ARE RODENT MODELS FIT FOR INVESTIGATION OF HUMAN OBESITY AND RELATED DISEASES?

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ARE RODENT MODELS FIT FOR INVESTIGATION OF HUMAN OBESITY AND RELATED DISEASES?

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Not only developed countries, but also most developing areas of the world, have experienced a surge in obesity prevalence over recent decades. Obesity complications are now among the leading causes of premature mortality, encompassing conditions such as coronary heart disease, stroke, and type 2 diabetes. This places a heavy burden on contemporary healthcare systems. While rodent models have limitations as experimental models of human obesity-related disease, study of rats and mice either spontaneously prone - or resistant - to obesity, or genetically engineered to illuminate underlying mechanisms has yielded key information about the metabolic defects linked to obesity, and their associated diseases. This topic includes both original research studies and reviews of the use of animal studies in specific areas of obesity-related disease. Various methodological approaches are discussed, with evaluation of the extent to which use of animal

models has facilitated progress, or, conversely, has proved a cul de sac in investigation of human disease mechanisms. Consideration is also given to future strategies to use such rodent models optimally to enhance comprehension and treatment of pandemic human obesity-related diseases.

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EDITORIAL
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Editorial: Are Rodent Models Fit for Investigation of Human Obesity and Related Diseases?

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Editorial on the Research Topic

Are Rodent Models Fit for Investigation of Human Obesity and Related Diseases?

Mice and rats provide the primary model systems in which the pathophysiology of obesity and its associated pandemic diseases are investigated. Rodent models have been instrumental in the garnering of numerous insights into fundamental pathophysiological mechanisms that are conserved across species. Nevertheless, in key respects rodent physiology is distinct from that of humans, and uncritical overreliance on rodent findings risks impeding translational progress toward improving human health. This research topic aimed to assess whether current use of mouse and rat models is appropriate to maximize generalizable insights and to minimize erroneous conclusions being drawn about human disease. It solicited 16 publications, encompassing 14 opinions or reviews and 2 original research articles, describing or evaluating many aspects of current research performed using rats or mice, covering both causes and consequences of obesity.

Type 2 diabetes is the main focus of three articles. Santosa et al. discuss the use of the rodent pancreatic beta cell to understand the signaling pathways involved in human beta cell differentiation. Laber and Cox and Thomsen et al. consider the successes for rodent modeling of human genome wide association studies for adiposity and type 2 diabetes mellitus (T2DM), encompassing similarities and differences between mouse and human genomes. They consider how animal models can be developed as more precise disease models of T2DM by targeted gene manipulation in the correct developmental and tissue context. Significant limitations of this strategy are also discussed.

Rodent modeling of two major obesity associated diseases, namely non-alcoholic fatty liver disease (NAFLD) and polycystic ovary syndrome (PCOS), is the focus of two articles. The use of monogenic mouse models to interrogate the pathophysiology and genetic predisposition to NAFLD is discussed by Mann et al. This points to interspecies differences and variability in experimental protocols as a limit for the extent to which results from rodent models can currently be extrapolated to humans. Huang-Doran and Franks reviewed the characteristics of the PCOS and assessed the adequacy of rodent models for investigation of this complex pathology. They highlight the variable recapitulation of the PCOS phenotype in rodents, and the relative lack of insulin resistance-related PCOS, quite unlike humans.

Rat models have been used for three decades to study facets of nutrition, endocrinology, the metabolic syndrome, obesity, lipid metabolism, vascular myocardial pathophysiology and pharmacology, and are the primary focus of several articles in the topic. Chusyd et al. review the physiological properties and metabolic profiles of rodent white adipose fat pads and compare these to white adipose depots in humans. The authors also elaborate on sexual dimorphism in

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adipose tissue depots and attempt to explain why premenopausal women generally have a healthier metabolic risk profile than men. Diane et al. discuss how the JCR:LA-cp rat has contributed to understanding of the gut's contribution to increased production of chylomicron particles, and how ruminant-derived trans fatty acids participate in regulating lipid metabolism. The authors also consider modeling of PCOS in this strain of rat. Bi and Moran focused on the insights into the neural basis of food intake and body weight control that have been yielded by studies of the obese OLEF-T rat, a CCK-1 receptor knockout model. The OLEF-T rat has also been valuable in dissection of interactions among exercise and food intake, and in investigating the role of the DMH in energy balance.

Giles et al. report lessons learned from study of the OP/OR rat, including the observation that OP rats have disturbances in hypothalamic signaling pathways involved in energy homeostasis before obesity develops, and while still on a low-fat diet. They also highlight the sexual dimorphism of obesity and point out that this is often ignored because most preclinical studies are performed on male rodent models. The authors have extended the study of the obese rat model to address the menopause and breast cancer using surgical ovariectomy in OP/OR female rats to mimic loss of ovarian function. Surgical ovariectomy was also used in mice by Chalvon-Demersay et al. to address the consequences of estrogen deficiency, however they suggest that differences observed even between rats and mice imply that extrapolations to humans must be made with caution. In the same article, the authors also discuss the "protein leverage hypothesis," which proposes that insufficient protein intake may be a key factor in obesity development. The authors discuss in detail mechanisms whereby dietary protein levels may affect food intake.

The brain is the focus of two further articles. Poon and Leibowitz discuss the various techniques used for the administration of substances to rodents in studies of the neuronal and molecular mechanisms determining the behavioral outcomes of gestational exposure to non-illicit substances of abuse, such as excessive dietary fat, ethanol, and nicotine. Münzberg et al. suggest that environmental factors and genetic predisposition, rather than personal choices, are at the root of the obesity pandemic, and critically evaluate how rodent models can help to understand the contribution of hedonic neural processes to body weight regulation. They summarize how these models helped to solidify the new view that homeostatic and hedonic controls are closely interrelated, often acting in unison at the unconscious level to affect biologically adaptive responses.

Lutz and Bueter review the advantages and limitations of studying mechanisms underlying the benefits of bariatric surgical approaches to obesity treatment (Roux-en-Y gastric bypass and vertical sleeve gastrectomy) in rats and mice. They conclude that most animal models recapitulate remarkably well findings in humans, but indicate that animals larger than rats and mice may offer additional specific advantages.

Thornton reviews experiments in favor of the specific hypothesis that increased hydration leads to body weight and fat loss through a decrease in feeding. The hypothesis derives from a broad association between chronic dehydration, raised levels of angiotensin II and chronic diseases such as obesity, diabetes, cancer, and cardiovascular diseases. Proposed mechanisms involve an increase in metabolism due to expansion of cell volume by hydration.

Two original studies were published in this topic. Even and Blais sound a cautionary methodological note by demonstrating that the cost of thermoregulation in mice housed at room temperature strongly affects attempts to estimate thermogenic responses to feeding accurately, due to heat transfer between diet-induced thermogenesis and non-shivering thermogenesis. They suggest that these observations undermine the use of mice housed below thermoneutrality to model human disorders of energy balance. Finally, Lombardo et al. show that use of a simple standard diet, without additive agents and without caloric restriction, is sufficient to rescue high-fat feeding-induced insulin resistance and prevent the evolution of diabetes without the need for a hypocaloric diet.

The articles in this topic offer a cross section of current approaches to studying obesity and its associated diseases in rodents. They highlight successes and failures, as well as illustrating important methodological issues and some emerging hypotheses in the field. Collectively, they attest to the enduring utility of highly tractable rodent models of complex human metabolic disease and to the importance of continued and careful adjustment of rodents to the disease being modeled. They further raise awareness of areas where species-specific pathophysiology appears irrevocably distinct. Appropriately critical use of rodent models will remain vital to interrogation of pandemic human metabolic disease.

AUTHOR CONTRIBUTIONS

All authors have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Knowledge Gaps in Rodent Pancreas Biology: Taking Human Pluripotent Stem Cell-Derived Pancreatic Beta Cells into Our Own Hands

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In the field of stem cell biology and diabetes, we and others seek to derive mature and functional human pancreatic β cells for disease modeling and cell replacement therapy. Traditionally, knowledge gathered from rodents is extended to human pancreas developmental biology research involving human pluripotent stem cells (hPSCs). While much has been learnt from rodent pancreas biology in the early steps toward Pdx1$^+$ pancreatic progenitors, much less is known about the transition toward Ngn3$^+$ pancreatic endocrine progenitors. Essentially, the later steps of pancreatic β cell development and maturation remain elusive to date. As a result, the most recent advances in the stem cell and diabetes field have relied upon combinatorial testing of numerous growth factors and chemical compounds in an arbitrary trial-and-error fashion to derive mature and functional human pancreatic β cells from hPSCs. Although this hit-or-miss approach appears to have made some headway in maturing human pancreatic β cells *in vitro*, its underlying biology is vaguely understood. Therefore, in this mini-review, we discuss some of these late-stage signaling pathways that are involved in human pancreatic β cell differentiation and highlight our current understanding of their relevance in rodent pancreas biology. Our efforts here unravel several novel signaling pathways that can be further studied to shed light on unexplored aspects of rodent pancreas biology. New investigations into these signaling pathways are expected to advance our knowledge in human pancreas developmental biology and to aid in the translation of stem cell biology in the context of diabetes treatments.

Keywords: pancreas, islet, beta cell, human, pluripotent stem cell

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INTRODUCTION

In the field of stem cells and diabetes, many scientists are actively pursuing the generation of insulin-secreting pancreatic β cells from human pluripotent stem cells (hPSCs) for β cell transplantation/replacement and treatment of diabetes (1–3). While insights from rodent pancreas developmental biology has guided the generation of PDX1$^+$ pancreatic progenitors from hPSCs, the specific

developmental principles thereafter remain murky. Henceforth, research groups have relied upon the transplantation of pancreatic progenitors, derived *in vitro*, into rodents for *in vivo* maturation (4–6). However, there has been considerable progress toward the generation of mature and functional human pancreatic β cells *in vitro* in the recent years. These β cells purportedly co-express cardinal β cell markers, such as PDX1, NKX6.1, musculoaponeurotic fibrosarcoma oncogene homolog A (MAFA), prohormone-processing enzymes, insulin, and C-peptide. Importantly, they are also monohormonal and glucose responsive.

Developmental biologists believe that there is much to be learnt from rodent developmental biology to guide hPSC-based generation of clinically useful cell types, such as pancreatic β cells. Owing to such efforts, the progression of definitive endoderm (DE) germ layer to PDX1$^+$ pancreatic progenitors has been well-explored. However, the investigations on the later steps of pancreatic endocrine development and β cell maturation have not been quite fruitful. The most substantial advances in stem cell biology have relied upon an arbitrary approach of iterative trial-and-error testing to achieve mature and functional pancreatic β cells *in vitro* (7). Therefore, several pertinent questions remain: why were we not able to extrapolate rodent developmental principles and apply them on hPSCs to derive mature and functional pancreatic β cells? Are there differences between rodent and human pancreas development that prevent such an application? In this review, we look at signaling pathways that have been activated or repressed in stem cell biology and retrospectively revisit existing knowledge about rodent pancreas biology. Our efforts highlight novel aspects of signaling pathways that can be further investigated in our translational efforts for diabetes.

INHIBITION OF TRANSFORMING GROWTH FACTOR-β SIGNALING IN THE LATER STAGES OF PANCREATIC DIFFERENTIATION

The transforming growth factor-β (TGF-β) superfamily of proteins regulates pancreas development and function (8). TGF-β1, TGF-β2, and TGF-β3 are expressed in pancreatic epithelial cells at E12.5 in mice. Thereafter, they become localized in the acinar cells (9). TGF-β1 can promote the development of mouse pancreatic β cells from pancreatic buds (10). Perplexingly, it also indirectly inhibits the formation of mouse pancreatic epithelial cells (11). In tandem, TGF-β2 has been demonstrated to inhibit *Hnf1*β and *Pdx1* gene expression. Hence, TGF-β can purportedly restrain the specification of pancreatic cell fate (12). TGF-β signaling effector SMAD3 can bind the *Ins* gene promoter to suppress its expression. In agreement, *Smad3*-deficient islets exhibit an active insulin signaling pathway (13). Collectively, these evidences suggest the requirement to inhibit TGF-β signaling for the derivation of mature and functional pancreatic β cells (**Figure 1A**).

In 2011, Nostro et al. used small molecule SB431542 (14), an Activin/TGF-β receptor antagonist, in their pancreatic differentiation protocol. SB431542 inhibits activin receptor-like kinases (ALK) 4/5/7 and the downstream TGF-β/Activin/Nodal signaling. SB431542 treatment was demonstrated to increase *INS* gene expression and the development of C-peptide$^+$ cells (15). Similarly, Cho et al. also utilized SB431542, in the presence of retinoic acid (RA), for pancreatic differentiation (16). Alternatively, Schulz et al. used TGF-βRI kinase inhibitor IV to obtain pancreatic progenitors from CyT49 hPSCs (17). Rezania et al. identified that the use of 2-(3-[6-Methylpyridin-2-yl]-1*H*-pyrazol-4-yl)-1,5-naphthyridine (ALK5iII) can effectively induce the expression of *NGN3*, *NEUROD1*, *INS,* and *GCG* transcripts to promote pancreatic endocrine specification (18). Rezania et al. further demonstrated that 1 μM ALK5iII is necessary for the induction of NEUROD1$^+$ cells, but it suppressed the proportion of NKX6.1$^+$ cells (4), a hallmark of functional β cells (19). Most recently, Rezania et al. compared the effects of several ALK5 inhibitors at a later phase of differentiation of hPSCs and found that only ALK5iII downregulated *NGN3* while increasing *INS*, *GCG,* and *SST* transcripts (6). Furthermore, 10 μM ALK5iII induced the expression of nuclear v-maf MAFA transcript, a critical mature β cell transcription factor, in diabetic rodents (20–22). Rezania et al. (6) concluded that ALK5iII was the most effective and specific inhibitor as it inhibited ALK5 but had minimal inhibition of other kinases. Similarly, Pagliuca et al. also employed 10 μM Alk5iII to derive mature and functional human pancreatic β cells from hPSCs (7) (**Figure 1B**; **Table 1**).

Overall, the inhibition of ALK5/TGF-βRI with ALK5iII appears to be more desirable as compared to the general inhibition of TGF-β signaling via the use of SB431542. Further studies are certainly required to investigate the intricacies of TGF-β signaling during pancreas development and β cell maturation.

PROTEIN KINASE C SIGNALING ENHANCEMENT

Protein kinase C (PKC) is a family of serine/threonine kinases that are involved in diverse cellular processes, including survival, apoptosis, cell cycle regulation, proliferation, migration, and differentiation (23). In maturing neonatal rat islets, PKCα was only found in β cells, PKCγ in α cells, and PKCε in δ cells (24). This differential expression of PKC isoenzymes (25) hints that PKC signaling may play a role in the functional maturation of pancreatic endocrine progenitors (**Figure 1A**).

Chen et al. (26) was the first to demonstrate that 300 nM (−)-indolactam V (ILV) or PKC agonists {500 nM [(2*S*,5*S*)-(*E*,*E*)-8-(5-(4-(trifluoromethyl)phenyl)-2,4-pentadienoylamino) benzolactam (TPB)] or 14 nM phorbol-12-myristate-13-acetate (PMA)} can efficiently increase the formation of PDX1$^+$ pancreatic progenitors from hPSCs via the activation of PKC signaling. ILV treatment resulted in an increased gene expression of several pancreatic progenitor markers, including *SOX9*, *PDX1*, *PTF1A*, *HNF6*, and *PROX1*, and endocrine progenitor markers, including *NGN3*, *NKX2.2,* and *NKX6.1*. The protein expression of pancreatic progenitor markers FOXA2, PTF1A, HNF6, and NKX6.1 were increased, whereas the expression of intestinal marker CDX2 and liver marker AFP were suppressed (26). In addition, they also found that ILV and PKC agonists, TPB or PMA, can synergize with FGF10 signaling to promote the proliferation of PDX1$^+$ cells derived from hPSCs. Interestingly, ILV treatment also works on

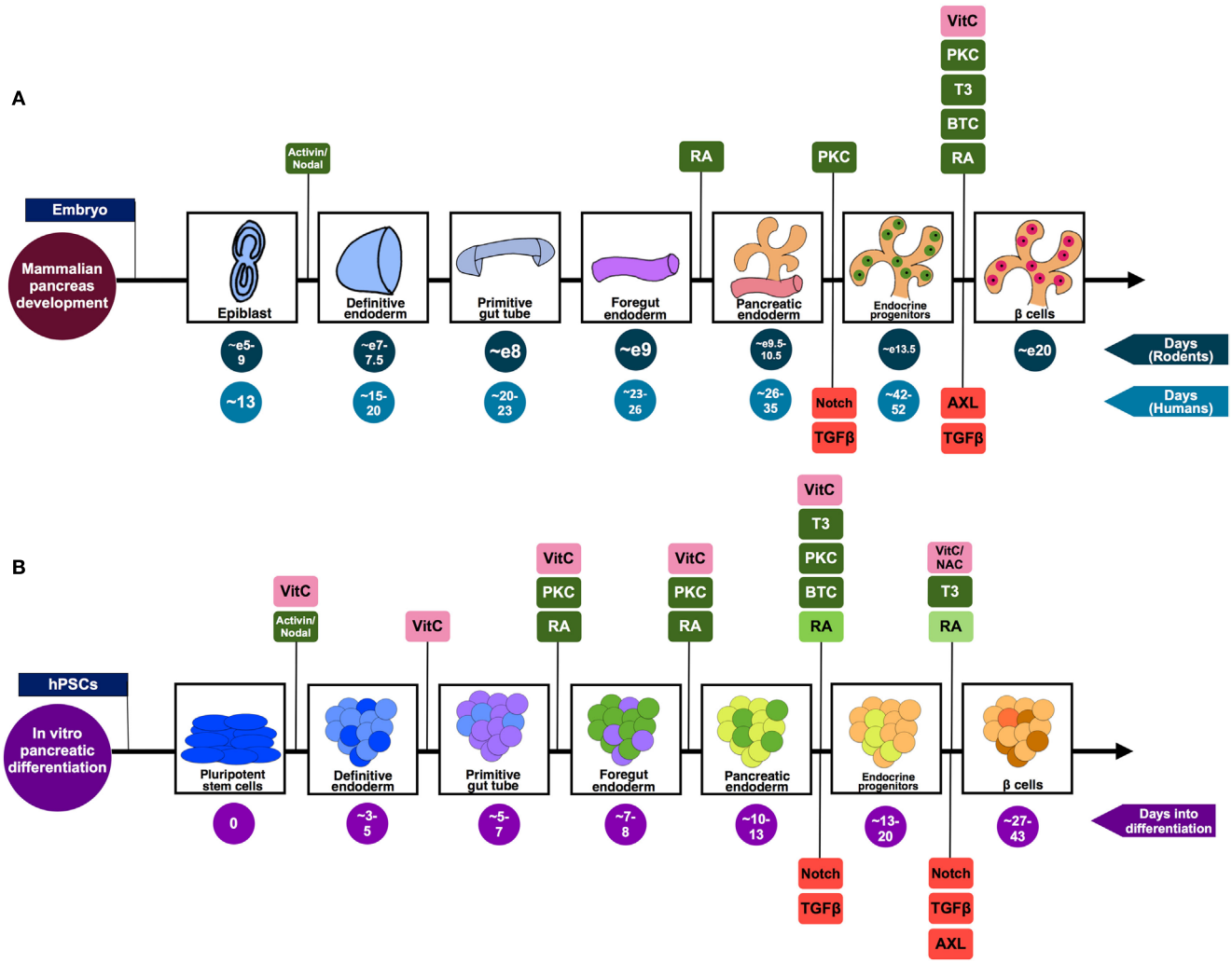

FIGURE 1 | A summary of the pivotal stages of pancreatic differentiation. (A) Mammalian pancreas development based on knowledge from both rodent and human studies. The signaling pathways suggested to be positively regulating the differentiation process are highlighted in green, while those found to be inhibited in order to drive differentiation toward pancreatic lineage are shown in red. **(B)** An *in vitro* pancreatic differentiation timecourse generating β-like cells from hPSCs. The signaling pathways (green) and antioxidants (pink) that are positively regulating the differentiation process at each phase of development are highlighted. Stage-specific signaling pathways that are inhibited to drive differentiation toward pancreatic lineage are shown in red. The decreasing doses of RA used during the differentiation process [as described by Rezania et al. (6) and Pagliuca et al. (7)] are represented by the decreasing shades of green. The differing colors co-existing in an aggregate illustrates the heterogeneity of cells prevalent in such a differentiation scheme. While some of the cells will transit from being endocrine progenitors (light orange) to Pdx1$^+$ insulin-producing β-like cells (brown), the end-product will include an assortment of maturing endocrine cell types (represented by orange and light orange).

mouse embryonic stem cells, suggesting a conservation of signaling pathway in both mouse and human cells (26) (**Table 1**).

Subsequently, Rezania et al. (4) employed two PKC activators in the mid-late stage of hPSC differentiation; 50 nM TPB (safer profile) and phorbol 12,13-dibutyrate (PDBu) (27), demonstrating that the activation of PKC signaling induces the gene expression of pancreatic lineage markers *PTF1A*, *NGN3*, *NEUROD1*, and *NKX6.1* while suppressing the expression of intestinal (*CDX2*) and liver (*ALB*) markers (4). This demonstrated that PKC signaling enriches the development of pancreatic progenitors while inhibiting intestinal and hepatic lineages. Similarly, Pagliuca et al. used 500 nM PDBu in their pancreatic differentiation protocol (7) (**Figure 1B**; **Table 1**). Although these data are encouraging, more studies remain to be done to thoroughly clarify the role of PKC signaling and the specific mechanisms in the maturation of pancreatic endocrine progenitors.

LOWER LEVEL OF RETINOIC ACID SIGNALING AS PANCREATIC DIFFERENTIATION PROGRESSES

It is well-established that RA signaling plays critical roles in the early and late stages of pancreas development (28). RA is a

TABLE 1 | Summary of some novel signaling pathways perturbed during pancreatic differentiation of hPSCs.

	Molecules	Mechanism	Induction of pancreatic lineage markers	Reference	Humans	Rodents
TGF-β inhibition	SB431542	Inhibits ALK 4/5/7	Upregulates *INS* gene expression and C-peptide$^+$ cells	Nostro et al. (15)	√	
	SB431542 + RA		Upregulates *PDX1* gene expression	Cho et al. (16)	√	
	TGF-βRI kinase inhibitor IV		Induces pancreatic progenitors from hPSCs	Schultz et al. (17)	√	
	1 μM ALK5iII	Inhibits ALK5	Downregulates *NGN3*. Upregulates *NEUROD*, *INS*, *GCG*, and *SST* transcripts Induces *Mafa* transcript expression in diabetic rodents	Rezania et al. (4, 6, 18)	√	√
	10 μM ALK5iII			Pagliuca et al. (7)	√	
Protein kinase C signaling	300 nM ILV	Activates PKC Synergizes with FGF10 signaling	Upregulates gene expression of pancreatic progenitor markers *SOX9*, *PDX1*, *PTF1A*, *HNF6*, *PROX1* Upregulates gene expression of endocrine progenitor markers including *NGN3*, *NKX2.2*, and *NKX6.1*	Chen et al. (26)	√	√
	500 nM TPB		Upregulates protein expression of FOXA2, PTF1A, HNF6, and NKX6.1		√	
	14 nM PMA		Downregulates protein expression of endoderm markers CDX2 and AFP		√	
	50 nM TPB	Activates PKC	Upregulates gene expression of pancreatic lineage markers *NGN3*, *NEUROD1*, *PTF1A*, and *NKX6.1* Downregulates gene expression of intestinal marker *CDX2* and liver marker *ALB*	Rezania et al. (4)	√	
	500 nM PDBu			Pagliuca et al. (7)	√	
Low retinoic acid (RA) signaling	1–3 μM RA	Activates RA receptors		Various	√	
	3 nM TTNPB			Schulz et al. (17)	√	
	1 μM → 100 nM → 50 nM RA			Rezania et al. (7)	√	
	2 μM → 100 nM → 25 nM RA			Pagliuca et al. (7)	√	
γ-secretase/Notch inhibitor	DAPT	Inhibits Notch signaling	Upregulates *NGN3* mRNA and protein expression in adult islets	Dror et al. (36)	√	√
			Upregulates *NGN3* and *NEUROD1* gene expression	Rezania et al. (18)	√	
	GSiXX		Upregulates expression of β cell maturation genes Downregulates expression of pancreatic exocrine marker *PTF1A*	Rezania et al. (6)	√	
	GSiXX + T3		Upregulates NKX6.1$^+$insulin$^+$GCG$^-$ β-like cells	Rezania et al. (6)	√	
	1 μM XXI		Upregulates β cell gene expression	Pagliuca et al. (7)	√	
T3	0.1 μM T3	Activates MAPK/ ERK signaling pathway	Induces rodent pancreatic β cell proliferation	Kim et al. (42)		√
	1 μM T3		Upregulates expression of *INS* and mature β cell markers	Rezania et al. (6)	√	
			Enhances co-expression of NKX6.1 and INS protein	Pagliuca et al. (7)		
AXL	2 μM BGB324 (R428)	Inhibits AXL	Upregulates MAFA protein expression	Rezania et al. (6)	√	
	GAS6	Activates AXL	Downregulates *Mafa* gene expression	Haase et al. (48)		√
Antioxidants	GPx-1	Antioxidants	Maintains protein expression of nuclear MAFA in diabetic rodents	Harmon et al. (22)		√
	NAC			Harmon et al. (21)		√
	Ebselen			Mahadevan et al. (20)		√
	1–2 mM NAC		Upregulates nuclear MAFA protein expression	Rezania et al. (6)	√	
	0.25 mM vitamin C		Generates mature and functional human pancreatic β cells	Pagliuca et al. (7)	√	
Betacellulin	BTC	Binds to ErbB-1 and ErbB-4 receptors to initiate PI3K/Akt, MAPK, STAT, and mTOR signaling pathways	Upregulates insulin secretion	Dahlhoff et al. (65)		√
			Upregulates mRNA and protein expression of IRS-2	Oh et al. (68)		√
			Induces *Pax4* gene expression in rat islets	Brun et al. (70)		√
			Sustains *PDX1* expression and induces β cell differentiation from hESCs	Cho et al. (71)	√	
	10 ng/ml BTC		Upregulates *Pdx1* gene expression and insulin production Downregulates amylase and glucagon production in mouse embryonic pancreas explants	Thowfeequ et al. (72)		√
	20 ng/ml BTC		Induces pancreatic differentiation	Pagliuca et al. (7)	√	
	50 ng/ml EGF		Preserves cell mass	Schulz et al. (17)	√	

BTC, Betacellulin; DAPT, N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester; GPx-1, glutathione peroxidase-1; GSiXX, γ secretase inhibitor XX; ILV, (–)-indolactam V; NAC, N-acetylcysteine; PDBu, phorbol 12,13-dibutyrate; PMA, phorbol-12-myristate-13-acetate; T3, L-3,3′,5-Triiodothyronine; TPB, [(2S,5S)-(E,E)-8-(5-(4-(trifluoromethyl)phenyl)-2,4-pentadienoylamino) benzolactam].

lipid-soluble vitamin A derivative synthesized from the oxidation of retinaldehyde via enzymes retinaldehyde dehydrogenase 1 (RALDH1), RALDH2, and RALDH3. RA produced at the splanchnic lateral plate mesoderm and Raldh2 expressed in the dorsal pancreatic mesenchyme promote Pdx1 induction in the dorsal foregut endoderm (**Figure 1A**). Raldh2 mutant mice exhibit dorsal pancreatic bud agenesis (29) as they fail to form pancreatic progenitors, indicated by the loss of Pdx1, Prox1, altered Isl1, and reduced Hlxb9 expression (29, 30).

Many existing protocols differentiating hPSCs into pancreatic cells utilize 1–3 μM RA. As an alternative, Schulz et al. replaced RA with 3 nM TTNPB/arotinoid acid – a more stable retinoid analog that can selectively activate RA receptors (RARs) (17). Interestingly, Rezania et al. started out using 1 μM RA during the early posterior foregut differentiation, subsequently reducing to 100 nM RA during pancreatic endoderm phase and further reducing to 50 nM during the pancreatic endocrine phase (6). Pagliuca et al. also reported a similar pancreatic differentiation protocol in which a decreasing dose of RA was used, starting with 2 μM followed by 100 nM RA at later stages, which was eventually reduced to 25 nM (7) (**Figure 1B**; **Table 1**).

While it is widely accepted that RA is crucial for pancreatic specification, using progressively lower doses of RA as practiced by Rezania et al. (6) and Pagliuca et al. (7) raises questions about the importance of RA concentrations during pancreas development both *in vitro* and *in vivo*. RA signals by binding RARs and retinoid X receptors (RXRs); so it is postulated that a decrease in RA signaling may be more conducive for subsequent pancreatic endocrine specification since retinoid receptors are upregulated in the pancreatic exocrine (31). However, given the lack of understanding in this regard, the significance of the dose of RA during human pancreas development warrants further studies.

INHIBITING γ-SECRETASE/NOTCH FOR ACCELERATED PANCREATIC ENDOCRINE DIFFERENTIATION

Notch signaling is essential for the proper development of pancreatic endocrine progenitors as it regulates their decision between differentiation and proliferation (32). The reduction of Notch signaling is known to promote accelerated pancreatic endocrine differentiation (33). Similarly, the inhibition of Notch signaling via γ-secretase (an intra-membrane protease) inhibitor can downregulate the expression of Notch target Hes-1, an inhibitor of pro-endocrine gene Ngn3 (34) (**Figure 1A**). Conversely, the activation of Notch in $PDX1^+$ pancreatic progenitors prevents pancreatic differentiation (35).

N-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine t-butyl ester (DAPT) is a commonly used γ-secretase inhibitor with an IC50 in the nM range. The inhibition of Notch signaling with DAPT can increase Ngn3 mRNA and protein expression in adult islets (36). Likewise, DAPT increases *NGN3* and *NEUROD1* gene expression in hPSC-derived pancreatic progenitors (18). In recent protocols developed by Pagliuca et al. (7) and Rezania et al. (6), other γ-secretase inhibitors have been employed to retard Notch signaling. Rezania et al. used γ-secretase inhibitor XX (GSiXX) that has an IC50 in the low nM range. They showed that GSiXX can induce the expression of β cell maturation genes but inhibit the expression of *PTF1A*, a marker of pancreatic exocrine lineage (6). GSiXX can also act in concert with triiodothyronine (T3) to increase the percentage of $NKX6.1^+INS^+GCG^-$ β-like cells (6). Alternatively, Pagliuca et al. employed the use of XXI at 1 μM (7), which has an IC50 in the picomolar range, and demonstrated that XXI worked with other factors to improve β cell gene expression (**Figure 1B**; **Table 1**). However, it remains unclear whether there are differences between DAPT, GSIXX, or XXI in the induction or suppression of key pancreatic transcription factors for the eventual promotion of pancreatic β cell formation.

TRIIODOTHYRONINE COULD PROMOTE PANCREATIC β CELL MATURATION

Studies in the 1980s suggest that thyroid hormones regulate insulin secretion, possibly via control over glucose oxidation and calcium uptake rates (37). T3, a thyroid hormone, can potentiate insulin signaling and increase insulin synthesis in diabetic rodents (38), in rodent islets (39), and in a rodent pancreatic β cell line (40) (**Figure 1A**). Mechanistically, T3 phosphorylates and activates AKT in pancreatic β cells, improving their survival (39, 41); 0.1 μM of T3 can increase rodent pancreatic β cell proliferation via the MAPK/extracellular signal-regulated kinase (ERK) signaling pathway (42). Interestingly, T3 apparently induces the transdifferentiation of human pancreatic ductal cell line (hPANC-1) into β-like cells, with an increased expression of *INS* transcripts (43). T3 can also increase both the mRNA expression of pro-endocrine gene Ngn3 and the number of β cells, indirectly inducing endocrine differentiation from exocrine cells; possibly via Akt signaling (44).

Of late, T3 has been shown to promote pancreatic β cell maturation and proliferation in rats (45). Based on these findings, Rezania et al. went on to demonstrate that 1 μM of T3 can actually induce the expression of *INS* and mature β cell markers, and enhance the co-expression of NKX6.1 and INSULIN protein (6). Similarly, Pagliuca et al. employed the same dose of 1 μM T3 in the later stages of their pancreatic differentiation protocol to generate human pancreatic β cells from hPSCs (7) (**Figure 1B**; **Table 1**). While the biology and role of T3 in pancreatic β cell maturation remains to be explored further, its inclusion in pancreatic differentiation protocols appears to serve a positive function.

INHIBITION OF TYROSINE KINASE RECEPTOR AXL INDUCES MATURE PANCREATIC β CELL MARKER (MAFA) EXPRESSION

AXL is a member of the Tyro3-Axl-Mer (TAM) transmembrane receptor tyrosine kinase (RTK) family that plays

an important role in essential cellular processes, such as cell survival, growth, proliferation, and differentiation. Its ligand, growth arrest specific 6 (Gas6), binds AXL to activate downstream signaling, including the phosphoinositide 3-kinase (PI3K), ERK, and signal transducer and activator of transcription 3 (STAT3) signaling (46). Interestingly, Rezania et al. performed small molecule and growth factor library screening to identify compounds that can induce mature β cell marker MAFA from hPSC-derived pancreatic progenitors and found that 2 μM BGB324 (R428), an inhibitor of AXL, can induce MAFA protein expression (6) (**Figure 1B**; **Table 1**). However, there is little information linking AXL to pancreas development and β cell maturation.

In 1999, Augustine et al. reported that the overexpression of AXL results in diabetes in mice. Furthermore, the administration of exogenous Gas6 exacerbated the condition (47). Haase et al. recently confirmed that GAS6 is expressed in pancreatic tissues and found that GAS6 reduced *Mafa* gene expression in rodents (48), likely due to the activation of AXL (**Figure 1A**). This corresponds with the increase in MAFA expression observed by Rezania et al. after the inhibition of AXL signaling (6). While there seems to be a *bona fide* association between AXL signaling and pancreas development, the cellular mechanism(s) remain a mystery.

