Inhalational Aesthetic Sevoflurane Exacerbates Eye Phenotype of SCA3 Transgenic Drosophila Model

CURRENT STATUS: POSTED

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DOI:
10.21203/rs.2.14482/v1

SUBJECT AREAS
Neurology
Neurosurgery

KEYWORDS
sevoflurane, SCA3, Drosophila
Abstract

Background: Spinocerebellar ataxia type 3 (SCA3) is an autosomal dominant inherited neurodegenerative disease. The features of SCA3 include extremely short life expectancies, motor functions, and eye phenotypes. Sevoflurane is one of the most frequently used inhalational anesthetics and shows both neuroprotective and neurotoxic effects. Previous studies showed neurotoxicity of sevoflurane exposure to Alzheimer’s disease models. However, the effect of sevoflurane inhalation on SCA3 is not clear.

Materials and Methods: Here, we exposed sevoflurane to SCA3-transgenic Drosophila model with clinically relevant concentrations and observed the consequent change of survival, motor function, and eye phenotype of the flies. Results: We found that sevoflurane exposure exacerbated eye phenotype but not survival or motor function of male SCA3-transgenic flies. The percentage of ommatidium retinal cell number of male SCA3-transgenic flies with 0%, 2.1%, or 3% of sevoflurane exposure was 70.2 ± 4.8%, 64.8 ± 4.5%, or 46.8 ± 2.9% respectively (ANOVA F = 27.86, total df = 10, p = 0.0002), while sevoflurane exposure did not show any harm to the control flies. Conclusions: Our results may acknowledge the need for caution of the potential hazard of sevoflurane application on patients with SCA3 or other poly-Q related neurodegenerative diseases.

Background

Spinocerebellar ataxia type 3 (SCA3), also known as the Machado–Joseph Azorean disease, Joseph’s disease, or the Machado–Joseph disease (MJD), is a rare autosomal dominant inherited neurodegenerative disease. The symptoms of MJD are caused by the expansion of a trinucleotide CAG repeat in the MJD1 coding region with more than 52 units, which encodes a polyglutamine (polyQ) tract in its translated protein product ataxin-3.[1] The MJD1 gene has 12 to 40 CAG repeats in healthy individuals, but this number expands to 55
to 86 repeats in one allele of affected patients and at-risk carriers.[2, 3] Ataxin-3 may be an ubiquitin-binding protein that interacts with the valosin-containing protein and Rad23 for endoplasmic reticulum-associated degradation.[4-6] Expanded pathogenic polyQ proteins tend to aggregate with various target molecules, including proteasome subunits or various transcription factors, such as the TATA-binding protein (TBP) and the CREB-binding protein (CBP).[7, 8]

The abnormal CAG repeat of SCA3 is incurable, but treatments for relief are available for some symptoms.[9] The features of SCA3 include motor functions, such as gait problems and tremor, speech difficulties, clumsiness, as well as eye phenotypes such as frequent visual blurring and diplopia.[10] Besides, SCA3 patients have extremely short life expectancies.[11]

Sevoflurane (2,2,2-trifluoro-1-[trifluoromethyl]ethyl fluoromethyl ether) is among the most frequently used inhalational anesthetics for general anesthesia during surgery. Anesthesia (2.1%) is a common clinically relevant concentration of sevoflurane, whereas 3.0% is a relatively high concentration.[12] Two opposite effects of sevoflurane have been reported, namely, the neuroprotective activity and neurotoxicity. On the one hand, Long-term exposure to sevoflurane could induce ER stress and further cause neuronal degeneration in aging rats[13] and could induce apoptosis and elevate Aβ levels, which may promote the neuropathogenesis of Alzheimer’s disease.[14] Moreover, sevoflurane shows developmental neurotoxicity.[15-17] On the other hand, sevoflurane shows neuroprotective effects, such as the improvement of cognitive ability and the protection against damage to cerebral cortical neurons after brain injury,[18] and transient forebrain ischemia.[19] The reduction of the calcium-dependent glutamate release was thought to be the underlying mechanism of protection against neuronal injury by sevoflurane.[20]

Since the inconclusive perspectives on the neurotoxic/neuroprotective role of sevoflurane,
its impact on neurodegenerative disease is not fully understood. It is clinically significant
to decipher the effects of the most popular anesthetic agent. Although sevoflurane
exposure increases apoptosis, inflammation, and Aβ levels in Alzheimer’s disease (AD),
[14, 15, 21] the effect of sevoflurane exposure on SCA3 is not clear.

