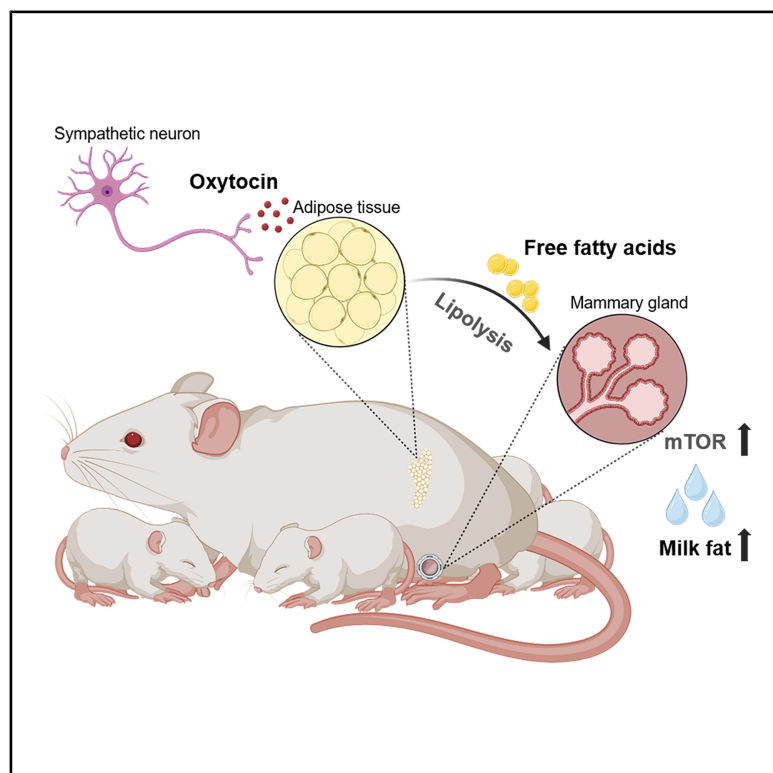


Cell Metabolism

Oxytocin signaling in adipocytes is required for normal milk fat production

Graphical abstract



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In brief

Li et al. show that sympathetic-derived oxytocin drives adipose lipolysis to supply triglycerides essential for neonatal growth during lactation. Adipocyte-specific oxytocin receptor (*Oxtr*) deletion impairs milk lipid content and offspring growth, revealing a new link between maternal oxytocin signaling in adipocytes, mammary metabolism, and neonatal nutrition.

Highlights

- Maternal loss of oxytocin signaling in adipocytes causes growth restriction in pups
- Milk lipid levels are reduced in mothers lacking adipocyte oxytocin signaling
- Loss of lipolysis causes metabolic reprogramming of mammary epithelial cells
- Maternal high-fat, but not sucrose, feeding rescues growth of pups

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Article

Oxytocin signaling in adipocytes is required for normal milk fat production

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SUMMARY

Milk triglycerides, a crucial nutrient source for newborn mammals, can be derived from adipose lipolysis, dietary sources, or *de novo* synthesis in mammary epithelial cells (MECs). Here, we identify a critical role for the neuropeptide oxytocin (OXT) in providing milk triglyceride needed to sustain neonatal growth, mediated by its actions on adipose lipolysis. Dams lacking OXT receptors (OXTRs) specifically in adipocytes (*Oxtr*^{ΔAd}) give birth to pups with reduced weight gain. Oxytocinergic sympathetic neurons are the relevant source of OXT mediating this effect. Milk from *Oxtr*^{ΔAd} dams was deficient in triglycerides, which could be rescued by liberalizing dietary fat intake. Finally, single-cell analysis of lactating mammary glands from *Oxtr*^{ΔAd} dams revealed a profound shift in the metabolic programming of MECs, indicative of reduced mTOR signaling, increased autophagy, and reduced lipid synthesis. These findings highlight the critical role of OXT-mediated adipose lipolysis in mammalian lactation, demonstrating that lipolysis-derived free fatty acids (FFAs) are essential for normal milk fat and neonatal health.

INTRODUCTION

Parturition represents a significant metabolic challenge to neonates, as their energy source transitions from maternal blood-borne nutrients to milk, which provides the calories required to support proper growth and development.¹ Milk is particularly enriched in fat, containing ~4% fat in human milk and up to 30% fat in murine milk.² Milk fat is predominantly comprised of triglycerides, which collectively constitute 98% of the total milk fat.^{3,4} Milk triglycerides originate from at least three sources: dietary intake, *de novo* synthesis in mammary epithelial cells (MECs) and liver, and adipose tissue lipolysis, although the relative importance of each of these sources, and their relationship to one another, is largely unknown. During lactation, adipose tissue lipolysis is intensified due to high energy demands, driven by neural, hormonal, and paracrine stimuli.^{5,6} Prior studies, mostly in dairy cattle, suggest that adipose lipolysis represents a major source of milk lipids. For example, during lactation, cows may mobilize 1 kg of adipose tissue lipid per day to produce milk.⁴

The mammary gland (MG) exhibits a highly heterogeneous stromal microenvironment, including adipocytes, preadipocytes, immune cells, endothelial cells, sympathetic nervous system (SNS) nerve fibers, and MECs.⁷ There is bidirectional cross-talk between adipocytes and MECs in the MG. For example, MECs secrete factors, termed “mammokines,” that regulate adipocyte functions such as thermogenesis.⁷ Conversely, adipocytes are essential for MG remodeling throughout the lactation cycle.⁸ However, the mechanisms by which mammary adipocytes affect MEC biology and milk production remain unclear.

Oxytocin (OXT) is a nine-amino-acid peptide that is primarily synthesized in the paraventricular nucleus and supraoptic nucleus of the hypothalamus and released by the posterior pituitary.^{9,10} The best-known actions of OXT are to cause uterine contractions at parturition and to promote myoepithelial cell contraction and subsequent milk let-down during lactation. OXT receptors (OXTRs) are also expressed at high abundance on the surface of adipocytes, and studies have linked OXT-OXTR signaling in adipocytes to a broad range of adipose functions, including lipolysis, insulin sensitivity, and energy



expenditure.¹⁰ Most recently, OXT was shown to signal through an ERK-mediated pathway to promote adipose lipolysis both directly and indirectly, by facilitating β -agonist-induced lipolysis.¹¹ Moreover, a subpopulation of oxytocinergic sympathetic neurons was found to be the relevant source of OXT mediating these effects.¹¹

In this study, we observed that offspring of adipocyte-specific *Oxtr* knockout (*Oxtr* ^{Δ Ad}) dams mated to wild-type (WT) males exhibit slower body weight gain than offspring from *Oxtr* ^{Δ Ad} sires mated to WT females. Sympathetic nerves innervating the MG emerged as the key source of OXT. By employing lipidomic analysis combined with *in vivo* physiological assessments, we demonstrate that lactating *Oxtr* ^{Δ Ad} dams produce milk with poorer triglyceride content than WT dams, resulting in growth restriction in their offspring. Furthermore, single-cell analysis of lactating MGs from *Oxtr* ^{Δ Ad} and WT dams revealed a profound metabolic shift in MECs, characterized by reduced mTOR signaling, increased autophagy, and decreased lipid synthesis. Our findings establish a direct link between maternal OXT signaling in adipocytes, lactation, and neonatal nutrition and growth.

RESULTS

Oxtr expression in adipocytes increases during lactation

We have previously shown that OXTR on the surface of adipocytes acts as a critical mediator of lipolysis. Isolated adipocytes from female mice exhibited significantly higher *Oxtr* mRNA levels compared with males in both depots examined (Figure S1A), corroborating previous findings,¹² and consistent with the fact that estrogen is a known inducer of *Oxtr* expression.¹³ Notably, *Oxtr* mRNA and protein levels increased significantly in adipocytes during early and late lactation compared with pregnancy and the virgin state, whereas OXTR on non-adipocytes (primarily lactocytes and myoepithelial cells) rose early in pregnancy and remained stable during lactation (Figures S1B–S1F). Because estrogen levels fall rapidly immediately following parturition,¹⁴ it is unclear what drives elevated *Oxtr* expression during lactation. Nonetheless, these data suggest that OXT actions on adipocytes might play an important role in lactation.

Offspring from *Oxtr* ^{Δ Ad} dams are growth-restricted

We noted that offspring from *Oxtr* ^{Δ Ad} dams displayed reduced body size at weaning compared with those from *Oxtr*^{fllox} dams (Figure 1A). We measured the body weight of pups from *Oxtr*^{fllox} and *Oxtr* ^{Δ Ad} dams on postnatal day (P)1 and again on P21, just prior to weaning. We observed a marked difference in body weight on P21 that was not apparent immediately after birth (Figure 1B). Further analysis revealed reductions in both fat and lean mass among offspring from *Oxtr* ^{Δ Ad} dams (Figure 1C), with significant decreases observed in white adipose tissue (WAT) and lung mass (Figure 1D). This phenomenon is also observed on subsequent breeding cycles (Figure 1E). Moreover, fewer pups were noted at weaning in litters belonging to *Oxtr* ^{Δ Ad} dams; again, this was not seen on P1 (Figure 1F). We considered whether this reduction in survival could be due to competition between littermates for nutrition; consistent with this, we noted that capping litter size at 4–5 suppressed excess mortality at weaning but did not affect growth restriction (Figures 1G and 1H).

Growth restriction of offspring from *Oxtr* ^{Δ Ad} dams is driven by maternal factors

We next asked whether the growth-restriction phenotype of offspring from *Oxtr* ^{Δ Ad} dams is dependent on factors intrinsic to the pups themselves, or whether it might reflect maternal issues. A similar degree of growth restriction was observed in both male and female offspring (Figure 2A). Similarly, the phenotype was not attributable to the genotype of the pup, as both *Oxtr* ^{Δ Ad} and *Oxtr*^{fllox} offspring from *Oxtr* ^{Δ Ad} dams exhibited lower body weight prior to weaning (Figure 2B).

To more rigorously test the hypothesis that the growth restriction phenotype is dependent on maternal factors, we performed a cross-fostering experiment, in which offspring born to *Oxtr*^{fllox} dams were fostered by *Oxtr* ^{Δ Ad} dams, and vice versa (Figure 2C). Reduced weight gain was seen in pups born to *Oxtr*^{fllox} dams but fostered by *Oxtr* ^{Δ Ad} dams, whereas pups born to *Oxtr* ^{Δ Ad} dams but fostered by *Oxtr*^{fllox} dams exhibited normal weight (Figures 2D, S2A, and S2B). The reduced body weight of affected pups does not persist into adulthood; consistent with this, fasting insulin, glucose, and insulin tolerance, and food intake are all indistinguishable between adult offspring of *Oxtr*^{fllox} and *Oxtr* ^{Δ Ad} dams (Figures S2C–S2G).

OXT from sympathetic nerves regulates growth of offspring

OXT is commonly thought of as being derived solely from the supraoptic and paraventricular nuclei of the hypothalamus, entering the bloodstream via the posterior pituitary. We have previously shown, however, that intra-adipose OXT derives in significant part from a subpopulation of oxytocinergic sympathetic neurons.¹¹ Mice in which the *Oxt* gene is conditionally ablated using tyrosine hydroxylase (TH)-Cre (*Oxt* ^{Δ TH}) have normal serum, but reduced intra-adipose OXT.¹¹ Here, we confirmed that serum OXT levels across lactation do not differ between *Oxt* ^{Δ TH} and control mice (Figure S3A). Pups born to *Oxt* ^{Δ TH} dams phenocopy those born to *Oxtr* ^{Δ Ad} dams, exhibiting severe growth restriction and poor neonatal survival when litter size is not capped (Figures S3B and S3C), indicating that sympathetic oxytocinergic neurons likely represent the relevant source of OXT in the MG.

Reduced lipid levels in the milk of *Oxtr* ^{Δ Ad} dams

Given that maternal factors drive the growth restriction observed in offspring of *Oxtr* ^{Δ Ad} dams, we speculated that lactation might be impaired in these animals. Milk volume, as determined by the weigh-suckle-weigh protocol, was not statistically different between *Oxtr*^{fllox} and *Oxtr* ^{Δ Ad} dams, although there was a trend toward reduced volume in *Oxtr* ^{Δ Ad} mice (Figure S4A). Similarly, levels of milk protein were not different between the genotypes, as assessed by total amino acid content, total protein content, and by western blotting of the major milk protein constituents, such as α - and β -casein, whey acidic protein, lactoferrin, and albumin (Figures S4B–S4E). Lactose levels were also not different between the two groups (Figure S4F). In contrast to these unchanged nutrients, we observed lower triglyceride levels in milk from *Oxtr* ^{Δ Ad} dams compared with those from *Oxtr*^{fllox} dams (Figure 3A).

To delve deeper into the impact of adipocyte OXT signaling on milk lipid composition, we performed an untargeted lipidomic

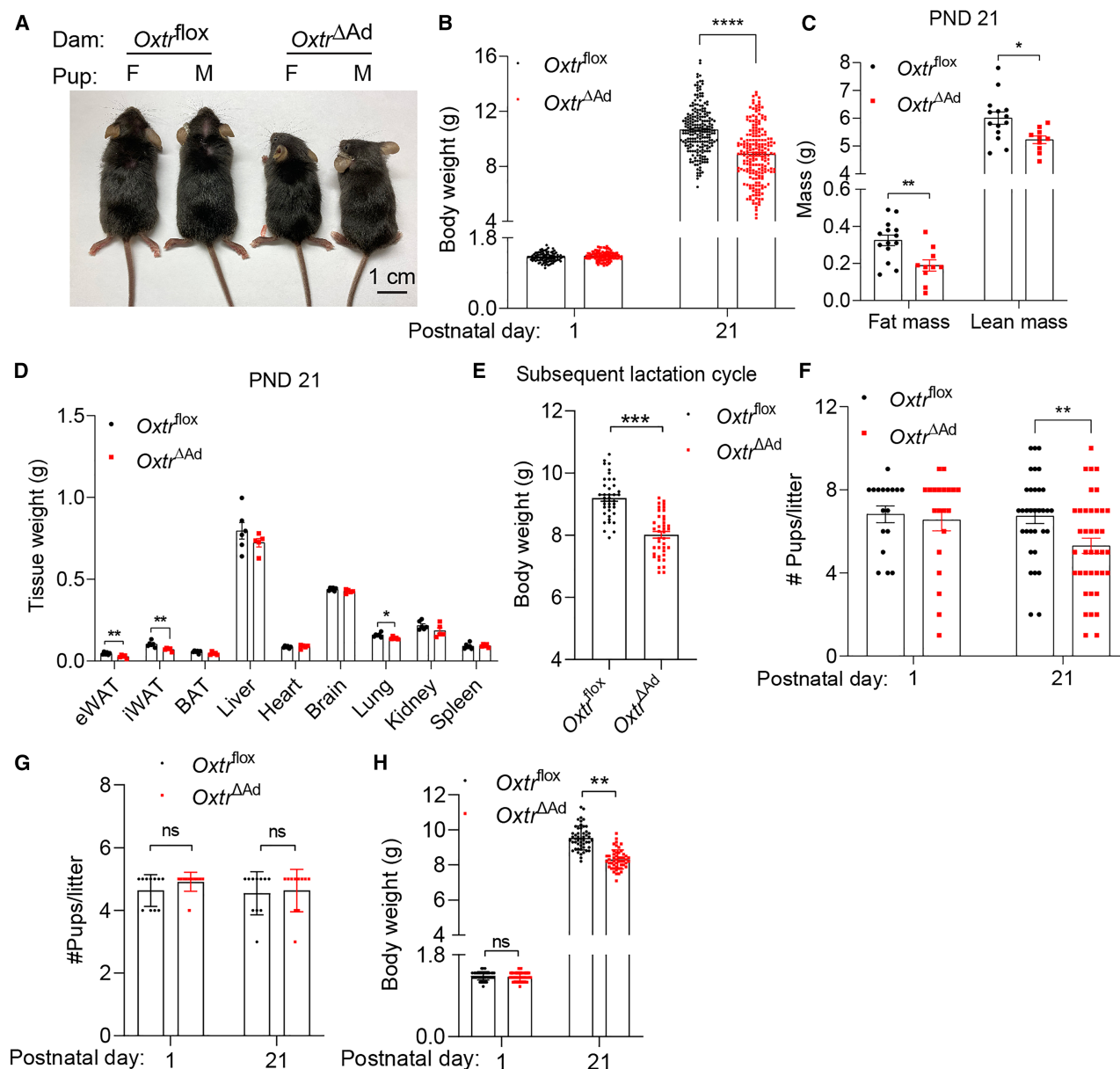


Figure 1. Pups of $Oxt^{\Delta Ad}$ dams are growth restricted and less likely to reach weaning age