ANTIOXIDANTS MAY BENEFIT PANCREATIC DIFFERENTIATION

Excessive levels of reactive oxygen species (ROS) have been implicated in glucotoxicity-induced pancreatic β cell destruction and dysfunction. In this regard, antioxidants play important defensive roles against ROS. In the endocrine pancreas, the antioxidant vitamin C is known to be an effective co-factor for the peptidyl α-amidation of several biologically active peptides and is necessary for optimal insulin secretion from pancreatic β cells (**Figure 1A**). In fact, high concentrations of ascorbic acid (vitamin C) were found in neonatal rat endocrine pancreas (49). Mechanistic studies involving other antioxidants, such as glutathione peroxidase-1 (GPx-1), *N*-acetylcysteine (NAC), and ebselen, were reported to maintain the protein expression of mature β cell marker MAFA (20–22) (**Table 1**).

Interestingly, Rezania et al. used 1–2 mM NAC during their pancreatic differentiation and found that it also increased nuclear MAFA protein expression (6). However, this was not replicated with another antioxidant, vitamin E. Pagliuca et al. also relied upon the use of 0.25 mM of vitamin C throughout S1–S5 phase of their pancreatic differentiation protocol to generate mature and functional human pancreatic β cells (7) (**Figure 1B**; **Table 1**). While the metabolism of vitamins C and E are altered before the onset of diabetes in rats, their contribution to the pancreas is unclear (50). Antioxidant treatments may preserve β cell function, exerting positive effects in diabetes (51), but their role in pancreas development and β cell maturation certainly remains elusive.

BETACELLULIN DIRECTS A PANCREATIC β CELL FATE

Betacellulin (BTC) is a member of the epidermal growth factor (EGF) family that plays a role in the differentiation of pancreatic β cells (52) (**Figure 1A**). It is largely expressed in the liver, kidney, small intestine, and pancreas (53), and is specifically expressed in 9- to 24-week-old human fetal pancreas (54). BTC binds to ErbB-1 and ErbB-4 receptors (55) to initiate downstream signaling pathways involving PI3K/Akt, MAPK, STAT, and mTOR signaling pathways (56).

Betacellulin appears to direct a pancreatic β cell fate. It can convert exocrine cells (57) and α cells (58) into insulin-secreting cells. It can also induce β cell neogenesis from ductal cells in diabetic mice (59). Li et al. demonstrated that exogenous BTC can promote β cell regeneration in 90% pancreatectomized rats (60) and convert δ to β cells in STZ-induced diabetic mice (61). Also, Yamamoto et al. observed that long-term administration of BTC reverses STZ-induced hyperglycemia in mice (62). Surprisingly, the loss of BTC in mice yielded no overt defect (63) despite their active roles in the pancreas. This could be explained by compensatory effects exhibited by the other EGFR ligands (64). The overexpression of BTC in transgenic islets does not affect islet structure, endocrine cell ratio, or β cell mass but enhances glucose-stimulated insulin secretion (65). However, ubiquitous overexpression of BTC in mice results in various pathologies (66). Intriguingly, gene variants and polymorphisms in the *BTC* gene have also been found to be associated with types 1 and 2 diabetes (54, 67).

The induction of β cell development/differentiation by BTC (52) could be an outcome of the downstream increase in insulin receptor substrate-2 (IRS-2) expression (68), an important mediator of β cell function (69). BTC can induce *Pax4* gene expression in rat islets, promoting β cell functionality (70). It can also sustain *PDX1* expression and induce β cell differentiation from hPSCs (71). Ten ng/ml BTC are sufficient to increase *Pdx1* gene expression, insulin production, and to inhibit amylase and glucagon production in mouse embryonic pancreas explants (72). Lately, Pagliuca et al. employed 20 ng/ml BTC in the later stages of their pancreatic differentiation protocol (7). Similarly, 50 ng/ml EGF was added to preserve cell mass (17) (**Figure 1B**; **Table 1**). These data strongly indicate that BTC and/or other EGF ligands are of importance in pancreas development and β cell maturation. Nonetheless, more detailed molecular mechanisms remain to be uncovered.

CONCLUDING REMARKS

Relating to the current most advanced human pancreatic β cell differentiation protocols, the biological mechanisms involved in the later stages of β cell development and maturation still remain elusive. The fact that the postnatal stage in rodents is grossly equivalent to the third trimester in human pancreas development (73) invites new approaches to study this aspect of β cell biology. In this review, we revisited some of the least-understood developmental signaling pathways in rodent pancreas biology. Our efforts unraveled interesting aspects of these signaling pathways that demand to be thoroughly elucidated at the mechanistic level.

Future studies should seek to highlight how immature β cells transit into mature and functional β cells. This would certainly advance our knowledge of human pancreas developmental biology and boost translational efforts in the use of stem cells for diabetes treatment.

AUTHOR CONTRIBUTIONS

MS: reviewed the literature, wrote and edited the paper. BL: edited the paper. NP: edited the paper and prepared the figure. AT: conceptualized the review topic and contents, wrote, edited, and approved the paper.

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OPINION
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Mouse Models of Human GWAS Hits for Obesity and Diabetes in the Post Genomic Era: Time for Reevaluation

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In recent years, genome-wide association studies (GWAS) have identified hundreds of loci and thousands of single-nucleotide polymorphisms (SNPs) associated with type 2 diabetes mellitus (T2DM) and obesity traits [such as body mass index (BMI) and waist–hip ratio (WHR)] in the human population (1–4). The vast majority of these SNPs are in non-coding regions of the genome and distal to promoters, suggesting they act through gene regulation which makes their functional interpretation challenging (5). Collectively, comparing the epigenetic landscape between mouse and human has established new pathways involved in obesity and diabetes, and in fact, inter-species conservation has successfully been used as criteria in finding functional and disease-relevant elements (6–8). By contrast, genome-wide comparative analysis of the mouse and human epigenome across tissues has highlighted the presence of cis-regulatory divergence (9, 10). New mouse engineering approaches together with bioinformatics dissection of trait-associated regions, for example, epigenetic modifications and genome interactions hold great promise to fully understand the underlying mechanisms of human disease-associated non-coding variants in T2DM and obesity.

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THE CONTEXT-SPECIFIC NATURE OF HUMAN GWA SIGNALS IN HUMAN

Over 80% of loci identified by GWAS are in intergenic and intronic regions and many of these genetic risk regions are enriched for histone modifications (5), suggesting they act as regulatory elements which appear to function in a highly cell-selective manner. Due to the tissue specificity as well as the developmental and epigenetic complexity of gene regulation, functional approaches require the study of the relevant tissue and cell type as well as genetic and bioinformatics approaches that reliably assess the regulatory role of non-coding variants (7, 11, 12). Ongoing progress in high-throughput sequencing and the development of new experimental tools are greatly advancing our capacity to study chromatin biology and genome function. In particular, ChIP-seq allows identification of transcription factor binding sites and chromatin states; chromosome conformation capture-based techniques (including 3C, 4C, 5C, CaptureC, and HiC) allow the study of chromatin interactions; and DNase hypersensitivity or ATAC-seq can identify accessible chromatin (13–16). Additionally, tools like HaploReg (17), Enlight (18), RegulomeDB (19), and The Islet Regulome Browser (11) are emerging that allow the integration of GWAS results with genetic and epigenetic annotations that can be used to dissect the gene regulatory networks that underpin genomic association signals.

By integrating the information gained from functional genomics efforts such as the ENCODE (5) and Roadmap Epigenomics projects (20) together with expression quantitative trait loci (eQTL) results and functional studies, it becomes increasingly clear that adipose tissue is one of several key effectors of genetic risk loci for T2DM and obesity trait associations, particularly for WHR signals (2, 7, 21, 22). However, there is currently still a lack of comprehensive maps linking distal elements that harbor disease-associated variants with their target genes in relevant tissues and developmental

stages. Furthermore, extensive fine-mapping of risk associations is crucial in order to narrow down the association signal to the likely causative variants which can then be functionally investigated (23). This has resulted in many studies being performed assuming that the closest gene to a given disease-associated signal is the causative one. Traditionally, target genes based on proximity to a signal were selected to model in the mouse using global or tissue-specific gene knockout or overexpression alleles to characterize gene function (24). However, with this approach, many target genes for GWA signals have potentially been overlooked, for example, in the case of the BMI-associated variants in *Fto* (25, 26). An additional level of complexity comes with the possibility for an association signal—that usually harbors dozens of SNPs—to potentially contain a number of disease-causing variants that might act in different tissues and/or at different times, affecting different genes. For example, there is currently evidence for intronic *FTO* risk variants to alter the expression of nearby genes in both adipose tissue and brain. An eQTL in human cerebellum links re9930509 to altered *IRX3* expression (25), rs1421085 has very convincingly been shown to be located within an enhancer for *IRX3* and *IRX5* in adipocyte precursors (7, 26) and rs1421085 and rs8050136 have been proposed to selectively alter *FTO* and *RPGRIP1L* expression in human-induced pluripotent stem cell-derived neurons (27). Therefore, mouse models which could help pinpoint variants, target genes, and relevant tissues would prove invaluable in the mechanistic dissection of human disease-associated sequence variants. However, whether it is possible to use the mouse for modeling regulatory variants (which is essential to capture the relevant spatiotemporal effects) will depend on the functional conservation of the regulatory circuitry of a given signal in human and mouse.

CONSERVATION BETWEEN HUMAN AND MOUSE

It is estimated that our last common ancestor with the mouse was about 90 million years ago (28). At this point, many of the core physiological regulatory mechanisms had evolved, for example, mouse and human share the same basic mechanisms for controlling food intake *via* leptin and hypothalamic anorexigenic and orexigeneic neurons, and similarly insulin and glucagon are core effectors in glucose homeostasis. However, there have clearly been many evolutionary changes over this long period of time. At the level of the genome, chromosome number and organization have changed, although it is striking how large tracts of DNA have conserved their order of genes and show high coding sequence conservation (29). Thus, if we wish to use the mouse as model of human metabolic disease we can rely on much of the core conservation of ancient metabolic pathways and their regulation but cannot ignore the fact of their continued evolution that adapts and changes these mechanisms for the survival of two very different organisms. The mouse ENCODE Consortium reported that comparative gene expression data from human and mouse reveals that some sets of genes tend to cluster more by species than by tissue and *vice versa* (29). More recently, it has been suggested that gene clustering by tissue rather than species is much stronger than originally thought (30). Interestingly, single-cell sequencing of human and mouse pancreatic alpha and beta cells showed good cross-species correlation of transcriptomes although with some important species differences (31). Finally, Breschi et al. (32) describe how transcriptomes show a continuum of variation from species dominated clustering to organ dominated clustering. Importantly, for modeling GWA signals in other species, genes that varied little between species (and are more organ-specific) are more likely to overlap with human risk variants (32).

EPIGENOMIC CONSERVATION BETWEEN HUMAN AND MOUSE—INSIGHTS FROM THE MOUSE ENCODE CONSORTIUM

Some of the other key findings of ENCODE in the mouse genome were that human and mouse trans-regulatory networks (transcription factor networks) are considerably more conserved than the cis-regulatory landscape, which in fact accounts for the majority of regulatory plasticity between human and mouse (28, 29). At the same time, the degree of divergence of regulatory elements varies widely between different types of elements that are active in different tissue contexts (9, 28). The Mouse ENCODE Consortium (29) demonstrated that 79.3% of mouse candidate enhancers (predicted by patterns of histone modifications) and 66.7% of transcription factor binding sites have sequence orthologs in humans. Further, 61.5% of tested candidate mouse-specific enhancers also show enhancer activity in human embryonic stem cells in a reporter assay (29), suggesting a degree of functional conservation between human and mouse gene regulation. Based on this level of conservation, it is intriguing to ask the question whether mouse chromatin states could be used to identify potential sites for functional characterization in mouse for human GWAS hits. Mapping 4,265 SNPs from human GWAS studies onto the mouse genome using 15 mouse samples revealed that human GWAS hits are associated with specific chromatin states in relevant mouse tissues (29). For example, in mouse kidney, H3K4me1 is enriched in specific GWAS hits associated with urate levels and metabolites. For mouse liver-specific H3K36me3, GWAS hits related to HDL cholesterol and triglyceride levels are enriched. Together, 55% of mapped SNPs overlapped with at least one histone mark in mouse (29). These results suggest that histone modification marks can be used to inform about human risk variants and for the identification of candidate functional sequences for characterization of human GWAS hits in mouse.

Furthermore, SNPs with high regulatory potential are enriched in conserved transcription factor binding sites (19). Cheng et al. (33) show that conserved sequences occupied by orthologous transcription factors in human and mouse are enriched for GWAS variants. When investigating whether this is true for individual phenotypes, they found that SNPs associated with type I diabetes and several other traits are significantly enriched in conserved transcription factor binding sites, with 13 out of 20 type 1 diabetes SNPs being in conserved binding sites. By contrast, all of the SNPs associated with pulmonary function

were found to be human-specific, suggesting that besides GWAS SNPs generally being enriched in conserved regulatory elements, that this enrichment is dependent on the trait (33). Whether this is the case for T2DM and obesity traits association is yet to be investigated. With continuous efforts and the increase in available mouse genome data sets, it will become possible to draw conclusions about the human–mouse conservation of transcription factor occupancy and enrichment of GWAS SNPs in adipose tissue. Indeed, on a cellular level, a systematic comparison between the human and mouse epigenome during adipocyte development and in different fat depots is largely missing, and Mouse ENCODE has currently only limited adipose tissue datasets that could be matched to human. Though, Mikkelsen et al. (34) generated a comparative analysis of chromatin state maps together with gene expression profiles from human adipose tissue and mouse 3T3-L1 at four time points during differentiation. They showed that although a significant amount of open chromatin in orthologous regions were shared between the two models (15–30%), most of them were species-specific. While we are not proposing that this affected the key findings of this study, it is worth pointing out that comparing a mouse cell line and primary human tissue-derived pre-adipocytes with their accompanying ontogenetic differences can potentially hinder the interpretation when using these data sets for dissecting specific GWAS loci with the aim to establish relevant functional sites.

Taken together, although the cis-regulatory landscape has substantially diverged between human and mouse on a global level, human trait-associated SNPs are enriched in sites that are conserved between the two species for the majority of traits investigated.

THE POTENTIAL FOR NEW STRATEGIES IN MOUSE MODEL ENGINEERING

With our current knowledge of the context-specificity of gene regulation and consequently the many layers of complexity of most GWAS signals, it becomes increasingly clear that it is necessary to study and understand the underlying regulatory network in the relevant human tissue (**Figure 1**). In the past,

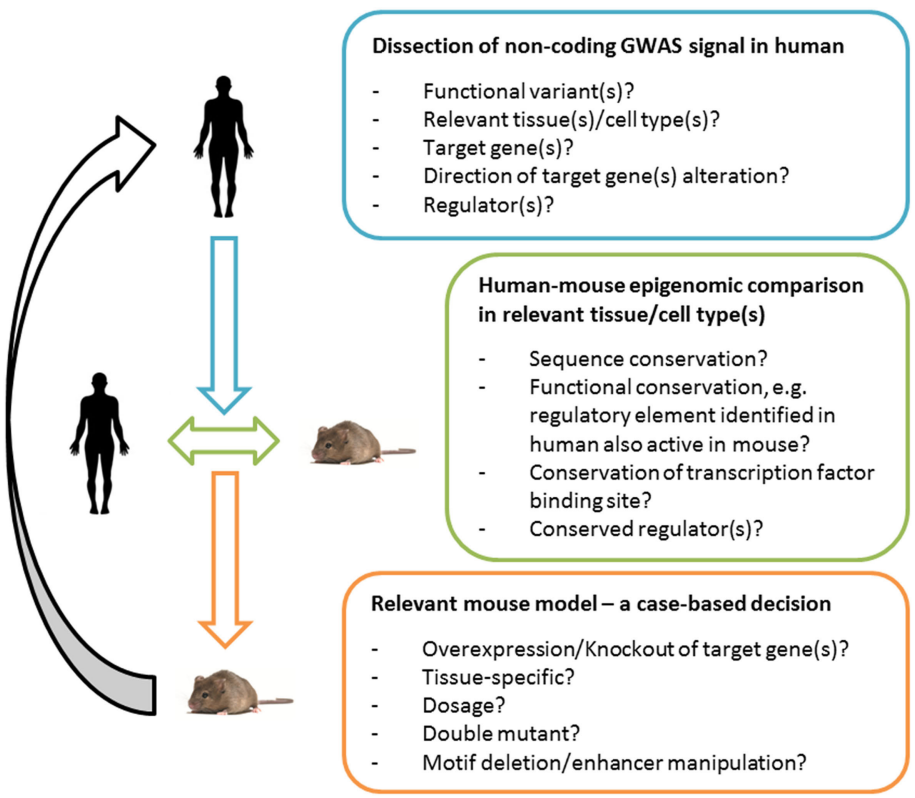

FIGURE 1 | Functional validation of variants in human and mouse. An integrative approach for the generation of meaningful and informative mouse models of human Genome-Wide Association Study (GWAS) signals. Deciphering the underpinnings of an association signal in the human context is essential. For mechanistic studies *in vivo*, a human-to-mouse epigenomic comparison can guide the choice of a relevant mouse model, e.g., in the case of low or insufficient functional conservation of a regulatory site (or the lack of data sets that can determine the latter) a classical tissue-specific target gene manipulation can prove valuable; in the case of a high functional conservation (based on genomics and bioinformatics dissection of the loci), a model that selectively manipulates the regulatory region can in principle be useful. Comparing the human–mouse epigenome can be expected to become increasingly powerful with the improvement of quality as well as the comprehensiveness of genomic data sets and tools. The translational utility will depend on the mouse model chosen and the information gained can feedback and help interpret human GWAS signals.

a successful approach to studying the function of individual candidate genes *in vivo* has been achieved by generating global knockout and overexpression models (24). However, these models do not resemble the tissue-specific nature of alterations in regulatory elements. Tissue-specific target gene manipulation using CRE drivers can be a powerful tool to overcome this problem. However, another challenge comes with the current lack of reliable pre-adipocyte-specific CRE lines that can be used to assess the tissue-specific effect of identified target genes in cases of pre-adipocyte-specific signals. Recent advances in genome engineering, namely CRISPR/Cas9, opened the opportunity to conveniently alter any regulatory sequence of interest (35). In other words, it is now possible to genome edit transcription factor binding sites and enhancer elements in the mouse which in principle has the potential to create mouse models of human risk variants that (i) are cell type-specific; (ii) alter all target genes; (iii) alter target genes at the relevant level and direction; and (iv) alter target genes at the relevant time of development (**Figure 1**).

CONCLUSION AND FUTURE DIRECTIONS

The majority of human genetic variants associated with common metabolic disease traits are located within distal regulatory elements. With our current knowledge of gene regulation and the context-specificity of the signal, it is necessary to understand the signal in human. Identifying targets and context is crucial in engineering a *relevant* mouse model. A comprehensive human-to-mouse epigenomic comparison can be informative about human risk variants. Although intriguingly, whether manipulation of regulatory elements will become a tool to dissect human obesity/T2DM risk variants in the mouse will depend on the functional conservation of a given signal. This is yet to be established and offers an exciting avenue to explore.

GLOSSARY

ATAC-seq—assay for transposase-accessible chromatin followed by high-throughput sequencing. This technique allows the identification of open chromatin.

BMI—body mass index. A measure of body weight that takes account of an individual's size and calculated by dividing body weight by height squared.

ChIP-seq—chromatin immunoprecipitation followed by high-throughput sequencing. This technique allows the identification of DNA fragments that are bound by a specific antibody.

Cis-regulatory—non-coding DNA sequences in or near a gene required for its spatiotemporal expression that characteristically contain transcription factor binding sites.

CRE—Cre recombinase recognizes DNA sequences known as LoxP sites and when a pair of sites is provided in the same orientation this leads to deletion of the intervening sequence. In this way, a segment of DNA such as a key exon (said to be floxed) can be deleted resulting in, for example, a null mutation. This can be done *in vivo* by gene editing to place LoxP sites in the required location and then crossing animals that carry this modification to Cre recombinase strains, which then results in recombination. The expression of Cre recombinase can be driven by a promoter of choice either as a transgene or knocked into an endogenous gene promoter. Thus, the recombinase can be expressed in specific tissues as required allowing cell- or tissue-specific recombination, i.e., for the generation of a conditional knockout.

Epigenome—a network of chemical compounds (for example, DNA methylation or histone modifications) surrounding DNA that modify the genome without altering the DNA sequence itself. These modifying elements play a role in determining which genes are active in a particular cell at a particular time.

eQTL—expression quantitative trait loci are genomic loci that contribute to variation in the expression levels of mRNAs. For example, in individuals in a population inheriting SNP allele A, the expression of gene Y is found to be quantitatively increased or decreased on average relative to the other SNP alleles inherited across the population assayed. This is a correlated trait rather than a direct functional link between a SNP and the expression of a gene. Further, any particular SNP marks a haplotype (a linked co-inherited group) of SNPs and as such represents a locus.

GWAS—Genome-Wide Association Study.

iPSC—induced pluripotent stem cell.

SNP—single-nucleotide polymorphism.

T2DM—type 2 diabetes mellitus.

Transcriptome—the entire mRNA expressed from the genes of a cell.

Trans-regulatory—in the context of transcriptional regulation, a trans-acting element is usually a DNA sequence that contains a gene. This gene encodes for a protein (or other molecules such as microRNA) that will regulate another target gene.

WHR—waist-to-hip ratio.

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The Importance of Context: Uncovering Species- and Tissue-Specific Effects of Genetic Risk Variants for Type 2 Diabetes

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Genome-wide association studies (GWAS) have been highly successful in identifying genetic variation associated with type 2 diabetes (T2D) risk and related quantitative traits (1–3). The vast majority of association signals are located in non-coding regions of the genome, influencing nearby genes through regulation of transcriptional, translational, or splicing activity (4). Due to the highly context-dependent nature of gene expression, the effects of many risk variants are restricted to specific cell types and produce more subtle effects than those observed in organism-wide (or "global") knockouts. In addition, identification of the underlying causal genes and target tissues is often a major challenge, hindering translation into disease mechanisms. Recent studies have shown that the intersection of genetic data and genomic annotations can be used to produce a cellular atlas with which to understand the phenotypes of GWAS signals. Through the generation of directed hypotheses, this integrated framework has the potential to bridge the gap between association signals and disease biology.

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THE BASIS FOR TISSUE SPECIFICITY

Across the human population, differences in complex traits, such as height and disease susceptibility, are influenced by the presence of single-nucleotide polymorphisms (SNPs). Some of these genetic variants modulate binding of transcription factors (TFs), which in turn drive differences in gene expression (5, 6). TF binding is also influenced by co-factors and chromatin state, which are highly dependent on cell type and developmental stage. To establish and maintain cellular identity, cell-type-specific TFs tend to bind in clusters, often referred to as cis-regulatory modules (7–9). Intriguingly, association signals for T2D have been found to show a significant overlap with islet-selective enhancer clusters (9, 10). Although other tissues have also been implicated in T2D susceptibility, this is consistent with physiological studies establishing islet dysfunction as a central mechanism of disease-associated variants (1, 11–13).

The overlap between T2D signals and enhancer clusters suggests that the effect of risk variants could be subject to the same tissue specificity observed at the level of regulatory activity (11). In other words, a motif-altering allele would only be expected to produce a molecular phenotype in those contexts (cell type or developmental stage) where the binding site has the potential to be occupied. In support of this notion, T2D risk variants were found to be enriched for nearby binding sites of the pioneer TF FOXA2 in islet and liver (14). T2D-association signals also show a significant overlap with SNPs affecting islet expression of regional transcripts, so-called cis-expression quantitative trait loci (cis-eQTL), most of which are not found to be eQTLs in other tissues (15). Together, these general observations outline some of the context-dependent effects that T2D risk variants can be subject to.

CONTEXT SPECIFICITY OF CAUSAL MECHANISMS FOR T2D SUSCEPTIBILITY

Recent studies have provided more specific evidence to support the notion that context specificity is a key aspect of GWAS causal mechanisms. The following cases collectively provide examples of mechanisms where studying the right tissue, species, and developmental stage proved critical to uncovering the relevant phenotypes.

At the *MTNR1B* locus, a convergence of evidence has pointed to effects of a non-coding T2D-association signal on the pancreatic β cell. Physiological studies have revealed a phenotype indicative of β-cell dysfunction in risk-variant carriers, with some evidence for additional effects on insulin action (12, 16, 17). Fine-mapping efforts identified a single likely causal variant that overlaps active islet and liver enhancers, and a cis-eQTL for *MTNR1B* in islets (14, 15, 18, 19). The risk allele, which increases islet *MTNR1B* expression, was predicted to create a NEUROD1 binding site and shown to selectively bind this key TF in human β cells. These results establish a likely causal mechanism for the non-coding risk allele, and illustrate how motif-altering alleles can generate highly tissue-specific effects. Surprisingly, exon re-sequencing of the *MTNR1B* gene has also shown coding loss-of-function (LOF) mutations to be associated with increased risk of T2D (20). The reason for the opposite directions of effect observed for coding and non-coding risk variants is unclear but may reflect differences between global and islet-specific roles of *MTNR1B.*

In the case of *PTF1A,* studying the right tissue proved necessary but not sufficient to elucidating the underlying mechanism for non-coding mutations in the region. Previous work had identified a group of patients suffering from unexplained isolated pancreatic agenesis, which includes neonatal diabetes as a clinical feature (21). To filter causal mutations from incidental variation, one study used pancreatic endoderm to define regulatory regions that are active during pancreatic development (22). Their strategy identified a distal enhancer that harbors mutations abolishing enhancer activity toward *PTF1A* (23). Coding LOF variants in *PTF1A* had previously implicated the gene in syndromic pancreatic agenesis, characterized by severe neurological features in affected individuals. The observation that the identified enhancer region is not active in any cell type other than pancreatic endoderm provides a plausible explanation for the absence of any cerebral defects (22). Remarkably, even adult pancreatic tissue did not show active chromatin marks in the region, highlighting that studying the right developmental stage was critical to the success of the approach.

The mechanisms underlying GWAS signals are sometimes studied using individuals that carry LOF mutations in positional candidate genes. The observed phenotypes will be a function of global effects across all the tissues where the gene is expressed, which may confound or mask the more context-dependent actions of regulatory risk alleles. At the *CDKN2A* locus, non-coding T2D signals have been robustly associated with measures of islet dysfunction, and a number of studies have established effects of *CDKN2A* on insulin secretion and cellular senescence in β-cells (12, 24, 25). By contrast, coding LOF mutations in *CDKN2A*, which are a cause of familial melanoma, were recently shown to result in a metabolic phenotype consistent with effects on both liver and β-cells (26). This discrepancy was proposed to arise from islet-specific TF binding of the enhancer region containing the T2D signals.

In animal studies, context-dependent knockouts can provide improved spatial and temporal resolution for targeting candidate causal genes. Even so, the disease relevance of the observed phenotypes is determined by the confidence with which the target tissue of the risk allele is known. One example is provided by an intronic T2D signal at the *TCF7L2* locus, which has been the focus of conflicting observations. Tissue-specific knockout studies have demonstrated primary roles of *TCF7L2* in a number of different tissues, including liver and islets, whereas the non-coding GWAS signal has been consistently associated with a relatively narrow insulin secretion defect. Genomic annotations provide a clue as to the underlying reason, with the risk variants being located in an islet-specific region of open chromatin (8, 10, 27). Furthermore, the region has chromatin marks indicative of regulatory activity in islets, but not in a wide range of other tissues (10). The annotations can, thus, be applied as a filter to exclude non-disease-relevant tissues, and guide efforts to study the effect of the risk allele in the most appropriate context.

Coding GWAS variants can also produce context-dependent mechanisms through the restricted expression of gene isoforms. A striking example of this is provided by a coding variant identified in the *TBC1D4* gene in a small founder population of Greenlandic Inuit (28). The risk allele, which produces a truncated transcript that results in nonsense-mediated decay, is positioned in an exon excluded from the short isoform of the transcript. Unlike the widely expressed short isoform, the long form is predominantly expressed in skeletal muscle (29). The decreased insulin sensitivity resulting from reduced expression of *TBC1D4* is, therefore, selectively imposed on muscle tissue. As a result, the risk variant has a different effect on fasting glucose from that observed in individuals carrying LOF mutations affecting both isoforms (28).

Similar to alternative spliceforms, gene homologs can contribute to concealing the primary effect of a GWAS signal in a specific context. At the *ADCY5* locus, T2D risk alleles have been linked to both decreased islet expression of the *ADCY5* gene and β-cell dysfunction, though the underlying molecular mechanism remains unclear (12, 15). Expression studies in rodents have shown *Adcy5* to be nearly undetectable compared with the closely related homolog *Adcy6* (30, 31). By contrast, human islets show roughly equal expression of these orthologous genes, hinting at a non-conserved function of *ADCY5* between the species (30, 32). It also highlights an underlying species-specificity that makes rodents less well suited as models for mechanistic studies.

LIMITATIONS OF TRADITIONAL APPROACHES

The examples above demonstrate that the molecular phenotypes of GWAS signals can be modulated by a multitude of context-dependent factors. In the case of non-coding variants, tissue- or developmentally restricted activity of the surrounding chromatin can limit effects on gene expression. For coding

variants, alternative splicing or expression of homologs can mask a broader phenotype to produce context-dependent effects. These insights have important implications for how we design studies to translate genetic signals into molecular mechanisms.

Traditionally, particular genes have been selected for follow-up studies based on a combination of known candidate-gene biology and proximity to the GWAS signal (**Figure 1**, *left*). For whole-organism gene knockouts, this could involve engineering

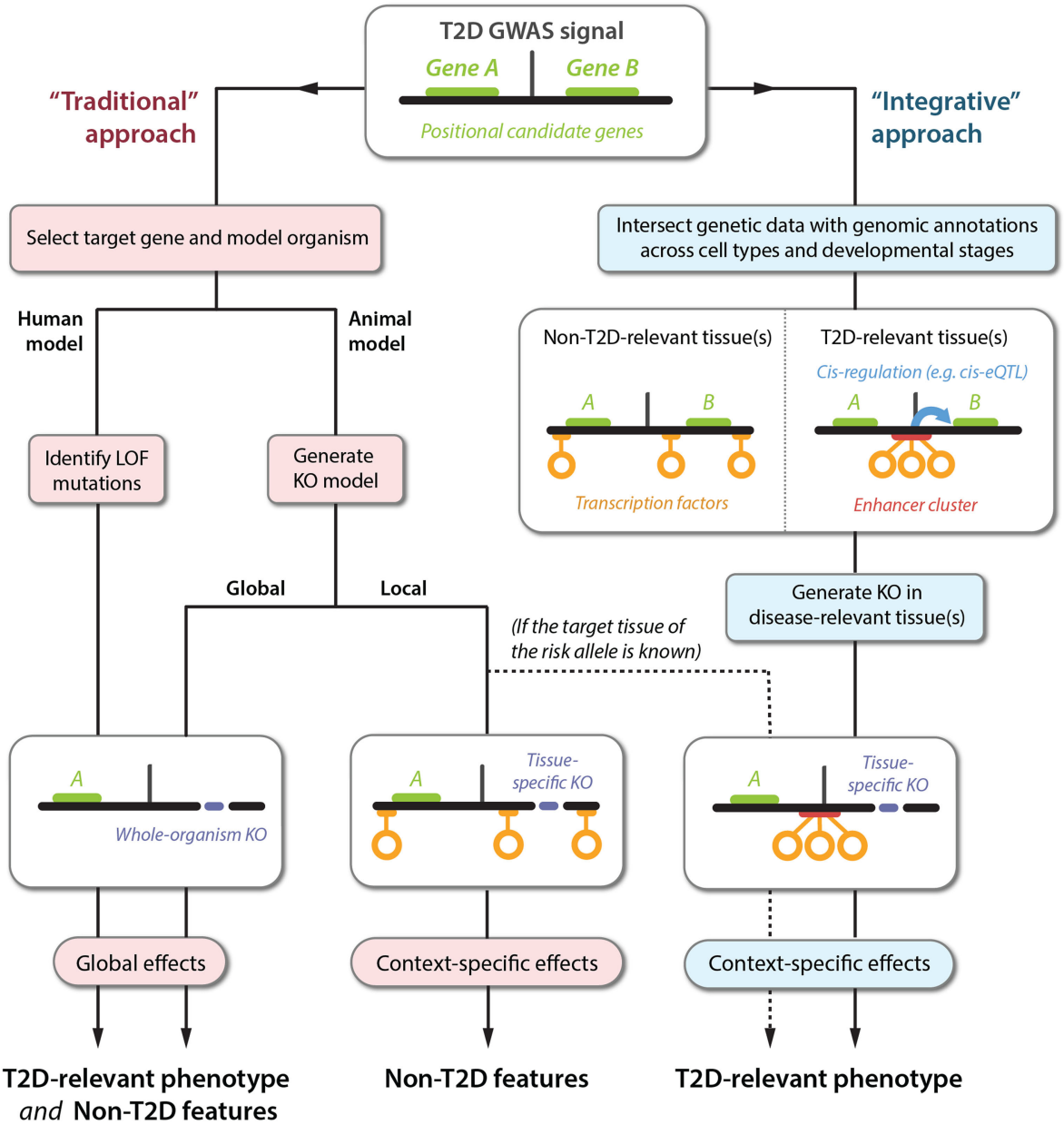

FIGURE 1 | Translating GWAS signals into disease mechanisms using traditional and integrative approaches. The top panel shows a schematic representation of a GWAS locus with a non-coding association signal for T2D risk located near two genes, *A* and *B*. The left-hand side represents the traditional approach followed for elucidating the causal mechanisms leading to a T2D phenotype. This approach can produce a range of phenotypes that are difficult to translate into causal GWAS mechanisms, because the selected genes are often knocked out globally (bottom left panel), or in a tissue-specific context that is irrelevant to understanding the effects of the T2D susceptibility phenotype (bottom center panel). Sequencing approaches can also be used to identify gain-of-function mutations (not shown), which are subject to the same limitations. The right-hand side outlines an alternative approach using integration of emerging datasets to produce directed hypotheses. For optimal resolution, several types of genetic and genomic datasets can be integrated, including TF binding, enhancer regions (defined based on chromatin state), and cis-regulatory relationships (e.g., identified by cis-eQTL or chromatin-conformation capture studies). In this example, the GWAS signal is observed to disrupt an enhancer cluster with cis-regulatory activity toward gene *B*. Importantly, the disruption exclusively affects enhancer activity in disease-relevant tissues. This proposes a follow-up experiment for manipulation of gene *B* in a specific context (bottom right panel), producing a phenotype that is likely to be directly relevant to understanding the molecular basis of T2D susceptibility.

of animal models or using genetic testing to identify individuals carrying LOF variants. As discussed, the relevance of these approaches for delineating disease-relevant mechanisms is limited by the potential for unspecific global phenotypes. Tissue-specific knockouts provide higher spatiotemporal resolution but require the target tissue(s) of the GWAS signal to be known. Human physiological associations can narrow down the list of likely relevant tissues, but these measures are often too crude to pinpoint specific cell types.

