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Elucidating the Role of Lipid Rafts on G Protein-Coupled Receptor Function in the Mouse Kidney: An In Vivo Approach

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Abstract

Numerous G protein-coupled receptors (GPCRs) and GPCR-signaling molecules reside in lipid rafts and thus, are inherently regulated in these microdomains. However, the limitations of current methods to investigate lipid raft biology and GPCR activity in situ have hindered the complete understanding of the molecular underpinnings of GPCR trafficking and signaling, especially in the whole organism. This book chapter details an innovative in vivo approach to study the crucial role of lipid rafts on the workings of GPCRs in the mouse kidney. This protocol involves the use of a modified mini osmotic pump to deliver an agent that selectively disrupts the lipid raft in the kidney.

Keywords

α -Cyclodextrin; Kidney; Lipid raft; Methyl- β -cyclodextrin; Mini osmotic pump; Mouse

1 Introduction

G protein-coupled receptors (GPCRs) constitute the largest family of cell surface receptors in the human genome. These receptors mediate a gamut of important cell signaling pathways, and, as such, are the targets of approximately 35% of the current drugs in clinical use [1]. Most of these receptors, including their cognate heterotrimeric G proteins and various accessory signaling molecules, preferentially cosegregate in highly organized plasma

membrane microdomains called lipid rafts. These are membrane islands that abound with phospholipids, glycosphingolipids, and cholesterol and serve as crucial signaling platforms for the organization, interaction, trafficking, and signaling of biomolecules involved in various biological processes.

The complete understanding of the intricacies of GPCR biology and how GPCR residency in lipid raft influences receptor activity is hampered by the lack of standardized methodology to study lipid rafts and the inherent drawbacks of current methods. One such drawback is the almost exclusive reliance on the use of mammalian cellular models, whether in the form of cell lines or primary cells grown in conventional 2D dishes or in 3D via Transwell® inserts. In vitro studies provide a rapid, relatively inexpensive, and highly controllable approach to investigate the molecular underpinnings of GPCR activity. Cell lines represent a homogeneous population of identical cells that are immortalized using viral genes or telomerases to perpetuate their continued survival. However, cultured cells are isolated from their natural environment and thus are removed from the interaction and compensatory mechanisms that are otherwise available to similar cells in their natural milieu and that may be critical to the hypothesis being tested [2]. As such, observations based on in vitro models may not always accurately reflect or translate into what is happening in the whole organism. For example, evaluating the molecular mechanisms involved in blood pressure control cannot be adequately carried out in cell models alone. The functional consequences of protein dysfunction on blood pressure and other physiologic parameters, such as water and electrolyte balance, sexual dimorphism, and biokinetics, can only be evaluated in whole organisms such as laboratory animal models.

The mouse is an excellent extension of in vitro studies on physiology, in general, and of lipid rafts, in particular. In addition to their relatively short gestation period, fecundity, short lifespan, and low-cost husbandry, the mouse has both a well-characterized biology and a well-developed system for genetic manipulation [3, 4]. Approximately 99% of the genes identified in the human genome are present in the mouse. In our case, mice have been shown to develop hypertension in response to the manipulation of genes that, in humans, are associated with elevated blood pressure [5-7]. Thus, a more comprehensive investigation of the importance of lipid raft in GPCR function warrants the use of animal models [8]. Here, we describe a proprietary strategy and discuss in detail the steps involved in using a mini osmotic pump to deliver an agent that disrupts the lipid raft microdomains only in the kidney. We targeted the kidney because it is the primary organ involved in the regulation of blood pressure and sodium excretion. Our innovative strategy can be modified and applied to other organs of interest.

Note: All animal studies were approved by the institutional Animal Care and Use Committee (IACUC) and were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALACi)-accredited facility.

2 Materials

2.1 Animals and Animal Husbandry

1. Laboratory mice from an approved vendor.

2. Cage (e.g., Tecniplast USA, Inc.) and accessories for water and food, and beddings.
3. Potable water and mouse chow (e.g., Envigo [Teklad] Laboratories).
4. Mouse ear tags (e.g., National Brand and Tag Co.).

