

Supplementary Information

Hypothalamic Prolactin Receptor⁺ Dopaminergic Neurons Regulate Energy Metabolism and Nociceptive Threshold in Mice During Lactation

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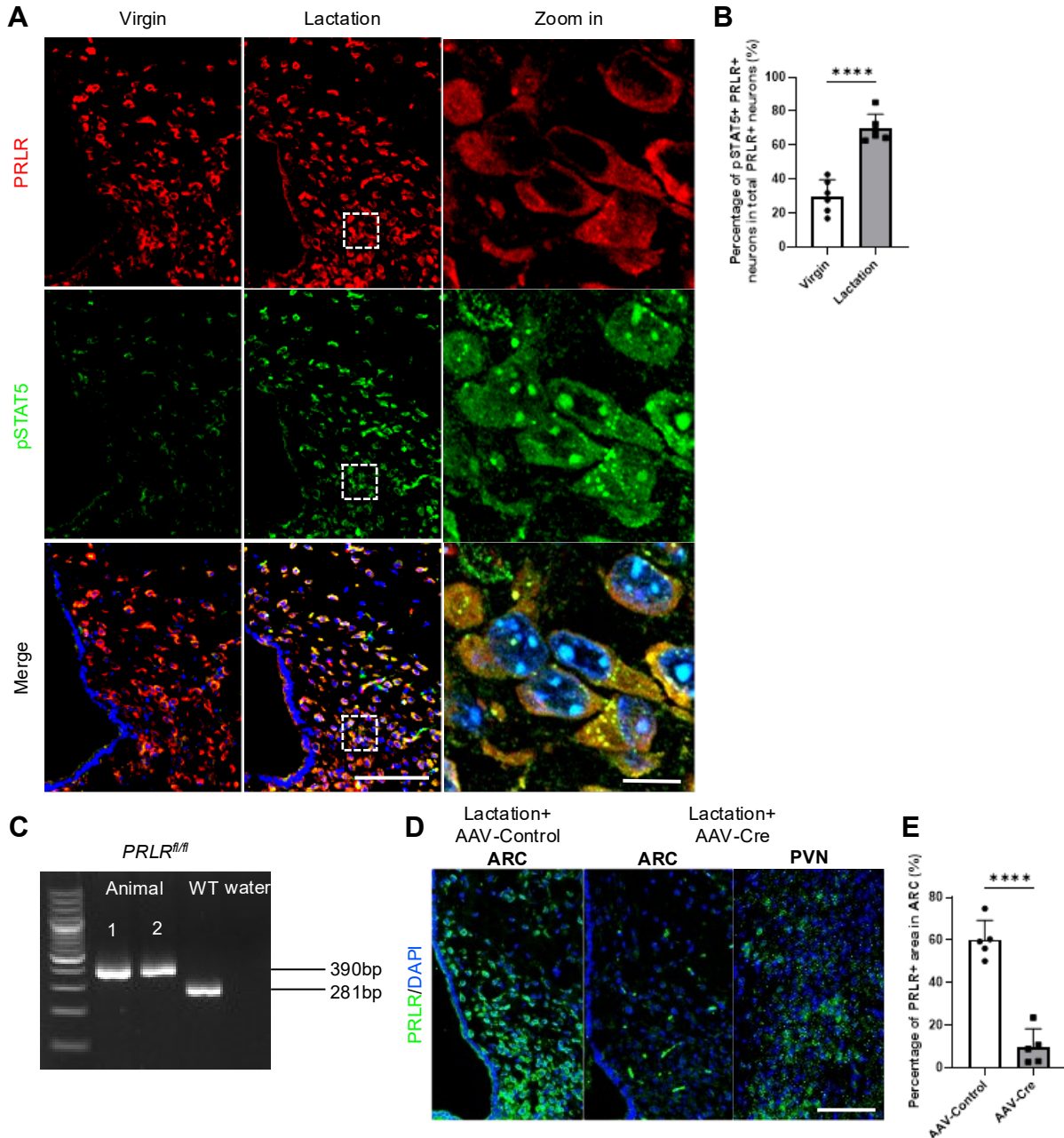
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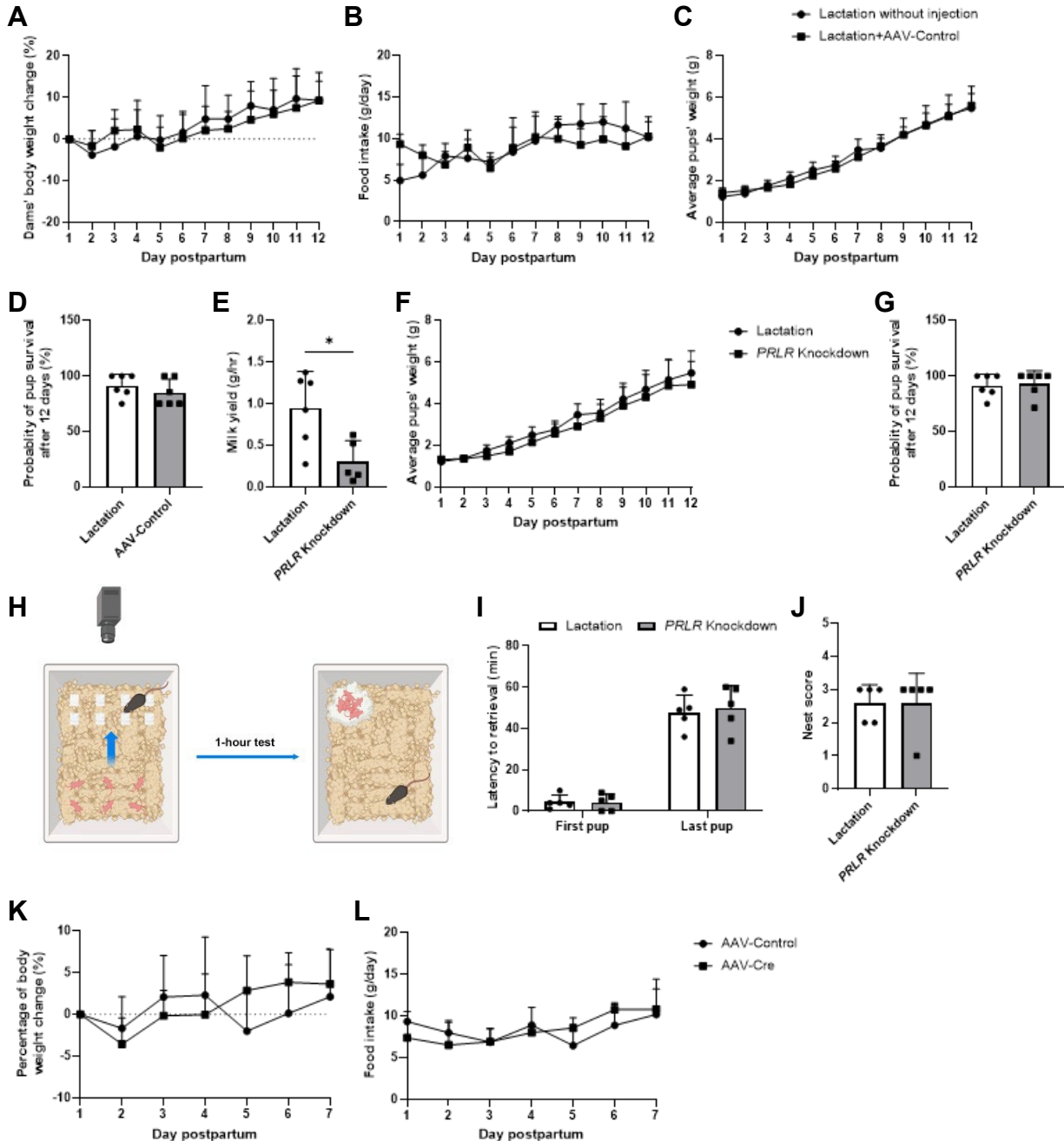
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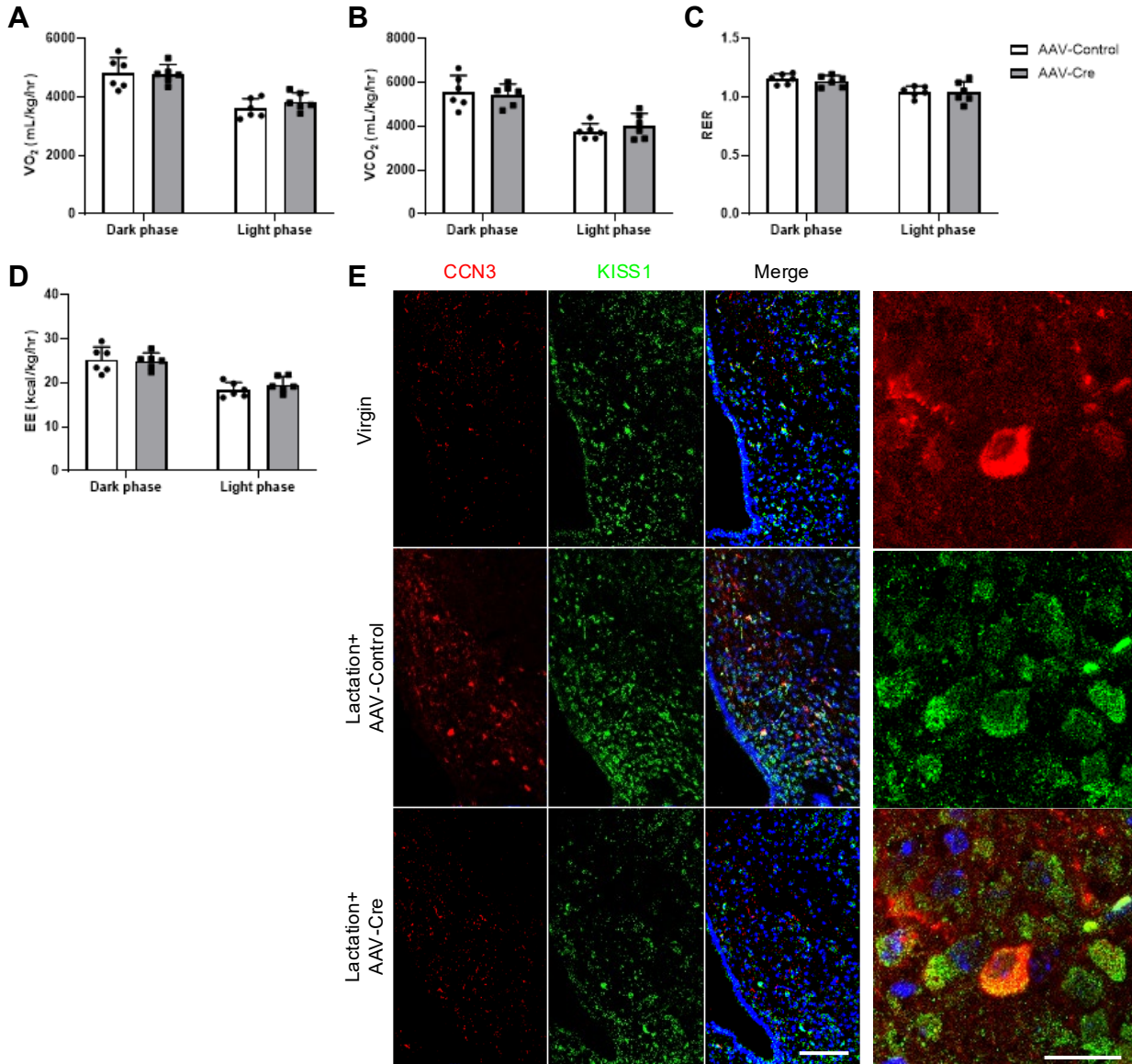
Supplementary Fig. 1 Validation of PRLR activation through pSTAT5 signaling and AAV-induced knockdown of *PRLR* in *PRLR^{fl/fl}* mice. (A and B) Representative immunostaining images (A) and quantitative analysis (B) showing PRLR⁺ (red) and pSTAT5⁺ (green) neuron expression and their overlap (yellow) in the hypothalamic ARC from virgin and lactating mice. Nuclei are counterstained with DAPI (blue). Scale bars, 100 μ m (low power images) and 10 μ m (high power images). n = 6 mice. (C) Genotyping of *PRLR^{fl/ox}* allele. Expected outcome: WT: 281 bp; Floxed: 390 bp. The PCR reaction was