In principle, using animal models to target non-coding regions could produce more precise disease models, but this strategy is constrained by the low conservation at the level of regulatory architecture. In a study of the *Cdkn2a* locus in mice, targeted deletion of a 70 kb non-coding interval established enhancer activity toward nearby genes, but the relevance to humans has been questioned by subsequent findings (33, 34). The region encodes a long non-coding RNA that has no clear ortholog in rodents, highlighting the possibility of divergent cis-regulatory mechanisms. More generally, TF binding sites have been shown to diverge even faster than the underlying sequence itself (35). For two key liver TFs, the majority of binding events were shown to be species specific, while only 10–30% of hepatic enhancer clusters have corresponding rodent orthologs (36, 37). Although subsets of conserved clusters may aid in the prioritization of causal variants, these observations suggest that a different approach is required to delineate GWAS mechanisms (37, 38).

Even in those cases where animal models do provide targeted gene manipulation in an appropriate context (whether through tissue-specific knockout or transcriptional dysregulation), the resulting phenotypes may not be directly relevant for understanding human disease. Though rodent models continue to be an important tool for studying type 2 diabetes pathogenesis, it has become increasingly clear that murine pancreatic islets differ in a number of ways from their human counterparts (39–41). Certain monogenic forms of diabetes are, therefore, not well recapitulated in rodents, and molecular mechanisms elucidated in animal models should be interpreted with caution (42).

TOWARD AN INTEGRATED UNDERSTANDING OF GWAS SIGNALS

To successfully study disease-relevant phenotypes, experimental designs can be guided by the integration of genetic association data and genomic annotations (**Figure 1**, *right*). At the core of this framework is the overlaying of a static dataset – a list of variants linked to disease susceptibility and/or physiological traits – with layers of highly dynamic functional information that provide spatial and temporal dimensions. These layers encompass diverse datasets, and include information centered on single variants, such as histone marks and TF binding sites, and higher-order information that signifies relationships between distinct elements, such as chromatin interactions and cis-eQTL data.

Genomic annotations provide a cellular atlas with which to navigate and interpret genetic data in the context of specific cell types and developmental stages. For instance, if a set of likely causal variants has been identified from GWAS or fine-mapping studies, the tissue of action may be inferred from comparing chromatin states across cell types (8–11, 43–46). Conversely, if the target tissue is known from physiological associations, the causal mechanism can be pinpointed by overlaying with relevant functional annotations (14, 22, 47–51). This process generates a plethora of directed hypotheses that can be followed up with specific functional experiments. Increasingly, such studies are likely to be focused on differentiated cells derived from human stem cells, which can provide disease models and chromatin maps that are both functionally and developmentally relevant.

The broader applicability of this approach is, in part, determined by the tractability of individual loci. For regions with extensive linkage disequilibrium, the arising complexity can hinder experimental follow-up. Starting from a limited set of credible variants is, thus, essential. More generally, the value in taking an integrative approach is dictated by the extent to which genomic annotations for disease-relevant tissues have been made available (or can be obtained). The construction of a truly integrated framework is an incremental and monumental effort, facilitated by the Encyclopedia of DNA Elements (ENCODE) and the NIH Roadmap Epigenomics project, which together cover hundreds of tissues and epigenetic annotations. Since each dataset is merely a snapshot of a given cell type in a particular metabolic and developmental state, this on-going process will continue to produce an atlas with ever-finer spatiotemporal resolution. For many hard-to-obtain organs, such as pancreatic islets, power to detect relevant genomic features is still limiting.

Even so, chromatin landscapes for tissues relevant to T2D have begun to emerge in recent years, enabling biological inferences to be made. As we have seen, this has successfully uncovered tissue- and species-specific effects of T2D risk variants. The insights have also provided compelling evidence to demonstrate that context-dependent phenotypes are not the exception but, in fact, a fundamental aspect of GWAS biology. In the coming years, we need to build on this paradigm to accelerate the translation of genetic findings into molecular mechanisms.

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How Useful Are Monogenic Rodent Models for the Study of Human Non-Alcoholic Fatty Liver Disease?

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Improving understanding of the genetic basis of human non-alcoholic fatty liver disease (NAFLD) has the potential to facilitate risk stratification of affected patients, permit personalized treatment, and inform development of new therapeutic strategies. Animal models have been widely used to interrogate the pathophysiology of, and genetic predisposition to, NAFLD. Nevertheless, considerable interspecies differences in intermediary metabolism potentially limit the extent to which results can be extrapolated to humans. For example, human genome-wide association studies have identified polymorphisms in PNPLA3 and TM6SF2 as the two most prevalent determinants of susceptibility to NAFLD and its inflammatory component (NASH), but animal models of these mutations have had only variable success in recapitulating this link. In this review, we critically appraise selected murine monogenic models of NAFLD, NASH, and hepatocellular carcinoma (HCC) with a focus on how closely they mirror human disease.

Keywords: steatosis, animal model, metabolic syndrome, steatohepatitis, genetic models

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a pandemic disorder associated with premature morbidity and mortality. Its etiology is multifactorial, with both genetic predisposition and environmental factors playing important parts (1). Animal models have been central to recent major translational research efforts, serving as discovery tools to identify new candidate pathogenic mechanisms, as a means of testing hypotheses arising from human studies, and as pre-clinical models in which to assess potential therapeutic strategies (2). There are multiple different experimental perturbations known to trigger NAFLD in animal models, which have been used to generate all components of the NAFLD spectrum (3–10). Such perturbations include genetic manipulation (e.g., ob/ob mouse), pro-steatotic or pro-inflammatory diets [e.g., methionine–choline deficient (MCD) diet], and toxic insults (e.g., streptozotocin injection). Combinations of these may be used to reflect the multifactorial nature of the human disease (e.g., ob/ob mice fed a MCD diet); however, a detailed discussion of rodent diets is beyond the scope of this review.

While animal models do potentially give key insights into the disease process that cannot be obtained through any other means, it is necessary to temper appreciation of this utility with understanding of the limitations of each of the models used.

We, now, briefly review the current understanding of the genetic architecture of human NAFLD, and the major approaches adopted to modeling it in animals, while critically appraising the utility of these models.

OVERVIEW OF NON-ALCOHOLIC FATTY LIVER DISEASE PATHOGENESIS

Human NAFLD is defined by the presence of macrovesicular steatosis in more than 5% of hepatocytes in individuals with a history of less than 20 g/day ethanol intake (11). The differentiation of macrovesicular from microvesicular steatosis is a qualitative histological assessment, and the two may coexist in more advanced disease (12). NAFLD exists as a pathological spectrum ranging from non-alcoholic fatty liver (NAFL, also known as "simple" steatosis), which denotes hepatic steatosis in the absence of steatohepatitis, through to non-alcoholic steatohepatitis (NASH), in which there is histological evidence of inflammatory infiltrates, hepatocyte ballooning (featuring Mallory-Denk bodies), and commonly fibrosis of varying severity. Cirrhosis can develop at the most severe end of the spectrum, conferring increased risk of hepatocellular carcinoma (HCC), portal hypertension, and liver failure (13). Longitudinal studies have shown 10–20% of patients with each stage of disease to progress to the following stage in, while there is also an element of reversibility, particularly in NAFL and NASH (14, 15).

A "two-hit hypothesis" has been suggested to account for the pathogenesis of advanced NAFLD, whereby the first "hit" drives development of steatosis and the second triggers inflammation and its sequelae, critically including fibrosis (16).

In principle, hepatic steatosis may result from pre-hepatic derangements, intra-hepatic derangements, or both, as illustrated in simplified form in **Figure 1**. A comprehensive review of pathways leading to hepatic steatosis is beyond the scope of this article and is provided elsewhere (17–19). In brief, pre-hepatic pathogenic factors encompass increased substrate flux to the liver (e.g., non-esterified free fatty acids (NEFA), monosaccharides, or amino acids) and dysregulation of hormones that act directly on hepatocyte metabolism (e.g., insulin, glucagon and related peptides, and adipokines). Increased "preload" in the form of NEFA delivery generally results from failure of adipose tissue adequately to buffer positive energy balance. This may be a consequence of hyperphagic obesity, in which even normal adipose buffering capacity is overwhelmed, or lipodystrophy, in which adipose energy buffering capacity is pathologically constrained. In some situations, a mixture of these is at play, as in generalized lipodystrophy, where the harmful results of absent adipose tissue are potentiated by concomitant lack of leptin.

Many pre-hepatic hormonal factors also influence propensity to NAFLD by acting on adipocytes to modulate lipolysis and/or through direct actions on hepatocytes (e.g., insulin, glucagon, glucagon-like peptides). There has been a particular focus on the ability of high levels of insulin, secondary to peripheral insulin resistance, to drive hepatic *de novo* lipogenesis. Another emerging influence on liver metabolism is the gut microbiome, which may affect gut hormone release and also signal directly through flux of bacterial metabolites such as acetate (20, 21).

Hepatocyte-autonomous (intra-hepatic) defects may also lead to triglyceride accumulation. Such defects may broadly be classified into: those increasing *de novo* synthesis of triglyceride; those perturbing lipid droplet dynamics, triglyceride mobilization and lipoprotein assembly or secretion; and those impairing catabolism of fatty acids by beta-oxidation. Although reduced ability to catabolize fatty acids *via* beta-oxidation (e.g., due to Mendelian disorders in key catabolic enzymes, or mitochondrial dysfunction) does result in hepatic steatosis, however, this is usually microvesicular in appearance and has a distinct clinical profile that often includes hypoglycemia, liver failure, and encephalopathy (22). These disorders will, thus, not be discussed further here.

Development of NASH is multifactorial; a comprehensive review of the inflammatory and fibrotic sequelae of hepatic lipid accumulation can be found elsewhere (23–26). Key elements of pathogenesis include oxidative stress (from lipid peroxidation and mitochondrial dysfunction) and activation of pro-inflammatory pathways (e.g., NF-κB) in hepatocytes, but other cellular pathways, including the endoplasmic reticulum stress response, have also been implicated (27). Coactivation of Kupffer cells, sinusoidal endothelium, and hepatic stellate cells gives rise to cytokines that augment inflammation [e.g., tumor necrosis factor alpha (TNFα), interleukin-1/-6] and drive fibrosis [e.g., transforming growth factor beta (TGFβ)] (19, 28, 29). These processes are also exacerbated by pre-hepatic factors, such as adipose inflammation/lipotoxicity, gut bacterial translocation, and endogenous alcohol production.

HUMAN GENETICS OF NAFLD

In the vast majority of patients, NAFLD is a multifactorial condition rooted in obesity and insulin resistance, based on strong clinical association and natural history studies in humans. Pandemic, idiopathic NAFLD is often referred to as "primary" NAFLD (30). Genetics can play a role in each stage of the pathophysiology of NAFLD, as illustrated both by rare monogenic conditions that feature severe NAFLD, and by the association of much more frequent single nucleotide polymorphisms (SNPs) with "common" NAFLD (31–33). The proliferation of recent human genetic findings puts their detailed treatment beyond the scope of this discussion; however, we select a series of mechanistically informative sentinel examples to appraise against rodent models.

"PRE-HEPATIC" NAFLD

Monogenic Hyperphagic Obesity

Flux of substrates, such as free fatty acids, amino acids, and lactate, provide the building blocks for hepatocyte triglyceride accumulation as well as the energy required for activation of anabolic pathways (see **Figure 1**). Excess flux can thus be a potent driver for NAFLD. Most attention has been paid to flux of free fatty acids, the product either of lipolysis of triglyceride

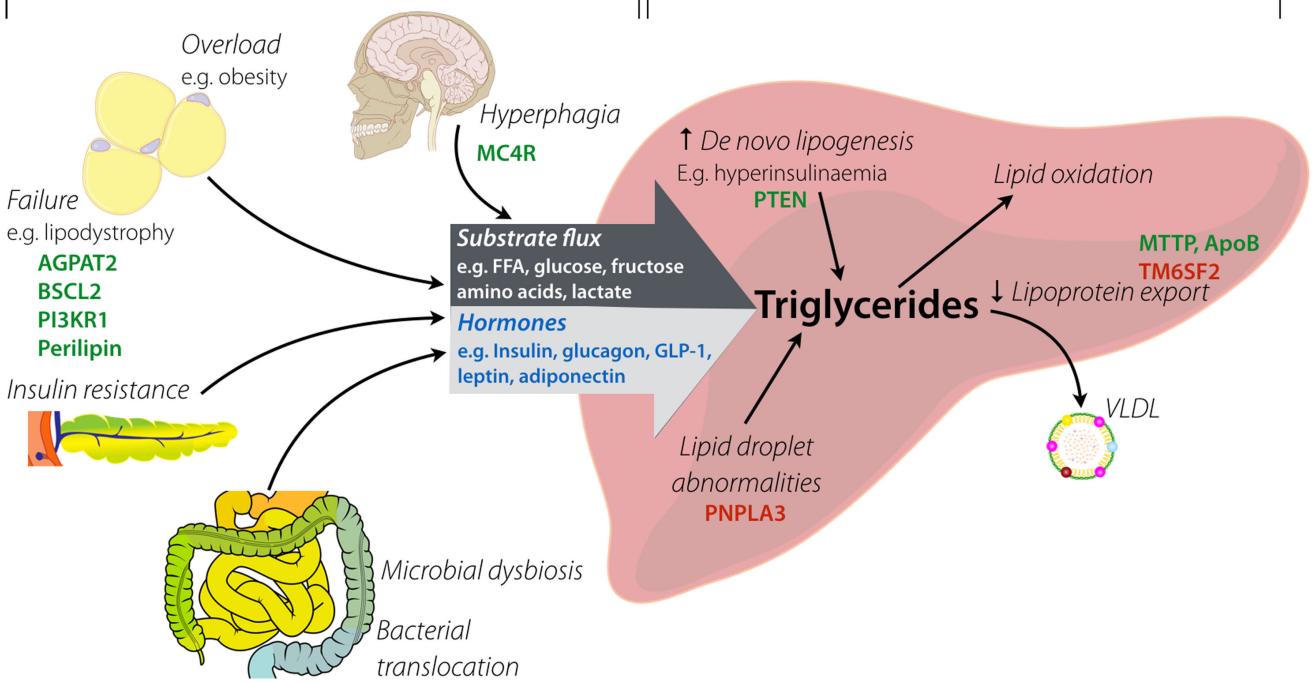

FIGURE 1 | Factors implicated in hepatic steatosis. Pre-hepatic factors lead to increased substrate flux to the liver by both increased load (e.g., raised fatty acid delivery) and altered composition (e.g., increased fructose). Hyperphagia causes provision of excess macronutrients, which contributes to overload of white adipose tissue (*via* obesity). Lipodystrophy causes functional or anatomical failure of adipose, with the resulting spill over of substrates passing to the liver. Insulin resistance contributes to hormonal changes (e.g., raised insulin, low adiponectin) that alter intra-hepatic metabolism of lipids. Intestinal dysbiosis influences both substrate delivery to the liver and generation of gut-derived hormones (e.g., elevated GLP-1). Key: hormones are in blue, examples of genes involved in monogenic disorders are in green, and examples of genes with pro-steatotic common polymorphisms are in red.

in adipose tissue or lipolysis of triglyceride in triglyceride-rich lipoproteins such as chylomicron remnants. Key determinants of free fatty acid flux to the liver are thus the dietary intake of fat and the efficiency of fatty acid trapping and storage in adipose tissue. Correspondingly, in states of hyperphagia or adipose tissue insufficiency, free fatty acid flux may be pathologically increased. Of note, "adipose tissue insufficiency" may arise from overload of adipose tissue that is normal or increased in amount, or may arise from frank anatomical deficiency of adipose tissue, or lipodystrophy.

Several human examples of monogenic hyperphagia exist. The first to be discovered was congenital leptin deficiency (34), with loss-of-function mutations in the leptin receptor gene discovered later (35). Leptin is an adipose-derived peptide adipokine that acts on the hypothalamus to signal replete energy stores and suppress appetite (36). It is expressed and secreted by white adipose tissue (WAT) in direct proportion to WAT volume, and serum levels fall commensurately with depletion of adipose stores during starvation. Primary leptin deficiency produces severe, hyperphagic obesity from a young age; however, liver fat accumulation has only rarely been commented on in published studies. In one study, severe NAFLD was identified in an obese leptin-deficient child that resolved quickly on introduction of recombinant leptin therapy (37). The long-term hepatic outcome of untreated human leptin deficiency or leptin receptor mutation is not known; however, leptin receptor polymorphisms have been associated with NASH and insulin resistance in patients with NAFLD (38, 39).

Several other forms of human monogenic obesity are now known. In contrast to genetic leptin or leptin receptor loss-of-function, which are extremely rare, heterozygous mutations in the melanocortin-4-receptor (MC4R) are the most common monogenic cause of obesity, accounting for around 6% of severe, early-onset obesity (40). There are no published reports of the impact of heterozygous MC4R mutations on hepatic steatosis in monogenic obesity, while polymorphisms in MC4R have been associated with alanine aminotransferase and BMI (41), but not with hepatic fat content in population-wide studies (42). Several other genes have been implicated in human monogenic obesity; however, in aggregate, these affect only a very small number of patients, and there is little information on the liver phenotype. Moreover, some of the gene products involved, such as prohormone convertase 1 and pro-opiomelanocortin have pleiotropic roles, not just in appetite control but also in the action of key peripheral hormones (peptide and steroid hormones respectively) which have independent effects on liver fat accumulation, so shall not be considered here.

The sparse attention paid in published human reports to the natural history of NAFLD in human monogenic obesity is likely

to be attributable to the rarity of the diseases, to the fact that most patients identified are children in whom clinically overt liver NAFLD has not yet had time to develop and to that fact that, in leptin deficiency, curative therapy with recombinant human leptin is the standard of care. This means that the long-term natural history of NAFLD in leptin deficiency will be extremely difficult to document in future, although determining the long-term liver outcome of MC4R loss-of-function should be both tractable and informative.

Monogenic Lipodystrophy

In the face of severe hyperphagia, the ability even of "normal" adipose tissue to buffer chronic positive energy balance by trapping and sequestering lipid is finite and is eventually overwhelmed, leaving the liver exposed to excess free fatty acid flux. However, some rare humans have a congenital deficiency of adipose tissue, which we shall consider only in its most severe, generalized form. In this situation, there is effectively absolute adipose failure, and complete lack of energy buffering is compounded by secondary lack of leptin and consequent hyperphagia, as the hypothalamus erroneously interprets very low or absent leptin as an indication of starvation. Around 95% of cases in humans congenital generalized lipodystrophy (CGL) are accounted for by biallelic mutations in either AGPAT2, encoding an enzyme involved in triglyceride synthesis, or in BSCL2, encoding an endoplasmic reticulum protein involved in lipid droplet regulation and adipocyte differentiation (43, 44). A clinical hallmark of CGL is very severe NAFLD, with early development of inflammation, fibrosis, and HCC. Indeed, clinical experience and published case series suggest that complications of advanced liver disease are among the major sources of mortality in CGL (45, 46). Observations in CGL and other acquired and genetic lipodystrophies establish unequivocally that primary disorders of adipose tissue are sufficient to cause the full spectrum of NAFLD in humans (47). This does not prove that adipose dysfunction is the primary mechanism at play in pandemic NAFLD; however, current population-wide data do not rule this out.

Endocrine Drivers

Many hormones exert a major influence on lipid accumulation within hepatocytes, although in each case this is only possible in the context of adequate energy charge and substrate flux to the liver, so these cannot easily be teased apart. Hormones of proven importance in altering liver lipid accumulation include insulin, glucagon, and the incretin gut peptides (e.g., GLP-1). In humans, insulin has attracted particular attention as several lines of evidence suggest that insulin, acting through the insulin receptor, stimulates *de novo* lipogenesis sufficiently to make a major contribution to liver fat accumulation in NAFLD (48–50). Some negative evidence in support of this model comes from the observation that humans with loss-of-function mutations in the insulin receptor itself, although showing very severe insulin resistance, are protected from dyslipidemia and liver fat accumulation (51). Preliminary evidence suggests that this also holds for patients with lipodystrophy and loss-of-function mutations in *PIK3R1*, a component of phosphatidylinositol-3-kinase involved in insulin signaling (52).

HEPATOCYTE-AUTONOMOUS NAFLD

Lipid Trafficking Abnormalities

Some genetic abnormalities can influence development of steatosis by hepatocyte-autonomous mechanisms. One of these mechanisms is impaired assembly and export of lipoproteins from hepatocytes, leading to intracellular accumulation of triglycerides. The best examples of this are heterozygous truncating mutations in APOB [encoding apolipoprotein B (ApoB)], causing hypobetalipoproteinaemia and biallelic mutations in MTTP (encoding microsomal triglyceride transfer protein), which cause abetalipoproteinaemia (53). These two conditions have a similar phenotype including NASH, low serum triglycerides, low LDL cholesterol, and neuropathy. Patients often develop progressive NASH with fibrosis even in the absence of diabetes, obesity, or other oxidative stresses (53, 54).

Further evidence in support of altered lipoprotein assembly and secretion as a significant player in NAFLD comes from GWAS of the commoner form of the disease. These have implicated a SNP in TM6SF2 (encoding transmembrane 6 superfamily member 2), which is needed for secretion of very-low density lipoproteins (VLDL), in NAFLD. The risk allele is found in around 1% patients with NAFLD (55) and correlates positively with serum aminotransferase levels (33), hepatic steatosis (31), NASH activity, and fibrosis (but not HCC) (56). Importantly, patients with this SNP have lower circulating LDL cholesterol, lower triglycerides, and reduced incidence of atherosclerotic disease, providing an example of dissociation of NAFLD from dyslipidemia and cardiovascular disease (57). Data relating to the association with insulin resistance and type 2 diabetes are currently inconclusive (58).

Population-wide human genetics has also implicated fundamental abnormalities in lipid droplets in NAFLD: for example, the p.Ile148Met SNP in PNPLA3, whose product is patatin-like phospholipase domain-containing 3, is associated with all stages of NAFLD, from simple steatosis, NASH, fibrosis, and development of HCC (59, 60). PNPLA3 (also known as adiponutrin) is a membrane-bound enzyme expressed at the surface of lipid droplets and on the smooth endoplasmic reticulum (61) and plays a role in lipid droplet dynamics. There has been conflicting evidence on whether this polymorphism is associated with insulin resistance, however.

Inflammation/Fibrosis

Inflammation and fibrosis are the hallmarks of complicated NAFLD, and a genetic basis for the susceptibility to these complications is often mooted as the "second hit" needed to drive progression from simple steatosis to inflammatory and fibrotic sequelae. Unsurprisingly, no single gene examples of a primary inflammatory or fibrotic disorders leading to end stage NAFLD have been reported (62, 63); however, the GWAS approach has identified common genetic variants associated with progression of fibrosis (e.g., near the gene for platelet-derived

growth factor alpha) or with the development of lobular inflammation (e.g., near genes encoding interleukin-6, or collagen type XIII alpha 1).

RODENT GENETIC MODELS OF NAFLD

The ideal "one stop" animal model of NAFLD would recapitulate the full progression from simple steatosis through NASH to fibrosis, cirrhosis, and HCC. Ideally, the disease process would progress rapidly (in contrast to the human condition) to maximize experimental tractability of the model. However, a valid alternative would be to have a series of different models for different stages of the disease sequence, which may be primarily metabolic, inflammatory, or fibrotic. It has been suggested that the primary goal of rodent models should be to mimic the pathology of NAFLD, including the underlying histopathology (3). On this narrow basis, it has been argued that rodents do not accurately model human NAFLD because of differences in ballooning degeneration and distribution of inflammatory infiltrate in NASH (4).

Many animal models have been described in which different degrees of NAFLD are seen, and we shall consider selected models only as examples, classifying them into two groups according to the broad pathogenic mechanism at play: (1) models of "extrahepatic" NAFLD (**Table 1**) and (2) models of "intrahepatic" NAFLD (**Table 2**).

MURINE "PRE-HEPATIC" NAFLD

Hyperphagic Models

The severely obese *ob/ob* mouse strain arose spontaneously in 1949, and was eventually discovered in 1994 to harbor a loss-of-function mutation in the gene encoding leptin, thereby ushering in the modern era of investigation of the neuroscience of appetite control. Given an *ad libitum* diet, *ob/ob* mice develop obesity, insulin resistance, hyperglycemia, and hepatic steatosis (74), and they have been become a highly popular model for many aspects of obesity and related disorders, either being studied in isolation, or crossed with other strains of interest in order to determine the effect of severe hyperphagia on mice with different genetic perturbations. The severe obesity of *ob/ob* mice is attributable to hypothalamic hyperphagia; however, several studies have provided evidence for additional peripheral effects of leptin to modulate insulin sensitivity and metabolism directly in tissues, such as muscle and liver. Whether such peripheral effects are also seen in humans remains to be established beyond doubt (75–77).

TABLE 1 | Examples of murine genetic models relevant to "pre-hepatic" NAFLD.

Model	Obesity	Insulin resistance	Hyper-lipidemia	Liver steatosis	NASH	Fibrosis	HCC
Hyperphagic models							
Ob/ob (64)	Y	Y	Y	Y	Y	Y (mild)	?
Db/db (64)	Y	Y	Y	Y	Y	Y (mild)	?
Mc4r$^{-/-}$ on HFD (65)	Y	Y	Y	Y	Y	Y	Y
Lipodystrophic models							
Agpat2$^{-/-}$ (66)	N	Y	N	Y	?	?	?
Bscl2$^{-/-}$	N	Y	N	Y	?	?	?
A-ZIP/F-1 (67)	N	Y	Y	Y	?	?	?
Adipose-specific *Insr* knockout (68)	N	Y	Y	Y	Y	Y	Y
Liver insulin action							
Liver-specific *Pten* knockout (69)	N	N	N	Y	Y (mild)	Y	Y

Features are based on ad libitum standard chow diet, unless otherwise stated. ob/ob is a leptin-deficient mouse; db/db is a leptin-resistant mouse; MC4R$^{-/-}$ mouse is a mouse with hypothalamic hyperphagia; Agpat2$^{-/-}$ and Bscl2$^{-/-}$ are lipodystrophic mice with functional and anatomical failure of white adipose; A-ZIP/F-1 is a lipodystrophic mouse due to specific failure of white adipose differentiation; adipose-specific insulin-receptor (Insr) knockout causes lipodystrophy; and liver-specific phosphatase and tensin homolog (PTEN) knockout disrupts hepatocyte insulin signaling.
HCC, hepatocellular carcinoma; HFD, high-fat diet; NASH, non-alcoholic steatohepatitis; Y, yes; N, no; ?, insufficient data.

TABLE 2 | Examples of murine genetic models relevant to "intrahepatic" NAFLD.

Model	Obesity	Insulin resistance	Hyper-lipidemia	Liver steatosis	NASH	Fibrosis	HCC
Impaired lipoprotein synthesis/secretion							
Apob$^{-/-}$ (70)	N	N	Y	Y	Y	Y	N
Fatty liver Shionogi (FLS) (71)	N	Y	N	Y	Y	Y	Y
FLS-*ob/ob* mouse (72)	Y	Y	Y	Y	Y	Y	Y
Tm6sf2 knockdown (32)	N	Y	Y	Y	N	N	N
Abnormal lipid droplet dynamics							
Hepatic-specific *PNPLA3* I148M expression (73)	N	Y	N	Y	N	N	N

Features are based on ad libitum standard chow diet. Apob$^{-/-}$ mouse is unable to secrete VLDL from hepatocytes; fatter liver Shionogi mice are thought to also have impaired VLDL secretion; FLS-ob/ob are FLS mice crossed with leptin-deficient mice; Transmembrane 6 superfamily 2 (Tm6sf2) knockdown mice have reduced triglyceride secretion; hepatic-specific expression of the human I148M allele of patatin-like phospholipase domain-containing 3 (PNPLA3) causes altered lipid droplet composition and storage.
NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; Y, yes; N, no.

The *ob*/*ob* mouse rapidly develops NAFL and at 20 weeks old early features of NASH are present (77, 78). NASH may been accelerated by a second "hit" of inflammatory/oxidative stress, using, for example, intraperitoneal injection of lipopolysaccharide (LPS) (79), dietary stressors such as a MCD diet (80), or crossing with another NASH-prone strain, such as the fatty liver Shionogi (FLS) mice, which are discussed later (81). Notably, however, leptin is required for normal immune function, and its deficiency dampens down both innate and acquired immune responses in humans (82, 83), though it seems to play a pro-inflammatory and pro-fibrogenic role in mice (78). This may explain why the inflammatory components of NAFLD appear relatively indolent in *ob*/*ob* mice than in more common NAFLD. On a related note, it has also be shown that the *ob*/*ob* mouse is relatively resistant to fibrosis (78, 80), partly due to reduced release of TNFα, which is necessary for activation of TGFβ, a key pro-fibrogenic molecule (84–86).

Collectively, these findings caution that, while the *ob*/*ob* mouse is a valuable model of primary hyperphagia-driven NAFLD, loss of specific actions of leptin on peripheral metabolism, coupled to some degree of immunosuppression, means that it is likely to deviate from pandemic NAFLD in key respects, especially related to the inflammatory and fibrotic end of the disease spectrum. Similar arguments may apply to leptin-resistant *db*/*db* mice, which harbor a splice site mutation abolishing expression of the long form of the leptin receptor; however, *db*/*db* mice are reported to exhibit more severe NAFLD than *ob*/*ob* mice (64, 80). In practice, a secondary stressor is also usually applied when using *db*/*db* mice to study NAFLD, with the MCD diet being widely used (87).

Mice in which *Mc4r* is genetically ablated have also been widely studied. Their primary defect lies within hypothalamic appetite control pathways, as the melanocortin 4 receptor responds to the neuropeptide α-MSH, which is generated in response to leptin action. *Mc4r*-null mice feature hyperphagic obesity without pathologically suppressed leptin levels, suggesting that they have the potential to model the extended spectrum of NAFLD more faithfully than *ob*/*ob* mice. In keeping with this, it has been reported that *Mc4r*-knockout mice, when exposed to a high-fat diet, develop not only steatosis but also exuberant NASH with established fibrosis after 20 weeks, progressing to HCC in all mice studied by 1 year (65).

Lipodystrophic Models

Many different murine genetic models of lipodystrophy have been described. Where direct comparison is possible between mice and humans, it has been found that the common forms of CGL are well modeled in mice, while the situation for human genetic forms of partial lipodystrophy is more complex, and will not be discussed further here (45).

Both *Agpat2*- and *Bscl2*-knockout mice have been described (88, 89). *Agpat2* encodes 1-acylglycerol-3-phosphate O-acyltransferase 2 and is needed for synthesis of triacylglycerols and glycerolphospholipids in white adipose. Many Agpat2$^{-/-}$ mice die in the first few weeks of life; however, the survivors accurately recapitulate the human condition of lipodystrophy with severe insulin resistance (66). Hepatic steatosis has also been reported to be a major feature of the mice before 16 weeks old, with liver weights twice normal, and modest inflammatory changes seen in addition to pronounced steatosis. More detailed time course studies of the liver disease in this model have not been reported, however.

Bscl2 encodes seipin, which is needed for both differentiation of white adipocytes and normal lipid droplet regulation, and deficiency has been suggested to shift the balance toward release of free fatty acids from adipocytes (89). Bscl2$^{-/-}$ mice display a lipodystrophic phenotype with near complete absence of WAT. Massive hepatic steatosis is a feature of all three Bscl2$^{-/-}$ models described. As in Agpat2 null mice, however, detailed natural history studies of liver pathology have not been reported: at 12 weeks, there is no evidence of NASH but there are no reported data beyond this age (90).

Although not directly modeling human disease, some other murine models have provided powerful evidence for the importance of adipose tissue in protection from NAFLD. One impactful model is the A-ZIP/F-1 (or "AZIP") mouse, which is an adipose-deficient mouse generated by transgenic overexpression of an artificially engineered dominant negative protein that interferes with critical adipogenic transcription factors. This causes deficiency of white adipose, severe insulin resistance, and hepatic steatosis, although it was said not to feature inflammation at the relatively young age studied (67). It is not known whether these mice develop NAFLD-related fibrosis, in part because of the reduced survival of the mice, which are severely diabetic. A much more recent model featured knockout of the insulin receptor selectively in adipose tissue, knockout being mediated by *cre* recombinase driven by the adipose-specific adiponectin promoter (68). These mice developed severe lipodystrophy and fatty liver disease very early, and by 12 weeks old, livers demonstrated not only steatosis but also increased ROS, lipid peroxidation, ballooning degeneration of hepatocytes, and elevated serum transaminase levels. By 1 year old the liver accounted for 25% of body weight and showed highly dysplastic liver nodules in addition to worsened inflammation and fibrosis. High fat feeding worsened the liver injury.

Liver Insulin Action

Although it is impossible to mimic a primary increase in insulin without inducing complex and potentially confounding changes to systemic metabolism, liver-specific knockout of the insulin receptor does afford the opportunity to test the proposition that liver insulin action is necessary for the development of hepatic steatosis. Indeed, in 2008, it was reported that ablation of the insulin receptor in mouse liver, although inducing severe systemic insulin resistance, did not increase liver triglyceride or liver weight (91).