(A) Representative appearance of male (M) and female (F) pups of Oxt^{fl} and $Oxt^{\Delta Ad}$ dams on postnatal day (P)21. Scale bars, 1 cm.
(B) Quantification of body weight of pups from Oxt^{fl} and $Oxt^{\Delta Ad}$ dams on P1 and P21. Pups of Oxt^{fl} dams on P1, $n = 96$; pups of Oxt^{fl} dams on P21, $n = 222$; pups of $Oxt^{\Delta Ad}$ dams on P1, $n = 108$; pups of $Oxt^{\Delta Ad}$ dams on P21, $n = 205$.
(C) Lean mass and fat mass of pups from Oxt^{fl} and $Oxt^{\Delta Ad}$ dams on P21; $n = 10$ –14.
(D) Tissue weight of pups from Oxt^{fl} and $Oxt^{\Delta Ad}$ dams on P21; $n = 5$ –6.
(E) Body weight of pups from Oxt^{fl} and $Oxt^{\Delta Ad}$ dams on P21 on a subsequent breeding cycle; $n = 40$.
(F) Number of newborn pups per litter from Oxt^{fl} and $Oxt^{\Delta Ad}$ dams on P1 and the number of pups per litter that survived through P21. Litters of Oxt^{fl} dams on P1, $n = 17$; litters of Oxt^{fl} dams on P21, $n = 33$; litters of $Oxt^{\Delta Ad}$ dams on P1, $n = 20$; litters of $Oxt^{\Delta Ad}$ dams on P21, $n = 39$.
(G) Number of newborn pups per litter from Oxt^{fl} and $Oxt^{\Delta Ad}$ dams on P1 and the number of pups per litter that survived through P21 when litter size was capped at 4–5; $n = 11$.
(H) Body weight of pups from Oxt^{fl} and $Oxt^{\Delta Ad}$ dams on P1 and 21 when litter size was capped at 4–5; $n = 50$ –54.
Data are presented as mean $\pm$ SEM. Statistical comparisons were made using a two-tailed Student's t test (B–E). * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

assessment of milk from Oxt^{fl} and $Oxt^{\Delta Ad}$ dams (Table S1). Principal component analysis (PCA) of the data revealed that lipid profiles diverge between milk samples from Oxt^{fl} and

$Oxt^{\Delta Ad}$ dams, with PC1 explaining 85.3% of the variance (Figure S4G). Among the 1,422 lipid species detected in murine milk, 412 were significantly altered in milk from $Oxt^{\Delta Ad}$ dams,

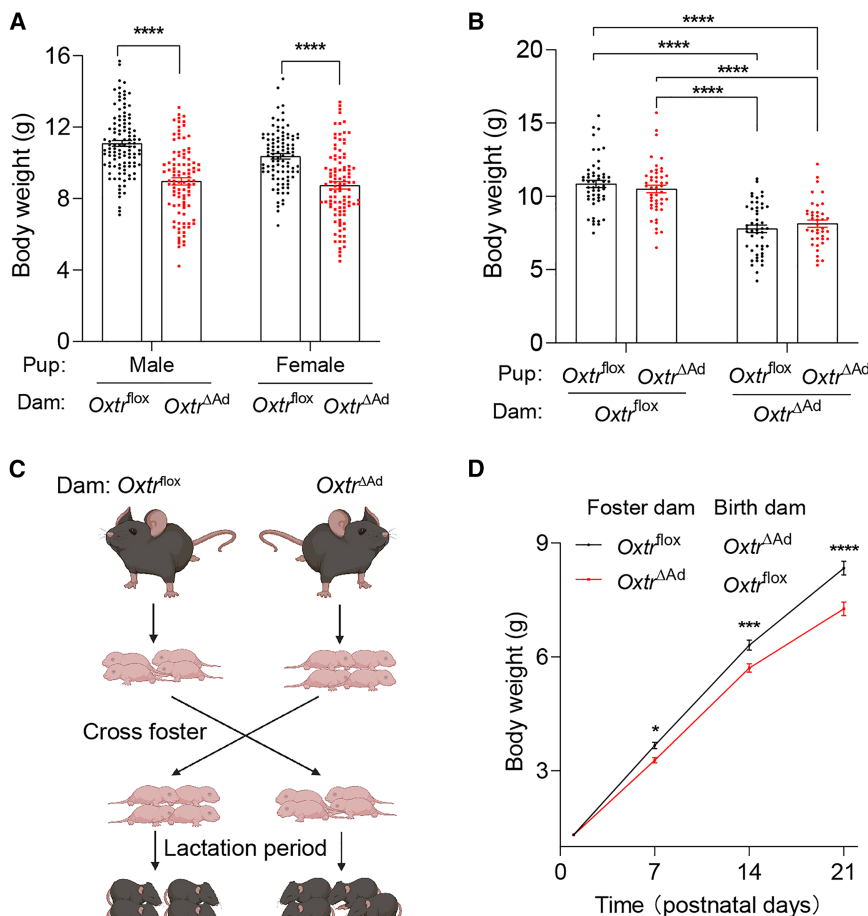


Figure 2. Growth restriction of offspring from *Oxttr^{ΔAd}* dams is caused by maternal factors

(A) Body weight of pups from *Oxttr^{flox}* and *Oxttr^{ΔAd}* dams on P21, separated by the sex of pups; $n = 97-112$.

(B) Body weight of pups of *Oxttr^{flox}* and *Oxttr^{ΔAd}* dams on P21, separated by the genotype of pups; $n = 40-52$.

(C) Schematic of the cross-fostering experiment determining the effect of dam genotype on pup growth during lactation. Pups from *Oxttr^{ΔAd}* dams crossed to *Oxttr^{flox}* sires on P1 were fostered by *Oxttr^{flox}* dams, and vice versa. The body weight of pups was measured weekly over the lactation period. Litter size was capped at 4–6.

(D) Trajectories of body weight of fostered pups over the lactation period; $n = 36-65$.

Data are presented as mean $\pm$ SEM. Statistical comparisons were made using two-tailed Student's *t* test (A and B) or two-way ANOVA (D). * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$.

mary-adjacent adipose tissue from *Oxttr^{flox}* and *Oxttr^{ΔAd}* dams, hypothesizing that the inability of *Oxttr^{ΔAd}* dams to efficiently mobilize adipose fat for milk lipid production would be reflected histologically. As predicted, deficient OXT signaling in adipocytes resulted in a more prominent adipose fraction adjacent to the mammary epithelium (Figures S5A–S5C). This finding aligns with diminished lipolysis seen in lactating *Oxttr^{ΔAd}* dams, as evidenced by reduced

with 81% of these (333 lipid species) being significantly reduced. Many major lipid classes, including triglycerides, diglycerides, phospholipids, ceramides, and sphingomyelins, were significantly depleted in milk from *Oxttr^{ΔAd}* dams (Figure 3B). The most striking difference was noted in a broad array of abundant triglycerides, which were reduced by 72% in milk from *Oxttr^{ΔAd}* dams (Figure 3C). Within milk triglycerides, we were able to detect 38 unique fatty acids (FAs), 29 of which were reduced (Figure 3D). This includes dramatic reductions in medium-chain FAs, as C8:0, C10:0, C12:0, and C14:0 were all reduced by 73%–78%. These lipids are unique in that they are produced endogenously by MECs, suggesting that they, too, are dependent on OXT-dependent signaling and lipolysis. Odd chain FAs (C13:0, C15:0, and C17:0, along with their monounsaturated versions) were among the only unchanged FAs in the triglyceride pool, potentially suggesting a reliance on dietary FAs, as these are thought to be obtained exclusively from the diet. Our lipidomics data do not enable us to assign stereospecificity, so we cannot conclusively distinguish n6 from n3 FAs.

Reduced delipidation in the MG of lactating *Oxttr^{ΔAd}* dams

Lactation is associated with significant delipidation of adipocytes, both within the inguinal depot that surrounds the MG and elsewhere.^{15–17} We examined the morphology of mam-

serum FAs (Figure S5D) and elevated fat mass (Figure S5E), and is consistent with our prior work demonstrating a role for OXT in lipolysis.¹¹ Following weaning, involution of the lactating MG is normal in *Oxttr^{ΔAd}* dams (Figures S5A and S5B).

Loss of OXTR in adipocytes reprograms the metabolism of MECs

MECs synthesize milk lipids almost exclusively in the form of medium-chain FAs, due to the presence of acyl CoA thioesterases that terminate chain lengthening earlier than in other lipogenic tissues.^{18,19} The observed growth restriction of pups, however, combined with the reduction of medium-chain FAs in milk from *Oxttr^{ΔAd}* dams, suggested to us that MECs were unable to compensate for the loss of adipose lipids by increasing local synthesis. To assess how metabolism might be reprogrammed in MECs in the absence of adipose lipolysis, we performed single-nucleus RNA sequencing (snRNA-seq) of MGs from *Oxttr^{flox}* and *Oxttr^{ΔAd}* dams, collected at 11 days post-partum. Overall, 13,035 nuclei were sequenced, enabling us to recover all expected cell types, including MECs (virtually all of which were lactocytes), basal-myoeptithelial (B-Myo) cells, adipocytes, adipose progenitor cells, luminal hormone-sensing (LHS) cells, luminal adaptive secretory precursors (LASPs),²⁰ vascular, and immune cells (Figures 4A and S6A). PCA showed clear demarcation between sample genotypes, accounted for primarily by increases

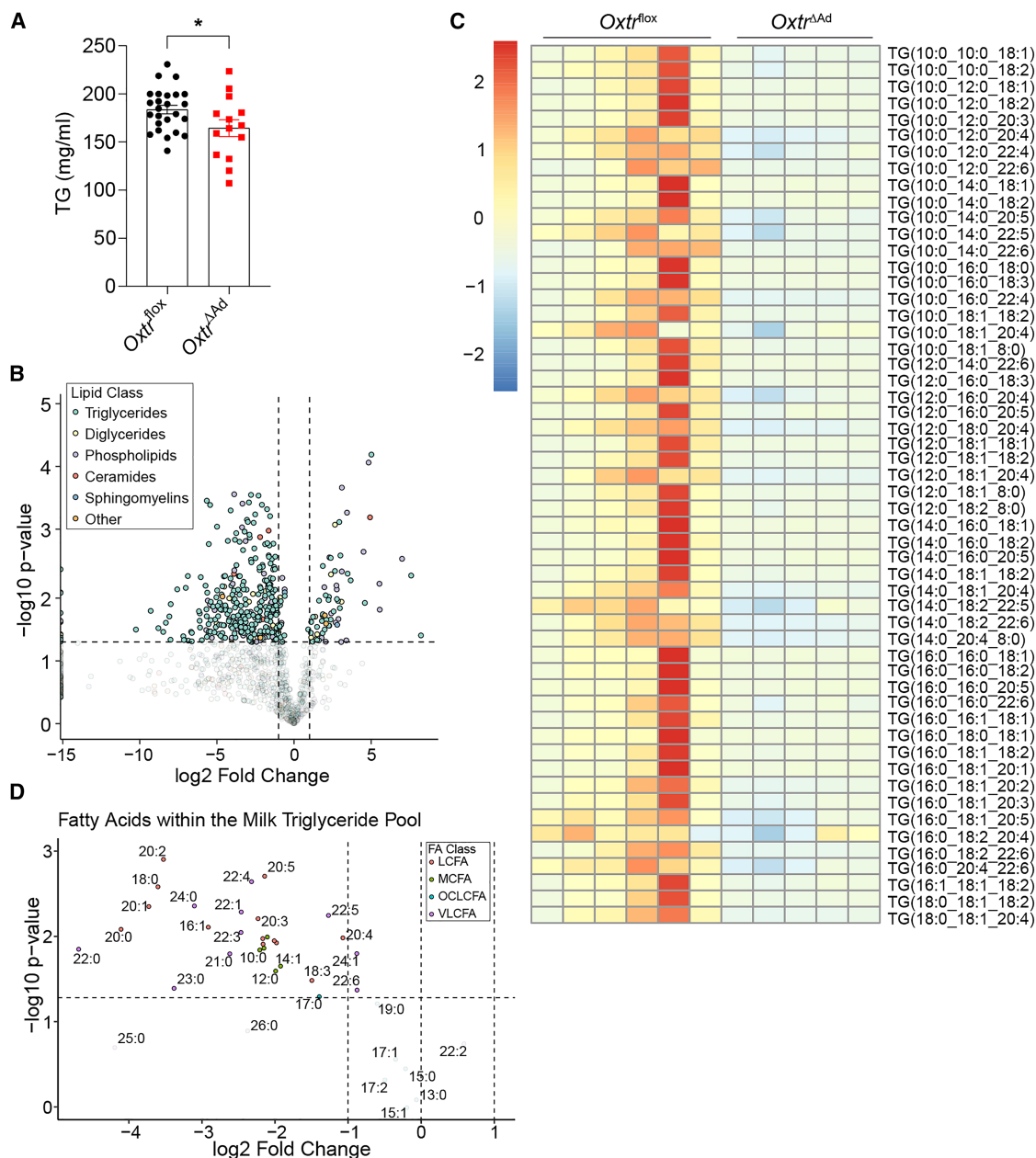


Figure 3. Lactating *Oxt^{ΔAd}* dams display reduced triglyceride levels in milk

(A) Triglyceride levels in milk collected from *Oxt^{fl}* and *Oxt^{ΔAd}* dams on P16; $n = 14\text{--}25$.

(B) Volcano plot showing the $\log_2$ fold change in milk lipid abundance versus $-\log_{10}(p \text{ value})$ in milk samples from *Oxt^{fl}* and *Oxt^{ΔAd}* dams on P16. The dotted line indicates the threshold of statistical significance (FDR-adjusted $p < 0.05$) and fold change ($\log_2\text{FC} > 1$ or < -1). Different lipid species are color-coded as shown in the plot; $n = 5$.

