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## The Genetics of Isoflurane-induced Developmental Neurotoxicity

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### Abstract

**Introduction**—Neurotoxicity induced by early developmental exposure to volatile anesthetics is a characteristic of organisms across a wide range of species, extending from the nematode *C. elegans* to mammals. Prevention of anesthetic-induced neurotoxicity (AIN) will rely upon an understanding of its underlying mechanisms. However, no forward genetic screens have been undertaken to identify the critical pathways affected in AIN. By characterizing such pathways, we may identify mechanisms to eliminate isoflurane induced AIN in mammals.

**Methods**—Chemotaxis in adult *C. elegans* after larval exposure to isoflurane was used to measure AIN. We initially compared changes in chemotaxis indices between classical mutants known to affect nervous system development adding mutants in response to data. Activation of specific genes was visualized using fluorescent markers. Animals were then treated with rapamycin or preconditioned with isoflurane to test effects on AIN.

**Results**—Forty-four mutations, as well as pharmacologic manipulations, identified two pathways, highly conserved from invertebrates to humans, that regulate AIN in *C. elegans*. Activation of one stress-protective pathway (DAF-2 dependent) eliminates AIN, while activation of a second stress-responsive pathway (endoplasmic reticulum (ER) associated stress) causes AIN. Pharmacologic inhibition of the mechanistic Target of Rapamycin (mTOR) blocks ER-stress and AIN. Preconditioning with isoflurane prior to larval exposure also inhibited AIN.

**Discussion**—Our data are best explained by a model in which isoflurane acutely inhibits mitochondrial function causing activation of responses that ultimately lead to ER-stress. The neurotoxic effect of isoflurane can be completely prevented by manipulations at multiple points in the pathways that control this response. Endogenous signaling pathways can be recruited to protect organisms from the neurotoxic effects of isoflurane.

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**Conflicts of Interest.** None

## Introduction

Jevtovic-Todorovic *et al.* and Young *et al.* demonstrated that commonly used anesthetics caused widespread apoptosis and neuronal degeneration in developing rat brains [1, 2]. These pathological changes were accompanied by a learning defect that persisted into adulthood in the rat. It is now established that, from nematodes [3] to rodents [1, 4, 5] and to primates [6, 7], volatile anesthetics *in isolation* are capable of inducing neurodegeneration in the developing nervous system [8–11]. It remains unclear how great a risk anesthetic exposure poses to the newborn human at clinical doses and lengths of time [9, 12, 13]. However, more than a million children undergo general anesthesia each year in the U.S. [14]; even rare developmental defects from their use during a critical window of vulnerability have potentially large implications for our current care of children. Since it is impossible to eliminate exposure of neonates to general anesthesia, it is critical that we develop a mechanistic understanding of the process in order to prevent anesthetic-induced neurotoxicity (AIN).

A potential productive approach to understand the mechanism of AIN is a genetic screen to detect the underlying molecular pathways that control its occurrence. In this manner, novel and otherwise unrecognized causes of AIN can be discovered and approaches to prevent AIN may be identified. Using changes in chemotaxis, we previously showed that early exposure to the volatile anesthetic isoflurane is neurotoxic to *C. elegans* [3]. Utilizing a genetic approach, we have identified two pathways, highly conserved from invertebrates to humans, that regulate AIN in the nematode, *C. elegans*. Identification of these novel interacting pathways allowed us to completely prevent AIN by genetic and pharmacologic interventions.

## Materials and Methods

### Strains

N2 Bristol, VC3201 *atfs-1(gk3094)* V, MT1522 *ced-3(n717)*, MT2547 *ced-4(n1162)*, MT5013 *ced-10(n3246)*, MT1082 *egl-1(n487)*, CB 845 *unc-30(e191)*IV, CB1338 *mec-3(e1338)*IV, CB1370 *daf-2(e1370)*III, CB3256 *mab-5(e1751)*III, CF1038 *daf-16(mu86)*II, CW532 *gas-1(fc21)*X, CX4 *odr-7(ky4)*X, CW859 *daf-16(mu86)*; *daf-2(e1370)*, CW860 *daf-16(mu86)*; *glr-1(n2461)*, GA187 *sod-1(tm776)*II, GA416 *sod-4(gk101)*III, GA503 *sod-5(tm1146)*II, GE24 *pha-1(e2123)*III, GS2477 *arIs37 I*; *cup-5(ar465)* III; *dpy-20(e1282)* IV, IK105 *pkc-1(nj1)*N, KG532 *kin-2(ce179)*X, KP4 *glr-1(n2461)*III, KU25 *pmk-1(km25)*IV, MC364 *ire-1(ok799)*II, MQ130 *clk-1(qm30)*III, MQ1333 *nuo-6(qm200)*II, MQ989 *isp-1(qm150)*IV, MR507 *aak-2(rr48)*X, MT1522 *ced-3(n717)*IV, MT1976 *unc-86(n946)*III, MT2246 *egl-43(n1079)*II, PY1589 *cmk-1(oy21)*IV, RB967 *gcn-2(ok871)*II (provided to the CGC by the *C. elegans* Gene Knockout Project at the Oklahoma Medical Research Foundation), SJ17 *xbp-1(zc12)*III; *zIs4* V, SJ4005 *zIs4[hsp-4::GFP]*N, SJ4058 *zIs9[hsp-60::GFP]*N, SJ4100 *zIs13[hsp-6::GFP]*N, TJ356 *zIs356[daf-16p::daf-16a/b::GFP + rol-6]*IV, TK22 *mev-1(kn1)*III, TU282 *lin-32(u282)*X, VC1099 *hsp-4(gk514)*II, VC1722 *skn-1(ok2315)*IV/*nT1 [qIs51]*(IV;V) (provided to the CGC by the *C. elegans* Gene Knockout Project at the Oklahoma Medical Research Foundation), VC433 *sod-3(gk235)*X,

VC498 *sod-2(gk257)I*, CW645 *sod-2(gk257);sod-3(gk235)*, VM487 *nmr-1(ak4)II*, and ZG31 *hif-1(ia4)V* were all obtained from the *Caenorhabditis* Genetics Center, Minneapolis MN. *gas-1(fc21)* was isolated in our laboratory [15]. CW859 (*daf-16;gir-1*) and CW860 (*daf-16;daf-2*) were constructed by crossing *daf-16(mu86)* with CB164 *dpy-17(e164)* to make *daf-16;dpy-17* before crossing into *glr-1(n2461)* or *daf-2(1370)* respectively. The resulting strains were allowed to self-fertilize to remove *dpy-17*. All mutations were confirmed by sequencing. All strains were grown as previously described on agar plates containing nematode growth media (NGM) with the *E. coli* strain OP50 as food [16].

### Synchronization

For each assay, cohorts of worms were synchronized on 35mm NGM plates by limiting egg laying to 2–4 hours at 20°C and then grown for 20 hours at 15°C or 20°C as necessary to obtain newly hatched L1 animals. The plates of newly hatched L1 animals were either exposed to isoflurane as described below or held at 15°C to slow development to match the isoflurane exposed cohorts.

### Isoflurane exposure

*C. elegans* L1 larvae were exposed to isoflurane at their clinical EC<sub>95</sub> (~6.5% isoflurane) for four hours at 20°C beginning at 20 hours after being laid as eggs. Generally, these animals were at hours 4–8 of L1 development. Some mutants developed slowly, such that the time of isoflurane exposure was adjusted to approximate hours 4–8 of normal L1 development. Isoflurane concentration was checked by gas chromatography as previously described [17]. Control animals were kept in room air at 15°C during that hour to slow development to match isoflurane exposure. After exposure, experimental and control animals were cultured at 20°C and tested for chemotaxis 3–5 days later, depending on the mutant strain, on day one of adulthood.