Conversely, liver-specific knockout of the phosphatase and tensin homolog (PTEN), which is a phosphatidylinositol-3,4,5-triphosphate (PIP_3) phosphatase that serves to antagonize insulin's metabolic actions, produces macrovesicular steatosis, NASH (including Mallory-Denk bodies), fibrosis, and HCC (69). Similarly, other modes of genetic activation of phosphatidylinositol-3-kinase pathway signaling also produce steatosis that progresses to HCC, but without such marked steatohepatitis (92, 93).

HEPATOCYTE-AUTONOMOUS MURINE NAFLD

Impaired Lipoprotein Synthesis/Secretion

Assembly and secretion of VLDL represents a major route for the final disposition of intrahepatocyte triglyceride. Apolipoprotein B (ApoB) is a core component of VLDL particles, and genetic partial deficiency in humans causes familial hypobetalipoproteinaemia. Homozygous loss-of-function mutations of ApoB in mice cause embryonic lethality due to exencephalus. However, several different lines of mice heterozygous for mutated ApoB have been described, and these accurately recapitulate the hepatic steatosis, low serum triglycerides, and low HDL cholesterol of the human condition, although little fibrosis has been reported in the absence of additional pro-inflammatory stimuli (70, 94). Nevertheless, an important caveat is that natural history of liver inflammation and fibrosis has not been reported beyond 12 weeks of age. Similar to humans with hypobetalipoproteinaemia, mice with reduced ApoB function do not show marked insulin resistance, which differentiates them from most patients with NAFLD.

The FLS mouse is a further NAFLD model, which arose spontaneously as a result of inbreeding. FLS mice are non-obese and only mildly insulin resistant, but show marked accumulation of macrovesicular triglyceride with mononuclear inflammatory infiltrate and fibrosis, which eventually results in development of HCC by 13–16 months even without additional carcinogenic stimuli (95, 96). The precise underlying genetic defect is not yet known; however, a defect in microsomal triglyceride transfer protein (MTTP) (97) has been suggested. Precisely, why this model is so susceptible to HCC is not known. Crossing of FLS mice with *ob/ob* mice results in a model with the full metabolic syndrome and progressive fibrosis (81, 98); this model most closely recapitulates the whole human spectrum of NAFLD in a practical, experimental timeframe.

As discussed, human GWAS have shown a SNP in TM6SF2 to be associated with NAFLD (99). Reminiscent of hypobetalipoproteinaemia, however, carriers of the risk-conferring T-allele seem relatively protected from atherosclerotic disease despite increased lipid fat content. TM6SF2 is a membrane-bound protein located on the endoplasmic reticulum involved in the secretion of VLDL from hepatocytes (100). Germline knockout of *Tm6sf*$^{-}$ has not yet been described in mice; however, adeno-associated virus-mediated knockdown selectively in mouse liver resulted in increased hepatic triglyceride content and reduced VLDL secretion (32). Moreover transgenic hepatocyte-specific expression of human wild-type TM6SF2 in mice caused an increase in serum LDL cholesterol and lower HDL cholesterol (101); however, the full spectrum of NAFLD has not been demonstrated in this model.

ABNORMAL LIPID DROPLET DYNAMICS

The p.Ile148Met variant in human PNPLA3, found in around 20% of the population, is associated with NAFLD progression (55). Therefore, efforts have been made to generate a rodent model that recapitulates this genetic predisposing factor and to elucidate the role of PNPLA3 in NAFLD. PNPLA3 is expressed in many tissues in mice, including WAT and liver (102). Expression is suppressed upon fasting (103) and upregulated following a carbohydrate load (104), which is believed to be mediated by steroid regulatory element-binding protein-1c (SREBP-1c) and carbohydrate-response element-binding protein (ChREBP). PNPLA3 was initially thought to function as a lipase and be involved in the release of triglycerides from intracellular lipid droplets (105–107), so it was hypothesized that reduced activity would increase hepatocellular triglyceride content (108).

If reduced PNPLA3 activity was to accelerate NAFLD, *Pnpla3* knockout mice would be expected to have severely fatty livers; however, in fact, they have no evidence of NAFLD (109). Indeed, there is no discernible difference between *Pnpla3*$^{-/-}$ and wild-type mice even when fed high-fat diet or crossed onto the *Lep*$^{ob/ob}$ background. It should be noted, however, that mice show the highest Pnpla3 expression in WAT, unlike humans, in whom expression is highest in the liver.

In contrast to the knockout mice, mice with hepatic overexpression of human PNPLA3 p.Ile148Met show increased hepatic triglyceride content and fatty acid synthesis (73, 110). They develop steatosis only on a normal chow or sucrose diet, but not on a high-fat diet, which suggests that the mutant PNPLA3 acts upon *de novo* fatty acids, rather than re-absorbed circulating non-esterified fatty acids (111). This is consistent with epidemiological data that suggests fructose-rich diets are more harmful than high-fat diets (112).

The cumulative data from these mouse models suggest that PNPLA3 functions as a lysophosphatidic acid acyltransferase, and that the p.Ile148Met polymorphism increases this activity, stimulating development of hepatic steatosis (113, 114). This would have not been identified without the initially surprising finding in the knockout model. Although mice with hepatic expression of human PNPLA3 p.Ile148Met develop NAFLD, it is not known whether these mice develop HCC with age as reported studies extend to only 12 weeks, however.

CONCLUSION

The spectrum of human NAFLD disease arises from complex environmental influences allied to genetic predisposition. Human monogenic diseases demonstrate unequivocally that adipose tissue failure and primary genetic defects in intrahepatocyte lipid handling can give rise to NAFLD and its sequelae. Genetic perturbation in rodents may be combined with a pro-inflammatory or pro-steatotic diet to accelerate liver injury and mimic the multifactorial nature of human NAFLD. In general, murine genetic models closely mimic monogenic forms of NAFLD, where mouse and human liver phenotypes have been described in sufficient detail to draw conclusions; however, many gaps exist in descriptions of the natural history of NAFLD associated with several of the monogenic diseases in mice or humans. Findings in both species indicate that there is more than one possible pathogenic route to NAFLD, meaning

that study of several different human conditions and their models will be important to tease out common mechanisms of liver damage. A critical advantage of mouse models over humans is that the tremendously powerful technologies available to perturb genes conditionally or in an organ-specific way permits isolation of only some parts of a highly interconnected system. So, while alignment of humans and their murine models could be further refined, murine models are a highly valuable tool in the study of NAFLD.

AUTHOR CONTRIBUTIONS

JM, RS, and MA were all involved in conceptualizing and writing the manuscript, in its critical review, and in approval of the final version.

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Genetic Rodent Models of Obesity-Associated Ovarian Dysfunction and Subfertility: Insights into Polycystic Ovary Syndrome

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Polycystic ovary syndrome (PCOS) is the most common endocrinopathy affecting women and a leading cause of female infertility worldwide. Defined clinically by the presence of hyperandrogenemia and oligomenorrhoea, PCOS represents a state of hormonal dysregulation, disrupted ovarian follicle dynamics, and subsequent oligo- or anovulation. The syndrome's prevalence is attributed, at least partly, to a well-established association with obesity and insulin resistance (IR). Indeed, the presence of severe PCOS in human genetic obesity and IR syndromes supports a causal role for IR in the pathogenesis of PCOS. However, the molecular mechanisms underlying this causality, as well as the important role of hyperandrogenemia, remain poorly elucidated. As such, treatment of PCOS is necessarily empirical, focusing on symptom alleviation. The generation of knockout and transgenic rodent models of obesity and IR offers a promising platform in which to address mechanistic questions about reproductive dysfunction in the context of metabolic disease. Similarly, the impact of primary perturbations in rodent gonadotrophin or androgen signaling has been interrogated. However, the insights gained from such models have been limited by the relatively poor fidelity of rodent models to human PCOS. In this mini review, we evaluate the ovarian phenotypes associated with rodent models of obesity and IR, including the extent of endocrine disturbance, ovarian dysmorphology, and subfertility. We compare them to both human PCOS and other animal models of the syndrome (genetic and hormonal), explore reasons for their discordance, and consider the new opportunities that are emerging to better understand and treat this important condition.

Keywords: androgen, fertility, insulin resistance, mouse models, obesity, PCOS

Abbreviations: FSH, follicle-stimulating hormone; GnRH, gonadotrophin-releasing hormone; IR, insulin resistance; LH, luteinizing hormone; PCOS, polycystic ovary syndrome; T2DM, type 2 diabetes mellitus.

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INTRODUCTION

The association between obesity, insulin resistance (IR), type II diabetes (T2DM), cardiovascular disease, and non-alcoholic fatty liver disease is well-established in the literature, discussed commonly in the clinic, and subject to intensive investigation in laboratories worldwide (1, 2). Perhaps less recognized is obesity's association with ovarian dysfunction, most commonly in the form of polycystic ovary syndrome (PCOS). Diagnostic criteria of PCOS incorporate three key features: biochemical and/or clinical evidence of androgen excess (including acne, hirsutism, and alopecia), ovarian dysfunction or anovulation (manifesting as absent or irregular menstruation), and the appearance of multiple peripheral cysts on ovarian ultrasonography (3). Rarer causes of raised androgen levels (such as an androgen-producing tumor) are first excluded. Metabolic dysfunction is common but not invariable in women with PCOS and so, although cross-sectional and longitudinal studies support a significant role for IR in the etiology of PCOS, diagnostic criteria do not currently incorporate metabolic parameters. Nevertheless, not only is PCOS the most common cause of anovulatory infertility and menstrual irregularity but (since it often manifests in the second and third decades) young women with PCOS also represent a large, identifiable group who may be at increased risk of metabolic (4–6) and cardiovascular diseases (7–10). Indeed, PCOS is a strong predictor of future T2DM (11). Women with PCOS therefore represent an important target for research and prevention.

The heterogeneous nature of PCOS, along with a lack of consensus over precise diagnostic criteria, has complicated efforts to understand its pathogenesis. Familial clustering studies and monozygotic twin concordance reveal an important genetic predisposition to the syndrome. Genetic variants identified from candidate gene screening and genome-wide association studies implicate insulin, growth factor, and gonadotrophin signaling, cellular proliferation, and DNA repair pathways; however, they so far account for less than 10 percent of the syndrome's heritability (12). The presence of PCOS-like features in animals exposed prenatally to androgens suggests that PCOS may have important developmental origins (13). Genetic and developmental influences likely interact with environmental factors in adolescence and adulthood to produce the complex physiological dysregulation that characterizes this syndrome.

Hormonal models, in which rodents, sheep, and non-human primates are treated during development or postnatally with androgens (testosterone, DHT, or DHEA), estrogens, aromatase inhibitors, or antiprogestins, are widely employed in PCOS research (14–19). Genetic rodent models offer a complementary albeit underutilized strategy in this field, allowing the contribution of individual genes to be evaluated on "clean" genetic backgrounds and providing tractable and affordable models in which to interrogate disease pathways (14, 20–23). Their value, however, depends on the fidelity of the model to human physiology and disease and the relevance of single-gene perturbations. After summarizing some main concepts relating to the pathogenesis of PCOS (**Figure 1**), we describe key rodent models relevant to the study of ovarian dysfunction in metabolic diseases (**Table 1**) and explore why their interpretation may be more complicated than initially apparent.

KEY PLAYERS IN PCOS PATHOGENESIS

Metabolic Features of PCOS

While PCOS is robustly associated with impaired insulin sensitivity and hyperinsulinemia (**Table 1**), this is independent of body weight, and a significant proportion of insulin-resistant women with PCOS are lean (44, 45). However, it is recognized that increased body weight exacerbates hyperandrogenism, oligomenorrhoea, and metabolic risk in PCOS (46, 47), and genetic studies have revealed a role for obesity-associated genes (48, 49).

Several observations suggest that IR, and associated compensatory hyperinsulinemia, may play a key pathogenic role in PCOS. Firstly, IR is more common in women with both hyperandrogenism and anovulation, compared to weight-matched hyperandrogenemic women with normal ovulatory cycles (50). Second, interventions that increase insulin sensitivity improve, independent of weight loss, ovulatory function, menstrual cyclicity, fertility, and hyperandrogenism in lean and obese patients (51–55). Third, a severe PCOS-like syndrome is a prominent (often-presenting) feature in patients with severe, genetic forms of IR (56) and is also reportedly associated with pancreatic insulinomata and excessive exogenous insulin in type 1 diabetes (57, 58).

Importantly, PCOS likely represents a state of "partial" IR, in which preserved insulin signaling in ovarian theca cells causes excessive androgen synthesis and theca cell proliferation, with subsequent hyperandrogenemia (**Figure 1**) (59–62). Other potential effects of hyperinsulinemia include reduced hepatic synthesis of sex hormone-binding globulin, thereby increasing free testosterone, hypersecretion of pituitary luteinizing hormone (LH), and reduced insulin-like growth factor-binding protein (63–65). This latter effect potentially modulates the paracrine growth factor-dependent regulation of early follicle development and dominant follicle selection (**Figure 1**).

Ovarian Dysmorphology

The abnormal appearance of the ovarian cortex in PCOS represents inappropriate and excessive initiation of follicle growth from the primordial follicle pool, followed by developmental failure and growth arrest at the medium-sized antral stage (5–10 mm) (66–68). Loss of coordinated follicle development results in fewer or absent ovulations, and therefore subfertility. Histologically, the ovary contains a reduced number of corpora lutea (representing fewer ovulations), more atretic follicles, stromal hypertrophy, and increased ovarian weight. As mentioned, hyperthecosis is prominent, with *in vitro* evidence suggesting that abnormal thecal cell proliferation contributes to excessive androgen biosynthesis (62, 69).

Hormonal Dysregulation

While IR and hyperinsulinemia may play a central, and in some cases primary, role in PCOS pathogenesis, the importance of hyperandrogenism should be stressed. Not only is it a defining

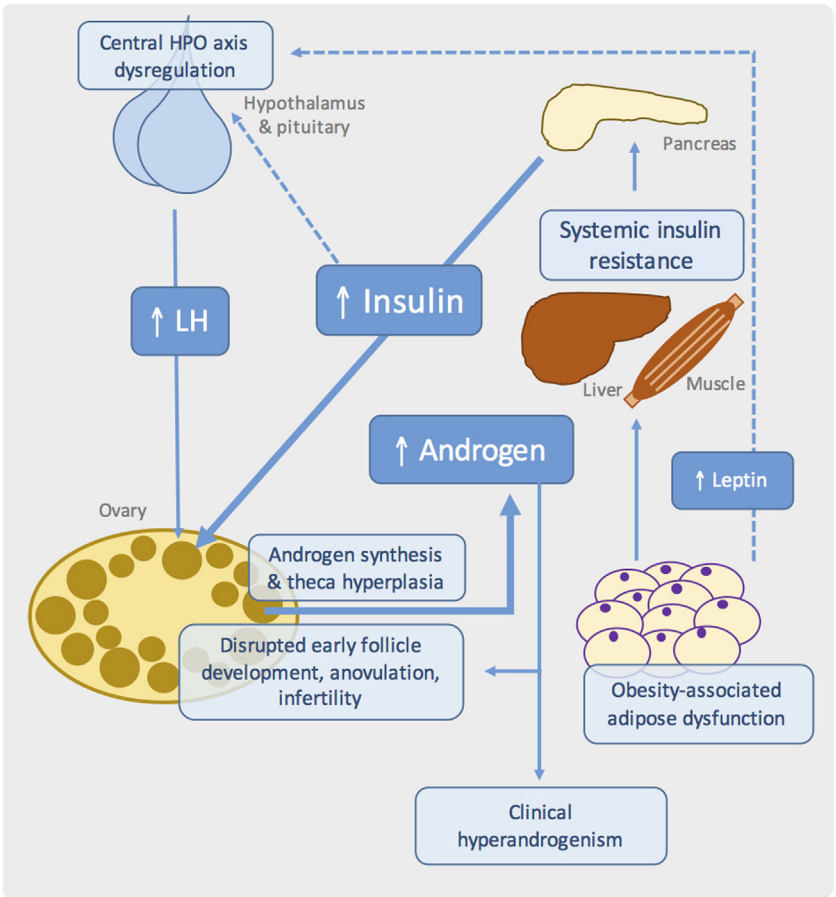

FIGURE 1 | Proposed pathogenic mechanisms in obesity-associated ovarian dysfunction and subfertility. Schematic showing the major metabolic and reproductive pathways involved in PCOS. Systemic insulin resistance, commonly due to adipose tissue dysfunction in the context of obesity, results in compensatory hyperinsulinemia. At the ovary, insulin synergizes with luteinizing hormone (LH) to drive androgen synthesis. Disrupted insulin, growth factor, gonadotrophin, and sex steroid signaling in the ovary leads to failure of follicle development and ovulation. Genetic and developmental influences are also likely to play an important role.

feature of the syndrome, both in ovulatory and anovulatory women, but other conditions associated with excessive androgen exposure (such as congenital adrenal hyperplasia and androgen-secreting tumors) also produce features of PCOS (70). Moreover, administration of androgens in rodents, sheep, and non-human primates results in pathophysiological changes that closely resemble features of PCOS in women. Androgens act at the ovary to disrupt follicular development and dominant follicle selection by promoting excessive early follicular growth, while systemic effects include development of IR and metabolic dysfunction (71–77). The role of androgens in PCOS may be particularly important during key developmental windows before the onset of IR (13). Prenatally, androgenized rhesus monkeys and sheep demonstrate ovarian hyperandrogenism and IR in adulthood, with increased follicle numbers, anovulation, and LH hypersecretion (78–81).

Dysregulation and reprograming of the hypothalamus–pituitary–ovarian (HPO) axis is common in PCOS, potentially driven by androgen exposure *in utero* and manifesting as hypersecretion of LH, persistently rapid LH pulse frequency, and below-normal levels of follicle-stimulating hormone (FSH) (82, 83). These alterations likely contribute to disrupted follicle development in PCOS, while high levels of LH also synergize with insulin to promote theca androgen production (**Figure 1**). However, it is noteworthy that many patients have normal LH levels, suggesting that elevated gonadotrophin levels is unlikely to be the primary defect in PCOS (84).

OVARIAN DYSFUNCTION IN GENETIC MODELS OF METABOLIC DISEASE

Rodent Models of Obesity

While there is no spontaneously occurring animal model of PCOS, transgenic and knockout rodent models widely used in metabolic research provide opportunities to study specifically the association between metabolic disease and ovarian dysfunction. However, it is important to note that key differences exist between human and rodent ovarian function. Whereas in humans, full follicular differentiation occurs in the later stages of fetal development, in rodents this occurs postnatally. The mouse estrus cycle

TABLE 1 | Reproductive features of rodent models of obesity and insulin resistance.

Model	Body weight	Associated metabolic phenotype	Sex steroids	Gonadotrophins	Fertility	Ovarian morphology	Menstrual cyclicity	Comments	Key reference
Human PCOS	↑	IR, ↑ insulin, T2DM, ↑ lipids (independent of BMI)	↑ T	↑ LH ↓ FSH	Subfertile	Multiple small, cortical cysts due to follicular arrest, follicular atresia, ↓ CLs	Oligo-/ amenorrhea	Insulin sensitizers improve menstrual regularity and hyperandrogenism.	(2)
High-fat diet mouse	↑	IR, ↑ insulin, ↑ FBG	N/R	↑ LH ↑ FSH	Subfertile	Diminished follicular development, old CLs	Irregular	Fertility restored after exogenous gonadotrophin (suggests HH).	(24, 25)
ob/ob mouse	↑	IR, ↑ insulin, ↑ FBG, glucose intolerance, ↑ lipids	↑ T ↑ E2	LH → ↓ FSH	Infertile	Ovarian atrophy, follicular atresia, ↓ CLs, no cysts	Acyclic, anovulatory	Ovarian interstitial cytolipema. Phenotype rescued with leptin.	(26–28)
db/db mouse	↑	IR, ↑ insulin, ↑ FBG, glucose intolerance	↓ E2/P	N/R	Subfertile	Ovarian atrophy, progressive follicular atresia	Irregular	Ovarian interstitial cytolipema.	(29–32)
Zucker rat	↑	IR, ↑ insulin, ↑ FBG, glucose intolerance	↓ T ↓ E2	LH → FSH →	Subfertile	↑ total follicle numbers, follicular atresia	Irregular (prolonged diestrus)		(33, 34)
Koletsky (JCR:LA-cp) rat	↑	↑ insulin, ↑ FBG, ↑ lipids	↑ T E2 →	N/R	Subfertile	Ovarian atrophy, cystic follicles, follicular atresia, thin GC layer, ↓ CLs	Irregular		(35, 36)
NZO rat (polygenic)	↑	IR, ↑ insulin, ↑ FBG, ↑ lipids	T → ↓ E2	↓ LH FSH →	Subfertile	↑ ovarian volume, ↑ total follicle numbers, follicular atresia, ↓ CLs, no cysts	Irregular		(37–39)
Neuron-specific IR deletion (mouse)	↑	Mild IR, ↑ insulin, ↑ TGs	N/R	N/R	Subfertile	Large, luteinized ovarian cysts, thecal-interstitial hyperplasia, ↓ CLs	Irregular		(40)
IR/LepR^{POMC} (mouse)	↑	IR, ↑ insulin, glucose intolerance	↑ T	↑ LH	Infertile	Occasional cyst-like follicles	Acyclic, anovulatory		(41)
Neuron-specific *IRS2* deletion (mouse)	↑	↑ FBG, glucose intolerance	↓ T ↓ E2	↓ LH	Infertile	Small ovaries, ↓ total follicle numbers	Acyclic, anovulatory		(42)
AKT2 deletion (mouse)	→	↑ insulin (older mice only)	↑ T (older mice only)	LH normal	Young mice fertile	Large luteinized cysts	N/R	Mice aged 120 weeks.	(43)

CL, corpus lutea; E2, estradiol; FBG, fasting blood glucose; FSH, follicle-stimulating hormone; IR, insulin resistance; LH, luteinizing hormone; N/R, not reported; P, progesterone; PCOS, polycystic ovary syndrome; T, testosterone; T2DM, type 2 diabetes mellitus; TGs, triglycerides.

lasts only 4–6 days, compared to 28 days in humans. Furthermore, rodents are polyovulatory, suggesting important differences in dominant follicle selection despite underlying similarities in the HPO axis.

In spite of these differences, various rodent models of obesity do display reproductive phenotypes comparable to PCOS (**Table 1**). Diet-induced obesity in wild-type mice is associated with disrupted estrus cyclicity, fewer corpora lutea, reduced fertility, and metabolic dysfunction, supporting the notion that obesity-associated metabolic dysfunction may contribute to PCOS (24, 25, 85). Among the genetic models, female *ob/ob* and *db/db* mice, which, due to loss-of-function mutations in leptin and leptin receptor, respectively, are hyperphagic, severely obese, hyperinsulinemic, and hyperglycemic are also infertile, acyclic, and anovulatory (**Table 1**). Morphologically, they show utero-ovarian atrophy, follicular atresia, apoptotic granulosa cells, deformed oocytes, absent corpora lutea, and no cystic structures (26, 27, 29–32). The endocrine profile of *ob/ob* mice includes elevated serum testosterone, estradiol, and progesterone, with reduced FSH but normal LH, while *db/db* mice have low estradiol and progesterone. The obese Koletsky and Zucker diabetic fat rats, both of which also lack functional leptin receptors, do exhibit estrus cycling (albeit irregularly) but are subfertile with increased follicle numbers, follicular atresia, and fewer corpora lutea. While androgen levels are elevated in the obese Koletsky, in Zucker, they are reportedly below normal (33–36). The New Zealand obese (NZO) mouse, notable for being a polygenic model of the human metabolic syndrome (37), also harbors leptin receptor variants and displays a reproductive phenotype similar to that of Zucker (**Table 1**) (38, 39).

In all of these models, reproductive dysfunction is at least partly attributable to loss of hypothalamic leptin signaling,

rather than obesity *per se*. Genetic leptin deficiency in humans is associated with low gonadotrophins and pubertal failure, which are restored with leptin replacement (86). Fertility, litter size, and estrus cyclicity of *ob/ob* mice were similarly ameliorated by human recombinant leptin (87, 88) or transplantation with wild-type adipose tissue (89, 90). Along with other peripheral signals, leptin is believed to modulate the activity of gonadotrophin-releasing hormone (GnRH) releasing neurons – and thus the entire HPO axis – in response to nutritional status (91). Indeed, low body weight is known to interfere with reproductive function and pubertal timing (92). Failure of central leptin action in rodent models of obesity therefore leads to infertility due to hypogonadotrophic hypogonadism and follicle development (**Figure 1**). Indeed, human obesity is also associated with a degree of hypothalamic leptin resistance, which may contribute to HPO dysregulation in PCOS (93, 94). Reports of excess lipid accumulation in follicular cells of *ob/ob* mice and the obese Koletsky rat suggest an additional "lipotoxic" mechanism by which extreme obesity may produce ovarian dysfunction, although there are no reports of such a phenotype in PCOS (30, 36).

In these models, the relative contribution of IR-associated hyperinsulinemia and central leptin resistance is difficult to disentangle, particularly since hypothalamic insulin signaling also regulates GnRH release and thus reproduction function (40, 41, 95–97). Mice with neuron-specific deletion of the insulin receptor gene (*Insr*) or hypothalamic POMC neuron-specific deletion of both leptin and *Insr* were hyperphagic, insulin resistant, and subfertile due to impaired follicular development (**Table 1**) (40, 41, 98). The combined knockout was notable for high levels of LH, hyperandrogenemia, and cyst-like follicles. POMC-specific deletion of leptin receptor alone produced only a subtle reproductive phenotype (99). Counterintuitively, pituitary-specific *Insr* knockout reportedly *rescued* the PCOS-like phenotype associated with diet-induced obesity (24). These observations highlight complex interactions between leptin and insulin in their regulation of reproductive function. Indeed, studies in mammals and non-mammalian species reveal that nutritional status and reproductive capacity are tightly intertwined, ensuring that reproduction only proceeds if nutritional status is optimal (100).

Genetic Models of Insulin Resistance

In humans, rare loss-of-function mutations in *INSR* not only cause extreme hyperinsulinemia but also oligomenorrhoea, hyperandrogenism, and excessive development of sex hormone-dependent tissues (56). Common genetic defects in insulin signaling are suggested to contribute to PCOS heritability (101, 102), and cellular studies reveal abnormalities in insulin-mediated insulin receptor autophosphorylation, IRS expression, PI3-kinase activation, GLUT4 expression, and insulin-stimulated glucose uptake in adipocytes and skeletal muscle from women with PCOS (103–107). However, the results of such studies are variable and need further verification.

Mice lacking functional insulin receptor develop profound metabolic abnormalities at birth and die within days. Of the tissue-specific knockouts, only those targeting the brain have a reported reproductive phenotype (108). Similar to the neuron-specific *Insr* knockout, global deletion of *Irs2* (but not *Irs1*) causes a combination of metabolic, reproductive, and ovarian features that likely result from disrupted central insulin and leptin action rather than abnormal systemic glucose metabolism (42) (**Table 1**). Thus, in addition to the impact of systemic hyperinsulinemia, the interpretation of global insulin signaling defects must consider the actions of insulin at the hypothalamus as well as disruption to the regulation of early follicle development by IGF1. There are no corresponding human syndromes of IRS dysfunction or deficiency with which to compare.

Downstream of IRS in the signaling pathway, non-functional mutations in human *AKT2* result in ovarian hyperandrogenism in the context of partial lipodystrophy, severe IR, diabetes, metabolic dyslipidemia, and fatty liver (109). In mice, global *AKT2* deletion produced a somewhat comparable ovarian phenotype, with increased androgenic steroidogenesis in the theca-interstitium, theca-interstitial hyperplasia, hyperandrogenemia, reduced corpora lutea, and ovarian cysts but normal LH levels (**Table 1**) (43). However, the large, luteinized, serous-filled cysts were quite distinct from the ovarian morphology characterizing human PCOS. For unclear reasons, reproductive features were absent in younger mice, although could be induced by treatment with LH, perhaps due to synergism with hyperinsulinemia.

Other human lipodystrophy syndromes (genetic or acquired) are similarly characterized by severe IR, ovarian hyperandrogenism, amenorrhea, and infertility (110–112). While genetic mouse models of generalized lipodystrophy manifest many metabolic features of the human diseases, "partial" lipodystrophy has been more challenging to model (113). Moreover, while the metabolic properties of these models have been interrogated in detail, their reproductive and ovarian phenotypes have not been reported widely. Studying these models may provide important new insights into the role of BMI-independent IR in PCOS-like ovarian dysfunction.

Genetic Models Targeting the HPO Axis

To better understand PCOS pathogenesis, rodent models of obesity and IR should be considered alongside those in which other implicated systems are targeted. Transgenic mice with chronically elevated gonadotrophin levels have a thickened theca cell layer, similar to PCOS, with correspondingly increased estrogen and testosterone levels (23, 114). However, unlike PCOS, their ovaries contain large, hemorrhagic cysts, as do those of mice lacking LH receptor (114, 115). Global or theca-specific deficiency of estrogen receptor subunits ERα or ERβ, or global deficiency of aromatase, produces chronically elevated gonadotrophins (due to lack of estradiol), arrested follicular growth, absent corpora lutea and anovulation (116–118). ERα knockout mice also show increased adiposity (without hyperphagia), IR, and diabetes (118, 119), whereas constitutive elevation of LH activity produces hyperphagic obesity with hyperleptinemia and hyperinsulinemia (120). These observations further illuminate the complexity of nutritional and reproductive cross talk in humans, again challenging the value of simplified rodent models.

TRANSGENIC RODENTS AND PCOS – NOT FIT FOR PURPOSE?

This discussion reveals that transgenic models of PCOS are complex, heterogeneous, and even the best examples deviate in important ways from the human syndrome. Models of obesity and IR have not typically been studied comprehensively from a reproductive perspective. Even when a reproductive deficit is noted, the ovarian and endocrinological phenotyping is often incomplete, with concerns raised over timing of the studies (relative to time of day, phase of the estrous cycle, and age of the animal), the rigor of morphological analyses, and the variability of ovarian appearances described as "cystic." Furthermore, as outlined above, important differences exist between human and rodent ovarian function. Such differences may explain why the reproductive consequences of androgen exposure are less consistent in rodents than in sheep or primates, and emphasize that results from rodent-based studies (genetic or hormonal) need to be extrapolated with caution to human PCOS (13, 23).

Emerging from this discussion is an important reminder that reproductive capacity and nutritional status are intertwined tightly through feedback and cross talk between reproductive and metabolic pathways. Across a wide range of species, including *Caenorhabditis elegans* and *Drosophila*, conserved mechanisms operate to regulate reproduction and energy homeostasis (121–123). In rodent models of obesity, the same lesions that produce hyperphagia also directly impact on the HPO axis, thereby complicating their interpretation. The bidirectional interaction between reproductive and nutritional signaling also operates systemically: while estrogen drives adipogenesis, and while testosterone drives food intake, both steroids in excess produce IR, hyperinsulinemia, high levels of circulating leptin, and reduced levels of adiponectin, all of which impact on the HPO axis and ovarian function. The hope of mimicking this complex network by perturbing single or a few genes is perhaps ambitious. Indeed, the notion that PCOS is precipitated by a single etiological factor is undoubtedly too simple. While monogenic perturbations in insulin signaling or adipose function in humans do produce PCOS-like syndromes, differences between human and rodent metabolism and reproduction mean that PCOS will not necessarily emerge from equivalent defects in mice. As in all complex human disease, the role of genetic, developmental, and environmental factors likely contribute heavily to the heterogeneity of human PCOS.

FUTURE OPPORTUNITIES

Complementary strategies are required to better understand this growing health problem. The combined use of hormonal treatments in transgenic animals may afford interesting, clinically relevant insights. Primary follicular cell and whole follicle cultures, including from transgenic animals, facilitate the study of tightly regulated paracrine and autocrine networks in early follicle development that become disordered in PCOS (124). The ease and efficiency of CRISPR-Cas9-based gene editing technologies will doubtless prove invaluable, particularly to explore new susceptibility loci emerging from large GWAS studies (48, 49, 101, 102, 125). Many of these loci implicate genes of largely unknown function. As they are investigated over the coming years, prudent selection of appropriate cell and animal systems will be imperative. The study of candidate genes in non-ovarian cell types is questionable, yet primary cultures are difficult to acquire and maintain, and ovarian cell lines are too atypical in their properties to be useful. Therefore, in spite of reservations highlighted above, transgenic rodent models will likely play an ongoing role in our effort to better understand and manage this challenging condition.

CONCLUSION

A clear relationship exists between obesity, metabolic dysregulation, and ovarian dysfunction. However, the mechanisms of this association are poorly understood. Without detailed knowledge of the etiology of PCOS, management is limited to empirical and symptomatic treatment. While hormonal models of PCOS demonstrate an important role for hyperandrogenemia, the reported genetic models incompletely replicate the PCOS phenotype. Their study has offered important insights into the interaction between metabolism and reproduction, but clear conclusions about PCOS pathogenesis have not been forthcoming. Nevertheless, specific models may prove useful for answering reductionist questions about aspects of the condition, such as disordered folliculogenesis or disruption of the HPO axis. Future efforts will benefit from ongoing combined study of humans, mouse models, and cells, driven by insights emerging from human genetic studies. These studies will continue to advance our understanding of this important condition and, with time, support new approaches to addressing both the metabolic and reproductive problems faced by affected women.

AUTHOR CONTRIBUTIONS

Both IH-D and SF contributed to the content, writing, and editing of this manuscript.