conducted with the primers shown in Supplementary Table 3. **(D)** Representative immunostaining images showing PRLR (green) expression in the hypothalamic ARC and PVN, respectively, after injection of AAV-Cre in the ARC of lactating *PRLR^{fl/fl}* mice. Scale bar, 100 μ m. **(E)** Quantitative analysis of PRLR expression in the ARC after injection of AAV-Control or AAV-Cre in the ARC of lactating *PRLR^{fl/fl}* mice. n = 5 mice. Data are presented as the mean $\pm$ SD. An unpaired, 2-tailed *t*-test was used for analyses. ****, *p* < 0.0001.



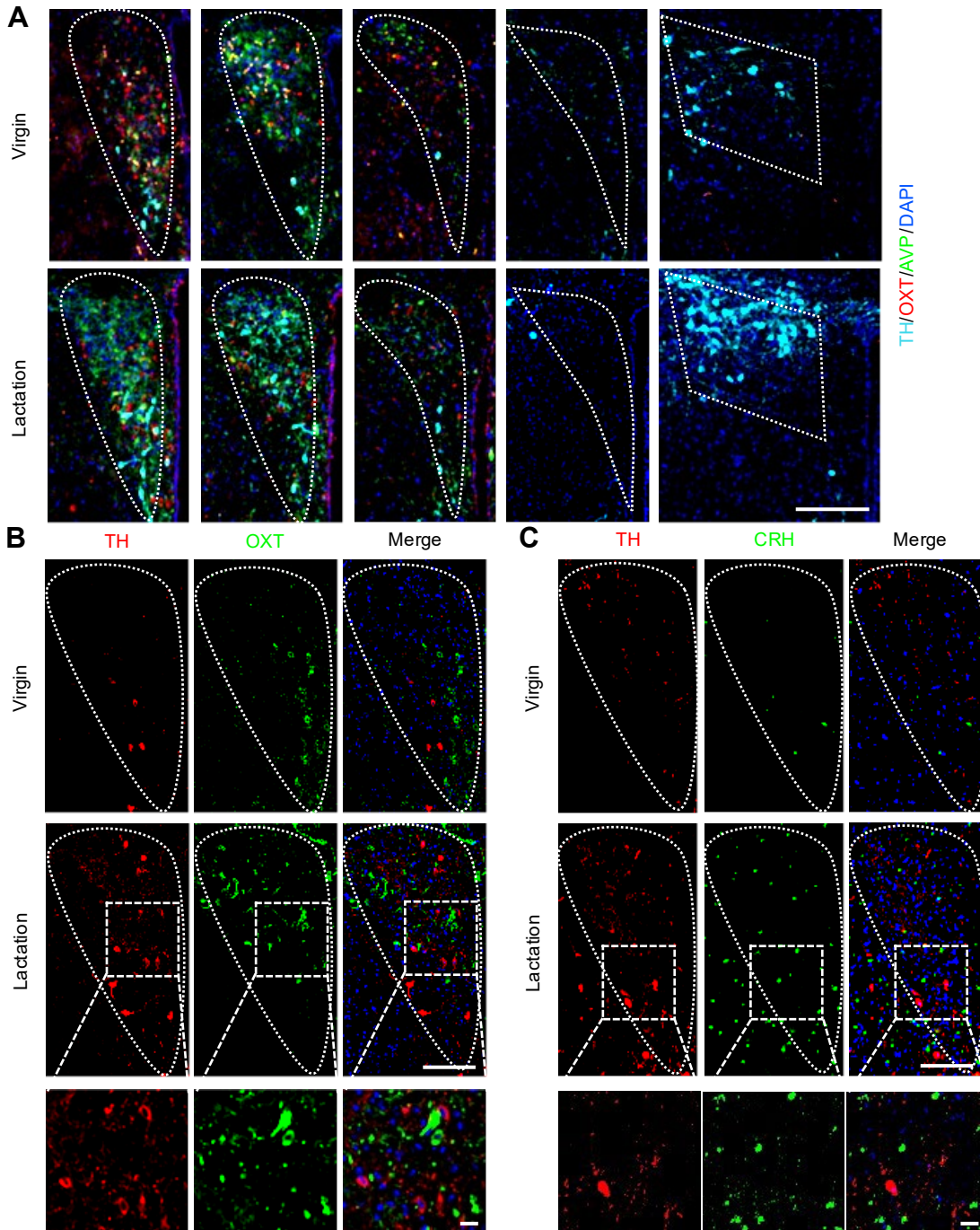
Supplementary Fig. 2 Impacts of AAV injection and *PRLR* knockdown on maternal and pups' health and development. (A and B) Body weight change (A) and daily food intake (B) of lactating mice without AAV injection and AAV-Control-injected lactating mice from postpartum day 1 to day 12. $n = 6$ mice. (C and D) Average pup weight from postpartum day 1 to day 12 (C) and pup survival rate on day 12 (D) of lactating mice without AAV injection and AAV-Control-injected lactating mice. $n = 6$ mice. (E) Milk yield on postpartum day 12 of lactating control mice and *PRLR* knockdown mice. $n = 6$ mice in

