Calreticulin Mediates Anesthetic Sensitivity in Drosophila melanogaster

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Background: Various species, e.g., Caenorhabditis elegans, Drosophila melanogaster, and mice, have been used to explore the mechanisms of action of general anesthetics in vitro. The authors isolated a Drosophila mutant, eth"311, that was hypersensitive to diethyl ether and characterized the calreticulin (crc) gene as a candidate of altered anesthetic sensitivity.

Methods: Molecular analysis of crc included cloning and sequencing of the cDNA, Northern blotting, and in situ hybridization to accomplish the function of the gene and its mutation. For anesthetic phenotype assay, the 50% anesthetizing concentrations were determined for eth"311, revertants, and double-mutant strains (wild-type crc transgene plus eth"311).

Results: Expression of the crc 1.4-kb transcript was lower in the mutant eth"311 than in the wild type at all developmental stages. The highest expression at 19 h after pupation was observed in the brain of the wild type but was still low in the mutant at that stage. The mutant showed resistance to isoflurane as well as hypersensitivity to diethyl ether, whereas it showed the wild phenotype to halothane. Both mutant phenotypes were restored to the wild type in the revertants and double-mutant strains.

Conclusion: eth"311 is a mutation of low expression of the Drosophila calreticulin gene. The authors demonstrated that hypersensitivity to diethyl ether and resistance to isoflurane are associated with low expression of the gene. In Drosophila, calreticulin seems to mediate these anesthetic sensitivities, and it is a possible target for diethyl ether and isoflurane, although the predicted anesthetic targets based on many studies in vitro and in vivo are the membrane proteins, such as ion channels and receptors.

TO understand the mechanisms of action of anesthetic agents, studies have shown two phenotypes of anesthetic sensitivity: hypersensitive and resistant to volatile anesthetics. The hypersensitive mutants require a lower (>20%) concentration of anesthetics, whereas resistant mutants need higher (>20%) concentrations than the wild type. A Drosophila mutant, EthAR201 (old name, Eth-29), is resistant to diethyl ether, chloroform, and halothane, and different genes control each anesthetic phenotype. Another Drosophila halothane-resistant mutant, bar, induced by a chemical mutagenesis, and many mutants hypersensitive and resistant to diethyl ether by using the mutagenesis of a transposon tagging of the P-element insertion, have been isolated. A number of mutated genes, hypersensitive and resistant to volatile anesthetics, have been identified in Drosophila, and Caenorhabditis elegans and mice.

In Drosophila, one of the candidate genes in the hypersensitive mutants is the para gene that encodes a voltage-gated sodium channel α subunit. The Na+ channel is known as the generator of the action potential and is expressed primarily in the nervous system of the fly. Mutations of the para gene have been isolated by heat-induced paralysis (29°–37°C), and some of them are temperature-sensitive to grow at lower temperatures than the wild type. Almost all mutants show hyper- sensitivity to diethyl ether. The mutant, para<sup>hdb</sup>38, is the most hypersensitive to diethyl ether, but it shows no paralysis at the lowest (29°C) temperature. It suggests that the underlying mechanisms are not identical, e.g., simple reduction of channel function, but rather distinct sites of the protein for diethyl ether anesthesia and heat-induced paralysis. In C. elegans, a candidate sodium ion channel subunit (UNC-8) and a close homolog of the mammalian stomatin (UNC-1) complex is considered a possible target molecule for diethyl ether and halothane anesthesia.

Stomatin is thought to regulate an as yet unknown ion channel to control sodium flux in a ball-and-chain fashion. Mutations in unc-1 and unc-8 genes alter sensitivities to volatile anesthetics, and some mutants of unc-1 suppress unc-8 mutation. In model animals, mutated genes encode the proteins that alter the sensitivity to volatile anesthetics, including the target molecules of volatile anesthetics. They are almost all...
membrane proteins such as sodium channel subunits and stomatin described above, Drosophila A kinase anchor protein 200 (DAP200), insulin-like growth factor type I receptor-like, syntaxin and syntaxin-binding proteins, G-protein α subunit (GOGA), the complex I of the nematode respiratory chain, γ-aminobutyric acid type A receptor, and α-2A adrenoceptor (reviewed in GamO19).