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REVIEW
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Relationships between Rodent White Adipose Fat Pads and Human White Adipose Fat Depots

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The objective of this review was to compare and contrast the physiological and metabolic profiles of rodent white adipose fat pads with white adipose fat depots in humans. Human fat distribution and its metabolic consequences have received extensive attention, but much of what has been tested in translational research has relied heavily on rodents. Unfortunately, the validity of using rodent fat pads as a model of human adiposity has received less attention. There is a surprisingly lack of studies demonstrating an analogous relationship between rodent and human adiposity on obesity-related comorbidities. Therefore, we aimed to compare known similarities and disparities in terms of white adipose tissue (WAT) development and distribution, sexual dimorphism, weight loss, adipokine secretion, and aging. While the literature supports the notion that many similarities exist between rodents and humans, notable differences emerge related to fat deposition and function of WAT. Thus, further research is warranted to more carefully define the strengths and limitations of rodent WAT as a model for humans, with a particular emphasis on comparable fat depots, such as mesenteric fat.

Keywords: rodents, humans, obesity, fat pads, fat depot, fat distribution

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INTRODUCTION

Obesity has largely been defined by a body mass index (BMI) >30 kg/m^2, or better still, a body fat percentage >25% in males and >35% in females (1). However, from a physiological standpoint, evidence indicates that body fat distribution, irrespective of BMI and/or body fat percentage, most strongly predicts risk of obesity-related diseases and complications (2). Risk can further be stratified by fat distribution, as individuals with a higher waist-to-hip ratio suffer disproportionately from obesity-related metabolic dysfunction (3). Indeed, individuals with gynoid obesity, characterized by subcutaneous fat in the gluteofemoral region, have minimal risk of developing metabolic dysfunction (3, 4); whereas individuals with the so-called android obesity, which is characterized by visceral fat accretion, suffer a greater risk of metabolic dysfunction (4, 5).

Abbreviations: BMI, body mass index; CR, caloric restriction; DSAT, deep subcutaneous adipose tissue; HOX, homeobox; IL-6, interleukin-6; SAT, subcutaneous adipose tissue; SSAT, superficial subcutaneous adipose tissue; TNF-α, tumor necrosis factor-α; VAT, visceral adipose tissue.

The metabolic consequences of body fat and its distribution have received extensive attention in the literature. As rodents are by far the most commonly used pre-clinical model of human obesity (6–9), further validation of important commonalities and differences between rodent and humans are needed. Specifically, investigators should seriously consider to what extent their experimental approach and findings are translational. For instance, rodent adipose tissue deposition is strikingly dissimilar to humans, and adipocytes in these depots display metabolic heterogeneity and are intrinsically different within a species. As such, further research needs to focus on how specific rodent fat pads correspond, if at all, with fat depots in humans. To our knowledge, only two studies have compared gene expression in mouse fat pads to gene expression in analogous human fat depots (10, 11). Thus, given the general lack of information or discussion on this highly relevant topic, a systemic review of the literature in our view, is warranted.

FAT DEPOTS VERSUS FAT PADS: ANATOMICAL CONSIDERATIONS

There are three main regional human anatomical fat depots: intra-abdominal, upper-body/abdominal subcutaneous, and lower body subcutaneous (**Figure 1A**) (12). Intra-abdominal refers to visceral adipose tissue (VAT), which surrounds the inner organs (13). VAT can further be divided into omental, mesenteric, retroperitoneal, gonadal, and pericardial. The upper-body subcutaneous adipose tissue (SAT) can be categorized depending on if it is situated superficial or deep to the fascia superficialis. The adipose tissue below the fascia is the deep subcutaneous adipose tissue (DSAT) compartment, whereas adipose tissue located superficially to this fascia is the superficial subcutaneous adipose tissue (SSAT) compartment (14, 15). Though SAT is distributed throughout the human body, the main depots are in the abdomen, buttocks, and thighs (16). The buttocks and thighs make up the lower body SAT and are termed the gluteofemoral depot (12, 13).

Like humans, adipose tissue in rodents is a multi-depot organ (**Figure 2**), but unlike humans, which have two main subcutaneous depots located in the abdominal and gluteofemoral region, rodents have two main subcutaneous pads located anteriorly and posteriorly. The anterior pad is located between the scapulae, descending from the neck to the axillae (17), while, the posterior pad, or inguinal fat pad, spreads from the dorsolumbar region to the gluteal region. The inguinal fat pad is comparable in terms of location to the large gluteofemoral subcutaneous depot in humans. Additionally, rodent SAT is separated from dermal adipose tissue by a smooth muscle layer, whereas, in humans, the SAT is continuous with dermal adipose tissue (17). Furthermore, there has been no evidence to our knowledge of multiple subcutaneous layers in rodents, such as is the case in humans.

Rodents harbor visceral fat pads in the perigonadal region, known as epididymal in males and periovarian in females, as well as retroperitoneal fat pads located on the kidneys, and the mesenteric fat pad located alongside the intestinal tract. The mesenteric fat pad is widely touted as the most analogous to human intra-abdominal adipose tissue both in its location and biology, because it has access to the portal vein. However, this depot is not well studied in rodents due to limitations in its surgical manipulation

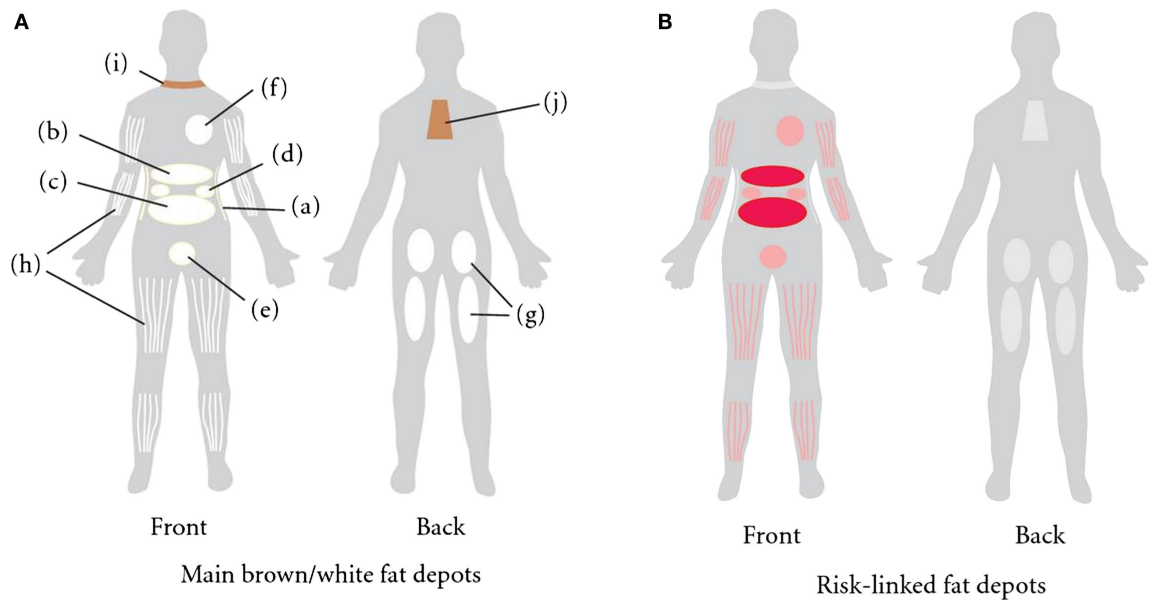

FIGURE 1 | **(A)** Distribution of fat depots in the human. The white adipose tissue (WAT) is found in subcutaneous adipose tissue (SAT) abdominally (a), gluteofemorally (g), and intramuscularly (h). WAT is also found in the visceral adipose tissue (VAT). The visceral depots are omental (b), mesenteric (c), retroperitoneal (d), gonadal (e), and pericardial (f). Brown adipose tissue is found supraclavicularly (i) and in the subscapular region (j). **(B)** Those fat depots linked to increased risk of developing obesity-related morbidities and mortality are colored in red (13).

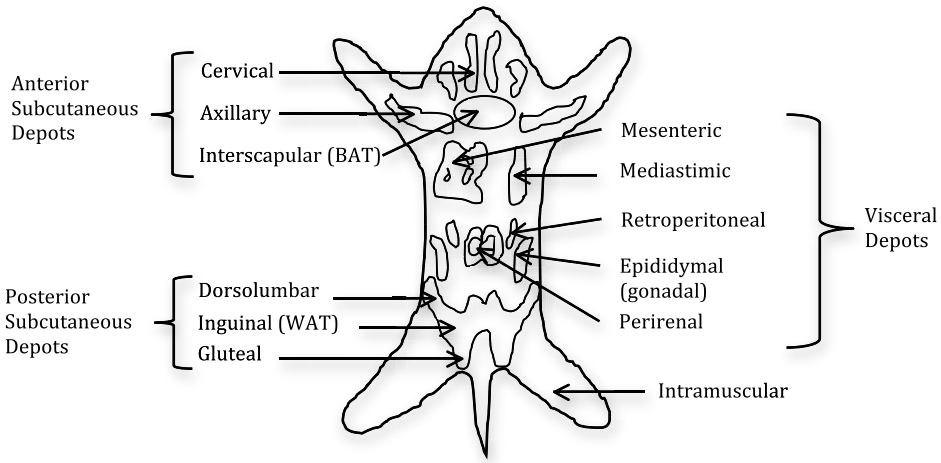

FIGURE 2 | **Distribution of fat pads in the mouse**. The fat is composed of two subcutaneous pads and several visceral pads. The main white adipose tissue (WAT) pads are the inguinal and epididymal, with the latter being the most frequent dissected pad. Brown adipose tissue (BAT) is distributed throughout the fat pads with the main BAT depot in the interscapular region (18).

and separation from contaminating vessels. The perigonadal fat pads are typically the largest and most readily accessible fat pads and, for these and other reasons, they are the most frequently used in the literature (19–21). However, humans do not harbor a fat depot analogous to these fat pads, which has led to the suggestion that these pads should be considered "peri-visceral" rather than true visceral fat pads. Another difference between the two species is the ambiguity when referencing the omental depot in rodents. Though it is clearly defined and developed in humans, it is less so in rodents, leading some investigators to not reference the omental depot at all (13). However, based on the literature, it seems that the omental depot is present in the mouse, albeit in small quantities, and is similarly connected to the stomach, as it is in humans (22). Thus, although adipose tissue is a multi-depot organ in both humans and rodents, there are anatomical differences that should be taken into consideration.

FAT PAD AND DEPOT DISTRIBUTION IN HEALTH AND DISEASE

In humans, increased VAT is associated with an increased risk for insulin resistance and dyslipidemias (23), while being an independent risk factor for type II diabetes (24), hypertension (25), and all-cause mortality (26) (**Figure 1B**). By contrast, SAT is associated with improved or preserved insulin sensitivity, and mitigated risk of developing type II diabetes and other metabolic derangements (27, 28). Alternatively, it has been challenged whether VAT accumulation increases the risk for metabolic dysfunction (29, 30). Some have asserted that abdominal subcutaneous fat plays an independent role in developing an unfavorable metabolic profile (14, 31), dependent on how the subcutaneous fat is distributed among abdominal and gluteofemoral subcutaneous fat depots. In rats and mice, respectively, employing a lipectomy model, whereby the epididymal and retroperitoneal fat pads are surgically removed, improves insulin action, reduces tumorigenesis and improves longevity, independent of confounding factors (32–35). It deserves mentioning that surgically ablating the mesenteric fat pad in rodents cannot be sufficiently performed with current techniques due to the heavy innervation and vascularization of this tissue. Thus, given the general sentiment that rodent mesenteric fat is most analogous to the human intra-abdominal depot, it is tempting to speculate that the true importance of VAT gleaned from rodent lipectomy studies, has if anything, underestimated the "true" hazards associated with visceral adiposity. By contrast, some studies have shown that removal of omental fat in obese men and women in conjunction with gastric bypass surgery provided no added benefit to metabolic endpoints (36, 37). However, unlike rodent studies, where ~75% of visceral fat was removed, the omentum in obese subjects likely accounts for a small fraction (<10%) of total visceral adiposity, an amount that may have been insufficient to observe meaningful benefits.

Mechanistically, the benefits associated with SAT may be attributed to its ability to act as a metabolic "sink" to buffer against the daily influx of nutrients by providing long-term energy storage (38), thereby protecting against ectopic fat deposition and associated lipotoxicity (3). Indeed, gluteofemoral fat in humans has been shown to have a protective role, such that it is independently associated with lower triglyceride levels (39), and higher concentration of high-density lipoprotein cholesterol (40, 41). Studies have shown that femoral fat is associated with an elevated adipose tissue lipoprotein lipase activity (40, 42). Though the mechanisms responsible for depot differences in metabolic profiles still remain unclear, speculatively, gluteofemoral fat secretes more beneficial adipokines, as supported by studies in rodents (43), thereby producing less pro-inflammatory molecules compared to VAT (3). Indeed, it is widely believed that the deleterious effects of

elevated VAT can be attributed in part to an enhanced secretion of pro-inflammatory cytokines and the release of FFA, which have direct portal access to the liver (41, 44).

In humans, Jensen et al. (5) demonstrated that in moderately obese women, body fat distribution is predictive of FFA dysfunction, such that upper body obese women, but not lower body obese women, had an increased adipose tissue FFA release, when normalized for lean body mass. Furthermore, upper body obese women released almost twice the amount of calories as lower body obese individuals. Speculatively, the differences in metabolic function between these two states could also be attributed to adipocyte hypertrophy, which occurred in the upper body obese women during weight gain, as opposed to hyperplasia, which was observed in the lower body obese. Therefore, the adipocytes in the lower body obese were still of normal size with more restrained lipolysis, while the upper body adipocytes were enlarged with increased lipolysis. Importantly, many of the hazardous metabolic sequelae secreted from adipose tissue have been linked to enlarged hypoxic adipocytes (45, 46).

In rodents, surgically transplanting subcutaneous fat has demonstrated either no deleterious effect or even proven beneficial to the recipient in some reports. By contrast, when subcutaneous fat was removed from mice, there was a significant increase in serum triglycerides and basal insulinemic index, which implies a quantitative role for subcutaneous fat acting as a metabolic "sink" (9, 47). Likewise, subcutaneous, but not visceral fat transplants, improved glucose metabolism in mice, particularly when they were placed in the viscera (43). It should be noted that large-volume liposuction (~10 kg) of subcutaneous fat in humans neither improved nor harmed the metabolic (48) or cardiovascular risk profile (49). Importantly, a redistribution of fat stores is a hallmark of aging and is characterized by a depletion of subcutaneous fat stores in older rodents and humans, which contributes to a simultaneous expansion of visceral and ectopic fat stores in sites such as liver, pancreas, bone, and skeletal muscle (50). Thus, taken together, these observations suggest that subcutaneous fat may be beneficial in both rodents and humans, not only via its own secretions but also by mitigating the accretion of fat in other more harmful sites with obesity and aging, including visceral and ectopic stores.

Beyond the contribution of VAT *per se*, an emerging association with specific sites of SAT have been linked with increased metabolic dysfunction in humans (30, 31, 51). As previously stated, subcutaneous fat is divided into two layers: superficial and deep (14, 15). Johnson et al. (15) investigated abdominal adipose tissue in obese women and determined that the area of DSAT was highly correlated with the area of VAT. Similarly, Kelley et al. (52) examined both lean and obese men and women, and found that DSAT and VAT were both strongly correlated with glucose, insulin, HDL, and triglycerides, whereas SSAT had a much weaker association, and more closely mirrored gluteofemoral SAT. Smith et al. (14) found that DSAT was correlated with VAT, as well as with fasting insulin levels. Because DSAT follows a pattern more associated with VAT, and SSAT parallels gluteofemoral adiposity, the location of adipose tissue sampling, either above or below the fascia, needs to be taken into account as these conclusions support the hypothesis that SAT is heterogeneous, having different physiological effects depending on depot location. Rodents do not harbor fat pads that are clearly analogous to the DSAT layer, though it is possible that the so-called peri-visceral fat pads (gonadal, retroperitoneal), which tracks with mesenteric fat mass, may be relevant candidates to explore in future studies.

SEXUAL DIMORPHISM IN ADIPOSE TISSUE REGULATION AND FUNCTION

It has been recognized that the development of adipose depots during positive energy balance differs according to sex. Independent of BMI, women typically carry 10% more body fat than their male counterparts (53, 54). Demerath et al. (55) found that women typically have greater total body fat than men. Additionally, compared to men, women have greater SAT in the abdominal and gluteofemoral depots, independent of total body fat (55, 56).

Not only does SAT area and volume differ between sexes, but also the spatial distribution of SAT. Regardless of race, women tend to harbor greater SAT in the lower abdomen, and in general, have lower visceral content than men (55, 56). One explanation for the increase in subcutaneous adiposity in women may be attributed to preferential increases in SSAT rather than in DSAT. The area of the deep subcutaneous depot is inversely associated with fasting insulin levels (14) and, in general, women have lower visceral content than men (55, 56). This may partially contribute to why, on average, pre-menopausal women tend to have healthier metabolic risk profiles compared to men, irrespective of total fat content. Nevertheless, it is acknowledged that additional factors, such as reproductive hormones and differential racial responses, influence both fat quantity and distribution.

As stated previously, women have more SAT than men, while men have more VAT than women (57). Thus, it is not surprising that leptin is a better correlate for body fat in females (58), as SAT secretes more leptin than VAT. By contrast, insulin is a better correlate for body fat in males (58), as insulin is more related to VAT than SAT (57). Because of the aforementioned sex differences in fat deposition, and their relation with various co-morbidities, it is important to consider whether these sex differences similarly exist in rodents, in order to promote their pre-clinical value as a guide for relevant translational research.

To date, the sexual dimorphism seen in humans is less documented in rodents (59, 60), although some inferences have been made. For example, similar to humans, female rats have higher plasma leptin levels compared to male rats (58), independent of body composition differences (61). Consistent with these observations is the brain sensitivity to these hormones, with male rats demonstrating greater central sensitivity to insulin, while females were more responsive to leptin (58).

Similar to humans, female rodents harbor greater fat mass compared to their male counterparts, but remain more insulin sensitive. Macotela et al. (60) showed that isolated adipocytes from subcutaneous fat of female mice were more insulin sensitive than male-derived subcutaneous fat. However, when further examining inter-depot insulin sensitivity in female mice, the periovarian fat pad proved to be more insulin sensitive than the inguinal fat

pad (60), a finding that is at odds with data from humans, where the gluteofemoral depot has proven to be the most sensitive SAT in females (62), and is also more insulin sensitive than the VAT (63).

An additional similarity between the species is spatial patterning of the adipose tissue with changes in sex hormones. Postmenopausal women preferentially redistribute adipose tissue from the gluteofemoral region to the abdomen, which mirrors the accrual of visceral fat observed with aging in men. Likewise, Grove et al. (59) demonstrated that ovariectomized female mice demonstrated a significant increase in adiposity similar to adipose tissue accumulation patterning in males, including an increase in total and visceral fat. However, whether ovariectomizing young rodents truly recapitulates female menopause in humans, which occurs during late-middle age, is debatable, and thus should be interpreted with caution.

Though similarities exist, other notable differences in regards to sex differences have been observed between species. One example is the fat pad composition and location associated with sex hormones (i.e., estrogen and testosterone) in rodents. For instance, the inguinal white adipose tissue (WAT) of female mice contains mammary glands, while the gonadal fat pad is situated near the reproductive tissue. This is not the case in humans, where the mammary glands are located in the breast tissue. Furthermore, unlike their male counterparts, several strains of female mice are relatively resistant to obesity on a high-fat diet (64, 65), but this protection can be removed by ovariectomizing the animal (66, 67). Thus, while fat patterning in adult females seems to be influenced strongly by reproductive hormones, overt protection against weight gain and obesity does not seem to be a shared characteristic between female rodents and humans.

WEIGHT LOSS

Regardless of sex, obese individuals undergoing weight loss preferentially decrease their VAT compared to SAT (68). Indeed, significant volumes of VAT are lost when subjected to caloric restriction (CR) and/or a physical activity intervention (69). Additionally, when SAT is mobilized in obese individuals, there is a greater increase in lipid mobilization in the abdominal region compared to the gluteofemoral region (8, 70, 71), which shows only a minimal change in mobilization (71). This preferential reduction in abdominal adipose tissue is consistent with the observation that adipocytes from the omental and mesenteric depots are more lipolytically active, when compared to adipocytes found in the gluteofemoral region (72–74).

When male rodents are subjected to CR or leptin treatment, an examination of weight loss effects on adipose tissue volume and spatial distribution (8, 75) reveals a preferential reduction in VAT compared with other adipose depots (76), while some have shown that male rodents lose both VAT and SAT (77). Similarly, female mice tend to conserve their SAT by preferentially reducing their visceral fat stores (77). This would be somewhat analogous to human studies, where both men and women reduce their visceral fat stores, with a greater extent occurring in men compared to women (78, 79).

Furthermore, as the majority of people who undergo diet-induced weight reduction regain the lost weight over time, it is important to determine if the same phenomenon is seen in rodents. Male mice exposed to *ad libitum* feeding following CR-induced weight loss demonstrated gains in both visceral and subcutaneous fat stores. However, female mice did not follow this same pattern, and were less capable of regaining visceral fat after weight loss (77). Speculatively, this may be attributed to reproductive hormones or to sex differences in leptin concentrations. Circulating leptin decreases with CR, but increases with *ad libitum* feeding in male mice, presumably due to increases in adiposity and food intake. In female mice, leptin patterns appear to differ from males. For example, while leptin concentrations are similarly reduced with CR in females (6, 80), they remain reduced after refeeding, as compared to *ad libitum*-fed controls (80), while others have shown leptin levels unchanged in response to either CR or refeeding (77). Irrespective of weight loss and body composition, women have higher levels of leptin compared to men (55), but both obese men and women who undergo CR, proportionately decrease leptin levels with the reduction of total fat, as well as visceral and subcutaneous fat (68). Interestingly, in children, when adjusting for body composition and fat deposition, sex has no independent effect on leptin concentrations (81). In addition, adults attempting to lose weight tend to engage in multiple episodes of weight loss and regain (e.g., weight cycling); thus, it is important to examine the effects of this flux in body composition and fat deposition on leptin concentration in adult humans. Interestingly, the association between leptin concentration and weight cycling holds true for females, but not for males (82). Importantly, the fact that both female and male mice show an increase in fat deposition after CR in a manner reminiscent of humans during weight regain suggests that rodents may be an informative model toward elucidating many sexually dimorphic traits related to fluctuations in energy balance and storage (83).

LIPOLYSIS

Lipolysis, which is the active breakdown of triglycerides to FFAs and glycerol, has been widely studied in both rodents and humans. Many striking similarities in the regulation of lipolysis exist among species, including stimulation by catecholamines, growth hormone, testosterone, and cortisol (corticosterone in rodents), as well as inhibition by insulin. Lipolysis can be stimulated in rodents and humans under similar physiological contexts, including fasting, exercise, and stress. While, activation of the sympathetic nervous system and subsequent release of epinephrine, norepinephrine, and cortisol represents a major driver of lipolysis in humans (84) and rodents (85), important differences between rodents and humans exist in how some of these pathways drive lipolysis.

Catecholamine-driven lipolysis, which occurs by activating three different β-adrenergic receptor (AR) subtypes, β1, β2, and β3, provides one important example. ARs are coupled to a Gα subunit, and catecholamine induced activation leads to a cascade of events culminating in lipolysis, including increased cAMP by adenylyl cyclase, activation of protein kinase A, and activation of hormone-sensitive lipase, enabling its translocation to the lipid

droplets to catalyze the hydrolysis of triglycerides (86). Both β1-AR and β2-AR are ubiquitously expressed in rodents and humans, whereas β3-AR expression is more tissue and species specific, with expression confined to WAT in rodents, while only being marginally expressed in human adipocytes (87). Indeed β3-adrenergic agonists induce a lipolytic response in rodents (88), but fail to significantly stimulate lipolysis in human adipocytes, *in vitro* (89) and *in vivo* (90).

In humans, α2-ARs, as compared to β-ARs, are highly expressed in SAT, and have a greater affinity for catecholamines (91), while this G-coupled protein receptor is absent in rodent adipocytes. In humans, α2-AR acts to inhibit lipolysis by decreasing cAMP levels. Indeed catecholamine activation of α2-ARs in non-obese humans has been shown to partially down regulate lipolysis in SAT (92). Further support of α2-AR regulating lipolysis was gleamed from rodent studies whereby the human α2-AR was expressed in transgenic mice on a β3-AR knockout background. Specifically, these animals exhibited a blunted catecholamine-stimulated lipolysis response and an obese phenotype (93). Therefore, it appears a balance between α2-AR and β-AR is necessary for lipolysis regulation, at least in humans (87).

Historically, the major circulating factors regulating lipolysis in human WAT have been appreciated to be catecholamines, growth hormone, cortisol, testosterone, and ghrelin (94). However, more recently, it has been shown that natriuretic peptide [extensively reviewed in Ref. (95)] induces lipolysis *in vitro* and *in vivo* (96), independently of the catecholamine-AR pathway (97), via a cGMP-dependent mechanism (96). However, natriuretic peptide lipolysis is apparently primate specific. Sengenès and colleagues showed that only primate adipocytes (i.e., humans and macaques) showed natriuretic peptide-induced lipolysis, while this effect was absent in rats, mice, hamsters, and other non-rodent mammals.

In summary, important similarities exist in the biology of rodent and human adipose tissue lipolysis, but as we have discussed, many important distinctions have been identified that warrant consideration when utilizing rodent models. Such differences are apparent in the species specificity of β-AR expression on adipocytes, and the distinct roles of α2-ARs and natriuretic peptide in regulating human, but not rodent adipose tissue lipolysis. Likewise, some lipolytic agents active in rodent adipocytes fail to have similar effects in human adipocytes (91). For example, adrenocorticotropic hormone (ACTH) induces lipolysis in mouse (98) and rat adipocytes (99, 100), respectively, while alpha-melanocyte-stimulating hormone (αMSH) was also shown to modulate murine adipocytes (101). However, neither ACTH or αMSH – induce lipolytic activity in human adipocytes (98, 102). Therefore, it is important to balance and account for these important differences against commonalities in the biology of adipose tissue lipolysis among species.

FAT PADS AND FAT DEPOTS AS AN ENDOCRINE ORGAN

Adipose tissue, which is made up of numerous cell types, including pre-adipocytes, adipocytes, T cells, B cells, and stromovascular cells, is now appreciated as an active endocrine organ, capable of secreting numerous humoral factors. Indeed, systemic levels of adipokines, including leptin, interleukin (IL) 1-β, IL-6, and tumor necrosis factor-α (TNF-α), are actively secreted and levels rise with increasing fat mass. On the other hand, adiponectin has been linked to many beneficial effects, but levels correlate negatively with increasing adiposity (103). Because these adipokines have pleiotropic actions, including extensive metabolic effects, it is important to determine if secretion is similar among humans and rodents with respect to depot location.

As mentioned previously, leptin is preferentially secreted in humans from peripheral subcutaneous depots at a higher rate than from VAT (103, 104), indicating that subcutaneous fat is a chief source of leptin production. Likewise, leptin expression was shown to be greater in rat inguinal as compared to epididymal fat pads (105). IL-6 is another important cytokine that is produced in significant amounts from adipose tissue. Mohamed-Ali et al. (106) showed that SAT is capable of producing IL-6, while Fontana et al. (103) demonstrated that IL-6 secretion is greater from VAT than from SAT in obese individuals (103). Furthermore, IL-6 levels are elevated in middle-aged men with visceral adiposity, as compared to lean men, and adjusting for visceral fat accounts for the rise in IL-6 with visceral obesity (107). Other fat-derived factors, such as TNF-α, show similar secretion patterns in VAT and SAT in humans (103), but adipose-derived TNF-α has been suspected to act in more an autocrine/paracrine rather than endocrine manner.

The data are less clear for adiponectin secretion from different human depots. Phillips et al. (108) showed that SAT secretes more adiponectin compared to VAT, while obese individuals demonstrate impaired total adiponectin secretion from SAT, but not from VAT depots (109). Meanwhile, some have reported no significant difference in total adiponectin secretion between subcutaneous and visceral adipocytes (103, 109), while others have shown that cultured visceral adipocytes secrete more adiponectin compared to subcutaneous adipocytes (104). Likewise, adiponectin expression from rat epididymal fat pads was significantly greater than that from inguinal fat (105).

In regard to evidence from rodents, a microarray study in isolated epididymal and inguinal fat pads from rats first showed marked differences in these tissues, including increased expression of resistin, angiotensinogen, adiponectin, and PPARγ in epididymal tissue, while inguinal fat demonstrated greater expression of PAI-1 and leptin. Studies have also assessed the effect of surgically removing the epididymal and perinephric fat pads on circulating levels of adipokines. Pitombo et al. (110) showed selective ablation of these fat pads restored insulin action and normalized TNF-α, IL-6, and adiponectin levels. By contrast, surgical removal of visceral fat pads in a male and female mouse model of intestinal cancer fed a high-fat diet led to sexually dimorphic responses. In males, lipectomy reduced adiponectin levels, but did not alter other adipokines, presumably due in part to a compensatory expansion of the mesenteric fat pad (6). By contrast, females had higher circulating levels of adiponectin than males, and while lipectomy reduced circulating levels, they remained at higher concentrations than observed in control males. Furthermore, no compensatory change was observed in

mesenteric fat pad mass in females, but leptin levels were significantly elevated, suggesting the expansion of subcutaneous fat stores. Unfortunately, the literature does not provide additional information regarding adipokine secretion in rodent models in relation to specific fat pads.

Finally, almost all studies evaluating circulating levels of adipokines are conducted according to clinical trial protocols, which often require the patient to fast overnight prior to blood sampling. However, measuring levels and expression patterns under basal conditions may be misleading as it is now recognized that nutrients can provoke the expression and production of adipokines from adipose tissue. Indeed, most humans (and rodents) spend the majority of their day in the postprandial state, and as a result, the expression and secretion of adipokines from fat depots and fat pads may be severely underestimated. Einstein et al. (111) first showed that when rats were exposed to hyperglycemia and hyperinsulinemia, expression of several peptides in fat, including resistin, adiponectin, leptin, PAI-1, and angiotensinogen, was markedly increased by 2- to 10-fold in epididymal fat pads, but less dramatically in subcutaneous fat. Similar patterns were also observed when animals were challenged with glucosamine (112) or FFA (113), and some of these responses were further exaggerated in aging rats. Similarly, Kishore et al. (114) showed in humans that adipose-derived factors from subcutaneous fat potentiate PAI-1 secretion from macrophages in response to FFAs. Furthermore, this response is similarly exaggerated in macrophages obtained from middle-aged versus younger-adult subjects (115). Mechanistically, these effects have been linked to increased flux through the hexosamine biosynthetic pathway as well as TLR4 receptors. It is also important to keep in mind that these comparisons, while informative, are made on a per milligram tissue or mRNA basis and do not necessarily account for the absolute fat pad size and, hence, overall contribution to whole body levels *in vivo*. Thus, given the inherent size of the visceral pads in relation to the inguinal tissue in rodents, the contribution of visceral fat to these secreted factors, particularly in response to nutrients, could be quite large. However, the relative contribution of visceral fat-derived cytokine release to the overall nutrient response *in vivo*, has not been carefully evaluated. Nevertheless, based on the current literature, it appears that adipokine secretion patterns in humans are predominantly similar to what has been shown in rodent studies.

HORMONAL AND GENETIC FACTORS GOVERNING FAT PAD DEVELOPMENT AND EXPANSION

As mentioned, many uncertainties remain regarding the mechanistic underpinnings responsible for the physiological differences among depots. During weight gain, different depots enlarge via hyperplasia, hypertrophy, or both (116), with new adipocytes generating more readily in some depots compared to others. The inter-depot physiological enlargement differences are likely influenced by both extrinsic and intrinsic factors.

Genetic factors have also been shown to influence the distribution of adipose tissue (117–119). The BMI of an individual is highly heritable and can possibly account for as much as 70% of the variance (117). However, C57BL/6 mice, which are inbred, and theoretically should be identical genetically, demonstrate marked variance in body mass, adiposity, and feeding behavior, even when the mice are fed the same diet type (120), perhaps due to epigenetic effects (121).

Although genetic factors have been implicated in fat pad growth and expansion, the genetic underpinnings controlling these processes are not as well understood. A few investigations have been conducted to elucidate the gene(s) that moderate the distribution of adiposity. Recently, Loh et al. (122) showed LRP5 is involved in fat distribution. Individuals with gain-of-function *LRP5* mutations are characterized with increased lower-body fat accumulation, compared to age-, sex-, and BMI-matched controls. However, more attention has focused recently on adipocytes from different depots, which have shown differential gene expression (105, 119, 123) and proliferative capacity (124, 125). When examining the transcripts that differed around a quarter were found to be developmental regulators (126–128), in particular the homeobox (HOX) superfamily of genes. Investigations have now started to actively examine differential HOX gene expression between depots in order to assist in determining the underpinnings of abdominal versus gluteofemoral adiposity (10, 123, 128, 129). A summary of the HOX gene network in rodent and human adipose tissue is provided in **Table 1**.

In addition to genetic contributors, structural and hormonal regulators have been shown to influence fat distribution. Scherer and colleagues (132) examined extracellular matrix components of adipose tissue, specifically collagen VI, under different metabolic conditions. In the absence of collagen VI, adipocytes were capable of unrestricted expansion, resulting in further lipid storage and a reduction in ectopic fat deposition (132). Interestingly, even with greater fat expansion, under both high-fat diet conditions and on an *ob/ob* background, collagen VI knockouts compared to controls had improved metabolic phenotypes. To determine if a similar relationship between increased collagen VI and metabolic stress existed in humans, Scherer and colleagues investigated an Asian Indian population due to their propensity to be more insulin resistant compared to BMI-matched Caucasians (133). Collagen VI alpha 3 (*col6a3)*, a major adipocyte-derived secretory protein with increased expression during states of metabolic stress in *ob/ob* and *db/db* mice, was compared between Asian Indian and control matched Caucasians. Similar to the rodent models, *col6a3* expression was significantly greater in abdominal and gluteal subcutaneous adipose depots in the investigated Asian Indians (132). Collectively, there is evidence that in terms of collagen VI, there may be similar adipocyte physiology in both rodents and humans that may inhibit expansion of adipose tissue.

