Mechanisms revealed through general anesthetic photolabeling

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Abstract

General anesthetic photolabels are used to reveal molecular targets and molecular binding sites of anesthetic ligands. After identification, the relevance of anesthetic substrates or binding sites can be tested in biological systems. Halothane and photoactive analogs of isoflurane, propofol, etomidate, neurosteroids, anthracene, and long chain alcohols have been used in anesthetic photolabeling experiments. Interrogated protein targets include the nicotinic acetylcholine receptor, GABA$_A$ receptor, tubulin, leukocyte function-associated antigen-1, and protein kinase C. In this review, we summarize insights revealed by photolabeling these targets, as well as general features of anesthetics, such as their propensity to partition to mitochondria and bind voltage-dependent anion channels. The theory of anesthetic photolabel design and the experimental application of photoactive ligands are also discussed.

Keywords

photoaffinity labeling; photolabeling; GABA$_A$ receptor; *Torpedo* nicotinic acetylcholine receptor; VDAC; voltage-dependent anion channel; leukocyte function-associated antigen; tubulin; PKC; protein kinase C; halothane; neurosteroid; propofol; etomidate; octanol; aminoanthracene; isoflurane; optoanesthesia

Introduction

General anesthetics are a chemically diverse class of drugs that cause clinically important endpoints through multiple molecular and neuronal mechanisms. The prevalence of general anesthetics in modern medicine mandates a greater understanding of their pharmacology. At clinical concentrations, these drugs can affect the function of proteins. Characterizing the interactions of anesthetics with proteins is a biochemical and biophysical challenge, especially as many targets are membrane proteins of lower abundance or without high-resolution structures. Injectable general anesthetics are typically of higher potency than the

volatile type, suggesting higher affinities for protein targets that influence consciousness; however, micromolar dissociation constants are common for all anesthetic-macromolecule interactions. Micromolar dissociation constants indicate rapid ligand unbinding, and one experimental approach that overcomes these dissociation kinetics is photoaffinity labeling, or simply "photolabeling".

General anesthetic photolabeling experiments have been performed on purified proteins, enriched subcellular fractions, tissue sections, and whole organisms, with variations on each biological system. After equilibration with a molecule, the biological system contains ligand-bound macromolecules, the concentration of which depends on the ligand concentration and the dissociation constants associated with the substrates in the system. Photolabeling specifies that the ligand undergo photolysis upon light exposure, generating chemically unstable intermediates that covalently attach to the macromolecules, converting ligand off-rates infinitely towards zero. This attachment (or "adduction") provides a tag in a site that can then be identified with mass spectrometry, or else traced if the ligand is radiolabeled. Anesthetic photolabeling has been used to identify protein targets and their binding sites, to study functional effects of ligand binding, and for discovery-based proteomic or distribution studies.

Anesthetic pharmacology encompasses mechanisms of hypnosis as well as those of numerous side effects, and therefore the photolabeling literature is broadly cast. Some consensus targets of general anesthetics have been extensively studied with photolabeling (e.g., gamma aminobutyric acid type A [$GABA_A$] receptors and nicotinic acetylcholine receptors [nAChRs]). Certain experimental approaches and some proteins, however, have been analyzed with specific ligands, particularly halothane, which was the first compound applied in anesthetic photolabeling experiments. The diversity of the photolabeling studies is reflected by incomplete information for all anesthetic chemotypes in this review. Herein, we overview the molecules used for anesthetic photolabeling, summarize studies utilizing the photolabeling technique, and describe molecular insights revealed with photoactive anesthetics. Future approaches, including *ex vivo* and *in vivo* photolabeling, are also discussed.

Development and detection of anesthetic photolabels

With ~254 nm exposure, halothane undergoes photodecomposition, primarily to reactive chlorotrifluoroethyl and bromine radicals, which permits its use as a photolabel [1,2]. An advantage of halothane photolabeling is the use of an unmodified clinical compound; however, the primary disadvantage is that the high energy UV that induces photolysis can be destructive to biological macromolecules. The chlorotrifluoroethyl radical, the distinguishing label, also demonstrates reaction selectivity towards aromatic residues, and the released bromine atom can propagate further radical reactions [3–9].

