Monosynaptic circuit mapping

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1 Works for me
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ABSTRACT

Investigate the preganglionic neurons in the intermediolaterol nucleus (IML) that innervate the postganglionic neurons innervating interscapular brown adipose tissue (iBAT) and inguinal white adipose tissue (iWAT).

We have used Adeno-associated virus (AAV) for monosynaptic retrograde tracing and Pseudorabies virus (PRV) for transsynaptic retrograde tracing. However, this experiment is monosynaptic circuit mapping where we combine monosynaptic retrograde tracing and transsynaptic retrograde tracing to only identify the neurons that directly innervate the sympathetic nervous system.

In this experiment, we injected 2 new viruses: 1) AAV6-FLEX-TVA-P2A-eGFP-2A-oG) and 2) G-Deleted Rabies-mCherry in the iBAT in two separate minor survival surgeries; we aimed to investigate which preganglionic neurons innervate the postganglionic neurons that innervate the interscapular brown adipose tissue. This was done by rabies guided monosynaptic circuit mapping, which labels exclusively monosynaptic input neurons.

We injected one cre-dependent helper AAV that expresses TVA (= receptor for avian sarcoma leucosis virus glycoprotein EnvA) and oG (optimized glycoprotein) AAV6-FLEX-TVA-P2A-eGFP-2A-oG) into the iBAT of cre driver mice (TH-ires-cre). After sufficient viral expression (4 weeks) mice received another iBAT injection with the modified rabies virus G-Deleted Rabies-mCherry that lacks glycoprotein gene (commercially available from Salk Institute GT3 Core) and thus cannot be further propagated into upstream neurons in the absence of TVA and oG expression. TVA and oG expression are required for rabies propagation and infection of upstream neurons and thus retrograde and transsynaptic transport of the mCherry labeled EnvA would only occur in upstream neurons with monosynaptic inputs. Primary infected neurons would be co-labeled with green TVA eGFP and red EnvA mCherry, while monosynaptic input neurons would only show red EnvA mCherry expression.

The 1st viral injections were performed in BSL1, and the 2nd viral injections were performed in BSL2 and all animals were then maintained in BSL2 housing until euthanasia followed by perfusion (10 days post-injection of the 2nd virus).

Mouse model: TH-ires-cre
Injection site: iBAT
Mouse numbers: 2 males and 2 females.
The injection dose for the 1st virus: around (5.92E^10 GC/ml)*12.5 ul = 7.4E^8 GC, and the volume would be 12.5 ul.
The injection dose for the 2nd virus was (4.89E^7 GC/ml)*12.5 ul = 6.1E^5 GC, and the volume would be around 12.5 ul.

We monitor the body weight of mice every day as we consider an excess weight loss (>20%) as a sign of sickness due to the viral infection. In this case, we would perfuse the animals as soon as we detect the loss of body weight. We do not expect the mice to die throughout the experiment, but only expect the IML labeling. We do the weight monitor because when we do similar experiments in the mice's CNS, we monitor their weight every day.

ATTACHMENTS

2017 Rabies Letter.doc Viruses.docx MSDS_AAV_2013, UPenn.pdf

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Jun 12, 2019	Rui Zhang	Pennington Biomedical Research Center, Louisiana State University
Dec 12, 2019	Clara Huesing	Pennington Biomedical Research Center

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GUIDELINES

The result from this protocol is not positive (This monosynaptic circuit mapping protocol didn't work for us).

MATERIALS TEXT

Supplies:

- BSL1 and BSL2 surgery room and animal housing
- Surgery cards and surgery record
- Personal protective equipment (PPE): coverall, bonnet, gloves mask, eye protection
- Eye lubricant
- Timer
- Biohazard stickers
- Bupivacaine/lidocaine
- 500nl blunt syringe with pulled glass pipette (Alternative is 1ul beveled Hamilton syringe)
- Aluminum foil (for easy cleanup of the virus work area)
- Kimtech paper
- Biohazard waste container for virus waste
- Sterile towels
- Surgery tools (scissors, hemostats, tissue forceps, teethed forceps)
- Sterile wound clip remover
- Sterile wound clips
- Sterile cotton swabs and gauze
- Isoflurane vaporizer, oxygen tanks, and anesthesia induction chamber
- Glass bead sterilizer
- Heating mats for surgery and recovery
- Virus stock
- Bupivacaine/lidocaine
- Carprofen
- Sterile Saline
- Novalsan
- 70% isopropyl rubbing alcohol
- 70% Ethanol
- Sterile H_2O
- 0.5ml syringes (for bupivacaine/lidocaine and carprofen)
- 3ml syringes (for saline)
- 70% ethanol spray

Mice:
- 2 males and 2 females TH-ires-cre mice were used.