(C) Heatmap showing levels of abundant milk triglycerides in *Oxt^{fl}* and *Oxt^{ΔAd}* dams on P16. Color intensity represents changes in triglyceride levels, with blue indicating a decrease and red indicating an increase in *Oxt^{fl}* or *Oxt^{ΔAd}* dams; $n = 5$.

(D) Volcano plot showing the $\log_2$ fold change in fatty acids within the milk triglyceride pool versus $-\log_{10}(p \text{ value})$ in milk samples from *Oxt^{fl}* and *Oxt^{ΔAd}* dams on P16. The dotted line indicates the threshold of statistical significance (FDR-adjusted $p < 0.05$), the separation of decrease and increase, and the fold change cutoff ($\log_2\text{FC} > 1$ or < -1). Different fatty acids are color-coded as shown in the plot; $n = 5$.

Data are presented as mean $\pm$ SEM. Statistical comparisons were made using two-tailed Student's t test (A); $*p < 0.05$. Milk lipids were log-transformed to normalize and then analyzed via Student's t tests between groups. p values were adjusted for multiple comparisons by the method of Benjamini and Hochberg (B–D).

in some MEC subpopulations (Figures S6B–S6D). Of note, MEC subpopulations were distinguished mainly by differences in the relative levels of key marker genes (Figure S6A); accordingly, we combined all MECs into a single bin for pseudo-bulk analysis.

A relatively small number of individual genes were significantly up- ($n = 20$) or downregulated ($n = 8$) in MECs from *Oxtr*^{ΔAd} samples (Figure 4B). Contrary to our expectations, we did not observe increased expression of genes of lipid transport or synthesis, apart from small increases in *Slc27a1* (encoding the FA transporter FATP1) and the docosahexaenoic acid (DHA) transporter *Mfsd2a* in selected MEC subtypes (Figure S6E). Pathway analysis, however, revealed significant alterations in several biochemical pathways (Table S2). Most notably, downregulated pathways included ribosome biogenesis, amino acid biosynthesis, and amino-acyl tRNA synthesis, whereas upregulated pathways included autophagy, amino acid catabolism, and FAD degradation (Figure 4C). Taken together, these findings suggested that mTOR activity might be diminished in MECs from *Oxtr*^{ΔAd} dams. Consistent with this, we noted reduced phosphorylation of many (e.g., AKT2S473, S6KT389, and 4E-BP1T70) mTOR targets (Figure 4D). Phosphorylation of ribosomal protein S6, a direct target of S6K that mediates the regulatory actions of mTOR signaling on protein synthesis, is also reduced in MGs of *Oxtr*^{ΔAd} dams (Figure 4D). Consistent with reduced mTORC1 signaling, enhanced autophagy was confirmed in *Oxtr*^{ΔAd} samples by assessment of LC-3 cleavage and lipidation (Figure 4E). We also noted upregulation of *Bnip3* mRNA and protein, which encodes a well-known inhibitor of mTOR signaling²¹ (Figures 4B and 4E). Taken together, these data indicate that MECs from *Oxtr*^{ΔAd} dams may be energy starved due to loss of free FAs (FFAs) from adipose lipolysis; subsequent reduction of mTORC1/2 activity may prevent *de novo* synthesis and secretion of milk lipids in these cells.

One possible explanation for altered mTOR activity in MECs could be altered insulin or prolactin levels; however, we found that these hormones did not differ between *Oxtr*^{ΔAd} and control dams (Figures S7A and S7B).

HFD feeding during lactation rescues the growth restriction of offspring from *Oxtr*^{ΔAd} dams

We considered the possibility that the growth restriction of pups from *Oxtr*^{ΔAd} dams might not result solely from the reduced lipid content of the milk. Alternatively, or in addition, we wondered whether there might be increased amounts of a “toxic” lipid or other substance in the milk. We reasoned that if excess dietary lipids could “rescue” growth in the offspring of *Oxtr*^{ΔAd} dams, it would argue in favor of reduced lipolysis as the cause of the growth restriction in these mice. To address this possibility, we placed timed pregnant *Oxtr*^{fllox} and *Oxtr*^{ΔAd} mice on a high-fat diet (HFD), starting 1 week before parturition. Some pregnant mice of both genotypes were continued on a chow diet as a control (Figure 5A). Maternal high-fat feeding during lactation is a well-documented method to enhance milk lipid content.^{22–25} As anticipated, administration of HFD effectively normalized the serum FFA levels and milk triglyceride levels of *Oxtr*^{ΔAd} dams (Figures 5B and 5C).

Consistent with our earlier observations, offspring of chow-fed *Oxtr*^{ΔAd} dams exhibited reduced weight gain compared with pups of *Oxtr*^{fllox} dams on the same diet (Figures 5D and 5E).

Notably, the introduction of HFD mitigated the growth restriction in the offspring of *Oxtr*^{ΔAd} dams (Figures 5D and 5E), suggesting that the added dietary lipids compensated for the loss of adipose lipolysis. Furthermore, HFD feeding restored both lean and fat mass, as well as the lower tissue weights previously observed in the offspring of chow-fed *Oxtr*^{ΔAd} dams (Figures S7C and S7D). High-fat feeding also reversed the defect in mTOR signaling in the mammary tissue of *Oxtr*^{ΔAd} dams but did not fully restore BNIP3 levels (Figures 5F and 5G). These results indicate that reduced milk fat content is a major contributor to the observed growth restriction in their offspring of *Oxtr*^{ΔAd} dams, and that lipid supplementation via the maternal diet can effectively reverse this phenotype. Interestingly, *Oxtr*^{ΔAd} dams do not consume more food than WT dams at baseline (Figure S7E), suggesting that there is no intrinsic drive to compensate for the reduced caloric content of their milk.

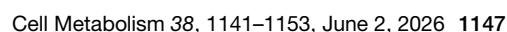
We wanted to know whether lipid is specifically required, per se, or whether any source of calories would rescue the pups of *Oxtr*^{ΔAd} dams. To that end, we supplemented lactating *Oxtr*^{ΔAd} dams with water containing sucrose (10% w/v), providing an equivalent number of calories as the HFD (Figure S7F). Notably, sucrose supplementation did not rescue the body weight of pups from *Oxtr*^{ΔAd} dams (Figures S7G–S7I). This experiment demonstrates that the growth restriction of these pups is due to a lack of adequate lipid from the mother, and not simply deficient calories, per se.

Offspring from *Pnpla2*^{ΔAd} dams are also growth-restricted

To confirm that the growth restriction of *Oxtr*^{ΔAd} pups is due to deficient lipolysis, and not some other action of OXT on adipocytes, we assessed whether another model of impaired adipose lipolysis would manifest the same phenotype. Adipose triglyceride lipase (ATGL, encoded by the *Pnpla2* gene) is a rate-limiting enzyme in adipose lipolysis. Adipocyte-specific *Pnpla2* knockout (*Pnpla2*^{ΔAd}) mice have been shown to exhibit significantly impaired lipolysis,²⁶ thus providing an orthologous model to assess the necessity of adipose lipolysis for offspring development. As with the offspring of *Oxtr*^{ΔAd} dams, the offspring of *Pnpla2*^{ΔAd} dams displayed reduced body weight gain compared with those of *Pnpla2*^{fllox} dams during lactation (Figures 6A and 6B). Growth restriction in this model was driven by a reduction in fat mass and a similar trend in whole-body lean mass (Figure 6C), with significant decreases observed in WAT, BAT, liver, heart, and lung (Figure 6D). Analogous to the *Oxtr*^{ΔAd} model, *Pnpla2*^{ΔAd} dams show defects in mTOR signaling in their mammary tissue, which are largely restored following high-fat feeding (Figures 6E and 6F). High-fat feeding also rescues pup weight in the offspring of *Pnpla2*^{ΔAd} dams (Figure 6G). These findings highlight the critical role of adipose lipolysis in the provision of milk fat, and thus normal pup growth.

DISCUSSION

OXT is an ancient hormone that has been co-opted to serve critical roles in mammalian reproduction, ranging from uterine contraction to the establishment of maternal behavior.^{27–31} OXT also has a well-established role in lactation, the defining feature of the mammalian lineage, by inducing contraction of



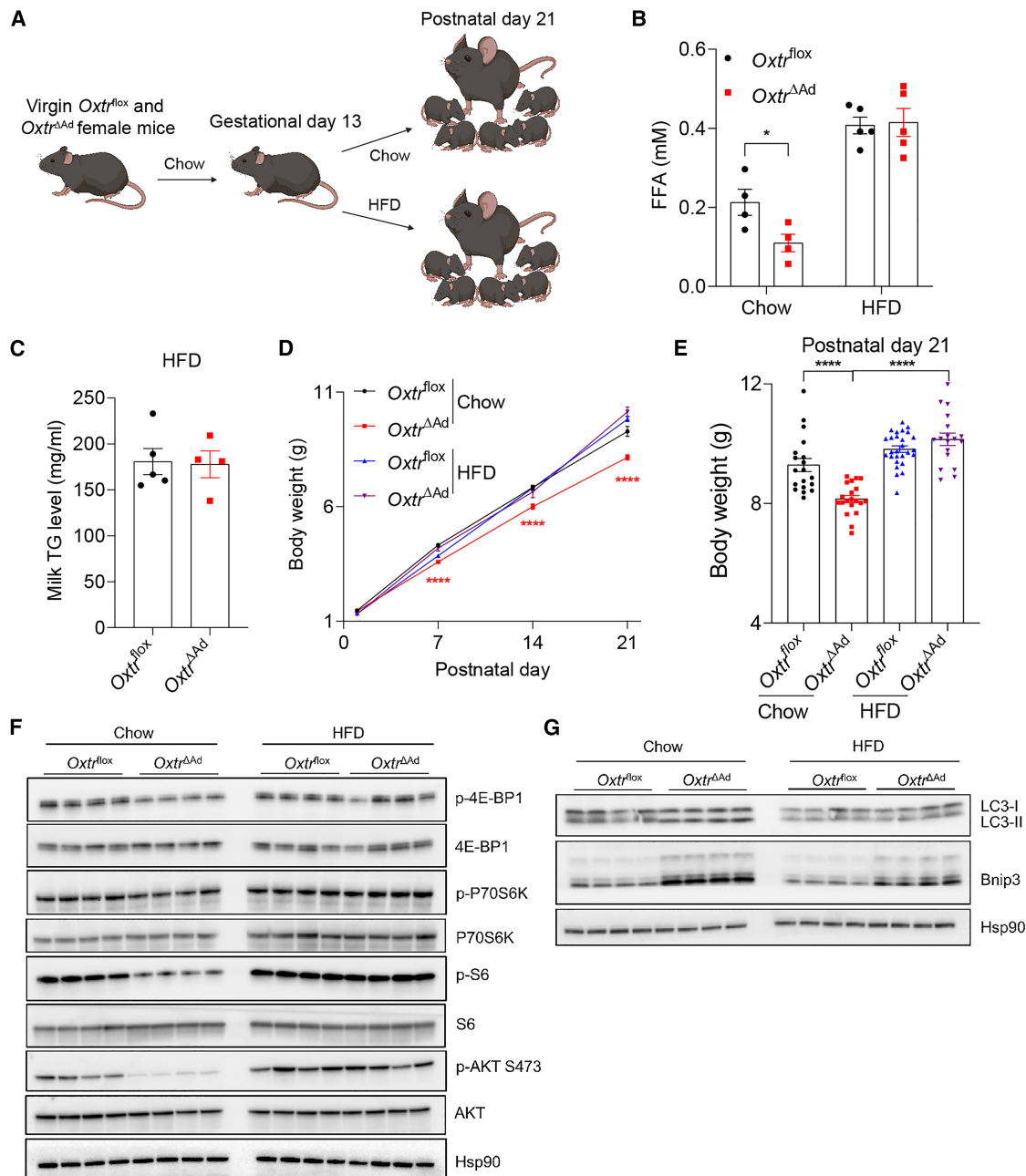


Figure 5. Adding lipid back to milk rescues the phenotype of pups of *Oxtr^{ΔAd}* dams

(A) Schematic of the dietary lipid supplementation experiment designed to test whether lipid addition to milk rescues the impaired development of offspring from *Oxtr^{ΔAd}* dams. Initially, *Oxtr^{flox}* and *Oxtr^{ΔAd}* female mice were maintained on a chow diet. On gestational day 13, they were divided into two groups—one continued on the chow diet, whereas the other was switched to a high-fat diet (HFD). Offspring body weight was then monitored throughout the lactation period.

(B) Serum FFA levels in lactating *Oxtr^{flox}* and *Oxtr^{ΔAd}* dams on chow or HFD on P11; *n* = 4–5.

(C) Triglyceride levels in milk collected from *Oxtr^{flox}* and *Oxtr^{ΔAd}* dams on HFD on P16; *n* = 4–5.

(D) Body weight of pups from *Oxtr^{flox}* and *Oxtr^{ΔAd}* dams on chow or HFD during lactation; *n* = 19–44.

(E) Body weight of pups of *Oxtr^{flox}* and *Oxtr^{ΔAd}* dams on chow or HFD on P21; *n* = 19–27.

(F) Western blots showing p4E-BP1 T70, 4E-BP1, pS6K T389, S6K, pS6 S235/236, S6, pAKT S473, and AKT protein levels in whole mammary gland lysates from lactating *Oxtr^{flox}* and *Oxtr^{ΔAd}* dams on either chow or HFD on P11 of lactation; *n* = 4.

(G) Western blots showing LC3-I, LC3-II, and Bnip3 protein levels in mammary glands from lactating *Oxtr^{flox}* and *Oxtr^{ΔAd}* dams on either chow or HFD on P11; *n* = 4.

Data are presented as mean ± SEM. Statistical comparisons were made using two-tailed Student's *t*-test (B, C, and E) or two-way ANOVA (D). **p* < 0.05; *****p* < 0.0001.