### Chemotaxis

Young adult worms were washed three times in chemotaxis buffer (5 mM potassium phosphate, 1 mM calcium chloride, and 1 mM magnesium sulfate) before being transferred in a minimal volume in the center of a 9cm NGM assay plate. The plates were divided into 3 regions in a modification of the technique described by Bargmann [3, 18]. One region contained an attractant (a 20ul spot of OP50) while the opposite region contained no attractant. The middle region served as the starting point for the animals. 2–3 plates of both control (unexposed) and exposed animals were assayed in parallel on a given day. Worms in each region were counted 1 hour after transfer. Scoring was done by an observer blinded to the exposure state of the strain but not to the strain being studied. A chemotaxis index (CI) was calculated using the formula:  $CI = 100 \times (\text{worms at food side} - \text{worms at control side}) / \text{total}$ . All results reported are new for this study (earlier results for N2, *ced-3* and *gas-1* were not included in this study).

### Isoflurane Preconditioning

Within one hour after hatching, synchronous L1s were exposed to 6.5% isoflurane for 1 hour, then allowed to recover for 3 hours, before being exposed to isoflurane as per the usual

protocol. Control animals were kept in room air at 15°C during that hour (to slow development to match isoflurane exposure). They were tested as adults as described above in the chemotaxis experiments. All preconditioning assays were performed in duplicate for control and exposed animals.

## Rapamycin

Rapamycin (LC laboratories) was dissolved in 100% dimethyl sulfoxide (DMSO) at 50 mg/ml and added to plate agar to 100uM with final DMSO concentration of 0.2% as described by Robida-Stubbs *et al.* [19]. Control plates contained 0.2% DMSO. Egg laying hermaphrodites were placed on rapamycin or DMSO plates, both with OP50, for 2 hours for synchronization, and then removed, as described above in *Synchronization*. Eggs were kept on rapamycin or DMSO plates until hatching and then exposed to isoflurane as L1s as described above. L1 animals were transferred to OP50 plates without rapamycin or DMSO the morning following isoflurane exposure, approximately 24 hours after hatching and 16 hours after isoflurane exposure.

## Statistical Analysis

Chemotaxis indices (CIs) are the mean of 6–9 experiments (except for N2 where n=15) each containing >50 animals (total animals for each strain, 300–450, except N2, total >800). Errors for CI are reported as the standard errors of the mean (SEM). Changes in CI (  $\Delta$  CI) between exposed and unexposed animals of a given genotype are calculated as the mean difference between the CI of the unexposed and the exposed animals. Error bars for  $\Delta$  CI were calculated by combining the standard deviations (SD) of the CIs and then calculating the SEM from the SD. Values for  $\Delta$  CI were compared by one-way ANOVA. If a significant difference was identified by ANOVA, then each mutant strain was compared to N2. Significance was defined as  $p < 0.01$ .

## Results

We compared the responses to isoflurane treatment of wildtype (N2) and 44 mutants that define different developmental pathways in *C. elegans*. The mutants were selected initially as those known to affect the nervous system and stress responses; ongoing results defined additional choices and refinements. For example, once the *daf-2/daf-16* pathway was identified as a possible underlying mechanism (see below), *daf-2* pathways that are independent of *daf-16* were also studied. Animals that moved extremely poorly without anesthetic exposure were unable to be tested. The genes were then broadly grouped according to their function (Supplementary Figure S1). The chemotaxis indices (CIs) of the unexposed and isoflurane-exposed mutants that significantly contributed to evaluation of the pathways we discuss are shown in Figures 1A,C,E,G,I. The effect of each genotype is expressed as the change in the CI (  $\Delta$  CI) between unexposed and exposed animals in Figures 1B,D,F,H,J.

As reported previously [3], adult N2 exposed to isoflurane as L1 larvae did not move toward food as well as unexposed worms (  $\Delta$  CI = 19.5  $\pm$  3.7;  $p = .00000008$ ); no other defects in locomotion were noted in exposed animals. Since *C. elegans* is immobilized at higher doses

of isoflurane than are mammals, we also tested a lower dose in the range of mammalian effective doses (2–2.5%). At these doses, the effect of isoflurane exposure on anesthetic-induced neurotoxicity (AIN) for N2 was similar to that at 6.5% (Supplemental Figure S2). To ensure that any mutation that eliminated AIN did so at the EC<sub>50</sub> for N2, thereby insuring a robust effect, all subsequent studies were formed at 6.5% isoflurane. CI was compared between mutants and N2. If a mutant had a CI not significantly different from the N2 value, we interpreted the result as the same AIN response as N2. An animal resistant to the effect of isoflurane would have a CI significantly less than that of N2, an indication of AIN inhibition.

### Genes affecting Apoptosis

We confirmed our earlier report that loss of the apoptotic caspase, *ced-3*, eliminated the neurotoxic effects of isoflurane in *C. elegans* [3]; after exposure to isoflurane as larvae, adult *ced-3* animals moved toward food as well as the unexposed animals (CI (*ced-3*) = 6 +/- 9.7). As a further test of the role of apoptosis in AIN, we tested three other genes involved in the apoptotic pathway in *C. elegans* (Supplemental Figure S3). Loss of *ced-4* (a cofactor of *ced-3*) or *egl-1* (an activator of *ced-4*) also eliminated AIN (CI (*ced-4*) = -2.4 +/- 10.9, p<0.01; CI (*egl-1*) = 6.1 +/- 12, p<0.01). However, loss of *ced-10* (required for engulfing apoptotic cells rather than effecting apoptosis) did not suppress AIN, as its CI was not different than that of N2 (CI (*ced-10*) = 32.8 +/- 9.1). These results show that, in *C. elegans*, is a critical component of AIN.

### Genes affecting Mitochondrial Function

Others have suggested that mitochondrial damage underlies at least part of AIN in rodents [20, 21], and that mitochondrial generation of reactive oxygen species (ROS) initiates AIN [6, 22]. We tested whether mitochondrial mutants had any effect on AIN in *C. elegans* (Figure 1A,B). In general, mitochondrial mutations caused defects in chemotaxis in unexposed animals; however, the CI was either not worsened (CI = app. 0) or improved by anesthetic exposure (a negative CI). The one exception was *clk-1*, a long-lived mutant with defective Coenzyme Q synthesis [23] and endogenous protection from chronic ROS damage [24]. While unexposed *clk-1* had a low CI, it responded to L1 isoflurane exposure similarly to N2. The CIs for all mitochondrial mutants are shown in Figure 1B; *clk-1* is the only mutant of this group with a CI similar to that of N2.

### Genes affecting ROS scavenging

To examine whether ROS scavenging plays a role in AIN, we tested mutations in superoxide dismutases (SODs) for their response to early anesthetic exposure (Figure 1C,D). Mutations in the rarely expressed extra-mitochondrial SODs, *sod-4* or *sod-5*, did not change the CI compared to wildtype, but loss of the primary cytoplasmic SOD, *sod-1*, lowered the CI compared to N2. Knockout mutations in either mitochondrial SOD, *sod-2* or *sod-3*, also suppressed the detrimental effect of isoflurane on chemotaxis. Surprisingly, the double mutant *sod-2;sod-3* had a CI similar to N2.