Viruses:
- AAV6-FLEX-TVA-P2A-eGFP-2A-oG (5.92E+10 GC/mL)and G-Deleted Rabies-mCherry (4.89E+7 GC/mL) ordered from the Salk Institute GT3 Core.

SAFETY WARNINGS

Institutional requirements before you start:
- Approval of Institutional Biosafety Committee (IBC) to work with the viruses AAV6-FLEX-TVA-P2A-eGFP-2A-oG and G-Deleted Rabies-mCherry
- Approval of Institutional Animal Care and Use Committee (IACUC) to perform these 2 virus injections in animals

BEFORE STARTING

Justification:
This is a part of our funded project 'Genetically-based neuro-modulation of adipose tissue functions'. From our other experiments, we observed the huge difference of the number of the PRV labeled sympathetic neurons due to transsynaptic retrograde labeling and that of the AAV labeled sympathetic neurons due to the direct monosynaptic retrograde labeling. We hypothesize that this big difference was due to the transsynaptic labeling within sympathetic ganglia. This method of experience is the only way to investigate this hypothesis by putting direct inputs in the sympathetic ganglia.

1st virus injection

1 1st virus injection.

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- We injected one cre-dependent helper AAV that expresses TVA (= receptor for avian sarcoma leucosis virus glycoprotein EnvA) and oG (optimized glycoprotein) AAV6-FLEX-TVA-P2A-eGFP-2A-oG) into the iBAT of cre driver mice (TH-ires-cre).

1.1 Place a sterile towel on the surgery platform over the heating mat, another sterile towel in the anesthesia induction chamber.

- The 1st virus injections were performed in BSL1.
- All containers that are in contact with virus waste need to be properly labeled as biohazard waste.
- Virus stock needs to be placed on ice during surgeries.

1.2 Record the mouse's body weight to prepare syringes for saline and analgesia treatment.
Warm saline (1-1.5 ml for 25 g body weight); bupivacaine/lidocaine (2.5-12.5 mg/kg body weight); carprofen (5-10 mg/kg body weight.)

1.3 Induce anesthesia with 5% isoflurane in the induction chamber.

1.4 Place the mouse on the surgery platform and maintain anesthesia via a nose cone at 1.5-2% Isoflurane/oxygen (adjust for each mouse accordingly to breathing and anesthetic depth (e.g. loss of withdrawal reflex)).

1.5 Apply eye lubricate to the mouse's eyes.

1.6 Disinfect the lower back of the mouse with 70% ethanol prior to subcutaneous carprofen injection.

1.7 Check for full withdrawal reflex to ensure anesthetic depth.

1.8 Use a clipper to shave the interscapular region of the mouse and remove all hair.

1.9 Disinfect the skin by three alternating scrubs of Novalsan followed by 70% Isopropyl rubbing alcohol.

1.10 Make a midline incision with sharp scissors from the scapula to the neck.

1.11 Remove connective tissue underneath the skin incision using a sterile cotton swab and teethed forceps until the interscapular brown adipose tissue (iBAT) depot is fully accessible.

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The iBAT pad (light brown color) is surrounded by a layer of white adipose tissue. We have successfully injected virus dorsally into the iBAT, however, we could increase our AAV infection success rate by placing the injections into the iBAT core and accessing the ventral iBAT site. The rostral portion of the iBAT depot is detached from the interscapular muscle and the iBAT lobe is flipped back from the rostral side. This will allow visibility of the main iBAT vein (Sulzer vein) and injections are placed from the ventral iBAT side (see Figure 1).

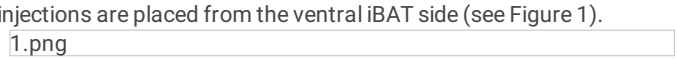

Figure 1: Schematic of iBAT depot.

1.12 Cover the iBAT with saline-soaked sterile gauze to keep the tissue from drying, then prepare the Hamilton syringe for injection.

Virus drawings are prepared in a biological safety area covered with aluminum foil, while we handle surgeries and injections on the surgery platform.

1.13 Fill the Hamilton syringe with 12.5 ul AAV6-FLEX-TVA-P2A-eGFP-2A-oG (5.92E+10 GC/mL) virus, return to the surgery platform with the Hamilton syringe to begin the injections.

