Response Index of 0.45 ± 0.02 after a 12-min exposure, whereas, $Shaker^{KS133}$ flies show an increased sensitivity to halothane and have a higher Response Index of 0.85 ± 0.09 after 12 min of exposure to halothane. On the other hand, mutations at the *narrow abdomen* (*na*) locus make flies resistant to the effects of halothane and they have a

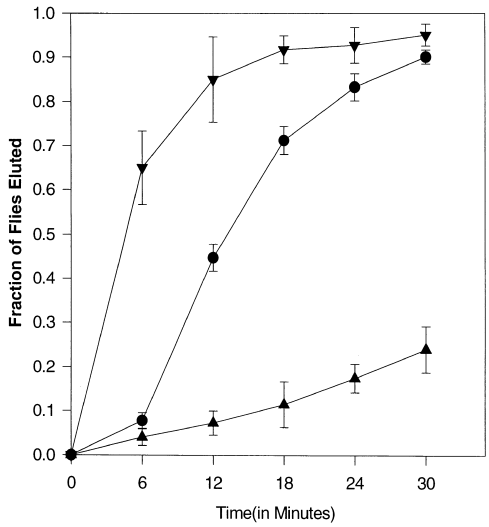

Fig. 1. Performance of two mutants compared to CS (●). Sh^{KS133} (▼) is hypersensitive to halothane and is eluted from the column very rapidly. na^{har38} (▲) is resistant to halothane and a majority of the population remains in the inebriometer column even after 30 min.

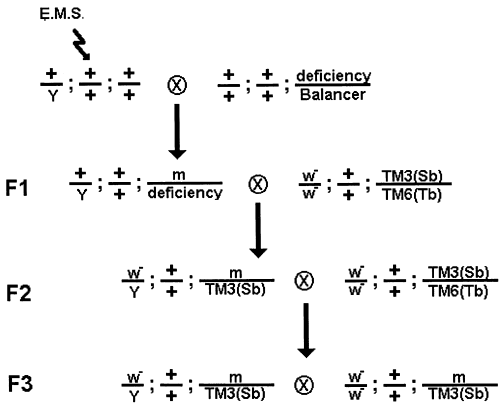

Fig. 2. Wild-type males were mutagenized using EMS and crossed to deficiency females. The F1 progeny that contained the mutation and a deficiency were crossed to a third chromosome balancer strain TM3(Sb)/TM6(Tb). The F2 males that contained the TM3 balancer were crossed to TM3(Sb)/TM6(Tb) females again and a sibling cross was performed in the F3 generation. The progeny of this sibling cross were run in the inebriometer and then scored based on the presence or absence of the Sb marker. Lines that consistently showed halothane resistance were used for further analysis.

Response Index of 0.24 ± 0.05 after a 30-min exposure in comparison to the CS Response Index of 0.90 ± 0.03 at that time point. Examining the Response Index of a population of flies at different time points allows us to differentiate between strains based on their sensitivity to halothane. In order to pick mutants that were resistant to the effects of halothane, we decided to screen for flies that showed significantly reduced response indices after 30 min of halothane exposure in the inebriometer.

3.1. Isolation of autosomal general anesthetic resistant (AGAR) mutants

We wished to obtain autosomal mutations that caused a halothane-resistant response similar to that of na^{har38}. Looking for resistance instead of hypersensitivity would allow us to eliminate mutations that generally weakened the fly and caused it to be more sensitive to external insult. Male CS flies were mutagenized using EMS and about 200,000 F1 progeny flies were screened in batches of 100 to 200 flies in the inebriometer. After two rounds of selection, 112 putative mutants, which remained for at least 30 min in the column at 0.75% halothane, were picked up and balanced lines were set up with them as shown in Fig. 2. Lines were set up only with male putative mutants to ensure that the mutation that caused the halothane resistance was autosomal. The X chromosomes in these males were inherited from the mother, and, therefore, the halothane resistance would have been the result of an autosomal mutation. These lines were retested in appropriate generations. Six such lines bred true in several subsequent generations. These autosomal general anesthesia resistant (AGAR) lines were labeled *AGAR-11*, *AGAR-21*, *AGAR-52*, *AGAR-53*, *AGAR-83* and *AGAR-211*. The response of these mutants to 0.5% halothane is shown in Fig. 3. The lines *AGAR-211* and *AGAR-83* showed the strongest