### Genes in the DAF-2 (insulin growth factor receptor) pathway

*daf-2* encodes a kinase that is the *C. elegans* insulin growth factor receptor (IGFR) orthologue [25]. The functional DAF-2 protein inhibits initiation of a stress-response protective pathway that is mediated by the FOXO transcription factor, DAF-16. Loss of function of the insulin-like receptor, DAF-2, completely eliminated the CI following isoflurane exposure (Figure 1E,F). In the absence of DAF-2, DAF-16 becomes localized to the nucleus and regulates longevity, fat metabolism, stress and innate immunity [26, 27]. Similarly, exposure to isoflurane causes nuclear localization of DAF-16 (Figure 2) within one hour after anesthetic exposure. If the loss of DAF-2 eliminates anesthetic-induced neurotoxicity by nuclear localization of DAF-16, then loss of DAF-16 should block the effect of DAF-2. *daf-2;daf-16* had the same CI as N2 and *daf-16* alone (Figure 1F). Thus, the *daf-2* effect on AIN is dependent on the presence of the DAF-16 protein.

### Genes affecting kinases independent of *daf-16* function

Defects in DAF-2 also induce a stress protective response via a DAF-16 *independent* pathway. Loss of DAF-2 activates the kinase AAK-2 (AAK-2 is orthologous to AMPK in humans) that in turn activates the kinase, PMK-1 (orthologous to the human *p38* MAPK). Activation of PMK-1 ultimately upregulates pathways of innate immunity and apoptosis [30, 31]. Somewhat surprisingly (since loss of DAF-2 should activate AAK-2 and PMK-1), mutations in either *aaak-2* or *pmk-1* also eliminated the CI (Figure 1E,F). However, both kinases (AAK-2 and PMK-1) are also known to mediate an ER-stress response by DAF-2 dependent and DAF-2 independent mechanisms [32, 33].

We tested 5 other kinases also known to affect stress responses in *C. elegans* (Figure 1G,H). Mutations in two kinases, CMK-1 (CaMK in humans) and GCN-2 (eIF2 $\alpha$  kinase in humans) eliminated the neurotoxic effects of isoflurane exposure. GCN-2 primarily decreases protein translation by phosphorylation of eIF2 $\alpha$  [34]; however, both GCN-2 and CMK-1 modulate the ER-stress response and apoptosis [35–37].

### Genes affecting transcription Factors

We tested the effect of transcription factors known to affect stress responses or neuronal development in *C. elegans* [38–41]. Mutations in four transcription factors (*skn-1*, *egl-43*, *mab-5*, *hif-1*) eliminated the CI of isoflurane exposure while maintaining the chemotactic response of the unexposed animals to at least 50% of that seen in N2 (Figure 1I,J). EGL-43 and MAB-5 are known to affect development of the sensory nervous system, here resulting in defective chemotaxis in the absence of isoflurane exposure [42, 43]. SKN-1 (Nrf2 in humans) and HIF-1 (hypoxia inducible factor) have well-defined interactions with other genes that affect neurotoxicity downstream of the effects of DAF-2 and DAF-16 (AAK-2 and PMK-1) [19, 44–46]. The SKN-1 pathway interacts with HIF-1 to mediate a stress response in the endoplasmic reticulum that is activated by mitochondrial ROS production [38, 39, 47, 48].

### Genes affecting the mitochondrial unfolded protein response (UPR<sub>MT</sub>)

The DAF-16-dependent effects of DAF-2 regulate multiple pathways, including those that operate in either mitochondria or endoplasmic reticulum [49, 50]. The unfolded protein

response of mitochondria (UPR<sub>MT</sub>) is activated by two overlapping pathways, one requiring the transcription factor ATFS-1 and the second pathway requiring the protein kinase, GCN-2 [51]. Both ATFS-1 and GCN-2 ultimately induce production of the mitochondrial-specific heat shock proteins HSP-6 and HSP-60. Loss of HSP-6 and HSP-60 did not affect anesthetic induced neurotoxicity in *C. elegans*. In addition, neither *Phsp-6::gfp* nor *Phsp-6::gfp* were upregulated following isoflurane exposure (Data not shown). Loss of ATFS-1 did not change CI (Supplemental Figure S1) but loss of the kinase GCN-2 eliminated CI (Figure 1G,H).

### Genes affecting the endoplasmic reticulum unfolded protein response (UPR<sub>ER</sub>)

In response to stress DAF-2/DAF-16 and GCN-2 also regulate the endoplasmic reticulum (ER) stress response [37, 50, 52], resulting in an upregulation of the ER-specific heat shock protein, HSP-4, one of two *C. elegans* heat shock proteins homologous to *hsp70* proteins in mammals. To delineate the role of the ER stress response in AIN, we tested the mutants *ire-1*, *xbp-1* and *hsp-4*. IRE-1 is a kinase and endoribonuclease that induces the ER stress response by splicing the mRNA of its downstream target, the transcription factor XBP-1 [53] which in turn upregulates expression of their downstream effector, HSP-4. The HSP-4 reporter (*Phsp-4::gfp*) was upregulated by larval isoflurane exposure at either the EC<sub>50</sub> for immobility (6.5%) (Figure 3, quantification Supplemental Figure S4) or the lower dose also affecting chemotaxis (2.5%) (Supplemental Figure S5). Similar to loss of GCN-2, loss of IRE-1, XBP-1 or HSP-4 each eliminated AIN (Figures 1D,H,J). When *Phsp-4::gfp* was placed in *xbp-1* or *ire-1* mutants, induction of the reporter by isoflurane was completely inhibited (Supplemental Figure S6). In addition, the *Phsp-4::gfp* reporter was also inhibited by the mutation *daf-2* (not shown) and by the mutant *gcn-2* (Supplemental Figure S7). The inhibition by *gcn-2* was restricted to isoflurane exposure during the L1 stage and was not true of heat shock which did induce *hsp-4* in agreement with Baker *et al.* [51]

### Effects of rapamycin

Both the *daf-2* and ER-stress pathways interact with the nutrient sensing complex, the mechanistic Target of Rapamycin (mTOR) [54, 55]. Prior studies have also indicated that isoflurane activates mTOR [56]. We therefore tested whether rapamycin, which inhibits mTOR, would have an effect on AIN. When nematodes containing *Phsp-4::gfp* were placed on rapamycin plates during the first day of life, induction of the reporter by isoflurane was inhibited (Figure 4A–D; quantification Supplemental Figure S8). Note that all plates had 0.2% DMSO on plates to facilitate uptake of rapamycin by the animals which moderately increased *Phsp-4::gfp* expression in the absence of isoflurane (compare Figure 4A with 3B). Treatment of nematodes during the first day of life (included the exposure to isoflurane) completely eliminated AIN as measured by chemotaxis (Figure 4E).

### Preconditioning with isoflurane

The involvement of the *daf-2* stress response and of *gcn-2* in the neurotoxic effects of isoflurane caused us to question whether these changes were sensitive to preconditioning similar to the effects seen in hypoxic preconditioning for both genes [57, 58]. Exposure of wildtype L1 larvae to isoflurane for one hour, followed by recovery for 3 hours before re-exposure, (Figure 5A), completely reversed the neurotoxic effects of isoflurane, *i.e.* the CI was eliminated (Figure 5B). We expected that the nuclear localization of DAF-16 seen with

isoflurane (Figure 2) exposure would be necessary for the preconditioning effect. However, *daf-16* mutants responded like N2 to preconditioning. This indicates that although isoflurane exposure does cause nuclear localization of DAF-16, successful preconditioning is not DAF-16 dependent. We also tested whether preconditioning inhibited the upregulation of *hsp-4* by isoflurane and found that it did so (Supplemental Figure S8).