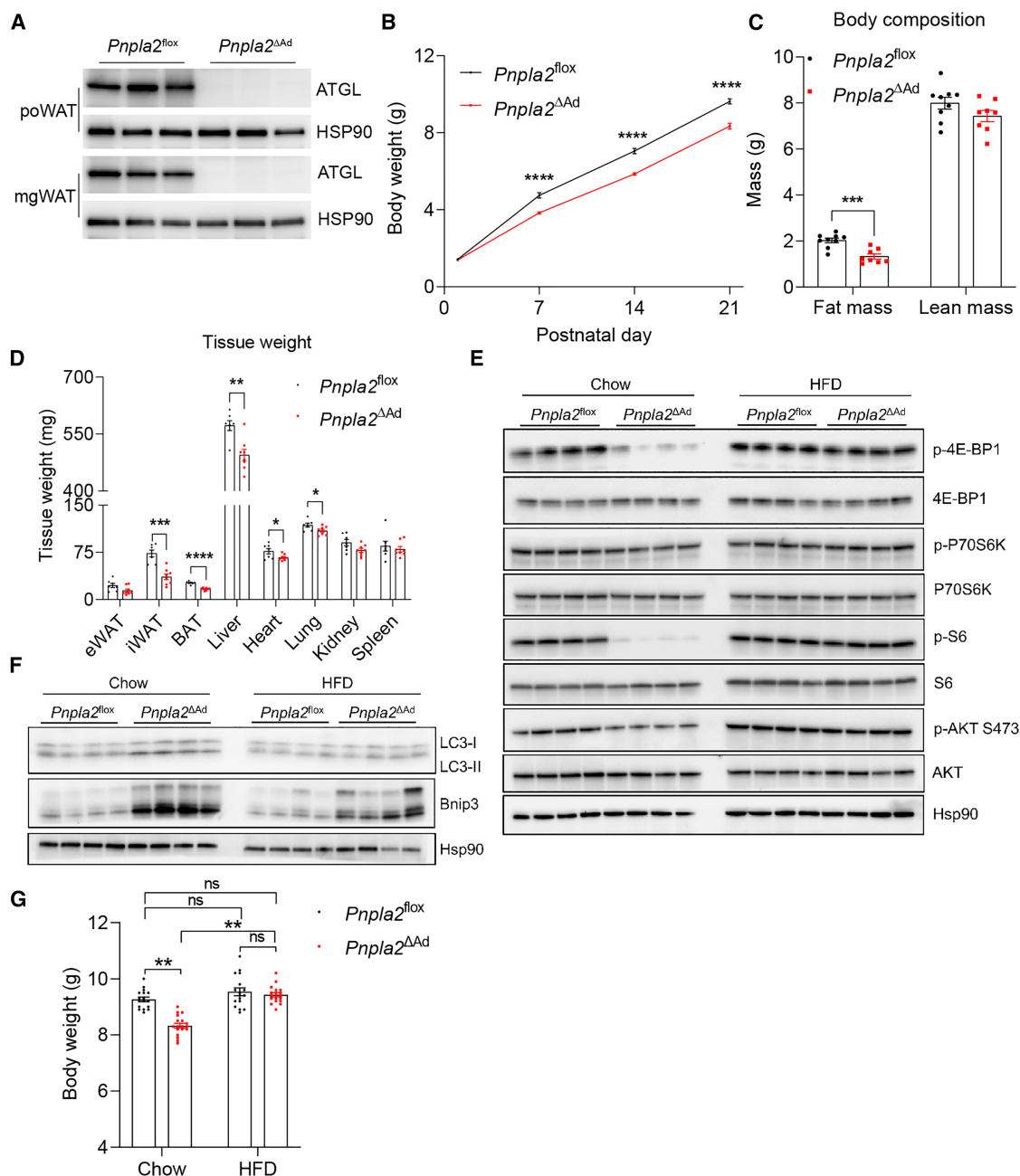


Figure 6. Offspring of *Pnpla2^{ΔAd}* dams exhibit impaired development during lactation

(A) Western blots showing ATGL protein levels in periovarian and mammary gland white adipose tissue from *Pnpla2^{lox}* and *Pnpla2^{ΔAd}* dams.
 (B) Body weight of pups of *Pnpla2^{lox}* and *Pnpla2^{ΔAd}* dams during lactation; *n* = 31–33.
 (C) Body composition of pups of *Pnpla2^{lox}* and *Pnpla2^{ΔAd}* dams on P21; *n* = 8–9.
 (D) Tissue weight of pups of *Pnpla2^{lox}* and *Pnpla2^{ΔAd}* dams on P21; *n* = 7–8.
 (E) Western blots showing p4E-BP1 T70, 4E-BP1, pS6K T389, S6K, pS6 S235/236, S6, pAKT S473, and AKT protein levels in whole mammary gland lysates from lactating *Pnpla2^{lox}* and *Pnpla2^{ΔAd}* dams on either chow or HFD on P11 of lactation; *n* = 4.
 (F) Western blots showing LC3-I, LC3-II, and Bnip3 protein levels in mammary glands from lactating *Pnpla2^{lox}* and *Pnpla2^{ΔAd}* dams on either chow or HFD on P11; *n* = 4.
 (G) Body weight of pups of *Pnpla2^{lox}* and *Pnpla2^{ΔAd}* dams on either chow or HFD on P21; *n* = 17–18.
 Data are presented as mean ± SEM. Statistical comparisons were made using two-tailed Student's *t* test (C, D, and G) or two-way ANOVA (B). **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001.

restriction in these pups could be rescued by liberalizing the intake of dietary fat. Finally, single-cell analysis of lactating MGs from *Oxtr*^{ΔAd} and WT dams revealed a profound shift in the metabolic programming of MECs, indicative of reduced mTOR signaling, increased autophagy, and reduced lipid synthesis.

OXT is involved in multiple aspects of mammalian reproduction, ranging from the promotion of uterine contraction to the transmission of maternal behavior.^{29–31,33,34} OXT has also been shown to affect lactation through its actions on myoepithelial cells of the MG, stimulating alveolar and ductal contractions by elevating intracellular calcium levels in myoepithelial cells, thereby facilitating milk ejection.³⁵ Our study extends these known functions of OXT by revealing its role in milk fat production through the stimulation of adipose lipolysis, which provides FFA and glycerol for triglyceride synthesis in MECs. Interestingly, the relevant source of OXT is TH+ sympathetic neurons. Previous studies have implicated sympathetic nerves as regulators of adult stem cell regeneration in the MG³⁶ and as drivers of bone resorption, thus providing calcium for milk.³⁷ Our work thus adds to the repertoire of sympathetic function in lactation.

Because of their high energy density, lipids in milk are the major calorie source for neonatal growth.³⁸ Milk lipids also enable the transfer of fat-soluble vitamins to the infant and provide substrates for membrane synthesis.³⁹ Perhaps unsurprisingly, much of our knowledge of milk lipid production comes from studies in dairy cows. In cattle, the importance of adipose tissue lipolysis to milk fat production is implied by mobilization of up to 1 kg of adipose tissue lipid per day,⁴ and there is generally a good correlation between milk fat percentage and plasma FFAs.⁴⁰ Genes associated with lipolysis are induced in bovine adipocytes in early lactation, and the lipolytic activity of fat explants is higher when harvested in the immediate post-partum period than when collected pre-partum.^{5,6} Lactating women have also been shown to have higher rates of lipolysis than women who use formula.⁴¹

Hormonal regulation of adipose lipolysis during lactation in non-ruminants has been ascribed mainly to a fall in serum insulin levels and increased insulin resistance.^{42,43} Our previous results demonstrating that endogenous OXT is also an important prolipolytic agent suggested a convergence of OXT activity in lactation, driving milk lipid synthesis in addition to its well-known ability to induce milk let-down. The increased expression of OXTR on adipocytes in lactation is consistent with the idea of enhanced sensitivity to OXT when lipolysis is most needed.

The total concentration of milk triglyceride levels varies between species but is relatively constant within a species. The specific identity of FA constituents of triglycerides, however, differs greatly depending on the diet and availability of fat stores. Thus, women consuming western HFDs (25%–40% calories as lipids) exhibit lower levels of medium-chain FFAs produced in MECs than women who eat a low-fat diet.⁴² In both humans and rodents, switching from a high-fat to a low-fat diet increases the proportion of medium-chain triglycerides in milk, without affecting overall triglyceride concentrations.^{42,44,45} This implies a level of molecular coordination, such that MECs can sense dietary or stored lipid and adjust their own lipid biosynthesis accordingly. Based on this, we predicted that impaired lipolysis caused by ablation of OXTR (or ATGL) in adipocytes would be

compensated by enhanced MEC lipid biosynthesis, with or without increased food intake. However, we saw neither of these adaptations; *Oxtr*^{ΔAd} dams did not eat more than controls, nor did their milk contain increased levels of medium-chain triglycerides. In fact, reduced levels of medium-chain triglycerides in their milk indicate that lipid synthesis in MECs is paradoxically repressed.

To assess what might be driving this, we utilized snRNA-seq to characterize the cellular composition and gene expression in lactating MGs from *Oxtr*^{ΔAd} and WT dams. Our results indicate significant reprogramming of MEC metabolism in the setting of reduced lipolysis, with a gene expression pattern suggestive of reduced protein synthesis, enhanced amino acid (especially branched-chain amino acid [BCAA]) degradation, and increased autophagy. BCAAs provide nitrogenous substrates for glutamate, glutamine, alanine, and aspartate synthesis in the MG, and also serve as an important energy source in MECs.⁴⁶ We speculate that, in the absence of robust lipolysis, MECs become starved for energy and thus consume BCAAs, obtained in part through enhanced autophagy. BCAAs are an important activator of mTOR,⁴⁷ and so the degradation of BCAAs might be expected to reduce mTOR activity, which was, in fact, observed in MGs from *Oxtr*^{ΔAd} dams. Reductions in mTOR activity could then account for the lack of compensatory *de novo* lipogenesis (DNL) in *Oxtr*^{ΔAd} MECs. Interestingly, lactose and major protein levels were spared in milk from *Oxtr*^{ΔAd} dams, suggesting that, in the setting of energy deprivation, MECs prioritize the formation of these sources of milk nutrition.

Another potential source of milk lipid is hepatic DNL. A recent study demonstrated that DNL is elevated in the livers of lactating mice, and blocking this by deletion of acetyl-coenzyme A synthetase 2 (ACSS2) reduces milk fat.⁴⁸ While we did not directly measure hepatic DNL in our model, it is noteworthy that dietary supplementation with sucrose, a major substrate for DNL, did not rescue the growth-restricted phenotype. Taken together, our data point to a critical adipocyte-MEC axis regulating the metabolism of the actively lactating MG, under the control of local OXT. These findings highlight the critical role of OXT-mediated adipose lipolysis in mammalian lactation, demonstrating that FFAs derived from lipolysis are essential for normal mammary milk fat and the health of offspring.

Limitations of the study

Our studies have certain limitations. First, mouse milk contains higher levels of lipid (up to 30%) than human milk (typically 4%), suggesting that newborn mice have a fundamentally different energy demand compared with humans and cows. Therefore, the quantitative impact of lipolysis on triglyceride content may differ in non-rodent species, which warrants further investigation. However, studies have shown that exogenous OXT affects milk yield and composition in cows.⁴⁹ It is also possible that oxytocin-induced adipose lipolysis may provide signaling lipids, presumably at lower levels than nutrient lipids, that may affect pup development. This represents an important area for future research. We also do not fully understand how loss of normal lipolysis leads to reprogramming of MECs, specifically by reducing mTOR activity. We do not see changes in systemic insulin or prolactin levels, and so the nature of the signal from adipocytes to MECs remains to be explored. Another

caveat relates to the TH-Cre mice used to assess the relevance of sympathetic-derived OXT in our model. TH can be expressed transiently during the development of some neuronal populations, raising the possibility that loss of OXT during development might affect lipolysis. We have previously used virally delivered Cre recombinase to demonstrate that loss of the *Oxt* gene in neurons that innervate the fat pad of adult male mice is sufficient to reduce lipolysis,¹¹ but we have not repeated that study in lactating mothers. Finally, our chow diet contains a lower percentage of calories as fat than typical breeder diets (16.5% versus 25%), which may have made the runting phenotype easier to detect.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Evan D. Rosen (erosen@bidmc.harvard.edu).

Materials availability

This study did not generate new, unique reagents.

Data and code availability

- Uncropped blots and source data are provided with this paper in [Data S1](#). The single-nucleus RNA-seq data have been deposited in GEO under accession number GSE289680 and are also available through the Broad Institute Single Cell Portal (https://singlecell.broadinstitute.org/single_cell/study/SCP2730).
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

Experimental design, E.L., Y.Y., H.S., J.J.A.-H., M.K., B.G., and E.D.R.; experimental execution, E.L., Y.Y., H.S., N.Z., J.J.A.-H., and K.P.; data analysis, E.L., J.J.A.-H., M.K., D.B., and S.N.; manuscript preparation, E.L., Y.Y., D.B., B.G., and E.D.R.; supervision and funding, B.G. and E.D.R.

DECLARATION OF INTERESTS

E.D.R. is on the Scientific Advisory Board of Source Bio, Inc. J.J.A.-H. and M.K. are employees of BPG Bio, Inc.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- Dietary sucrose supplementation experiment
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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
OXTR	Santa Cruz	Cat# sc-515809; RRID: AB_2891171
ATGL	Cell Signaling Technology	Cat# 2138; RRID: AB_2167955
HSP90	Cell Signaling Technology	Cat# 4874; RRID: AB_2121214
p-4E-BP1	Cell Signaling Technology	Cat# 13396; RRID: AB_2798206
4E-BP1	Cell Signaling Technology	Cat# 9644; RRID: AB_2097841
pS6K	Cell Signaling Technology	Cat# 9234T; RRID: AB_2269803
S6K	Cell Signaling Technology	Cat# 34475; RRID: AB_2943679
pAKT S473	Cell Signaling Technology	Cat# 9271S; RRID: AB_329825
AKT	Cell Signaling Technology	Cat# 4685; RRID: AB_2225340
LC3	Cell Signaling Technology	Cat# 4108; RRID: AB_2137703
Snip3	Cell Signaling Technology	Cat# 3769; RRID: AB_2259284
pS6	Cell Signaling Technology	Cat# 2211; RRID: AB_331679
S6	Cell Signaling Technology	Cat# 2217; RRID: AB_331355
Perilipin A	Abcam	Cat# ab61682; RRID: AB_944751
Donkey anti-mouse Alexa Fluor 594	Thermo Fisher Scientific	Cat# R37115; RRID: AB_2556543
Donkey anti-goat Alexa Fluor 488	Thermo Fisher Scientific	Cat# A11055; RRID: AB_2534102
Sheep Anti-Mouse IgG – Horseradish Peroxidase	Cytiva	Cat# NA931; RRID: AB_772210
Donkey Anti-Rabbit IgG H&L (HRP)	Cytiva	Cat# NA934; RRID: AB_772206
Chemicals, peptides, and recombinant proteins		
Oxytocin	Sigma-Aldrich	Cat# O3251
cOmplete Protease Inhibitor Cocktail	Roche	Cat# 11697498001
Phosphatase inhibitor cocktail 2	Sigma-Aldrich	Cat# P5726
Phosphatase inhibitor cocktail 3	Sigma-Aldrich	Cat# P0044
Laemmli sample buffer 4x	Boston BioProducts	Cat# BP-110R
Calcium chloride solution	Sigma-Aldrich	Cat# 21115
Glycerol Standard Solution	Sigma-Aldrich	Cat# G7793
PBS	Thermo Fisher Scientific	Cat# 10010049
Chloroform	Sigma-Aldrich	Cat# C2432
UltraPure DNase/RNase-Free Distilled Water	Thermo Fisher Scientific	Cat# 10977023
BSA	Sigma-Aldrich	Cat# A8022
Formalin, Buffered, 10% Phosphate Buffer	Fisher Scientific	Cat# SF100-4
Coomassie Brilliant Blue R-250	Bio-Rad	Cat# 1125530025
DMEM	Fisher Scientific	Cat# 11995073
Fetal Bovine Serum	Sigma-Aldrich	Cat# F2442
Penicillin-Streptomycin	Gibco	Cat# 15140163
0.05% Trypsin	Corning	Cat# MT25052CI
Proteinase K	Thermo Fisher Scientific	Cat# 25530049
KCl	Sigma-Aldrich	Cat# P9541
HEPES	Sigma-Aldrich	Cat# H43375
Tris	Sigma-Aldrich	Cat# 11814273001
DTT	Sigma-Aldrich	Cat# 43816
SDS	Thermo Fisher Scientific	Cat# AM9820
EDTA	Sigma-Aldrich	Cat# E5134