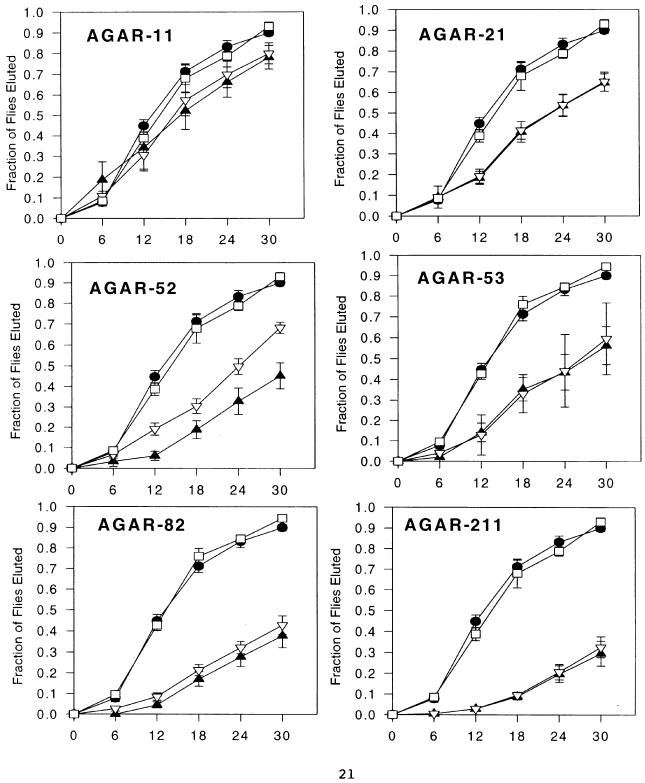

Fig. 3. Inebriometer profiles of various AGAR lines. Each panel shows the elution profiles of the AGAR lines. AGAR lines were maintained over a balancer, TM3 *Sb* in the case of *AGAR-11*, *AGAR-21*, *AGAR-52* and *AGAR-211*. *AGAR-53* and *AGAR-82* were maintained over TM6 *Tb*. Homozygous AGAR lines (▲) and balanced AGAR (▽) lines show significantly reduced halothane sensitivity when compared with balancer (□) or CS (●) flies.

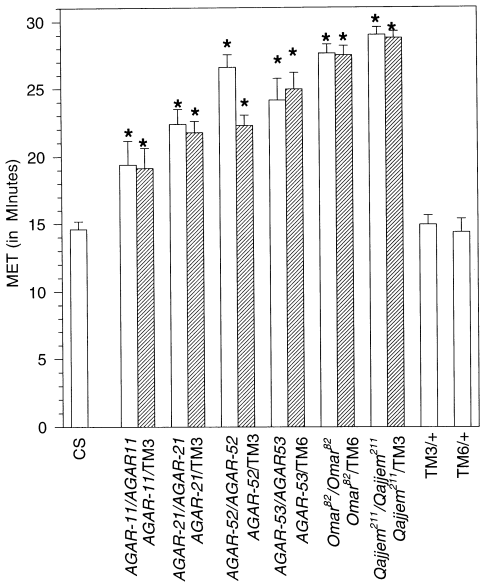

Fig. 4. METs of homozygous and heterozygous AGAR mutants. * Represents METs that are statistically significant from that of CS ($P < .05$).

responses and were subsequently mapped to clearly distinct loci. They have been named *Qajjem* (Maltese for awake) and *Omar* (to keep the poet's name together).

3.2. Characterization of AGAR mutants

The AGAR stocks were isogenized by repeatedly outcrossing them to the CS wild-type strain and then selecting the mutants based on their phenotypes. This cantonization process helped remove secondary mutations from the background of the mutation that caused the anesthesia resistance phenotype. During the cantonization, recessive lethalities that were initially associated with the *AGAR-21* and *AGAR-53* lines were separated out. The isogenized mutants were then maintained using third chromosome balancers.

