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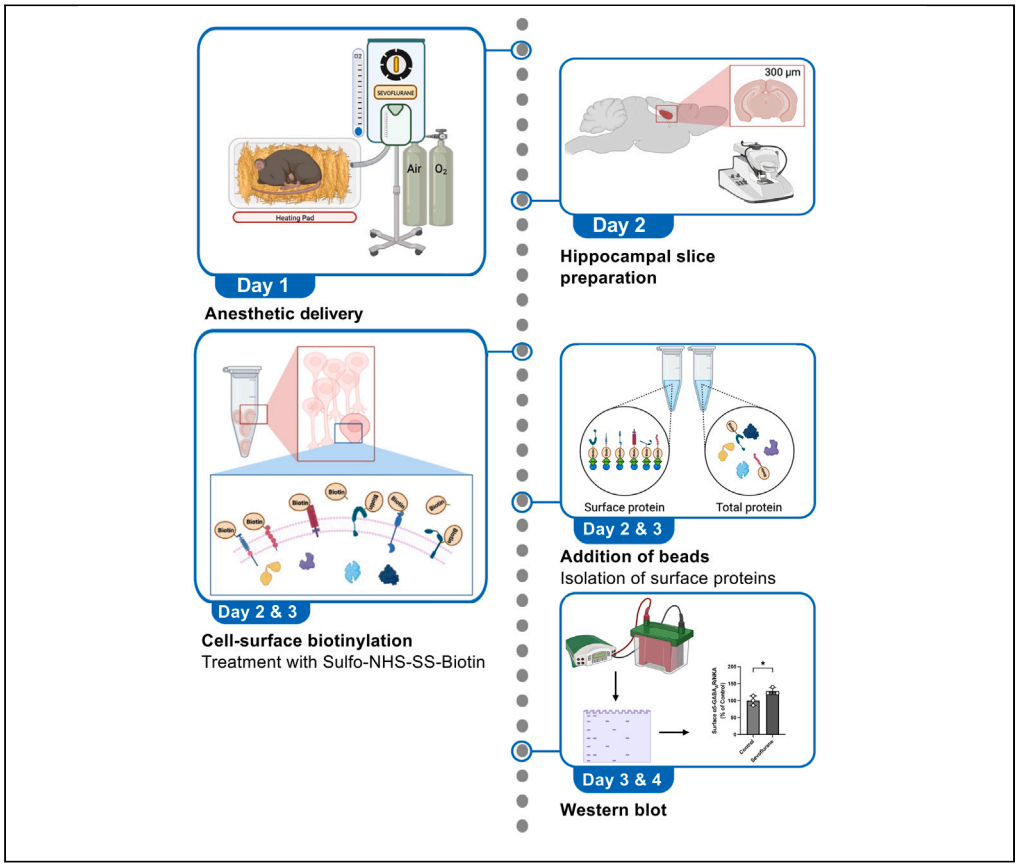
Cell-surface biotinylation of $GABA_A$ receptors in mouse hippocampal slices after sevoflurane anesthesia



Here, we present a protocol for studying the cell-surface proteins in hippocampal slices after *in vivo* administration of sevoflurane, an inhaled general anesthetic drug, to mice. We describe steps for anesthetic delivery, hippocampal slice preparation, and cell-surface biotinylation. We then detail the isolation of surface proteins and their quantification through Western blotting. This protocol can be adapted to study changes in other surface proteins following exposure to various general anesthetic drugs.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for biotin labeling of cell-surface $GABA_A$ receptors in hippocampal slices

Isolation of labeled proteins allows quantification of surface protein levels

Strategy for detection of $GABA_A$ receptors after sevoflurane anesthesia

Quantification of surface proteins in the mouse hippocampus using Western blotting

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Cell-surface biotinylation of $GABA_A$ receptors in mouse hippocampal slices after sevoflurane anesthesia

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SUMMARY

Here, we present a protocol for studying the cell-surface proteins in hippocampal slices after *in vivo* administration of sevoflurane, an inhaled general anesthetic drug, to mice. We describe steps for anesthetic delivery, hippocampal slice preparation, and cell-surface biotinylation. We then detail the isolation of surface proteins and their quantification through Western blotting. This protocol can be adapted to study changes in other surface proteins following exposure to various general anesthetic drugs.

For complete details on the use and execution of this protocol, please refer to Wang et al. (2012),[1] Zurek et al. (2014),[2] and Yu et al. (2019).[3]

BEFORE YOU BEGIN

Exposure to commonly used general anesthetic drugs changes the expression levels of various proteins on the cell surface of neurons, and these changes persist long after the drugs are eliminated from the brain. The increase in cell-surface expression of a subtype of inhibitory $GABA_A$ receptors likely contributes to persistent cognitive deficits that are observed after general anesthesia.[2,4–7] To understand such cognitive impairments, robust methods are needed for studying changes in the cell-surface expression of proteins. Here we describe a protocol for *in vivo* administration of sevoflurane, a commonly used inhaled general anesthetic drug, in mice; followed by biotinylation and purification of cell-surface proteins present in *ex vivo* hippocampal slices. Cell-surface proteins are labeled with Sulfo-NHS-SS-biotin and then isolated by a pull-down assay with deglycosylated avidin beads. These steps are followed by Western blotting to measure levels of cell-surface $GABA_A$ receptors. Specifically, we have repeatedly used this protocol to measure changes in the cell-surface expression of α5 subunit-containing $GABA_A$ receptors after treatment with a variety of injectable and inhaled general anesthetic drugs (e.g., etomidate and isoflurane) as these receptors contribute to post-anesthetic memory disorders in mice.[1,2,7] Although this protocol focuses mainly on $GABA_A$ receptors, it can be easily adapted to study other cell-surface proteins in alternative animal models (e.g., rats, guinea pigs) after exposure to other general anesthetic drugs.