We have isolated another hypersensitive mutant, eth31, which has a transposon in the Drosophila calreticulin gene, crc. It is the first fertile mutation of the crc gene. Calreticulin is known to have multiple functions and diverse cellular distribution (reviewed in Michalak et al.20 and Krause and Michalak21). Briefly, the protein has three domains, N, P, and C, and resides mainly in the lumen of the endoplasmic reticulum in nonmuscle cells. On the basis of several in vitro and in vivo mammalian studies, the N-domain is unique to calreticulin and functions outside of the endoplasmic reticulum. That is, calreticulin modulates both the α-integrin adhesive function and integrin-initiated Ca^{2+}-signaling into the cell and binds to DNA-binding domain of steroid hormone receptors to regulate the gene expression. Recently, calreticulin was found to mediate nuclear export of glucagon receptors, and glucagon receptor export is facilitated by its DNA-binding domain, which is shown to function as a nuclear export signal.22 The P-domain has the chaperoning action of glycoproteins, coworking with calnexin and protein–protein interacting action with sarcoplasmic/endoplasmic reticulum Ca^{2+}-adenosine triphosphatase and inositol triphosphate (InsP_{3}) receptor in endoplasmic reticules. The C-domain has a high-capacity Ca^{2+}-binding site. The P- and C-domains play a central role in intracellular Ca^{2+} homeostasis.

To confirm that the mutated crc gene of eth31 causes altered phenotype (hypersensitivity to diethyl ether), we cloned and sequenced the crc cDNA and examined the expression of crc at various developmental stages. We also examined the relations between the crc gene and phenotype in the mutant, revertants, and transgenic flies. Furthermore, we determined the 50% anesthetic concentrations (EC_{50}s) of other anesthetics, isoflurane and halothane, and speculated on the possible relation between the phenotype and functions of crc.

Materials and Methods

Drosophila Stocks
The Drosophila mutant hypersensitive to diethyl ether, eth31, is named as an ether-anesthetic sensitive mutant, is found as a mutation on chromosome 3, and is the 11th mutant in our laboratory. The mutant that has a P-element in the crc locus and the P-element excision-induced revertants, revertant-2, -3, -5, and -44, have been isolated previously.3 CyO/Sp;Sbp[fry^{+}Δ2-3 99B]/TM6 (jump starter), w^{+};PrDr/TM3 (balancer), Canton-S (wild type), and w (white) were used in this study. 25 CyO, TM6, and TM3 are balancers that inhibit the crossing over. P[fry^{+}Δ2-3 99B] is a P-element construct to produce transposase and inserted in the third chromosome at 99B. The flies were raised on a standard medium (agar, yeast, glucose, corn grits) or on an egg-collecting medium (grape juice, whisky, agar) for collecting embryos at 25°C. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the participating institutions in Department of Earth and Life Sciences Osaka Prefecture University.

cDNA Cloning and DNA Sequence Analysis
The general cloning techniques described by Sambrook et al.24 were followed. A 2.1-kb genomic DNA fragment (pr-clone in fig. 1A) was isolated by the plasmid rescue method and used as a probe to isolate cDNAs from the crc locus. Using the pr-clone as a probe, wild-type cDNA clones were isolated from a Canton-S adult male library in the phage λ gt10.26 We determined the complete nucleotide sequence of the cDNA clone on both strands. The cDNA insert was isolated from the phage vector, subcloned into the Bluescript KS" vector (Stratagene, Burlingame, CA), and sequenced by a dideoxynucleotide chain termination procedure using Dye Deoxy Terminator Cycle Sequencing Kit in a model 373A autosequencer (Perkin Elmer ABI, Foster City, CA). Analysis of sequence data was searched using BLAST programs provided by the NCBI server (National Institutes of Health, Bethesda, MD) and programs provided by the Berkeley Drosophila Genome Project server (Berkeley, CA). All reagents used in this study were supplied by Nacalai tesque Inc. (Nara, Japan) unless otherwise mentioned.

