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Induced Changes in Protein Receptors Conferring Resistance to Anesthetics

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Abstract

Purpose of review—While general anesthetics have been provided effectively for many years, their exact molecular underpinnings remain relatively unknown. In this manuscript, we discuss the recent findings associated with resistance to anesthetic effects as a way of shedding light on these mechanisms.

Recent findings—The original theories of anesthetic action based upon their effects on cellular membranes have given way to specific theories concerning direct effects on ion channel proteins. These molecular targets are intimately involved in the conduct of neuronal signaling within the central nervous system and are thought to be essential in the modulation of conscious states. It is the lack of a thorough understanding of unperturbed consciousness that fosters great difficulty in understanding how anesthetics alter this conscious state. However, one very fruitful line of analysis in the quest for such answers lies in the examination of both *in vitro* and *in vivo* ion channel systems which seem to maintain variable levels of resistance to anesthetics.

Summary—Information about the possible targets and molecular nature of anesthetic action is being derived from studies of anesthetic resistance in GABA receptors, tandem pore potassium channels, and an apparently wide variety of protein systems within the nematode, *C. elegans*

Keywords

anesthetic mechanism; ligand-gated ion channel; GABA receptor; tandem pore potassium channel; mutation; anesthetic resistance; *C. elegans*

Introduction and Background: The Evolution of Anesthetic Mechanism Theories Toward Protein Targets

For over 160 years, general anesthesia has provided for the safety and comfort of a wide variety of invasive procedures. Early investigations into the mechanisms of anesthetic action

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by Meyer and Overton [1] demonstrated a significant correlation of anesthetic potency with solubility in olive oil. These studies were interpreted to mean that anesthetics must act by altering cell membranes. This became known as the Meyer-Overton Hypothesis.

The Meyer-Overton correlation of anesthetic potency with lipid solubility remains a robust relationship between lipid solubility and anesthetic potency. However, while the correlation has yet to be completely explained, the original hypothesis stemming from that correlation now has several significant exceptions. Koblin and Eger [2,3] have demonstrated several classes of compounds that deviate from the Meyer-Overton rule. These include many polyhalogenated linear and cyclic hydrocarbons, whose lipid solubilities would predict reasonable anesthetic potency but whose actual anesthetic effect is minimal. In particular, with regard to the minimum alveolar concentration (MAC) of anesthetic gas required to suppress motion to surgical incision in 50% of the patient population, these substances can be classified into those with partial anesthetic effect (the transitional compounds) and those with no effect at all (the nonimmobilizers). In fact, within the series of transitional anesthetic compounds, potency correlates with a compound's hydrophilicity and not its lipophilicity. Other exceptions to this correlation include the differing potencies of various anesthetic stereoisomers despite similar hydrophobic characteristics [4,5], as discussed below as an example of relative anesthetic resistance.

While hypotheses to explain anesthetic mechanisms involving the direct effects of anesthetics on lipid bilayers have not been fully satisfactory, other investigations into the direct effect of anesthetics on proteins have proven more enlightening. Among the first to demonstrate the interaction of an anesthetic with a functional protein was the work of Franks and Leib [6,7]. They demonstrated the enzymatic suppression of firefly luciferase, the enzyme responsible for the light of the firefly, due to the formation of the anesthetic-protein complex. While luciferase itself may not be directly relevant to the underpinnings of anesthetic mechanisms, this was the first work demonstrating an effect on protein function that was the direct result of its interaction with an anesthetic.

This seminal work emphasizing a direct role of protein-anesthetic interactions has justified the pursuit of many others to show the electrophysiologic consequences of direct anesthetic binding to a variety of membrane-bound proteins. In particular, the latter proteins in question are actually associated with neuronal conductance and the possible modulation of conscious states. These include anesthetic effects on both voltage-gated and ligand-gated ion channels, as well as a variety of G-protein coupled receptors. Of paramount importance, the class of ligand-gated ion channels has been the subject of great focus because of its implication in the mediation of anesthetic-like states when interacting with other drugs (*i.e.*, the benzodiazepine and barbiturate effects on gamma amino butyric acid receptor-GABAaR), as well as several key studies demonstrating how they are fundamentally affected by the presence of anesthetics. Mihic *et al*. [8] first demonstrated the role of specific amino acids necessary for the anesthetic effect on the human glycine alpha one receptor (GlyRa1) in an oocyte preparation. They showed that mutation of serine 267 to a variety of other amino acids caused significant effects on the way in which anesthetics enhanced glycine-mediated chloride ion conductance.