Ethical approval

All animal procedures relating to this study were approved by the Animal Care Committee of the University of Toronto.

Animal habituation

Timing: 1 week

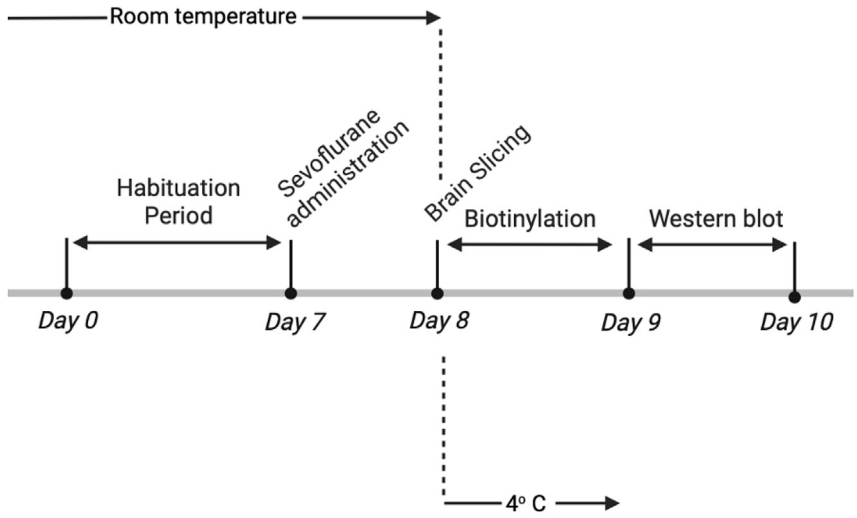

Figure 1. Timeline for the experimental procedure

1. If purchasing mice from a commercial vendor, allow the mice to acclimatize to the animal facility for at least one week after arrival to avoid the confounding effects of stress and transportation before starting the experimental work (Figure 1).

Setting up the anesthetic delivery machine

Timing: 2–3 h

The anesthetic gas delivery machine together with the appropriate vaporizer, pressurized gases and tubing, should be checked and the concentration of anesthetic gas calibrated prior to drug delivery to the mice. This step is only performed initially. It is important to replace the gas tanks when necessary and check the pressure of medical air and oxygen, and monitor the delivery of sevoflurane to the induction chamber before each experiment.

2. To prevent hypoxia and hyperoxia, sevoflurane is delivered in a gas mixture containing 30% O_2 combined with 70% medical air.

   Note: Most rodent anesthesia machines do not accommodate the delivery of medical air; however, the gases supplied from pressurized cylinders can be mixed downstream of the vaporizer to achieve the desired concentration of oxygen using an arrangement of generic tubing and connectors as shown in Figures 2A and 2B.

3. Connect the anesthetic drug delivery tubing to the gas anesthesia induction chamber (Figure 2C).
4. Place a heating pad underneath the induction chamber to maintain core body temperature.
5. Turn on the gas analyzer and measure the concentration of oxygen and anesthetic drugs.
6. Because O_2 and medical air are mixed downstream of the vaporizer, the sevoflurane concentration in the mixed gas will be lower than the concentration set on the vaporizer. Before anesthetizing the mice, use the gas analyzer to create a calibration curve and determine the sevoflurane concentration delivered to the induction chamber (Figures 2C and 2D).