2.2 Preparation of Reagents and Mini Osmotic Pump

1. Methyl- β -cyclodextrin (β -MCD).
2. Sodium chloride, 0.9% (w/v) Aqueous, Isotonic Saline (e.g., ThermoFisher Scientific™).
3. *TransIT*®-EE Delivery Solution or *TransIT*®-QR Delivery Solution (e.g., Mirus Bio LLC).
4. α -cyclodextrin (α -CD).
5. Mini osmotic pump (100 μ l capacity for 7-day infusion) with catheter (e.g., ALZET®).

2.3 Anesthesia, Analgesia, and Surgical Preparation

1. Buprenorphine (e.g., Reckitt Benckiser, Inc.).
2. Pentobarbital sodium (e.g., Oak Pharmaceuticals, Inc.).
3. 70% ethyl alcohol.
4. Tuberculin syringes.
5. Electric clipper or depilatory cream.
6. 3 M Micropore tape.
7. Circulating Water Heating pad (e.g., Stryker or Gaymar).
8. Povidone®-iodine scrub.

2.4 Laparotomy and Implantation of Mini Osmotic Pump

1. 3 M Steri-Drape.
2. Surgical scissors.
3. Surgical retractors.
4. Dumont micro-blunted forceps.
5. Tuberculin syringe.
6. Mouse jugular catheter (e.g., ALZET®).
7. Surgical glue (e.g., 3 M Vetbond™ Tissue Adhesive).
8. 4-0 Ethilon® suture.

2.5 Wound Closure and Recovery

1. 4-0 Vicryl® suture.
2. 5-0 Ethilon® suture.
3. Needle holder.
4. Staple applicator and staples.
5. Wessels Induction/Warming Chamber (e.g., Vivarium Electronics).

2.6 Post-surgical Analgesia and Monitoring

1. Buprenorphine (e.g., Reckitt Benckiser, Inc.).
2. Surgical scissors or staple remover.

2.7 Euthanasia and Tissue Fixation

1. Pentobarbital sodium (e.g., Oak Pharmaceuticals, Inc.).
2. Tuberculin syringe with G26 or G27 needle.
3. Sterile normal saline solution.
4. Isopentane (2-Methylbutane).
5. 4% Paraformaldehyde.
6. Dry ice.

2.8 Verification of Lipid Raft Integrity

1. 10% bovine serum albumin.
2. Cholera Toxin Subunit B (CTxB) with Alexa Fluor® 488 (e.g., ThermoFisher Scientific™).
3. *Lotus tetragonolobus* lectin (LTL), biotinylated (e.g., Vector Laboratories).
4. Streptavidin with Alexa Fluor® 647 (e.g., ThermoFisher Scientific™).
5. Fluoro-Gel with Anti-Fading Mounting Medium.
6. Slide holder.
7. Confocal microscope.

2.9 Other Considerations

2.9.1 Ventilation

1. Polyethylene (PE-90) tube.
2. 4-0 Ethilon® suture.
3. Needle holder.
4. SomnoSuite Mouse Anesthesia System with MouseVent Automatic Ventilator (e.g., Kent Scientific Corporation).

2.9.2 Femoral Artery/Vein Catheterization

1. Scalpel.
2. PE-50 tube.
3. Blood Pressure Analyzer (e.g., Cardiomax II).
4. 4-0 Ethilon® suture.
5. Venous clip.
6. Iris scissors.
7. 3 M Micropore Tape.
8. 11-0 PROLENE® suture.
9. 5-0 surgical silk suture.
10. Needle holder.

2.9.3 Urinary Bladder Catheterization (Nonsurvival Surgery)

1. Surgical scissors.
2. PE-90 tube.
3. 4-0 Ethilon® suture.
4. 3 M Surgical staples.

2.9.4 Preparation of Surgical Instruments

1. Autoclave.
2. 5.75% o-phthalaldehyde.
3. Sterile normal saline solution.
4. Glass bead sterilizer (e.g., Harvard Apparatus).

2.9.5 Use of Metabolic Cages

1. Metabolic cages (e.g., Harvard Apparatus).
2. Potable water and mouse chow (e.g., Envigo [Teklad] Laboratories).
3. Blood Pressure Analyzer (e.g., Cardiomax II).
4. Weighing scale.
5. Soap.