RNA Isolation, Northern Blot Hybridization, and 5'RACE
Total RNA was isolated from the embryos, larvae, pupae, and adult flies. Poly(A)^+ RNA was separated on oligo(dT)-cellulose columns (Amersham Life Science, Buckinghamshire, United Kingdom). Next, 5 μg per lane of poly(A)^+ RNA was loaded per lane of the formaldehyde-agarose gel. After electrophoresis, the RNA was transferred to a Hybond-N membrane (Amersham). Flourescein labeling of the probes, hybridization, and detection of mRNA were performed according to the instructions provided by the supplier (Amersham). Furthermore, to determine the 5' ends of the mRNA transcripts, 200 ng poly(A)^+ was applied for 5' RACE using the RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) kit (Ambion Inc., Austin, TX). Briefly, the RNA is treated with calf intestinal phosphatase in a 20-μl reaction mixture to remove free 5'-phosphates and to remain the cap structure found on intact 5' ends of mRNA. After phenol:chloroform extract, the
The C-domain binds more than 25 mol of Ca\(^{2+}\) ribosomal protein 49 (rp49) as a positive control. 41 (E), respectively. A 1.4 kb is a transcript of three sequence repeats, and a high-affinity binding site. The N-domain binds to KXGFFKR of steroid hormone receptors, retinoic receptors, and \(\alpha\)-integrin. The P-domain has two sets of amino acid residues, and a signal sequence (1–19 residues).40

**Expression of mRNA from the crc Locus**

To study the expression patterns of the crc mRNA, we synthesized digoxigenin-UTP-labeled single-stranded DNA probes. Sense and antisense probes were synthesized by polymerase chain reaction using each N, P, and C domain primers of crc cDNA subclone: sense primers, N-1 (5′-tctgaaggagaatttcgac-3′, 148–169 nt), P-1 (5′-aggacagtctggactctg-3′, 672–691 nt), and C-1 (5′-gttaagtcggcaacttcct-3′, 1043–1063 nt); and antisense primers, N-2, P-2 (5′-tttgctgccagctcctt-3′, 915–896 nt), and C-2 (5′-caatctgctgtctgcttga-3′, 1300–1279 nt). Probes were hybridized in situ to whole mounts of embryos and developing brains according to the method of Tautz and Pfeifle.27 The preparation mounted in glycerol was viewed using an Olympus BX60 light microscope (Olympus Optical Co., Ltd., Osaka, Japan).

**Isolation of P-element Excision-induced Revertants of crc**

Recently, a lethal excision derivative, revertant-s20-1, was isolated by dysgenic cross.28 Briefly, flies carrying the crc with inserted P-element of eth\(^{as}311\) (\(w^{+}\)) were crossed to \(+;CyO\text{r}^{y}\text{B}^{y}\text{Sb}^{y}\text{y} \Delta 2-3 \text{ 99B}/\text{TM6} \). Dysgenic progeny carrying CyO:Sb were crossed to \(w^{+}; PrDr/\text{TM3} \) flies twice. \(w^{+}p^{+} /\text{TM3} \) flies (\(p^{+}\) represents excised P-element) progeny were selected for lines.5 revertants-s20-1 is a line in which no homo \(p^{+/p^{+}}\) progeny emerged, i.e., lethal.

**Transgenic crc Lines**

Transgenic lines with the wild-type crc gene were produced. A new P-factor for driving genes behind the hsp70 promoter of the plasmid, pCaspeRhs, was constructed with \(w^{+}\) as the selectable marker.29 This vector, containing an EcoRI fragment of the crc cDNA, was injected into \(w^{+}\) embryos. Chromosomal localization of the transgenes and the generation of homozygotes for the transgenes were performed by standard crosses. The crc\(^{+}\) transgene resides on the second chromosome (crc-T1 and crc-T2) and mutated crc gene on the third chromosome in crc-T1;eth\(^{as}311\) and crc-T2;eth\(^{as}311\), respectively.

**Anesthetic Techniques and Statistical Analysis**

For the estimation of anesthetic concentration, each of 20 1-day-old female and male flies were administered a given concentration of anesthetics, and their responses were scored using avoidance reflex from stimulation by a fine brush as an endpoint.3 Diethylether, isoflurane
(Forane; Abbott Laboratories Inc., Park, IL), and halothane (Fluothane; Takeda Chemical Industries, Osaka, Japan) were used. The dose–response curves from at least four to nine data points were linearized by log-probit transformation to estimate EC$_{50}$ with 95% confidence limits. Comparison of EC$_{50}$s was conducted by analysis of covariance.18