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Triton-X100	Thermo Fisher Scientific	Cat# BP151
Tween20	Thermo Fisher Scientific	Cat# BP337
Collagenase, Type 1	Worthington Biochemical	Cat# LS004196
DMSO	Sigma-Aldrich	Cat# 472301
Protector RNase Inhibitor	Roche	Cat# 3335399001
Bovine Serum Albumin, Molecular Biology grade	New England Biolabs (NEB)	Cat# B9200S
NP-40 Detergent Solution	Thermo Scientific	Cat# 28324
RNase Inhibitor	Thermo Scientific	Cat# AM2684
Dispase II	Roche	Cat# 4942078001
Methanol	Greenfield Global	Cat# 339000000CSGL
Acetic acid	Sigma-Aldrich	Cat# A6283
2-Propanol, ACS Reagent Grade	Sigma-Aldrich	Cat# 190764
Sodium hydroxide (NaOH), reagent grade	Sigma-Aldrich	Cat# S5881
NaCl	Sigma-Aldrich	Cat# S7653
MgCl ₂	Sigma-Aldrich	Cat# M2393
NucBlue	Invitrogen	Cat# R37605
Sucrose	MP Biomedicals	Cat# 821713

Critical commercial assays

BCA Protein Assay Kit	Thermo Fisher Scientific	Cat# 23227
4–15% Criterion TGX Precast Midi Protein Gel	Bio-rad	Cat# 5671084
PrimeScript RT Reagent Kit	Takara Bio	Cat# RR037A
SYBR green PCR master mix	Thermo Fisher Scientific	Cat# 4312704
Free Glycerol Reagent	Sigma-Aldrich	Cat# F6428-40ML
Free Fatty Acid Quantitation Kit	Sigma-Aldrich	Cat# MAK044-1KT
Oxytocin ELISA Kit	Enzo Life Sciences	Cat# ADI-900-153A-000
Mouse Insulin ELISA Kit	Crystal Chem	Cat# 90080
Mouse Prolactin ELISA Kit	RayBiotech	Cat# ELM-Prolactin-1
Infinity Triglycerides reagents	Thermo Fisher Scientific	Cat# TR22421
Trizol	Life Technologies	Cat# 15596-018
Direct-zol RNA Miniprep Kits	ZYMO Research	Cat# R2052
Qubit dsDNA High Sensitivity assay kit	Thermo Fisher Scientific	Cat# Q32851

Deposited data

Single cell RNA seq of mammary gland samples from lactating Oxt ^{fllox} and Oxt ^{Ad} dams	This paper	GEO: GSE289680
Lipidomic analysis of milk samples from Oxt ^{fllox} and Oxt ^{Ad} dams	This paper	Table S1
DEGs in Mammary epithelial cells from lactating Oxt ^{fllox} and Oxt ^{Ad} dams	This paper	Table S2

Experimental models: Organisms/strains

Mouse: C57BL6J mice	Jackson Laboratory	Cat# 000664
Mouse: Oxt ^{fllox} mice	Jackson Laboratory	Cat# 008471
Mouse: Adipoq-Cre mice	Jackson Laboratory	Cat# 028020
Mouse: Oxt ^{fllox} mice	Li et al. ¹¹	N/A
Mouse: Th-Cre mice	European Mouse Mutant Archive	EM: 00254
Mouse: Pnpla2 ^{fllox} mice	Jackson Laboratory	Cat# 024278

Oligonucleotides

A full list of qPCR primers in STAR Methods	This paper	N/A
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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and Algorithms		
Biorender	Biorender	Biorender.com
Seurat (version 4.1.0)	Stuart et al. ⁵⁰	N/A
Scrublet	Wolock et al. ⁵¹	N/A
GraphPad Prism 10	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
AIVIA AI image analysis software	AIVIA	https://www.aivia-software.com/leica-aivia
Cell Ranger program (version 7.2.0)	Zheng et al. ⁵²	N/A
DemuxEM (version 0.1.7)	Gaublomme et al. ⁵³	N/A
CellBender (version 0.2.0)	Fleming et al. ⁵⁴	N/A
'ScDbfFinder' function (version 1.8.0)	Germain et al. ⁵⁵	N/A
Seurat sctransform	Hafemeister et al. ⁵⁶	N/A
edgeR package (version 3.36.0)	Robinson et al. ⁵⁷	N/A
Other		
Standard Diet	LabDiet	Cat# 5008
Rodent Diet With 60 kcal% Fat	Research Diets	Cat# D12492
BIDMC Functional Genomics Core	Beth Israel Deaconess Medical Center	RRID: SCR_024877

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**Animals**

Animal care and experiments were performed with approval from the BIDMC Institutional Animal Care and Use Committee. Mice were housed on a 12-h light/dark cycle at constant temperature (23°C) and 45% average humidity, with free access to food and water. C57B6/J mice (Jax 000664) were purchased from Jackson Laboratory and used for experiments after an acclimatization period of several weeks. Experiments involving adipocyte-specific *Oxtr* knockout mice and *Pnpla2* knockout mice used littermate controls. For studies in lean mice, 8–12-week-old male or female mice were used. The chow diet used was purchased from LabDiet (Catalog # 5008; 16.5% kcal from fat); high fat diet was from Research Diets (catalog #D12492; 60% kcal from fat). *Oxtr^{fllox}* mice (Jax 008471) were crossed to Adipoq-Cre mice (Jax 028020) to generate *Oxtr^{ΔAd}* mice. *Pnpla2^{fllox}* mice (Jax 024278) were crossed to Adipoq-Cre mice⁵⁸ (Jax 028020) to generate *Pnpla2^{ΔAd}* mice. *Oxtr^{fllox}* mice were generated in the Rosen lab as described,¹¹ and crossed to Th-Cre mice (European Mouse Mutant Archive; EM:00254) to generate sympathetic neuron-specific *Oxtr* knockout mice.

METHOD DETAILS**Mouse adipocyte isolation**

Inguinal and perigonadal adipose tissue were dissected from 8–12-week-old C57BL/6J male and female mice, minced and digested in PBS (Thermo Fisher Scientific, 10010049) with 10mM CaCl₂, 1.5U/ml collagenase D (Sigma, 11088882001) and 2.4U/ml dispase II (Roche 4942078001) for 30 min at 37°C. The cell suspension was then filtered through a 100 μm cell strainer (Corning 352360), centrifuged at 800 rpm for 5 min. Floating adipocytes were collected for RNA or protein extraction.

Metabolic studies

Breeding cages were set up using 8-week-old male and female mice. Food intake of dams was measured daily. Body weight of pups was measured at four time points, including postnatal day 1, 7, 14 and 21. Fat and lean mass was determined by the body composition analyzer EchoMRI (Echo Medical Systems). To measure serum glycerol and Free fatty acid (FFA) levels in lactating female mice, tail vein blood samples were collected. FFA levels were measured using a free fatty acid quantitation kit (Sigma, MAK044). Tissue weight of pups was measured using a scale (Mettler Toledo AG104) immediately after manual dissection. For the second round of breeding, the same dams were maintained in their home cages for 3 weeks after separation from pups on postnatal day 21 (PND21) to allow recovery, and were then paired again for breeding.

Measurement of maternal serum oxytocin, insulin and prolactin

Blood samples were collected from dams at the indicated time points by tail vein bleeding. For lactating dams, pups were removed for 2 h on postnatal day 11 (PND11) prior to blood collection to standardize suckling-induced hormone release. Serum oxytocin,

insulin, and prolactin concentrations were measured using a mouse oxytocin ELISA kit (ADI-900-153A-0001, Enzo Life Sciences), a mouse insulin ELISA kit (90080, Crystal Chem), and a mouse prolactin ELISA kit (ELM-Prolactin-1, RayBiotech), respectively, according to the manufacturers' instructions.

Cross-fostering experiment

Female *Oxtr^{fllox}* and *Oxtr^{ΔAd}* mice were bred with male *Oxtr^{ΔAd}* and *Oxtr^{fllox}* mice, respectively. Breeding cages were monitored every day, and male mice were taken out of cages once female mice were found pregnant. The day on which pups were born was considered P0, and pups were cross-fostered on P1. All the pups of *Oxtr^{fllox}* dams were housed with lactating *Oxtr^{ΔAd}* dams. Similarly, same-day born pups of *Oxtr^{ΔAd}* dams were housed with lactating *Oxtr^{fllox}* dams. Litter size was capped at 4-6 in all cages, and body weight of pups was monitored every week. After 3 weeks, the cross-fostered pups were weaned and divided into two groups. One group was used to measure fat mass and lean mass, while the other was placed on a chow diet and used to measure weekly body weight.

Weigh-suckle-weigh experiment

Dam milk output during a feeding was measured using a modified weigh-suckle-weigh paradigm.⁵⁹ To minimize variability, only dams with 5 pups were used for these experiments. Dams were separated from pups for 2 hours on postnatal day 10 (P10) to allow the pups to digest the food in their system and let the dam accumulate milk at same time. We then recorded the weight of the dam and pups before and after separation. After 2 hours, we reunited the pups and dam for 1 hour. Following the 1 hour feed period weight changes were assessed in the dam and pups.

Milk protein, lactose and triglyceride measurement

Milk protein was assessed using a milk gel technique.²⁴ Whole mouse milk was diluted 1:3 in 1X PBS+EDTA and centrifuged at 4°C for 10 minutes at 10,000 rpm. The supernatant was collected and centrifuged for a second time at 4°C for 10 minutes at 10,000 rpm. If the supernatant was sufficiently clear, 2.5 uL of skim milk was loaded into a 12% SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad 1610436, Hercules, CA, USA) blue. Destain (methanol, acetic acid and DI water in a 50:10:40 ratio) was then applied. Images were captured using the Syngene G box system (Bangalore, India).

Milk fat was assessed by using a triglyceride assay.²⁴ Milk and tissue TG were analyzed by the Infinity Triglycerides reagents (Thermo TR22421). Glycerol standard solution was from Sigma G7793 (Livonia, MI, USA). Milk lactose content was measured using a YSI analyzer. To prepare skim milk, the whole milk was diluted 1:3 with water and centrifuged twice at 4°C for 10 minutes at 10,000 rpm. Milk was incubated in beta-galactosidase buffer for 1 hour at 37°C in a water bath. Glucose was then measured with a YSI 2300 STAT plus machine and a conversion applied to determine the lactose concentration.

Milk amino acids quantification

Milk total amino acids were measured by the University of Michigan Metabolomics Core, as previously demonstrated.²⁴ Proteins were hydrolyzed prior to amino acid analysis using the Ezfaast kit. The analytes were measured using liquid chromatography-triple quadrupole mass spectrometry operating in multiple reaction monitoring mode. The coefficient of variation for amino acid analysis is 10%.

Mammary gland involution experiment

Age-matched female mice were allowed to nurse their litters until postnatal day 21. Following weaning, dams were maintained in their home cages without pups to induce mammary gland involution. Mammary glands 4 and 5 were collected and fixed for hematoxylin and eosin staining and further adipose area quantification at the time of weaning (PND 21), or at 3 days (early involution) and 3 weeks (late involution) after weaning.

Immunofluorescence staining in mammary gland

Mammary glands 4 and 5 were collected from female mice at the indicated reproductive stages, including virgin, early pregnancy (P3), late pregnancy (P16), early lactation (PND3), and late lactation (PND16). Tissues were fixed, paraffin-embedded, and sectioned at a thickness of 5 μm. After gradually rehydrated, permeabilized with 0.1% Triton X-100 (28314, Thermo Fisher Scientific) in PBS for 15 min, and blocked with 5% donkey serum in PBS for 30 min, tissue sections were then incubated overnight at 4°C with mouse monoclonal anti-OXTR antibody (sc-515809, Santa Cruz Biotechnology; 1:200) and the adipocyte marker perilipin (ab61682, Abcam; 1:1000). After washing with PBST, sections were incubated with donkey anti-mouse Alexa Fluor 594 (R37115, Thermo Fisher Scientific; 1:1000) and donkey anti-goat Alexa Fluor 488 (A11055, Thermo Fisher Scientific; 1:1000) for 4 h at room temperature. Sections were then washed, counterstained with DAPI, and mounted with antifade mounting medium. Immunofluorescence images were acquired using a Zeiss LSM 880 upright confocal microscope and analyzed using ImageJ software (version 1.52a).

Mammary adipose and duct area quantification

Mammary glands 4 and 5 were excised and placed in 10% neutral buffered formalin over 72 hours for fixation. Paraffin embedded tissues were sectioned at 5μm and placed on glass slides. Sections were de-paraffinized using graded xylene and gradually rehydrated. They were then stained with hematoxylin and eosin according to standard protocols. Sections were analyzed using the AIVIA AI image analysis software. With the guidance of the Michigan Diabetes Research Center Microscopy Core the software was trained

to recognize either mammary ductal tissue or adipose tissue. Once the training was optimal the sections were then quantitated. One section was counted per dam.

Milk sample collection and lipidomic analysis

Prior to euthanasia, dams were separated from their pups for two hours. During this time, the pups were euthanized and the dams had access to food and water. After a two-hour separation, the dam was placed under general anesthesia by injecting ketamine and xylazine (5 μ L/g) into the hind leg muscle. Once the dam stopped moving, 0.5 U/ μ L (2 units total) of oxytocin was injected into the upper arm muscle. The milk was manually collected and 20 μ L milk was aliquoted for downstream analysis, and immediately frozen at -80°C . The samples remained at -80°C before shipping for analysis.

Total lipids were extracted from breast milk using a modified Folch extraction⁶⁰ with 2:1:0.5 chloroform:methanol:water (v/v/v) in the presence of known amounts of isotopically-labeled internal standards (Equisplash Lipidomix, Avanti Polar Lipids). Extracts in chloroform were dried fully under nitrogen gas, reconstituted in 100 μ L isopropanol and stored in autosampler vials at 4°C until analysis. Nontargeted lipidomics was completed using an Agilent 1290 LC pump equipped with an Acquity Premier CSH C18 column (2.1 mm $\times$ 150 mm, 1.7 μ M; Waters), coupled to a Thermo Q-Exactive Plus mass spectrometer. The mobile phase consisted of A: 60:40 acetonitrile:water (v/v) and B: 90:10 isopropanol:acetonitrile (v/v), both with 10 mM ammonium formate, 0.1% formic acid and 0.1 μ M reserpine (lock mass). The flow rate was 230 μ L/min and the column compartment temperature was 55°C . The gradient elution program was as follows: starting at 32% B, linear increase to 55% B from 0–5 min, followed by linear increase to 65% from 5–8 min, hold at 65% B from 8–10 min, linear increase to 80% B from 10–14 min, linear increase to 90% B from 14–25 min, linear increase to 100% B from 25–25.5 min, hold at 100% B until 27 min, drop to 32% B at 27.1 min and equilibration until the 30 min mark. Breast milk samples were analyzed using a polarity-switching full-scan mass spectrometry method at 17,000 resolution with spray voltages +3.5 kV and -2.5 kV in the positive and negative ion modes, respectively, with scan range m/z 120–1600. Centroided spectra were automatically lock-mass corrected during acquisition (reserpine $[\text{M}+\text{H}]^{+}$ m/z 609.28121 and $[\text{M}+\text{formate}]^{-}$ m/z 653.27104). Representative breast milk pools were used as targets in pseudo top-40 data-dependent tandem mass spectrometry experiments with iterative exclusion lists using IE-Omics.⁶¹ Lipids were putatively identified using LipidMatch software⁶²; semiquantitative values (normalized by sample volume and internal standard subclass amount) were generated as described earlier.⁶³

Milk lipids were log-transformed to normalize and then analyzed via Student's t tests between groups. P-values were adjusted for multiple comparisons by the method of Benjamini and Hochberg. Lipid classes and fatty acids were abstracted from individual lipidomic data, which included the class and the fatty acids within that species.