3 Methods

3.1 Animals and Animal Husbandry

1. The choice of the mouse strain and age to be used depends on the objective of the study. Sex- and age-matched controls must be included in the study.

2. House the mouse in ventilated caging system in an AAALACi-accredited animal holding room (*see* Note 1).
3. Provide the mouse with food (*see* Note 2) and fresh potable water ad libitum.
4. On the day of the experiment, transport the mouse to the surgery room. The mouse is weighed and rested for 1 h prior to surgery.

3.2 Preparation of Reagents and Mini Osmotic Pump

1. Reconstitute β -MCD with sterile water (50 mg/ml) with stirring for 30 min at room temperature (*see* Note 3). Other strategies that can be used for in vivo lipid raft analysis are summarized in Table 1.
2. Prepare a working solution of β -MCD (40 mg/kg/day for 7 days in 100 μ l of sterile normal saline solution [NSS]). Alternatively, an in vivo transfection reagent may be used (e.g., *TransIT*[®]-EE Delivery Solution or the *TransIT*[®]-QR Delivery Solution, which are originally used for DNA or siRNA transfection) [19, 20]. Use α -CD and/or sterile NSS as negative control (*see* Note 4).
3. Using a tuberculin syringe fitted with a G27 blunt needle load the β -MCD working solution into a 7-day mini osmotic pump (with a capacity of 100 μ l and flow rate of 0.5 μ l/h. Fit the pump with a modified polyethylene delivery tubing (Fig. 1). Prime the pumps by placing the prefilled pumps in sterile 0.9% saline or PBS at 37 °C for at least 4–6 h (preferably overnight) prior to implantation.

3.3 Anesthesia, Analgesia, and Surgical Preparation

1. One hour prior to the induction of anesthesia, administer buprenorphine via subcutaneous route. Clean the injection sites with 70% ethanol prior to injection.
2. Induce anesthesia using pentobarbital sodium given via intraperitoneal injection. Other anesthetic agents that may be used are listed in Table 2.
3. Shave the abdomen and inner thigh areas using an electric shaver or depilatory cream to prevent contamination. This is performed away from the surgical table. The shaved area should be 2–3 $\times$ the size of the anticipated surgical field. It is best to remove the hair immediately prior to surgery.

¹The health and welfare of the mice [23] should be monitored carefully by a licensed veterinarian. Sentinel mice should be used to monitor for pathogenic organisms in the colony. Mice, rats, and hamsters should be socially housed, as a standard practice. Single housing may sometimes be necessary for scientific, medical, or behavioral reasons. When this occurs, the singly housed animal should be provided with additional inanimate enrichment (e.g., nesting materials, housing, or exploratory manipulanda) to help offset the lack of social housing.

²The mice are normally fed with regular rodent laboratory chow, which is formulated for normal life cycle nutrition. The mouse diet can be custom-made with regard to chemical composition, depending on the objective of the experiments. For example, mice can be given low salt (<0.04% NaCl), normal salt (0.80% NaCl), and high salt (4–8% NaCl) diet [24] to test for salt sensitivity. The concentration of other minerals (e.g., potassium, calcium, and magnesium) should be taken into consideration.

³Sonication with cooling may be also employed. Solutions may be stored for several months at 4 °C. β -MCD powder should be stored tightly sealed at room temperature.

⁴ α -CD (40 mg/kg/day for 7 days in 100 μ l of sterile PBS) does not bind to cholesterol and thus does not disrupt the lipid raft and may be used as a control for β -MCD [25].

4. Tape down the mouse in a supine position over a thermostatically controlled warm water-circulating pad to maintain the mouse's rectal temperature at ~37.5 °C.
5. Disinfect the shaved area with alternate swabs of 70% ethyl alcohol and povidone-iodine (Betadine®) scrubs 3×. Work from clean to dirty, that is, scrub starting at the center of the surgical site and then slowly work toward the periphery of the surgical field, making sure not to move backward.
6. Place the mouse in the supine position by taping the legs down on the heat pad to maintain the rectal temperature between 36.5 and 38 °C during surgery.
7. Monitor the following parameters every 5 min during the entire surgical procedure:
 - a. Respiratory rate
 - b. Response to noxious stimulus (i.e., lack of withdrawal response to deep toe and tail pinch or lack of blink reflex to palpebral stimulation).
 - c. Lack of spontaneous movement.