## Discussion

### AIN Pathways

In *C. elegans*, exposure of L1 animals to isoflurane vastly upregulates expression of the HSP-4 reporter, a marker of ER stress. In addition, it causes translocation of DAF-16 to the nucleus, setting off a chain of events in a classic stress protective pathway. This apparent paradoxical set of responses is clarified by the genetic loss of key molecules in these two pathways. Loss of wild type products of multiple activators of ER stress, as well as loss of *daf-2<sup>+</sup>*, which induces movement of DAF-16 to the nucleus, completely blocks AIN. We interpret the data to show that these two pathways have opposing roles in AIN. If set in motion prior to the environmental stress of a VA, the *daf-2* pathway is protective, and can compensate for the simultaneous activation of a neurotoxic response that is ultimately mediated by ER stress. We propose a model which explains all our data in Figure 6. Isoflurane induces the two highly conserved signaling pathways by inhibition of mitochondrial function, similar to their known responses to a variety of other environmental stresses like heat shock, caloric restriction, and hypoxia [52–54]. Others have shown that mitochondrial ROS plays an important role in AIN [22]. We propose that isoflurane inhibits mitochondrial dysfunction, which acutely increases ROS production. This in turn, activates the protective DAF-2 pathway depicted on the left of the figure in blue ovals. It also initiates a neurotoxic ER-stress pathway that is shown on the right in red ovals. The isoflurane target of mitochondria/ROS production is at the bottom in green.

### AIN Induction- role of mitochondria

Boscolo *et al.* suggested that acute ROS generation causes AIN [20–22]. Isoflurane has been shown to inhibit mitochondrial function in nematodes and mammals [59–61], and been shown by others to increase mitochondrial ROS production [62, 63]. The unexposed mitochondrial mutants, other than *clk-1*, were significantly defective in chemotaxis, but did not worsen after isoflurane exposure as L1s. We interpret this finding to indicate that isoflurane and mitochondrial mutations are affecting behavior via the same pathway, inhibition of electron transport and increased ROS damage. A chronic increase in ROS damage, as seen in some mitochondrial mutants, can lead to ROS damage that results in a defect in chemotaxis. However, the mitochondrial mutants initiate a cascade of defense mechanisms in response to this chronic stress [64]. A similar rationale has been applied to the SOD mutants for their paradoxical effect of lengthening lifespan, interpreted as a low level of increased ROS signaling which induces a chronic protective response that improves health [65, 66]. This may position both mitochondrial and SOD mutants to be relatively immune to further damage from the brief increase in ROS generated by an acute exposure to isoflurane.



In contrast, *clk-1*, which has a severe defect in electron transport, responds to isoflurane exposure like N2. *clk-1* is a unique mutant that accumulates *less* ROS damage to mitochondrial protein than N2 despite producing increased ROS [24, 67]. Increased scavenging by an intermediate in CoQ synthesis is proposed to be responsible for this remarkable lack of chronic ROS damage [24, 68]. Taken together, the simplest explanation of our data is that anesthetics inhibit mitochondria at a critical point in development, generating an acute increase in ROS signaling that ultimately transduces neurotoxicity in the adult *C. elegans*. *clk-1* does not accumulate *chronic* ROS damage and, like N2, is unable to sufficiently scavenge *acute* isoflurane induced ROS production to avoid AIN. We conclude that acute mitochondrial inhibition by isoflurane induces AIN by increased ROS production in agreement with Jevtovic-Todorovic and colleagues [20–22].

## ER-Stress

Isoflurane exposure induces a striking upregulation of HSP-4, with no effects on HSP-6 or HSP-60 expression. AIN is eliminated when *hsp-4* is lost, but there is no effect of loss of *hsp-6* or *hsp-60*. Coupled with the results of others [62, 63], we conclude that the acute increase in ROS signaling by isoflurane must exert its AIN effects outside the mitochondrion. This sets in motion multiple responses that contribute to AIN, including activation of *aak-2*, *pmk-1*, *skn-1*, *hif-1*, and *gcn-2*, discussed below. These genes have multiple, sometimes opposing effects on ER-stress [69]. The end result of their activation is a clear increase in expression of UPR<sub>ER</sub> and ER-stress. Since activation of IRE-1, XBP-1 and HSP-4 are components of the final pathway activating ER-stress, downstream from HIF-1 and SKN-1, and their losses each eliminate AIN, the UPR<sub>ER</sub> apparently causes AIN. Prior studies in mammals have also indicated that ER-stress plays a role in AIN in both young and old [70–73]; these studies clarify the mechanisms by which isoflurane induces that response. Inhibition of ROS-induced components of ER-stress eliminates AIN.

Linking an acute increase in ROS signaling to a neurologic defect following isoflurane exposure is unquestionably complicated. Transient mitochondrial ROS production activates PMK-1, SKN-1 [48] and HIF-1 [74] and the loss of PMK-1, SKN-1, HIF-1 and GCN-2 all eliminated AIN. PMK-1 can induce apoptosis directly [75] or by phosphorylation of SKN-1, resulting in its translocation to the nucleus [76]. While SKN-1 is usually considered to improve response to stress, its activation has also been shown to result in defects in neuromuscular function [77, 78] by altering expression of the synaptic cell adhesion molecule, neuroligin. Given these activities, loss of either PMK-1 or SKN-1 could play a role in protecting animals from the neurotoxic effects of isoflurane. Activation of HIF-1 by isoflurane has been shown to play a role in AIN and anesthetic-induced preconditioning [79–81] and to be activated by inhibition of mitochondrial respiration and ROS production [82]. We conclude that developmental stage-specific isoflurane exposure inappropriately activates HIF-1, resulting in activation of ER-stress. Loss of GCN-2 also eliminated the neurotoxic effects of isoflurane. Activated GCN-2 phosphorylates the cytosolic translation initiation factor, eIF2 $\alpha$ , decreasing protein translation to restore homeostasis during times of cellular stress [34]. GCN-2 is activated by ROS formation and contributes to activation of both the UPR<sub>MT</sub> and the UPR<sub>ER</sub> [51, 58]. Since we detected no increase in UPR<sub>MT</sub>, it appears that under these conditions GCN-2 primarily activates the UPR<sub>ER</sub>.

Finally, removing the ER chaperone protein HSP-4 eliminates AIN, indicating that HSP-4 levels are detrimentally upregulated by isoflurane beyond what is necessary for cellular homeostasis. Since loss of GCN-2 should increase protein translation, such an increase may be necessary to alleviate the effects of an increase in the UPR<sub>ER</sub>. Further studies to determine if HSP-4 levels are dependent on *gcn-2* following isoflurane exposure will help indicate the precise step at which isoflurane is exerting its effect on the UPR<sub>ER</sub>.

## DAF-2

If the far-reaching DAF-2 stress response is in place *prior* to the acute increase in ROS signaling caused by isoflurane exposure, the effects of mitochondrial inhibition by isoflurane are eliminated. This protective pathway is DAF-16-dependent. In contrast to molecules whose activation contributes to AIN, this protective pathway is induced by DAF-16-dependent loss of DAF-2 [29, 83]. Nuclear translocation of DAF-16 protects mitochondria and the cell from ROS damage by induction of superoxide dismutases and catalases, as well as other protective mechanisms [83, 84]. We expected that the preconditioning we observed with a brief exposure to isoflurane prior to the neurotoxic dose would activate stress protective pathways in a manner similar to genetic loss of *daf-2*. However, since preconditioning was seen in the *daf-16* mutant, *e.g.* is *daf-16* independent, other, yet unidentified mechanisms, also are at play.

