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The worm sheds light on anesthetic mechanisms

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One hundred and sixty five years have passed since the first documented use of volatile anesthetics to aid in surgery, but we have yet to understand the underlying mechanism of action of these drugs. There is no question that, in vitro, volatile anesthetics can affect the function of numerous neuronal and non-neuronal proteins. In fact, volatile anesthetics are capable of binding such diverse proteins as albumin and bacterial luciferase. The promiscuity of volatile anesthetic binding makes it difficult to determine which proteins are modulated by anesthetics to cause the state of anesthesia. Consequently, despite a great deal of in vitro data, the fundamental physiological process that volatile anesthetics perturb to effect neuronal silencing is not yet identified. Recently, data has increasingly indicated that membrane leak channels may play a role in the anesthetic response. Here we comment on the use of optogenetics to further support such a model.

Keywords: anesthetics, halothane, optogenetics, cholinergic, potassium channels, leak channels

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Genetics

It remains unknown how volatile anesthetics function in vivo.[1-3] In vivo studies are critical to determine which proteins, when modulated, cause the components of an anesthetic state, which includes unconsciousness, analgesia, amnesia, and immobility.[4,5] However, in vivo screens for genetic mutants that affect responses to volatile anesthetics have never identified a mutant that is completely resistant to their effects. This inability to find a completely resistant mutant can be explained by two different hypotheses. First, volatile anesthetics function by affecting multiple targets; therefore a mutation in any single gene is not sufficient to render the animal completely resistant. Second, the underlying process that volatile anesthetics affect is so critical to the survival of the animal that a truly resistant mutant is not viable.

However, there are mutants that have shown moderate resistance to anesthetic induced immobility, and others have shown significant hypersensitivity. Mice lacking TREK-1, a potassium leak channel in the two-P domain potassium channel (K2P) family, required 48% more halothane, a volatile anesthetic, than wild-type mice for lack of response to tail clamp.[6] Similarly, loss of the related K2P channels, TASK-1 and TASK-3 also decreased sensitivity to volatile anesthetics, though to a more modest degree than did TREK-1.[7,8] Conversely, both *C. elegans* and Drosophila mutants that lack a sodium leak channel [*nca(lf); nca-2;nca-1* in *C. elegans* and *narrow abdomen (na)* in Drosophila] are hypersensitive to halothane (a 70% reduction in the dose of halothane required to anesthetize wild type), but have no change in sensitivity to isoflurane, another volatile agent.[9] Both the potassium and sodium leak channels (known as NALCN in mammals) are critical for establishing the neuronal resting membrane potential.[10,11] It stands to reason that mutants that show some change in anesthetic sensitivity perhaps represent elimination of one of many anesthetic targets, as in the first hypothesis. Alternatively, if the second hypothesis is true, perhaps the vital physiological process that anesthetics perturb is affected mildly in these

mutants–enough to change sensitivity and yet allow the animal to survive. Both of these hypotheses imply that volatile anesthetics perturb an underlying physiological process to produce anesthesia.

It follows that discovering the critical physiological process is the first step in determining the mechanism of action of volatile anesthetics. If anesthetics affect a specific underlying physiological process, then when this same process is modulated by other mechanisms in a whole animal, the effect of anesthesia must be either overcome or enhanced. Since ion channels involved in setting the resting membrane potential change sensitivity to halothane in multiple model organisms, we hypothesized that halothane may function by blocking the Na^+ current through the *nca* channels or by increasing the K^+ current through the K2P channels or both. This hyperpolarization would lead to neuronal silencing and anesthesia (**Fig. 1**). In fact, both an increased K^+ current and a reduced Na^+ current leading to hyperpolarization have been reported when cultured mammalian neurons were exposed to halothane.[12] However, whether these in vitro modulations are important for anesthesia in a whole animal is not known.

