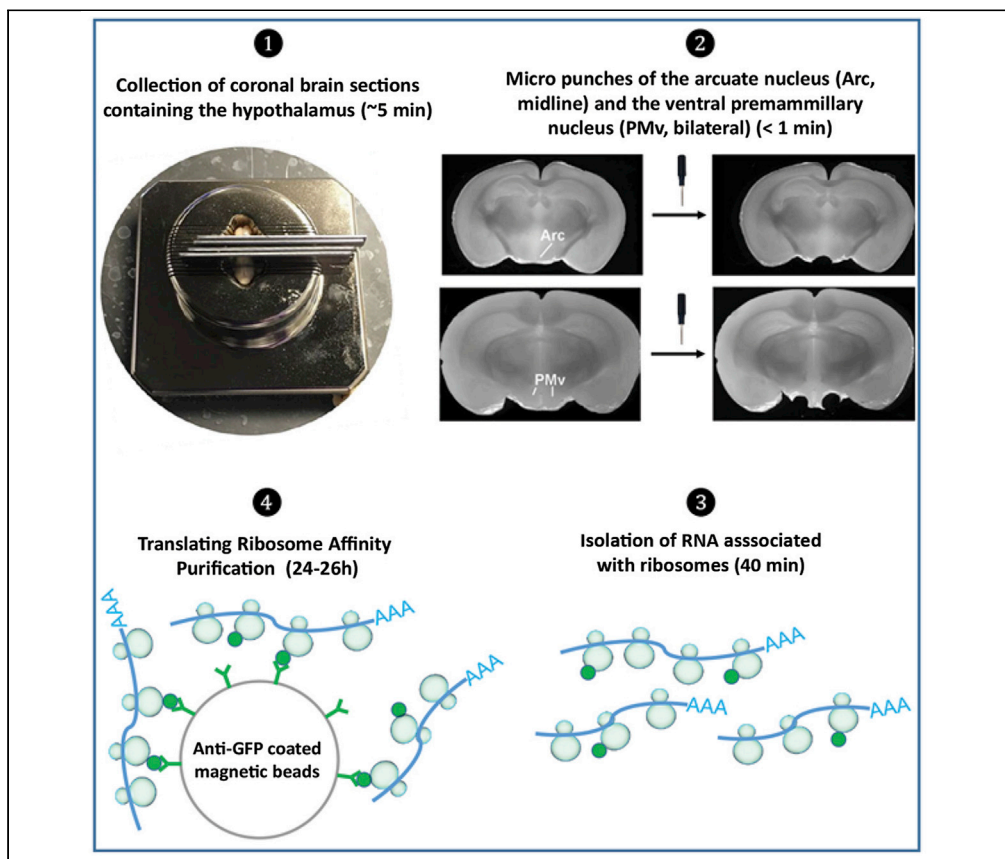


## Protocol

# Protocol to extract actively translated mRNAs from mouse hypothalamus by translating ribosome affinity purification



Here, we present an in-depth protocol for extracting ribosome-bound mRNAs in low-abundance cells of hypothalamic nuclei. mRNAs are extracted from the micropunched tissue using refined translating ribosome affinity purification. Isolated RNAs can be used for sequencing or transcript quantification. This protocol enables the identification of actively translated mRNAs in varying physiological states and can be modified for use in any neuronal subpopulation labeled with a ribo-tag. We use leptin receptor-expressing neurons as an example to illustrate the protocol.

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### Highlights

A protocol to isolate actively transcribed RNAs from low-abundance cells in mouse brain

Isolation of translating RNAs using eGFP-labeled L10a ribosomal protein

A procedure to harvest small brain nuclei avoiding RNA degradation

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## Protocol

## Protocol to extract actively translated mRNAs from mouse hypothalamus by translating ribosome affinity purification

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## SUMMARY

Here, we present an in-depth protocol for extracting ribosome-bound mRNAs in low-abundance cells of hypothalamic nuclei. mRNAs are extracted from the micro-punched tissue using refined translating ribosome affinity purification. Isolated RNAs can be used for sequencing or transcript quantification. This protocol enables the identification of actively translated mRNAs in varying physiological states and can be modified for use in any neuronal subpopulation labeled with a ribo-tag. We use leptin receptor-expressing neurons as an example to illustrate the protocol. For complete details on the use and execution of this protocol, please refer to Han et al. (2020).

## BEFORE YOU BEGIN

Generate enough *LepRb*<sup>eGFP-L10a</sup> transgenic animals that express GFP-labeled L10a ribosomal protein (Heiman et al., 2014; Heiman et al., 2008; Krashes et al., 2014) targeted to *LepRb* neurons by crossing *LepRb*<sup>Cre</sup> mice (Leshan et al., 2006) with *Rosa26*<sup>eGFP-L10a</sup> mice (Allison et al., 2015) for use. For *LepRb* hypothalamic neurons, we pooled 3 mice per biological replicate and used 4 biological replicates (n=12 mice/experimental group). We found that using mice homozygous for both *LepRb*<sup>Cre/Cre</sup> and *Rosa*<sup>eGFP-L10a/eGFP-L10a</sup> increases efficiency of the method (Allison et al., 2018; Allison et al., 2015; Garcia-Galiano et al., 2017; Han et al., 2020). Make sure to screen for germline recombination in your mouse model to avoid harvesting the wrong cells and that Cre is expressed in the correct or expected cells by analysis of eGFP expression. Note that other mouse models expressing ribosome tags are available with protocol described elsewhere (Sanz et al., 2019). In this protocol, we used eGFP-L10 that show overexpression of L10 potentially increasing the signal in low abundant hypothalamic cells (Krashes et al., 2014).

Stock solutions should be prepared fresh on the day before experimental day 1. Refer to the [key resources table](#) for a complete list of materials.

Make sure the bench, all the tubes and equipment are clean and RNase-free before use (e.g., clean bench and pipettes with RNase ZAP or similar).



1. Prepare DTT at stock (200×) concentration 100 mM in RNase free or DEPC-treated water. This can be stored long term (approximately a year) in aliquots (~250 μL) at –20°C. DTT will be used in prepared solutions at a concentration of 0.5 mM. Alternatively, use the 0.1M DTT that is usually included in Reverse Transcriptase kits.
2. Prepare cycloheximide (CHX) at stock (1000×) concentration 100 mg/mL in methanol (MeOH) and store at 4°C for up to one day. CHX will be used in solutions at a final concentration of 100 μg/mL; there is no need to make more than 1 mL.

**Note:** CHX is toxic and an environmental pollutant. Consult your institution on how to properly dispose solutions containing CHX and supplies that may have been in contact with it (e.g., pipette tips, tubes, weigh boats).

3. Prepare 07:0 PC (DHPC) at a stock concentration of 300 mM in RNase-free water. Let powder reach room temperature before reconstitution. Rehydrated powder needs to sit at room temperature with occasional vortexing for about 30 min for full solubilization. Each biological replicate will require 100 μL. Make an appropriate number of aliquots and store at –20°C in glass vials up to 3 months. Do not use plastic vials.
4. Prepare 1 mg/mL of biotin-protein L in RNase free water. Biotin-Protein L instead of A is recommended by the Memorial Sloan Kettering Cancer Center (MSKCC) antibody core. Make 200 μL aliquots and store at –20°C for up to 6 months.
5. Prepare 1 × PBS containing 3% bovine serum albumin (IgG-Free, Protease-Free) and store at 4°C (approximately 1 month).
6. Prepare buffer solutions (see [materials and equipment](#) section).