To overcome these problems, and further broaden application, photoactive anesthetic analogs containing diazirine or aryl azide moieties have been synthesized for a range of anesthetics. The chemotypes represented by these photolabels include haloalkanes [10], long chain alcohols [11,12], etomidate [13–16], neurosteroids [17], haloethers [18,19], anthracene [20], alkylphenols [21–23], and barbiturates [24] (Figure 1). Incorporated photoactive groups undergo photolysis at lower energy wavelengths than halothane (~300–375 nm), limiting damage to biological samples upon irradiation. The chemical and pharmacologic properties of these analogs, most notably general anesthetic potency, are initially characterized to ensure reasonable mimicry of the parent compounds. Photolabeling studies with analogs are strengthened by confirmation that pharmacologic properties are conserved with the parent compound through parallel functional assays or competition experiments.

Competition experiments hint at target specificity or saturability, easing subsequent experimental investigations, but not necessarily designating a relevant role for the functional effects of the compounds.

The diazirine moiety has generally been favored in anesthetic photolabel design. Diazirine photolysis ideally results in a single reactive intermediate, a singlet carbene, and an inert by-product, dinitrogen. A singlet carbene is a neutral divalent carbon species having an empty *p*-orbital and a hybrid orbital that contains two paired valence electrons [25]. By virtue of this electronic arrangement, the carbene intermediate simultaneously displays carbocation and carbanion characteristics, allowing both electrophilic and nucleophilic reactions, and enabling covalent attachment to proteins orders-of-magnitude faster than carbon-centered radicals (e.g., chlorotrifluoroethyl radical) [26,27]. While initially formed in the singlet state, many carbenes will convert to a triplet state that has one electron of the same spin in the two valence orbitals. This species will act as a diradical and has much lower reactivity than the singlet carbene. The promiscuous reactivity of singlet carbenes is desired to reliably photolabel equilibrium binding sites, which can consist of chemically diverse side chain and backbone atoms. Additionally, photolysis of diazirines often generates alternative intermediates, including diazonium and carbocation species that display undesired selectivity for nucleophilic residues [22,28–30]. Placing the diazirine on an electron deficient carbon or adjacent to an electron withdrawing group, especially a trifluoromethyl group, promotes the desired singlet carbene reactivity; this feature is integrated into multiple photoactive ligands of diverse chemotypes (Figure 1). Photolabel reactivity is suggested by recovering a broad range of adducted residues, including aliphatic amino acids [19,21,24,30,31].

In addition to suggesting broad reactivity of the photolysis intermediate, photolabel attachment to multiple and diverse residues lining a pocket indicates a considerably dynamic ligand in the site. It is likely that general anesthetics favorably assume multiple orientations, defined primarily by steric hindrance, in a pocket where interactions are mediated by hydrophobic or van der Waals forces. An experimental method to more completely probe an anesthetic cavity is photolabeling with isomers or analogs that contain the substituted photoactive group in different positions of the molecule [12]. In addition to defining steric limitations, this approach can provide evidence for critical higher-energy interactions, such as an intermolecular hydrogen bond, if a consistent relationship (e.g., molecular distance) is identified between the location of the adducted residues, the placement of the photoactive group, and the predicted intermolecular interaction.

To detect residue-level attachment of a photolabel to a protein site, Edman degradation and liquid chromatography-tandem mass spectrometry (LC-MS/MS) are most commonly used. Edman degradation is a technique in which N-terminal residues are chemically cleaved from a peptide in repeated cycles, and the released, derivatized amino acids and peptide fragments can be isolated, identified, and scintillation counted. A radioactive probe is essential for identifying a residue with an attached photolabel using Edman degradation. LC-MS/MS avoids the use of radioactivity and identifies photolabeled peptides by mass (note: mass spectrometers actually detect mass-to-charge ratios [*m/z*], but "mass" suffices for this discussion). In LC-MS/MS, peptide masses are detected before trapping and fragmenting the peptides into random pieces, the masses of which are also detected. Computer algorithms assist manual interpretation of mass spectra by identifying peptides and amino acids with a mass modification that is equivalent to that of the adducted ligand.