Buffers and reagents

Timing: 1 h

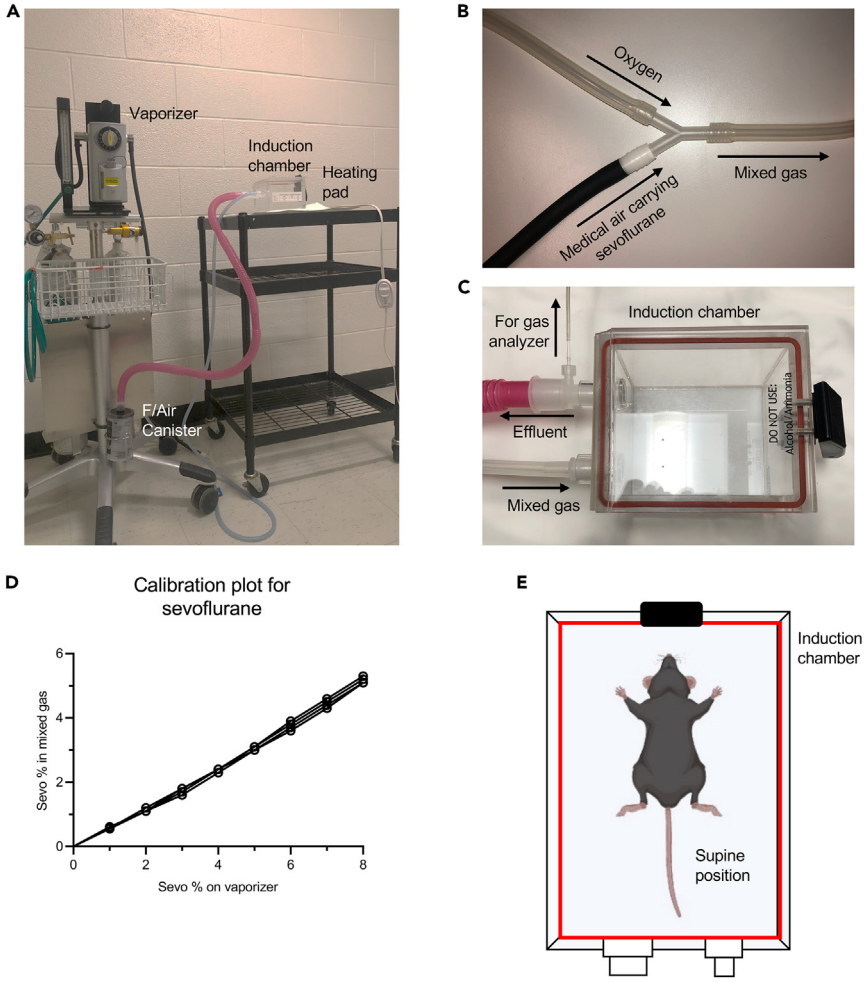

Figure 2. Setup of the anesthetic delivery machine and assessment of sevoflurane concentration

(A) The anesthetic drug delivery machine setup: Sevoflurane is vaporized in medical air before being mixed with oxygen. The gas mixture is delivered to the induction chamber, which is placed on top of a heating pad. The effluent gas from the chamber is then scavenged using a F/Air canister.

(B) Example of the tubing and connectors used for gas mixing.

(C) Zoomed-in image of the induction chamber with the gas analyzer output.

(D) Calibration plot for sevoflurane, showing readings on the vaporizer versus readings in the mixed gas analyzer (n = 8).

(E) The supine position of the mouse, indicating loss of consciousness.

7. On the day of the experiment, prepare buffers and other reagents as needed. Refer to the materials and equipment for a list of recipe tables.
8. Prepare 500 mL artificial cerebrospinal fluid (aCSF) containing 125 mM NaCl, 10 mM D-glucose, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2.5 mM KCl, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, and adjust the pH to 7.4 with 0.5 M HCl.
9. After adjusting the pH, keep the aCSF on ice for at least 30 min. Keep this solution constantly bubbling with carbogen gas (95% O_2, 5% CO_2).
10. To make the biotinylation solution, prepare 1 mL of 0.5 mg/mL Sulfo-NHS-SS-biotin in aCSF per sample and store the solution on ice.
11. To make the quenching buffer, prepare 1 mL of 5 mg/mL Bovine Serum Albumin (BSA) in aCSF per sample and add glycine (25 mM final concentration). Keep it on ice.
12. Prepare 10 mL of Lysis buffer (150 mM NaCl, 20 mM HEPES, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, protease and phosphatase inhibitors), and adjust the pH to 7.4 with 0.5 M HCl.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
GABRA5 monoclonal antibody	Thermo Fisher Scientific	MA5-27700
Na/K-ATPase Antibody	Cell Signaling Technology	3010
THE™ beta Actin Antibody [HRP]	GenScript	A00702
Chemicals, peptides, and recombinant proteins		
Oxygen (O_2)	Messer	100354
Medical air	Messer	100034
Carbogen gas	Messer	100904
Sodium chloride (NaCl)	BioShop	SOD002
N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES)	BioShop	HEP001
Ethylenediaminetetraacetic acid (EDTA)	BioShop	EDT001
Triton X-100	Sigma-Aldrich	X-100
10% Sodium dodecyl sulfate (SDS)	Promega	V6551
cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail	Sigma-Aldrich	11836170001
Pierce™ Phosphatase Inhibitor Mini Tablets	Thermo Fisher Scientific	A32957
Sodium chloride (NaCl)	BioShop	SOD002
Potassium chloride (KCl)	BioShop	POC308
Magnesium chloride ($MgCl_2$)	BioShop	MAG510
Calcium chloride ($CaCl_2$)	BioShop	CCL302
Monosodium phosphate (NaH_2PO_4)	Sigma-Aldrich	S9638
Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich	S6014
D-glucose	BioShop	GLU501
EZ-Link™ Sulfo-NHS-SS-biotin	Thermo Fisher Scientific	21331
Bovine serum albumin	Sigma-Aldrich	A7030
Glycine	Bio Basic Canada Inc.	GB0235
2× Laemmli sample buffer	Bio-Rad	1610737
NeutraAvidin beads	Thermo Fisher Scientific	29202
Hydrochloric acid (HCl)	BioShop	HCL333.500
Experimental models: Organisms/strains		
Mouse C57BL/6, 8–9 weeks, male	Charles River	N/A
Software and algorithms		
Image Lab 6.0	Bio Rad	www.bio-rad.com/en-ca/sku/1709690-image-lab-software
Prism 9	GraphPad	www.graphpad.com/features
Other		
1.5 mL Eppendorf tubes	Axygen	MCT-150-C
F/Air Canister	Benson Medical	20522
F/Air Canister Bracket	Benson Medical	20526
Scavenging tubing 19 mm magenta	Benson Medical	10024
"Y" Piece Adapter (7 mm Ends)	Benson Medical	30614-55
Silicone tubing	Benson Medical	20810
Gas analyzer	Datex Engstrom	ULT-Svi-29-08
Heating pad	Sunbeam	731-500-000R
Induction chamber	Benson Medical	941443
Microcentrifuge	Beckman Coulter	8043-30-1145
Rotating Mixer	Benchmark Scientific	1190B06
Probe sonicator	Active Motif	www.activemotif.com/catalog/719/epishear-probe-sonicatorwww.activemotif.com