Similar to the collagen VI model, overexpression of adiponectin in *ob/ob* mice lead to an increase in adipocyte cell number and, thus, increased adipose tissue mass, specifically SAT (134). However, even with the observed hypertrophy, the unrestricted expansion of adipose tissue associated with elevated adiponectin levels resulted in a major improvement in the overall metabolic phenotype, even in an obese state (134). The authors speculated that the improvement in metabolic parameters was partially attributed to increased PPARγ activity in adipocytes, leading

TABLE 1 | Expression patterns of the HOX gene network in human fat depots and mouse fat pads.

	Human		Mouse	
GENE	**SubQ**	**Visceral**	**SubQ**	**Visceral**
HOXA1[a]				
HOXA3[a]		X		
HOXA4[a]	X	X		
HOXA5[a]	X			
HOXA10[a]				
HOXA11[a]				
HOXB1[a]	X			
HOXB2[a]	X			
HOXB5[a]		X		
HOXB8[a]	X			
HOXB13[a]		X		
HOXC4[a]				
HOXC6[a]	X	X		
HOXC11[a]	X			
HOXA10[b]	X			
HOXC6[b]	X			
HOXA2[c]		X		
HOXA3[c]		X		
HOXA4		X		
HOXA5[c]		X		
HOXA9[c]		X		
HOXA10[c]	X			
HOXB7[c]		X		
HOXB8[c]		X		
HOXC8[c]		X		
HOXC13[c]	X			
IRX2[c]				
HOXA5[d]		X		
HOXC8[d]		X		X
HOXC9	X[f]	X[d]		X[d]
Nr2f1	X		X	
Gpc4[d]		X		X
Thbd[d]	X			X
shox2[d]		X		X
Tbx15[d]	X		X	
En1[d]		X	X	
Sfrp2[d]	X		X	
EN1[e]		X	X	
HOXA2[e]	X			
HOXA4		X		
HOXA5[e]		X		
HOXA9[e]		X		
HOXA10[e]	X			
HOXC6[e]	X			
HOXC8[e]	X			
HOXC10[e,f]	X			

Data were obtained from Cantille et al. (130)[a], Vohl et al. (123)[b], Karastergiou et al. (128)[c], Gesta et al. (10)[d], Tchkonia et al. (126)[e], and Brune et al. (131)[f].

to a redistribution of lipids from ectopic sites to SAT. Likewise, increased adiponectin levels may also play a role in human obesity. Bouatia-Naji and colleagues (135) investigated common single nucleotide polymorphisms (SNPs) in the ACDC adiponectin encoding gene in French Caucasians and concluded that hyper-adiponectinemia may be associated with severe obesity.

Adiponectin levels are also increased in growth hormone receptor knockout (GHRKO) mice (136), likely because adiponectin is negatively regulated by GH (137). Similar to the above transgenic models, GHRKO mice are characterized by greater relative amounts of body fat in both males and females, with a disproportionate amount of fat deposition in SAT (138). In spite of harboring greater amounts of adipose tissue, GHRKO mice are metabolically healthy, an effect attributed to their increased adiponectin levels (139). Interestingly, Laron dwarfism, which is a human syndrome characterized by defective GH signaling, is characterized by obesity, in spite of a small stature, but these individuals are protected against type 2 diabetes (140) and have elevated adiponectin levels (141). Thus, humans and rodents may have a similar response to reduced GH/IGF-1 signaling and associated increased levels of adiponectin on fat accretion and patterning as well as glucose metabolism.

Glucocorticoids also influence adipose tissue differentiation, function, and distribution. High levels of glucocorticoids partially contribute to visceral obesity in conjunction with diabetes, hyperlipidemia, and hypertension (142, 143). One mechanism for the production of glucocorticoids is through the enzyme 11-β-hydroxysteroid dehydrogenase type 1 (11β HSD-1). Interestingly, Masuzaki and colleagues (144) created a mouse model overexpressing 11β HSD-1 selectively in adipose tissue that reflected 11β HSD-1 levels observed in adipose tissue from obese humans, who are reported to have increased 11β HSD-1 activity. They observed that a modest increase in 11β HSD-1 activity was sufficient to induce hyperphagia and increased VAT accumulation. Furthermore, the increased VAT accumulation was attributed to significantly greater glucocorticoid receptor alpha isoform expression in mesenteric compared to SAT. In addition, increased corticosterone release into the portal vein of rodents may contribute to the observed rise in portal FFA levels, which parallels the increase in FFA levels in humans characterized by high circulating cortisol and metabolic syndrome (145–148). Collectively, it appears that structural and hormonal factors associated with increases in adipose tissue are largely similar in both humans and rodents. Therefore, rodents appear to be a viable model of human adipose tissue regulation by many common hormonal factors.

CONCLUSION

It is understood that not all obese individuals are at the same risk for developing metabolic perturbations and that body fat distribution is an important determinant of obesity-related complications. Individuals with increased upper-body adiposity are disproportionately burdened by obesity-related diseases, compared to lower-body obese individuals. Thus, it is paramount that studies continue to elucidate the pathways linking various adipose pads and depots in relation to health and disease, as well as the mechanistic underpinnings dictating how body fat is distributed in order to answer fundamental questions. Rodents are commonly used to model features of human metabolism and obesity, yet it is unclear to what extent rodent fat pads are a suitable model of human fat depots. Here, we have highlighted examples of both shared and divergent traits among rodent fat pads and human fat depots. Given some of the stark differences in adipose tissue location and function among species, we urge

careful consideration in experimental design and interpretation when attempting to draw definitive parallels between rodent fat pads and human fat depots.

AUTHOR CONTRIBUTIONS

DC, DW, DH, and TN participated in the writing of this review paper. DC and TN conceived the original idea.

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REVIEW
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Mechanisms of Comorbidities Associated With the Metabolic Syndrome: Insights from the *JCR:LA-cp* Corpulent Rat Strain

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Obesity and its metabolic complications have emerged as the epidemic of the new millennia. The use of obese rodent models continues to be a productive component of efforts to understand the concomitant metabolic complications of this disease. In 1978, the *JCR:LA-cp* rat model was developed with an autosomal recessive corpulent (*cp*) trait resulting from a premature stop codon in the extracellular domain of the leptin receptor. Rats that are heterozygous for the *cp* trait are lean-prone, while those that are homozygous (*cp/cp*) spontaneously display the pathophysiology of obesity as well as a metabolic syndrome (MetS)-like phenotype. Over the years, there have been formidable scientific contributions that have originated from this rat model, much of which has been reviewed extensively up to 2008. The premise of these earlier studies focused on characterizing the pathophysiology of MetS-like phenotype that was spontaneously apparent in this model. The purpose of this review is to highlight areas of recent advancement made possible by this model including; emerging appreciation of the "thrifty gene" hypothesis in the context of obesity, the concept of how chronic inflammation may drive obesogenesis, the impact of acute forms of inflammation to the brain and periphery during chronic obesity, the role of dysfunctional insulin metabolism on lipid metabolism and vascular damage, and the mechanistic basis for altered vascular function as well as novel parallels between the human condition and the female *JCR:LA-cp* rat as a model for polycystic ovary disease (PCOS).

Keywords: obesity, thrifty genotype, metabolic syndrome, immune function, inflammation, cardiovascular diseases, pcos, JCR rat

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INTRODUCTION

Obesity and the Clinical Problem for Our Generation

Obesity and its metabolic complications have emerged as the epidemic of the new millennia. Fueled by a caloric-dense (and nutrient-poor) food chain that is readily available in most developed countries, the current generation reflects the expression of a human phenotype plagued by the "obesogenic" environment. We have become so efficient at creating foods that target brain reward

pathways to stimulate addictiveness and palatability, that we now suffer from the health consequences of not being able to alter our behavior away from this food environment. So too, we are just as guiltily of bestowing this new found talent on the next generation of young adults.

For many scientists, this problem has become a life-long commitment to understand how overnutrition and nutrient-poor choices on the backdrop of the existing food chain could result in such a devastating change to our metabolic prognosis in such a short period of time. Animal models have been a prominent tool in this endeavor. One such model, the *JCR:LA-cp* rat (re-derived in Edmonton, AB, Canada in 1978 by Dr. James C. Russell), has received significant attention. For the last three decades, this model has appeared in the literature every year, often in numerous forms spanning facets of nutrition, endocrinology, metabolic syndrome (MetS), obesity, lipid metabolism, vascular myocardial pathophysiology, and pharmacology (**Figure 1**).

The *JCR:LA-cp* Rat

In 1978, at the fifth backcross to the LA/N strain [including elements of the corpulent (*cp*) trait], initial breeding stock was sent from National Institutes of Health (NIH) by Dr. Carl T. Hanson to the laboratory of one of the authors (James C. Russell) at the University of Alberta. These rats were the founders of a colony that retained ~3% of the genome derived from the obese spontaneously hypertensive rat (or SHROB). Unlike other NIH colonies, at the time, that were maintained inbred and congenic, the *JCR:LA-cp* strain has been maintained as a closed outbred colony at the University of Alberta to retain the unknown genetic elements leading to early development of cardiovascular disease (CVD).

The formidable contributions that have originated from this rat model have been well documented and reviewed extensively up to 2008 (1–3). The premise of the early studies focused on characterizing the pathophysiology of MetS-like phenotype that was spontaneously apparent in this model. Highlights of the work from this period included useful descriptions of the biochemical profile, careful reports of glucose and insulin metabolism, endothelial function and its impairment, and unique observations of early vascular atherogenesis.

Contributions of the *JCR:LA-cp* Rat and Recent Advancements

The purpose of this review is to celebrate the major contributions that this rat model has served over many years and to highlight recent advancements. In a unique way, this model has been

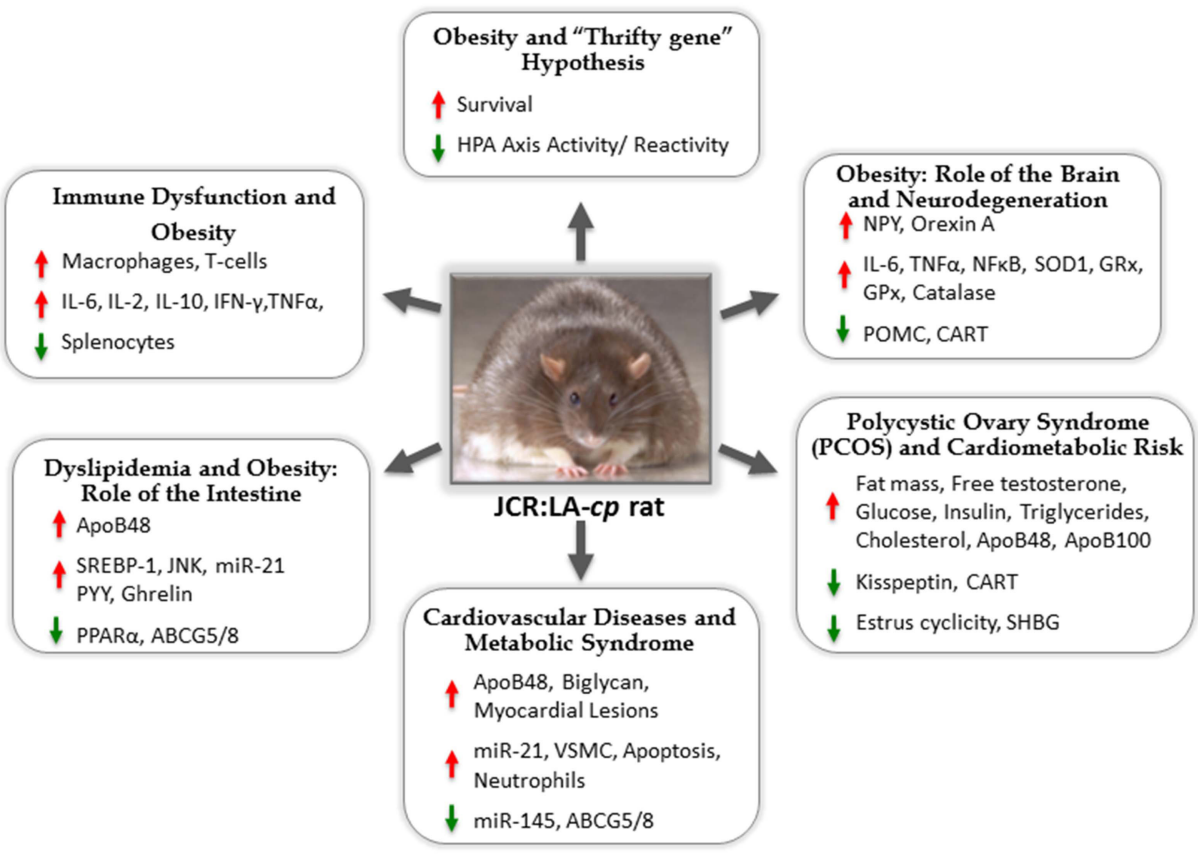

FIGURE 1 | The *JCR:LA-cp* rat: useful animal model for studies of metabolic syndrome and mechanisms of associated comorbidities.

carefully studied, yet successfully pervasive into the broad areas of research that underpin our understanding of obesity and the MetS.

We wish to highlight areas of advancement made possible by this model including; emerging appreciation of the "thrifty gene" hypothesis in the context of obesity, the concept of how chronic inflammation may drive obesogenesis, the impact of acute forms of inflammation to the brain and periphery during chronic obesity, the role of dysfunctional insulin metabolism on lipid metabolism and vascular damage, the mechanistic basis for altered vascular function as well as novel parallels between the human condition and the female *JCR:LA-cp* rat as a model for polycystic ovary disease (PCOS).

OBESITY: TESTING THE "THRIFTY GENE" HYPOTHESIS OF ADAPTATION TO DIETARY ENERGY INTAKE

The prevalence of obesity is continuing to increase at an alarming rate in both economically advanced Western societies and developing countries in economic–nutrition transition (4). Obesity is linked to adverse short-term and long-term health outcomes, including increased risk of CVD and insulin resistance leading to overt type 2 diabetes (T2D) (5, 6). Genetic differences between individuals explain a major proportion of the within-population variation in body mass index (7). However, genetic susceptibility alone may not result in obesity without other environmental influences (8). A positive energy balance beyond meeting energy requirements results in excess dietary energy intake being preferentially stored as triglycerides (TG) in adipose tissue, resulting in an increase in body weight and fat mass. Obesity can be viewed as a result of physiological dysfunction or perturbation in normal feedback mechanisms relating to feeding behavior and energy balance. The adaptive response or "thrifty gene" theory of obesity explains how genes favoring the efficient use of energy store in periods of feast followed by famine result in obesity in the food-rich or "obesogenic" environment of prosperous societies (9, 10). This "thrifty gene" hypothesis has been the dominant theory of the developmental origins of obesity and related chronic disease. The "thrifty gene" hypothesis provides a potential explanation for the dramatic rate of T2D and obesity prevalence in Pima Indian population of Arizona relative to the genetically similar Pima Indians in Mexico (11). However, there are only limited experimental studies that have tested the adaptive-survival value of an obese-prone genotype. Our ongoing research is based on the *JCR:LA-cp* rodent model that expresses the corpulent (*cp*) autosomal recessive trait (*cp*), a nonsense Tyr763Stop mutation in the Ob-R gene, resulting in a total absence of functional leptin receptors (12). Animals that are homozygous for the *cp* trait (*cp*/*cp*) are obese-prone, hyperphagic, and develop features of the MetS, similar to the metabolic aberrations observed in the clinical setting (13). Animals that are heterozygous for the *cp* trait (+/*cp*) or wild type (+/+) are lean-prone, have normal food intake, and do not develop the MetS. To verify the adaptive response hypothesis that an obese-prone genotype confers a survival advantage when challenged with dietary energy restriction and food-seeking behavior induced activity, juvenile (35–40 days) male *JCR:LA-cp* rats (obese-prone and lean-prone) were exposed to 1.5 h/day of feeding and 22.5 h/day of voluntary wheel running (14). We have shown that this experimental design leads to increased wheel running or food-seeking behavior and self-starvation (14). Initial body weights were similar in both groups of animals; however, the obese-prone animals survived twice as long (8.2 ± 1.1 vs. 3.5 ± 0.2 days) and ran similarly, compared to their lean-prone counterparts (14). A follow-up study showed that prior conditioning with dietary energy restriction for 1 week provided a survival advantage to the obese-prone *JCR:LA-cp* phenotype (15). Dietary energy restriction primed the animals to regulate energy homeostasis pathways following exposure to continued energy restriction and food-seeking-related activity (15). The obese- and lean-prone phenotypes have metabolic differences, which we have shown are associated with alterations in feeding-related neuropeptide gene expression in the arcuate nucleus of the hypothalamus including NPY, Orexin A, CART, and POMC. In addition, hypothalmic pro-inflammatory cytokines and oxidative stress markers IL-6, Tnf-α, and NF-kβ, superoxide dismutase-1 (SOD1), glutathione reductase (GRx), glutathione peroxidase (GPx), and catalase mRNA are elevated in the obese-prone phenotype (16). Interestingly, when the obese- and lean-prone animals are exposed to the same degree of dietary energy restriction, these hypothalamic inflammatory and oxidative stress markers were improved in the obese-prone phenotype, while they are exacerbated in the lean-prone phenotype (16). These findings demonstrate that, in juvenile *JCR:LA-cp* rodents, the metabolic and neural adaptation to energy restriction are dependent on the phenotype, and this can confer a survival advantage (16). Overall, our research findings suggest that obesity is a developmental outcome dependent on the interrelationship of an obese-prone genotype and feeding conditions/"obesogenic" environment. The *JCR:LA-cp* rodent offers a unique opportunity to study the underlying mechanisms of the behavioral and physiological pathways involved in the development of obesity and the possible conference of evolutionary survival traits.

CHARACTERIZATION OF THE ALTERED IMMUNE FUNCTION IN OBESITY USING THE *JCR:LA-cp* RAT MODEL: MODULATION WITH AGE AND DIETARY FAT

Obesity is often recognized as a chronic inflammatory state with altered immune responses, including T cell dysfunction (17–19). The *JCR:LA-cp* rat model of the MetS shares altered immune function, similar to that observed clinically in obese individuals (17–19). The obese-prone rodent model has chronic low-grade systemic inflammation (20–22) and, recently, has been shown to have inflammation in the brain (23, 24). Our group has also demonstrated significant alterations in the acquired immune system of the obese-prone phenotype (19, 25–27). The altered T cell function in the *JCR:LA-cp* rat appears to be dependent on the age of the animal and length of time of exposure to a high-fat diet. When a high-fat diet is introduced at 8 weeks of age and fed for 3 weeks, the obese-prone rats have fewer splenocytes (an indicator of the peripheral lymphopenia) compared to their lean-prone counterparts (27). The obese-prone animals also have a

higher proportion of macrophages, T-cells (primarily CD8+ and CD4+) and CD4+CD25+ (T regulatory cells) (27). This inflammatory T cell response would contribute to the overall chronic inflammation observed in these animals. When splenocytes from the *JCR:LA-cp* rats are stimulated *ex vivo* with Concanavalin A (ConA, polyclonal T cell mitogen), they produce the same amount of IL-2 (proliferative cytokine) but have increased levels of the inflammatory cytokine IL-6 (27). In mesenteric lymph nodes (MLN), which are part of the gut-associated lymphoid tissue and visceral adipose tissue, the obese-prone rats have a lower proportion of T-cells (both CD4+ and CD8+ cells) and fewer mature T cells (CD3+CD90+), with a higher proportion of CD4+CD25+ (T regulatory cells) (27). Despite lower T cell numbers, *ex vivo*, MLN from obese-prone rats produce higher amounts of IL-2, IL-10, IFN-γ, and Tnf-α after stimulation with Con A (27), suggestive of a highly reactive pro-inflammatory state in the intestine. When a high-fat diet was fed for 3 weeks to older *JCR:LA-cp* animals (aged 14 weeks), splenocytes had a lower inflammatory response in IL-6 and IL-10 production in the unstimulated condition. In stimulated conditions [with ConA, lipopolysaccharide (LPS), or pokeweed], a lower production of IFN-γ and decreased IL-1β (LPS) and IL-10 (Con A) was observed (25). This suggests, similar to the human condition, that the animals have some degree of immunosuppression and which would make them susceptible to infection. In splenocytes, there was a greater number of CD4+ cells and fewer CD4+CD25+ cells, B cells, and macrophages in obese-prone animals. In MLN, a greater production of IL-4 following Con A stimulation, and IL-1β, IL-10, and IFN-γ after stimulation with LPS was observed (26), again suggestive of intestinal inflammation or increased exposure to antigens (perhaps microbiome) from the intestine. In addition, there was a higher proportion of CD3+CD8+ cells and a lower proportion of CD4+CD25+ in the obese-prone animals (26), consistent with chronic inflammation. When a high-fat diet is introduced at 3 weeks of age and fed for 13 weeks, the obese-prone rats have a lower proportion of CD3+ cells (both CD4+ and CD8+), a higher proportion of CD4+CD25+. In addition, these animals have naive or less mature CD4+ cells and activated B cells in the spleen compared to lean-prone control animals (20), which would leave the animals more prone to infection. With the exception of IL-2 production, which did not differ between obese- and lean-prone animals, the *ex vivo* production of cytokines after stimulation was exacerbated in this group compared to previous studies in older animals or fed for shorter periods of time. After Con A stimulation, splenocytes from obese-prone animals produced lower levels of IL-1β (43%), IL-4 (53%), Tnf-γ (31%), and IFN-γ (31%), and twofold greater IL-6 compared to lean-prone animals (20).

In summary, the obese-prone *JCR:LA-cp* rat displays an immune dysfunction that is similar to that reported in obese individuals (17–19, 28), which suggest lymphopenia and immunosuppression and abnormal inflammatory responses to immune challenges. Differences in immune cell type and function are observed in both the intestinal lymphoid tissue and peripheral immune system suggesting that there is both systemic and intestinal involvement. Interestingly, we have learned that the altered immune profile of the *JCR:LA-cp* rat is also modified by the age and length of feeding period of a high-fat diet. Additionally, we have also demonstrated that immune dysfunction in this model can be improved by dietary supplementation with long-chain polyunsaturated fatty acids (PUFA), including trans-vaccenic acid (20, 27) and fish oil containing docosahexanoic and eicosapentanoic acid (25, 26).

INFLAMMATION, STROKE, AND INFECTION IN OBESITY

We and others have contributed to the fact that high levels of circulating biomarkers of inflammation are present in the obese state (29). We know that obese individuals are more likely to develop other chronic inflammatory conditions, including certain forms of cancer, diabetes, cardiovascular, and cerebrovascular disease. Mechanisms by which obesity alters immune and inflammatory responses and how these changes contribute to the development of other diseases are still not clear.

Stroke is a major cause of morbidity and mortality, and the incidence of an ischemic episode has been associated with peripheral and central immune dysfunction (30). Chronic systemic inflammatory conditions, such as infections, atherosclerosis, diabetes, and obesity are associated with increased risk of stroke, suggesting that these conditions and associated inflammation may contribute to the development of stroke (30). In this respect, aged *JCR:LA-cp* rats have been shown to have increased brain inflammation using PET imaging (23). Furthermore, patients with multiple risk factors for stroke, in the absence of any brain pathology, have also been shown to have increased brain inflammation, and this may be associated with increased risk for cerebral ischemia (23). Despite research efforts, there has been a lack of translation of these findings from bench to bed side in stroke patients. One reason for this may be the failure to consider clinical comorbidities in experimental models of stroke. IL-1 is altered peripherally in obesity and is also an important mediator of ischemic brain injury (31). IL-1 receptor antagonist (IL-1Ra) is protective against ischemic brain damage in healthy animals (31). After cerebral ischemia, aged *JCR:LA-cp* rats showed increased blood brain–barrier disruption and brain inflammation compared to their lean-prone counterparts, and this was reduced following systemic administration of IL-1Ra. IL-1Ra treatment was also shown to significantly reduce the infarct volume (measured by MRI) in obese-prone animals, further supporting IL-1Ra as a lead candidate for the treatment of ischemic stroke (24).

Bacterial infections have been proposed to contribute to stroke development and may worsen ischemic event outcomes (32). In this setting, a sustained pulmonary infection (*Streptococcus pneumoniae* isolate) induced in *JCR:LA-cp* was used to investigate the effect of infection on vascular and inflammatory responses prior to and after cerebral ischemia (33). The results showed that the pneumonia infection augmented atherosclerosis and exacerbated ischemic brain injury *via* IL-1 and platelet-mediated systemic inflammation pathways (33). Targeting these mechanisms could be therapeutically useful to prevent infection-induced thrombo-inflammatory responses that may predispose individuals to ischemic vascular events and adversely affect stroke outcomes.

DEVELOPMENT OF DYSLIPIDEMIA DURING OBESITY AND HYPERINSULINEMIA: OVERPRODUCTION OF LIPIDS BY THE INTESTINE AND BIOACTIVE TRANS-FATTY ACIDS

Intestinal Contribution to Hypercholesterolemia and Diabetic Dyslipidemia

One of the most significant advances that have occurred over the last 5 years with respect to intestinal lipid metabolism is the understanding of how the gut contributes to whole body cholesterol homeostasis (34). In particular, we now appreciate how these intestinal-based mechanisms become dysfunctional under conditions of obesity and insulin resistance. Using the *JCR:LA-cp* rat model, we have shown that circulating hyperinsulinemia (as is the case during conditions of MetS or prediabetes) can lead to increased absorption of both lipid and sterol from intestine that can, in turn, exacerbate dyslipidemia and CVD risk. Specifically, we have demonstrated that insulin resistance results in a chronic overproduction and excessive secretion of intestinal-derived apoB48-containing chylomicron particles. This finding is consistent with studies from the *JCR:LA-cp* rat (35), the hamster (36), and, more recently, humans (37).

Mechanisms responsible for the overproduction of intestinal-derived lipoproteins have been reviewed elsewhere (38) but can include stimulation by excess free fatty acids, manipulation of both GLP-1 and GLP-2, increased circulating Tnf-alpha, increased stability of apoB48, and the downregulation of enterocytic insulin receptor (IR) substrate-1. Work from our own group utilizing the *JCR:LA-cp* rat has also confirmed that this is likely multifactorial resulting from; suppression of enterocytic Pparα (39), increased SREBP-1 (40), decreased ABCG5/G8 (39), and increased phosphorylation of JNK (41). New emerging data also suggest that increased enterocytic Tnfα may also contribute to increased apoB *per se* (42). In addition, Parnell and Reimer have elegantly shown that there is an increase in satiety hormone (proglucagon, PYY, and ghrelin) mRNA expression in the *JCR:LA-cp* rat gut that may potentially contribute to intestinal lipid overproduction (43).

Importantly, these observations provide valuable mechanistic explanation for why those with the MetS and diabetes typically have a unique lipoprotein phenotype. Left untreated, those with obesity and early insulin resistance develop atherogenic dyslipidemia that renders a significantly increased CVD risk profile. Other efforts from our laboratory have used the *JCR:LA-cp* rat to better understand potential nutritional means to curb these metabolic impairments.

Impact of Dietary Long-Chain Fatty Acids, PUFA, and Ruminant Trans-Fatty Acids to Lipoprotein Metabolism in the *JCR:LA-cp* Rat

Pharmaceutical compounds that activate peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ (e.g., fibrates and thiazolidinedione, respectively) have been successful as potent lipid-lowering and insulin-sensitizing therapies for CVD and diabetes-related dyslipidemia (44, 45). Activation of PPAR-α can effectively reduce plasma hypertriglyceridemia possibly *via* modulating fatty acid oxidation and energy homeostasis pathways. Upregulation of PPAR-γ activity has also been shown to normalize insulin sensitivity, improve lipid metabolism, and the clearance of lipoproteins, as well as restore vascular contractility and endothelial function (46). We became interested in the fact that a number of natural PPAR-α ligands exist among which include long chain and PUFA (e.g., oleic acid, arachadonic acid, eicosapentaenoic acid, and docosahexaenoic acid) (47, 48). More specifically, conjugated linoleic acid (CLA) is another naturally occurring agonist of the PPAR-α pathway, which has been proposed to be a primary mechanism *via* which CLA elicits pleiotropic effects (49, 50). It has now been recognized that aside from being converted to *c*9,*t*11-CLA *in vivo*, its precursor vaccenic acid (VA) may also have independent bioactivity in regulating lipid metabolism. However, few studies have attempted to explore the metabolic pathways that are potentially modulated by VA. Using the obese *JCR:LA-cp* rat, we have offered a number of contributions to better understand the potential role of VA in activating and thereby regulating lipid metabolism (51, 52). Most recently, our group has obtained data indicating that VA is a potent PPAR-α and PPAR-γ agonist and strongly binds to the ligand-binding domain of both receptors. In accordance, we found a substantial elevation in energy expenditure during both light and dark cycle in VA-supplemented obese *JCR:LA-cp* rats (40, 53). The observed change in energy metabolism may partially be accredited to enhanced citrate synthase activity (an indicator of fatty acid oxidation) in liver and adipose tissue of VA/CLA-fed obese rats. Our evidence implies an active involvement of these nuclear receptors in regulating lipid metabolism, which is shared by other bioactive fatty acids such as DHA and *c*9,*t*11-CLA. These findings have been important in understanding the role of ruminant derived trans-fatty acids as a class, and how they differ from pro-inflammatory industrial produced trans-fatty acids through the partial hydrogenation of vegetable oils (54).

In addition to PUFA and ruminant trans-fatty acids, prebiotic fibres have also been proposed to promote weight loss and lower plasma lipids; yet, the mechanisms are not fully understood. By using the *JCR:LA-cp* rat, Parnell and Reimer have demonstrated that a 10% dietary blend of prebiotic fibres (inulin and oligofructose) can lower total cholesterol concentrations. The dietary blend is thought stimulate cholesterol excretion in the form of bile as well as reduce hepatic steatosis through a FAS independent mechanism (55).

ARTERIOGENESIS IS MEDIATED BY microRNA IN THE CARDIOVASCULATURE AND IS ASSOCIATED WITH INTESTINAL LYMPHATIC LIPOPROTEINS IN THE METABOLIC SYNDROME

Coronary collateral growth (arteriogenesis) is an important adaptive process in transient, repetitive coronary artery occlusion, and myocardial ischemia, which occurs in stable *angina*

pectoris. Well-developed collateral networks are associated with lower incidence and severity of myocardial infarction (56). In contrast to angiogenesis, which is characterized by new vessel (capillary tube) formation, arteriogenesis is defined by remodeling and enlargement of small arterioles (with very low blood flow) to connect with larger conducting arteries. This process critically depends on both normal endothelial (57, 58) and vascular smooth muscle cell (VSMC) function (59). Arteriogenesis has shown to be severely impaired in animal models exhibiting endothelial dysfunction, including the Zucker obese pre-diabetic (ZOF) rat (60) and the MetS obese-prone *JCR:LA-cp* rat (59, 61, 62). Human patients with impaired endothelial function and the MetS also exhibit impaired arteriogenesis (63–65).

We have recently shown that microRNA (miR)-145 and miR-21 are important regulators of arteriogenesis. miR-145 levels are reduced in the *JCR:LA-cp* obese-prone rat compared to the lean-prone metabolically normal animal, and when miR-145 levels are upregulated in the obese-prone animal, the result is complete restoration of coronary collateral growth (59). This effect was mediated by conversion of the aberrant synthetic VSMC phenotype to the normal contractile VSMC phenotype in the obese-prone model (59). miR-21 levels were markedly increased in the heart of obese-prone animals, shown to be positively correlated with VSMC proliferation (66) and decreased apoptosis of neutrophils (66). Conversely, downregulation of miR-21 to levels found in lean-prone animals resulted in significant collateral growth recovery in obese-prone animals, associated with a decrease in VSMC proliferation (66); concomitant with a restoration of apoptosis of neutrophils (67). miR-21 is well accepted as a major pro-survival and pro-proliferative miR. Moreover, elevated miR-21 levels were shown to be positively correlated with: increased expression of pro-proliferative markers (G1/S and G2/M cyclins and cyclin-dependent kinases); low expression of "tumor suppressors" (p21, p27, and pRb); high expression of anti-apoptotic Bcl-2/Bcl-2 dimers; low expression of pro-apoptotic Bcl-2/Bax dimers (caspases 9 and 3); and decreased cytochrome *c* release from the mitochondria. Collectively, this study established that miR-21 can regulate neutrophil apoptosis and is a required component for successful collateral enlargement.

A Role for the Lymphatic Expression of microRNA in Lipid Metabolism?

Interestingly, miR-21 expression was also significantly increased in jejunal enterocytes isolated from obese-prone *JCR:LA-cp* rat. The concentration of miR-21 in these animals was also increased in intestinal lymph and in the high-density lipoprotein (HDL) fraction isolated from the lymph (66). miR-21 levels in enterocytes were examined because, in the obese-prone animal, the intestine has overproduction of lipids associated with increased secretion of chylomicrons (CM), and HDL is also found in the lymphatics isolated from the intestine (68). This intestinal secretion of lipids contributes significantly to the lipid–lipoprotein content of the lymphatics (68). Our results further indicated that 95% of miRs in intestinal lymph was associated with the HDL fraction, rather than the CM fraction (68). HDL has also been identified as a major transporter of miRs in the circulation (69, 70). More recently, the lymphatic system was revealed to be critical for the metabolic turnover of HDL and the reverse cholesterol transport system (71). These results suggest that altered enterocyte lipid and lipoprotein metabolism and/or HDL-dependent lymphatic cholesterol transport may be inter-related to the elevated cardiac miR-21 levels observed in the obese-prone *JCR:LA-cp* model.