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Chemicals, peptides, and recombinant proteins</b>		
Biotin-Protein L	GeneScript	Cat#M00097
β-Mercaptoethanol	Sigma	Cat#63689
Bovine Serum Albumin (IgG-Free, Protease-Free)	Jackson ImmunoResearch	Cat#0001-000-162
Cycloheximide (CHX)	Sigma	Cat#C7698
DL-Dithiothreitol (DTT)	Sigma	Cat#D9779
D(+)-Glucose	Sigma	Cat#G7528
07:0 PC (DHPC)	Avanti Polar Lipids	Cat#850306P
1,2-diheptanoyl-sn-glycero-3-phosphocholine		
cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail	Roche	Cat#11836170001
HBSS (10×), calcium, magnesium, no phenol red	Invitrogen/Life Technologies	Cat#14065-056
HEPES (1 M)	Affymetrix/Fisher	Cat#16924
KCl (2 M), RNase-free	Invitrogen	Cat#AM9640G
Methanol, anhydrous, 99.8%	Sigma	Cat#322415
MgCl <sub>2</sub> (1 M)	Invitrogen	Cat#AM9530G
Protein Solubilizer 40, Sterile 10% of NP-40 Ampoules	AG Scientific	Cat#P1505
PBS (10×) Phosphate-Buffered Saline pH 7.4, RNase-free	Invitrogen	Cat#AM9625
RNasin Ribonuclease Inhibitor (40 U/μL)	Promega	Cat#N2515
Protector RNase Inhibitor (40 U/μL)	Roche	Cat#3335402001
SUPERase <sup>in</sup> RNase Inhibitor (20 U/μL)	Invitrogen	Cat#AM2694
Nuclease-Free Water (not DEPC-Treated)	Invitrogen	Cat#AM9937
Sodium azide, ReagentPlus	Sigma	Cat#S2002
Sodium bicarbonate, BioXtra	Sigma	Cat#S6297
Dynabeads MyOne Streptavidin T1	Invitrogen	Cat#65601
RNaseZap RNase Decontamination Solution	Invitrogen	Cat#AM9782

(Continued on next page)

<b>Continued</b>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
GFP Ab, C8 (AB_2716736)	Memorial-Sloan Kettering Monoclonal Antibody Facility	Cat#HTZ-GFP-19C8
GFP Ab, F7 (AB_2716737)	Memorial-Sloan Kettering Monoclonal Antibody Facility	Cat#HTZ-GFP-19F7
<b>Critical commercial assays</b>		
RNeasy Micro Kit	QIAGEN	Cat#74004
SuperScript II Reverse Transcriptase	Invitrogen	Cat#18064022
<b>Experimental models: organisms/strains</b>		
LepR <sup>cre</sup> mice	<a href="#">Leshan et al., 2006</a>	N/A
Rosa <sup>eGFP-L10a/eGFP-L10a</sup> mice	<a href="#">Krashes et al., 2014</a>	N/A
<b>Other</b>		
Refrigerated microcentrifuge	Eppendorf	Cat#5417R
Mini-centrifuge or small benchtop centrifuge	Thermo Fisher Scientific	Cat# 12-006-901
RNase-free 1.5 mL Eppendorf tubes	Eppendorf	Cat#0030120086
RNase-free 50 mL conical tubes	Thermo Fisher Scientific	Cat#AM12501
Sterile round cell culture dish	Thermo Fisher Scientific	Cat# 172931
MagneSphere Magnetic Separation Stand	Promega	Cat#Z5342
RotoFlex Tube Rotator	Argos Technologies	Cat#FV-04397-33
KIMBLE BIOMASHER II	DWK Life Sciences	Cat#749625-0010
Closed System Tissue Grinder		
Pellet Pestle Cordless Motor	Thermo Fisher Scientific	Cat# 12-141-361
RNaseAlert	Integrated DNA Technologies	Cat# 11-02-01-02
Adult Mouse Brain Slicer Matrix	Zivic Instruments	Cat# BSMAS001-1
Hard carbon steel razor blade	VWR	Cat# 55411-050
Palkovits punch (1 or 1.25 mm of diameter)	Ted Pella Inc.	15112-100 or -125
Stereomicroscope	Zeiss	OPMI 1-FC
Fisherbrand™ High Precision Dissecting Micro Scissors	Fisher Scientific	Cat# 08-953-1B
Micro forceps	Roboz	Cat# RS-5070

**Alternatives:** Any refrigerated centrifuge that achieves 20,000 × g and have a cooling system can be used. Other magnetic tube holders, tube rotators, surgical tools and stereo microscopes are suitable for this protocol.

## MATERIALS AND EQUIPMENT

Buffers should be prepared before experiments and stored at 4°C during experiment time. To maintain RNase free conditions, these solutions were prepared for single use in 50 mL conical tubes. When necessary, final solutions should be prepared from RNase-free stock solutions using sterile disposable serological pipettes or pipettes tips with filter.

<b>1. Low salt buffer</b>		
Reagent	Final concentration	Volume (mL)
KCl (2 M)	150 mM	3.75
MgCl <sub>2</sub> (1 M)	10 mM	0.50
HEPES (1 M)	20 mM	1.00
NP40 (10%)	1%	5.00
Water	N/A	39.75
Total	N/A	50.00

**Note:** Filter, sterilize and store at 4°C for up to 1 month. Immediately before use, add DTT to a final concentration of 0.5 mM and CHX to a final concentration of 100 µg/mL.

### 2. High salt buffer

Reagent	Final concentration	Volume (mL)
KCl (2 M)	350 mM	8.75
MgCl <sub>2</sub> (1 M)	10 mM	0.50
HEPES (1 M)	20 mM	1.00
NP40 (10%)	1%	5.00
Water	N/A	34.75
Total	N/A	50.00

**Note:** Filter, sterilize and store at 4°C for several months. Immediately before use, add DTT and CHX to 0.5 mM and 100 µg/mL respectively. Approximately 4 mL of high salt buffer is required per biological replicate, therefore, 50 mL should be enough for 11–12 biological replicates. If processing more than 12 samples scale up the amount of buffer accordingly.

### 3. Tissue lysis buffer

Reagent	Final concentration	Volume (mL)
KCl (2 M)	150 mM	3.75
MgCl <sub>2</sub> (1 M)	10 mM	0.50
HEPES (1 M)	20 mM	1.00
Water	N/A	44.75
Total	N/A	50.00

**Note:** Filter, sterilize and store at 4°C for several months. Immediately before use, in 10 mL Tissue Lysis Buffer add 1 mini EDTA-free protease inhibitor tablet, 0.77 mg of DTT (to 0.5 mM) and 1 g of CHX (to 100 µg/µL). Add 62.5 µL of RNasin (6.25 µL/mL = 250 U/mL), 62.5 µL of SUPERase.In (6.25 µL/mL = 125 U/mL), and 125 µL Protector RNase Inhibitor (12.5 µL/mL = 500 U/mL). We only make as much lysis buffer with the RNase inhibitors as necessary, due to cost. We found that rare hypothalamic cell types (e.g., LepRb and GnRH neurons) require increased concentration of RNase inhibitors in the lysis buffer (Burger et al., 2018; Han et al., 2020).