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
AE150 Benchtop pH meter	Fisher Scientific	www.fishersci.com/shop/products/accumet-ae150-ph-benchtop-meter/13636AE153
Sigma Delta Vaporizer	Penlon	www.penlon.com/Product-Groups/Vaporizer/Sigma-Deltawww.penlon.com
Leica VT1200 Microtome	Leica	www.leicabiosystems.com/en-ca/research/vibratomes/leica-vt1200/www.leicabiosystems.com
ChemiDoc XRS+	Bio-Rad	www.bio-rad.com/en-ca/product/chemidoc-xrs-system

MATERIALS AND EQUIPMENT

The following is a list of solutions and reagents:

Note: We recommend making all buffers and solutions fresh on the day of the experiment.

- Artificial Cerebrospinal Fluid (aCSF)

Reagent	Final concentration	Amount (Stock)
Sodium Chloride (NaCl)	125 mM	3.65 g
D-glucose	10 mM	0.90 g
Sodium Bicarbonate ($NaHCO_3$)	25 mM	1.05 g
Monosodium Phosphate (NaH_2PO_4)	1.25 mM	0.075 g
Potassium Chloride (KCl)	2.5 mM	0.093 g
Magnesium Chloride ($MgCl_2$)	1.3 mM	0.65 mL (1 M stock)
Calcium Chloride ($CaCl_2$)	2.5 mM	1.25 mL (1 M stock)
Total	N/A	500 mL

Note: Adjust the pH to 7.4 using 0.5 M HCl and keep it on ice for at least 30 min before use. Keep aCSF constantly bubbling with carbogen gas (95% O_2, 5% CO_2) throughout the procedure.

- Biotinylation solution (Sulfo-NHS-SS-biotin)

Reagent	Final concentration	Amount
Artificial Cerebrospinal Fluid (aCSF)	N/A	5 mL
Sulfo-NHS-SS-biotin	0.5 mg/mL	2.5 mg
Total	N/A	5 mL

Note: Prepare 1 mL of biotinylation solution per sample using fresh aCSF that was previously prepared. Keep on ice until use.

Alternatives: A lower concentration of Sulfo-NHS-SS-biotin (Thermo Fisher Scientific, Cat#21331) also provides good results. Test a range from 0.3–1.0 mg/mL Sulfo-NHS-SS-biotin for the amount of tissue collected.

- Quenching buffer

Reagent	Final concentration	Amount (Stock)
Artificial Cerebrospinal Fluid (aCSF)	N/A	5 mL
Bovine Serum Albumin (BSA)	5 mg/mL	25 mg
Glycine	25 mM	125 µL (1 M stock)
Total	N/A	5 mL

Note: Prepare 1 mL of quenching buffer per sample using fresh aCSF that was previously prepared. Keep on ice until use.

- Lysis buffer

Reagent	Final concentration	Amount (Stock)
Sodium Chloride (NaCl)	150 mM	1250 µL (1.2 M stock)
N-2-Hydroxyethylpiperazine-N′-2-Ethanesulfonic Acid (HEPES)	20 mM	1000 µL (200 mM stock)
Ethylenediaminetetraacetic Acid (EDTA)	2.5 mM	100 µL (250 mM stock, pH 7.5)
Triton X-100	1%	100 µL
10% Sodium dodecyl sulfate (SDS)	0.1%	100 µL
Complete™, Mini, EDTA-free Protease Inhibitor Cocktail	N/A	1 tablet
Pierce™ Phosphatase Inhibitor Mini Tablets	N/A	1 tablet
Double distilled water	N/A	7350 µL
Total	N/A	10 mL

Note: Adjust the pH to 7.4 with 0.5 M HCl. The lysis buffer should be left on ice and only be used when it is cold.

Alternatives: Other strong or mild lysis buffers can also be used, such as NP-40 or RIPA. Different lysis buffers may provide varying yields. Therefore, it is highly recommended to determine which lysis buffer provides the highest detection range for the protein of interest. We found that this lysis buffer is suitable for membrane proteins, particularly for $GABA_A$ receptor proteins.

STEP-BY-STEP METHOD DETAILS

Sevoflurane administration in live animals

Timing: 2–2.5 h

Mice are anesthetized with sevoflurane, which is delivered in a gas mixture that contains supplemental oxygen for 2 h while being maintained in a warm environment. Control mice receive only supplemental oxygen and medical air for 20 min. Previous studies have shown that the procedure described below maintains pH, O_2, and CO_2 saturation with minimal cardiorespiratory disruption.[2,6,8]

1. Turn on the heating pad underneath the induction chamber to ensure that mice are kept warm during anesthesia (Figures 2A and 2C).
2. Adjust the airflow on the anesthetic delivery machine to deliver 2.3% sevoflurane, in 30% O_2 and 70% medical air mixture, at a rate of approximately 1 L/min.

⚠ CRITICAL: Use a gas analyzer to monitor expired gases (effluent from the chamber or from a sampling catheter placed in close proximity to the animal's nose) and ensure that O_2 is delivered at the appropriate concentration (Figure 2C).