## Mechanistic Target of Rapamycin (mTOR)

Interestingly, both DAF-2 and ER-stress pathways interact with the nutrient sensing complex, mTOR, which has been shown to be activated by isoflurane [56] and ROS. mTOR broadly controls the metabolic state of cells [69]. Since rapamycin completely eliminates AIN and blunts expression of *hsp-4*, our data indicate that mTOR plays a central role in transducing AIN. This is consistent with a model in which an increase in ROS production, caused by isoflurane, activates mTOR directly, thus modulating the metabolic state of the cell, and upregulating ER-stress. mTOR may also connect the two pathways in Figure 6, representing a direction for future studies and a mechanism amenable to treatment. Our immediate plans are to characterize other markers of mTOR activation and ROS production along with determining functional changes in the nervous system of *C. elegans* following isoflurane exposure.

## Summary

We have identified highly conserved pathways that regulate AIN in *C. elegans*. Our data are best explained by a model in which isoflurane acutely increases ROS signaling by inhibiting mitochondrial function and activates mTOR. This results in activation of responses that ultimately lead to ER-stress. Defense mechanisms can be put in place by a preconditioning dose of isoflurane, mTOR inhibition, activation of a stress protective pathway or inhibition of the ER-stress pathway. We have shown that it is possible to recruit endogenous stress pathways to protect the organism from the neurotoxic effects of isoflurane.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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### Summary Statement

Two highly conserved pathways regulate isoflurane induced neurotoxicity in *C. elegans*. The data are best explained by a model in which isoflurane acutely activates the mechanistic Target of Rapamycin (mTOR) resulting in activation of responses that lead to endoplasmic reticulum related stress and cell death.

### Highlights

- DAF-2 pathway inhibits anesthetic induced neurotoxicity (AIN)
- ER-stress controls AIN
- Inhibition of mTOR with rapamycin blocks AIN
- Preconditioning with isoflurane blocks AIN