3.4 Laparotomy and Implantation of Mini Osmotic Pump

1. Place a sterile drape or dressing over the animal. Drapes may be paper, cloth, or plastic products with an adequate central cutout for access to the surgical site (see Note 6).
2. Make a midline incision using a scalpel or surgical scissors along the *linea alba* to expose the abdominal cavity.
3. Retract the abdominal muscles to the side using retractors and carefully isolate one of the kidneys (Fig. 2).
4. Using a pair of Dumont forceps (or any pair of atraumatic forceps), delicately lift the renal capsule away from the kidney.
5. Puncture the lifted renal capsule using a tuberculin syringe fitted with a G35 needle, filled with warm normal saline solution. Slowly infuse 100 µl of warm (37 °C) normal saline solution underneath the renal capsule to separate completely the capsule from the kidney.
6. Quickly withdraw the needle from the capsule and replace the needle with the pump delivery tubing connected to the mini osmotic pump. Make sure that the end of the pump delivery tubing is positioned securely in the subcapsular space and held in place using a drop of surgical glue at the puncture site (Fig. 3).

⁶.Using appropriate intraoperative technique is crucial for a good surgical outcome, including aseptic techniques and conscientious tissue-handling during surgery. Aseptic techniques include (a) donning nonsterile surgical items including face-masks, hairnets, and shoe covers prior to scrubbing, (b) keeping hands within the sterile field, (c) handling the instruments with utmost care to maintain sterility, and (d) keeping the surgical drapes clean, dry, and secured in place using towel clamps, sutures, or adhesives to provide appropriate protection. Moreover, limited and gentle handling of tissues using atraumatic forceps is essential to prevent infection, delayed healing, and dehiscence of the incision site.

7. Position the mini osmotic pump at the contralateral side of the abdominal cavity and secure the body of the mini osmotic pump by suturing (using a 4-0 Ethilon®) onto the dorsal abdominal wall to prevent dislodgement.
8. Reposition the intestines and omentum back to their normal location.

3.5 Wound Closure and Recovery

1. Close the abdomen using an absorbable 4-0 Vicryl® suture for the abdominal muscles and 5-0 Ethilon® suture for the skin, respectively (Fig. 4).
2. Remove the tapes from the extremities and maintain the mouse in the prone position on the thermostatically controlled warm (37 °C) water-circulating pad.
3. Once ambulatory, that is, the mouse exhibits spontaneous movement and can maintain sternal recumbency, carefully transfer the mouse into a dedicated Wessels induction/warming chamber maintained at 37 °C, or alternatively over a clean cage with paper beddings set on a circulating water heating pad heated to 37 °C, to help maintain the proper body temperature of the mouse for 24 h (see Note 7).
4. Transfer the mouse to the animal holding room and perform adequate post-surgical monitoring.

3.6 Post-surgical Monitoring

1. Monitor daily the health and general welfare of the mouse. Assess the health of the mouse visually twice daily for the first 72 h post-operatively and then decrease to once daily. Parameters include:
 - a. General attitude/activity level
 - b. Redness, swelling, and discharge at the incision site
 - c. Appetite and amount of feces in the cage
 - d. Change in body weight taken daily and body condition score. A loss of 20% of the post-operative body weight indicates a need for euthanasia (*vide infra*)
 - e. Observations of clinical signs
2. Administer buprenorphine for postoperative analgesia every 6–12 h for 36 h. Continued pain is manifested as (see Note 8):
 - a. Lethargy and nonambulation
 - b. Failure to access feed and water (no feces in the cage)

⁷In the absence of a Wessels Induction/Warming Chamber, ensure to protect the animals from hypothermia during post-surgical recovery by not placing them on naked metal surfaces. The mouse cage should be covered such that half of the cage rests on a thermostatically controlled circulating water heating blanket to conserve body temperature and allow the mouse to thermoregulate.