### 4. Dissection buffer

Reagent	Final concentration	Volume (mL)
HBSS, Hank's Balanced Salt Solution (10 ×)	1 ×	5.00
HEPES	2.5 mM	0.13
Glucose	35 mM	1.50
NaHCO <sub>3</sub>	4 mM	0.22
Water	N/A	43.15
Total	N/A	50.00

**Note:** Filter, sterilize and store at 4°C for up to one month. Immediately before use, add CHX to a final concentration of 100 µg/mL.

If desired, confirm final solutions are RNase free by checking for RNase activity using RNaseAlert.

## STEP-BY-STEP METHOD DETAILS

### Preparation of the affinity matrix (day 1)

⌚ Timing: 2–2.5 h

**Note:** This protocol is modified from previous publication (Heiman et al., 2014).

1. Thaw Biotin-Protein L and anti-GFP antibodies on ice.
2. Resuspend the Dynabeads MyOne Streptavidin T1 thoroughly in the original bottle by gentle pipette mixing. The magnetic beads are iron colored (reddish-brown); when the beads are dispersed, the solution become opaque. When the beads are collected on a magnet, the solution becomes clear.
3. Calculate the required beads amount of based on the ratios below. Each biological replicate (ARC or PMv punches from 3 mice) requires: 250  $\mu$ L Dynabeads MyOne Streptavidin T1, 100  $\mu$ L Biotin-Protein L (1  $\mu$ g/ $\mu$ L in 1X PBS), and 50  $\mu$ g of each GFP antibody, 19C8 and 19F7.
4. To wash the magnetic beads, transfer the required bead volume to a 1.5 mL tube and place it on a magnetic tube holder. After 30 s, the beads will bind to the magnet and the solution will clear. Remove the supernatant.
5. Add 1X PBS (1 mL/1.5 mL tube) to the tube(s) and invert several times.
6. Place the tube(s) on magnetic holder, wait 30 s for the solution to clear and remove supernatant. If necessary, perform a quick spin to collect drops from the wall and lid of the tube(s). Repeat the procedure twice (three washes total).
7. Conjugate Biotin-Protein L to the Streptavidin MyOne T1 Dynabeads. Resuspend the washed beads in an appropriate volume of 1X PBS (original bead volume minus volume of Biotin-Protein L to be added) and 100  $\mu$ L Biotin-Protein L per purification. *For example:* For an original bead volume of 250  $\mu$ L, add 100  $\mu$ L Biotinylated Protein L (1  $\mu$ g/ $\mu$ L in 1X PBS) + 150  $\mu$ L of 1X PBS.
8. Incubate the beads for 35 min at 18°C to 25°C (room temperature) with gentle end-over-end rotation in a tube rotator.
9. After 35 min, place the tube(s) back on the magnet holder, wait 30 s for the solution to clear and remove supernatant.
10. Block the magnetic beads. Wash beads 5 times with 1 volume of 1X PBS containing 3% Bovine Serum Albumin (IgG-Free, Protease-Free) (1 mL if in a 1.5 mL tube).
11. After the last wash, place the tube(s) back on the magnetic holder, wait for 30 s for the solution to clear, remove supernatant, and proceed to antibody binding in Low Salt Wash Buffer.
12. Conjugate the anti-GFP to the beads via Biotin-Protein L. Add 50  $\mu$ g each of anti-GFP antibodies 19C8 and 19F7 (100  $\mu$ g total) in 1 mL final volume of Low Salt Wash Buffer and incubate at room temperature for 1 h using gentle end-over-end rotation in a tube rotator.
13. Wash the antibody conjugated beads 3 times with Low Salt Wash Buffer (1 mL for all washes if in a 1.5 mL tube). After the last wash, resuspend the beads in a volume of Low Salt Buffer such that each purification will receive an aliquot of the listed components – beads/Biotin-Protein L/Ab (the affinity matrix in ratios described above) in a 250  $\mu$ L final aliquot volume.

**Note:** After antibody conjugation to the Streptavidin magnetic beads, combine aliquots and then dispense from a common source. The rationale is to have a consistent affinity matrix across all samples and to remove variability.

**Note:** Once prepared, the affinity matrix can be used immediately, or can be stored for up to two weeks at 4°C with the addition of sodium azide to final concentration of 0.02%. If using pre-prepared affinity matrix stored in sodium azide, wash three times in Low Salt Buffer before use.