3. Allow the air in the induction chamber to be replaced with the delivered gas and monitor the concentration of anesthetic drug with the gas analyzer.
4. Once saturated, quickly open the chamber and place the mouse inside. Troubleshooting 1.
   a. Wait for the mouse to lose consciousness, which can take several minutes, depending on the concentration of anesthetic drug.
   b. The loss of righting reflex (LORR) can be used to confirm the loss of consciousness. To assess LORR, gently tilt the induction chamber until the mouse is supine.
   c. When the mouse turns supine, with all four paws not touching the floor, LORR is considered to be achieved. If the mouse rights itself, wait an additional 30 s and assess the LORR again.
5. Continuously deliver sevoflurane, medical air and oxygen for 2 h, taking care to visually monitor the animal for cyanosis and hypoventilation.

   Note: Under optimal anesthetic conditions, the breathing rate should be approximately 55–65 breaths per minute. Cyanosis, characterized by a bluish-purple hue, can be observed in the extremities of the mouse. Cyanosis can be difficult to detect and physiological monitors including pulse oximetry, heart rate and blood pressure monitors are strongly recommended.[4]

   ⚠ CRITICAL: Ensure that the mouse is positioned supine throughout the anesthetic treatment, as this position helps to keep the airway patent that is unobstructed (Figure 2E).

6. After 2 h have elapsed, turn off the sevoflurane vaporizer.
7. Increase the air flow rate to 3 L/min to rapidly flush the remaining sevoflurane from the induction chamber.
8. Allow the mouse to recover in the warmed chamber for at least 10 min or until it regains consciousness before returning it to the home cage.

Preparation of hippocampal slices

Timing: 1–2 h

Twenty-four hours after anesthetic drug treatment, prepare coronal hippocampal slices from a mouse 8–9 weeks old according to standard procedures.[9]

9. Sacrifice mice using decapitation. Quickly extract the brain and keep it in ice-cold carbonated aCSF (Figure 3A).
10. Make two coronal cuts in the mouse brain as denoted by the dotted lines using a razor blade, as shown in Figure 3B.
11. Use super glue to adhere the rostral end of the brain onto the metal vibratome plate (Figure 3C).
12. Attach the metal plate to the vibratome such that the brain is submerged in the vibratome reservoir containing ice-cold aCSF, and surrounded by ice (Figures 3C and 3D).

    ⚠ CRITICAL: Ensure that while collecting the brain slices, the vibratome reservoir chamber is constantly bubbled with carbogen (Figure 3D).

13. Cut coronal slices with a thickness of 300–350 μm.

    Note: A setting of 300 μm of thickness will allow the collection of at least 3 complete coronal slices that include the hippocampi (Figure 3E).

    Note: Using regular dissecting scalpels, carefully remove the hippocampal slices from the coronal slices.