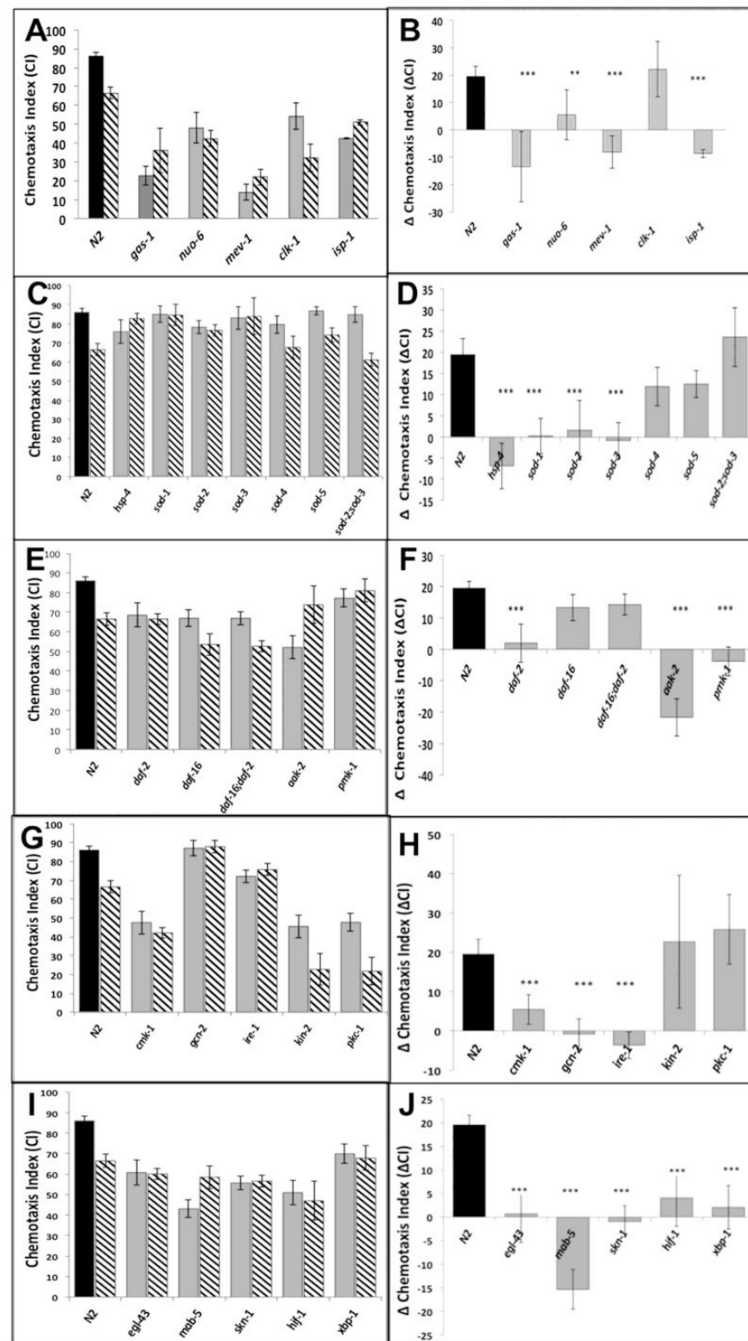
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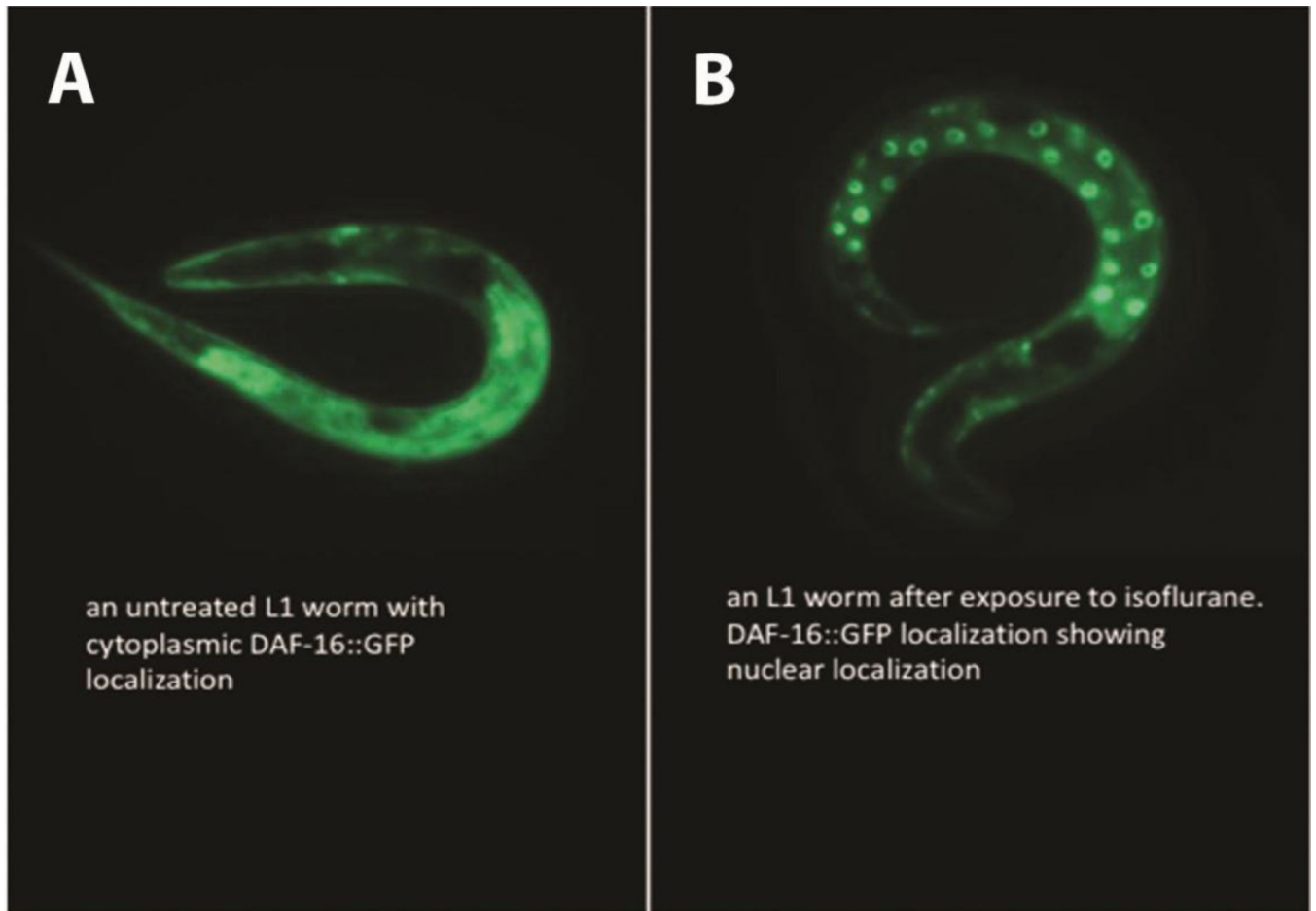
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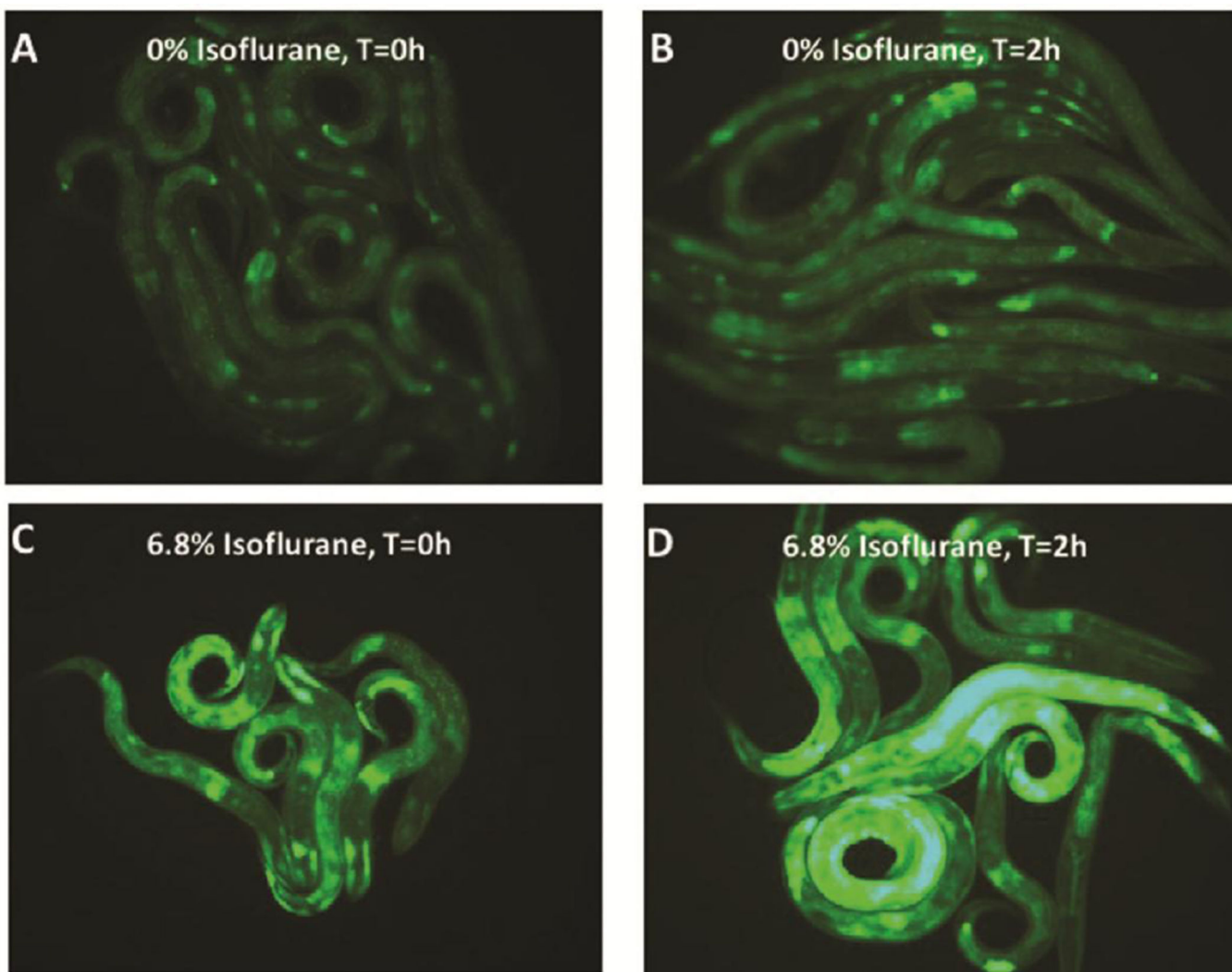


**Figure 1.**  
**A,C,E,G,I.** Chemotaxis indices (CIs) in adults after exposure to isoflurane as L1 larvae. Unexposed animals (solid fill), exposed animals (angled hatching). For all graphs, error bars denote SEM values, N>300 animals for each value. **B,D,F,H,J** Differences in CIs (  $\Delta$ CI) between exposed and unexposed animals. Difference between CI of N2 (19.5  $\pm$  3.7) and each mutant was compared to determine if the mutant affected AIN. \*\* = CI different from N2, p<0.01, \*\*\* = CI different from N2, p<0.005. **A,B.** Mitochondrial mutants. Chemotaxis in unexposed mitochondrial mutants (*gas-1*, *nuo-6*, *mev-1*, *isp-1*) was not

worsened by isoflurane exposure. The exception was *clk-1* which had a CI similar to that of N2. **C,D. ROS scavengers/ ER Stress.** The effects of defects in ROS scavenging on AIN in *C. elegans*. CIs of five superoxide dismutase mutants and *hsp-4* (loss of the ER-specific heat shock protein HSP-4). **E,F. DAF-2 dependent pathway.** The effects of the *daf-2* stress pathway on AIN. Loss of DAF-2 removed the AIN effect. The *daf-16* mutation removed the effect of *daf-2* on AIN. **G,H. Kinases.** Effects of 5 kinases on neurotoxicity. Loss of *cmk-1* and *gcn-2*, both involved in innate immunity and ER-related stress, eliminated AIN. Loss of *ire-1* also eliminated AIN and is discussed later. **I,J. Transcription factors.** The transcription factors *skn-1*, *hif-1* and *xpb-1* all eliminated AIN.