Results

Drosophila Calreticulin Locus

eth$^{as}311$, a hypersensitive mutant to diethyl ether anesthesia, was screened by determining EC$_{50}$ of diethyl ether. It has a modified P-element between the eight base repeats (CTGACTGA) of 26–33-bp upstream from the initiation codon (ATG) within the first exon of the crc locus.2 We obtained a 1.4-kb cDNA, sequenced it, and performed a 5’ RACE analysis (EMBL sequence accession No. AB000718). We identified a 1420-bp full-length cDNA including the 5’ and 3’ untranslated regions of crc, 82-bp and 117-bp, respectively (fig. 1A). The open reading frame and deduced amino acid sequence were similar to those predicted from the genomic sequencing reported by Smith.30 Drosophila calreticulin has high homology (~ 70%) to calreticulins of other eukaryote species, indicating structural and functional similarities among them. The model of Drosophila calreticulin shown in figure 1B is built from a mammalian model of calreticulin.20,21 Northern blotting analysis showed that only a 1.4-kb transcript was found at all developmental stages in the wild type and eth$^{as}311$ (fig. 1C). In the wild type, the highest expression was observed at pupa stage; however, the mRNA level was much lower in the mutant than the wild type at all developmental stages, including pupal stage (fig. 1D). These results show that eth$^{as}311$ is a low-expression mutation in the crc gene and support that eth$^{as}311$ is a fertile mutation. In fact, the endogenous calreticulins were detected in the wild type and the mutant (Sumiko Gamo, M.D., and Dai Keyakidani, M.S., Department of Earth and Sciences, Osaka Prefecture University, Sakai, Osaka, Japan, unpublished data, April 1999). However, another Drosophila mutant, $l(3)s114307$, and the knockout mice of calreticulin gene are lethal.51,52

crc mRNA Expression at Various Embryonic Stages

crc mRNA is expressed at all developmental stages in wild-type Drosophila. Whole-mount in situ hybridization of the crc transcripts was performed to identify the cells and tissues that are stained by the antisense cDNA of the probe at various embryonic stages. At the cellular blastoderm, where embryonic cells distribute on the egg surface, the perinuclear cytoplasm was stained, but no staining of the cell nucleus (fig. 2A-1) was found compared to a sense RNA probe (fig. 2A-2). The cells on the egg surface were stained, but the yolk granules were not stained at the central region of the egg. At embryonic stage 9, the crc mRNA was expressed ubiquitously, especially at pericerebral and ventral neuroblast regions of the central nervous system (fig. 2B-1), but no expression was detected when a sense probe of crc was used (fig. 2B-2). Strong expression was detected in the head region, ventral nervous systems, trachea, and hind gut at embryonic stage 15 in the wild type (fig. 2C-1), but disruption of the crc expression was found in the mutant embryo (data not shown); the pattern was similar to that of the wild-type embryo when a sense RNA probe was used (fig. 2C-2).

crc mRNA Expression in the Central Nervous System

We performed in situ hybridization to find when and where the highest level of the crc transcript appears. At the third instar larvae, the outer surface neurons in the brain of the wild type were stained most strongly (fig. 3A-1). In addition, the leg and wing disks and ring gland were stained but very weakly stained in the thoracic and abdominal ganglia (data not shown) as well as the ventral ganglion (fig. 3A-1). No strong crc mRNA expression was observed when sense probes were used in wild-type brain (fig. 3A-2) and in the brain of eth$^{as}311$ by using antisense probes (fig. 3A-3). We focused on the crc expression in the brains of pupae and adults. Unlike most other larval organs, the central nervous system persists into the adult stage in Drosophila. The staining intensity of newly emerged adult type neurons (outside of brain) was stronger than the larval type neurons (inside of brain) in the 19-h-old pupae (fig. 3B-1). The strongest expression was observed at 19 h after puparization in the wild type in comparison to the control staining (fig. 3B-2), and the level of crc mRNA was markedly low in the eth$^{as}311$ brain even in 19-h-old pupae (fig. 3B-3). Subsequently, the expression decreased gradually in wild-type brain (fig. 3C-1) and was still clearer than the control of a sense probe (fig. 3C-2). In 48-h-old pupae, more differentiation of optic lobes occurred and the staining of neuronal cell bodies was stronger than the axons in the optic lobes, brains, and the subesophageal ganglia that develop from the larval part of the ventral ganglion and esophagus (fig. 3C-1). In 90-h-old pupae (figs. 3D-1 and -2), the brain was morphologically similar to the adult brain, and the staining was markedly weak (fig. 3D-1). In 1-day-old adult brains, staining was found at the limited small regions (fig. 3E-1), no staining was found in the control brain (fig. 3E-2), and little was found in the mutant brain (fig. 3E-3). During the formation of adult brain, the maximum expression is apparent in the earlier pupal stage, but it decreases gradually to minimal expression in the adult brain.
Therefore, these staining patterns are due to expression of crc mRNA and consistent with the results of Northern blot analysis of crc (fig. 1D).