A primary consideration for both sequencing methods is the length of peptide that can be analyzed. Edman degradation is limited to peptides ~30 amino acids or less, due to the efficiency of the chemical reaction, while LC-MS/MS optimally sequences peptides ~7–20

amino acids in length; therefore, proteases or chemical cleavage methods must be chosen to optimize peptide length and also to maximize sequence coverage of the analyzed protein. Further, the hydrophobic nature of anesthetic binding sites often leads to hydrophobic, sometimes insoluble peptides to analyze for adducts. This is particularly problematic for mass spectrometry, which only detects charged, and presumably soluble, peptides and fragments. Elaborate methodologies, aided in part by advances in mass spectrometer technology, have been developed to comprehensively sequence some membrane proteins with LC-MS/MS [32]. The accuracy and throughput of LC-MS/MS makes this technique important for the future of photo-adduct analysis, yet the reliability of the more laborious Edman degradation has been critical thus far in identifying photolabel binding sites.

Supramolecular binding of a volatile anesthetic

Halothane, an early anesthetic photolabel, has been used to characterize tissue-level distribution of volatile anesthetics. Halothane photolabeling of brain slices *ex vivo* demonstrated widespread anatomic binding with specificity in synapse-dense regions compared to cell bodies and white matter [33]. This reflects concentrated binding of the anesthetic in a protein-rich compartment. At least 15% of neuronal proteins are photolabeled by halothane, in agreement with the number of proteins that contain cavities large enough to accommodate the ligand [34]. Subcellular halothane binding and lipid distribution are not correlated [35], although membrane proteins can increase anesthetic binding to surrounding lipids [6]. Together, these suggest preferential binding of the general anesthetic to proteins, with lipid binding generally nonspecific, with the exception of lipids at some protein-membrane interfaces.

In agreement with widespread binding, no intracellular microenvironment becomes saturated within the clinical range of halothane (0–300 μM) [35]. Interestingly, halothane preferentially and saturably partitions to mitochondria [36]. Protein components of respiratory complexes and multiple mitochondrial enzymes have been identified with photolabeling as specific binding partners of halothane [37,38]. General anesthetics affect mitochondrial respiration [39,40], and genetic modulation of respiratory complexes alters mammalian sensitivity to anesthetics, including injectable drugs [41]. This suggests mitochondrial energetics modulate sensitivity to anesthesia, with specific ligand-protein interactions a plausible mechanism of altering mitochondrial energetics.

Despite localization of halothane to mitochondria, anesthetics also elicit their endpoints through mechanisms that do not involve energetic depression. Most current theories emphasize additive or synergistic interactions with multiple targets, each contributing variably to a given endpoint. Thus, bulk partitioning and/or saturable binding by themselves do not specify relevance, as a high concentration of ligand may be required for a small contribution to an endpoint and vice versa, on both molecular and physiologic levels. Further, isoflurane was relatively ineffective at competing halothane binding in brain slices or synaptosomes, arguing against a unitary mechanism of anesthesia dependent on bulk binding [2,33,34].

Protein targets

Photolabeling enriched subcellular fractions or purified protein allows interrogation of general anesthetic targets, including ligand specificity and binding site character. Significant findings regarding specific proteins are summarized here.

nAChR

Nicotinic acetylcholine receptors are heteromeric ligand-gated cation channels. Neuronal subtypes are widely distributed in the central nervous system, but with low abundance and diverse subunit stoichiometries. The readily available *Torpedo* muscle-type nAChRs with a fixed stoichiometry and medium resolution structure has served as a model to study anesthetic interactions [42]. The subunits are symmetrically arranged around the pore in the order of α*–γ–α*–δ–β, and extracellular interfacial acetylcholine sites are located between subunits denoted by the asterisks.

Halothane photolabeling of *Torpedo* nAChRs identified the transmembrane helices as the preferential anesthetic binding domain on Cys-loop receptors [3]. Halothane labeling was saturable and partially inhibited by isoflurane. Intrasubunit labeling was concentrated in the α subunit, but the identification of residue-level binding sites was confounded by the chemical selectivity of the photolysis intermediate [9]. Intrasubunit binding was demonstrated for azi-isoflurane and *m*-azi-propofol in a water-solubilized version of the nAChR α subunit transmembrane domain [43]; an analogous site for desflurane and propofol was crystallographically identified in a homologous cationic bacterial Cys-loop channel [44]. Indeed, photolabeling of *Torpedo* receptors in native membranes identified a δ intrasubunit site for *m*-azi-propofol and *p*TFD-etomidate [45,46].