The question that arises from such work is whether the effect of mutations actually involved the anesthetic binding site, or merely a related site that produced enough allosteric change to indirectly alter the anesthetic binding site. This issue was taken up by Mascia and colleagues [9] using cysteine mutagenesis within the GlyRa1 along with exposure to a series of sulfhdryl derivatized anesthetics. The GlyRa1 could be mutated from serine to cysteine at the same 267 position noted above without significantly altering ion channel function. The mutated GlyRa1 could then be exposed to the anesthetic, propanethiol (the sulfhydryl analog of the anesthetic alcohol, propanol), which produced its potentiating effect on ion conductance. This effect could be reversed with simple washout maneuvers. The mutated GlyRa1 was then exposed to propanethiol in the presence of oxidizing agents that would catalyze the formation of a covalent disulfide bond between the cysteine at position 267 and the propanethiol. Propane and propanol are anesthetics as is the sulfhydryl derivative, propanethiol. This propanethiol binding could now potentiate the ion channel in a manner that was of the same quality as that produced by the previous application of propanethiol alone. However, this potentiating effect became irreversible with the oxidation step, even when all remaining reagent was removed from the system, including the lipid bilayer, with washout maneuvers similar to those previously performed. Both the same quality and magnitude of anesthetic effect was produced when the mutated system was exposed to propyl-MTS, the methanethiosulfonate derivative of the anesthetic propanethiol, forming the same disulfide bond more efficiently and without the additional oxidizing agents that could otherwise produce toxicity in the oocyte preparation. This demonstrated that, at least for the anesthetic propanethiol, it was both necessary and sufficient for the anesthetic to interact with the protein alone to produce its quantifiable effect since all residual anesthetic had been washed from the membrane. Likewise there was no means by which the anesthetic could have been covalently linked to any membrane component. This clearly demonstrated that the modified protein site was an actual anesthetic binding site, thereby avoiding the need to invoke any lipid component in the mechanism of action.

Since that time, the race has been on to examine other anesthetic-protein interactions in an effort to better understand the molecular mechanism of anesthetic action, gain greater insight into the fundamental mechanisms of consciousness, and perhaps design a more potent yet safer anesthetic agent. Among the many analyses, the exploration of systems that introduce relative anesthetic resistance has emerged as both popular and potentially enlightening. Studies of various human populations are underway in an effort to discern specific genetic polymorphisms associated with decreased anesthetic sensitivity and possibly identify their underlying protein/mechanistic correlates [10]. However, these studies require large numbers and long periods of time for data collection. Additional work has focused on the molecular modeling of anesthetic binding sites within ligand-gated ion channels [11-13]. More immediate data has come through the examination of receptor systems that demonstrate altered anesthetic sensitivity. As noted in the study from Mihic et al. above, the initial idea for creating chimeras and point mutations in the GlyRa1 subunit came from noting the reduced anesthetic effects in preparations of the homologous GABA rho1 subunit[14]. More recent studies of anesthetic resistance have now been carried out on key ion channel systems thought to be intimately involved in modulating the conscious state. As discussed in this manuscript, these have involved the GABA receptor, the class of tandem

pore potassium channels, and several protein sites within the nematode, *C. elegans*. The "holy grail" of such research would be to introduce such mutations from *in vitro* studies into whole organism preparations and produce an animal with resistance to anesthetic administration. This, too, has been achieved in isolated settings with modest success as noted below.

Resistance to anesthetic effects in the Gamma Amino Butyric Acid Receptor (GABAaR)

Among the first works to discuss resistance to volatile anesthetics was that of Franks and Lieb who described the nearly twofold greater potency of the (+) stereoisomer of isoflurane over that of its (-) counterpart [15]. Such a stereoisomer effect implied an anesthetic binding site with more stringent 3D spatial requirements than mere bulk solvent could provide, quite probably satisfied by well defined amino acid sidechain orientations within a protein binding site. There have since been multiple studies of protein mutations, both of a chimeric and single point nature, that have decreased isoflurane and other volatile anesthetic sensitivity for a variety of protein ion channels. In particular, Jenkins et al. demonstrated how the point mutations of specific amino acids in the GABA alpha 2 receptor could confer decreased sensitivities to larger anesthetics like isoflurane as the size of the point mutation increased in sidechain volume and the volume of the putative anesthetic binding site decreased [16].