**Low crc Expression Is Associated with Hypersensitivity to Diethylether**

We demonstrated here that in the ethas311, the insertion of a P-element into the crc gene results in a decrease in mRNA levels. To confirm that the inserted P-element causes the mutant phenotype (hypersensitivity to diethylether anesthesia), we determined EC50s in the excised P-element revertants. The revertants of ethas311 were isolated, and their genomic DNA were analyzed by Southern blot hybridization to show complete removal of the P-element from the ethas311 mutants to revert wild type.3 In the revertant-2, -3, -5, and -44, EC50s were of similar values to those of wild-type strains, Canton-S and w, 1.94% and 1.95% atm in these female flies and 1.91% and 1.98% atm in the male flies, respectively (table 1). These results strongly indicate that the hypersensitivity to diethylether of the mutant phenotype is caused by the inserted P-element in the crc locus, which induced a lower expression of the gene.

Furthermore, we prepared transgenic lines, crc-T1 and crc-T2, by introducing wild-type crc gene under a heat shock promoter, which expressed the gene by heat treatment and examined whether they could be rescued by heat shock from the mutant phenotype to wild type. Transgenic lines crossed to ethas311 to emerge as double mutants, crc-T1;ethas311 and crc-T2;ethas311. Their EC50s for diethylether were compared to the wild-type and mutant strains (table 1). Double-mutant strains without heat shock had significantly lower EC50s than those of the wild type, although their ED50s were higher than those of ethas311. When exposed to heat (37°C), their EC50s were not significantly different from those of the wild type. Thus, the transgenic flies were rescued from the mutation phenotypes to the wild type. The results also strongly indicate that crc is the gene that causes the hypersensitivity to diethylether in ethas311. Three types of heat treatment were performed: heata, heatt, and heatc were performed 1 h/day for the whole life and 4 days and 3 days before EC50 determination, respectively. However, their EC50s were not different from one another. A double mutant with a crc+ transgene plus a lethal revertant-s20-1 (crc-T1;s20-1) with heat treatment recovered and was still alive at adulthood in the homozy-
The results indicate that a transgene could be expressed by heat treatment.

crc Mutant Phenotypes to Volatile Anesthetics

Previous genetic studies have identified several mutated genes that alter the sensitivity to various volatile anesthetics. In some cases, the mutant phenotypes to volatile anesthetics are not parallel, and some are different among themselves. Therefore, we compared the sensitivity to isoflurane and halothane between eth^as^311 and the wild type. The dose-response curves for isoflurane were plotted to determine the EC_{50}s by using loss of avoidance response as an endpoint (fig. 4). The EC_{50}s of isoflurane were higher in eth^as^311 than in the wild type in both sexes. eth^as^311 was resistant to isoflurane but hypersensitive to diethylether. To confirm that the inserted P-element causes the isoflurane-resistant phenotype, we determined EC_{50}s in the revertant and transgenic strains as well as hypersensitivity to diethylether anesthesia. In the revertant-2, -3, -5, and -4, EC_{50}s were of similar values to those of wild-type strains, Canton-S and w, 0.75% and 0.77% atm in these female flies and 0.80% and 0.79% atm in the male flies, respectively (table 2). crc-T1, eth^as^311 without heat shock had significantly lower sensitivity to isoflurane than wild type female flies.