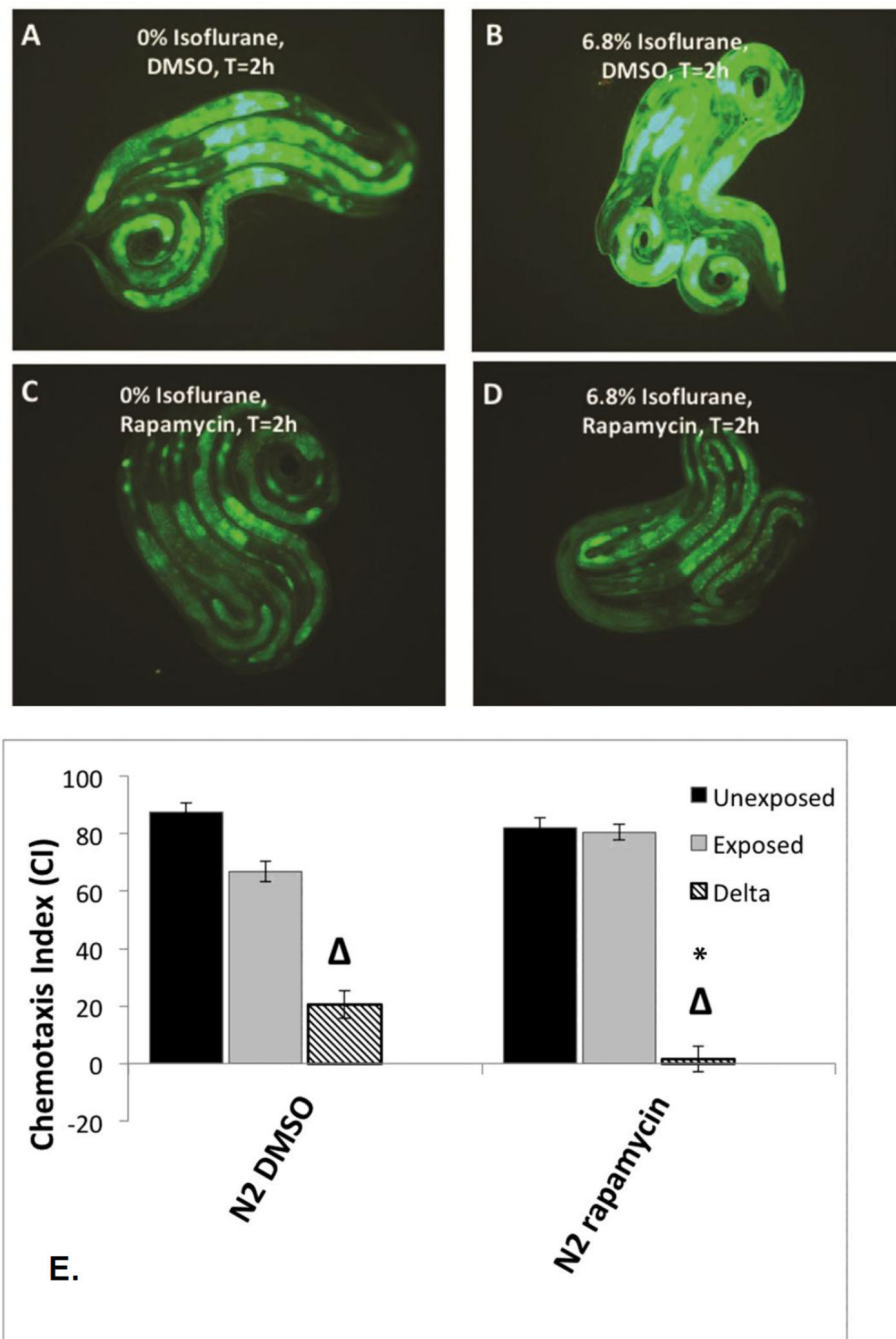


**Figure 2. Expression of an DAF-16::GFP fusion protein following exposure to isoflurane**  
**A.** A GFP fusion protein with DAF-16 is distributed throughout the cytoplasm when not exposed to isoflurane. **B.** One hour after exposure to isoflurane, the DAF-16::GFP fusion protein is translocated to the nucleus. These results show that isoflurane exposure leads to nuclear localization of DAF-16 similar to mutations in either DAF-2 or GAS-1 [28, 29].



**Figure 3. Expression of an HSP-4 reporter following exposure to isoflurane**

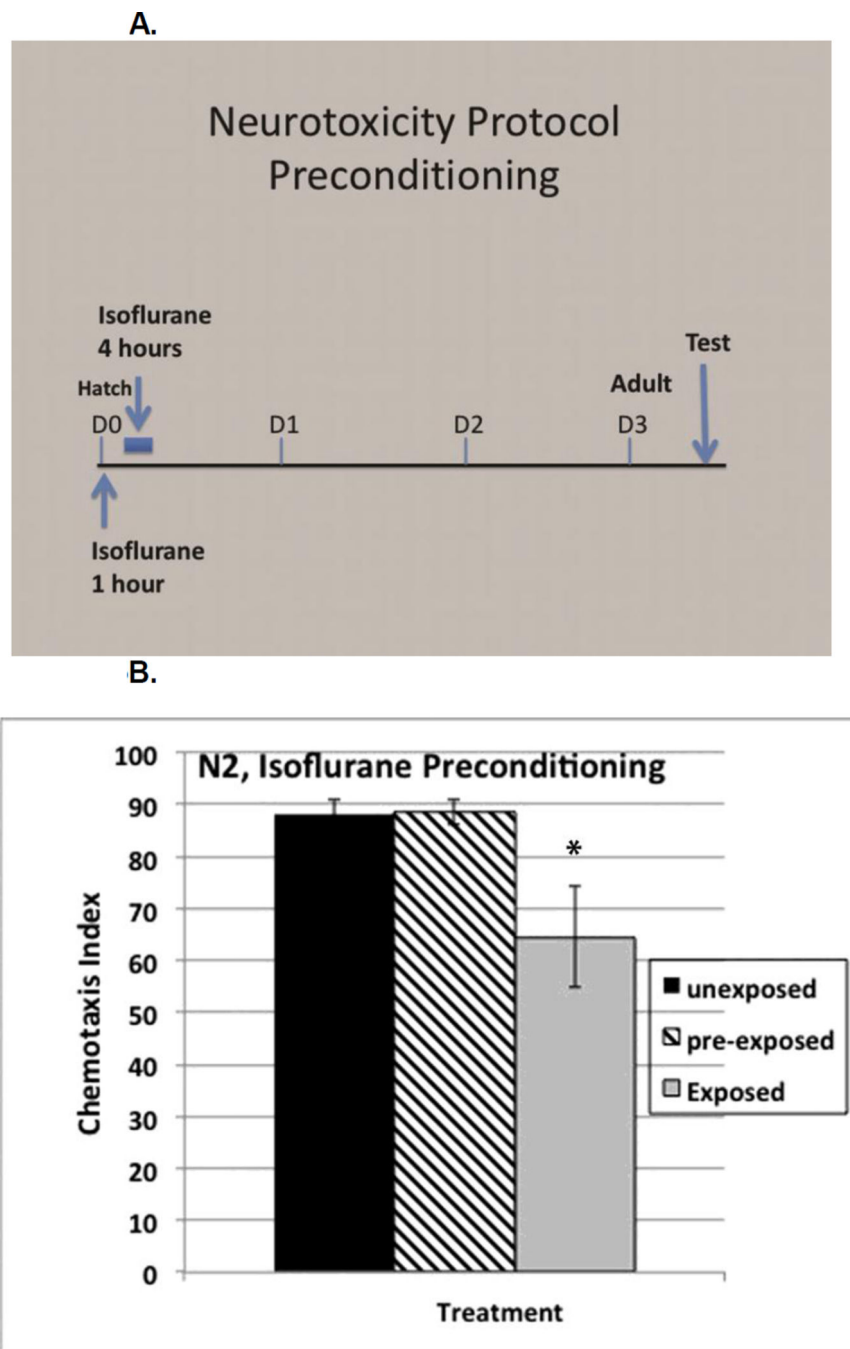
The ER specific heat shock protein HSP-4 reporter is upregulated by isoflurane exposure. **A.** HSP-4::GFP expression at t=0 hours (after removal from anesthetic chamber) without isoflurane exposure. **B.** HSP-4::GFP expression at t=2 hours (after removal from anesthetic chamber) without isoflurane exposure. **C.** HSP-4::GFP expression at t=0 (after removal from anesthetic chamber) after isoflurane exposure. **D.** HSP-4::GFP expression at t=2 hours (after removal from anesthetic chamber) with isoflurane exposure. Expression peaked at 2–3 hours and then slowly decayed (not shown). The reporters for mitochondrial UPR heat shock proteins (HSP-6 and HSP-60) were not upregulated by isoflurane exposure.