lactation group, $n = 5$ mice in knockdown group. $p = 0.0177$. **(F and G)** Average pup weight from postpartum day 1 to day 12 (F) and pup survival rate on day 12 (G) of lactating control mice and *PRLR* knockdown mice. $n = 6$ mice. **(H)** Diagram depicting the pup retrieval test. Typically, eight pieces of nesting material and six pups were laid out in a rat cage arena prior to the beginning of each trial. After 1 hour of each trial, all the pups of each dam were retrieved regardless of groups. Created in BioRender. Wang, C. (2026) <https://BioRender.com/cjxs0op>. **(I and J)** Latency to retrieve the first pup and the last pup (I) and nest quality (J) of lactating control mice and *PRLR* knockdown mice. $n = 5$ mice. **(K and L)** Body weight change (K) and daily food intake (L) of lactating control mice and *PRLR* knockdown mice from postpartum day 1 to day 7. $n = 6$ mice in AAV-Control group, $n = 5$ mice in AAV-Cre group. Data are presented as the mean $\pm$ SD. A two-way ANOVA with Bonferroni's post hoc test was used for analyses involving two factors (A, B, C, F, I, K and L). An unpaired, 2-tailed *t*-test was used for analyses between two groups (D, E and G). A 2-tailed Mann-Whitney test was used for analyses between two groups without normal distribution (J).



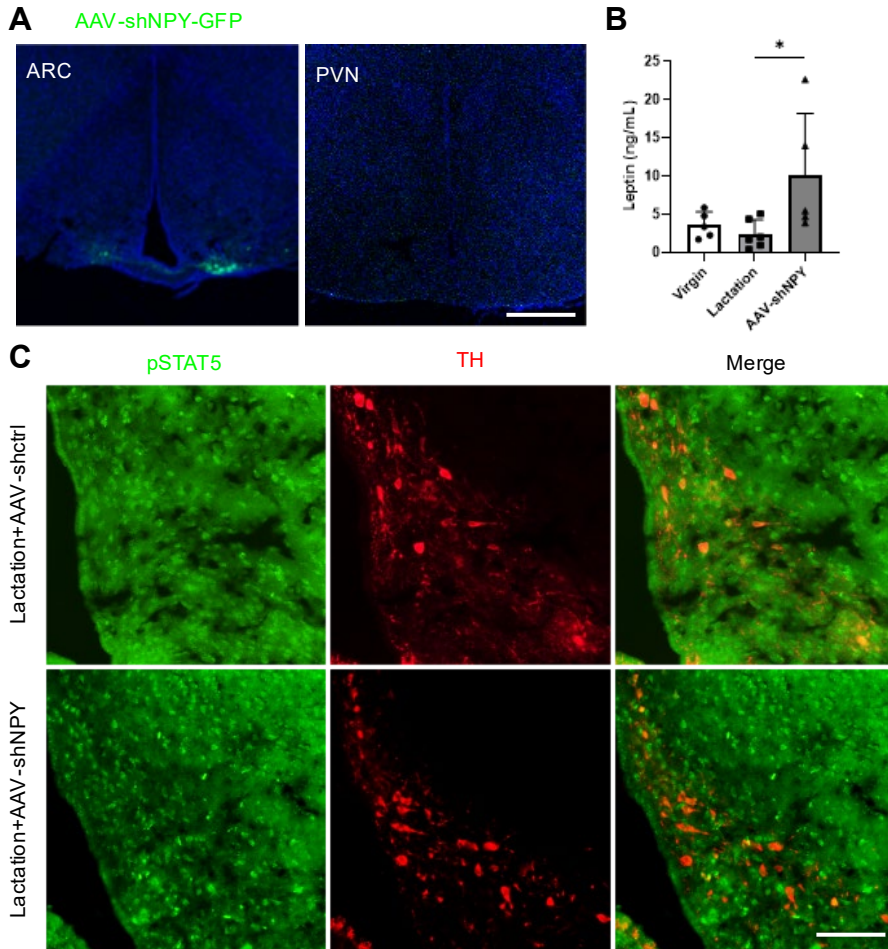
Supplementary Fig. 3 Energy expenditure and CCN3 secretion from KISS1 neurons in *PRLR* knockdown mice. (A-D) Energy expenditure test of AAV-Control-injected lactating mice and AAV-Cre-injected lactating mice around postpartum day 11-13, showing the parameters of VO_2 (A), VCO_2 (B), RER (C) and EE (D) in dark phase and light phase. $n = 6$ mice. **(E)** Representative immunostaining images showing CCN3 (red) and KISS1 (green) expression and their overlap (yellow) in the hypothalamic ARC from virgin, AAV-Control-injected or AAV-Cre-injected lactating mice. Nuclei are counterstained with DAPI (blue). Scale bar, 100 μm for lower magnification and 20 μm for higher magnification. The experiment was repeated three times independently with similar

