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Optoanesthesia: Use of Anesthetic Photolabels *In Vivo*

A.R. McKinstry-Wu^{*,1}, M.B. Kelz^{*}

^{*}Department of Anesthesiology and Critical Care, University of Pennsylvania, Philadelphia, PA, United States

Abstract

Investigation of how anesthetics produce hypnosis requires knowledge of their effects at the molecular, neuronal, circuit, and whole-brain network level. Anesthetic photolabels have long been used to explore how anesthetics bind and affect known protein targets, but they could potentially assist in investigation of anesthetic effects at higher organizational levels of the central nervous system. Here, we advocate the use, and provide detailed methods for the application of anesthetic photolabels in slice electrophysiology and in intact animals as a means of investigating anesthetic effects on distinct circuits and brain centers.

Keywords

photolabel; anesthetic; electrophysiology; EEG; mouse; *in vivo*

1. Introduction

Investigating the mechanism of anesthetic action is a particularly thorny problem, not only because general anesthetics can effect changes at multiple organizational levels in the central nervous system, but also because discerning which changes are necessary or sufficient to produce an anesthetic state are not yet clear. Any approach that hopes to tackle how anesthetics produce hypnosis necessarily must address effects at these various organizational levels: specific proteins affected, changes in neuronal function, circuit changes, and network changes. It must also tie those effects to the ultimate behavioral phenotype, hypnosis itself. Existing strategies to address complexities of anesthetic effects on higher-order structures such as circuits and networks are mostly comprised of local administrations by microinjection or infusions of anesthetics at specific structures in the central nervous system (CNS).^{1–3} This strategy accounts for anesthetic effects possibly arising from combined effects on multiple neuronal types and has previously proven relatively effective. The primary disadvantages of this approach are poor control of concentrations, and diffusion of anesthetic away from the area of investigation resulting in effects from drug at areas external to the region of interest (particularly relevant if the area under investigation is near to a ventricle.)

¹Corresponding author's: andrew.mckinstry-wu@uphs.upenn.edu.

Here, we outline methods for investigation of mechanisms of anesthetic-induced hypnosis using anesthetic photolabels *in vivo* in specific regions of intact animals or in slice, a top-down approach we have dubbed optoanesthesia. Anesthetic photolabels are hypnotics that convert to highly reactive species with exposure to a specific wavelength of light—converting the typical extremely transient anesthetic-protein-target interaction to a covalent linkage. While the inhaled anesthetic halothane has intrinsic photolabel properties, other photolabel anesthetics have been created by modifying the base structure of anesthetic compounds to include photoactive groups. These agents have been successfully used *in vitro* to identify anesthetic binding sites on known relevant targets of anesthetics.^{4–7} Photolabel anesthetics that additionally contain a chemically modifiable tag have been used to identify previously unrecognized protein targets of anesthetics (as well as labelling known targets.)⁸ Anesthetic photolabels have further been employed to explore functional changes in known protein targets of anesthetics using artificial expression in electrophysiologic models.⁹ Weiser et al. first described the use of anesthetic photolabels in an *in vivo* system, *Xenopus laevis* tadpoles, where illuminating the entire organism extended hypnosis in an illumination-duration-dependent manner.¹⁰ We have extended this *in vivo* approach to a mammalian (rodent) model where illumination of limited CNS regions causes irreversible covalent binding of anesthetic photolabel in those regions, while in all other regions drug diffuses away and is eliminated. We additionally propose an extension of this method in slice electrophysiology to limit drug effect to one portion of the slice. These approaches can be extended to use previously described anesthetic photolabel molecular target identification methods such that organismal effects may be translated to regional brain anesthetic effects and molecular targets within those relevant regions.⁸

2. Anesthetic Photolabel Availability.

These novel compounds are not available commercially, Small amounts are available, generally as a collaboration, from a limited number of academic laboratories. Validated volatile anesthetic and propofol analogues can be obtained from the Eckenhoff lab at the University of Pennsylvania, etomidate, barbiturate and alcohol analogues from the Miller lab at Harvard, and the neurosteroid analogues from the Evers Lab at Washington University. The amounts made available are typically tiny, and great care must be taken in their deployment.

3. Anesthetic Effects in a Circumscribed Region

The use of photolabel anesthetics in slice and *in vivo* have the advantage of acting on anesthetic targets in a similar method to their parent compounds, while being able to limit the extent of that action to regions of photoadduction after washout of anesthetic drug from other parts of the brain or slice occurs.¹¹ Our *in vivo* and slice photolabelling studies employ *m*-azipropofol (aziPm), an alkylphenol with a photoactive diazirine ring. Peak absorbance of the diazirine ring occurs at 367 nm, with sufficient near-ultraviolet (UVC) light converting the diazirine ring into nitrogen gas and a highly reactive carbene that undergoes rapid covalent binding with virtually any nearby moiety. The limit of the extent of photoadduction is thus primarily dependent on the spread, duration, and intensity of UVC illumination. Fortunately, brain tissue readily absorbs wavelengths in the UVC spectrum, such that spread

by both penetration and scatter is limited, even with an intracerebral light source (figure 1.) For slice electrophysiology, UVC light can either be directed through the microscope optics in order to illuminate only the portion of the slice within view, when photoadducting the neuron under recording, or a separate external fiber may be mounted and used to illuminate a portion of the slice external to the field of view.