1.14 Remove the saline-soaked gauze from the brown adipose tissue and locate the brown adipose tissue pad.

1.15 Place injections (5x2.5 ul) into the right BAT and hold the syringe in place for ~30 seconds after each injection to minimalize backflow, slowly pull the syringe out of the BAT while holding a sterile cotton swab on the site of injection to dry of any possible virus leakage.

1.16 Place a sterile saline-soaked gauze on top of the brown adipose tissue and clean the syringe under the biological safety cabinet with 70% ethanol. Flush the syringe about 10x with 70% ethanol. After that flush the syringe about 10x with sterile H_2O.

Collect all liquid as biohazard waste.

1.17 Remove the saline-soaked gauze from the brown adipose tissue and with a sterile cotton swab and teethed forceps, reposition the overlaying white adipose tissue back to its original location prior to your incision.

1.18 With the teethed and tissue forceps align both sides of the incision properly, and apply wound clips (~25 mm apart, to ensure proper blood circulation).

1.19 Inject bupivacaine/lidocaine subcutaneously around the incision site.

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1.20 Inject warm saline intraperitoneal for rehydration.

1.21 Place the unconscious mouse in a fresh cage with one cage side on a heating mat and monitor until startling reflexes are restored.

1.22 Use 70% ethanol to clean surgery tools and place in glass bead sterilizer for 15-20 seconds before additional surgeries.

2nd virus injection

2 2nd virus injection.
- After sufficient viral expression (4 weeks) mice received another iBAT injection with the modified rabies virus G-Deleted Rabies-mCherry that lacks glycoprotein gene (commercially available from Salk Institute GT3 Core) and thus cannot be further propagated into upstream neurons in the absence of TVA and oG expression. TVA and oG expression are required for rabies propagation and infection of upstream neurons and thus retrograde and transsynaptic transport of the mCherry labeled EnvA would only occur in upstream neurons with monosynaptic inputs. Primary infected neurons would be co-labeled with green TVA eGFP and red EnvA mCherry, while monosynaptic input neurons would only show red EnvA mCherry expression.

The 2nd viral injections were performed in BSL2 and all animals were then maintained in BSL2 housing until euthanasia followed by perfusion (10 days post-injection of the 2nd virus).

We monitor the body weight of mice every day as we consider an excess weight loss (>20%) as a sign of sickness due to the viral infection. In this case, we would perfuse the animals as soon as we detect the loss of body weight. We do not expect the mice to die throughout the experiment, but only expect the IML labeling. We do the weight monitor because when we do similar experiments in the mice's CNS, we monitor their weight every day.

2.1 Place a sterile towel on the surgery platform over the heating mat, another sterile towel in the anesthesia induction chamber.

2.2 Record the mouse's body weight to prepare syringes for saline and analgesia treatment.
Warm saline (1-1.5 ml for 25 g body weight); bupivacaine/lidocaine (2.5-12.5 mg/kg body weight); carprofen (5-10 mg/kg body weight.)

2.3 Induce anesthesia with 5% isoflurane in the induction chamber.

2.4 Place the mouse on the surgery platform and maintain anesthesia via a nose cone at 1.5-2% Isoflurane/oxygen (adjust for each mouse accordingly to breathing and anesthetic depth (e.g. loss of withdrawal reflex)).

2.5 Apply eye lubricate to the mouse's eyes.

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2.6 Disinfect the lower back of the mouse with 70% ethanol prior to subcutaneous carprofen injection.

2.7 Check for full withdrawal reflex to ensure anesthetic depth.

2.8 Use a clipper to shave the interscapular region of the mouse and remove all hair.

2.9 Disinfect the skin by three alternating scrubs of Novalsan followed by 70% Isopropyl rubbing alcohol.

2.10 Make a midline incision with sharp scissors from the scapula to the neck.

2.11 Remove connective tissue underneath the skin incision using a sterile cotton swab and teethed forceps until the interscapular brown adipose tissue (iBAT) depot is fully accessible.

2.12 Cover the iBAT with saline-soaked sterile gauze to keep the tissue from drying, then prepare the Hamilton syringe for injection.

> Virus injections are prepared under a biological safety cabinet, while we handle surgeries and injections outside the biological safety cabinet.

2.13 Fill the Hamilton syringe with 12.5ul G-Deleted Rabies-mCherry virus, return to the surgery platform with the Hamilton syringe to begin the injections.

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2.18 With the teethed and tissue forceps align both sides of the incision properly, and apply wound clips (~25 mm apart, to ensure proper blood circulation).

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2.20 Inject warm saline intraperitoneal for rehydration.

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2.22 Use 70% ethanol to clean surgery tools and place in glass bead sterilizer for 15-20 seconds before additional surgeries.