BCA assay for milk protein quantification

The BCA assay for skim milk was performed following a trichloroacetic acid (TCA) precipitation procedure to remove interfering substances. For each 5 μ L of skim milk, 45 μ L of water was added, followed by 10 μ L of 0.15% (w/v) sodium deoxycholate and 10 μ L of 72% (w/v) TCA. The mixture was incubated at room temperature for 10 minutes. After centrifugation at maximum speed for 10 minutes, the supernatant was discarded. The protein pellet was then dissolved in 10 μ L of 5% (w/v) SDS prepared in 0.1 N sodium hydroxide (NaOH). The protein concentration was determined by measuring absorbance at 562 nm using the BCA assay.

Dietary lipid supplementation experiment

Breeding cages were established using 8-week-old male and female mice, with all cages initially maintained on a chow diet. Female mice were checked for vaginal plugs the next morning after pairing, and those with plugs were considered potentially pregnant. To prevent further mating, males were removed from the breeding cages once pregnancy was confirmed. On gestational day 13, pregnant $\text{Oxtr}^{\text{flox}}$ and $\text{Oxtr}^{\Delta\text{Ad}}$ mice were randomly divided into two groups: one group remained on the chow diet, while the other was switched to a high-fat diet (HFD). These diets were maintained throughout the duration of the experiment, and food intake was measured every other day. On PND1, litter sizes were standardized to 4–5 pups per cage. Pup body weight was recorded weekly. Blood samples from dams were collected from the tail vein on postnatal day 11 to measure serum FFA, insulin, and prolactin. Milk samples were collected on postnatal day 16 for triglyceride analysis. Pup lean mass, fat mass, and tissue weights were measured on postnatal day 21.

Dietary sucrose supplementation experiment

Breeding cages were established as described above. Upon confirmation of pregnancy, males were removed from the cages. Pregnant $\text{Oxtr}^{\text{flox}}$ and $\text{Oxtr}^{\Delta\text{Ad}}$ dams were maintained on a chow diet throughout the experiment. Beginning on gestational day 13, dams were randomly divided into two groups: one group received drinking water supplemented with 10% (w/v) sucrose, while the control group continued to receive regular drinking water. Sucrose supplementation was maintained during lactation, and both food intake and water consumption were measured every other day. On postnatal day 1, litter sizes were standardized to 4–5 pups per cage. Pup body weight was recorded weekly. Pup body composition and tissue weights were assessed on postnatal day 21. Caloric intake for both HFD and sucrose experiments were calculated from the day mice were switched to a high-fat diet (HFD) or sucrose solution until the weaning at PND21. Energy intake was calculated based on the following caloric values: chow diet (LabDiet 5008), 3.54 kcal/g; high-fat diet (D12492), 5.24 kcal/g; and 10% sucrose solution, 0.4 kcal/ml.

Single nucleus RNA-seq of mammary glands of *Oxtr^{flox}* and *Oxtr^{ΔAd}* dams

Mammary gland collection

Breeding cages were established using 8-week-old male and female mice, with all animals maintained on a standard chow diet. To prevent further mating, males were removed from the cages once pregnancy was confirmed. On postnatal day 1 (P1), litter sizes were standardized to 4–5 pups per cage. Inguinal white adipose tissue (iWAT) was harvested from lactating *Oxtr^{flox}* and *Oxtr^{ΔAd}* dams on postnatal day 11 (P11). Lymph nodes were carefully removed from the iWAT fat pads, which were then flash-frozen in liquid nitrogen and stored at -80°C for subsequent single nucleus RNA-seq analysis.

Single nucleus RNA-seq (snRNA-seq)

Nuclei were isolated from frozen mouse mammary gland samples for 10x snRNA-seq using a slightly modified approach to what was previously described.^{64,65} Samples were kept frozen on dry ice until immediately before nuclei isolation, and all sample handling steps were performed on ice. Each flash-frozen adipose tissue sample was placed into a gentleMACS C tube (Miltenyi Biotec, 130-096-334) with 2 ml freshly prepared TST buffer (0.03% Tween 20 (Bio-Rad 1610781), 0.01% Molecular Grade BSA (New England Biolabs B9000S), 146 mM NaCl (ThermoFisher Scientific AM9759), 1 mM CaCl_2 (VWR 97062-820), 21 mM MgCl_2 (Sigma Aldrich M1028), and 10 mM Tris-HCl pH 7.5 (ThermoFisher Scientific 15567027) in Ultrapure water (ThermoFisher Scientific 10977-023)) with or without $0.2\text{ U }\mu\text{l}^{-1}$ of Protector RNase Inhibitor (Sigma Aldrich 3335399001). gentleMACS C tubes were then placed on the gentleMACS Dissociator (Miltenyi Biotec 130-093-235) and tissue was dissociated by running the program *mr_adipose_01* twice, and then incubated on ice for 10 min. Lysate was passed through a $40\text{ }\mu\text{m}$ nylon filter (CellTreat 229481) and collected into a 50 ml conical tube (Thomas Scientific 1149Z03). Filter was rinsed with 3 ml of freshly prepared ST buffer (146 mM NaCl, 1 mM CaCl_2 , 21 mM MgCl_2 , 10 mM Tris-HCl pH 7.5) with or without $0.2\text{ U }\mu\text{l}^{-1}$ RNase Inhibitor, and collected into the same tube. Flow-through was centrifuged at $500g$ for 5 min at 4°C with brake set to low. Following centrifugation, supernatant was removed, and the nuclear pellet was resuspended in $50\text{--}200\text{ }\mu\text{l}$ PBS pH 7.4 (ThermoFisher Scientific 10010049) with 0.02% BSA, with or without $0.2\text{ U }\mu\text{l}^{-1}$ RNase inhibitor. In order to reduce ambient mRNA, the nuclear pellets of some samples were washed 1–3 times with 5 ml PBS, 0.02% BSA before final resuspension. An aliquot of nuclei from each sample was stained with NucBlue (Invitrogen R37605) counted in a haemocytometer using fluorescence to identify intact nuclei, and then immediately loaded on the 10x Chromium controller (10x Genomics) according to the manufacturer's protocol.

For each sample, 10,000–16,500 nuclei were loaded in one channel of a Chromium Chip (10x Genomics). The Single Cell 3' v3.1 chemistry was used to process all samples. cDNA and gene expression libraries were generated according to the manufacturer's instructions (10x Genomics). cDNA and gene expression library fragment sizes were assessed with a DNA High Sensitivity Bio-analyzer Chip (Agilent 5067-4626). cDNA and gene expression libraries were quantified using the Qubit dsDNA High Sensitivity assay kit (ThermoFisher Scientific Q32851). Gene expression libraries were multiplexed and sequenced on the Nextseq 500 (Illumina) with a 75-cycle kit and the following read structure: Read 1: 28 cycles, Read 2: 55 cycles, Index Read 1: 8 cycles.

Single-nuclear RNA-seq data analysis

Raw sequencing reads were converted to FASTQ format files using *bcl2fastq* (Illumina; version 2.20.0). A single mis-match was allowed for each sequence index. Reads were simultaneously aligned and quantified into cell-by-gene count matrices using the Cell Ranger⁵² program (version 7.2.0), using the GRCh39 reference genome assembly and a corresponding GENCODE-vM33 mouse genome annotation. Hashtag oligonucleotide sequences (HTOs) were counted using the Cumulus Tool on Feature Barcoding (https://github.com/lilab-bcb/cumulus_feature_barcoding, version 0.11.1), and DemuxEM⁵³ (version 0.1.7) was used to collate nuclei (hereafter “cells”) into individual samples based on their associated HTO content. In order to adjust for downstream effects of ambient RNA expression within these cells, we used CellBender⁵⁴ (version 0.2.0) to remove counts strongly associated with ambient RNA molecules from the count matrices and to further estimate the “true” cells, i.e. droplets estimated to be cells by Cell Ranger, assigned to only a single sample based on HTO demultiplexing, and with $>50\%$ posterior probability of containing a cell, according to CellBender. All samples were assessed for doublet content using ‘*scds*’ function⁶⁶ (version 1.10.0), a threshold of hybrid score 0.6 and cells called as doublets using ‘*ScDblFinder*’ function (version 1.8.0) were removed before further analysis. Genes were filtered such that only genes detected in two or more cells were retained. For this data, the median number of UMIs detected per cell was 4106 and the median number of genes detected per cell was 1829. Integration, clustering and subclustering analysis were performed using Seurat⁵⁰ (version 4.1.0). The gene counts for individual samples were normalized and regressed on mitochondrial read percentage, using the ‘*SCTransform*’ function. Samples were then integrated and clustered using the standard Seurat *sctransform*⁵⁶ methodology. Clusters were manually annotated as described based on gene-signature and marker-gene expression (‘*FindMarkers*’).

Pseudo-bulking snRNA-seq and differential expression analysis

Single-nucleus RNA-seq counts were first filtered to retain only MEC clusters, then aggregated by sample and cluster to create pseudo-bulk RNA-seq profiles for downstream differential expression analysis (DEA). Normalization and DEA were conducted using the edgeR⁵⁷ package (version 3.36.0). Prior to normalization, low-abundance genes were filtered using the ‘*filterByExpr*’ function. Blocking groups were assigned based on the number of pups. Genes were considered significantly differentially expressed if they met a $\log_2$ fold change threshold of ≥ 1 and a false discovery rate threshold of ≤ 0.05 . Gene set enrichment analysis was performed using built-in functionality of edgeR (‘*fry*’⁶⁷ and ‘*camera*’⁶⁸ functions) using KEGG pathways as our gene set list.

RNA isolation and quantitative PCR

Total RNA was extracted from isolated adipocytes using the Direct-zol RNA MiniPrep Kit (ZYMO Research). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). RNA expression levels were quantified using the

ABI PRISM 7500 system (Applied Biosystems). Quantitative PCR (qPCR) was performed on the QuantStudio Real-Time PCR System (v1.2). Relative gene expression was calculated using the ddCt method, with 36B4 serving as the internal control. The following primers were used for qPCR: *Oxtr*-forward GCACGGGTCAGTAGTGTCAG, *Oxtr*-reverse AAGCTTCTTTGGGCGCATTG; *36B4*-forward CAGCAGGTGTTTGACAACGG, *36B4*-reverse GATGATGGAGTGTGGCACCG.

Western blotting

Adipose tissue samples and isolated adipocytes were homogenized in RIPA buffer containing protease (Roche, 11697498001) and phosphatase inhibitors (Sigma-Aldrich, P5726 and P0044) using the Qiagen TissueLyser II (Qiagen, 85300). Protein lysates were separated by SDS-PAGE using 4–15% Criterion TGX Precast Midi Protein Gel (Bio-rad, 5671084) and transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were then incubated with the appropriate primary and secondary antibodies for detection. Primary antibodies used: mouse monoclonal anti-OXTR (1:1000, Santa Cruz sc-515809), anti-HSP90 (1:1000, Cell Signaling Technology #4874), anti-ATGL (1:1000, Cell Signaling Technology #2138), anti-p-4E-BP1 (1:1000, Cell Signaling Technology #13396), anti-4E-BP1 (1:1000, Cell Signaling Technology #9644), anti-pS6K (1:1000, Cell Signaling Technology #9234T), anti-S6K (1:1000, Cell Signaling Technology #34475), anti-pAKT S473 (1:1000, Cell Signaling Technology #9271S), anti-AKT (1:1000, Cell Signaling Technology #4685), anti-LC3 (1:1000, Cell Signaling Technology #4108S), anti-Bnip3 (1:1000, Cell Signaling Technology #3769S), anti-pS6 (1:1000, Cell Signaling Technology #2211S) and anti-S6 Ribosomal Protein (1:1000, Cell Signaling Technology #2217). Secondary antibodies used: Sheep Anti-Mouse IgG - Horseradish Peroxidase (1: 1000, Cytiva #NA931) and Donkey Anti-Rabbit IgG H&L (HRP) (1: 1000, Cytiva #NA934). Western blotting data were collected using the ChemiDoc Imaging System (BIO-RAD).

QUANTIFICATION AND STATISTICAL ANALYSIS

Wild-type mice were randomly assigned to treatment groups. **Blinding was not performed for any animal experiments.** All data were normally distributed, and **no data points were excluded from the analysis.** Results are presented as mean $\pm$ s.e.m. Statistical analyses were conducted using GraphPad Prism (Version 8). Comparisons between two groups were performed using a two-tailed paired Student's t-test or unpaired Student's t-test, as specified in the figure legends. For comparisons involving multiple groups, two-way ANOVA followed by Tukey's post hoc analysis was applied. Statistical significance was defined as $P < 0.05$, with asterisks denoting the significance levels ($*P < 0.05$). The definition of "n" is provided in the figure legends.