M-azi-propofol and *p*TFD-etomidate, as well as TDBzl-etomidate, also bind an *inter*subunit site, likely at the γ–α non-agonist interface [45–47]. Both the intra- and inter-subunit sites might enhance or inhibit channel function, depending on the ligand. The most common mechanism for channel inhibition by anesthetics, however, is pore block. *M*-azi-propofol, *p*TFD-etomidate, TDBzl-etomidate, azi-etomidate, and 3-azi-octanol all bind within the nAChR ion channel [29,45–48]. The inhibitory influence of binding to pore residues was further demonstrated electrophysiologically by photolabeling functional receptors with 3-azi-octanol and azi-etomidate to produce irreversible pore block [49,50].

The *Torpedo* muscle-type nAChR has been critical in establishing the presence of multiple specific binding sites for anesthetics on Cys-loop receptors. However, translating this information to mammalian neuronal-type nAChRs will be challenging, especially without high-resolution structures of multiple receptor subtypes. In addition, the most potent anesthetic analog discussed (TDBzl-etomidate) actually potentiates this excitatory receptor [47], in contrast to etomidate and most clinical general anesthetics and their analogs [16,51]. This limits the usefulness of muscle-type nAChR inhibition as model for anesthetic pharmacology, and demonstrates limitations associated with subtle changes in the chemical structure of the ligands.

GABA$_A$ receptor

Most intravenous and volatile anesthetics positively modulate the anionic GABA$_A$ receptor, also a Cys-loop ligand-gated channel. Rational design of a general anesthetic ligand would likely consider GABA$_A$ specificity, and receptor modulation is a standard validation of photolabel pharmacology. Neuroanatomic expression of GABA$_A$ subunits essentially reflects receptor function. In phasic receptors, synaptic inhibition is largely mediated through $(\alpha 1–3)_2(\beta)_2(\gamma 2)$ subunit stoichiometries, while $(\alpha 4–6)_2(\beta)_2(\delta)$ subtypes mediate tonic, extrasynaptic inhibition [52]. Anesthetic-sensitive GABA$_A$ receptors have subunits arranged in order of β*–α–β*–α–γ/δ, with extracellular interfacial GABA sites denoted by the asterisk, and a benzodiazepine site specific to the α–γ interface.

Similar to nAChRs, anesthetics bind the GABA$_A$ receptor transmembrane domain. Site identification and competition experiments are complicated by allosteric interactions

between multiple transmembrane sites, which also couple to the extracellular GABA site. GABA enhances azi-etomidate and *m*TFD-mephobarbital photolabeling of receptors [53,54]; similarly, propofol, pentobarbital, isoflurane, octanol, etomidate, as well as azi-etomidate, TDBzl-etomidate, and *m*TFD-mephobarbital, enhance muscimol binding to the agonist sites [30,54,55]. Positive allosteric coupling has also been demonstrated with halothane photolabeling of brain slices co-incubated with GABA [56].

In contrast to nAChRs, a GABA$_A$ intrasubunit site has not been identified with anesthetic photolabeling; however, intersubunit binding is conserved amongst ligands. Azi-etomidate and TDBzl-etomidate photolabel residues at the β*–α interfaces in γ-containing receptors, with competitive binding by etomidate and isoflurane [30,53–55]. Alternatively, *m*TFD-mephobarbital photolabels analogous sites at the α–β and γ–β non-agonist interfaces, with photolabeling inhibited by pentobarbital and phenobarbital [54]. Interestingly, *p*TFD-etomidate preferentially binds to the *m*TFD-mephobarbital site, while bulkier barbiturates such as thiopental and brallobarbital show decreasing preference for any subunit interface [54]. Propofol inhibits both azi-etomidate and *m*TFD-mephobarbital binding, suggesting propofol could bind to at least four subunit interfaces [54]. Photolabeling with *ortho*-propofol diazirine confirmed alkylphenols bind at least to interfacial sites that contain a β subunit [23]. Neurosteroids, including allopregnanolone and alphaxalone, enhance azi-etomidate and *m*TFD-mephobarbital binding specifically in the absence of GABA [54,57]; lack of competition by neurosteroids is consistent with the identification of a 6-azi-pregnanolone site on a β transmembrane helix, located deeper in the membrane towards the cytoplasm [31].

Chiara and colleagues clearly demonstrated that general anesthetics have varying affinities for the cavities photolabeled by azi-etomidate and *m*TFD-mephobarbital [54]. Structural features of these interfaces are conserved, and it is likely that the allosteric mechanisms of potentiation overlap. The allosteric energetics of TDBzl-etomidate are particularly interesting, as this ligand potentiates the cationic nAChR and anionic GABA$_A$ receptor by binding to non-agonist and agonist interfaces, respectively. Essential will be the extension of GABA$_A$ photolabeling to other ligands and receptor subtypes, including δ-containing receptors believed to contribute to the hypnotic effects of injectable drugs [58].