**Figure 4. *Phsp-4::gfp* expression in L1 larvae with or without concurrent treatment with rapamycin and isoflurane**

**A.** *Phsp-4::gfp* expression at t=0 hours (after removal from anesthetic chamber) without isoflurane exposure. **B.** *Phsp-4::gfp* expression at t=2 hours (after removal from anesthetic chamber) with isoflurane exposure. **C.** *Phsp-4::gfp* expression at t=0 hours (after removal from anesthetic chamber) without isoflurane exposure with rapamycin treatment. **D.** *Phsp-4::gfp* expression at t=2 hours (after removal from anesthetic chamber) after 6.5% isoflurane exposure with rapamycin exposure. All plates had 0.2% DMSO on plates to

facilitate uptake of rapamycin by the animals. The presence of DMSO mildly increased *Phsp-4::gfp* expression in the absence of isoflurane. **E. Chemotaxis indices (CIs) in adults after exposure to isoflurane as L1 larvae during concurrent treatment with rapamycin.** Unexposed animals (dark fill), exposed animals (light fill) and delta values ( ) for control (left) and rapamycin (right) groups are shown. For all graphs, error bars denote SEM values, N>800 animals for each value. These results show that concurrent treatment with rapamycin completely inhibits AIN in *C. elegans*.



**Figure 5.**  
**A.** Protocol timeline used to test for neurotoxicity and preconditioning in *C. elegans*. First stage larva (L1) are first exposed to isoflurane at their EC<sub>95</sub> at age zero hours (arrow) and then again at four to eight hours (blue bar). They are then allowed to grow to adulthood (3 days for wildtype, D3). Chemotaxis is then tested as described in the methods and in Figure 1. **B.** Chemotaxis Index showing the neurotoxic effect in wildtype (N2) *C. elegans* following isoflurane exposure (black and gray bars). Preconditioning eliminated the neurotoxic effect caused by isoflurane (cross hatched bar). p value compares the difference between

preconditioned animals and non-preconditioned animals exposed to isoflurane (cross hatched and gray bars). Error bars are SEM.

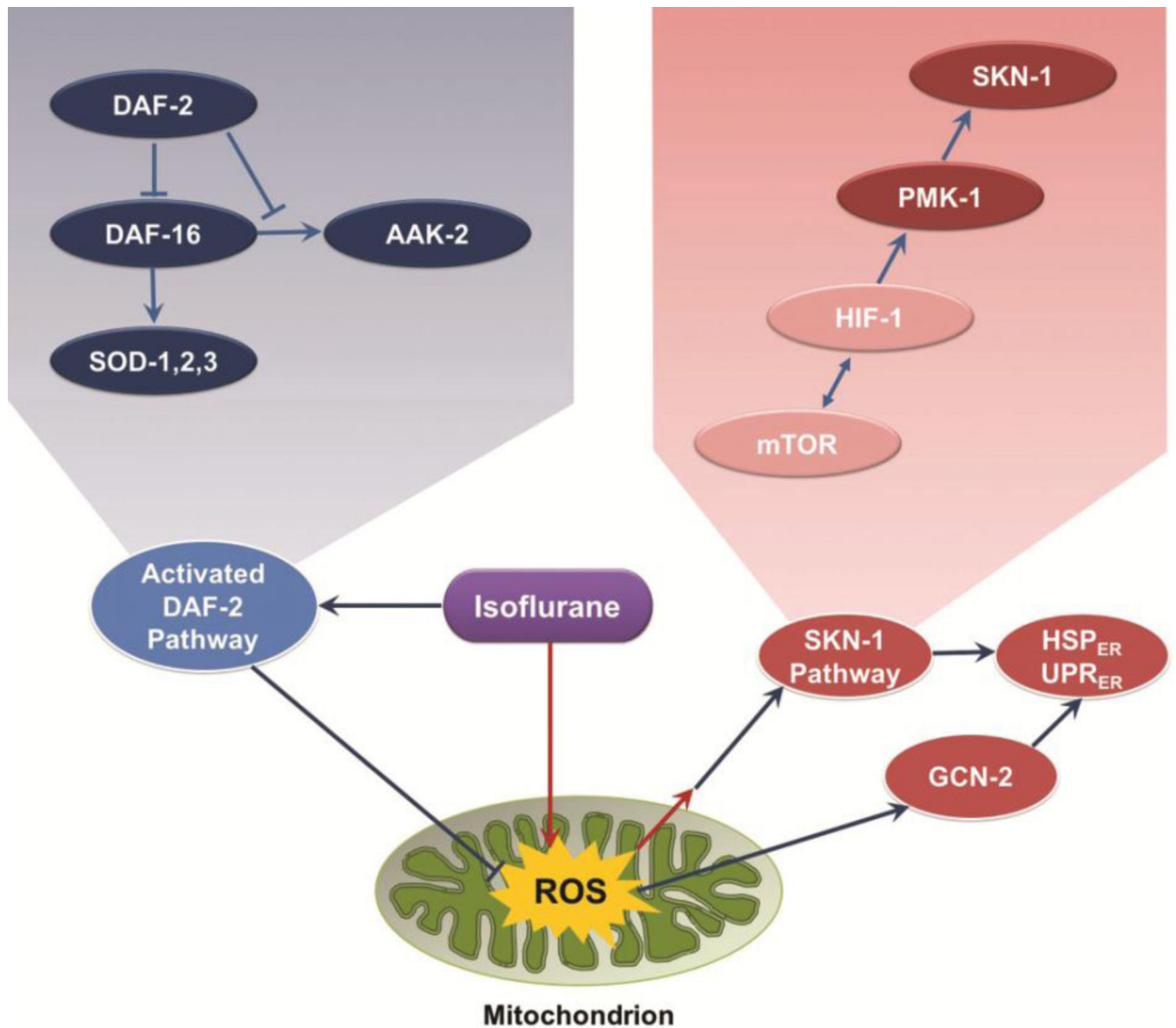
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**Figure 6.**

Two intersecting pathways that affect AIN in *C. elegans*. The grouping on the left of the figure (blue ellipses) represents the DAF-2 pathway which, when induced, is capable of inhibiting AIN. The genes on the right (red ellipses) are activated by ROS, leading to ER-stress, apoptosis and causing AIN. The mitochondrial/ROS isoflurane target is at the bottom in green. The pathways are expanded in the upper inserts.