⁸If pain is evident after 72 h, as characterized by failure to feed, hunched appearance, and ruffled fur, withdraw the animal from the study and euthanize by an overdose of Veterinary Euthanasia Solution, followed by cervical dislocation or by exsanguination, followed by bilateral thoracotomy to confirm death.

- c. Hunched appearance
 - d. Vocalization
 - e. Failure to groom (ruffled fur, piloerection)
 - f. Excessive licking/scratching
 - g. Self-mutilation
 - h. Other signs (twitching, tremors, convulsions, weakness, hyperventilation, open-mouth breathing, and excessive nasal/ocular discharge)
3. For survival surgery, remove the skin staples or sutures at 8–10 days post-operatively.

3.7 Euthanasia

1. Perform euthanasia via the use of the veterinary euthanasia solution (e.g., pentobarbital sodium, 100 mg/kg body weight, via intraperitoneal or intravenous route), followed by bilateral thoracotomy or cervical dislocation to confirm death (*see* Note 9).
2. Extract the kidneys and other organs and flash-freeze in liquid nitrogen or isopentane in dry ice or fix with paraformaldehyde (*see* Note 10).

3.8 Verification of Lipid Raft Integrity

Visualization of the lipid raft microdomains is the easiest approach to determine the success of the *in vivo* lipid raft disruption in the kidney. There are now commercially available kits that have been developed for labeling the lipid rafts using the Cholera toxin subunit B (CTxB) that is tagged with fluorophores. CTxB binds to the pentasaccharide chain of ganglioside GM₁, which selectively partitions into lipid rafts [21, 22]. Alternatively, antibodies that specifically target the lipid raft protein markers, such as caveolin-1, caveolin-3, and flotillin-1, may be used. However, isolating the lipid rafts from the nonlipid rafts through sucrose gradient ultracentrifugation and fractionation followed by immunoblotting for marker proteins to demonstrate their distribution should be performed to verify lipid raft integrity.

1. Cut a small piece of the fixed kidney and prepare tissue sections using standard protocols (*see* Note 11).

⁹Alternatively, euthanasia may be performed by asphyxiation using CO₂ dispensed from a pressure-regulated cylinder, followed by cervical dislocation, or by exsanguination followed by bilateral thoracotomy. Performing two methods of euthanasia is recommended. Confirmation of death is made by the observation of cessation of heart beat and respiration accompanied by fixed and dilated pupils and loss of corneal reflex.

¹⁰When the best tissue morphology is required, the mouse should be deeply anesthetized, as previously described (Subheading 3.4), and subjected to cardiac perfusion with saline, the vena cava is severed to drain the infusate, followed by a 4% paraformaldehyde flush. The harvested organs must be flash-frozen using liquid nitrogen or isopentane in a beaker placed in dry ice, or immersed in an adequate volume (e.g., fixative volume is ~20× that of the organ on a weight per volume or use 2 ml per 100 mg of organ) of 4% paraformaldehyde. Organs should be fixed for a minimum of 48 h at room temperature. Store the organs at –80 °C until subsequent experimentation. If plasma or serum is required, the mouse has to be anesthetized first, as previously described (Subheading 3.4), before blood is extracted via the femoral artery or through direct cardiac puncture. Thereafter, perform thoracotomy to confirm death.

2. Block the tissue with 1% bovine serum albumin for 30 min at room temperature.
3. Immunostain the tissue for CTxB conjugated with Alexa Fluor[®] 488 for 1 h at 37 °C. To visualize the renal proximal tubules, use the LTL conjugated with Alexa Fluor[®] 647 for 1 h at 37 °C to target the lectin-rich brush border and plasma membranes of the renal proximal tubules. Use DAPI to visualize the nuclei.
4. Apply a mounting medium over the tissue and gently mount a cover slip over the tissue specimen. Air-dry at room temperature for a few minutes or at 4 °C overnight.
5. Image the lipid raft, brush border of the proximal tubule, and nuclei in the various segments of the nephron, sequentially in separate channels to avoid bleed-through using a confocal microscope (Fig. 5).