### Micropunch sampling of PMv and Arc (day 1)

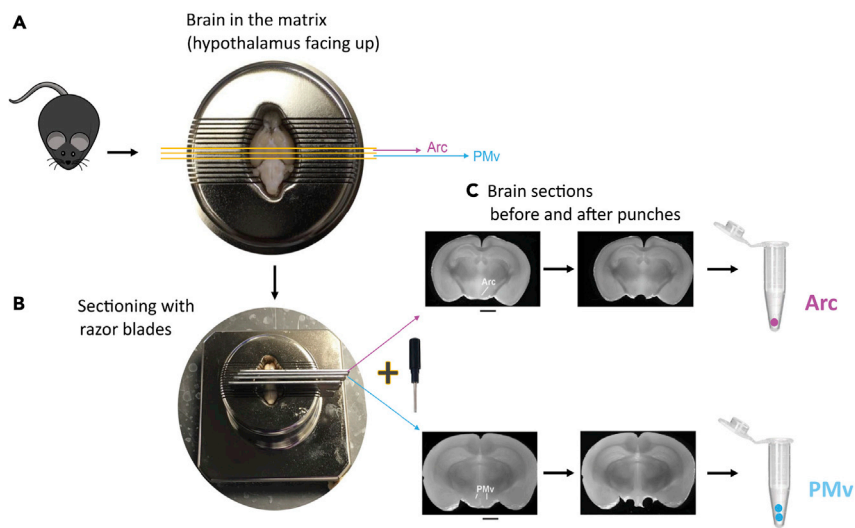
⌚ Timing: ~10 min

14. Add the following to 10 mL Tissue Lysis Buffer on ice.

- a. 1 tablet of EDTA-free protease inhibitors
- b. 10  $\mu\text{L}$  100 mg/mL CHX (Final: 100  $\mu\text{g}/\text{mL}$ )
- c. 5  $\mu\text{L}$  100 mM DTT (Final: 0.5 mM)
15. Each biological replicate requires 500  $\mu\text{L}$  of Tissue Lysis Buffer containing CHX, DTT, protease inhibitors and the adequate RNase inhibitor described below.
  - a. 6.25  $\mu\text{L}/\text{mL}$  of RNasin Ribonuclease Inhibitor (40 U/ $\mu\text{L}$ )
  - b. 6.25  $\mu\text{L}/\text{mL}$  of SUPERase•In RNase Inhibitor (20 U/ $\mu\text{L}$ )
  - c. 12.5  $\mu\text{L}/\text{mL}$  of Protector RNase Inhibitor (40 U/ $\mu\text{L}$ )
16. Add 500  $\mu\text{L}$  Tissue Lysis Buffer in a 1.5 mL tube on ice and place aside.
17. Add 10  $\mu\text{L}$  100 mg/mL CHX to 10 mL Dissection buffer on ice and set aside. Place two 3.5 cm round cell culture dishes (one for Arc and one for PMv) on ice and set aside.
18. Clean the mouse brain matrix, and the stage of stereo microscope with 70% ethanol and/or with RNase ZAP to decrease RNAase contamination.
19. Fill a styrofoam or plastic box with ice. Then insert the mouse brain matrix into the ice keeping only the working area outside the ice.
20. To aid in visualizing the tissue we found it helpful to place one or two freezing ice bag (s) on top of the bench and cover with a black background to improve visual contrast with the brain slices.
21. Clean the black background with 70% ethanol and/or RNase ZAP.
22. Depending on institutional guidelines and animal protocol you may be required to anesthetize the mouse before euthanasia. We lightly anesthetized adult 10 weeks old female mice with isoflurane and then euthanized by decapitation. The brain was then quickly removed taking care to preserve the Arc and median eminence.
23. Immediately insert the brain into the matrix with cortex side down (Figure 1A).
24. Adjust the brain's position in the matrix with cold forceps so that the sagittal sinus and transverse sinus line up with the perpendicular grooves of the block.
25. Once the brain is in position, place a chilled razor blade through the optic chiasm and another chilled blade through the caudal end of the hypothalamus (caudal to the mammillary bodies). This will help to hold the brain in place.
26. Continue to add a chilled blade at 1 mm interval at the level of interest (Figure 1B).
27. Once the two blades are in place, press them down to the bottom of the slots with your fingers.
28. Grasp each side of the two blades and move out of the matrix by rocking back and forth.
29. Once the blades are out of the slots, separate them gently and transfer the section to a pre-chilled cell culture dish placed on top of the black background using chilled forceps.
30. To remove blood and other debris, quickly rinse the brain slice with approximately 500  $\mu\text{L}$  ice-cold dissection buffer (containing CHX and DTT).
31. Harvest the Arc (one punch in the midline, 1–1.25 mm diameter) and, in another section, the PMv (bilateral, one punch in each side, 1–1.25 mm diameter) with the help of a stereo microscope (Figure 1C).
32. Once harvested, immediately place the micropunch from each brain region into an ice pre-chilled tube with 500  $\mu\text{L}$  Tissue Lysis Buffer.
33. Repeat the steps to harvest more Arc and PMv micropunches from two additional mice and pool them together as one biological replicate. It takes about 3 min to collect both PMv (two punches) and Arc (one punch) from one mouse, or about 10 min for all three mice. Because we had three groups, we made the micropunches in three mice from each group (~30 min total) before processing the tissue for TRAP. This strategy resulted in RNA Integrity Number (RIN) = 10 for most of the samples, meaning the degradation of RNA was minimal.

**△ CRITICAL:** For optimal polysome integrity and high RNA yields, perform TRAP purification immediately with fresh tissue, as approximately half of the monosomes and the polysome aggregates are lost after flash freezing of tissue in liquid nitrogen, storage at  $-80^{\circ}\text{C}$  and thawing (Heiman et al., 2014). If not possible, due to the requirement of large numbers





**Figure 1. Micropunch sampling of the mouse arcuate and ventral premammillary nuclei (Arc and PMv, respectively)**

(A) Brains were inserted into the matrix with hypothalamus facing up immediately after harvesting. (B) Once the brain is in position, chilled razor blades were placed along the extension of the hypothalamus. (C) The PMv and Arc were collected separately from the left and right sides of each individual mouse brain by micropunches. Scale bar: 2 mm.

of transgenic animals or different time-points following treatments, the isolated micropunches can be stored at  $-80^{\circ}\text{C}$  after flash freezing in liquid nitrogen.

△ **CRITICAL:** To avoid experimental artifacts, all samples in an experiment should be processed in the same way (fresh or frozen).

**Note:** Both the dissection buffer and the lysis buffer contain CHX. Follow your institutions' guidelines for disposing of solutions (and/or solids) contaminated with CHX.

### Translating ribosome isolation (day 1)

⌚ **Timing:** ~40 min

**Note:** RNA fraction definitions: Input RNA = RNA isolated from tissue lysates before S2 centrifugation (this will contain RNA from all the cells in the punch). Depleted/Cleared RNA = RNA isolated from non-GFP-labeled polysomes. Enriched RNA = RNA isolated from the GFP-labeled polysomes bound to the immunoprecipitation matrix.

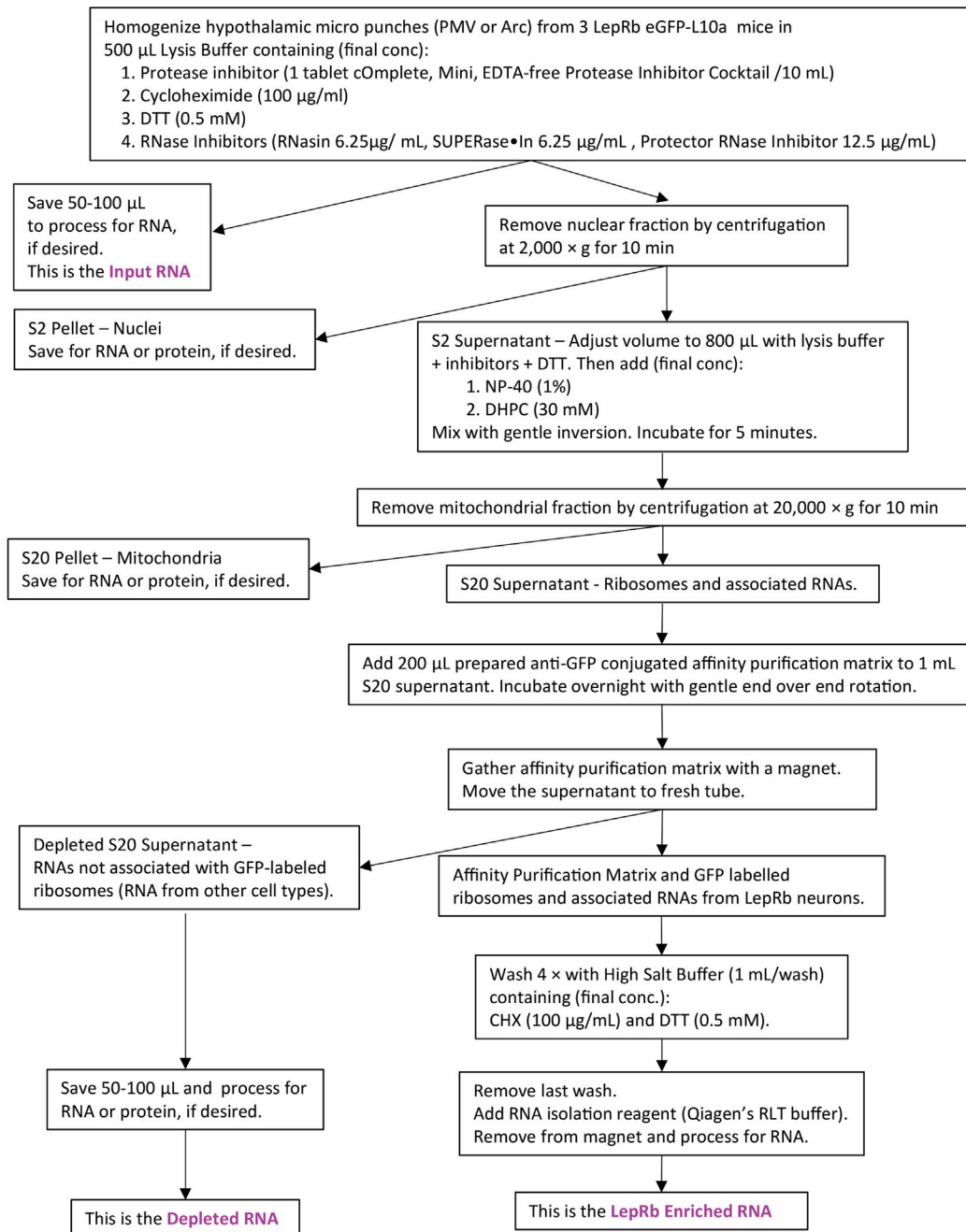
34. Set centrifuge to  $4^{\circ}\text{C}$ ; refer to [Figure 2](#) for a flow chart.
35. Combine the micropunches from one biological replicate together (i.e., six micropunches for the PMv and three for the Arc). Homogenize the pooled PMv or Arc punches in 500  $\mu\text{L}$  (or more if needed) of Tissue Lysis Buffer with a single use pestles and a small, motorized homogenizer.