In order to localize these mutations to a chromosome, flies from the balanced lines were crossed to CS, and then the progeny were tested for anesthesia resistance. If the mutations were on the third chromosome, then the progeny that carried the balancer would not show an anesthesia resistant behavior. However, if the mutations were on the second chromosome, then the population of balanced flies would show a phenotype that was intermediate to that of the AGAR mutant and the control. In the case of all the AGAR mutants that were crossed to CS, the population of flies that carried the balancer showed Response Indices in the range of 0.84–0.97. Their siblings that did not carry the balancer, showed the Response Indices that were similar in range to the heterozygous response indices of the respective AGAR mutants. This confirmed that all the AGAR mutations were on the third chromosome.

As seen in Fig. 4, wild-type flies have a MET of 14.59 ± 0.57 min. The AGAR mutants have METs that are about 1.3 to 2 times as high as that of the CS, even when they have only one copy of the mutant gene. When these mutations are present in two copies, neither their Response Indices nor METs are statistically significant from flies that carry only one copy of these mutations. The only exception is in the case of *AGAR-52* where there is a statistically significant difference ($P < .05$) in the METs and Response Indices of flies that carry either one or two copies of the mutation. Since the AGAR mutants are dominant we have not been able to do a complementation test between the various mutants to ascertain if any of them are allelic.

3.3. Mapping Omar and Qajjem

We proceeded with the mapping of the strongest AGAR mutants — *Omar* and *Qajjem*. A mutant locus can be mapped by comparing its linkage to other loci on the same chromosome. The AGAR mutants have a dominant phenotype, which was used for the initial mapping. The strategy used for this mapping is summarized in Fig. 5. AGAR flies were crossed to a marker strain *rucuca*, which contained eight recessive markers spread across the third chromosome.

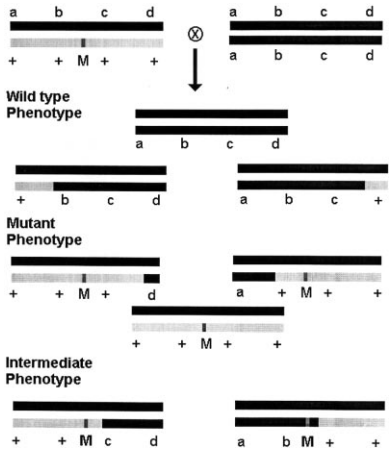

Fig. 5. Scheme for mapping AGAR mutations. A female fly, that carries a dominant mutation M (the mutant chromosome is represented in gray) and a multiply marked chromosome carrying four recessive markers a, b, c and d (chromosome in black) is crossed to a male carrying two copies of the multiply marked chromosome. The various recombinant classes of progeny are assayed for the presence or absence of the mutant phenotype, and then scored based on the markers they carry. Recombinant classes that never carry the M mutation will show wild type phenotype (classes a b c d, + b c d and a b c +), whereas those recombinant classes that always carry the M mutation will show mutant phenotype (classes a + + +, + + + d and + + + +). However, if recombination events occur between markers that flank the mutation M (b and c), the mutant phenotype will be seen only in those cases where the recombination event has picked up the M mutation. In other cases, the recombination event between b and c will produce flies that show a wild-type phenotype. Therefore, recombinant classes + + c d and a b + +, when assayed en masse would produce a phenotype intermediate to wild-type and mutant phenotypes. This result would indicate that mutation M lies between markers b and c and is confirmed by setting up lines with flies from each of the classes that show intermediate phenotype.

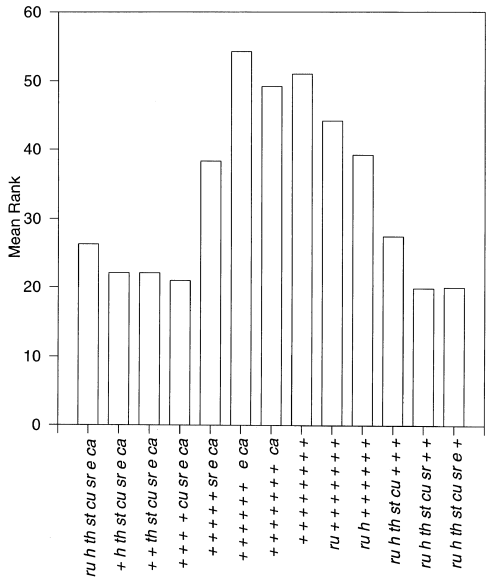

Fig. 6. Rank order of the METs of various recombinant classes obtained during the mapping of *Qajjem*^{211}. + + + + + *sr e ca* and *ru h th st cu* + + + are lines that showed intermediate phenotypes.