EARLY INTIMAL ATHEROGENESIS, ARTERIAL LIPID RETENTION, AND NOVEL THERAPEUTICAL TARGETS

Impact of Remnant Dyslipidemia to Atherosclerotic Vascular Disease during Obesity and Insulin Resistance

One of the major complications of obesity and T2D is the dramatic increased risk for CVD [specifically, atherosclerotic vascular disease (ASVD)], the reasons for which are multifactorial. We have been very interested in understanding the role that chronic metabolic disease has in exacerbating dyslipidemia and how this mechanistically translates into greater lipid deposition within the arterial wall. Atherogenic cholesterol-dense lipoproteins (for example, low-density lipoprotein, LDL-C) are thought to permeate both intact and/or damaged arterial endothelium, become entrapped within the sub-endothelial space, and accumulate, resulting in inflammation and atheroma (72, 73). Although the literature documents a significant epidemiological and/or genetic (i.e., GWAS) association between raised circulating fasting LDL-C and ASVD risk, a large proportion of subjects diagnosed with CVD are either normolipidemic (normal levels of LDL) or have substantial residual risk (74–76). These data suggest that clinically, atheromata-associated cholesterol is derived from alternate sources, including non-fasting remnant lipid fractions as originally proposed by Zilversmit (77). Indeed, the International Atherosclerosis Society has recognized non-fasting measurements of remnant cholesterol as a major target in their "Global Recommendations for the Management of Dyslipidemia" (78).

Interestingly, a series of recent publications authored by the Copenhagen Heart Study group have provided evidence for a major shift in the paradigm of atherogenesis (and potentially its therapy), suggesting that remnant (non-fasting) cholesterol is a major causative factor for ischemic heart disease (IHD) (79, 80).

Using the *JCR:LA-cp* rat, we have also collected a substantial array of preclinical data showing that remnant lipoproteins carry substantially more cholesterol (due to their size and enrichment) compared to other atherogenic fractions. Using well-established arterial perfusion methodology, we have conducted comparative dual labeling lipoprotein experiments. *Cy5*-labeled remnants were isolated, purified, and perfused simultaneously with *Cy3*-labeled LDL (designed to expose equivalent LDL-derived apoB100 and remnant-derived apoB48). We observed a significantly greater number of LDL particles delivered to the vessel ($4.5 \pm 1 \times 10^{-9}$ μg/μm^2 tissue) as compared to remnants ($0.48 \pm 0.15 \times 10^{-9}$ μg/μm^2 tissue) (81). However, after extensive washout (a further 60 min) with lipoprotein-free buffer (i.e., representing residual *retention*), we observed significantly fewer (55% decrease) LDL particles remaining in the tissue

($2.9 \pm 0.42 \times 10^{-9}$ μg/μm^2 "retention" vs. $4.5 \pm 1 \times 10^{-9}$ μg/μm^2 "delivery," $p < 0.05$). Additional studies have gone on to show that there is an increased deposition of remnant lipoproteins in arteries from *JCR:LA-cp* rats as demonstrated by arterial perfusion of equivalent numbers of particles. Mechanistically, we have proposed that this phenomenon may be due to (a) dyslipoproteinemia and/or (b) perturbations in the vessel wall. Crossover perfusion experiments have revealed that increased retention of the *number of remnant lipoproteins* during insulin resistance was due to differences in arterial vasculature and independent of other factors effecting particle dysfunction *per se*.

Impact of Obesity and Insulin Resistance on the Extracellular Matrix and Arterial Remodeling

Our group has also shown that intestinal-derived remnant lipoproteins can colocalize with arterial biglycan in an insulin-deficient model of type I diabetes *ex vivo* (82). Subsequently, we have demonstrated, in the *JCR:LA-cp* rat, that in the prediabetic milieu, aortic biglycan protein core content increases significantly with age and correlates linearly with increasing hyperinsulinemia. We know that the expression of biglycan protein core has been shown to be increased with fatty acids (83, 84), angiotensin II (85), and transforming growth factor-β (TGF-β) (86, 87). Consistent with this, obese *JCR:LA-cp* rats have been shown to have elevated concentrations of TGF-β (88) and non-esterified free fatty acids (51).

Arterial Retention of Remnant Lipoproteins and Associated Cholesterol Deposition in Response to Ezetimibe and Simvastatin

Ezetimibe (EZ) is a pharmaceutical compound that selectively reduces intestinal cholesterol absorption by inhibiting the Niemann-pick C1-like 1 (NPC1L1) transporter (89) while Simvastatin (SV) is a HMG-CoA reductase inhibitor. Using the arterial perfusion approach in the *JCR:LA-cp* rat, we have shown that EZ treatment can ameliorate the deposition of arterial remnants and associated cholesterol *ex vivo* (90). It is also intriguing, that the addition of SV to EZ appeared to have an additional benefit reducing arterial cholesterol deposition, suggesting a synergism of independent modes of action.

Combination of Ezetimibe with Simvastatin Improves Fasting and Postprandial Lipids

A study by Bozzetto et al. reported that the combination of EZ with SV in T2D subjects can beneficially impact both fasting and postprandial triglyceride-rich lipoproteins (91). They found that the addition of EZ to SV reduced the number of circulating CM particles (apoB48) in the postprandial state, while also lowering both fasting and postprandial chylomicron cholesterol (88). Postprandial data from studies in *JCR:LA-cp* rats are consistent with benefits of either EZ + SV therapy on both remnant particle metabolism and corresponding cholesterol (81).

Ischemic myocardial lesions constitute a critical end point in CVD. Previous studies using the *JCR:LA-cp* rat strain have demonstrated a correlation between the frequency of myocardial lesions with hyperinsulinemia (92). Hearts isolated from *JCR:LA-cp* rats treated with either EZ (−84%) or EZ + SV (−84%) have displayed a significant reduction in the frequency of early stage 2 myocardial lesions or very recent ischemic lesions undergoing scavenging and repair, characteristic for this strain at this age.

ESTABLISHING *JCR:LA-cp* RODENT RAT AS A MODEL OF SPONTANEOUS LEFT VENTRICULAR HEART DYSFUNCTION

The *JCR:LA-cp* rat has previously been identified as a model that also includes pathological complications of endothelial dysfunction and myocardial ischemia, in addition to other dysfunctional complications consistent with the MetS (41, 92, 93). We have recently demonstrated that *JCR:LA-cp* rats exhibit significant cardiac dysfunction and present as a useful animal model of spontaneous LV dysfunction (94). *JCR:LA-cp* rats were subjected to Doppler echocardiography analysis (*Vevo 770 Micro-Imaging system*). 2-D parasternal long- and short-axis images of the left ventricle (LV) were obtained using a 25-MHz linear-array transducer and doppler probe in anatomical M-mode at the level of papillary muscles at a sweep speed of 150 mm/s. We observed that *JCR:LA-cp* rats exhibited distinct signs of cardiac dysfunction, and that hearts exhibited a marked increase (~40%) in LV mass vs. their lean counterparts. Echocardiographical analysis also revealed that hearts from *JCR:LA-cp* rats had an increased early (MV-E) filling velocity (~40%) and reduced late (MV-A) filling (~15%) velocity compared to age-matched lean rats. We further revealed that *JCR:LA-cp* rats had a restrictive filling pattern, shown by a significantly shortened isovolume relaxation time (IVRT) (~30% decrease). Finally, we found that *JCR:LA-cp* rats exhibited progressive worsening of diastolic filling properties, with a 1.6-fold increase in the ratio of early to late filling velocity (E/A).

THE FEMALE *JCR:LA-cp* RAT AS A MODEL OF POLYCYSTIC OVARY SYNDROME AND CARDIOMETABOLIC RISK

The *JCR:LA-cp* Rat as a Spontaneous Model of PCOS

Polycystic ovary syndrome (PCOS) has become an increasing public health concern given its association with menstrual dysfunction, infertility, MetS, T2D, and CVD risk (95, 96). The syndrome afflicts 5–18% of women in their reproductive years, in adolescents to premenopausal women. PCOS is diagnosed by the presence of clinical or biochemical hyperandrogenemia, menstrual irregularity, and/or polycystic ovaries (97). The incidence of PCOS is twofold to threefold greater in overweight-obese adolescents and women and is coassociated with features of the MetS including obesity, impaired insulin sensitivity, and dyslipidemia predisposing women to increased risk of prematurely developing T2D and CVD (96, 98, 99). Animal models of PCOS have been used to further understand the role of androgens, in particular

testosterone, on the development of the metabolic aberrations in this condition, and the features of these models have been previously reviewed (100, 101). These models have primarily used testosterone to induce PCOS; however, the *JCR:LA-cp* rodent model is the only model to spontaneously develop PCOS in conditions of the MetS (102). The significance of the *JCR:LA-cp* rat in this context is the similarity to the human condition in which the development of the PCOS phenotype is preceded by increased adiposity and insulin resistance. Interestingly, we have known, for some time, that female homozygous *JCR:LA-cp* rats are infertile but have only recently begun to appreciate the metabolic impactions. In humans, females that carry a predisposition for insulin resistance due to family history often see a clinical presentation that antagonizes endocrine–reproductive dysfunction, a feature that is also observed in female *JCR:LA-cp* rats (96, 103, 104).

Cardiometabolic Risk, Dyslipidemia, and the Hypothalamic–Pituitary–Gonadal Axis

The pathogenesis of PCOS is linked to altered hypothalamic–pituitary–gonadal axis function and perturbed insulin and testosterone metabolism (105, 106). One of the major areas of research in the PCOS-prone *JCR:LA-cp* rodent model is understanding the distinct mechanisms of androgens and insulin in the cardiometabolic manifestations, particularly dyslipidemia and CVD risk (102, 107, 108). Dyslipidemia occurs in greater than 70% of PCOS patients and is positively correlated with increasing quartile of plasma hyperandrogenemia (97, 99). We have characterized the dyslipidemic profile of the PCOS-prone *JCR:LA-cp* rodent model, which has markedly elevated fasting and non-fasting plasma TG, total cholesterol (TC), apoB48, and apoB100 (markers of intestinal CM and hepatic very low-density lipoprotein and low-density lipoproteins, respectively) compared to control animals (35, 104). PCOS-prone animals have twofold the intestinal triglyceride, cholesterol, and apoB48 secretion in the fasted state compared to their lean-prone control counterparts, and this is associated with increased mRNA expression of SREBP-2, LDLR, and apoB (102, 107). When given dietary lipid, this elevated CM lipoprotein particle (apoB48) and lipid (cholesterol and TG) secretion is further exacerbated (107). We have further shown, in this model, that plasma testosterone and insulin are positively correlated with fasting and non-fasting plasma TG and apoB48, consistent with the role of these lipogenic mediators in the development of dyslipidemia in PCOS, insulin resistance, and obesity (106, 107, 109).

Intervention with flutamide, an androgen receptor (AR) inhibitor, has confirmed that testosterone action *via* the AR mediates apoB-hyperlipoproteinemia and hypertriglyceridemia, and this appears to be independent of effects on insulin (110). Fasting plasma apoB100, apoB48, and TG concentrations were lowered by 25–50% in animals treated with flutamide. Flutamide–metformin combination treatment similarly lowered these parameters; however, metformin treatment alone had no effect on fasting plasma lipids, indicating a predominant effect of the AR inhibitor to mediate lowering of plasma lipids. Additionally, the intestinal secretion of TG, cholesterol, and the cholesterol/apoB48 and TG/apoB48 (a marker of lipid per CM particle secreted from the intestine) were markedly reduced following flutamide treatment. Hepatic and intestinal lipogenic gene expression showed that flutamide may lower hepatic SREBP-1, LDLR, and HMGCR in PCOS-prone animals (110). Interestingly, PCOS-prone animals have reduced IR, MAPK1, AKT2, and PTPN1 mRNA expression in the intestine, but not the liver. However, flutamide and metformin treatment appeared to favor hepatic upregulation of the IR mRNA, as well as MAPK1 and protein kinase B (AKT2); however, in the intestine, MAPK1 was downregulated, and no effect on AKT2 mRNA expression was observed in PCOS-prone animals. Overall, these findings indicate that lipogenic and insulin signaling gene expression is altered in PCOS-prone animals compared to lean-prone controls. Effects of AR inhibition and insulin-sensitizing treatments appear to modify these pathways in association with improvements in plasma and intestinal secretion of lipids (110).

Energy Restriction and Exercise Intervention in the Female *JCR:LA-cp* Rat

In the PCOS-prone model, we have shown that energy restriction and voluntary exercise intervention (4 h/day) at an early life stage can significantly attenuate reproductive and cardiometabolic aberrations (111). The combination of diet and exercise was shown to lower total body weight gain and body fat mass by 30% in PCOS-prone animals. Consistent with our ongoing studies on dietary energy restriction and food-seeking-induced exercise (112, 113), we have also found that energy restriction independently induces food-seeking behavior related activity in the PCOS-prone animal, favoring an increase in energy expenditure. In terms of cardiometabolic risk, the combination of energy restriction and exercise decreased fasting plasma TG and apoB48 in PCOS-prone animals. In addition, the combination of exercise and dietary energy restriction increased serum hormone-binding globulin and free androgen index, and normalized mRNA expression of hypothalamic CART and Kisspeptin. Collectively, these findings were associated with improvements in follicular morphology and estrus cyclicity. In similar exercise conditions, we have shown that the addition of metformin–flutamide treatment lowers total body weight and body fat-pad weight and tends to lower fasting plasma lipids. Interestingly, a combination of both metformin and flutamide treatment, in addition to exercise further reduces free Testosterone and improves estrus cyclicity compared to exercise alone (113).

The findings of this work have revealed voluntary exercise has modest effects on cardiometabolic risk factors, and the inclusion of medications that specifically target insulin resistance and dyslipidemia are required to modulate these risk factors in this obese PCOS-prone model. These results have also highlighted the necessity for early intervention with combinations of lifestyle and/or dietary pharmaceutical medication to modulate the hypothalamic–pituitary–ovary axis.

IMPACT OF *JCR:LA-cp* RAT MODEL TO TRANSLATIONAL OUTCOMES FOR OBESITY

One measure of impact for the appropriateness of animal models to research is the usefulness of preclinical outcomes for

clinical translation. Perhaps the most successful application of the *JCR:LA-cp* rat model has been its suitability to study the progression of metabolic disease from initial stages of overnutrition (without the need for dedicated high caloric diets), resulting in the development of insulin resistance through to the phenotypic complications of hyperinsulinemia and hallmark conditions of the MetS. Many of the advances discussed in this review have made an impact to a better understanding of the human clinical condition. One such example is the revelation of how the intestine integrates and coordinates whole body lipid metabolism more prominently than previously appreciated (114). The discovery that the intestine will contribute to dyslipidemia through unregulated overproduction of lipids has been confirmed clinically in those with insulin resistance and T2D (115, 116). This in turn has provided a new platform for modes of action of different classes of pharmaceutical compounds; including intestinal cholesterol transporter inhibition and incretion blockade. At the same time, the understanding of remnant cholesterol metabolism and how this relates to early conditions of childhood obesity has also come to the fore in the context of potential subclinical risk of CVD (117, 118).

The vision for this research sector will be to continue to strive for ways to aid the younger generation to become more aware of the comorbidities of obesity in childhood, and how they will progress into adulthood. In order to achieve this, we will have to target methodologies that identify metabolic risk of obesity in the younger generation that can have usefulness in the clinic and beyond.

AUTHOR CONTRIBUTIONS

Introduction to the clinical problem: SP, WP, and JR. Obesity: testing the "thrifty gene" hypothesis of adaptation to dietary energy intake: AD and WP. Characterization of the altered immune function in obesity using the *JCR:LA-cp* rat model: modulation with age and dietary fat: CF and MR. Inflammation, stroke, and infection in obesity: JP and SA. Development of dyslipidemia during obesity and hyperinsulinemia: overproduction of lipids by the intestine and bioactive trans-fatty acids: RM, MJ-S, and SP. Arteriogenesis is mediated by microRNA in the cardiovasculature and is associated with intestinal lymphatic lipoproteins in the metabolic syndrome: RH and PR. Early intimal atherogenesis, arterial lipid retention, and novel therapeutical targets: RM and SP. Establishing *JCR:LA-cp* rodent rat as a model of spontaneous left ventricular heart dysfunction: FB, SK, and SP. The female *JCR:LA-cp* rat as a model of polycystic ovary syndrome (PCOS) and cardiometabolic risk: DV and AD. Impact of *JCR:LA-cp* rat model to translational outcomes for obesity: SP.

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REVIEW
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Obesity in the Otsuka Long Evans Tokushima Fatty Rat: Mechanisms and Discoveries

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Understanding the neural systems underlying the controls of energy balance has been greatly advanced by identifying the deficits and underlying mechanisms in rodent obesity models. The current review focuses on the Otsuka Long Evans Tokushima Fatty (OLETF) rat obesity model. Since its recognition in the 1990s, significant progress has been made in identifying the causes and consequences of obesity in this model. Fundamental is a deficit in the cholecystokinin (CCK)-1 receptor gene resulting in the absence of CCK-1 receptors in both the gastrointestinal track and the brain. OLETF rats have a deficit in their ability to limit the size of meals and in contrast to CCK-1 receptor knockout mice, do not compensate for this increase in the size of their spontaneous meals, resulting in hyperphagia. Prior to becoming obese and in response to pair feeding, OLETF rats have increased expression of neuropeptide Y (NPY) in the compact region of the dorsomedial hypothalamus (DMH), and this overexpression contributes to their overall hyperphagia. Study of the OLETF rats has revealed important differences in the organization of the DMH in rats and mice and elucidated previously unappreciated roles for DMH NPY in energy balance and glucose homeostasis.

Keywords: cholecystokinin, neuropeptide Y, CCK-1 receptor, dorsomedial hypothalamic nucleus, food intake, obesity

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INTRODUCTION

Rodent obesity models have been critical to our understanding of the neural systems involved in the controls of food intake and body weight. Dissection of the genetics underlying the obesity of ob/ob and db/db mice led not only to the discovery of leptin but also contributed greatly to the understanding of multiple hypothalamic peptide systems involved in energy balance. Another example of a genetic model that has increased our understanding of the neural systems involved in energy balance is the Otsuka Long Evans Tokushima Fatty (OLETF) rat. This rat obesity model was derived from a spontaneous obesity in an outbred colony of Long Evans rats. OLETF and a control Long Evans Tokushima Otsuka (LETO) lines were then developed by selective breeding. OLETF rats were initially studied primarily as a model of late onset type 2 diabetes, as older OLETF rats were not only obese but also hyperglycemic and insulin resistant (1).

Characterization of overall pancreatic function in OLETF rats demonstrated the absence of a pancreatic amylase response to administration of the brain gut peptide cholecystokinin (CCK) (2). Further studies revealed that OLETF rats had a >6 kbp deletion in the gene for the CCK-1 receptor that spanned the first and second exons and resulted in the absence of expression of a functional CCK-1 receptor (3). Thus, the OLETF rat is a CCK-1 receptor knockout model.

CHOLECYSTOKININ AND CHOLECYSTOKININ RECEPTORS

Cholecystokinin is a gut/brain peptide that plays a variety of roles. Gut CCK is released from I cells in the upper intestine in response to the intraluminal presence of nutrients and plays a variety of roles in the overall digestive function. Exogenously administered and endogenously released CCK slow gastric emptying, modulate intestinal motility and stimulate gall bladder and pancreatic secretions. CCK also plays a role in the control of food intake by contributing to meal termination. Exogenously administered CCK reduces food intake and does so by reducing meal size (4–6). A role for endogenously released CCK in the controls of meal size is demonstrated by the ability of CCK receptor antagonists to increase food intake by prolonging eating – increasing meal duration and size (7, 8). The primary mechanism of action of CCK in the inhibition of food intake is paracrine, acting on local vagal afferent terminals in close apposition to the intestinal I cells (9, 10). CCK receptors are expressed in vagal afferent cell bodies in the nodose ganglion and transported to abdominal vagal endings (11). CCK both directly activates vagal afferent fibers and also sensitizes vagal fibers to signals, transmitting information about gastric and intestinal luminal volume (12, 13).

In the brain, CCK acts as neurotransmitter/neuromodulator. CCK-producing neurons are widely distributed in the brain, and CCK neurons have been reported to be the most ubiquitous of all peptidergic neurons. Cell bodies are found throughout all layers of the cerebral cortex and are widely distributed throughout olfactory and limbic systems and in multiple hypothalamic nuclei. In the midbrain, CCK cell bodies are found in the substantia nigra, the ventral tegmental area, and the raphe nucleus (14, 15), and CCK modulates both dopaminergic and serotonergic function (16).

There are two CCK receptor subtypes (17, 18). These were initially identified based on their relative affinity for various CCK fragments and analogs. CCK-1 receptors require the sulfated tyrosine, and these were originally characterized in rat and guinea pig pancreas. CCK-1 receptors exist in both low capacity, high affinity and high capacity, low affinity states. CCK-2 receptors have high affinity for unsulfated CCK and various CCK fragments and were initially characterized in brain. Both receptors are members of the G-coupled super family of receptors. As well as found in pancreas and gall bladder, CCK-1 receptors are expressed in the nodose ganglion (and transported in vagal afferent fibers) and in a number of specific brain sites, including the dorsomedial hypothalamus (DMH) (17). There are important species-specific differences in the expression patterns of CCK-1 and CCK-2 receptors, including the expression of CCK-2 and not CCK-1 receptors in human pancreas. However, the expression of CCK-1 receptors in vagal afferent neurons and in specific brain sites appears to be similar in rat and man (not in the mouse as will be discussed later).

The satiety actions of CCK depend on the interactions with CCK-1 receptors. Sulfated CCK-8 or sulfated longer forms (i.e., CCK-33, CCK-58) inhibit food intake in a dose-related fashion, while unsulfated CCK or shorter CCK fragments do not (4, 19). Furthermore, specific CCK-1 antagonist administration increases food intake while CCK-2 antagonists do not (7). This pharmacological specificity has been demonstrated across multiple species.

CHARACTERIZATION OF THE HYPERPHAGIA IN OLETF RATS

The initial discovery that OLETF rats had a deletion in the gene for the CCK-1 receptor led to experiments examining whether CCK could inhibit their food intake. OLETF rats lacking functional CCK-1 receptors were shown to be insensitive to the feeding inhibitory actions of exogenously administered CCK. Characterization of their daily food intake revealed that OLETF rats ate meals that were about twice as large as those of LETO controls and, in response to this increase in the size of their meals, they at fewer meals. However, the decrease in meal frequency was not sufficient to normalize their food intake resulting in a chronic hyperphagia or overconsumption (**Figure 1**) (20). Evidence for the hyperphagia is evident even prior to weaning. In independent ingestion tests, in which rat pups are consuming milk off the floor of a test chamber, OLETF pups as young as 2 days of age consume significantly more sweetened milk than age-matched LETO controls (21). In tests assessing nursing behavior, OLETF pups also gain more weight during a suckling bout indicative of increased intake (22).

The food intake of OLETF rats is also characterized by higher preferences for high fat (23), sucrose and other sweet tastes (24). This can be demonstrated in both real feeding and sham feeding paradigms, implicating taste mechanisms in the preferences.

Pair feeding experiments in which the daily intake of OLETF rats was limited to that of paired LETO control rats revealed that the obesity in the OLETF rats was completely attributable to their hyperphagia. Pair feeding completely normalized their rates of body weight gain (**Figure 2**) as well as the size of their fat mass and their glucose regulation (20). Thus, the OLETF rat is an obesity model of disordered food intake.

CHARACTERIZATION OF HYPOTHALAMIC FUNCTION IN OLETF RATS

The lack of compensation for the increase in meal size in OLETF rats requires explanation. Chronic administration of CCK at meal onset results in chronic decreases in meal size but an increase in meal frequency such that overall food intake is not affected (5). These data suggest a role for CCK in meal termination, but not in overall food intake. Knockout of CCK-1 receptors in the mouse produces results that are consistent with this interpretation. CCK-1 knockout mice have increased meal size, but the decrease in meal frequency compensates for this so that CCK-1 KO mice have normal body weight (25, 26). Why does the absence of CCK-1 receptors result in obesity in the OLETF rat, but not in a mouse KO?

Part of the answer comes from the examination of hypothalamic signaling in the OLETF rat. While mRNA expression for arcuate POMC and neuropeptide Y (NPY) was appropriate in obese or lean pair-fed OLETF rats [elevated POMC and reduced NPY in the obese state and normal expression in lean

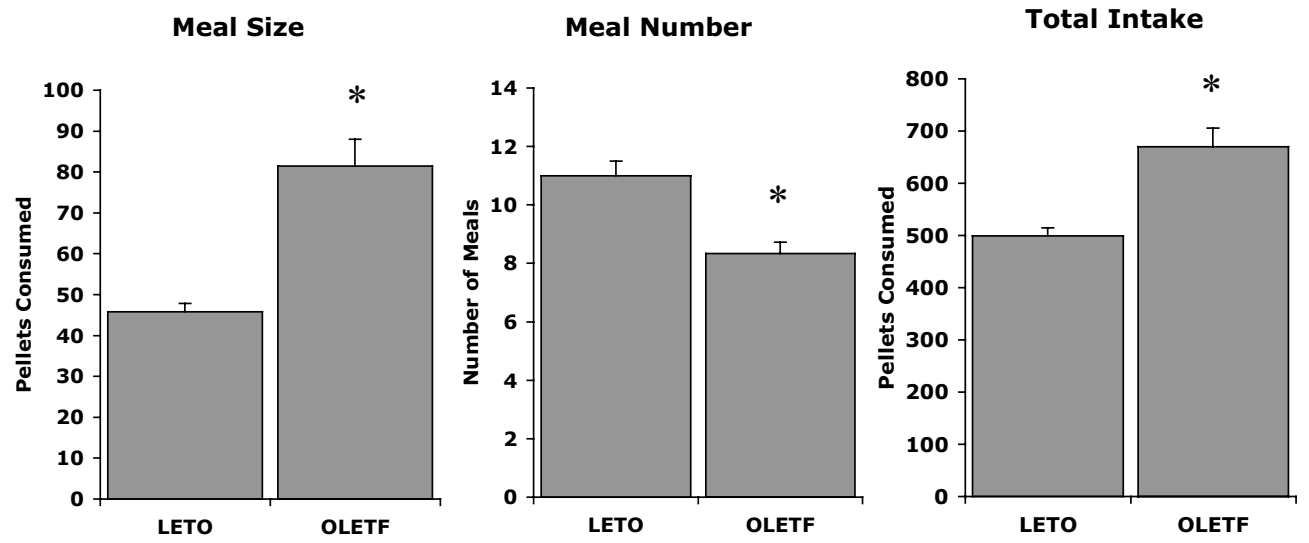

FIGURE 1 | **Meal patterns in LETO and OLETF rats (20).**

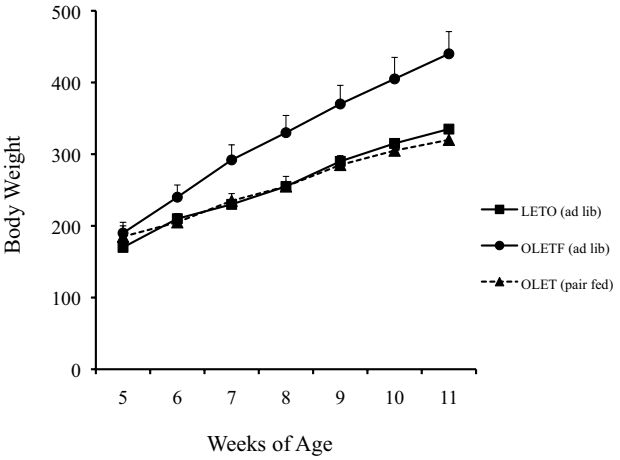

FIGURE 2 | **Pair feeding normalized body weight in growing OLETF rats (20).**

OLETF rats pair-fed to amounts consumed by control LETO rats (27)], NPY expression in the compact subregion of the DMH was significantly elevated in pair-fed OLETF rats and normalized in ad lib-fed rats (27). These data suggested the possibility that elevations in DMH NPY might be driving the hyperphagia on OLETF rats. Analyses of NPY expression levels in juvenile OLETF rats prior to obesity were consistent with such an explanation. Five-week-old pre-obese OLETF rats had greatly elevated DMH NPY expression. Importantly, the same neurons expressing NPY in the DMH also expressed CCK-1 receptors representing one of the populations of brain CCK-1 receptors identified in the original autoradiography studies (27). Furthermore, direct injection of CCK into the DMH both reduces food intake and downregulates NPY mRNA expression without affecting ARC NPY expression, suggesting a role for CCK in modulating DMH NPY (28). In the absence of CCK-1 receptors, DMH NPY is upregulated.

An examination of NPY expression in the mouse revealed that although NPY expression was evident in the ARC, its expression was not evident in the compact region of the DMH. NPY receptors are evident in the dorsal and ventral medial subregions of the DMH, and NPY expression increases in response to exposure to a high-fat diet. A role for these in the lasting hyperphagia that occurs in diet-induced obesity has been suggested (29). In contrast to rats, mouse DMH does not contain CCK-1 receptors as neither binding activity nor mRNA expression, for CCK-1 receptors are detected in the DMH (23). These data have led to the hypothesis that the obesity in the OLETF rats results from a combination of disordered satiety signaling due to the lack of vagal afferent CCK-1 receptors and an upregulation of DMH NPY that prevents complete compensation for the increased meal size. The CCK-1 receptor knockout mouse has similar deficits in the control of meal size, but in the absence of altered DMH signaling, appropriately compensates for chronically consuming larger meals.

This hypothesis was directly tested in the rat using viral-mediated knockdown of DHM NPY in OLETF rats (30). Forty percent knockdown of DMH NPY mRNA expression in response to bilateral administration of an AAV-expressing short hairpin RNA (AAVshNPY) significantly reduced the food intake and weight gain trajectory of OLETF rats. The alteration in food intake was expressed as a partial reduction in the size of consumed meals such that the meal size deficit in OLETF rats with DMH injections of AAVshNPY was similar to the meal size deficits in CCK-1 receptor KO mice. DMH NPY overexpression in control rats had the opposite effect. Overexpressing DMH NPY resulted in increased food intake, especially on a high-fat diet, and significantly elevated weight gain (30).

EXERCISE AND OLETF OBESITY

The study of the OLETF rat has led to a number of important insights about interactions between exercise and food intake and the role of DMH signaling in energy balance. Providing OLETF rats access to a running results in a normalization of their body weight (31) and prevention if hyperinsulinemia (32). This is not simply due to the increased energy expenditure as their daily food intake is also greatly reduced by running wheel access, and their meal patterns are normalized (33). The long-term effects of running wheel activity depend upon the timing of access. In adult OLETF rats, running wheel access normalizes food intake and body weight, but at the cessation of access, food intake greatly increases, and body weight returns to levels of comparably aged OLETF rats that did not have access to running wheels. Thus, the effects of exercise are temporary and only evident during the time of running wheel access. In contrast, providing access to running wheels for a 6-week period beginning at 8 weeks of age had long-lasting effects on both food intake and body weight in OLETF rats. Although food intake and body weight increased somewhat when access to the running wheels was stopped, OLETF rats did not regain weight to levels of control OLETF rats without running wheel access (33). Effects of exercise on other rodent obesity phenotypes have now been demonstrated as well (34–36). The age-dependent aspect of the effects of exercise may depend on epigenetic effects in pathways undergoing maturation and thus increasing the possibility of lasting effects when the exposure is at a younger age.

NOVEL ACTIONS OF DMH NPY

The observation of altered DMH NPY signaling in the OLETF rat and how DMH knockdown rescues the obese phenotype has led to extensive studies of the roles of DMH NPY in various aspects of energy balance. As mentioned above, overexpression of DMH NPY leads to increased food intake and body weight, especially when rats are presented with a high-fat diet (30).

These data led to a more careful examination of the consequences of altered DMH NPY signaling. Knockdown of NPY in the DMH in normal weight Sprague-Dawley rats has been demonstrated to reduce the size of fat depots and ameliorate high-fat diet-induced hyperphagia and obesity. Furthermore, DMH NPY knockdown resulted in the development of brown adipocytes in inguinal white adipose tissue that was characterized by increased uncoupling protein 1 expression. DMH NPY knockdown also increased energy expenditure and enhanced the thermogenic response to a cold environment. This knockdown also enhanced insulin sensitivity. These data identified novel roles for DMH NPY in modulating adipose tissue, thermogenesis, insulin sensitivity, and energy expenditure (37).

Further work has revealed a novel modulator of DMH NPY signaling. Gene expression profiling of the DMH in response to exercise revealed an elevation of the expression of transthyretin (TTR), best known as a blood and cerebrospinal fluid transporter of thyroxine and retinol. To test the hypothesis that TTR may play a role in modulating signaling-related energy balance in the DMH, we examined the effects of brain TTR on food intake and body weight and have further determined hypothalamic signaling that may underlie its feeding effect in rats. We found that icv administration of TTR in normal growing rats decreased food intake and body weight. Furthermore, TTR administration decreased NPY levels in the DMH. Chronic icv infusion of TTR in OLETF rats reversed their hyperphagia and obesity. Overall, these studies examining factors that might modulate DMH NPY demonstrated a novel anorectic action of central TTR in the control of energy balance (38), providing a potential novel target for obesity treatment.

SUMMARY

Work with the OLETF rat has not only been focused on identifying the mechanisms underlying its obesity but also served as a vehicle for uncovering multiple novel mechanisms involved in the overall controls of energy balance.

AUTHOR CONTRIBUTIONS

Both authors have approved the final version of the manuscript.

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REVIEW
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Modeling Diet-Induced Obesity with Obesity-Prone Rats: Implications for Studies in Females

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Obesity is a worldwide epidemic, and the comorbidities associated with obesity are numerous. Over the last two decades, we and others have employed an outbred rat model to study the development and persistence of obesity, as well as the metabolic complications that accompany excess weight. In this review, we summarize the strengths and limitations of this model and how it has been applied to further our understanding of human physiology in the context of weight loss and weight regain. We also discuss how the approach has been adapted over time for studies in females and female-specific physiological conditions, such as menopause and breast cancer. As excess weight and the accompanying metabolic complications have become common place in our society, we expect that this model will continue to provide a valuable translational tool to establish physiologically relevant connections to the basic science studies of obesity and body weight regulation.