Several studies have been performed utilizing similar stereospecificity rationale that eventually resulted in a mutant mouse with partial resistance to isoflurane. Tomlin et al. demonstrated stereoselective effects of etomidate on GABAaR channels [17]. Further studies by Belleli et al. defined single point mutations within the GABAaR that clearly altered the effects of etomidate modulation on GABAaR mediated currents[18,19]. Similar results were also obtained by Siegwart et al. for two point mutations in the heteromeric GABAaR[20]. This result led them to study enantiomeric effects on GABAaR currents as well as their subsequent effects on IPSC's in the reticulothalamic neurons of mice and the fictive swimming reflexes in tadpoles[21]. This clearly demonstrated a role for the GABAaR in the modulation of anesthetic effects. This result led Jurd and colleagues to introduce a single point mutation into mice to examine anesthetic effect on an entire organism. They produced a single point mutation in the GABA beta 3 receptor subunit that made mice virtually insensitive to both propofol and etomidate, and partially insensitive to volatile anesthetics [22]. While the results for propofol and etomidate were quite dramatic, the effect of the mutation on the modulatory capabilities of the volatile anesthetics was less pronounced. In fact, the effect was only partial in nature at best, suggesting that the volatile anesthetics must have mechanisms that extend beyond a single ligand-gated ion channel effect. Liao et al. have tested the same GABAaR beta 3 knockin mice and concluded that this receptor subunit is not involved in the amnestic effect and only modestly involved in the immobility effect of isoflurane [23].

Another interesting set of mutations that confers modest isoflurane resistance *in vitro* has also been introduced into the mouse in an effort to examine overall phenotypic effects. Specifically, within the GABAaR alpha 2 subunit, the S270H mutation seems to introduce isoflurane resistance while conferring abnormal response to GABA [24]. However, the

simultaneous introduction of an L277A point mutation preserves isoflurane resistance while restoring GABA sensitivity. Mutant knockin mice with the double point mutation were created which were phenotypically grossly normal overall, though with enhanced mortality and fear conditioning response. Decreased isoflurane effect on dentate granule neurons in brain slices from these animals were confirmed via electrophysiologic recordings. However, the overall loss of righting reflex as a measure of anesthetic-induced hypnosis, the immobility response to tail clamp, and the amnestic effect to fear conditioning did not markedly differ between genotypes. These same double point mutations were also introduced into the GABAaR alpha one subunit of mice. These mice were further evaluated for immobility and electrophysiologic responses of lumbar neurons to noxious stimulation (via hindpaw pinch). Isoflurane MAC did not differ between wild-type and knock-in mice. Similarly, isoflurane depressed neuronal responses to noxious stimulation in both wild-type and knock-in mice. While these two sets of experiments show mutations in the GABAaR that confer clear *in vitro* resistance to isoflurane, the translation of this effect into a like response in the whole organism has proven less than satisfying. Reasons for this may include those related to developmental changes in mutant mice and the possibility of compensatory system alterations, as well as the possible lack of this specific receptor subtype being important for the anesthetic effect in the whole organism. Additionally though, there is the very real possibility of the specific mobility endpoint of anesthetic effect not being modulated by this receptor system, while some other very important anesthetic-associated phenotypes (i.e. learning and memory) may be markedly affected as noted below.

In two separate works, Rau et al. made similar attempts to introduce mutations into the mouse GABAaR in an effort to observe overall anesthetic response phenotype/resistance including endpoints of immobility, hypnosis, learning and memory. In the first work, they created mutant mice with a knockout for the GABAaR alpha 4 subunit [25]. Knockout mice and their wild-type controls were assessed via three separate behavioral tests: conditional fear (to assess amnesia), loss of righting reflex (to assess hypnosis), and MAC for response to noxious stimulation (to assess immobility). Their results indicated that knockout of the alpha4 subunit reduced the amnestic effect of isoflurane, minimally affected loss of righting reflex, and had no effect on immobility. In a similar vein a few years later, they produced a mouse line with a knockout mouse for the GABAaR beta 3 subunit that was tissue specific to the forebrain [26]. They subsequently conducted behavioral performance analyses of these animals. This included assessment of learning and memory via tests of fear conditioning responses to situational context in addition to auditory stimuli. Further testing included testing mobility responses to painful stimuli. These tests were then repeated in the presence of either the intravenous anesthetic, etomidate, or the volatile anesthetic, isoflurane. Their results show that while etomidate equally suppressed the fear response behaviors in both wild type and knockout mice, the beta3 knockout showed a statistically significant lessening of its conditioned fear response due to the presence of isoflurane. These results suggest that the GABAaR beta 3 subunit within the mouse forebrain seems to contribute to the amnestic effects of isoflurane while not to that of etomidate. No effect of genotype was noted for any of the mobility assays for either etomidate or isoflurane. These results are somewhat in contrast to the results obtained by Jurd et al. regarding the GABA beta 3 mouse knockin and its resistance to etomidate with only partial resistance to

isoflurane effects [22] This may be due to baseline genotypic variances and alterations in developmental compensations in the two preparations, but is a significant difference that is yet to be completely explained.