Supplemental information

**Oxytocin signaling in adipocytes is required
for normal milk fat production**

Erwei Li, Yangmian Yuan, Haijing Sun, Khushi Patel, Nan Zhang, Soumya Nagesh, Juan J. Aristizabal-Henao, Michael Kiebish, Christopher Jacobs, Dave Bridges, Brigid Gregg, and Evan D. Rosen

Figure S1

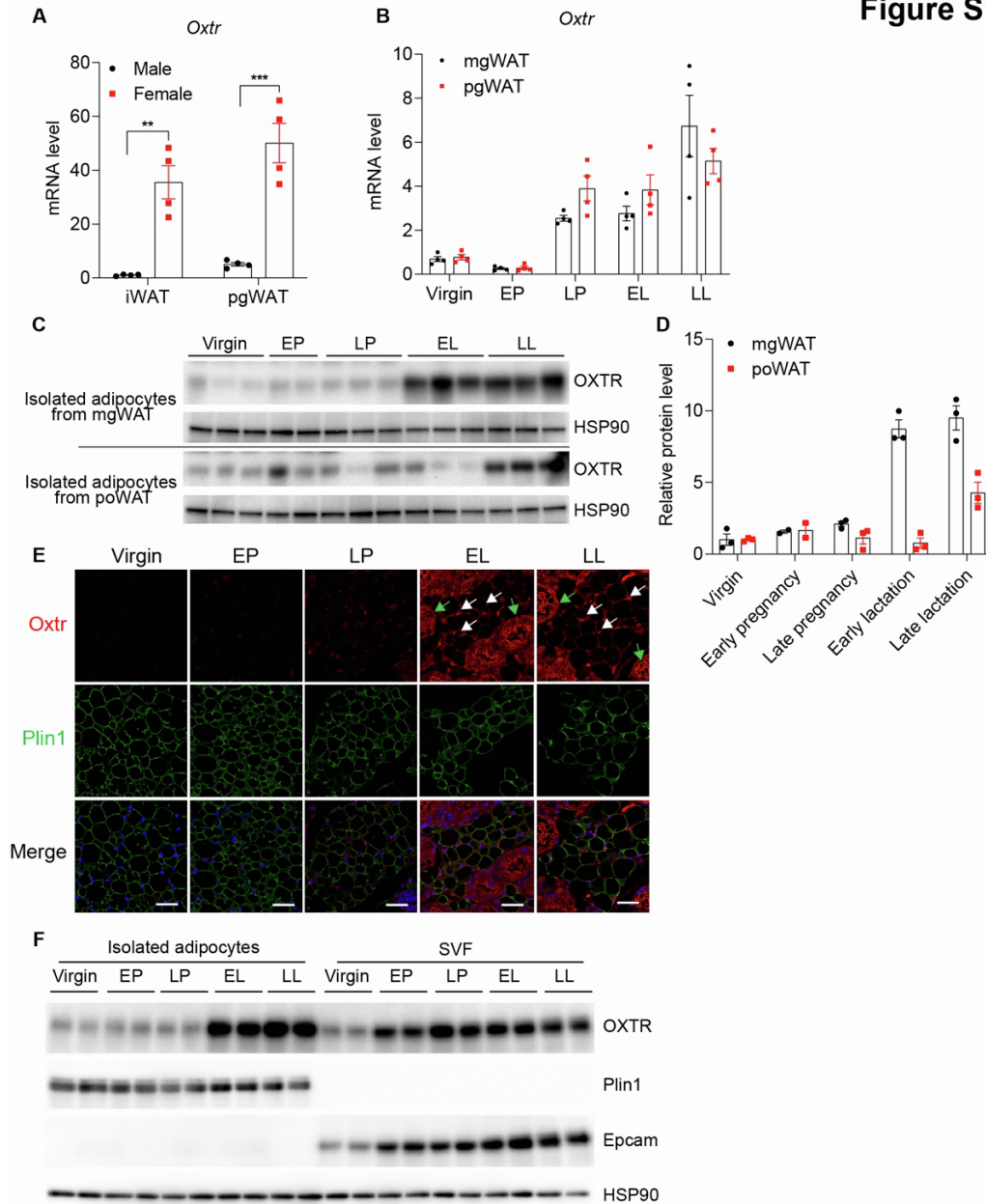


Figure S1. OXTR expression is significantly higher in female adipocytes and further increases during lactation, related to Figure 1. (A) *Oxtr* mRNA levels in male and female adipocytes from inguinal (iWAT) and perigonadal (pgWAT) white adipose tissue; n=4. **(B)** *Oxtr* mRNA levels in female adipocytes from mammary gland (mgWAT) and periovarian (poWAT) white adipose tissue across different reproductive stages, including virgin, early pregnancy, late pregnancy, early lactation and late lactation; n=4. **(C)** OXTR protein levels in female adipocytes from mgWAT and poWAT across different reproductive stages, including virgin, early pregnancy, late pregnancy, early lactation and late lactation; n=2-3. **(D)** Quantification of western blots in (C) based on densitometry. **(E)** Representative images of mgWAT of WT female mice across different reproductive stages immunostained for PLIN1 and OXTR. Green arrows indicate OXTR signals on mammary epithelial cells and white arrows indicate OXTR signals on adipocytes. Scale bars, 50um. **(F)** Western blot analysis of OXTR, PLIN1 and EPCAM in isolated adipocytes and stromal vascular fraction from mgWAT of mice at virgin, early pregnancy (EP), late pregnancy (LP), early lactation (EL), and late lactation (LL) stages; n=2. Data are presented as mean $\pm$ s.e.m. Statistical comparisons were made using 2-tailed Student's t test (A). **P < 0.01; ***P < 0.001.

Figure S2

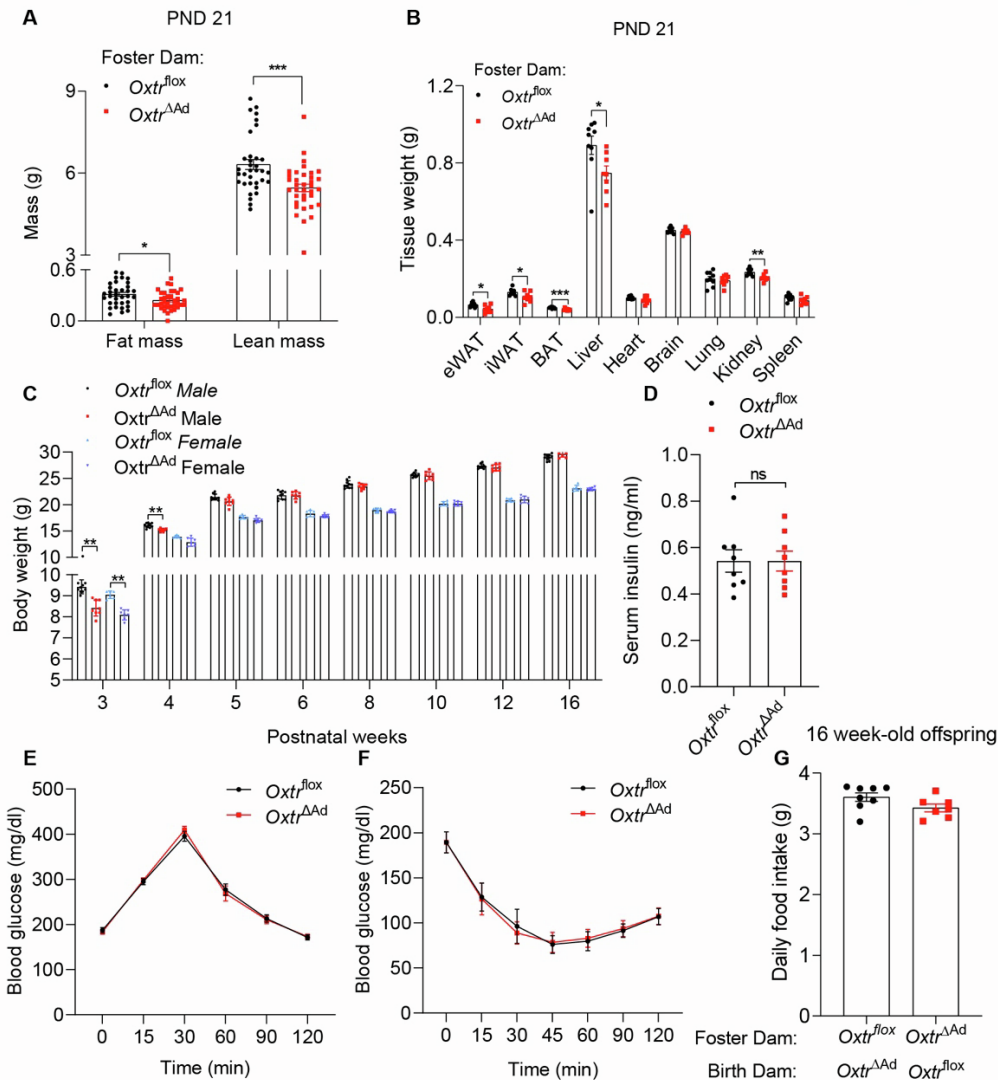


Figure S2. The phenotype of pups is determined by the genotype of dams, related to Figure 2. (A) Fat mass and lean mass of fostered pups on postnatal day 21; n=34-36. **(B)** Tissue weight of fostered pups on postnatal day 21; n=8-9. **(C)** Body weight of male and female pups of Oxt^{fllox} and $Oxt^{\Delta Ad}$ dams at postnatal weeks 3, 4, 5, 6, 8, 10, 12, 16; n=6-10. **(D)** Serum insulin levels of 10-week-old male offspring of Oxt^{fllox} and $Oxt^{\Delta Ad}$ dams; n=8. **(E)** GTT in 10-week-old male offspring of Oxt^{fllox} and $Oxt^{\Delta Ad}$ dams; n=8. **(F)** ITT in 10-week-old male offspring of Oxt^{fllox} and $Oxt^{\Delta Ad}$ dams; n=8. **(G)** Food intake of fostered mice after weaning (16 weeks old); n=7-8. Data are presented as mean $\pm$ s.e.m. Statistical comparisons were made using 2-tailed Student's t test (A-C). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

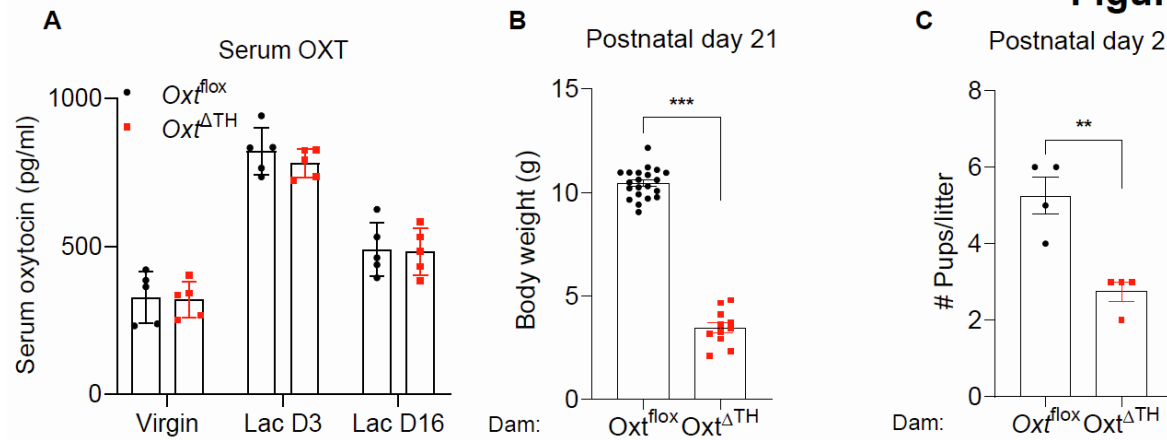
Figure S3

Figure S3. Pups of *Oxt^{ΔTH}* dams display impaired development during lactation, related to Figure 2. (A) Serum oxytocin levels in female *Oxt^{flox}* and *Oxt^{ΔTH}* mice at stages of virgin, lactation day 3 and lactation day 16; n=5. (B) Quantification of body weight of pups from *Oxt^{flox}* and *Oxt^{ΔTH}* dams on postnatal day 21; pups of *Oxt^{flox}* dams n=21, pups of *Oxt^{ΔTH}* dams n=11. (C) Number of pups per litter from *Oxt^{flox}* and *Oxt^{ΔTH}* dams that survived through postnatal day 21; litters of *Oxt^{flox}* dams n=4, litters of *Oxt^{ΔTH}* dams n=4. Data are presented as mean $\pm$ s.e.m. Statistical comparisons were made using 2-tailed Student's t test (B and C). **P < 0.01; ***P < 0.001.

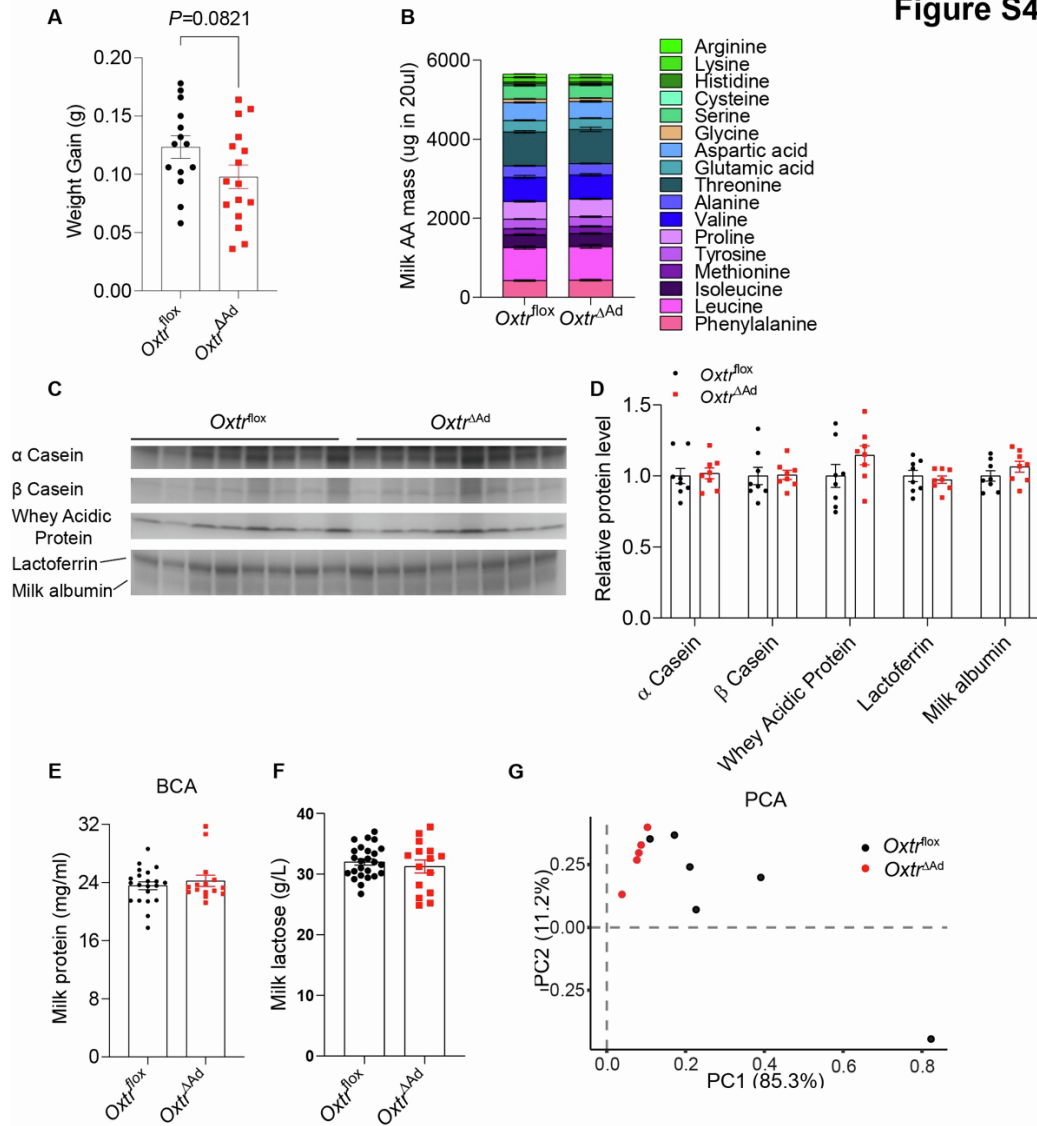


Figure S4. Milk protein and lactose levels are comparable between *Oxt^{flox}* and *Oxt^{flox}ΔAd* dams, related to Figure 3. (A) Volume of milk produced within 3 hours by lactating *Oxt^{flox}* and *Oxt^{flox}ΔAd* dams on postnatal day 10, measured using weigh-suckle-weigh assays; n=14-16. **(B)** Stacked plot showing the mass distribution of total amino acids in milk samples from *Oxt^{flox}* and *Oxt^{flox}ΔAd* dams on postnatal day 16; n=6. **(C)** Protein levels of α casein, β casein, whey acidic protein, lactoferrin and albumin in milk samples collected from *Oxt^{flox}* and *Oxt^{flox}ΔAd* dams on postnatal day 16; n=8. **(D)** Quantification of (D) based on densitometry. **(E)** Total protein levels in milk collected from lactating *Oxt^{flox}* and *Oxt^{flox}ΔAd* dams, n=15-21. **(F)** Lactose levels in milk collected from *Oxt^{flox}* and *Oxt^{flox}ΔAd* dams on postnatal day 16; n=15-25. **(G)** PCA of lipids from milk samples of *Oxt^{flox}* and *Oxt^{flox}ΔAd* dams on postnatal day 16. Each dot indicates an individual biological replicate; n=5. Data are presented as mean ± s.e.m. Statistical comparisons were made using 2-tailed Student's t test (A and D-F).