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Fig. 3. The highest crc expression in the brains of early pupae. The crc antisense was used as a probe in A-1, B-1, C-1, D-1, and E-1 in Canton-S and A-3, B-3, and E-3 in eth^as^311, and the sense probes were used in A-2, B-2, C-2, D-2, and E-2 in Canton-S. (A) Larval brain hemispheres (bb) including the optic lobes (ol) and ventral ganglion (vg) in the third instar larvae. (B) The brain of a 19-h-old pupa. (C) Eye ganglia (arrows), subesophageal ganglia (sub), and esophagus (es) in the head region of a 48-h-old pupa. (D) Staining of the brain and optic lobes of a 90-h-old pupa. (E) In the adult brain, staining was limited to arrow regions (arrows). For names of organs and developmental stages, refer to Hartenstein. Bar = 50 µm.
higher EC₅₀s than those of the wild type; their EC₅₀s
with heat shock did not differ significantly from those of
the wild type. Thus, the transgenic flies were rescued
from the isoflurane-resistant phenotypes to the wild
type. These results also strongly indicate that crc
is the gene that causes resistance to isoflurane anesthesia in
eth₃11. On the other hand, the EC₅₀s of halothane
were not different in eth₃311 and wild type (table 2),
suggesting that calreticulin is not involved in the halo-
thane anesthesia pathway or halothane does not seem to
affect the functional site of the protein.

Discussion
We had collected Drosophila mutants with altered
sensitivity to diethylether anesthesia, which are transpo-
son tagging strains to facilitate gene cloning. In
the current study, we demonstrated that the 1.4 kb of crc
mRNA is expressed at all developmental stages and is
widely distributed in tissues, including the central ner-
vous system. In the cellular blastoderm, the crc mRNA
was already expressed in the peripheral nuclei in the
cells. Increased expression in the ectoderm was ob-
served especially in the central nervous system and then
increased in mesoderm tissues with advancement of
embryonic stage. crc gene was expressed in the cell
proliferation regions. At late larval and early pupal
stages, its expression markedly increased in the brain;
the highest expression of the mRNA was detected in
newly developed adult neurons at 19 h after puparia-
tion and then decreased until adult stage. During the
formation of adult brain and optic lobes, the mRNA
expression was stronger in cell bodies of neurons than in
axons. Considered together, our results suggest that cal-
reticulin is required for all development stages of Dro-
osphila and especially for the formation of the brain and
optic lobes at the late larval and early pupal stages (figs.
3A-1 and B-1).
We also showed that eth₃311, which is the first fertile
mutation in crc, has lower transcriptional levels of the
crc gene at all developmental stages and leads to the
mutant phenotypes. The crc mutants, revertant-s20-1
(this study) and l(3)s114307,31 cause death during em-
byronic maturation and are probably null mutation.
In addition, knockout mice for the calreticulin gene die
during embryonic development.32 In this regard, calreti-
culin is important for the development, organization,
and pathfinding of the peripheral nervous system in
Drosophila embryos31 and for formation of the heart,
brain, and ventral body wall closure in mice.32 In fibro-
blasts, the proteins influence cell migration in calcium-
and substrate-dependent manners.33 The crc may play a
role in cellular adhesion during cell proliferation, differ-
entiation, movement, and synapse formation in the
brain, similar to knockout mice and Aplysia.32-34
The eth₃311 shows different sensitivities to various
volatile anesthetics, i.e., hypersensitivity to diethylether,
resistance to isoflurane, and wild-type sensitivity to halothane (tables 1 and 2). These findings support the possibility that diethylether and isoflurane interact with the different sites of calreticulin to induce anesthesia, but halothane does not interact with any functional site of the protein. In *crc-T1;etha*311, a transgene of *crc* controlled by a heat shock promoter is expressed only by heat shock. Transgenic flies that were not exposed to heat shock showed hypersensitivity to diethylether and resistance to isoflurane (tables 1 and 2). The transgenic flies that were exposed to heat shock were rescued to show the wild-type anesthetic responses. *crc-T1;etha*311 exposed to heat (37°C) for 1 h a day for 3 days just before anesthesia showed wild-type sensitivities to diethylether and isoflurane. This suggests strongly that the mutant phenotypes are caused by low expression of calreticulin in the adult flies.

At clinical concentrations, halothane and isoflurane cause a leak of calcium into the cytosol, deplete InsP₃-sensitive calcium stores, and prevent the increase in cytosolic calcium concentration.³⁵ Halothane reduces endoplasmic reticulum Ca²⁺ content in airway smooth muscle cells via increased Ca²⁺ leak through both InsP₃ and ryanodine receptors, and it alters InsP₃ receptors directly and indirectly via modulating InsP₃ levels.³⁶ These results suggest that halothane targets as InsP₃ receptor, but it does not interact with calreticulin because lower levels of *crc* expression cause diethylether hypersensitivity and isoflurane resistance but normal response to halothane in *Drosophila*. The reduced amount of calreticulin in the mutant may induce higher cellular Ca²⁺ levels in resting cells and require a higher concentration of isoflurane to induce anesthesia, causing isoflurane resistance.