4. Slice Electrophysiology

Anesthetic photolabels facilitate investigation of circuit-level anesthetic effects through slice electrophysiology. Whereas drugs are generally introduced into the artificial cerebrospinal fluid and thus applied across the entire slice during recording, photoadducting drug in one portion of the slice and subsequently allowing washout can extend the drug effect for the illuminated portion of the slice while the remainder of the slice no longer experiences direct effects of the drug. UV illumination is achieved either through an integrated 120 mW 365 nm LED lightsource (Thorlabs M365L2-C1, Newton, NJ) that illuminates through the microscope optics, or through a multimode fiber attached to a second arm on the recording stage, which is in turn coupled to 200 mW 375 nm laser (Power Technology Inc., Alexander AK.) The investigation of two regions that project to each other requires careful selection plane in which to cut the slice so that one can preserve those connections.¹² Slice electrophysiological recordings are generally performed as described previously,^{13,14} in brief:

- Sacrifice mice by cervical dislocation followed by decapitation, place the head in ice-cold pre-oxygenated sucrose solution containing of 248 mM sucrose, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 2.0 mM MgSO₄, 2.5 mM CaCl₂, 10 mM dextrose, and 26 mM NaHCO₃.
- Rapidly remove the brain and slice 200 μ m thick slices in the appropriate plane using a Leica microslicer (Leica, Allendale, NJ) while continuously oxygenating the brain in the ice-cold sucrose solution.
- Transfer sections to a holding chamber containing artificial cerebrospinal fluid (aCSF- consisting of 124 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 2.0 mM MgSO₄, 2.5 mM CaCl₂, 10 mM dextrose, and 26 mM NaHCO₃) that is continuously bubbled with 95% O₂/5% CO₂. Keep the slices in the chamber at 37°C for one hour, followed by room temperature until recording.
- Whole cell voltage and current clamp recordings are conducted as described previously.^{13,14} The slice is continuously perfused with oxygenated aCSF at 2 ml/min at 32°C. After visualizing a cell in the area of interest, make a gigaohm seal with a recording electrode and rupture the membrane to obtain a whole-cell recording. The electrodes should have a resistance of 5–8 M Ω when filled with an intracellular solution of 130 mM K-gluconate, 5 mM NaCl, 10 mM phosphocreatine disodium salt, 1mM MgCl₂, 10 mM HEPES, 0.02 mM EGTA, 0.5 mM Na₂GTP, 2 mM MgATP, and 0.1% biocytin (pH 7.3, 280–290 mOsm)

- After patching, switch to current clamp recording and cells should be characterized as previously described, followed by 10 minutes of baseline recording obtained.
- Photolabel anesthetics should be diluted from stock in fresh oxygenated aCSF immediately prior to use.
- After baseline recording, expose the cells of interest to UVC stimulation alone. For either source of illumination, the goal is a thirty second exposure at 1 Hz with a 80% duty cycle and an intensity of 60 mW/mm².
- Allow for another 3 minutes of recording after UVC exposure to ensure cell remains at baseline. · Turn on injection pump and provide the slice with 3 minutes of anesthetic photolabel in aCSF, followed by a 20 minute washout period.
- Once cell has returned to baseline, turn on the injection pump again to give 3 minutes of anesthetic photolabel in aCSF, this time illuminating for 30 seconds as above once slice has been exposed to drug for 1 minute.
- Perform washout, recording for two hours or cell is no longer viable.

5. Photoadduction in Intact Animals

5.1 Measures of Hypnosis and Sedation in a Mouse Model

The singular advantage of working in a top-down manner for investigating anesthetic action is the possibility of evaluating the most relevant anesthetic endpoint, hypnosis, which as a behavioral phenotype can *only* be evaluated at the level of the intact organism. While the classic behavioral measure in animal models that serves as a proxy for loss and return of consciousness is the righting reflex, it is limited. The binary output can fail to capture sub-maximal sedation effects that contribute to but are not sufficient to produce hypnosis, and may produce false positives if there is loss of motor tone without sedation or hypnosis. To address these limitations, we propose examining three behavioral measures of hypnosis and sedation. In addition to the righting reflex, we use video tracking to record distance travelled by, and speed of, the mouse, and a response to a very mild aversive stimulus, an adhesive sticker placed on the mouse's nose. We additionally use electroencephalographic (EEG) recording to look for known signatures of sedation, including increased power in the delta frequency range (0–4 Hz). Finally, we also continuously record cervical electromyograms (EMG) in order to distinguish a general loss of motor tone from sedation or hypnosis.