VDAC

Voltage-dependent anion channels (VDACs) are ubiquitous targets of general anesthetics. These β-barrel porins, typically in an open conformation, gate metabolites and ions traversing the mitochondrial outer membrane. VDAC isoforms bind halothane [37,38], neurosteroids [17,31,59], long-chain alcohols [11,48], alkylphenols [22,60], anthracene [20], and etomidate [13,14,16,29,45]. Saturable, competitive binding has been demonstrated for all chemotypes, and VDAC has been photolabeled in tissue originating from mammals (including humans), amphibians, insects, and fish. Anesthetic effects on VDAC function have not been reported; characterization and measurement of this mitochondrial ion channel is technically challenging and requires specialized systems. VDAC and cys-loop receptors associate *in vitro* [17,59,61,62], though the *in vivo* relevance of this is unclear, and knockout of VDAC isoforms 1 and 3 does not affect rodent sensitivity to neurosteroids [59]. The necessity of VDAC2 for cellular viability renders these channels difficult to study *in vivo*, as the isoforms compensate for the loss of each other, and a variety of specific pharmacologic modulators are not yet available.

Tubulin

As the primary component of microtubules, α- and β-tubulin isoforms are expressed in every cell type, and have been proposed as relevant targets for all general anesthetics [63]. 6-azi-

pregnanolone binds neuronal β-tubulin in a site conserved with colchicine, a potent inhibitor of microtubule stability [64]. 6-azi-pregnanolone and its parent anesthetics, pregnanolone and allopregnanolone, also inhibit tubulin polymerization, suggesting modulation of neuronal microtubule dynamics or stability by endogenous neurosteroids. The fluorescent anesthetic 1-aminoanthracene [65] and its analog 1-azidoanthracene also bind tubulin, occupying the colchicine site and destabilizing microtubules [20]. Co-administration of the potent microtubule stabilizer epothilone D increased the concentration of allopregnanolone or 1-aminoanthracene required to achieve the immobility endpoint in tadpoles [20]. This suggests microtubule polymerization dynamics can alter general anesthetic sensitivity, albeit through mechanisms that remain undefined. Halothane also photolabels tubulin [37,38], yet stabilizes microtubule polymerization similar to propofol and isoflurane [20,66], casting uncertainty as to whether tubulin represents a unitary target for anesthetic hypnosis.

Protein Kinase Cδ

The protein kinase C (PKC) family of proteins consists of serine/threonine kinases involved in signal transduction, including regulation of pre- and post-synaptic ion channels and GPCRs. "Conventional" and "novel" PKC isoforms contain four domains, termed C1–C4. The C1 domain consists of conserved subdomains, C1A and C1B, which each bind diacyglycerol and phorbol esters (PE) with varying affinities [67]. Diacylglycerol and PE sufficiently activate novel isoforms while conventional PKCs also require calcium, which binds the C2 domain. Conventional and novel PKCs, in addition to "atypical" PKCs that are both calcium and diacylglycerol/PE independent, contain a C3 ATP binding domain and a C4 catalytic domain [68].

General anesthetics variably affect PKC activity, causing inhibition or activation depending on the isoform, anesthetic, and experimental conditions [68]. The isolated catalytic domains of PKCs appear unaffected by anesthetics [69]. In contrast, anesthetics, most notably alcohols, allosterically interact with the diacylglycerol/PE sites on the C1 domain [70]. Photolabeling experiments with azi-alcohols have been performed with PKCδ, a "novel" isoform, in part because a high-resolution structure of its C1B subdomain is available.

Separately, the PKCδ C1A and C1B subdomains were isolated and photolabeled with 3-azi-octanol, 7-azi-octanol, and 3-azi-butanol, and specific binding sites allosteric to the diacylglycerol/PE sites were identified on each subdomain [71,72]. The allosteric coupling between butanol or octanol and a phorbol ester was maintained even for the isolated C1B domain [71]. The azi-alcohols photolabeled a single residue in C1B, corresponding to tyrosine-236 of the full-length protein, nearby the PE binding cleft [71]. Further demonstrating the importance of this site, PKCδ C1B was crystallized in complex with the anesthetic cyclopropylmethanol [73], which non-competitively inhibits PE stimulated PKCδ activity, and was revealed to hydrogen bond with tyrosine-236. Mutation of this residue abolished binding of cyclopropylmethanol and its inhibitory effects on PE stimulated activity [73].