Figure S5

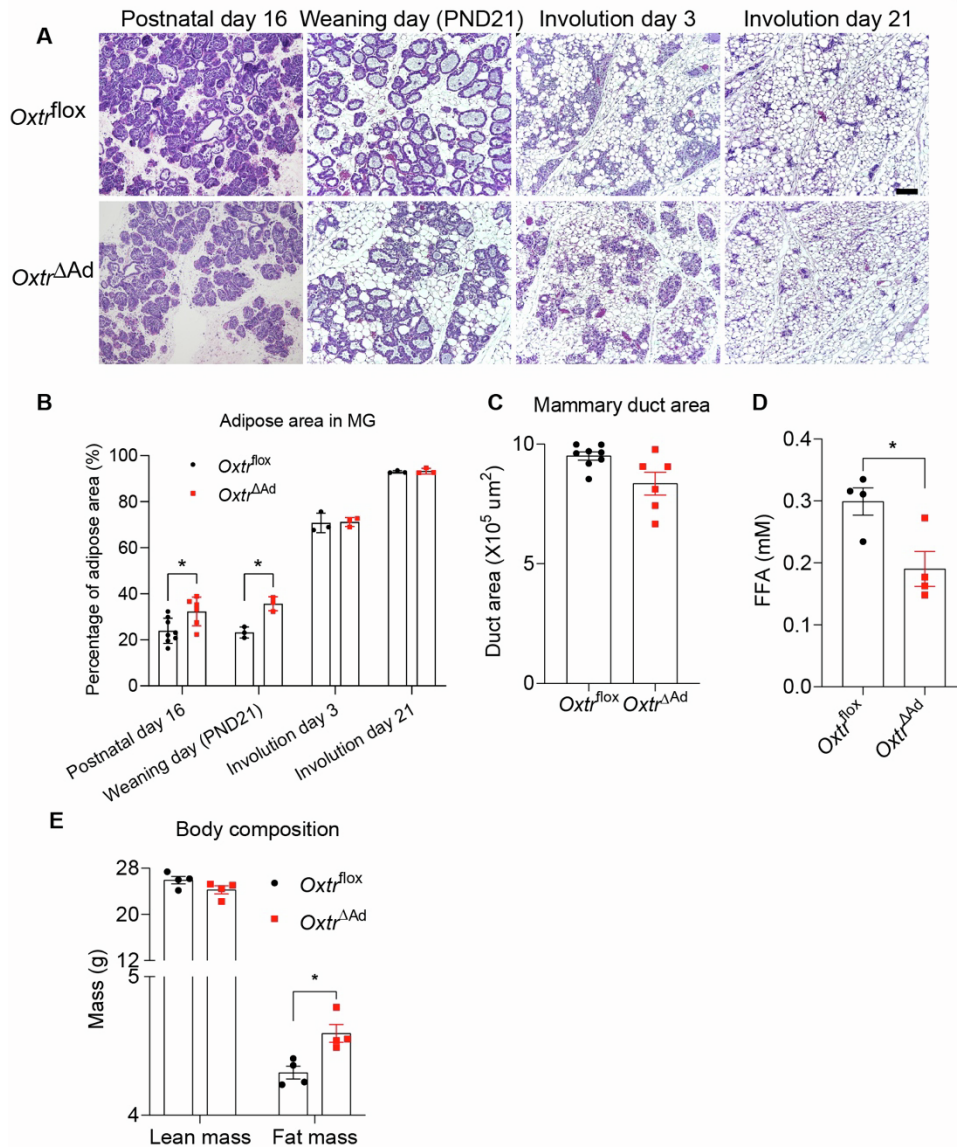


Figure S5. More fat is reserved in lactating *Oxtr^{ΔAd}* dams, related to Figure 3. (A) Representative histology sections of mammary glands dissected from *Oxtr^{flox}* and *Oxtr^{ΔAd}* dams on postnatal day 16, weaning day (PND21), involution day 3 and involution day 21. **(B)** Quantification of adipose area in mammary gland sections from *Oxtr^{flox}* and *Oxtr^{ΔAd}* dams on postnatal day 16, weaning day (PND21), involution day 3 and involution day 21; n=6-8. **(C)** Quantification of duct area in mammary gland sections from *Oxtr^{flox}* and *Oxtr^{ΔAd}* dams on postnatal day 16; n=6-8. **(D)** Serum free fatty acid levels in lactating *Oxtr^{flox}* and *Oxtr^{ΔAd}* dams on postnatal day 11; n=4. **(E)** Body composition of lactating *Oxtr^{flox}* and *Oxtr^{ΔAd}* dams on postnatal day 10; n=4. Data are presented as mean $\pm$ s.e.m. Statistical comparisons were made using 2-tailed Student's t test (B-E). *P < 0.05.

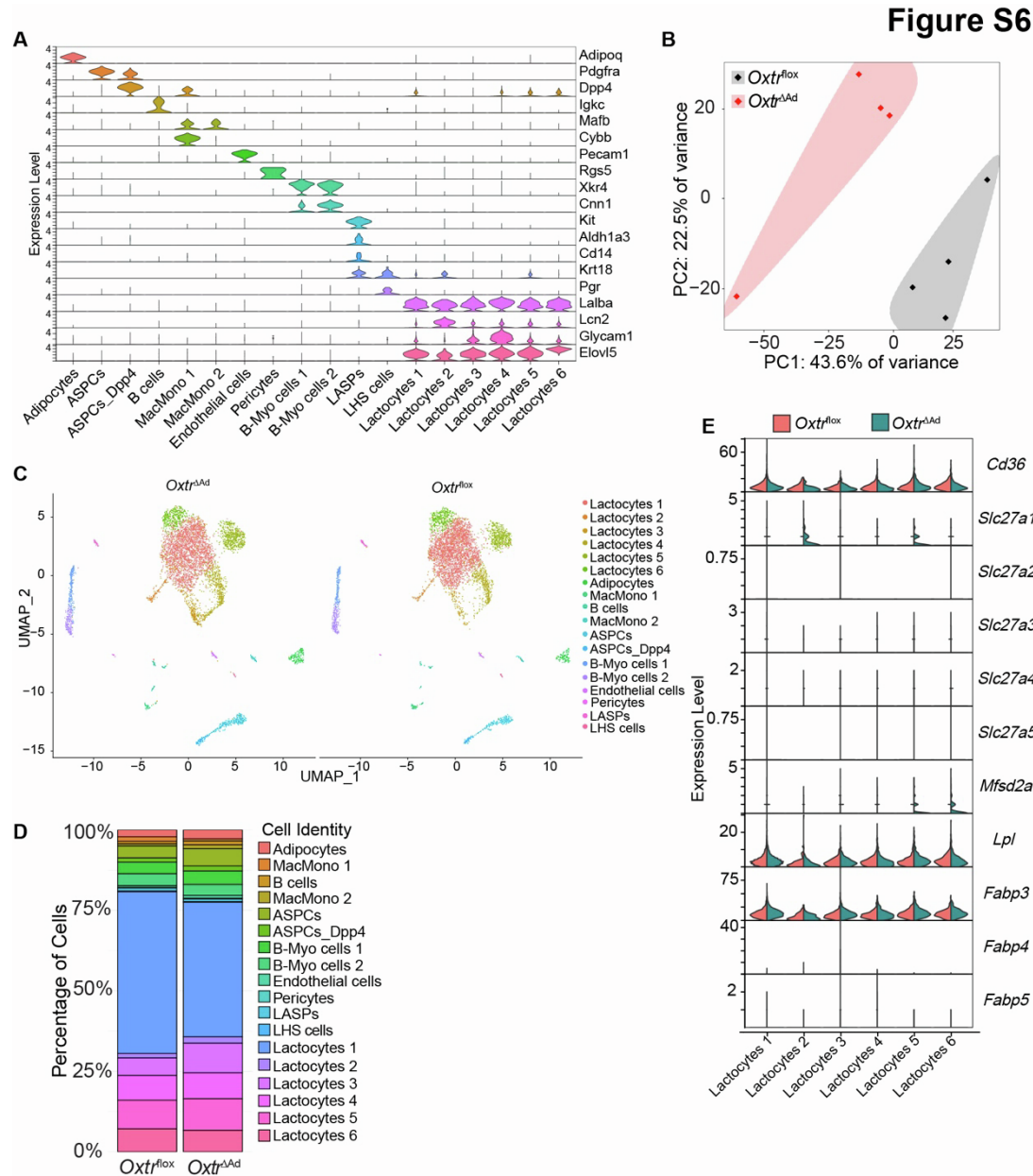


Figure S6. The proportions of each cell type within the mammary glands are comparable between *Oxt^{fllox}* and *Oxt^{ΔAd}* dams, related to Figure 4. (A) Marker genes for each cell type in lactating mammary glands from *Oxt^{fllox}* and *Oxt^{ΔAd}* dams. (B) PCA of pseudo-bulk single cell RNA-seq data of mammary glands showing cluster formation of lactocytes between *Oxt^{fllox}* and *Oxt^{ΔAd}* dams. (C) UMAP projection of all 13,035 sequenced mammary gland nuclei split by genotype. (D) Stacked plot showing the proportions of each cell type in mammary glands from lactating *Oxt^{fllox}* and *Oxt^{ΔAd}* dams on postnatal day 11. (E) Stacked violin plot showing the expression of genes related to lipid transport and uptake in lactocytes of *Oxt^{fllox}* and *Oxt^{ΔAd}* dams.

Figure S7

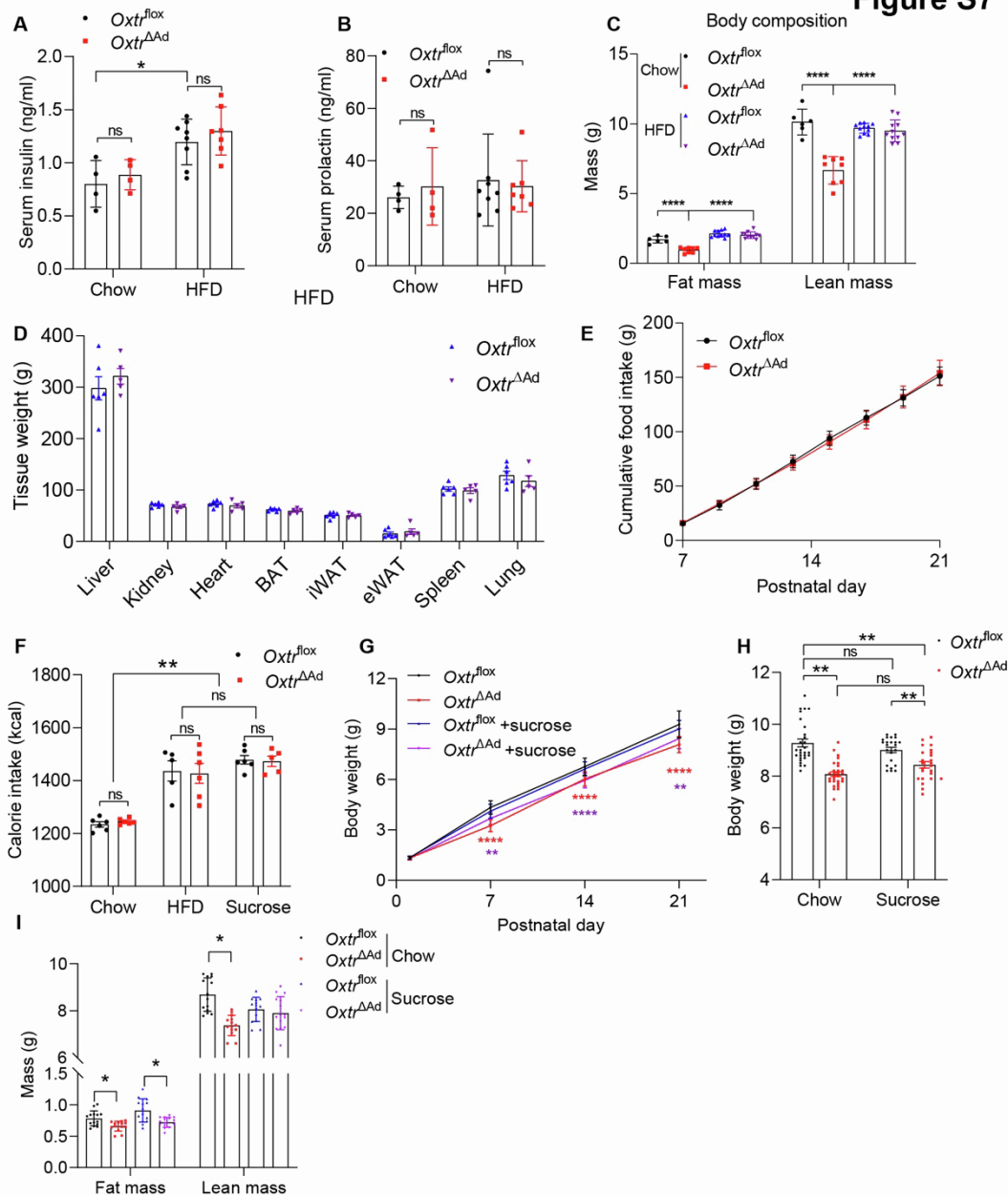


Figure S7. Dietary lipid supplementation rescues the developmental phenotype of pups of *Oxt^{ΔAd}* dams, related to Figure 5. (A) Serum insulin levels in *Oxt^{fllox}* and *Oxt^{ΔAd}* dams on either chow or HFD; n=4-8. **(B)** Serum prolactin levels in *Oxt^{fllox}* and *Oxt^{ΔAd}* dams on either chow or HFD; n=4-8. **(C)** Body composition of pups of *Oxt^{fllox}* and *Oxt^{ΔAd}* dams on chow or HFD on postnatal day 21; n=6-8. **(D)** Tissue weight of pups of *Oxt^{fllox}* and *Oxt^{ΔAd}* dams on HFD on postnatal day 21; n=5-6. **(E)** Cumulative food intake of *Oxt^{fllox}* and *Oxt^{ΔAd}* dams during lactation; n=4-6. **(F)** Accumulated calorie intake of *Oxt^{fllox}* and *Oxt^{ΔAd}* dams on chow, HFD or chow plus *ad libitum* sucrose water during lactation; n=5-6. **(G)** Body weight of pups from *Oxt^{fllox}* and *Oxt^{ΔAd}* dams either on chow or chow plus *ad libitum* sucrose water during lactation; n=23-30. **(H)** Body weight of pups of *Oxt^{fllox}* and *Oxt^{ΔAd}* dams either on chow or chow plus *ad libitum* sucrose water during lactation on postnatal day 21; n=23-29. **(I)** Body composition of pups of *Oxt^{fllox}* and *Oxt^{ΔAd}* dams either on chow or chow plus *ad libitum* sucrose water during lactation on postnatal day 21; n=14-15. Data are presented as mean ± s.e.m. Statistical comparisons were made using 2-tailed Student's t test (A, B, D-F, H, and I) or 2-way ANOVA (C and G). ****P<0.0001.