Meiotic recombination would occur in progeny females that contained one copy of the AGAR mutation and one copy of the marker chromosome. These females were crossed back to males of the *rucuca* strain. The progeny of this cross would have one chromosome that had all the *rucuca* markers and one chromosome that would be either a recombinant or parental chromosome. Since the markers are recessive, only the markers present on the recombinant chromosome, as well as the *rucuca* chromosome, could be scored visually. These recombinants were ''run'' through the inebriometer en masse and then scored individually on the basis of markers they carried and the time at which they were eluted from the column. METs were then calculated for each recombinant class. Since the recombinants were tested together and not as separate recombinant classes, a more precise estimate of the sensitivity of each class to halothane could be obtained by a rank order analysis of their METs. The classes that do not carry the AGAR mutation would have lower ranks, whereas those that carried the AGAR mutation would have higher ranks. Recombination events between markers that flanked the AGAR mutation would at times pick up the AGAR mutation and at times not do so. The resulting recombinant classes would therefore have a rank that was intermediate to those that always contained the AGAR mutation and those that did not carry the AGAR mutation. Lines were set with males from each recombinant class to confirm their AGAR phenotypes. A larger number of lines were set up with flies from the classes that showed an intermediate phenotype.

The rank order analysis of *Qajjem*^{211} shown in Fig. 6 indicates that the mutation lies between the markers *cu* and *sr,* which are located at recombination positions 50 and 62, respectively. In order to confirm this position, lines were set up with males of all recombinant classes and then progeny were tested for the dominant phenotype. Of the 79 lines set up, 50 lines were set with flies that had a recombination event around the *cu* or *sr* loci. The METs for these 50 lines are summarized in Table 1. Recombination events that occurred between the *cu* and *sr* loci carry the *Qajjem*^{211} mutation 23 out of 35 times. These 23 lines have a combined mean MET of 28.28 min, and they are statistically indistinguishable from 26 other lines that carried the *Qajjem*^{211} mutation. These 26 lines consisted of 10 lines from flies from the ++++++ *e ca* recombinants, five lines from the +++++++ *ca* and *ru h th st* +++ + class, and two lines each from the + + + + + + + , *ru* + + + + + + + , and *ru h* +++++ + classes. All these lines carried the *Qajjem*^{211} mutation and showed METs ranging between 26.63 and 30.57 min. The 10 recombinant lines that had recombination events between *cu* and *sr*, but no *Qajjem*^{211} phenotype had a combined mean MET of 14.8 min, which was statistically indistinguishable from other recombinant lines that did not carry the *Qajjem*^{211} mutation. The 18 recombinant lines that showed no resistance to halothane consisted of five lines set with the *ru h th st cu sr* + + and + ++ + *cu sr e ca* classes

Table 1
METs of recombinant lines used to map *Qajjem*

Recombinant	Number of lines	Average MET	Highest MET ± S.E.M.	Lowest MET ± S.E.M.
+ + + + + *sr e ca*	18[a]	28.32	29.65 ± 0.65	27.53 ± 0.57
	2[b]	14.87	15.53 ± 0.65	14.22 ± 0.89
+ + + + + + *e ca*	10[a]	28.52	29.42 ± 0.36	27.75 ± 0.67
ru h th st cu + + +	5[a]	28.16	29.55 ± 0.25	27.53 ± 0.66
	10[b]	14.78	16.35 ± 0.65	13.12 ± 0.61
ru h th st cu sr + +	5[b]	15.44	16.35 ± 0.48	14.56 ± 0.68

The various recombinant lines fell into two categories marked by superscripts a and b. Column 2 indicates the number of lines of a given genotype that fell into each phenotypic category. Column 3 shows an average MET for the lines in each category. Columns 4 and 5 show the highest and lowest METs of lines in each category. A total of 18 out of the 20 lines in the + + + + + *sr e ca* class fell in the resistant category while two lines fell in the sensitive category. All lines in the + + + + + + *e ca* showed the resistant phenotype. Five out of the 15 lines in the *ru h th st cu* + + + recombinant class were resistant and all the five lines in the *ru h th st cu sr* + + recombinant class were sensitive. These results confirm that the *Qajjem* locus is located between *cu* and *sr*.