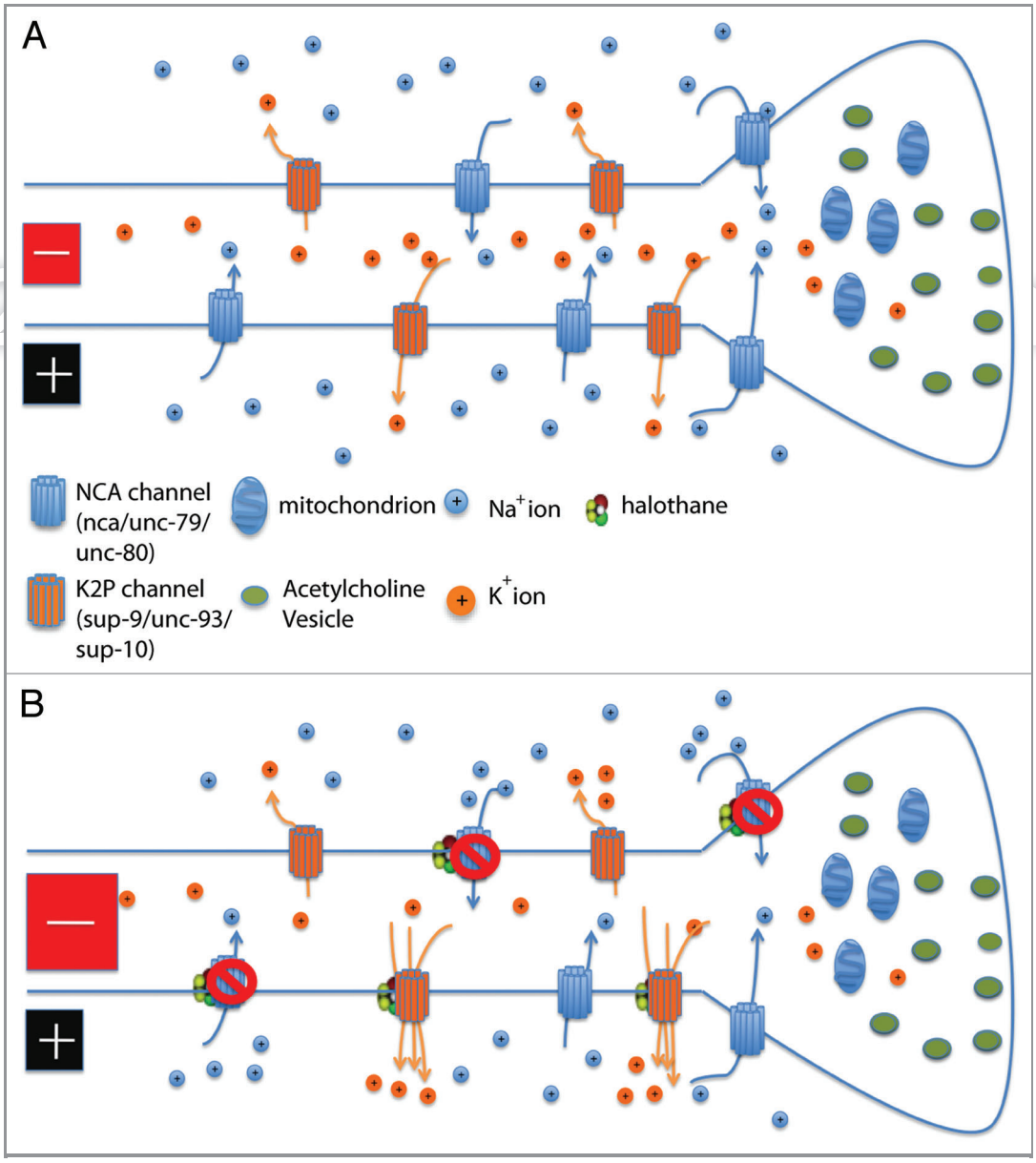

Figure 1. Model for halothane function. (A) The Resting Membrane Potential (RMP) of a neuron is dependent on Na^+ and K^+ movement across the neuronal membrane. The NCA and K2P channels are responsible for Na^+ and K^+ leak in neurons necessary to establish RMP. The intracellular region is negatively charged compared with the extracellular milieu. (B) In our model, halothane causes anesthesia by hyperpolarizing neuronal RMP by binding to both K2P and NCA channels. Here halothane is shown to inhibit the Na^+ influx and increase the K^+ efflux from the neuron. Both of these actions will lead to a net reduction of positive ions in the intracellular lumen, which causes neuronal hyperpolarization (the intracellular region becomes more negative) and silencing.

Recently, we tested the above model in several ways.[13] First, we determined the sensitivity of *C. elegans* K2P channel mutants to halothane. The *C. elegans* K2P channel most similar to mammalian K2P channels was SUP-9, which forms a functional channel with the UNC-93 and SUP-10 proteins. We found that loss of function mutants in *sup-9* or *unc-93* caused an 8.7% resistance to halothane, while gain of function mutants of *unc-93* or *sup-10* reduced the dose of halothane required for immobility by approximately 60% compared with wild type. Next, we constructed strains that harbor both *nca* loss of function (*nca(lf)*) and K2P gain or loss of function (*gf* or *lf*) mutations, to discover whether mutations in the K2P channels can add to or subtract from the effect of *nca(lf)* on anesthetic sensitivity. We found that combining the hypersensitive *nca(lf)* mutations with the *K2P(gf)* mutations had a synergistic effect, and resulted in an animal that was 17-fold more sensitive than wild type.