△ **CRITICAL:** Keep the homogenates ice cold; pre-chill pestle with Tissue Lysis Buffer (+protease and RNase inhibitors) and make sure the pestle does not rise above the level of the solution to avoid bubbles

36. Bring the final homogenate volume to 800  $\mu\text{L}$  by adding 300  $\mu\text{L}$  of ice-cold Tissue Lysis Buffer (with protease and RNase inhibitors). If desired, save an aliquot (50–100  $\mu\text{L}$ ) of homogenate and store at  $-80^{\circ}\text{C}$  for RNA extraction. This is the input total RNA fraction.



## TRAP Flow Chart



**Figure 2. TRAP flow chart**

Summary of the protocol for TRAP organized in individual steps for concision and clarity.

37. Prepare the post nuclear supernatant (S2) by centrifugation at 4°C for 10 min, 2,000 × g.

**Note:** The S2 pellet is the nuclear fraction, which you can keep at –80°C for RNA or protein, if desired.

**Note:** S2 indicates the pellet post 2000 × g (S2) centrifugation.

38. Transfer the S2 supernatant to a pre-chilled tube and add 1/9 sample volume of each 10% NP-40 (final concentration = 1%) and 300 mM DHPC (final concentration = 30 mM). Mix gently by hand inversion of tubes. For ~800  $\mu$ L of supernatant this is 100  $\mu$ L of each 10% NP-40 and 300 mM DHPC.
39. Incubate on ice for 5 min.
40. Pellet the mitochondria by centrifugation at 4°C, 10 min, 20,000  $\times$  g (S20). The ribosomes and their associated RNAs remain in S20 supernatant.
41. Transfer the S20 supernatant to a new pre-chilled tube on-ice and proceed immediately to translating ribosome affinity purification (TRAP). The S20 supernatant can be stored on ice for several hours while additional samples are harvested before proceeding to TRAP.

### Translating ribosome affinity purification (TRAP) (day 1–2)

⌚ Timing: ~18–20 h

42. Thoroughly resuspend the pre-prepared affinity matrix by gentle pipetting. If the affinity matrix was pre-prepared and stored with sodium azide, it should be washed 3 times in Low Salt Buffer before use. Follow your institutions guidelines for disposing of solutions contaminated with sodium azide; refer to [Figure 2](#). TRAP flow chart.
43. Add 200  $\mu$ L affinity matrix to each sample (now at a volume of ~ 1 mL).

**⚠ CRITICAL:** Always resuspend the affinity matrix thoroughly by gentle pipetting immediately before use.

44. Incubate at 4°C with gentle end-over-end mixing in a tube rotator overnight (16–18 h).
45. Pre-chill the magnet holder on ice (or overnight at 4°C).
46. The next day, prepare High Salt Buffer by adding CHX and DTT to 100  $\mu$ g/mL and 0.5 mM final concentrations, respectively. Set aside on ice. You will need ~4 mL high salt buffer/biological replicate. Prepare enough volume for all samples (e.g., 5 biological replicates will need ~20 mL).
47. Prepare the RNA isolation buffer. For QIAGEN RNeasy® Micro kit, add 10  $\mu$ L  $\beta$ -mercaptoethanol ( $\beta$ -ME)/mL RNeasy RLT buffer. Set Aside.

**Note:** If using another kit, follow manufacturer's instructions.

48. Prepare a set of microcentrifuge tubes to collect the post S20 lysate not associated with the anti-GFP coated beads.
49. The washing and collecting of the GFP-labeled ribosomes bound to the magnetic beads must be done either in a cold room or on ice. Collect the beads on a cold magnet holder. If necessary, use a mini centrifuge to spin down beads from caps in between washes.

**⚠ CRITICAL:** Save either an aliquot (100–200  $\mu$ L) or the entire unbound fraction S20 lysate, this can be stored at –80°C to compare to enrichment of transcripts in purified material by downstream assays. RNA isolated from this fraction should be depleted (or cleared) of RNAs of your cell type of interest.

50. Add 1 mL of ice-cold High Salt Buffer and resuspend with gentle pipetting or 1–2 min end-over-end washing. After each wash, collect the beads with magnet and discard the supernatant.

**⚠ CRITICAL:** All washes should be performed by carefully pipetting to avoid the introduction of bubbles.

51. Repeat High Salt Buffer wash 3 times (1 mL/wash for a total of 4 washes).

52. After the last wash, remove wash buffer, remove the tube from the magnet and resuspend beads in 350  $\mu\text{L}$  RLT with  $\beta$ -ME and then bring to room temperature (10 min) before processing (to avoid crystalizing the guanidine, or to redissolve the crystals).
53. Place the tube back on the magnetic holder, wait for 30 s for the solution to clear. Remove the RNA (now in RNeasy RLT buffer) from the beads and place in a new collection tube. Proceed immediately to RNA clean-up following kit manufacturer's instructions (QIAGEN RNeasy<sup>®</sup> Micro kit) or store at  $-80^{\circ}\text{C}$  and process at a later date. RNA isolated from this fraction should be enriched for RNAs of your cell type of interest.