Unraveling the complex interactions of alcohols with PKCδ has only begun. For instance, while azi-octanol, azi-butanol, and cyclopropylmethanol bind an identical site on C1B, the binding modes likely differ. This is first suggested by the positive coupling between butanol or octanol and PE, and the conversely negative coupling between cyclopropylmethanol and PE [71,73]. Additionally, binding modes conserved with cyclopropylmethanol were not observed in structures of PKCδ C1B co-crystallized with longer chained alcohols [73]. The structural and functional effects of clinical general anesthetics on PKCδ as well as other PKC isoforms should be the subject of further investigation.

LFA-1

Leukocyte function-associated antigen-1 (LFA-1) is a heterodimeric adhesion protein, composed of αL and β2 subunits, that mediates leukocyte arrest at endothelial inflammatory sites. The activated, "open" conformation of this integrin has an exposed intercellular adhesion molecule (ICAM) binding domain, also known as the αI domain, on the αL subunit. The β2 subunit is homologous to αL, but functions as a regulatory subunit to facilitate conformational changes of the protein, including transitions from the resting, "closed" conformation to the activated form. General anesthetics, especially the volatile type, have been shown to suppress leukocyte accumulation at inflammatory sites [74,75]. A critical antagonistic mechanism is suggested by *in vitro* inhibition of LFA-1 binding to ICAM by volatile anesthetics and propofol [76–78].

NMR and x-ray crystallography studies with isoflurane and sevoflurane identified an anesthetic binding site on the αI domain of the αL subunit, allosteric to the ICAM binding site, that is photolabeled by azi-isoflurane and *m*-azi-propofol and is conserved with other LFA-1 antagonists (e.g., lovastatin) [19,77,79–81]. Azi-isoflurane photolabeling also identified a homologous binding site on the β2 subunit in the βI domain [76]. Subsequent *in vitro* experiments suggested that isoflurane inhibits leukocyte arrest by binding to both the αL and β2 subunits and stabilizing the LFA-1 closed conformation [76].

In addition to LFA-1, other adhesion molecules, including platelet integrins that mediate clot formation and stability, are also inhibited by volatile anesthetics and have been probed with azi-isoflurane [82]. Identifying mechanisms associated with volatile anesthetic integrin inhibition, and the apparent lack of inhibition by propofol, is of clinical interest [82].

Photoactivation of ligands *in vivo*

The *in vivo* efficacy of anesthetic analogs is routinely tested in tadpoles by dissolving the compound in water, from which it is absorbed by passive diffusion. In these tadpole assays, animal immobility is the conventional and fairly unambiguous anesthetic endpoint [83]. In a recent study, tadpoles anesthetized with *m*-azi-propofol were photolabeled *in vivo* [60]. This assured photolabeled targets were functional and equilibrated with ligand concentrations aligned to the anesthetic endpoint. Covalent attachment of ligand in target sites dramatically lengthens drug off-rates, thereby increasing apparent affinity and potency of a compound [60]. Similarly, light-induced attachment of 1-azidoanthrance to tadpole forebrain targets converted this relatively inefficacious anesthetic analog into a potent immobilizer [20]. In both cases, "optoanesthesia" was reversible, indicating that *in vivo* mechanisms exist for terminating even covalent drug action.

Ex vivo photolabeling of GABA$_A$ receptors with azi-etomidate demonstrated that covalent attachment of ligand can irreversibly modulate the ion channel [84]. This included potentiation of GABA currents after washout of unattached compound, as well as increased receptor sensitivity to direct activation by etomidate and propofol [84]. Similarly, *in vivo* attachment of *m*-azi-propofol to relevant targets, likely including the GABA$_A$ receptor, prolonged emergence of anesthetized tadpoles following washout of unattached compound, and after emergence, tadpoles were more sensitive to propofol [60]. Time-resolved proteomic identification of proteins photolabeled *in vivo* provides molecular candidates associated with the immobility endpoint of general anesthesia.