Optogenetics

These data further implicated the resting membrane potential as the process that anesthetics perturb. However, all the mutant analyses suffer from a confounding factor–compensatory genetic or biochemical changes in response to the genetic mutations in the mutant animals. Furthermore, measuring the actual resting membrane potential in a live animal is still not feasible. Despite advances in calcium indicators, voltage sensing proteins and dyes, none provided the resolution needed to measure the changes in membrane potential of neurons in a live animal. However, the advent of optogenetics allowed us a powerful opportunity to actually manipulate the membrane potential acutely in an otherwise wild-type worm only in specific instances. If hyperpolarization is the ultimate effect of volatile anesthetics, we reasoned that an artificial depolarization of the neurons of anesthetized animals should reverse anesthetic effects. Thus, we examined the anesthetic behavior of animals expressing the light sensitive proteins channelrhodopsin-2 (ChR2) or halorhodopsin, proteins that will either depolarize or hyperpolarize the cells where they are expressed[14,15] in the presence of stimulating light. We showed that halothane induced immobility can be reversed by ChR2 induced depolarization of cholinergic neurons (see video S3 in reference 13). Similarly, immobility can be achieved at a much lower concentration of halothane when the acetylcholine neurons are hyperpolarized via halorhodopsin. In a surprising result, immobility induced by two other volatile anesthetics, isoflurane and sevoflurane, is not reversed by activation of ChR2 channels. The difference in sensitivities to isoflurane and halothane was seen previously in *nca(lf)* mutants as well.[9] Given the differences in sensitivity to isoflurane and halothane in both the *nca (lf)* mutants and the ChR2 containing animals, it was important to note that the expression patterns of ChR2 and *nca-1* were largely overlapping (**Fig. 2**). This indicates that the mechanisms of their effects on halothane sensitivity are mediated through a specific subset of neurons in *C. elegans*. It may be that immobility in isoflurane requires activation of these channels in a different set of cells.

The reversal of anesthetic induced immobility by ChR2 is not complete. While sinusoidal movement is seen upon activation of ChR2, it is neither as vigorous as wild-type movement, nor is it sustainable for longer than 25 sec. These differences may be due to the limitations of ChR2 function and activation. The current through ChR2 decays upon long-term stimulation[14] and therefore may not be sufficient to counteract the hyperpolarization driven by halothane over a long period of time. This hypothesis is supported by the fact that upon providing the worm with a minute to recover between pulses of blue light, more movement can be elicited by subsequent light pulses. However, it is also entirely possible that halothane perturbs other aspects of neuronal or muscular function in addition to hyperpolarization of cholinergic neurons; therefore the reversal of immobility is incomplete.

We also found that depolarizing cholinergic neurons in either *nca(lf)* or *K2P(gf)* mutants rescued the movement and swimming defects of these mutants, suggesting that an underlying hyperpolarization was at least partially responsible for the mutant phenotypes (videos S1 and S2 in ref. 13). Finally, the anesthetic sensitivity of these mutants was also rescued by depolarization of cholinergic neurons—in particular the *nca(lf)* mutants were able to move at halothane concentrations in which wild-type animals would be immobile when ChR2 was activated.

Discussion

Reversal of anesthetic effects was first observed only after exposure of anesthetized organisms to atmospheric pressures in excess of 150 atm.[16,17] At similar pressures other drugs, such as morphine, ethanol, and lidocaine,[14] also lost their effect, suggesting that this was a non-specific effect.[16] Therefore, pressure reversal was abandoned as a mode of research to determine mechanism of action of volatile anesthetics. In the past decade, Alkire and colleagues have performed intriguing experiments to identify the site in the brain that is responsible for consciousness, as well as targets for volatile anesthetics. Microinjection of nicotine into the central medial thalamus of rats restored righting and mobility in the face of sevoflurane anesthesia.[18] In addition, an antibody to a voltage gated potassium channel, when applied to the same specific region of the brain, aroused the animal from anesthesia by volatile anesthetics.[19] In this admittedly more complex animal model, these results have been interpreted in multiple ways. As the authors point out, mice lacking the receptor targeted by nicotine in their experiments do not have a change in response to anesthetics. Nor would it be predicted that antibody blockade of a potassium channel that is known to be blocked by volatile anesthetics would produce arousal. Nevertheless, these elegant experiments raised many important questions to understand loss of consciousness in a mammalian model.