5.2 Method for *In vivo* Photoadduction with Behavioral and EEG Sedative-Hypnotic Endpoints

- Adult mice are implanted with stereotactically-targeted guide cannulae (PlasticsOne, Roanoke VA) directed bilaterally to structures of interest and simultaneously implanted with dural EEG leads and with cervical electromyographic wires using techniques previously described, though with reduced lead number to accommodate the cannula.¹⁵ We also implant an

indwelling jugular catheter tunneled to the mouse's back, as described by Kmietek et al.¹⁶

○ If the targeted structures are cortical, rather than deep, bilateral cranial windows can be created by carefully drilling out the skull above the cortical region of interest and placing miniature circular coverslips with high UVC transmittance (Warner Instruments, Hamden CT) over the windows. The coverslips must be secured by careful placement of dental cement along their edges. Placement of these coverslips will likely require altering your EEG lead locations to accommodate the windows.

- Mice are allowed to recover for one week. In the second week after surgery, they are habituated to the testing box (a plexiglass box 15 × 25 cm with an overhead video camera [Dinolite VGA, New Taipei City, Taiwan] capturing 5 fps of 640×480 pixel images) while tethered to an EEG recording cable. They additionally undergo daily training for nasal tape removal. (Figure 2.)
- Note: Details of laser exposure and drug dosing are specific to aziPm, other photolabels may require different wavelengths, exposure power, or exposure time.
- Two weeks after surgery, the mouse is restrained, bilateral multimode fibers (Thorlabs, Newton NJ) coupled to a 200 mW 375 nm laser (Power Technology Inc., Alexander AK) are inserted into the guide cannulae and secured, the mouse is tethered to the recording cable via an amplifier chip also containing a 3-axis accelerometer (Intan Technologies, Los Angeles, CA) and the central venous catheter is attached to a syringe pump containing the anesthetic photolabel.
 - For cortical windows, the laser is manually targeted at the window rather than placing the fiber in the guide cannula.
 - All acquisition of biopotentials (EEG/EMG) used open-source Open-Ephys hardware (www.open-ephys.org) via Intan amplifier chips.
- The mouse is placed in the testing box, video recording is started, and baseline activity and EEG/EMG are recorded for 10 minutes. At the end of the 10 minutes, a sticker is placed on the mouse's nose for a tape test trial.
- The laser is turned on at 10 mW at 1 Hz with a 50% duty cycle for one minute. After laser administration, the tape test is repeated and another 10 minutes of activity and EEG/EMG are recorded to ensure that UVC illumination alone does not affect the animal.
- 48 mg/kg aziPm bolus (a hypnotic dose [ED95] of the anesthetic photolabel) is administered via the syringe pump, and once the mouse is unresponsive it is placed on its side and a sticker placed on its nose.
- In addition to continuous EMG/EEG, accelerometry, and video, the mouse's time to return of righting reflex and sticker removal are recorded.

- The mouse is allowed to recover under constant monitoring before being detached and returned to its home cage (at least one hour to allow full recovery.)
- The mouse is allowed two days to recover before further testing.
- On the second testing day, the mouse is attached to the recording, drug, and laser setup. Another 10 minute baseline is taken, followed by anesthetic photolabel administration with simultaneous laser administration (as detailed above) to photoadduct the drug at the region of interest.
- The mouse is placed on its side and a sticker placed on its nose, and recovery endpoints are recorded as above. Note that we record for a minimum of 2 hours after the removal of the sticker, as we have seen continued behavioral sedation, including slowed movement, for significant amounts of time after sticker removal.
- Video for return of right reflex and sticker removal is manually scored by blinded investigators. All video and biopotentials can be analyzed using Matlab 2016b with the Signal Processing Toolbox and Image Processing Toolbox.

6. Potensial Limitations and Pitfalls

A strength of using anesthetic photolabels *in vivo*, their effect on actual anesthetic targets in a defined physical space, is also a potential limitation. The anesthetic photolabels, like their parent compounds, affect many targets in many neuron types. This lack of cell specificity could potentially make it difficult to identify the populations contributing to the anesthetic effect. It is for this reason that this technique is truly complementary with existing cell-type specific methods, such as optogenetics and the use of designer receptors activated by designer drugs, that can target specific neuronal populations, but do not necessarily capture the effects of anesthetics on their native targets. While we did not encounter significant effects of near-UV illumination alone in either slice recordings or in intact animals, it remains a possibility that light alone may have an effect in certain regions or in specific neuronal populations, potentially damaging or killing neurons in the exposed areas. This is the reason we have included all the light alone controls, and it is also why it is critical to ensure that a cell or animal has in fact returned to a true baseline before progressing with testing. Finally, experimentally determining the minimum necessary photoadduction time and intensity for a given anesthetic photolabel is critical given the theoretic possibility of photoconcentration. Photolabelling in excess could produce functional concentrations of drug significantly above the physiologically relevant concentration. This could be similar to the problem encountered with microinjections, where at the injection point, the concentration of drug is higher than could ever be administered peripherally. The times and powers given here to minimize that possibility are for aziPm, but will vary with other compounds.

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Fig. 1. Penetration of ultraviolet light through brain tissue. 375 nm light absorbance as a function of tissue depth. A 50 mW laser was projected through varying thicknesses of murine brain tissue and the resulting decrease in power plotted.

Fig. 2. Optoanesthesia in the intact animal. The mouse is restrained for optical fiber placement and attaching the syringe pump to the venous catheter (A) and subsequently allowed to move freely in the cage (B).