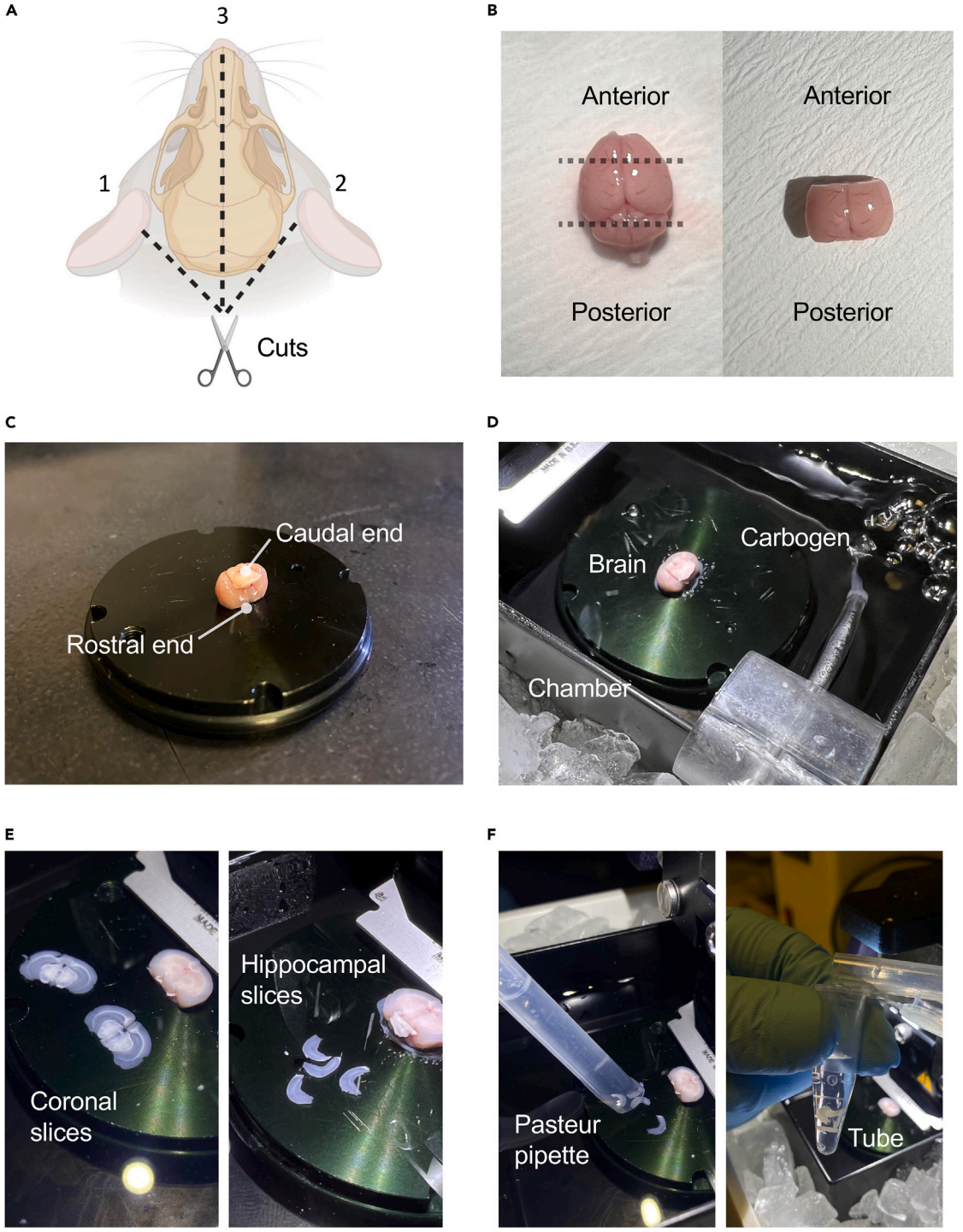

Figure 3. Dissection of mouse hippocampal slices

(A) Schematic representation of the suggested cuts in sequential order to expose the brain.

(B) Isolated mouse brain showing the removal of rostral and caudal brain sections.

(C) Zoomed-in image of the mouse brain attached to the metal plate.

(D) Photograph of the vibratome chamber containing ice-cold aCSF.

(E) Coronal slices and isolation of hippocampal slices.

(F) Transfer of hippocampal slices into an Eppendorf tube.

14. Using a Pasteur pipette, transfer four hippocampal slices into a 1.5 mL Eppendorf tube. Two hippocampal slices can be obtained from a single coronal slice (Figure 3F).

Pause point: Allow the slices to recover on ice with 1 mL of cold aCSF for 5–10 minutes before starting the biotinylation procedure.

Cell-surface biotinylation of hippocampal slices

Timing: 3 h - overnight

Isolation of surface proteins from mouse hippocampal slices using Sulfo-NHS-SS-biotin. It is recommended to prepare the biotinylation solution, quenching and lysis buffers before starting the Preparation of hippocampal slices.

15. After the recovery, keep the four hippocampal slices in the same Eppendorf tube (Figure 3F).
16. Wash the slices with 500 μL of ice-cold aCSF. Repeat twice.

Note: Handle the hippocampal slices with minimal agitation; allow the slices to settle to the bottom of the tube without moving it before proceeding.

⚠ CRITICAL: When removing aCSF from the tubes during the washes, exercise caution to ensure that none of the slices are aspirated into the pipette. First, use P1000 tips to remove aCSF from the top, then use P200 tips to remove the remaining aCSF near the slices at the bottom. Pipette the solution slowly to prevent tearing the slices.

17. Aspirate the remaining aCSF in the tube and add 1 mL of biotinylation solution (aCSF containing 0.5 mg/mL of Sulfo-NHS-SS-biotin). Incubate on ice for 1 h, swirling gently every 15 min.
18. Carefully remove the biotinylation solution and wash the slices with 500 μL of ice-cold aCSF. Repeat twice.
19. Carefully aspirate the remaining aCSF and add 1 mL of quenching buffer to each tube to remove any unbound Sulfo-NHS-SS-biotin. Incubate for 5 min.
20. Aspirate the quenching buffer and carefully wash the slices with 1 mL of ice-cold aCSF twice.

⚠ CRITICAL: This washing step is of utmost importance as it helps to remove all residual biotin from the solution. Failure to adequately wash can result in an increase in the number of cytoplasmic proteins in the biotinylated protein fraction.

21. Remove aCSF from the tubes and add 500 μL of ice-cold lysis buffer to each tube.
22. Sonicate the samples twice, each time for 5 s, with a 5-s rest in between. Use a probe sonicator (sonicator setting: 5 ms pulse, 70% amplitude) while keeping the tubes on ice.
23. Rotate the tubes (using an end-over-end mixer) containing the lysate at 4°C for 10 min.
24. Centrifuge the tubes containing the lysate for 30 min (microcentrifuge setting: 12,500 RPM, 4°C).
25. Transfer the supernatant to new 1.5 mL Eppendorf tubes and discard the pellet. Keep the tubes on ice. Troubleshooting 5.
26. Transfer 50 μL of supernatant to new 1.5 mL Eppendorf tubes and add 50 μL of 2× Laemmli buffer to each tube. Store the tubes in a freezer at −30°C until further analysis.

Note: These tubes contain the total protein fraction (cytoplasmic and surface proteins).

27. Add 100 μL of NeutraAvidin beads (no prior washing required) to the Eppendorf tubes containing the remaining supernatant. Rotate tubes at 4°C for 2 h or overnight.

Note: Make sure that an equal volume of beads is added to each tube. Agitate the bottle containing beads vigorously for 30 s. After adding the beads to the tubes, wait until they are precipitated and visually confirm that the quantity of beads is equal in all tubes. Failing to achieve an equal quantity of beads may affect the level of surface proteins being pulled down.

Note: Better results can be achieved with overnight incubation.

Washing NeutraAvidin beads

Timing: 1 h

Washing of NeutraAvidin beads with lysis buffer to remove unbound proteins.

28. Centrifuge the tubes containing the NeutraAvidin beads for 3 min (centrifuge setting: 2,500 RPM, 4°C).
29. Carefully remove the supernatant.
30. Add 500 μL of lysis buffer. Rotate for 5 min at 4°C. Troubleshooting 3.
31. Repeat steps 28 through to 30 three times.