In this study, we used *Drosophila* as our model to study whether sevoflurane ameliorates
or exacerbate SCA3 phenotypes. Previous studies have shown that the pan-neuronal
expression of human *MJD1-Q84* with the pathogenic expanded polyQ stretch of 84 CAG
repeats, but not the expression of human *MJD1-Q27* with 27 CAG repeats, results in SCA3
phenotypes, such as shortened lifespan, attenuated motor function, and eye phenotype.
[22, 23] This was achieved by utilizing the GAL4-UAS system.[24] Specifically, the first
filial generation of the offspring of the parental flies each carrying *elav-Gal4* (pan-
neuronal driver) and *UAS-MJD1-Q84* (or *UAS-MJD1-Q27* or *w1118* for control) was used as
the SCA3 model and control respectively. Adult flies were used to avoid potential
developmental neurotoxicity of sevoflurane exposure. Three doses of sevoflurane
exposure, namely 0%, 2.1%, and 3%, were tested to simulate the clinical usage. Survival
rate, motor function, and eye phenotype were selected as the indicators to judge the
effectiveness of sevoflurane exposure on SCA3 *Drosophila* model.

Methods

*Drosophila stocks and genetics*

All stocks were obtained from the Bloomington Stock Center. Flies were raised on standard
cornmeal food at 25 °C and 60% humidity in a 12 h light/dark cycle. The GAL4/UAS system
was used for the overexpression of transgenic UAS in *Drosophila* as previously
described[24]. Four strains of *Drosophila* were used: *elav-Gal4*, *UAS-MJD1-Q84*, *UAS-MJD1-
Q27*, and *w1118* (wild type). Virgin female flies carrying the driver *elav-Gal4* on the X
chromosome were crossed with males carrying \textit{UAS-MJD1-Q84} or \textit{UAS-MJD1-Q27}, or \textit{w^{1118}}. All F1 offspring expressed Q27 or Q84 in the nervous system, thereby producing a model for SCA3. The virgin female flies carrying the driver \textit{elav-Gal4} were crosses with \textit{w1118} male flies and their F1 offspring were used as controls. The F1 offspring of \textit{elav-Gal4 > UAS-MJD1-Q84}, abbreviated as \textit{elav > UAS-Q84} in the text, expresses an expanded polyQ stretch with 84 CAG repeats in the eye or nervous system inducing the pathogenic phenotypes in SCA3 patients, as previously described.[22] The F1 offspring of \textit{elav-Gal4 > UAS-MJD1-Q27}, abbreviated as \textit{elav > UAS-Q27} in the text, expresses an expanded polyQ stretch with 27 CAG repeats, and does not present the disease phenotype.[25] The F1 offspring of \textit{elav-Gal4 > w^{1118}}, abbreviated as \textit{elav > w^{1118}} in the text, was used as another wild type control. Fly food was changed every 3–4 d to maintain the freshness of the surroundings.

\textbf{Sevoflurane exposure}

All flies were aged 6 days after eclosion and received 0\%, 2.1\% or 3\% sevoflurane plus oxygen for 1 h in identical anesthetizing chambers. The flies were anesthetized once daily, and the process was performed for two times. Fly eye dissection and the tests of fly survival rate and climbing ability were performed on the day after the instances of anesthetization.

\textbf{Survival and climbing assay}

Flies were maintained at 25 \textdegree C in a 12 h light:12 h dark cycle at 60\% humidity. A total of 30 to 35 flies were placed in one culture vial, and three vials for each genotype of each sex. Culture food was replaced every 3–4 d by transferring flies into new vials. The number of dead flies was recorded while transferring them. For the climbing assay, the vials were gently tapped on the table and left to stand for 18 s. The number of flies that
climbed to a height of at least 5 cm was recorded. The number of flies that could not climb and failed to reach 5 cm in each vial was also counted. At least 100 flies of each genotype were tested.