3.9 Other Considerations

3.9.1 Ventilation

1. For proper ventilation and prevention of fluid buildup during long procedures, insert an endotracheal tube. Secure the tube via a purse string ligature using a 4-0 Ethilon[®] suture. For a surgical procedure that lasts about 30 min, ventilation will be dependent on spontaneous breathing.
2. In the event of a surgical procedure of longer duration, assisted ventilation should be performed, for example, using the SomnoSuite mouse anesthesia system with MouseVent automatic ventilator. The machine is designed to deliver a gas anesthetic (i.e., isoflurane). The machine is fully automatic and will adjust for the maintenance of anesthesia, ventilation rate, tidal volume, and other parameters.

3.9.2 Femoral Artery/Vein Catheterization

1. After attaining the correct anesthetic plane, make an incision at the inguinal area and isolate the femoral artery. Cannulate the femoral artery with a PE-50 heated and stretched to 180 µm (inner diameter) attached to Cardiomax II Blood Pressure Analyzer for blood pressure determination (Fig. 6) (*see* Note 12).
2. Cannulate the femoral vein for fluid replacement of blood loss, using saline solution, if needed. Isolate the vein and place a distal ligature using a 4-0 Ethilon[®] suture. Place a venous clip about 1–2 cm proximal to the ligature.

¹¹Tissue section is prepared following a series of steps: embedding in paraffin blocks, sectioning the tissue (3–4 µm thick) using a microtome, and deparaffinization. In paraformaldehyde-fixed tissues, methylene bridges form during fixation, which crosslink proteins, and therefore, mask antigenic sites. To enhance the visualization of proteins, perform an antigen retrieval step, which may be either enzymatic or heat-mediated method. Both serve to break the methylene bridges and hence, expose the antigenic sites for the antibodies to bind. The enzymatic method is a much gentler process than the heat-mediated method and is best suited to more sensitive tissues. However, the enzymatic method tends to take much longer and is more technically demanding. Frozen tissue sections do not need an antigen retrieval step.

¹²Blood pressure may also be monitored via radio-telemetry [26]. This is preferred to blood pressure measurement under anesthesia because it allows continuous, pain-free and restraint-free, and real-time monitoring of blood pressure in conscious mice, and hence, reflects more physiological readings. Blood pressure measured under pentobarbital anesthesia is generally lower than telemetry readings [27-30].

3. Using a pair of iris scissors, make a small incision between the clip and ligature and gently insert a PE-50, with one end cut at a 45° angle.
4. Tie another ligature around the vessel and the inserted cannula in the area next to the insertion site to secure the cannula in place.
5. Remove the venous clip.
6. Secure the exposed part of the cannula by taping it into the surgical board.
7. Close the skin using surgical staples to prevent insensible fluid loss from evaporation.
8. At the end of the experiment, remove the catheter and repair the femoral artery using an 11-0 PROLENE® suture. Close the incision using 5-0 surgical silk suture.

3.9.3 Urinary Bladder Catheterization (Nonsurvival Surgery)

1. Using a pair of surgical scissors, make a small midline abdominal incision to expose the urinary bladder.
2. Perform a cystostomy by making a small incision on the dome of the urinary bladder.
3. Insert a flanged-ended PE-90 catheter and secure with a purse string 4-0 Ethilon® suture. This is for urine sample collection.
4. Close the skin using surgical staples to prevent insensible fluid loss.

3.9.4 Preparation of Surgical Instruments

1. Steam-autoclave all surgical instruments at 122 °C or cold sterilize (particularly sharp instruments) by soaking in 5.75% o-phthalaldehyde for 5 min at 50 °C.
2. Rinse the chemically soaked instruments in sterile saline prior to use to ensure that no chemical comes in contact with the animal tissues.
3. If the same set of instruments is used in several surgeries (serial surgery), maintain sterility between animals through the use of a glass bead sterilizer. Briefly, remove all organic debris with sterile normal saline-moistened gauze and place the instrument tips in a bead sterilizer for 10–20 s. Then place the instrument on a sterile field to cool for at least 20 min prior to use for the next mouse.