⚠ CRITICAL: Be careful when removing lysis buffer from the tubes to ensure the beads at the bottom of the tube are not disturbed. These beads are bound to surface proteins and any disturbance could potentially affect the quantification of proteins in later steps.

32. Remove the lysis buffer, being as careful as possible to avoid disturbing the beads.
33. Add 50 μL of 2× Laemmli sample buffer to each tube.
34. Store in a freezer at −30°C until use.

Pause point: These tubes contain the biotinylated protein fraction (isolated surface proteins). Store in a freezer at −30°C until use.

Western blotting

Timing: 2 days

For subsequent Western blot analysis, prepare a 10% Polyacrylamide Tris-HCl gel. We use a standard Western blotting protocol[3,7,9] with a few modifications for biotinylated proteins.

35. Heat both the biotinylation and total protein fraction on a heating block at 70°C for 7 min.

Note: We recommend using 70°C for α5-$GABA_A$ receptors, as boiling membrane proteins in the loading buffer may lead to aggregation. It is important to note that other membrane proteins may require heating at different temperatures.

⚠ CRITICAL: Avoid loading beads into the wells. Spin down the samples briefly to ensure the beads are settled at the bottom of the tube. Carefully collect the sample without touching the beads inside the tube.

36. Load 10 μL of each protein fraction in the same gel for comparison by Western blotting.
37. Preferably run the total and biotinylated protein fraction in the same gel. This will help to easily compare both fractions and ensure the performance of the procedure using internal control proteins (β-actin) in later steps (Figure 4).

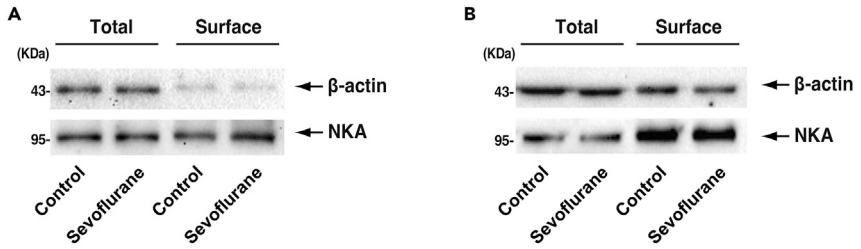

Figure 4. Comparison of two experiments showing different results after biotinylation

(A) Representative Western blot from a successful biotinylation procedure. An internal control protein, β-actin, is shown in the total protein fraction and only minimally detected in the surface protein fraction. Na/K-ATPase expression, a non-GABA receptor expressed in the surface membrane, is also shown in the total and surface protein fractions.

(B) An example of an unsuccessful biotinylation procedure is shown as excessive levels of β-actin in the surface protein fraction are detected.

Note: Proteins are separated via SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes are blocked with 5% skim milk in T-TBS.

38. Optimize and select primary antibodies whose molecular weights do not overlap. We use antibodies against Na/K-ATPase (Cell Signaling Technology, 3010) as a control for cell-surface proteins and an antibody for $GABA_A$ receptor α5 subunits (Thermos Fisher Scientific, MA5-27700) for quantification. Troubleshooting 4.

Note: Primary antibodies for the α5 subunit of $GABA_A$ receptors, Na/K-ATPase and β-actin are used at dilutions of 1:1000 and incubated overnight. Anti-rabbit or anti-mouse secondary antibodies are used at dilutions of 1:5000 and incubated for one hour.

EXPECTED OUTCOMES

This protocol allows the quantification of expression levels of both the total and the cell-surface subtypes of $GABA_A$ receptors in mouse hippocampal slices (total and biotinylated protein fractions, respectively). Using this protocol, we have shown that general anesthetic drugs, when administered *in vivo*, increase cell-surface expression of α5 subunits (Figure 5).

For more details see Wang et al.,[1] Zurek et al.,[2] and Yu et al.[3]

QUANTIFICATION AND STATISTICAL ANALYSIS

Blots were imaged using the ChemiDoc XRS+ system (BioRad) and quantified using Image Lab software 6.0 (BioRad). Levels of α5 subunits were normalized to their respective protein loading controls, which are Na/K-ATPase for surface protein. Blots containing surface proteins were probed for β-actin to determine the purity of the biotinylated proteins fraction. Data are presented as a percentage of the mean of the control group.

LIMITATIONS

Conventional biotinylation methods for labeling cell-surface proteins are commonly used when studying protein expression in neuronal cultures for *in vitro* experimental paradigms.[10,11] Here, we present a versatile protocol for studying changes in cell-surface levels of $GABA_A$ receptors in hippocampal slices after *in vivo* administration of an inhalational general anesthetic drug in mice.

A limitation of this protocol is that it does not provide information on the spatial distribution of $GABA_A$ receptors in the membrane. $GABA_A$ receptor subunits are mainly expressed in either the synaptic or extrasynaptic sites in neurons,[12,13] which cannot be distinguished using this protocol. To

Figure 5. *In vivo* administration of sevoflurane increases cell-surface expression of α5 subunits

(A) Sevoflurane was administrated to 8–9 weeks-old mice for 2 h. Control mice receive only supplemental oxygen and medical air for 20 min. After, 24 h later, hippocampal slices were collected and surface proteins were labeled with Sulfo-NHS-SS-biotin. The biotinylated cell-surface proteins were isolated by pull-down assay, and total and surface fractions were Western blotted.