**△ CRITICAL:** Buffers from alternative RNA purification kits can be used, but the buffer used to release bound RNA from the beads must contain the denaturant guanidine thiocyanate. Guanidine thiocyanate can form crystals at low temperatures. Be sure to purify and clean up RNA at  $18^{\circ}\text{C}$  to  $25^{\circ}\text{C}$  (room temperature) to avoid crystallization.

### RNA cleanup and quantitation (day 3)

⌚ Timing: 4–6 h

54. Proceed to RNA extraction follow the QIAGEN RNeasy<sup>®</sup> Micro Kit, or similar manufacturer's instructions. Elute RNA in 30  $\mu\text{L}$  RNase-free water. Purified RNA should be kept at  $-80^{\circ}\text{C}$  for long-term storage. The RNeasy Micro Kit allows for elution of RNA in small volumes (as little as 10  $\mu\text{L}$ ). This is the cell specific or enriched ribosome-bound RNA.
55. For the depleted (or input) RNA, isolate RNA by adding 100–150  $\mu\text{L}$  lysate to 350  $\mu\text{L}$  RLT and following the protocol for RNA cleanup.
56. To assay the concentration and integrity of the RNA, analyze 1  $\mu\text{L}$  of each sample on a Bioanalyzer 2100 using an RNA Pico/Nano chip (follow Agilent's protocol for running chips). As RNA concentration in TRAP samples is likely to be low ( $<1$  ng/ $\mu\text{L}$ ), we do not recommend wasting RNA to nanodrop.

### cDNA synthesis and qPCR to screen for cell-type-specific RNA enrichment (day 3)

⌚ Timing: 4–5 h

57. As cell specific enhanced RNA concentrations are likely to be low, if necessary, reverse transcribe cDNA blind to concentration.
58. Reverse Transcribe 3–5  $\mu\text{L}$  RNA of both enriched RNAs and depleted RNAs with the Reverse Transcriptase of your choice. We used Superscript II (Invitrogen) and the reaction was primed with both oligo dT and random hexamers (Burger et al., 2018; Burger et al., 2020b; Han et al., 2020; Ruka et al., 2013).
59. We also recommend creating a standard curve. This could be a dilution series of the RNA of interest, such as a pool created from either input or depleted RNA. We have shown previously that a range of 0.005 ng/ $\mu\text{L}$  to 5 ng/ $\mu\text{L}$  of hypothalamic RNA can be reverse transcribed and PCR amplified with high efficiency (Ruka et al., 2013).

**Note:** Creating a standard curve provides an opportunity to determine the lower limit of RNA that can be both reverse transcribed and PCR amplified successfully from the tissue of interest. However, other options for determining PCR efficiency (e.g., dilution series of amplicon) are also acceptable.

60. cDNA from the depleted and enriched RNAs were amplified for *Lepr* (cell specific transcript) and *Tnf* and *Ccl3* (micro glial transcripts, potential glial contaminants), and  $\beta$ -actin (*Actb*; example of a housekeeping transcript) using Taqman qPCR technology (Han et al., 2020). *Lepr*, *Tnf3* and *Ccl3* were normalized to *Actb* using normalized relative expression, comparative cycle threshold

method (Bustin, 2002) and enrichment calculated as normalized relative expression in the enhanced RNA sample, divided by the normalized relative expression in the depleted sample. qPCR traces from TRAP enrichment from rare hypothalamic cell types can be found in previous publications (Burger et al., 2018; Krashes et al., 2014).

61. If desired, pre-amplification of remaining cDNA may facilitate examination of further transcripts of interest either by qPCR (Burger et al., 2018; Ruka et al., 2013) or PCR array.

**Note:** Once the technique is working it may be unnecessary to collect the input RNA.

### EXPECTED OUTCOMES

RNA yield from TRAP performed on PMv/Arc LepRb containing cells pooled from three mice is compatible with RNA-seq and TaqMan qPCR techniques. We observed Pico chip RNA concentration for TRAP samples ranged from 301–2488 pg/ $\mu$ L for Arc and from 171–1000 pg/ $\mu$ L for PMv. The enrichment of *LepR* in TRAP fraction vs unbound fraction as assayed by TaqMan qPCR, was around 4.5 and 6.6 for Arc and PMv, respectively (Figure 3A). And the Integrity Number (RIN) of RNA for most of the samples was above 9.0 (Figure 3B). Ribosomal RNAs at 2 kb (rRNA 18S) and 4 kb (rRNA 28S) should be visible by capillary electrophoresis (Figure 3C). For different genotypes of adult mice, e.g., wild type, *ob/ob*, *ob/ob* mice treated with leptin, the Pico chip RNA concentrations varied with genotype.

See Figures 3D and 3E for expected outcomes on TRAP purity for Arc and PMv, respectively. Transcripts known to be expressed in LepRb cells, such as *LepR*, *Stat3*, *Npy*, *Agrp* are enriched in TRAP fractions, while the genes that are not expressed in LepRb cells, such as *Tnf*, *Ccl3* specific to microglia (Allison et al., 2015; Campbell et al., 2017) are de-enriched in the TRAP fraction (as compared to the input RNA).

### LIMITATIONS

This protocol was optimized for isolation of the translated mRNAs from LepRb-expressing cells. It should work in the same conditions for extraction of mRNAs from LepRb containing cells in other brain nuclei or regions, but the yield and purity of LepRb cell-specific mRNA extraction may vary. You may need to adjust the volume of the affinity matrix. For other hypothalamic cell populations, such as the rare GnRH neuron (Wray et al., 1989), you may benefit from reducing the volume of the affinity matrix and/or increasing the number of mice from which to pool micropunch samples (Burger et al., 2018; Burger et al., 2020b; Heiman et al., 2014). RNA yields from TRAP of *Gnrh1* expressing cells were exceedingly low (~50 pg/ $\mu$ L). Nonetheless, enrichment for *Gnrh1* was at 270–290 fold high as GnRH neurons invest heavily in making GnRH peptide (Burger et al., 2018; Burger et al., 2020a, 2020b). Therefore, it is difficult to generalize what RNA yields or amounts of enrichment you might expect from this technique as it is highly dependent on the abundance on the cells of interest, the CRE driver, expression levels of the target gene, etc.