Diethylether might act on calreticulin directly; calreticulin easily loses its function in the mutant, leading to hypersensitivity to diethylether. A possible explanation of the hypersensitivity to diethylether and isoflurane resistance is that the two volatile anesthetics act on different regions of calreticulin to induce opposite effects. We speculated previously that isoflurane interacts with the P- and C-domains of calreticulin, which control intracellular Ca²⁺ levels. Another function in the N-domain of calreticulin is as a modulator of both α-integrin adhesion and integrin-initiated Ca²⁺ influx signaling in fibroblasts of knockout mice.³⁵ Furthermore, the N-domain does not only decrease tyrosine phosphorylation of β-catenin, resulting in the modulation of cell adhesiveness,³⁷,³⁸ but it also is known as the receptor for thrombospondin, one of the de-adhesive matricellular proteins.³⁹ The low level of calreticulin in the *crc* mutant may lead to hypersensitivity to diethylether via weakness of the adhesion complexes and the signaling pathway.

Last, the highest *crc* expression was noted during development in the newly formed adult brain at the pupa stage of 19 h. In the insect, steroid hormone ec dysone induces metamorphosis. The marked reduction of calreticulin in the pupa stage may affect the formation of adult tissues by nuclear export of the glucocorticoid receptor,²² down-regulation of steroid hormone receptor functions, and chaperoning function to glycoproteins.²⁰,²¹ These effects may be disregarded as the cause of sensitivity to diethylether and isoflurane directly, but because of their indirect effects they may not be ruled out. However, we cannot ignore the interaction between the anesthetics and other proteins, particularly in the case of halothane.

### References


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**Table 2. Concentrations of Isoflurane and Halothane (EC₉₀) in Drosophila Mutants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoflurane</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canton-S</td>
<td></td>
<td>0.75 (0.71–0.79)</td>
<td>0.80 (0.76–0.84)</td>
</tr>
<tr>
<td>w</td>
<td></td>
<td>0.77 (0.71–0.84)</td>
<td>0.79 (0.73–0.85)</td>
</tr>
<tr>
<td>etha*311</td>
<td></td>
<td>1.18 (1.13–1.23)†</td>
<td>1.08 (1.04–1.12)†</td>
</tr>
<tr>
<td>revertant-2</td>
<td></td>
<td>0.78 (0.74–0.82)</td>
<td>0.76 (0.71–0.81)</td>
</tr>
<tr>
<td>revertant-3</td>
<td></td>
<td>0.85 (0.78–0.91)</td>
<td>0.67 (0.63–0.71)</td>
</tr>
<tr>
<td>revertant-5</td>
<td></td>
<td>0.72 (0.67–0.75)</td>
<td>0.71 (0.67–0.73)</td>
</tr>
<tr>
<td>revertant-44</td>
<td></td>
<td>0.80 (0.74–0.86)</td>
<td>0.77 (0.74–0.79)</td>
</tr>
<tr>
<td><em>crc-T1;etha</em>311</td>
<td>Without heat</td>
<td>0.98 (0.92–1.04)†</td>
<td>0.93 (0.88–0.98)‡</td>
</tr>
<tr>
<td><em>crc-T1;etha</em>311</td>
<td>With heat</td>
<td>0.84 (0.77–0.90)</td>
<td>0.84 (0.78–0.89)</td>
</tr>
<tr>
<td>Halothane</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canton-S</td>
<td></td>
<td>0.44 (0.43–0.45)</td>
<td>0.35 (0.33–0.37)</td>
</tr>
<tr>
<td>etha*311</td>
<td></td>
<td>0.48 (0.46–0.50)</td>
<td>0.34 (0.32–0.36)</td>
</tr>
</tbody>
</table>

EC₉₀ₘₙ (95% confidence limits) are shown as % atm.

* Heat treatment at 37°C for 1 h a day for 3 days before the anesthetic assay. † P < 0.05, ‡ P < 0.01 compared with Canton-S.

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