**Fly eye dissection and immunohistochemistry**

All flies were age- and sex-matched to assess the modification of eye phenotype. For immunocytochemistry, eyes samples from the flies anesthetized with temporary sevoflurane exposure (0%, 2.1% or 3.0%) for the subsequent 2 d were dissected and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). Fixed samples were washed thrice in PBST (0.3% PBS with Triton X-100) for 10 min, and samples were incubated in the respective primary antibody in PBST with 5% goat serum at 4 °C overnight. Subsequently, the samples were washed thrice in PBST for 10 min and incubated in the secondary antibody in PBST with 5% goat serum at room temperature for 2 h. After incubation, samples were washed thrice for 10 min in PBST and mounted in 80% glycerol. Samples were analyzed under a Leica TCS SP2 confocal microscope. The procedures for whole-mount adult fly retinal immunohistochemistry were described in previous studies.[26, 27] The following primary antibodies were used in this study: lamin (1:20; DSHB) and rhodamine-conjugated phalloidin (1:20; Sigma). The secondary antibody anti-mouse IgG was conjugated to Alexa Fluor 488 (1:100, Molecular Probes). The number of duplicates of each genotype under 0%, 2.1% or 3.0% sevoflurane exposure of male flies respectively: *elav-Gal4 > w1118, 5, 4, 4; elav > UAS-Q27, 5, 4, 3; elav > UAS-Q84, 5, 3, 3. And that of female flies: *elav-Gal4 > w1118, 4, 3, 4; elav > UAS-Q27, 5, 4, 5; elav > UAS-Q84, 4, 6, 8.*

**Statistical analysis**

The number of phalloidin-stained photoreceptors was counted manually for statistical analysis. We calculated the total number of ommatidia then inferred the total number of
photoreceptors in the confocal sections by counting the remaining number of photoreceptors in the same confocal sections. The degenerative ratio is the ratio of the remaining photoreceptors to the inferred total number of photoreceptors (that is 7 X number of ommatidia). We used one-way ANOVA with Tukey’s multiple comparison tests to determine the significant values. Statistical significance was set at p < 0.05. For the comparison of survival or climbing curves, the log-rank (Mantel-Cox) test was used.

Results

**Exposure to sevoflurane did not influence survival rate in SCA3-transgenic flies**

To access whether sevoflurane ameliorates or exacerbate SCA3 phenotypes, the SCA3 Drosophila model of elav>Q84 and two control groups, namely elav>Q27 and elav>w1118, were treated with 0%, 2.1%, and 3% of sevoflurane and observed for subsequent survival rate, motor function, and eye phenotype for both male and female respectively. For survival rate, as an internal control of 0% sevoflurane treatment, the SCA3 model showed significantly deteriorated survival for both male (Fig. 1A, Supp. Table 1) and female (Fig. 1B, Supp. Table 2) compared to both control groups. However, both doses of sevoflurane neither ameliorated nor exacerbated survival of SCA3 model for both male (Fig. 1C, Supp. Table 1) and female (Fig. 1D, Supp. Table 2) compared to 0% control groups. Sevoflurane treatment also showed no harm to control groups with these dosages for both male (Supp. Fig. 1A and 1B) and female (Supp. Fig. 1C and 1D).

**Exposure of sevoflurane did not influence the motor function of SCA3-transgenic flies**

For motor function, as an internal control of 0% sevoflurane treatment, the SCA3 model showed significantly deteriorated climbing ability for both male (Fig. 2A, Supp. Table 3) and female (Fig. 2B, Supp. Table 4) compared to both control groups. As the case of survival rate, both doses of sevoflurane neither ameliorated nor exacerbated climbing
ability of SCA3 model for both male (Fig. 2C, Supp. Table 3) and female (Fig. 2D, Supp. Table 4) compared to 0% control groups. Sevoflurane treatment also showed no harm to control groups with these dosages for both male (Supp. Fig. 2A and 2B) and female (Supp. Fig. 2C and 2D).