### TROUBLESHOOTING

#### Problem 1

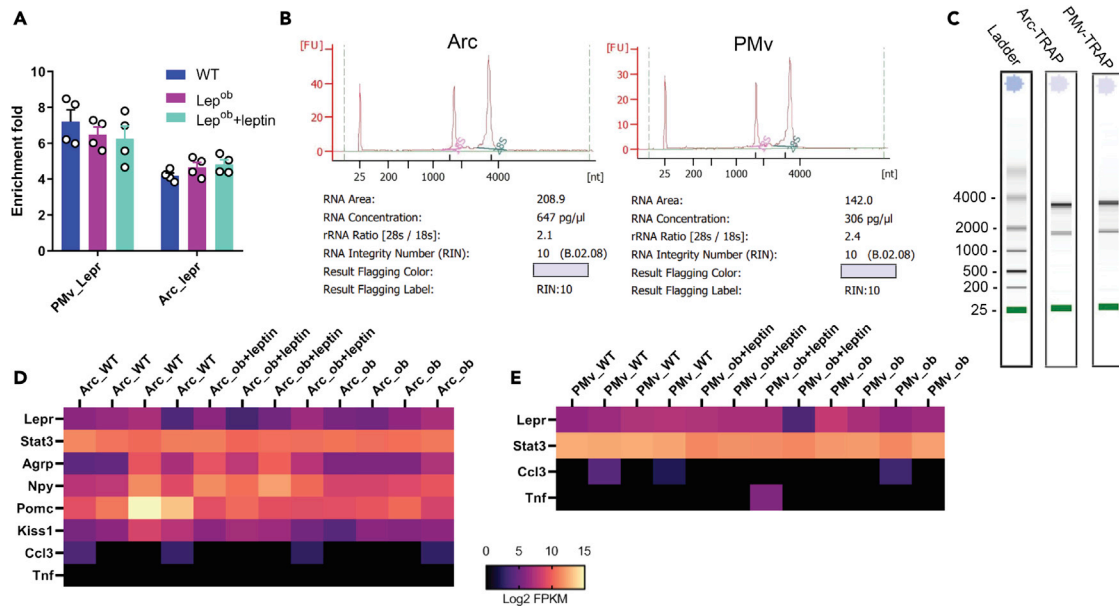
Contamination by cells from other brain nuclei (in Micropunch sampling of PMv and Arc, step 31)

#### Potential solution

A small contamination is difficult to avoid, but you can adjust the diameter of the micropunch accordingly. In our studies, we used 1.25 mm diameter. Different sizes are commercially available. You can also use a fluorescent stereoscope to identify the GFP cells to be harvested. Alternatively, you can compare the expression of genes associated with the cell populations to verify if the data is reliable.

#### Problem 2

The yield of mRNA is too low and below Nano drop or Pico chip threshold of detection (in Micropunch sampling of PMv and Arc, step 33).



**Figure 3. Expected outcomes of TRAP purification**

(A) TaqMan qPCR evaluates the enrichment of *LepRb* in PMV/Arc TRAP fraction versus unbound fraction. The expression of *LepRb* was normalized to  $\beta$ -actin (figure reprinted with permission from Han et al., 2020).

(B) TRAP-purified RNA quality evaluated with Agilent 2100 and Pico Chip.

(C) Capillary electrophoresis of RNAs extracted from *LepRb*-specific cells in Arc and PMv.

(D) Heatmap of RNA-seq data from Arc (D) and PMv (E) *LepRb* specific cells harvested by TRAP.

### Potential solution

If the yield of mRNAs is low, this could be caused by low RNase and/or poor RNase-free technique. RNase activity (in the solutions prior to homogenization and/or in the tissue homogenate) can be checked with RNaseAlert. If solutions are RNase-free, reverse transcribe the TRAP RNA blind to concentration (using a fixed volume of RNA) and check for enrichment of RNA of interest by qPCR (vs input or depleted RNA). If enrichment is high despite low or non-detectable RNA, the method is working but you need to increase number of mice per biological replicate. This is a delicate balance however as increasing input tissue should increase yield, but it will also increase RNase load. An increase in RNase inhibitor may be necessary.

In addition, we suggest performing RNA isolation with fresh brain samples, as approximately half of the monosomes and the polysome aggregates are lost after flash freezing tissue in liquid nitrogen, storage at  $-80^{\circ}\text{C}$  and thawing (Heiman et al., 2014). It is also very important to add CHX and magnesium to stabilize translating ribosomes on intact mRNA once tissue is harvested and to add RNases inhibitors to avoid or reduce RNA degradation during RNA extraction.

Check RNA yield and integrity (RIN) of input RNA and/or depleted RNA. If one or both are low and/or degraded, you may have issues with high RNase activity. If this is the case, improve RNase free techniques and/or increase RNase inhibitor concentrations in lysis buffer.

### Problem 3

The enrichment of gene driving Cre is low (cDNA synthesis and qPCR for screen for cell type RNA enrichment, step 60).

### Potential solution

If enrichment is poor despite quality input RNA and depleted RNA, you may have problems with either ribosomal integrity or the affinity matrix. The quality of the affinity matrix should be tested

using western blotting of the lysates and the beads for GFP protein (Heiman et al., 2014). For rare hypothalamic cell types increase the concentration of RNase inhibitors in the lysis buffer (Burger et al., 2018; Han et al., 2020).

### Problem 4

Contamination by adjacent brain nuclei detected by expression of genetic markers (cDNA synthesis and qPCR for screen for cell type RNA enrichment, step 60).

### Potential solution

When harvesting small nuclei, e.g., PMv and Arc, consult the Allen Mouse Brain Atlas or other references and find landmarks necessary to identify the area of interest. For precisely targeted micro-punch samples, smaller Palkovits punches ([https://www.tedpella.com/section\\_html/precision-brain-punches.htm.aspx](https://www.tedpella.com/section_html/precision-brain-punches.htm.aspx)) may be preferable. Note that some nuclei contamination from other regions is unavoidable. If this is a limiting issue, we suggest using laser microdissection to harvest the brain nuclei of interest.

### Problem 5

Contamination by non-LepRb cell transcripts, i.e., *Tnf*, *Ccl3*. (cDNA synthesis and qPCR for screen for cell type RNA enrichment, step 60).

### Potential solution

Make sure to fully resuspend the beads during wash, because high background contamination by non-LepRb cell transcripts can result from insufficient beads resuspension during wash. Non-LepRb cell transcript contamination can be assessed by qPCR, RNA-seq or in situ hybridization. Also, a known problem with TRAP is nonspecific binding of RNAs to the affinity purification matrix (Burger et al., 2018; Burger et al., 2020b; Dougherty, 2017; Heiman et al., 2014). Optimizing the volume of affinity purification matrix per biological replicate will help to minimize this. However, we strongly recommend including a negative control that is the same tissue from mice that do not express L10a-GFP that is processed in an identical manner. By this method we identified that approximately 25% of the enriched genes in the GnRH neuron were erroneous (Burger et al., 2020a(Burger et al., 2020b)). The RNAs that bound nonspecifically to the immunoprecipitation matrix tended to be high molecular weight and were associated with the nuclear compartment and DNA binding proteins (Burger et al., 2020a(Burger et al., 2020b)). These erroneously enriched RNAs were then removed from the RNASeq datasets. Authors using the related technique RiboTag (Sanz et al., 2009) have recently amended their protocol to include the ionic detergent sodium deoxycholate in the homogenization buffer to minimize such false positive RNAs (Sanz et al., 2019). Alternatively, it has also been suggested that nonspecific RNA binding to the affinity purification matrix can be minimized by preabsorbing the lysate with, in this case, antibody free streptavidin coated magnetic beads and/or IgG-coated beads. Testing contamination by incubating your sample with beads coated with no antibodies or with IgGs and assess by qPCR the expression of these mRNAs can provide information on contamination levels. Some mRNAs tend to stick nonspecifically to beads or IgGs. A suggested solution is to pre-incubate the brain lysate on non-coated beads for 30 min at 4°C, then on IgG-coated beads at 4°C for 30 min before the real immunoprecipitation on GFP-coated beads to make sure your non-specific mRNAs are removed (Bertin et al., 2015; Mazare et al., 2020a; Mazare et al., 2020b).