results. Data are presented as the mean $\pm$ SD. A two-way ANOVA with Bonferroni's post hoc test was used for analyses.

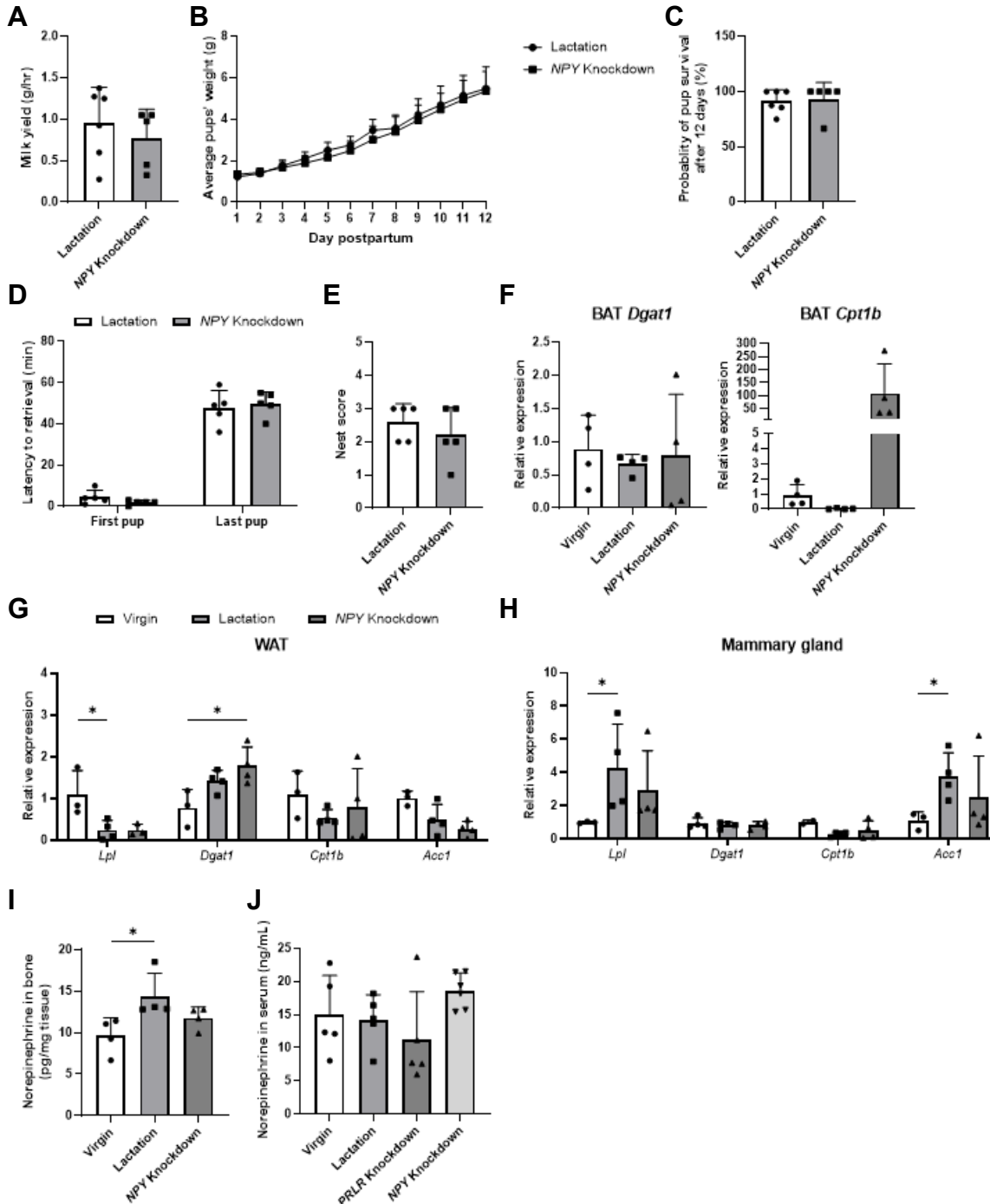


Supplementary Fig. 4 Identification of TH-expressing neurons' type in the Ma subdivision of PVN. (A) Representative immunostaining images showing TH (cyan), OXT (red) and AVP (green) expression in PVN of virgin and lactation female mice across the A/P axis with an interval of ~120 μm between each image. Ma subdivision spans from the first to the third slides in each group, which is composed of OXT and AVP neurons. Nuclei are counterstained with DAPI (blue). Scale bar, 200 μm. **(B)** Representative

immunostaining images showing TH⁺ (red) and OXT (green) neuron expression in the Ma subdivision from virgin and lactating mice. Scale bars, 100 μ m (low power images) and 20 μ m (high power images). **(C)** Representative immunostaining images showing TH⁺ (red) and corticotropin-releasing hormone (CRH) (green) neuron expression in the Ma subdivision from virgin and lactating mice. Scale bar, 100 μ m. All the experiments were repeated three times independently with similar results.