Resistance to anesthetic effects in Potassium Channels

While potassium channels have not been studied as extensively as the GABAaR and other ligand-gated ion channels with respect to anesthetic effects, another site of anesthetic action that has demonstrated volatile anesthetic resistance is the family of tandem pore potassium channels. A novel anesthetic-activated potassium current was first described in a single molluscan neuron by Franks and Lieb [27,28]. Along these lines, animals with knockouts for certain tandem pore potassium channels have demonstrated resistance to volatile anesthetics [29]. Within the tandem pore potassium channel family, TREK-1 is sensitive to anesthetic gases Xe, N2O, and cyclopropane, while another member of this potassium channel family, TASK-3, seems insensitive to the latter gases while retaining sensitivity to the volatile anesthetics [30]. Additional studies producing single point mutations in TREK-1 seem to confer relative resistance to certain volatile anesthetics [31]. Indeed, certain aspects of deep sleep are similar to the anesthetized state.Through the generation of TASK-3 knockout mice, Pang et al. have shown a significant role for the TASK-3 potassium channel in the theta oscillations of the cortical EEG that are associated with both sleep and anesthetized states [32]. In particular, TASK-3 knockout animals show marked alterations in both anesthetic sensitivity and natural sleep behavior.

Resistance to anesthetic effects in *C. elegans* proteins

Among the many organisms studied for their variable effects of anesthetic agents, the nematode, Caenorhabditis elegans, has been a valuable source of information. First described by Morgan et al., volatile anesthetics have multiple effects on the relatively less complicated nervous system of this organism, making it more amenable to dissection and discovery [33]. In particular, the mutant strains known as unc-1, unc-13, unc-79, unc-80, mev-1 and GAS-1 show altered responses to a series of volatile anesthetics [34-37]. Resistance to halothane and isoflurane has been shown in mutants of the syntaxin protein . Variable resistance within *C. elegans* has also been shown to the stereoisomers of isoflurane [37,38]. The Goalpha protein, a homolog of mammalian stomatin protein, has also been shown to modulate isoflurane resistance in *C. elegans* [39]. The preconditioning effect due to isoflurane within *C. elegans* seems to be blunted by the Apaf-1 homolog CED-4 protein [40]. A syntaxin suppressor mutant (js127) revealed that adenylate cyclase acts in neurons within a Galphas-PKA-UNC-13-dependent pathway to modulate isoflurane sensitivity [41]. Such activation of neuronal adenylate cyclase appears to antagonize isoflurane inhibition of locomotion in *C. elegans* and thereby enhances isoflurane resistance. The sensitivity of *C. elegans* to halothane is significantly altered by either manipulation of membrane conductance with optogenetic methods or generation of mutations in leak channels that alter the resting membrane potential [42]. Interestingly, immobility induced by isoflurane is not affected by optogenetic modulation, suggesting that different volatile anesthetics, while possibly having some mechanisms in common, may have many modulatory effects mediated by separate mechanisms.

Conclusion

General anesthetics have been provided safely and effectively for many years and yet their exact molecular underpinnings remain relatively unknown. Theories of anesthetic action rooted in bilayer membrane perturbations have given way to specific theories concerning direct effects on ion channel proteins. These molecular targets are intimately involved in the conduct of neuronal signaling within the central nervous system and are thought to be essential in the modulation of conscious states. Better molecular characterization of anesthetic mechanisms is actively being derived from the examination of both *in vitro* and *in vivo* ion channel systems which seem to maintain variable levels of resistance to anesthetics, specifically the GABA receptors, tandem pore potassium channels, and a variety of protein systems within the nematode, *C. elegans*. Continued analyses along these lines will shed greater line on the exact molecular nature of anesthetic interactions with relevant receptor targets. This will hopefully aid in the future design of better anesthetics as well as enhance our understanding of consciousness itself.

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Key Points

a. While general anesthetics have been provided safely and effectively for many years, their exact molecular underpinnings remain relatively unknown.

b. One quest for such answers lies in the examination of both *in vitro* and *in vivo* ion channel systems which seem to maintain variable levels of resistance to anesthetics.

c. Valuable information about the possible targets and molecular nature of anesthetic action is being derived from studies of anesthetic resistance in GABA receptors, tandem pore potassium channels, and several protein systems within the nematode, *C. elegans*.