**Exposure of sevoflurane exacerbated eye phenotype of male SCA3-transgenic flies**

For eye phenotype, the completeness of ommatidium retinal cell was quantified by counting the cell number of ommatidium retinal cell normalized to 7 times of ommatidium number, since seven retinal cells can be observed in a wild type ommatidium on a confocal section. Thus, a 100% ratio denotes a normal phenotype, while a 50% ratio indicates an ommatidium with only half retinal cells averagely. As an internal control of 0% sevoflurane treatment, the SCA3 model showed significantly deteriorated eye phenotype with nearly 30% or 40% for male (Fig. 3A left panel) or female (Fig. 3C left panel) respectively compared to both control groups. Moreover, sevoflurane treatment showed no harm to control groups with these dosages for both male (Fig. 3A the first two rows, and 3B) and female (Fig. 3C the first two rows, and 3D). Interestingly, sevoflurane treatment significantly exacerbated eye phenotype in the male SCA3 Drosophila model in a dose-dependent manner (Fig. 3A bottom row, 3B, and Table 1), but not the female counterpart (Fig. 3C bottom row, 3D, and Supp. Table 5).

**Discussion**

In this study, we found that sevoflurane exposure with clinically relevant concentration exacerbated eye phenotype but not survival or motor function of SCA3-transgenic flies. Furthermore, this phenomenon is sex-dependent and restricted to male flies. Our findings identified heterogeneous effects of sevoflurane exposure on distinct SCA3 phenotypes, and this may imply a board set of indicators, but not a single one should be used to judge
the impact of a treatment on a disease. Since flies are much more tolerant to ischemic anesthesia,[28, 29] our results may acknowledge the need for caution of the potential hazard of sevoflurane application on patients with SCA3 or other poly-Q related neurodegenerative diseases. Meanwhile, for the wild type or overexpression of normal MJD1 models, sevoflurane exposure showed no harm to survival, motor function, and eye phenotype. These results confirmed the safety of sevoflurane application on healthy subjects.

The sex-biased effect of sevoflurane exposure on SCA3 flies may be caused by the bias of the expression of MJD1-Q84, rather than the difference in the hormone system. A previous study of SCA3 using a similar Drosophila model of SCA3 reported much more severe phenotype in the male SCA3 model and identified the X chromosome dosage compensation to be the cause of this consequence.[25] Although the genetic instability of CAG repeat units in male SCA3 patients has been mentioned,[30] this phenomenon might be not universal.[31] Therefore, this may imply that the potential hazard of sevoflurane on patients with SCA3 is not sex-dependent, but MJD1-polyQ expression dependent.

In contrast to our previous findings of protective effects of sevoflurane on AD Drosophila model,[32] this study identified the deleterious one on SCA3 flies. These opposite effects highlight the complex nature of sevoflurane on neurodegenerative diseases. Further investigations are essential to deepen our understanding in this field to ultimately solve the long-standing paradoxical role of sevoflurane on neurotoxicity.

Previous studies identified sevoflurane exposure decreases extracellular signal-regulated kinase (ERK) phosphorylation; however, this event induces toxicity in the developing but not adult brain.[33, 34] Follow-up studies showed that sevoflurane-induced cognitive dysfunction could be rescued by regulation of Tau/GSK3β and ERK/PPARγ/CREB signaling,[35] and PPARγ dysregulation may induce microglia-mediated neuroinflammation.[36]
Another line of evidence showed that reduction of the glial cell-derived neurotrophic factor might be the cause of the anesthesia-induced cognition deficits.[37] These shreds of evidence address the ERK-PPARγ-microglia cascade to the neurotoxicity induced by sevoflurane exposure. However, preconditioning with sevoflurane or isoflurane showed protective effects against ischemic stress,[38, 39] probably through inducible NO synthesis[40] and activation of mitochondrial ATP-sensitive potassium channels.[41] In this study, we showed that sevoflurane exposure exacerbated SCA3 phenotype; however, the relevance of the ERK-PPARγ-microglia cascade has not been addressed in SAC3 pathology and is a target for future research.

Conclusions

In conclusion, we identified that sevoflurane exposure with clinically relevant concentration exacerbated eye phenotype of SCA3-transgenic Drosophila. Our results may acknowledge the need for caution of the potential hazard of sevoflurane application on patients with SCA3 or other poly-Q related neurodegenerative diseases.