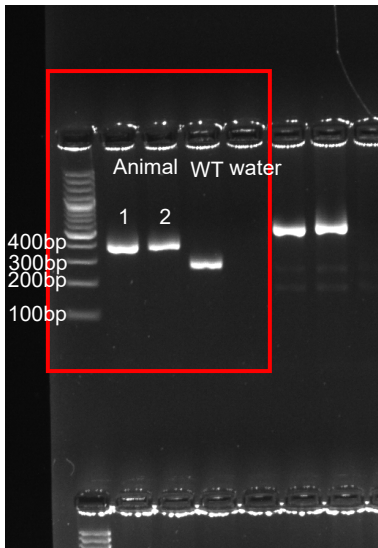
Supplementary Fig. 5 Validation of no change in PRLR activation and TH expression in the ARC in *NPY* knockdown mice. (A) AAV-shNPY-GFP and AAV-shctrl-GFP injection sites at the ARC. Nuclei are counterstained with DAPI (blue). Scale bar, 400 μ m. **(B)** ELISA measurement of serum leptin concentration (in ng/mL) on the 12th day postpartum in virgin mice, lactating mice, and AAV-shNPY-injected lactating mice. $n = 5$ mice in virgin and AAV-shNPY groups, $n = 6$ mice in lactation group. $p = 0.0459$. **(C)** Representative immunostaining images showing pSTAT5⁺ (green) and TH⁺ (red) neuron expression and their overlap (yellow) in the hypothalamic ARC from AAV-shctrl-injected lactating mice and AAV-shNPY-injected lactating mice. Scale bar, 100 μ m. The experiment was repeated three times independently with similar results. Data are presented as the mean $\pm$ SD. A one-way ANOVA with Tukey's post hoc test was used for analyses.



Supplementary Fig. 6 Impacts of *NPY* knockdown on mothers and pups, lipid metabolism and sympathetic outflow in bone or serum after *PRLR/NPY* knockdown. (A) Milk yield on postpartum day 12 of lactating control mice and *PRLR* knockdown mice. $n = 6$ mice. (B and C) Average pup weight from postpartum day 1 to day 12 (B) and pup survival rate on day 12 (C). $n = 6$ mice. (D and E) Latency to retrieve the first pup and the last pup (D) and nest quality (E). $n = 5$ mice. (F-H) mRNA expression of key enzymes

related to lipid metabolism in BAT (F), WAT (G) and mammary gland (H). F, n = 4 mice. G, n = 3 mice in virgin group, n = 4 mice in lactation group, n = 3 mice in knockdown group of *Lpl* measurement and 4 mice in knockdown group of other genes measurement. H, n = 3 mice in virgin group of *Lpl* and *Acc1* measurement, 4 mice in virgin group of *Dgat1* measurement and 2 mice in virgin group of *Cpt1b* measurement, n = 4 mice in lactation and knockdown groups. G, $p = 0.0421$ and 0.0133 . H, $p = 0.0137$ and 0.0482 . **(I)** ELISA of NE concentration (in pg/mg tissue) in bone on the 12th day postpartum. n = 4 mice. $p = 0.0332$. **(J)** ELISA of NE concentration (in ng/mL) in serum on the 12th day postpartum. n = 5 mice in virgin, lactation and *PRLR* knockdown groups, n = 6 mice in *NPY* knockdown group. Data are presented as the mean $\pm$ SD. An unpaired, 2-tailed *t*-test was used for analyses between two groups (A and C). A two-way ANOVA with Bonferroni's post hoc test was used for analyses involving two factors (B and D). A 2-tailed Mann-Whitney test was used for analyses between two groups without normal distribution (E). A one-way ANOVA with Tukey's post hoc test was used for analyses among more than two groups (F, I and J). A two-way ANOVA with Tukey's post hoc test was used for analyses involving two factors (G and H).

PRLR^{fl/fl} genotyping



Supplementary Fig. 7 Raw gel in Supplementary Fig. 1C

Supplementary Table 1: Bacterial and virus strain information

AAV virus	Vendor	Catalog
AAV-Syn-Cre	SignaGen Laboratories	Cat# SL116034
pAAV-shNPY-GFP	Origene	Cat# TR30034 and TR513932