[a] Resistant.
[b] Sensitive.

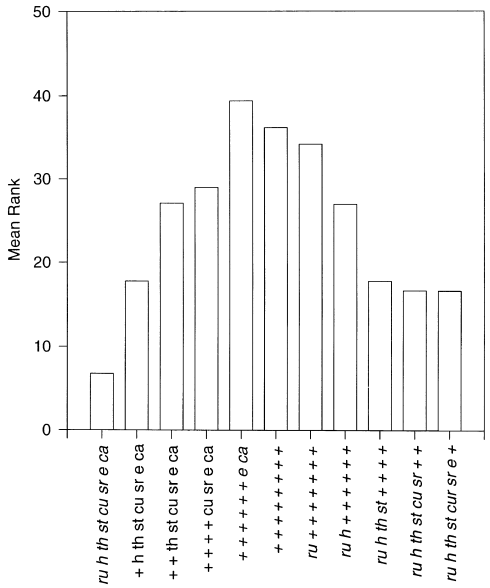

Fig. 7. Rank order analysis of the METs of various recombinant classes obtained while mapping *Omar*^{82}. *+ + th st cu sr e ca*, *+ + + + cu sr*, *e ca* and *ru h + + + + + +* are recombinants that showed ranks that were intermediate to the lines that showed anesthesia resistance and to those that showed sensitivity.

and two lines each with the *ru h th st cu sr e ca*, *+ h th st cu sr e ca*, *++ th st cu sr e ca* and *ru h th st cu sr e +* recombinants. These lines had METs that ranged between 12.57 and 17.15 min indicating that these lines do not contain the *Qajjem*^{211} mutation. This data thus clearly identifies the *Qajjem*^{211} locus to be located between the markers *cu* and *sr*.

A similar rank order analysis of the *Omar*^{82} recombinants in Fig. 7 shows that the mutation may be located between the markers *h* and *st*. The marker *th* lies very close to *st*, and very few flies with recombination events between these two markers were obtained and therefore not considered for this analysis. A total of 65 lines were set up with male from various recombinant classes, and the data from 40 lines that were in the *h − st* region have been tabulated in Table 2. Of the 30 lines that had a crossover event in-between *h* and *st*, only three lines showed wild-type phenotype while 27 lines showed the *Omar*^{82} phenotype. The three lines that showed sensitivity to halothane had a combined mean MET of 15.18 min and ranged from 12.72 to 17.11 min, which was similar to the METs of 16 lines from other recombinant classes that did not carry the *Omar*^{82} mutation. Of these 16 lines, five lines were from the *+ h th st cu sr e ca* and *ru h th st + + + +* recombinant classes each, while there were two lines each from the *ru h th st cu sr e ca*, *ru h th st cu + + +*, and *ru h th st cu sr + +* recombinant classes. Nineteen lines from various recombinants that did show the *Omar* phenotype — five lines of the *+ + + + cu sr e ca* and *+ + + + + sr e ca*, three lines of the *ru + + + + + +* and two lines of the *+ + + + + + e ca*, *+ + + + + + ca*, and *+ + + + + + +* recombinant classes each showed *Omar* phenotype and were indistinguishable from the 25.66 min combined mean MET of the 27 lines that had recombination events between *h* and *st*. These data indicated that the *Omar*^{82} locus is between the markers *h* and *st*.