3.9.5 Use of Metabolic Cages

1. Prepare a single-mouse metabolic cage with premeasured water and food (*see* Note 13).

¹³The metabolic cage is designed to house an individual mouse for a 24-h period for the purpose of studying the animal's metabolic function or effects of an intervention on the animal's metabolism. These include, but are not limited to, daily food and water intake, effects of different drug therapies, urinary protein excretion, and renal function studies. The metabolic cage is specifically designed to separate urine from feces for analysis. The metabolic cage should be housed in a climate-controlled room with limited access and lights that are on a timer to provide the appropriate light–dark cycle (circadian rhythm). The mouse should be studied in metabolic

2. Place a preweighed mouse into the metabolic cage (*see* Note 14).
3. After 24 h in the metabolic cage, weigh the mouse and transfer into its regular cage.
4. Measure the remaining water and food. Collect and measure the mouse feces and urine samples, as well.
5. Disassemble and clean the metabolic cages between use with mild soap and water. Air-dry the components and reassemble prior to next use. No chemicals should be used to clean the metabolic cage.

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cages at least twice: first to measure the baseline values of the parameters of interest, and second to measure the changes in the parameters after/during treatment or intervention (including vehicle as negative control). The mouse should be transferred back to its regular housing between the first and second metabolic cage studies for at least 3 days.

¹⁴There is no need to acclimatize the mouse prior to the 24-h metabolic cage collection phase [31]. However, acclimatization of the mouse may be required for an extended period in the metabolic cage; for example, mice in metabolic cages for 3 weeks have increased hypothalamic–pituitary–adrenal axis activity, oxidative stress, and overall metabolism. Thus, caution must be exercised in the interpretation of data obtained from mice housed in metabolic cages for a long period of time, as their condition may not be representative of a normal physiology, in this regard [31].

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Fig. 1. Mini osmotic pump. The mini osmotic pump is fitted with a modified polyethylene mouse jugular catheter

Fig. 2.
Exposure of the kidney. The abdominal muscle flap is retracted to expose the kidney gently

Fig. 3. Insertion of the catheter connected to the mini osmotic pump into the kidney. The modified catheter tip attached to the mini osmotic pump is gently inserted into the subcapsular space of the kidney. *The drape was removed to expose the entire surgical field when the photo was taken*

Fig. 4. Closure of laparotomy. The laparotomy is closed using a simple continuous suture technique. *The suture is tightened at the end of the procedure before the final knot is made*

Fig. 5.

Disruption of lipid rafts in the kidneys of C57Bl/6 J mice. Adult (8–10 week) male mice on normal salt (0.8% NaCl) diet were uninephrectomized prior to a 7-day renal subcapsular minipump infusion of the cholesterol depletor β -MCD to disrupt the lipid raft in the remaining kidney. α -CD and vehicle were used as negative controls. The mice were sacrificed, and the kidneys were flash frozen in isopentane, fixed with 4% paraformaldehyde, sectioned, and immunostained. The brush border (using LTL) and lipid raft (using CTxB) were visualized, via confocal microscopy, using a Carl Zeiss LSM 510 META with $\times 63/1.4$ NA oil-immersion objective and processed using Zeiss 510 META with Physiology 3.5 and Multiple Time Series 3.5 software. $630\times$ magnification, scale bar = 20 μm

Fig. 6. Femoral artery cannulation. In some cases, the femoral artery needs to be catheterized for blood pressure measurement and monitoring