Abbreviations

Spinocerebellar ataxia type 3, SCA3; Machado-Joseph disease, MJD; polyglutamine, polyQ; TATA-binding protein, TBP; CREB-binding protein, CBP; Alzheimer’s disease, AD; extracellular signal-regulated kinase, ERK.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material
The data generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors certify that there is no competing interest with any financial organization regarding the material discussed in the manuscript.

**Funding**

This work was supported by grants from the Ministry of Science and Technology in Taiwan (MOST 106-2320-B-039-023, MOST 107-2314-B-039 -042 -MY2, MOST 104-2320-B-039-009, MOST 105-2632-B-039-002, MOST 106-2632-B-039-001) and grants from China Medical University & Hospital (CMU107-S-51, CMU107-S-08, CMU104-S-14-03, CMU105-S-53, DMR-101-065, and DMR-103-099).

**Authors' contributions**

C-W C. and W-Y L. designed the model and the computational framework and analyzed the data. K-B C. and Y-C K. carried out the implementation. J.C. performed the calculations and wrote the manuscript with input from all authors. C-Y L. and H-P L. conceived the study and were in charge of overall direction and planning.

**Acknowledgements**

Not applicable.

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Table

Table 1. ANOVA analysis of the percentage of ommatidium retinal cell number of male Drosophila of elav>Q84 treated with sevoflurane.
## ANOVA summary

| F         | 27.86 |
|------------|-------|
| P value summary | 0.0002 |
| P value summary | *** |
| R square | 0.8745 |
| Number of treatments (columns) | 3 |
| Number of values (total) | 11 |

## ANOVA table

|                      | SS    | DF | MS    | F (DFn, DFd) |
|----------------------|-------|----|-------|--------------|
| Treatment (between columns) | 1051  | 2  | 525.5 | F (2, 8) = 27.86 |
| Residual (within columns)  | 150.9 | 8  | 18.86 |
| Total                 | 1202  | 10 |       |

## Tukey's multiple comparisons test

|                      | Mean Diff. | 95.00% CI of diff. | Adjusted P Value |
|----------------------|------------|--------------------|------------------|
| 0% vs. 2.1%          | 5.337      | -3.726 to 14.4     | 0.2693           |
| 0% vs. 3%            | 23.38      | 14.32 to 32.45     | 0.0002           |
| 2.1% vs. 3%          | 18.05      | 7.916 to 28.18     | 0.0024           |

## Figures
Exposure to sevoflurane did not influence survival rate in SCA3-transgenic flies. (A–B) The life span of SCA3-transgenic flies was shorter than that of control flies for both male A) and female B). (C–D) Sevoflurane treatment did not alter the survival curve for both male C) and female D) SCA3 flies. (elav>W1118 and elav>Q27 were used as controls; elav>Q84 was the SCA3 disease line.)
Exposure of sevoflurane did not influence the climbing ability of SCA3-transgenic flies. (A–B) The climbing ability of SCA3-transgenic flies was declined faster than that of control flies for both male A) and female B). (C–D) Sevoflurane treatment did not alter the climbing curve for both male C) and female D) SCA3 flies. (elav>w1118 and elav>Q27 were used as controls; elav>Q84 was the SCA3 disease line.)
Exposure to 2.1% or 3.0% sevoflurane exacerbated SCA3 eye phenotype in male flies. (A–B) Exposure to 2.1% or 3.0% sevoflurane did not cause any eye phenotype in control flies (elav>W1118, and elav>Q27), but exacerbated SCA3
eye phenotype in male flies, as revealed in A) the representative photo of a confocal section and B) the completeness of ommatidium retinal cell. (C–D) Exposure to 2.1% or 3.0% sevoflurane did not cause any eye phenotype in control (elav\textgreater w1118 and elav\textgreater Q27) or SCA3 female flies, as revealed in C) the representative photo of a confocal section and D) the completeness of the ommatidium retinal cell. The completeness of ommatidium retinal cell was quantified by counting the cell number of ommatidium retinal cell normalized to 7 X ommatidium number. Values represent mean ± SEM, ** for \( p < 0.01 \), and *** for \( p < 0.001 \) (one-way ANOVA with Tukey's multiple comparisons test). Con. indicates 0% sevoflurane exposure. (Red, phalloidin; green, lamin.)

Supplementary Files

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