4. Discussion

We presented the isolation and characterization of six lines that show a halothane resistant phenotype in the foregoing sections. We focused on the onset of anesthesia rather than the hypnotic state since the inebriometer is best suited for analyzing differences in loss of postural control. All the mutants isolated, except *AGAR-52*, have a dominant phenotype that is virtually indistinguishable from their homozygous phenotypes. All the AGAR lines — *AGAR-11*, *AGAR-21*, *AGAR-52*, *AGAR-53*, *Omar*^{82} and *Qajjem*^{211} map to the third chromosome. We have used the dominant phenotype of two of these lines, *Omar*^{82} and *Qajjem*^{211}, to further map them to two separate loci on the third chromo-

Table 2
METs of recombinant lines set up to map *Omar*

Recombinant	Number of lines	Average MET	Highest MET ± S.E.M.	Lowest MET ± S.E.M.
+ + th st cu sr e ca	18[a]	25.69	27.58 ± 0.37	23.55 ± 0.75
	2[b]	14.60	15.43 ± 0.84	13.78 ± 0.13
+ + + + cu sr e ca	5[a]	25.77	27.45 ± 0.83	23.98 ± 0.53
ru h + + + + + +	9[a]	25.60	26.81 ± 0.68	24.13 ± 0.59
	1[b]	16.32	16.32 ± 0.34	
ru h th st + + + +	5[b]	14.89	16.38 ± 0.24[b]	13.44 ± 0.54

All lines tested fell into two categories marked by superscripts a and b. Column 2 shows the number of lines of a given genotype that fell into a given phenotypic category. Columns 3, 4 and 5 are average METs of the various lines and the range of the METs of lines in each category. In the *++ st cu sr e ca* recombinant class, 18 of 20 lines showed the *Omar* phenotype whereas two lines showed halothane sensitivity. Among the *+ + + + cu sr e ca* lines, all five lines showed resistance to halothane and all five *ru h th st + + + +* lines showed sensitivity to halothane. Nine out of 10 *ru h + + + + + +* lines showed the halothane resistant *Omar* phenotype. From these results, we conclude that *Omar* maps between *h* and *st*.

[a] Those that showed the *Omar* phenotype.

[b] Those that showed wild-type sensitivity to halothane.

some. *Omar*^{82} has been mapped to the region between *h* and *st* while *Qajjem*^{211} has been mapped to the region between *cu* and *sr*.

Although the screen was designed to pick recessive mutants in the F1 generation, we did not pick up any truly recessive mutants. In addition, the dominant mutations obtained seem to be all on the third chromosome. The inability to obtain recessive mutants could have been due to the fact that the deficiencies used by us only covered about 30% of the third chromosome. The absence of second chromosome mutants from this screen was primarily due to the fact that we focused on third chromosome mutations and set lines with a third chromosome balancer. Lines that contained a mutation on the second chromosome would have their phenotypes diluted with respect to a third chromosome balancer and, hence, would have been lost in subsequent retesting.

It is unlikely that the limitations of the screen preclude the identification of recessive mutants whose phenotypes were uncovered by the deficiency stocks used. In an earlier screen, two alleles of the *narrow abdomen* (*na*) gene that show recessive halothane resistance were isolated using an identical assay [8]. The behavioral phenotype of the recessive *na*^{har38} allele is much stronger than that of any AGAR mutant. We therefore believe that the absence of recessive phenotypes cannot be attributed to ceiling effects of the inebriometer.

The dominant phenotypes of these mutants are possibly due to neomorphic or antimorphic effects of the mutation. We have characterized all the deficiency stocks used for the mutagenesis and have not found any significant resistance to halothane and therefore do not believe that the dominant phenotype of these mutants are due to haploinsufficiencies. Pharmacokinetic differences, increased metabolism of halothane, lowered absorption and lowered efficiency of halothane reaching the right concentrations in the brain are all possible explanations for the halothane resistance phenotype. However, we have designed our assay and screen to minimize the number of mutants isolated that may be affected in the absorption of halothane. We have also tried to minimize effects due to metabolic degradation of halothane. We flow 6 l of halothane per minute through the inebriometer, and this large excess of fresh halothane should negate effects due to metabolic degradation. Genetic analysis, mapping the foci of action and molecular analysis will reveal the true nature of these mutant genes.

The AGAR mutants are a valuable addition to previously isolated mutants and will be particularly useful for interaction studies with *har* mutants, on the X chromosome, and with VA hypersensitive mutants like ion channel mutants. Such studies could be extended to physiological preparations from animals carrying mutations and combinations of mutations. We hope that molecular and cell biological studies of such mutants will be feasible in the future and that these techniques will help dissect the neuronal pathways and mechanisms involved in the action of volatile general anesthetics.

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