Supplementary Table 2: Antibody information

Antibody	Vendor	Catalog
Rabbit monoclonal anti-phospho-Stat5	Cell Signaling Technology	Cat# 4322; RRID:AB_10544692
Chicken polyclonal anti-tyrosine hydroxylase	Abcam	Cat# ab76442; RRID:AB_1524535
Rabbit polyclonal anti-tyrosine hydroxylase	Sigma	Cat# AB152; RRID:AB_390204
Mouse monoclonal anti-PRLR	Invitrogen	Cat# MA1-610; RRID:AB_2171291
Rabbit polyclonal anti-phospho-Stat5	Cell Signaling Technology	Cat# 9351; RRID:AB_2315225
Rabbit polyclonal anti-PRLR	Bioss	Cat# bs-6445r
Mouse monoclonal anti-beta-Actin	Cell Signaling Technology	Cat# 3700; RRID:AB_2242334
Rabbit monoclonal anti-neuropeptide Y	Cell Signaling Technology	Cat# 11976; RRID:AB_2716286
Chicken polyclonal anti-GFP	Abcam	Cat# ab13970; RRID:AB_300798
Goat polyclonal anti-NOV/CCN3	R&D Systems	Cat# AF1976; RRID:AB_355085
Rabbit polyclonal anti-KISS1	Proteintech	Cat# 18375-1-AP; RRID:AB_11182705
Guinea pig polyclonal anti-vasopressin	Synaptic Systems	Cat# 403 004; RRID:AB_2725758
Rabbit polyclonal anti-CRF	ImmunoStar	Cat# 20084; RRID:AB_572228
Goat polyclonal anti-oxytocin/neurophysin I	Novus Biologicals	Cat# NBP2-76346; RRID:AB_3403503
Rabbit monoclonal anti-phospho-AMPK α	Cell Signaling Technology	Cat# 2535; RRID:AB_331250
Goat polyclonal anti-perilipin	Novus Biologicals	Cat# NB100-60554; RRID:AB_922242
Alexa 488 secondary anti-rabbit	Jackson ImmunoResearch Laboratories Inc.	Cat# 711-546-152
Alexa 594 secondary anti-rabbit	Jackson ImmunoResearch Laboratories Inc.	Cat# 711-296-152
Alexa 647 secondary anti-rabbit	Jackson ImmunoResearch Laboratories Inc.	Cat# 711-605-152
Alexa 488 secondary anti-chicken	Jackson ImmunoResearch Laboratories Inc.	Cat# 703-546-155
Alexa 594 secondary anti-chicken	Jackson ImmunoResearch Laboratories Inc.	Cat# 703-586-155
Alexa 594 secondary anti-mouse	Jackson ImmunoResearch Laboratories Inc.	Cat# 715-546-151

Alexa 488 secondary anti-goat	Jackson ImmunoResearch Laboratories Inc.	Cat# 705-546-147
Alexa 594 secondary anti-goat	Jackson ImmunoResearch Laboratories Inc.	Cat# 705-586-147
Alexa 488 secondary anti-guinea pig	Jackson ImmunoResearch Laboratories Inc.	Cat# 706-546-148

Supplementary Table 3: PCR primer information

Oligonucleotides	Source	Citation
ChIP-qPCR primer (<i>Th</i> promoter): Binding Site I forward: 5'-GGGTGAGAGCTCTGTCCTTT-3'	This paper	N/A
ChIP-qPCR primer (<i>Th</i> promoter): Binding Site I reverse: 5'-CCTTCAGGCAATGGTTCAGG-3'	This paper	N/A
ChIP-qPCR primer (<i>Th</i> promoter): Binding Site II forward: 5'-GTCCAAGCCCTGTCTAGAGG-3'	This paper	N/A
ChIP-qPCR primer (<i>Th</i> promoter): Binding Site II reverse: 5'-CTCACCCCTGAAAATGGCCAC-3'	This paper	N/A
ChIP-qPCR primer (<i>Th</i> promoter): Binding Site III forward: 5'-GCCCTGGTTTGATTAGAGAGC-3'	This paper	N/A
ChIP-qPCR primer (<i>Th</i> promoter): Binding Site III reverse: 5'-GCCTCCGTCCCATTAGATCT-3'	This paper	N/A
ChIP-qPCR primer (<i>Th</i> promoter): Binding Site IV forward: 5'-TGTCCTGAACCATTGCCTGA-3'	This paper	N/A
ChIP-qPCR primer (<i>Th</i> promoter): Binding Site IV reverse: 5'-GATCCACCCTAGCTTCCTCA-3'	This paper	N/A
PCR primer for genotype: PRLR-floxed forward: 5'-CATCTGGCATCCAAAGATGA-3'	Banerjee et al.	https://doi.org/10.2337/db15-1527
PCR primer for genotype: PRLR-floxed reverse: 5'-TGGGACCATCAATAATGCAG-3'	Banerjee et al.	https://doi.org/10.2337/db15-1527
PCR primer for genotype: NPY-Cre common forward: 5'-TCCATGATTTGCCTCTTG-3'	The Jackson Laboratory	
PCR primer for genotype: NPY-Cre reverse: 5'-ACACCGGCCTTATTCCAAG-3'	The Jackson Laboratory	
PCR primer for genotype: NPY-Cre wild type reverse: 5'-CTGAGACATAGATTTCGTTCCAAGG-3'	The Jackson Laboratory	
PCR primer for genotype: Sun1-GFP forward: 5'-ACACTTGCCTCTACCGGTTTC-3'	The Jackson Laboratory	
PCR primer for genotype: Sun1-GFP reverse: 5'-CTGAACTTGTGGCCGTTTAC-3'	The Jackson Laboratory	
PCR primer for genotype: Sun1-GFP wild type forward: 5'-AAGGGAGCTGCAGTGGAGTA-3'	The Jackson Laboratory	
PCR primer for genotype: Sun1-GFP wild type reverse: 5'-CAGGACAACGCCACACA-3'	The Jackson Laboratory	
ChIP-qPCR primer (<i>Ccn3</i> promoter): Binding Site I forward: 5'-TGTTTCCTTCTCACGAGCTG-3'	This paper	N/A
ChIP-qPCR primer (<i>Ccn3</i> promoter): Binding Site I reverse: 5'-TGTGTGTGTGAGTGAGTGGA-3'	This paper	N/A
ChIP-qPCR primer (<i>Ccn3</i> promoter): Binding Site II forward: 5'-GTCCTTTCCTCCTACCCACC-3'	This paper	N/A
ChIP-qPCR primer (<i>Ccn3</i> promoter): Binding Site II reverse: 5'-CATATGAGCTGGGCAGGGAT-3'	This paper	N/A
ChIP-qPCR primer (<i>Ccn3</i> promoter): Binding Site III forward: 5'-AGCTCTGTTTGAAGTGTGA-3'	This paper	N/A
ChIP-qPCR primer (<i>Ccn3</i> promoter): Binding Site III reverse: 5'-CCTCTTCTCTGGGTGGTTG-3'	This paper	N/A
qPCR primer (<i>Ucp1</i>) forward: 5'-CTGCCAGGACAGTACCCAAG-3'	Guo et al.	https://doi.org/10.1002/advs.202207602
qPCR primer (<i>Ucp1</i>) reverse: 5'-TCAGCTGTTCAAAGCACACA-3'	Guo et al.	https://doi.org/10.1002/advs.202207602