In vivo photolabeling in mammalian systems has not been reported in the literature; however, the potency of photoactive analogs in rodents indicates this is a feasible approach. Propofol and etomidate, administered intravenously, anesthetize mice with two to three times the potency of their corresponding analogs, *m*-azi-propofol, *ortho*-propofol diazirine

and azi-etomidate (our unpublished data and [23, 85]). Photolabeling defined anatomic coordinates *in vivo* in rodents or tadpoles could identify higher and lower brain structures that are functionally related to specific anesthetic endpoints.

Conclusions

The continued and improved application of anesthetic photolabeling techniques will further the goal of unraveling the pharmacology of these drugs. Through photolabeling, progress has been made in understanding anesthetic-protein interactions and the complex mechanisms that cause general anesthesia. The relevance of revealed targets and binding sites must continue to be examined through *in vitro* functional and *in vivo* physiologic assays, including through mutagenesis and animal knock-in/knockout approaches. To comprehensively understand the causes of hypnosis and drug side effects of any single chemotype is ambitious, especially as research naturally transitions to currently used clinical compounds. However, it is clear that the information generated from anesthetic photolabeing experiments will be invaluable in rational drug design and the continued elucidation of general anesthetic mechanisms.

Acknowledgments

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• Of importance

•• Of major importance

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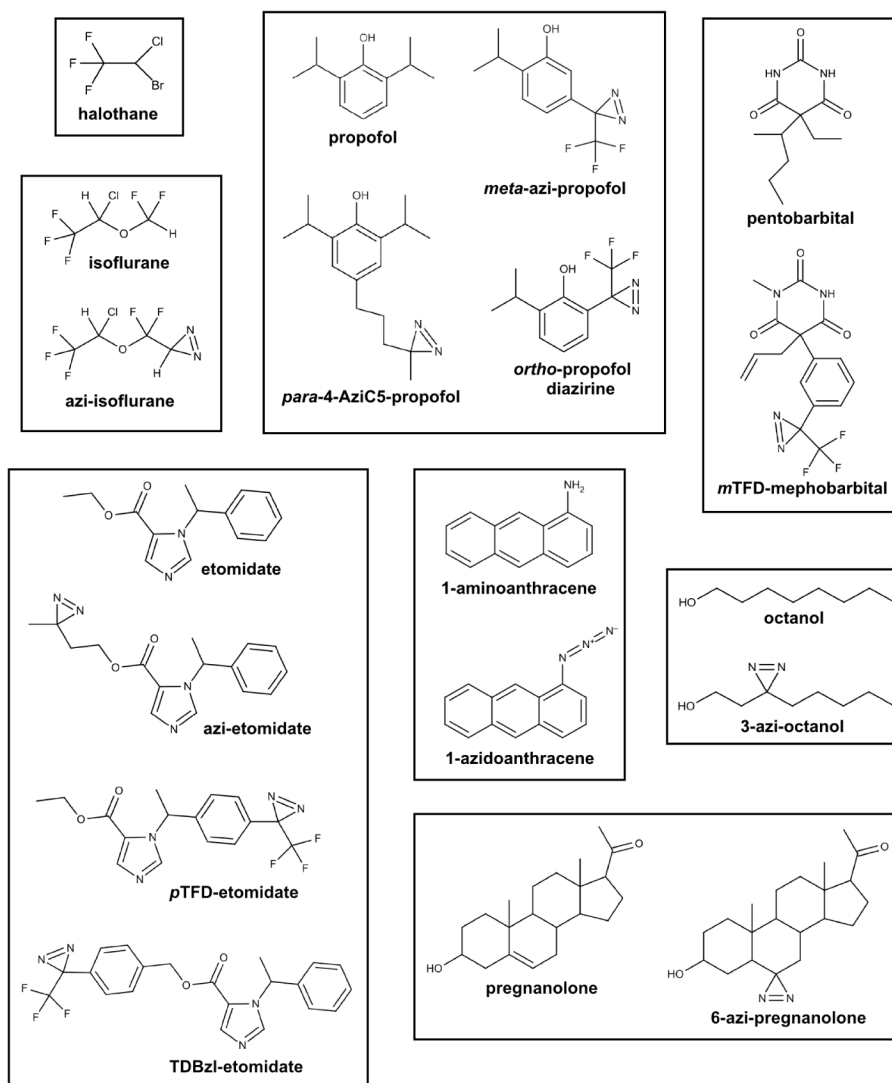
Figure 1.
Chemical structures of general anesthetics and their photoactive analogs. With the exception of halothane, a diazirine or azido group serves as the photoactive moiety.