Perfusion

3 Mice were perfused with 0.9% saline first and then 10% formalin, immediately after euthanization.

3.1 Prepare perfusion setup in the biological safety hood. (This includes laying out tools, filling 50 ml beaker with saline and formalin solutions, setting out animal carcass bags, and cardboard sheets where needed, and charging up the 1000mL beaker with isoflurane.)

3.2 In the biological safety hood, each mouse is euthanized in a 1000mL beaker filled with isoflurane by pouring isoflurane into a 30 ml tube 2/3 filled with pissue towel.

> Monitor the mouse's breathing. Pay particular attention to snap breathing, once this has stopped; the mouse is ready for perfusion!

3.3 Pin the mouse belly up to the Styrofoam block and spray it off with 70% ethanol. (This helps to potentially decontaminate the mouse and keep its fur out of the way.)

3.4 Make an incision using the M/medium scissors by pulling up part of the mouse's skin above the abdomen. Be careful to not cut too deep; only cut deep enough to open up the mouse's organ space (peritoneum, you can see the mouse's intact intestines if done properly).

3.5 Continue to open the peritoneum by sliding the scissors along and cutting up the sides of the mouse until you reach and can clearly see its entire rib cage.

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3.6 Pierce the sack (diaphragm) that covers the bottom of the rib cage and cut it completely away using the S/small scissors. Be careful not to poke/pierce the heart as you do this!

3.7 Cut each side of the rib cage to completely open up the chest cavity and clamp the sternum with a mosquito forceps/hemostat to retract it up and away from the heart. The heart should be completely exposed and clearly visible.

3.8 Prepare the 50mL syringe for perfusion by filling up with at least 30mL of ice-cold saline and connecting it with the butterfly needle by attaching the tubing to the tip of the syringe. Once attached, depress the plunger on the syringe to expel any air bubbles in the tubing. You should clearly see a jet of saline shoot from the tip of the butterfly needle.

3.9 With the other mosquito forceps/hemostat gently grasp the heart to position the tip of the left ventricle so that it just barely peeks out from the edge of the forceps/hemostat. (It is important to not clamp too much of the heart as this will impede flow and reduce the effectiveness of the perfusion.)

3.10 With your free hand insert the butterfly needle into the tip of the left ventricle and fully clamp the mosquito forceps/hemostat. (Insert the needle as shallow as possible, you don't want to stab through the heart and destroy the integrity of the left ventricle.)
2.JPG

3.11 Slowly depress the plunger of the 50mL syringe to perfuse at least 30mL of ice-cold saline. Monitor the liver to ensure proper perfusion. (It should slowly change from deep red to a grey/beige color.)

3.12 Once the 50mL syringe of is empty, disconnect it from the butterfly needle and fill it with 20mL of ice-cold 10% NBF. Reconnect the syringe carefully to prevent air bubbles and continue to slowly perfuse. (Properly perfused mice will "dance" when 10% NBF reaches the muscles in their limbs and tail.)

3.13 Once the 50mL syringe is empty again, remove the needle from the left ventricle by unclamping the mosquito forceps/hemostat. Also, remove the set of mosquito forceps/hemostat clamped to the sternum.

3.14 Clean bench surfaces with 10% bleach, dispose of all remaining mouse tissue in the carcass bag in the biohazard freezer.

3.15 Dispose of the perfusion run off (blood, saline, and formalin) mixture that has collected in the appropriate waste container.

3.16 For tools and surfaces that came in contact with blood spray with 10% bleach, wash with soap, spray with 70% ethanol, dry, and return to them to their original location.

3.17 After fixing, carefully discard formalin in the appropriate waste container.

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Dissections

4 Mice are dissected in the chemical hood for exposing sympathetic chain ganglia by that first remove all the inner organs, except the kidneys and diaphragm, and then remove surrounding tissues that block the view of sympathetic ganglia that innervate iBAT, especially the stellate ganglion.

4.1 Remove all the inner organs below diaphragm, except the kidneys.

4.2 Remove the heart and lungs

4.3 Remove the surrounding tissues that block the view of the sympathetic chain ganglia that innervate iBAT.

Check virus expression in sympathetic chain ganglia under stereomicroscope

5 Expression of the viruses in sympathetic chain ganglia is checked under the stereomicroscope in the red channel. We didn't observe promising red signals in the iBAT-related sympathetic chain ganglia.

If we observed successful virus expression, and then we would further dissect the mice to expose the back side of the spine for better visualization of the preganglionic neurons that innervate the postganglionic neurons that innervates iBAT, and process them with iDISCO staining and then confocal and light sheet microscopy imaging.

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