qPCR primer (<i>Lpl</i>) forward: 5'- ACTCGCTCTCAGATGCCCTA-3'	Ranganathan et al.	https://doi.org/10.1194/jlr.M600248-JLR200
qPCR primer (<i>Lpl</i>) reverse: 5'- TTGTGTTGCTTGCCATTCTC-3'	Ranganathan et al.	https://doi.org/10.1194/jlr.M600248-JLR200
qPCR primer (<i>Dgat1</i>) forward: 5'- CCTCAGCCTTCTTCCATGAG-3'	Ranganathan et al.	https://doi.org/10.1194/jlr.M600248-JLR200
qPCR primer (<i>Dgat1</i>) reverse: 5'- ACTGGGGCATCGTAGTTGAG-3'	Ranganathan et al.	https://doi.org/10.1194/jlr.M600248-JLR200
qPCR primer (<i>Cpt1b</i>) forward: 5'- TTCAACACTACACGCATCCC-3'	He et al.	https://doi.org/10.1161/CIRCULATIONAHA.111.075978
qPCR primer (<i>Cpt1b</i>) reverse: 5'- GCCCTCATAGAGCCAGACC-3'	He et al.	https://doi.org/10.1161/CIRCULATIONAHA.111.075978
qPCR primer (<i>Acc1</i>) forward: 5'- ATTGTGGCTCAAACCTGCAGGT-3'	Guilherme et al.	https://doi.org/10.1016/j.celrep.2023.112488
qPCR primer (<i>Acc1</i>) reverse: 5'- GCCAATCCACTCGAAGACCA-3'	Guilherme et al.	https://doi.org/10.1016/j.celrep.2023.112488
qPCR primer (<i>Npy</i>) forward: 5'- CACGATGCTAGGTAACAAG-3'	Wang et al.	https://doi.org/10.1111/jnc.12150
qPCR primer (<i>Npy</i>) reverse: 5'- CACATGGAAGGGTCTTCAAG-3'	Wang et al.	https://doi.org/10.1111/jnc.12150

Supplementary Table 4: Commercial assay information

Assay Name	Vendor	Catalog
RNAscope® Multiplex Fluorescent Reagent Kit v2 with TSA Vivid Dyes	Advanced Cell Diagnostics	Cat# 323270
RNAscope® Probe - Mm-Prlr	Advanced Cell Diagnostics	Cat# 430791
RNAscope® Probe - Mm-Th-C2	Advanced Cell Diagnostics	Cat# 317621-C2
Pierce Magnetic ChIP Kit	Thermo Scientific	Cat# 26157
Mouse Prolactin ELISA Kit	Thermo Scientific	Cat# EMPRL
Mouse Leptin ELISA Kit	Thermo Scientific	Cat# KMC2281
Free Fatty Acid Assay Kit	Abnova	Cat# KA1667
Leukocyte Acid Phosphatase (TRAP) Kit	Sigma	Cat# 387A
Norepinephrine ELISA Kit	Abnova	Cat# KA3836

Supplementary Table 5: Software information

Name	Company	Version
Zen Black	Carl Zeiss Microscopy	https://www.zeiss.com/microscopy/
Photoshop 2020	Adobe	Version 21.0.2
ImageJ	National Institutes of Health	https://imagej.net/ij/
GraphPad Prism	GraphPad Software	http://www.graphpad.com/
Vector NTI Advance	Invitrogen	https://www.thermofisher.com/us/en/home/life-science/cloning/vector-nti-software.html
NRecon	Bruker MicroCT	Version 1.6
DataViewer	Bruker MicroCT	Version 1.5
CTAn	Bruker MicroCT	Version 1.9
CTvox	Bruker MicroCT	Version 3.3
Biorender	Biorender	https://biorender.com/