In our studies, the ability to reverse immobility induced by halothane under physiologic conditions in a wild-type animal suggests that an underlying hyperpolarization is the cause of halothane-induced immobility in the

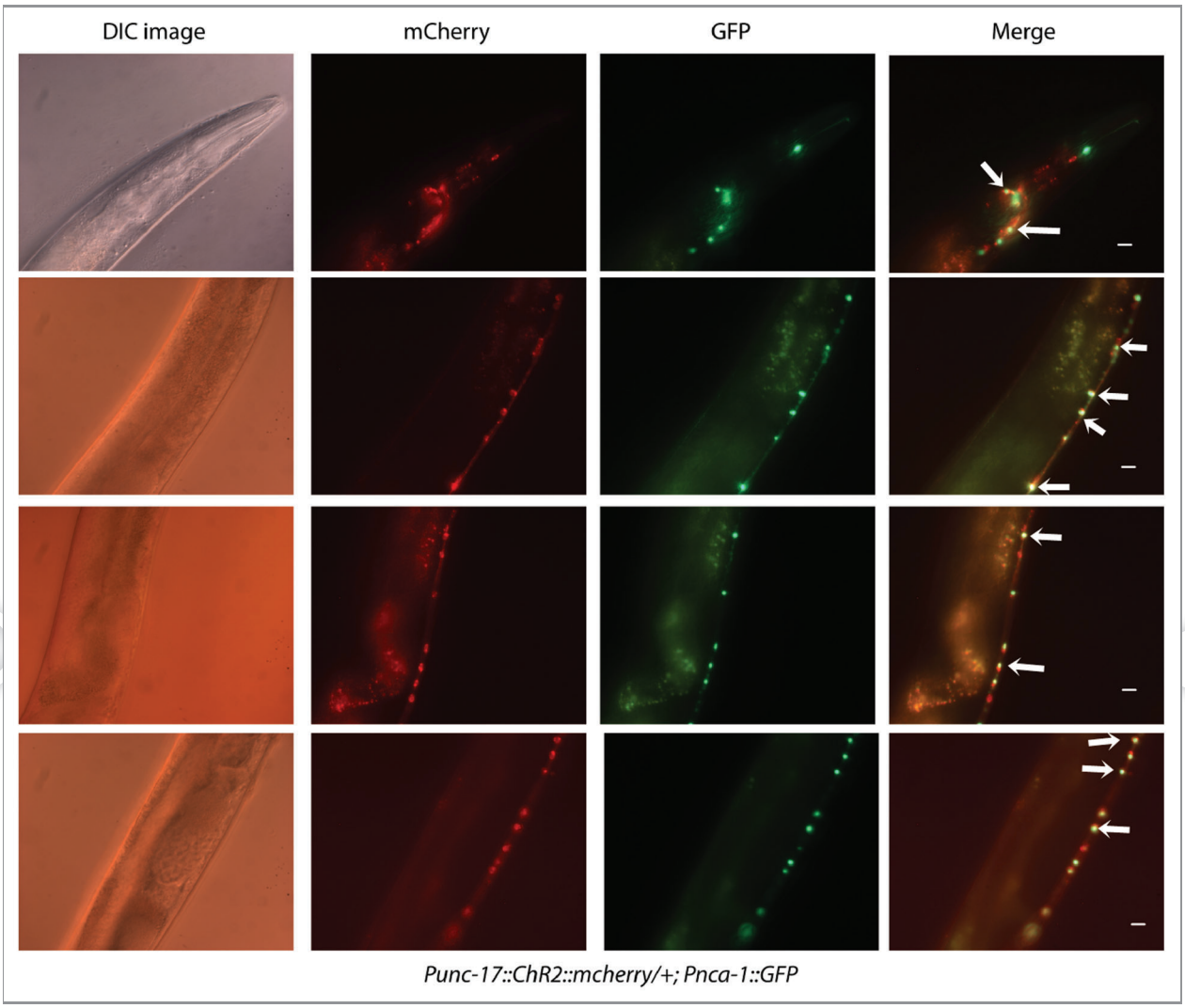

Figure 2. *nca-1* is expressed in cholinergic neurons. From left: column 1, nematode portion shown with Nomarski optics; column 2, *Punc-17::ChR2::mcherry* marks cholinergic neurons with mCherry fluorescence, shown in red; column 3, the *Pnca-1::GFP* reporter shows the expression pattern of the NCA-1 channel, shown in green; column 4, co-expression of mCherry and GFP can be seen in cell bodies of neurons around the nerve ring and retrovesicular ganglion (top row) and through the ventral nerve cord (rows 2–4) of *Punc-17::ChR2::mcherry/+; Pnca-1::GFP/+* heterozygote. Scale bars, 10 um. Arrows denote cell bodies around the nerve ring (top row) and ventral nerve cord (rows 2–4) and show the co-expression of ChR2 and NCA-1.

nematode. It must also be noted that isoflurane induced immobility is not reversed by ChR2 induced depolarization, arguing against a non-specific mechanism. The ability to reverse halothane-induced immobility but not isoflurane-induced immobility, suggests that these anesthetics function at least partially via disparate mechanisms. However, differences between the effects of isoflurane and halothane have long been noted for *unc-79* and the *nca* mutants in *C. elegans* and their orthologous mutants in Drosophila (*dunc79* and *narrow abdomen*).[9]

We previously reported that a mutation [(*gas-1(fc21)*] in a subunit of complex I of the electron transport chain was hypersensitive to all volatile anesthetics.[20] Gene expression studies in *gas-1* indicated that K2P channels were broadly upregulated in this mutant.[13] While the ability of ChR2 activation to reverse immobility was preserved in the *nca(lf)* and *K2P(gf)* mutants, ChR2 activation did not reverse immobility in the halothane hypersensitive strain, *gas-1*. The trivial explanation for this result is that ChR2 function is inhibited in *gas-1* animals. However, both anesthetized and non-anesthetized *gas-1(fc21)* animals carrying ChR2 in either cholinergic neurons or muscle do respond to blue light by contracting their muscles, showing that ChR2 function remains in these animals. The degree of ChR2 function may however still be reduced in *gas-1*, accounting for the lack of anesthetic reversal. Alternatively, *gas-1* is expressed pan-neuronally and therefore other classes of neurons may be important for immobility reversal in a *gas-1* background. Since we earlier noted that a broad class of potassium channels was upregulated in *gas-1* animals, it may be that the increase in potassium leak channel activity is too great to be overcome by the ChR2-induced currents. A final possibility is that anesthetics may also directly affect the mutant complex I to cause immobility by a second mechanism.