## 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. Carol F Elias ([cfelias@umich.edu](mailto:cfelias@umich.edu))

### Materials availability

Dataset and reagents generated in the original study are available upon request.

### Data and code availability

The study did not generate unique datasets or code.

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### AUTHOR CONTRIBUTIONS

C.F.E. contributed with the conceptualization, funding acquisition, supervision, validation, visualization, and review and editing of the original draft. X.H. contributed with the conceptualization, investigation, validation, visualization, and writing of original draft; L.L.B., S.M.M., and D.G.-G. contributed with the methodology, supervision of procedures, and review and editing of the original draft; S.M.M., M.G.M.J., and D.P.O. contributed with resources and editing of the original draft.

### DECLARATION OF INTERESTS

The authors declare no competing interest.

### REFERENCES

- Allison, M.B., Pan, W., MacKenzie, A., Patterson, C., Shah, K., Barnes, T., Cheng, W., Rupp, A., Olson, D.P., and Myers, M.G., Jr. (2018). Defining the transcriptional targets of leptin reveals a role for Atf3 in leptin action. *Diabetes* 67, 1093–1104.
- Allison, M.B., Patterson, C.M., Krashes, M.J., Lowell, B.B., Myers, M.G., Jr., and Olson, D.P. (2015). TRAP-seq defines markers for novel populations of hypothalamic and brainstem LepRb neurons. *Mol. Metab.* 4, 299–309.
- Bertin, B., Renaud, Y., Aradhya, R., Jagla, K., and Junion, G. (2015). TRAP-rc, translating ribosome affinity purification from rare cell populations of *Drosophila* embryos. *J. Vis. Exp.* 103, 52985.
- Burger, L.L., Vanacker, C., Phumsatitpong, C., Wagenmaker, E.R., Wang, L., Olson, D.P., and Moenter, S.M. (2018). Identification of genes enriched in GnRH neurons by translating ribosome affinity purification and RNAseq in mice. *Endocrinology* 159, 1922–1940.
- Burger, L.L., Wagenmaker, E.R., Phumsatitpong, C., Olson, D.P., and Moenter, S.M. (2020a). Prenatal androgen exposure alters the developmental trajectory of both GnRH neuron and preoptic area RNA transcripts in female mice (Mlibrary Deep Blue Data: Deep Blue Data). <https://doi.org/10.7302/7ybd-c428?>
- Burger, L.L., Wagenmaker, E.R., Phumsatitpong, C., Olson, D.P., and Moenter, S.M. (2020b). Prenatal androgenization alters the development of GnRH neuron and preoptic area RNA transcripts in female mice. *Endocrinology* 161, bqaa166.
- Bustin, S.A. (2002). Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J. Mol. Endocrinol.* 29, 23–39.
- Campbell, J.N., Macosko, E.Z., Fenselau, H., Pers, T.H., Lyubetskaya, A., Tenen, D., Goldman, M., Verstegen, A.M.J., Resch, J.M., McCarroll, S.A., et al. (2017). A molecular census of arcuate hypothalamus and median eminence cell types. *Nat. Neurosci.* 20, 484–496.
- Dougherty, J.D. (2017). The expanding toolkit of translating ribosome affinity purification. *J. Neurosci.* 37, 12079–12087.
- Garcia-Galiano, D., Borges, B.C., Donato, J., Jr., Allen, S.J., Bellefontaine, N., Wang, M., Zhao, J.J., Kozloff, K.M., Hill, J.W., and Elias, C.F. (2017). PI3K $\alpha$  inactivation in leptin receptor cells increases leptin sensitivity but disrupts growth and reproduction. *JCI Insight.* 2, e96728.
- Han, X., Burger, L.L., Garcia-Galiano, D., Sim, S., Allen, S.J., Olson, D.P., Myers, M.G., Jr., and Elias, C.F. (2020). Hypothalamic and cell-specific transcriptomes unravel a dynamic neuropil remodeling in leptin-induced and typical pubertal transition in female mice. *iScience* 23, 101563.
- Heiman, M., Kulicke, R., Fenster, R.J., Greengard, P., and Heintz, N. (2014). Cell type-specific mRNA purification by translating ribosome affinity purification (TRAP). *Nat. Protoc.* 9, 1282.
- Heiman, M., Schaefer, A., Gong, S., Peterson, J.D., Day, M., Ramsey, K.E., Suarez-Farinas, M., Schwarz, C., Stephan, D.A., Surmeier, D.J., et al. (2008). A translational profiling approach for the molecular characterization of CNS cell types. *Cell* 135, 738–748.
- Krashes, M.J., Shah, B.P., Madara, J.C., Olson, D.P., Strohlic, D.E., Garfield, A.S., Vong, L., Pei, H., Watabe-Uchida, M., Uchida, N., et al. (2014). An excitatory paraventricular nucleus to AgRP neuron circuit that drives hunger. *Nature* 507, 238–242.
- Leshan, R.L., Bjornholm, M., Munzberg, H., and Myers, M.G., Jr. (2006). Leptin receptor signaling and action in the central nervous system. *Obesity (Silver Spring)* 14 (Suppl 5), 208S–212S.
- Mazare, N., Oudart, M., Cheung, G., Boulay, A.C., and Cohen-Salmon, M. (2020a). Immunoprecipitation of ribosome-bound mRNAs from astrocytic perisynaptic processes of the mouse hippocampus. *STAR Protoc.* 1, 100198.
- Mazare, N., Oudart, M., Moulard, J., Cheung, G., Tortuyaux, R., Maily, P., Mazaud, D., Bemelmans, A.P., Boulay, A.C., Blugeon, C., et al. (2020b). Local translation in perisynaptic astrocytic processes is specific and changes after fear conditioning. *Cell Rep.* 32, 108076.
- Ruka, K.A., Burger, L.L., and Moenter, S.M. (2013). Regulation of arcuate neurons coexpressing kisspeptin, neurokinin B, and dynorphin by modulators of neurokinin 3 and kappa-opioid receptors in adult male mice. *Endocrinology* 154, 2761–2771.
- Sanz, E., Bean, J.C., Carey, D.P., Quintana, A., and McKnight, G.S. (2019). RiboTag: ribosomal tagging strategy to analyze cell-type-specific mRNA expression *in vivo*. *Curr. Protoc. Neurosci.* 88, e77.
- Sanz, E., Yang, L., Su, T., Morris, D.R., McKnight, G.S., and Amieux, P.S. (2009). Cell-type-specific isolation of ribosome-associated mRNA from complex tissues. *Proc. Natl. Acad. Sci. U S A* 106, 13939–13944.
- Wray, S., Grant, P., and Gainer, H. (1989). Evidence that cells expressing luteinizing hormone-releasing hormone mRNA in the mouse are derived from progenitor cells in the olfactory placode. *Proc. Natl. Acad. Sci. U S A* 86, 8132–8136.