Table 1**Other strategies to target the lipid raft in vivo**

Strategy	Comments
High-fat diet	C57BL/6J mice fed with high fat diet (42% fat, 4 weeks vs. control diet with 4% kJ of fat) led to the loss of lipid raft among hematopoietic stem cells and early progenitor cells, but not in more mature progenitor cells [9]. High saturated fat (60% kcal from lard, 12 weeks) or high-fat diet with 50:50 lard and n-3 polyunsaturated fatty acid-enriched menhaden oil, but not normal fat (low fat, 13% kcal fat) shifted the endothelial nitric oxide synthase to nonlipid rafts in wild-type C57BL/6J mouse aorta but remained in lipid rafts in <i>Cav-1</i> knockout mouse aorta [10].
Methyl- β -cyclodextrin (β -MCD)	Acute renal interstitial infusion of β -MCD (200 μ g/kg/min) in Sprague-Dawley rats blocked the fenoldopam-induced natriuresis, while long-term (3 days) lipid raft disruption via continuous renal interstitial infusion of β -MCD (80 μ g/kg/min) decreased renal cortical caveolin-1 expression and increased blood pressure [11].
2-Hydroxypropyl- β -cyclodextrin (HP- β -CD)	Seven-week-old male C57BL/6J mice were administered with 20 μ g ovalbumin with or without 3 mg HP- β -CD (a promoter of lipid raft formation) through a hind footpad. HP- β -CD induced dendritic cell maturation and activation, which was prevented by treatment with filipin [12].
Ursodeoxycholic acid (UDCA)	UDCA (a membrane stabilizer; 0.5 μ M for 1 h) partially reduced ethanol- or EPA-induced injuries in the liver of Zebrafish larvae [13].
Pravastatin	Pravastatin (0.5 mM, a lipid raft disrupter which acts through inhibition of cholesterol synthesis) protected the liver of Zebrafish larvae against both ethanol- and EPA-induced injury [13].
Sodium pentobarbital	Pentobarbital (50 mg/kg, 15 min, intraperitoneal) treatment in male Wistar adult rats led to a reduction of the total protein associated to lipid rafts, with a higher reduction of the NMDA receptor compared with the GABA _A receptor [14].
α -Synuclein	Perfusion of α -synuclein (10 μ M in an artificial cerebrospinal fluid solution at the rate of 0.25 μ l/min) into the striatum reduced the amount of plasma membrane cholesterol, altered the partitioning of Cav2.2 channels, and resulted in an acute release of dopamine [15].
Docosahexaenoic acid (DHA)	In male C57BL/6J mice fed for 4 weeks with a polyunsaturated fatty acid docosahexaenoic acid-enriched diet or a control diet, cholesterol shifted from raft to nonraft domains, accompanied by a decrease in γ -secretase activity without affecting presenilin1 protein levels in the brain [16].
Ischemia/reperfusion of the intestines	In adult male Wistar rats, ischemia (occlusion of the superior mesenteric artery for 60 min), followed by reperfusion resulted in disruption of tight junction proteins and redistribution of the lipid raft marker caveolin-1, relocation of claudins 1, 3, and 5, from detergent-resistant to detergent soluble fractions, and an increase in intestinal permeability [17].
n-3 polyunsaturated fatty acids (PUFA)	n-3 polyunsaturated fatty acid (PUFA) feeding in mice (vs. n-6 PUFA, 2 weeks) altered colonic caveolae microenvironment by increasing phospholipid n-3 fatty acyl content and reducing both cholesterol (by 46%) and caveolin-1 (by 53%), without altering total cellular levels, and decreased the localization of caveolae-resident signaling proteins GTPase H-Ras and endothelial nitric oxide synthase in colonic caveolae by 45 and 56%, respectively [18].

Table 2Anesthetic or analgesic agents used for mice (*see* Note 5)

Drug	Dose and rate	Route	Frequency
Ketamine–Xylazine	80–100 mg/kg 8–10 mg/kg	Intraperitoneal	Once
Isoflurane	3–5% (induction) 1–5% (maintenance)	Inhalation	Continuous
Buprenorphine	0.05–0.10 mg/kg BW	Subcutaneous or intramuscular	Every 6–12 h or as needed
Pentobarbital sodium	40–60 mg/kg	Intraperitoneal or intravenous	Once or continuous

⁵.Pentobarbital sodium is the drug of choice when performing blood pressure measurements. If ketamine–xylazine is used and additional doses are needed, the first repeat dose will be ketamine (30–50 mg/kg body weight) only since xylazine has a much longer duration of action than ketamine. Subsequent additional doses would be of both ketamine (20–50 mg/kg body weight) and xylazine (2–5 mg/kg body weight). If isoflurane is used, it should be dispensed via precision vaporizer and all waste gases should be scavenged with an activated charcoal filter.