Our work demonstrates the use of a system to test in vivo the importance of hyperpolarization and depolarization of the membrane potential for volatile anesthetic sensitivity (**Fig. 1**). This system can be used to test whether other anesthetics also function in a manner similar to halothane. If anesthetics do function by different mechanisms, how then do we account for the additivity of anesthetic effects? In humans,[21] rats[22] and *C. elegans*[23] combining half the concentration of halothane required to fully anesthetize with half the concentration of isoflurane required to fully anesthetize animals has the same effect as a 100% of each anesthetic. The additivity and disparate mechanisms of different anesthetics suggests that the underlying process that the anesthetics perturb may be related. For instance, each anesthetic may affect different parts of neuronal function, but both ultimately cause neuronal silencing. In *C. elegans*, using uncoordination as the anesthetic endpoint, it has been suggested that anesthetics, in particular isoflurane, may function by preventing synaptic vesicle release.[24] It is feasible that a combination of a half-maximal hyperpolarization by halothane and a half-maximal inhibition of synaptic release by isoflurane can cause neuronal silencing and anesthesia when a subanesthetic dose of each is used.

The additive nature of volatile anesthetic interaction has been interpreted to mean that anesthetics function by the same mechanism.[21,22] However, our results (and those of others) contradict this assertion. The ability to reverse only halothane induced immobility, and not isoflurane-induced immobility, implies that these two volatile anesthetics function via disparate mechanisms, and therefore probably different sites. Theoretical analysis of anesthetic mechanisms suggests that additivity can also be explained by actions of volatile anesthetics at different sites as long as receptor occupancy is below 50%.[25] Future experiments to understand the basic physiologic processes perturbed by volatile anesthetics will need continued use of whole animal models to explain what continues to be an enigmatic phenomenon. The availability of optogenetic constructs in mice is one such intriguing possibility. Additionally, restricting the expression of genes such as *nca-1* and *nca-2* or expressing channelrhodopsin-2 in a smaller subset of neurons to determine reversal should further identify cells important to the determining anesthetic state.

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