(B) Levels of α5 subunits were normalized with Na/K-ATPase and expressed as a percentage of the control. N = 3 experiments. Data are shown as mean ± SD. Unpaired, 2-tailed Student's *t*-test; *p < 0.05.

overcome this limitation, complementary imaging techniques can be used to study the co-localization of $GABA_A$ receptors with synaptic markers.[13,14] Additionally, electrophysiological studies can be conducted to record changes in synaptic and tonic inhibitory currents, which can provide insights into the location of the $GABA_A$ receptor subtypes of interest. For example, α5 and δ subunit-containing $GABA_A$ receptors are mainly found extrasynaptically, where they generate a tonic inhibitory current that regulates neuronal excitability, and memory, and likely contribute to a variety of neurological disorders.[2,3,8]

Finally, this protocol studies all cell types that are present in the hippocampus such as neurons, astrocytes, and microglia. However, it does not provide information on the identity of cells that express $GABA_A$ receptors. Alternatively, *in vitro* astrocytes or neuronal cultures can be used to study specific neural cells, but these methods often fail to replicate the pharmacokinetics of anesthetic drugs. While using hippocampal slices after *in vivo* administration of anesthetic drugs is more physiologically relevant, it should be considered that the inability to identify specific neural cells expressing $GABA_A$ receptors is a limitation of this approach.

TROUBLESHOOTING

Problem 1

Inadequate anesthesia in animals (step 4 - step-by-step method details).

Potential solution

Verify that the desired concentration of sevoflurane set on the vaporizer is accurately being delivered by using a gas analyzer. Check the volume of sevoflurane in the vaporizer, and refill as necessary. Given that anesthetic potency varies between animal strains,[15] the concentration of sevoflurane may need to be modified to ensure the loss of righting reflex in the animal under investigation.

Problem 2

Variation in gas delivery from the anesthesia machine during the procedure (step 4 - step-by-step method details).

Potential solution

This issue is likely related to changes in gas flow caused by low gas pressure in the tanks. Check the pressure on the manometer and replace the tank if necessary. Blockages in the delivery tubing can also cause this issue. Ensure that the tubing is not twisted or blocked along the delivery line.

Problem 3

Presence of cytoplasmic proteins in biotinylated protein fraction during Western blotting (step 30 - step-by-step method details).

This is a recurrent problem during Western blotting. Although the detection of small amounts of cytoplasmic proteins is unavoidable (Figure 4A), an excessive presence of cytoplasmic control proteins (e.g., β-actin, tubulin or GAPDH) can sometimes be detected in the biotinylated protein fraction (Figure 4B). This contamination could be due to inadequate washing or insufficient time in the quenching buffer.

Potential solution

Optimize the time in the quenching buffer by increasing the incubation time or consider changing the quenching buffer (e.g., 100 mM NH_4Cl). Include a 5-min rotation after adding the quenching buffer. Carefully wash with abundant aCSF after quenching to completely remove residual biotin. An incomplete washout of biotin can result in biotin interacting with cytoplasmic proteins during the lysis steps of the protocol. Additionally, keep all reagents on ice and keep the tubes at lower temperatures during biotinylation, washing, and quenching steps. Use an ice bucket or incubate in a cold room during the process if necessary.

Problem 4

Detection of undesired bands or lack of detection of the primary antibody (step 38 - step-by-step method details).

When analyzing a Western blot, it is possible to detect an intense band in the total protein. This excess protein could be due to excessive BSA used in the quenching buffer, which can interfere with the detection of $GABA_A$ receptors with a molecular weight close to 60 kDa.

Potential solution

We recommend reducing the amount of BSA used when preparing the quenching buffer. Test a range from 2.0–5.0 mg/mL of BSA. Experimenters can also try other quenching buffers to reduce the presence of undesired bands around 60 kDa. Additionally, if the protein of interest is not detected, the amount of $GABA_A$ receptor subunit protein in the total protein fraction may be lower than in the biotinylated protein fraction due to the high concentration of surface protein pulled down. To increase detection levels of the protein of interest, increase the total protein volume when loading samples into the gel.

Problem 5

Levels of surface control proteins are not equal (step 25- step-by-step method details).

Potential solution

Include a Bicinchoninic acid Protein Assay (BCA) or any other method for protein quantification after collecting the supernatant. By quantifying the total proteins in this step, it will be possible to select an equal amount of protein to be used as input for the biotinylation.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Beverley A. Orser (beverley.orser@utoronto.ca).

Materials availability

This protocol did not generate new reagents.

Data and code availability

All data generated using this protocol will be shared by the lead contact upon request. This protocol has not generated any code.

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AUTHOR CONTRIBUTIONS

A.A., S.M.N., and S.K. wrote and all authors edited the manuscript.

DECLARATION OF INTERESTS

B.A.O. serves on the Board of Trustees of the International Anesthesia Research Society (San Francisco, CA, USA) and is the co-director of the Perioperative Brain Health Centre (Toronto, ON, Canada http://www.perioperativebrainhealth.com). She is a named inventor on a Canadian patent (2,852,978) and two US patents (9,517,265 and 10, 981, 954). The patents, which are held by the University of Toronto, are for new methods to prevent and treat delirium and persistent neurocognitive deficits after anesthesia and surgery and to treat mood disorders. B.A.O. also collaborates on clinical studies that are supported by in-kind software.

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