Keywords: obesity resistance, weight regain, menopause, breast cancer, exercise, adipose, high-fat diet, sex differences

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INTRODUCTION

Worldwide obesity rates have more than doubled since the 1980s, and in 2014 more than 39% of adults were overweight, and 13% were obese (1). This translates to an estimated 1.9 billion adults who are overweight, of whom more than 600 million are obese. Further, 41 million children under the age of 5 are also overweight or obese, making obesity a worldwide epidemic. While obesity was once only a problem for high-income countries, there are now more deaths worldwide attributed to overweight and obesity than underweight. Obesity negativity affects virtually every system of the body and increases the risk for cardiovascular disease, diabetes, osteoarthritis, and many cancers. While childhood obesity increases the risk of obesity in adulthood, it also poses immediate risks to children, such as breathing difficulties, increased risk of fractures, and psychological effects. Further, markers of metabolic disease that were once considered limited to adults are now appearing in children with obesity, including hypertension, insulin resistance, and early markers of cardiovascular disease. Clearly our current efforts to stop this growing epidemic are not working, and there is an urgent need to understand the etiology and to develop new strategies, interventions, and therapies to prevent and/or treat this disease.

It is generally accepted that the problem of obesity reflects the merger of environmental, biological, and psychosocial pressures (2). Underlying the biological component of this issue is the genetic and epigenetic foundation that establishes the systems controlling energy homeostasis

and body weight regulation. The heterogeneity of this foundation imparts variability in how these systems function and respond to external challenges and stressors. Even in the current obesogenic environment, not all individuals become obese. While some individuals have a predisposition to accumulate excess weight, others have a predisposition to remain lean. For the vast majority of the population, the variability in the predisposition for obesity is not linked to a single mutation or epigenetic insult. Rather, numerous genes and epigenetic events are involved in generating a polygenic predisposition that favors leanness, obesity, or some level of adiposity between the two extremes, when faced with obesogenic environmental pressures.

One environmental pressure believed to be promoting obesity is the availability of energy dense diets. In animal models, this has been shown to increase energy intake (3–6) and eventually leads to the development of obesity and insulin resistance (7–10). However, the susceptibility to obesity in response to this challenge is highly dependent upon the strain of the animal (11–18). The purpose of this review is to summarize the development, nuances, and applications of an approach that models this polygenic susceptibility to the development of obesity in response to the common environmental pressure of a freely available energy dense diet. This experimental paradigm has been, and continues to be, a valuable tool to translate the wealth of knowledge from basic science studies of energy balance to physiological relevant aspects of the human condition.

DIET-INDUCED OBESITY (DIO) FROM OBESITY-PRONE ANIMALS

DIO in the Context of Alternative Models of Obesity

Numerous types of animal models have been employed to study obesity. Certain mutations, as with leptin or the leptin receptor, give rise to monogenic forms of obesity with extreme phenotypes (19). These models have proven very valuable in elucidating the function of specific factors involved in energy homeostasis and body weight regulation. It has become clear, however, that the biological contribution to obesity in humans involves numerous factors in a number of tissues that coordinately favor the accumulation and maintenance of an excess amount of adiposity. Translating these observations to the human condition requires applying this information in a more physiologically relevant context.

As an alternative, different strains of animals with a known genetic disposition for leanness or obesity have been studied. The Osborne–Mendel (OM) and S5B/P1 model is one such example. In studies comparing several strains of rats on a high fat (HF) diet, OM rats were identified as prone to the development of obesity, while S5B/P1 rats were resistant (8). Using such models has helped researchers identify genetic differences that contribute to the predisposition to develop obesity. However, the heterogeneity between these two strains also imposes unwanted variability in comparative studies that may be irrelevant or confounding.

To overcome the potential confound of strain differences, we and others have utilized an approach that yields a range of adiposity phenotypes within the same strain. Because a diet high in fat has been linked to obesity in both humans and animal models, this has become the most common challenge used to select the extremes of adiposity phenotypes. The strengths of this approach are that the polygenic predisposition for obesity reflects a differential response to the diet, while minimizing the extraneous differences between strains. This general approach has been used with both inbred and outbred strains of rats and mice to select those that become lean or obese with the same dietary challenge. Selection from inbred strains, which are considered to be isogenic, provides a more modest range of phenotypes that presupposes less genetic variability and emphasizes epigenetic variability in driving the phenotype. In contrast, selection from outbred strains presupposes greater variability in both genetic and epigenetic differences, which likely better reflects the susceptibility to obesity in humans. The diet-induced obesity (DIO)/diet-resistant (DR) model of obesity that we emphasize in the discussion of this review are derived from outbred rats (15, 18).

Over the past two decades, some confusion has emerged in the terminology used in describing outbred models. We and others have often referred to them as obesity-prone (OP) and obesity-resistant (OR), rather than DIO and DR. The two different naming schemes have often been used interchangeably, while others have inferred some distinction between them. Here, we propose that both classifications have value and are inherently related, in that the polygenic predisposition (OP/OR) ultimately leads to the respective phenotype (DIO/DR), when challenged with the obesogenic selection diet. To minimize the confusion in future studies, we propose the following definitions that distinguish the use of these terms:

- OP: a person or animal that has a predisposition to become obese when challenged with an obesogenic environmental pressure;
- OR: a person or animal that has a predisposition to remain lean when challenged with the same obesogenic environment;
- DIO: the OP person or animal that has become obese in response to the obesogenic diet; and
- DR: the OR person or animal that has remained lean in response to the obesogenic diet.

Evolution of the Selection Process – A Historical Perspective

Selection and study of OP/OR phenotypes began over 25 years ago in both Sprague-Dawley and Wistar outbred rats (18, 20). In general, a relatively large group of outbred rats was challenged with a HF diet for a defined period of time, and the cohort was stratified by the amount of weight gained. For the early studies from Hill et al. (21, 22), Wistar rats were obtained from Harlan Laboratories (Madison, WI, USA; now Envigo). The low fat (LF) acclimation diet consisted of a 20% LF diet (20% kcal from fat; 20% protein; 60% carbohydrate) for 2 weeks. A 60% HF diet (60% kcal from fat; 20% protein; 20% carbohydrate) was then fed for 4–5 weeks, and the top and bottom 25% of weight

gainers were identified as either OP or OR. At the end of the 10-week study, body composition and fat pad weights confirmed the DIO and DR phenotypes. Subsequent modifications to the protocol selected the top and bottom tertiles (rather than quartiles) to represent the extremes of weight gain (23). During a similar time period, Levin published similar findings using a different strain and a different obesity-inducing diet. In these studies, male Sprague-Dawley rats were fed a diet consisting of chow, corn oil, and sweetened condensed milk [~31% kcal fat and 45–53% kcal carbohydrate (primarily sucrose)]. Like the Wistar rats, only about half of the rats consuming this diet for 3–5 months gained excess weight compared to chow-fed controls (20, 24–26).

Over the years, there have been a number of different modifications to the design of the selection diet that are particularly relevant to note. Levin's group has consistently employed a diet that contains sucrose, in addition to being high in fat. In some studies, Levin's group also used the highly palatable liquid diet Ensure (27, 28). In contrast, Hill's research team moved to LF and HF diets with 12 and 45% kcal from fat, with the protein component held constant at 20%. Further, the sucrose component of the original HF diet was replaced with starch (29). Finally, in conjunction with a move to the University of Colorado in 1997, Charles River Laboratories (Wilmington, MA, USA) became the source for Wistar rats, while maintaining the 12 and 45% fat for the LF and HF diets, respectively (14).

In 1994, Pagliassotti et al. modified the selection protocol to demonstrate that weight gain during the first week on the HF diet was highly predictive of weight gain over the four subsequent weeks. Specifically, they reported a strong correlation between weight gain after 1 and 5 weeks of HF feeding ($r = 0.87$; $n = 200$) (12). Based on this finding, most subsequent studies have relied on this shorter duration of HF diet screening for identifying OP and OR rats. In collaboration with Hill's group, a study out of the Leibowitz lab (30) used a similar model in Sprague-Dawley rats to identify measures in prepubertal animals that were predictive of adult adiposity. Similar to studies in Wistar rats from the Hill lab, they found that weight gain across a 5-day interval from 30 to 35 days of age on a HF diet (45–60% kcal fat) was strongly and positively correlated ($r^2 = 0.71$–0.82) with accumulated body fat in four depots after 4–6 weeks of HF feeding. Screening in younger animals did not show this same correlation, suggesting that waiting until 4–5 weeks of age is necessary to identify OP and OR phenotypes using this model (30). Similarly, Levin's group have also shown that the DIO and DR phenotypes are not different between 3 and 5 weeks of age (31).

Overall, the diet and the timeframe of selection has evolved and varied between groups over the past 25 years. A reduced time frame for selection allows for animals to be studied earlier in the development of the DIO/DR phenotypes. Diet composition has evolved to better reflect a more reasonable and relevant amount of fat (40–50% kcal), and either to include or not include sucrose as a portion of the carbohydrate component. It should be noted that the addition of sucrose can lead to more severe metabolic derangements in the DIO animals that are generated, even to the extent of developing diabetes in some cases.

Levin's DIO/DR Model – Selective Inbreeding

One limitation of a model that requires stratification based on weight gain is that differences between OP and OR phenotypes cannot be studied prior to the dietary challenge. To circumvent this limitation, Levin et al. developed inbred lines of DIO and DR rats that were derived from OP and OR Sprague-Dawley rats. Briefly, outbred Sprague-Dawley rats that were either susceptible or resistant to weight gain after 2 weeks on a HF, high energy diet were identified and inbred (15). After five generations of selective inbreeding, the resultant lines breed true to their respective phenotypes, with a bimodal distribution of weight gain in response to the high energy diet. The resulting lines of animals were termed DIO or DR for those that were either susceptible or resistant to DIO, respectively. These valuable lines have provided the means to examine preexisting differences between OP and OR animals, as well as the differential responses that occur within the first few days of exposure to a HF and/or high energy diet.

Current Selection Protocol for DIO/DR Studies of Obesity

In recent years, we have employed a standardized dietary screen of outbred rats (**Figure 1**) to identify those rats that have a polygenic predisposition for resistance or propensity to become obese under environmental pressures that are thought to contribute to obesity in humans: consumption of a HF diet and limited physical activity (18, 32–35). While we typically use Wistar rats, outbred Sprague-Dawley rats have also been used with success. A number of vendors are available that commercially produce semi-purified diets, but we have typically used Research Diets D12344 and D11724 as our HF (46%) and LF (12%) diets, respectively.

As a general protocol, rats arrive at our facility at ~5 weeks of age. They are individually housed in wire bottom cages and provided free access to a LF diet for an acclimation period of ~2 weeks. This allows time for any institution-required quarantine, recovery from the stress of transport, and an opportunity to perform any baseline measurements. Following acclimation, rats are provided *ad libitum* access to the HF diet, which results in a heterogeneous distribution of body weight gain in the outbred animals. Rats have traditionally been ranked according to their change in body weight in response to HF feeding; those with the lowest weight gain are classified as OR, whereas those with the greatest weight gain are classified as OP. At this point, animals in the middle group are removed from the study. The HF diet is a critical component for the separation of rats into the OP and OR phenotypes as there is no difference in body weight gain when the animals are consuming an LF diet.

In addition to feeding a HF diet, another key requirement for emergence of the OP and OR phenotypes in this model is individual housing in wire bottom cages. Individual housing serves several purposes. First, it limits the physical activity of the animals compared to animals housed in a group environment (36), as group-housed animals are constantly engaged with one another. Second, it allows for more precise measures of food intake, which is often an outcome in studies of this nature. Third, it prevents dominant animals from influencing

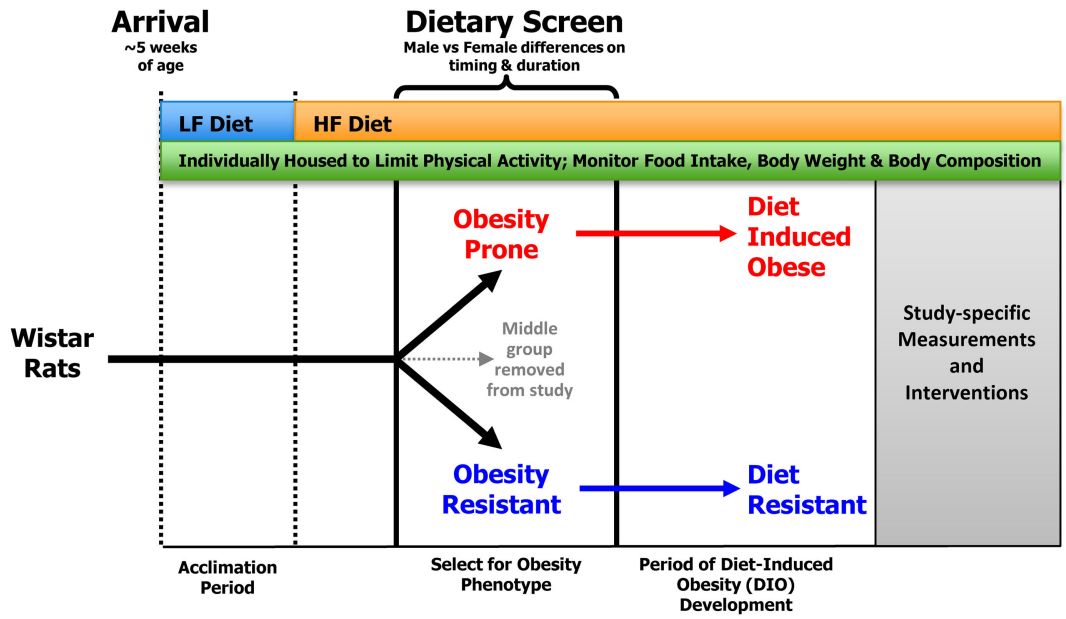

FIGURE 1 | General screening procedure for selecting OP and OR rats. Outbred Wistar rats that arrive at ~5 weeks of age are placed in individual wire bottom cages that limit physical activity and allow for accurate measurement of food intake and spill. Food intake, body weight, and body composition are monitored for the duration of the study. Following a brief acclimation period, rats are provided *ad libitum* access to the HF diet, which results in a heterogeneous distribution of body weight gain in the outbred animals. Obesity-prone (OP) and -resistant (OR) animals are identified as described in the text and matured into diet-induced obese (DIO) and diet-resistant (DR) animals, respectively.

food intake patterns of other rats in the group (37). Finally, it prevents coprophagia that can be common in studies of energy restriction and weight loss. In most of our studies, we use rat cages measuring ~9.25″(L) × 6.75″(W) × 6.75″(H), although we have also used slightly larger cages with success.

Developing the DIO/DR Model for Studies in Females

Sex-specific effects and sex differences in outcomes have received greater attention over the last decade as the scientific community has developed an appreciation for the importance of the biological variable of sex. Barry Levin's inbred DIO/DR lines have been valuable in this regard, as females could be derived directly from the respective inbred lines. The use of outbred strains presented a challenge with the selection of OP and OR rats, in that the unique aspects of female physiology and the associated variability confounded the screening process. Even so, the expense of maintaining Levin's inbred lines and the tenuous nature of their commercial availability led us to develop a more consistent screening protocol for the more readily available outbred strains. Our initial goal with this work was to combine the OR/OP model with established approaches for studying the loss of ovarian function and mammary tumors to develop paradigm for examining the impact of obesity on postmenopausal breast cancer. In addition to our intended use, this model has applicability to the study of numerous obesity-associated comorbidities in females.

We used the same source for female Wistar rats as we had for male rats in our previous studies: Charles River Laboratories (Wilmington, MA, USA). Identical to our male protocol, the female rats were individually housed in metabolic caging designed to allow for monitoring food intake while minimizing physical activity. Rats were fed the same HF purified diet (46% kcal fat; Research Diets, New Brunswick, NJ, USA; RD#12344) with free access to water.

In early studies with females, Hill's group successfully separated female rats into OP and OR after 4 weeks on a 60% fat diet containing sucrose (18). In more recent studies, we have observed that separating females into their respective phenotypes is more complicated when the sucrose-free, 46% fat diet is used. In our group's first published study in females (38), we stratified the rats into tertiles using the male-specific protocol based on change in body weight in response to HF feeding early in life. However, we found body composition in the mature females to be more variable than in the males, such that larger animal could have a lean phenotype and smaller animals could present with a higher level of adiposity. This necessitated the use of a retrospective analysis to identify rats as lean, mid-weight, and obese based on their weight gain from ~8 to 19 weeks of age. A 19-week time point was chosen because this was the time at which weight gain began to taper off, which we interpreted to indicate that the animals were fully mature and likely no longer depositing lean body mass.

In two subsequent studies with females (39, 40), we adjusted our approach and ranked animals by their rate of weight gain in the obesogenic environment from 10 to 18 weeks of age. Using this criteria resulted in a clean separation into obese and lean phenotypes in the mature animals (studied between 20 and 30 weeks of age), in which obese rats had significantly higher body weight,

body fat, and circulating triglycerides when compared to their lean counterparts.

Although our 8-week screening protocol generated a clean separation of the OP and OR phenotypes, it was far more labor intensive and costly than the 1-week protocol used in males, where the middle tertile of animals could be transferred out of the study early in life, rather than at 4–5 months of age. Thus, as part of a recent large obesity/menopause/breast cancer study with >300 female rats spread over three cohorts (currently unpublished), we measured body weight and composition at several time points to determine the earliest and shortest period of time that could be used to predict the OP and OR phenotypes in females with an acceptable level of accuracy. Female Wistar rats were individually housed and fed our standard HF diet (46% fat) for the duration of the study. Body weights were measured weekly for the duration of the study, and body composition measurements (qMR, EchoMRI, Houston, TX, USA) were performed at 9, 14, and 18 weeks of age. Body composition was also measured in all animals immediately prior to undergoing surgical ovariectomy (OVX). This occurred at an average of 26.5 ± 0.6 weeks of age and was therefore used as a measure of adult adiposity for the analysis.

As shown in **Figure 2A**, the correlation between the % BF at the time of OVX and weight gained from 8.5 to 9.5 weeks of age was poor ($r = 0.43$). This improved slightly when change in body weight was measured over 2 weeks (8.5–10.5 weeks of age, $r = 0.59$, data not shown) and was further improved when extended to 8 weeks (8.5–18.5 weeks of age, $r = 0.69$, data not shown). However, these changes in body weight were still not as predictive of adult adiposity as the <1 week weight gain was in numerous studies in the males described above. Thus, we investigated other potential measures that could be used to accurately screen for the OP and OR phenotypes in females. Change in % BF over the various time points were measured, but these correlations were no better than changes in body weight ($r = 0.35$–0.63, depending on the cohort of animals and time points measured). However, % BF at 14 weeks of age was highly correlated with adult adiposity (**Figure 2B**; $r = 0.70$–0.81, depending on the cohort). These correlations appeared to be stronger when controlling for the age at the time OVX body composition was analyzed ($r = 0.79$–0.84). At 18 weeks of age, the correlation strengthened even further ($r = 0.89$). However, in our opinion, the added time and cost associated with the additional month of animal housing to delay the separation is likely not warranted. Importantly, our data indicate that % BF early in life (9 weeks of age in this study) is not an accurate predictor of % BF at maturity ($r = 0.54$), and approximately one-third of rats would have been incorrectly categorized (OR, mid, OP) if this early marker of body composition was used. Additional studies will be required to further determine if a time point between 9 and 14 weeks is predictive of long-term adiposity. In the meantime, our approach is to refrain from screening until ~14 weeks of age to identify the OR and OP phenotypes in females.

LESSONS LEARNED FROM OP/OR RATS

Preexisting Differences That May Contribute to Their Predisposition for Obesity

As previously mentioned, one limitation of an OP/OR model that requires stratification based on weight gain is that it is difficult to assess differences between OP and OR phenotypes prior to the dietary challenge. However, an inherent assumption that has been repeatedly validated by our group is that once classified as either OP or OR, rats remain within their respective groups for the remainder of the experimental protocol, suggesting that there are likely preexisting differences between these animals even before

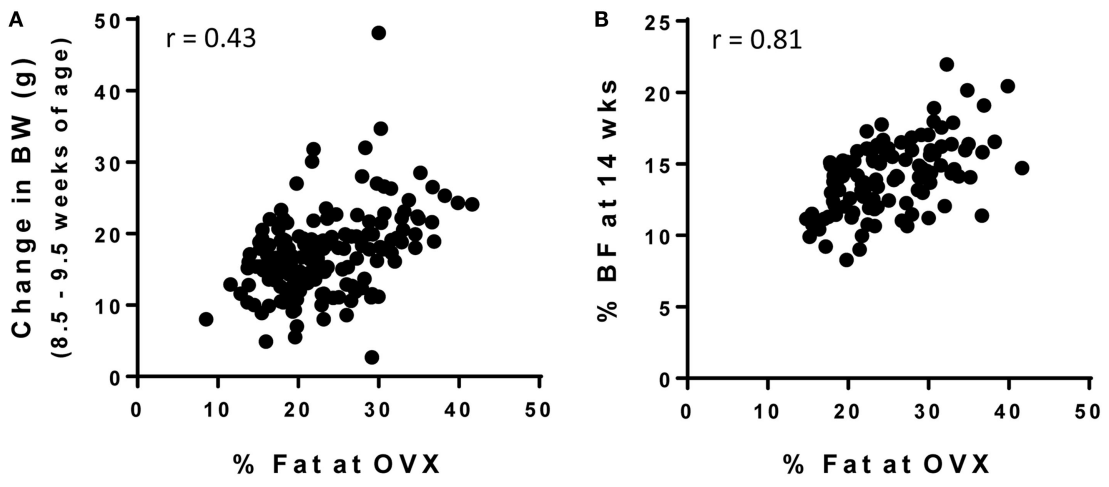

FIGURE 2 | Screening strategies for OP and OR phenotype in female Wistar rats. Female rats were individually housed in wire bottom cages and fed a HF diet (46% kcal fat) for the duration of the study. Body weight and body composition were measured throughout the study and was correlated with % body fat at the time of OVX (maturity; 26.5 ± 0.6 weeks of age). **(A)** Change in body weight early in life (8.5–9.5 weeks of age) is not a strong predictor of adult adiposity, but **(B)** % body fat at 14 weeks of age does predict adult adiposity.

the obesogenic challenge. Although our data on differences in OP/OR rats prior to a HF diet challenge are limited, one study did examine preexisting differences in skeletal muscle, and it was observed that OR rats had a significantly higher proportion of type I muscle fibers in the medial head of the gastrocnemius muscle than OP rats (muscle biopsy), suggesting that differences in muscle fiber composition may play a role in determining susceptibility to diet-induced obesity (22).

Additional knowledge in this field comes from studies by Levin et al. Initially, they identified Sprague-Dawley rats as being prone to become DIO or DR on the basis of high vs. low 24-h urine norepinephrine (NE) output (26). Using this approach, DIO-prone rats were observed to have significant reductions in heart, pancreas, and hypothalamic NE turnover, potentially indicating that differences in NE metabolism may be involved in the development of DIO on high energy diets (41). DIO-prone rats also exhibited greater arcuate nucleus NPY mRNA expression, fewer arcuate nucleus projections, leptin resistance, and abnormalities in serotonin turnover compared to DR-prone rats under pre-obese chow-fed conditions (42–45). Similarly, leptin receptor mRNA expression (46), counter regulatory responses to insulin-induced hypoglycemia (47), and central insulin signaling (48) have all been observed to be lower in the inbred line of DIO rats compared to DR rats. Collectively, these observations are indicative of abnormalities in hypothalamic pathways involved in energy homeostasis, all of which may contribute to the development of DIO when presented with a high energy diet.

While the research focus of Levin's group has primarily been the brain, we have directed our efforts toward differences in whole body and the periphery. Briefly, we have observed that regardless of gender, LF diet fed inbred lines of DIO and DR rats do not differ with respect to total energy expenditure (TEE), food intake, or physical activity (49). Others have also observed no differences in physical activity across the DIO and DR phenotypes on chow diet (50). Interestingly, despite similar intakes and energy expenditure on the LF diet, a greater rate of lipid disappearance was observed in the DR rats compared to DIO rats, suggesting greater basal lipid oxidation in DR rats (49).

Differential Response to an Obesogenic Diet

In general, following provision of a HF diet, both OP and OR rats initially experience a positive energy imbalance. The OR rats, however, appear to sense the nutrient overload and adjust their food intake and increase their energy expenditure to reestablish energy balance. In doing so, OR rats exhibit an increase in the oxidation of dietary fat. In contrast, OP rodents continue to eat to excess until expenditure increases from their accumulated mass to reestablish energy balance. Although OP rats have a markedly higher food intake, greater intake explains some, but not all of the variance in body weight gain between the OP and OR phenotypes (18, 51). These differences in food intake suggest that there are preexisting differences in regions of the brain that regulate feeding behavior. The findings from Levin's group with respect to these neuronal differences are beyond the scope of this review; however, we will briefly summarize some of their key findings. One week of HF feeding results in OP rats having elevated leptin, insulin, triglycerides, and glucose, along with increased lipoprotein lipase activity (LPL) in adipose tissue and galanin expression in the paraventricular nucleus (30, 49, 51). It is also noteworthy that OP rats have lower skeletal muscle LPL activity and a decline in the ratio of beta-hydroxyacyl-CoA dehydrogenase to citrate synthase activity, indicating a rapid decline in the capacity for lipid transport and the muscle to metabolize lipids (30, 52). OP rats are also characterized by a preferential trafficking of dietary lipid to adipose tissue for storage, whereas OR rats have greater trafficking of dietary lipid to skeletal muscle after 1 week of HF feeding (52). After 4–5 weeks of HF feeding, OP rats continue to consume more than OR rats, have a higher 24-h respiratory quotient (RQ) (indicating lower relative fat oxidation), and higher plasma levels of free fatty acids (FFA) (18). Insulin sensitivity is also lower in OP rats, which is the result of both lower glucose uptake and lower glucose disposal in skeletal muscle (18, 23). Although we have observed no differences in spontaneous physical activity (SPA) following 1 week of HF feeding (49), others have shown that DIO rats have lower SPA after both 4 and 10 weeks on a HF diet, findings that have been linked to the function or orexin signaling (50, 53, 54).

Established Obesity – Differences between DIO and DR Rats

Once obesity has been established and the rate of weight gain declines, there are concomitant reductions in the differences in RQ and the measured energy imbalance between DIO and DR rats (51). Coincident, or possibly the cause of the normalization of energy and nutrient balances, is the finding that many hypothalamic differences/abnormalities are also normalized between DIO and DR rats (44). Regardless, DIO rats generated from the aforementioned screening protocol exhibit many of the metabolic derangements and hallmarks that are linked to obesity in humans, including insulin resistance, glucose intolerance, lower spontaneous physically activity, and impaired fat oxidation (40, 49, 52, 55, 56).

DIO/DR Differences Unique to Females

Aside from the initial studies that showed no difference in EI, TEE, or activity levels between DIO and DR rats, our work with the female-specific aspect of this model has not specifically addressed the differences between male and female rats in terms of their propensity to become obese. We have, however, performed comprehensive metabolic studies of the mature lean and obese animals across the estrous cycle, and during the initial stages of weight gain following surgical OVX (40). We observed that obese animals experienced greater fluctuations in energy balance across the 4-day estrous cycle than their lean counterparts, and this was driven by greater variability in food intake across the cycle (**Figure 3**). A rise in estrogen levels during the proestrus phase of the cycle underlies a reduction in food intake, and this estrogen-mediated response appears to be delayed in obese animals (**Figure 3**). While circulating estradiol levels were not significantly different between the DIO and DR animals, we suspect that the inherent impairment in leptin and/or insulin sensitivity in the obese may impart reduced sensitivity to the effects of estrogen at this stage of their cycle. Additional

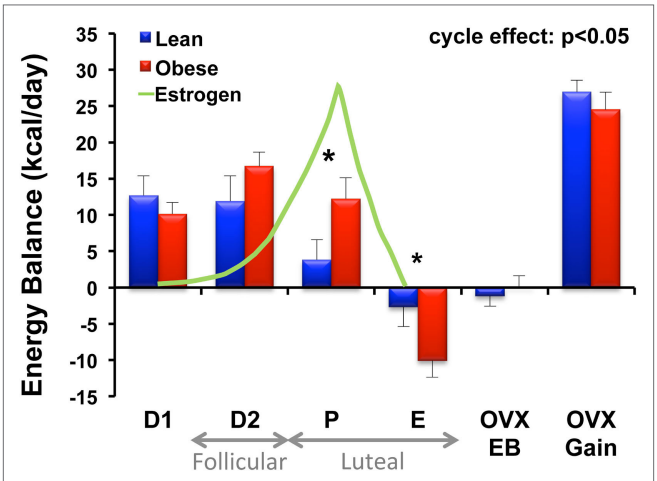

FIGURE 3 | Energy balance across the estrous cycle and following OVX in lean and obese rats. Energy balance (intake – expenditure) was measured in lean and obese rats during each phase of the estrous cycle [diestrus 1 (D1), diestrus 2 (D2), proestrus (P), and estrus (E)], immediately following surgical ovariectomy (OVX) while in energy balance (OVX-EB), and during OVX-induced rapid weight gain (OVX-Gain). Relative circulating estrogen levels across the cycle (D1–E) and corresponding follicular and luteal phases of the human menstrual cycle are indicated. Obese rats experience greater fluctuations in energy balance across the cycle compared to lean rats. A rise in circulating estrogens is associated with a decrease in food intake (and energy balance) in the lean; however, this response is delayed in the obese. *Significant difference between lean and obese groups ($P < 0.05$). Modified from Ref. (40).

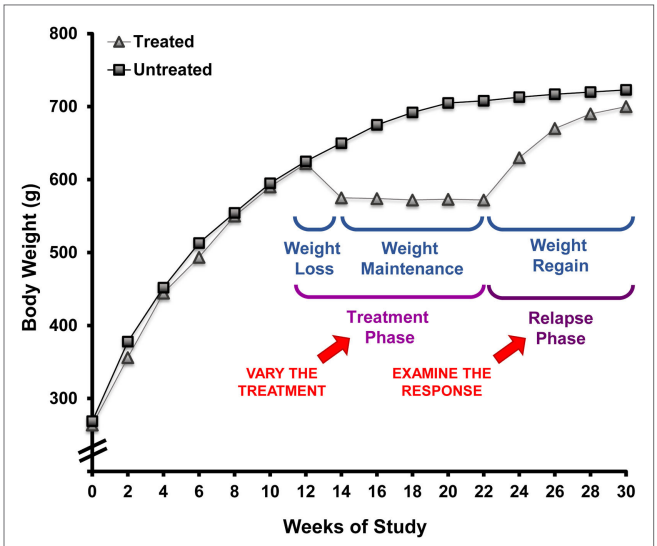

FIGURE 4 | Rodent paradigm to study the metabolic propensity to regain weight after weight loss. This paradigm, employing obesity-prone rats, has well-defined primary and secondary outcomes that describe the metabolic propensity to regain weight after prolonged weight reduction. This approach can be applied to test weight reduction strategies for their ability to overcome the metabolic pressures driving weight regain by modifying the environmental conditions in the treatment phase and examining the response during the relapse phase.

studies are needed to examine this possibility. We further found that cycling obese rats were less active, expended more energy per movement, and oxidized more carbohydrate than lean rats. Despite these phenotypic differences across the cycle, OVX induced a large positive energy imbalance in both obese and lean rats, which resulted primarily from an increase in energy intake in both groups. TEE was not altered in either group, despite the fact that they were eating more food. Our interpretation of these observations is that the increased thermic effect of food (from the greater food intake) is essentially balanced out by any reduction in the non-resting energy expenditure (NREE) that occurs from the decline in physical activity levels.

Characterizing the Metabolic Propensity to Regain Weight after Weight Loss

Over the past decade, we have employed the OR/OP model to study the phenomenon of weight regain after weight loss. The paradigm we developed to model the human condition is shown in **Figure 4**. Following the standardized screen to identify the OR and OP phenotypes described above, the young rats are maintained in the obesogenic environment (HF diet; limited physical activity) for 16 weeks, during which excessive weight gain occurs in what would be equivalent to childhood and adolescence. As the rats mature, growth rates slow, the gain in body weight and fat-free mass plateaus, and further weight gain comes slowly and primarily in the form of fat mass. The rats are then given a two-step treatment regimen (weight loss followed by weight maintenance) that reflects the most common approach used in humans: restricted consumption of an LF diet. The rats are fed a calorie-restricted LF diet that induces a 10–15% loss in weight that is primarily fat mass. A LF provision, adjusted on a daily basis, is given so that weight is maintained at this reduced level. In some studies, weight reduction has been sustained with intake-regulated maintenance for up to 16 weeks, a period of time that would reflect several years of weight reduction in humans (57). The propensity to regain weight is then characterized by allowing the rats to have free access to a specific diet while monitoring body weight, body composition, and pertinent aspects of metabolism as they relapse to the obese state. Our assertion is that this paradigm reflects the human condition with respect to:

(1) Genetic pressures – polygenic predisposition to become obese under obesogenic conditions;
(2) Obesogenic conditions during formative development – maturation in an obesogenic environment;
(3) The most common approach to weight reduction – restricted/controlled intake of an LF diet; and
(4) The most common failure – not controlling intake.

In our first set of studies, we examined weight regain immediately after weight reduction and assessed how energy balance and fuel utilization were altered with prolonged weight reduction. As expected, the drive to regain could be described by an increased rate of gain and a return to the previous level of body weight and fat mass. We observed that prolonged (8-week) weight reduction was accompanied by a persistent reduction in TEE that was due in part to a sustained reduction in resting energy expenditure (REE).

By adjusting for the variability attributed to variations in fat-free mass, we observed an enhancement in metabolic efficiency, meaning the suppression in resting metabolic rate was greater than what would be predicted based the decreased mass of metabolically active tissues that occurred with weight reduction. Like adult humans that are obese, fluctuations in weight were primarily due to changes in fat mass rather than changes in fat-free mass. Furthermore, relapsing rats had a tendency to burn carbohydrate rather than fat. Importantly, this study established the weight loss/weight regain paradigm, describing the basic approach and technical tools used for its application (34).

The Role of Timing in the Weight Regain Process

Length of Time in Weight Maintenance

Based upon observations from the National Weight Control Registry, the paradigm was applied to address whether increasing the time of weight maintenance would attenuate the metabolic drive to regain weight. Given the sustained reduction in TEE with prolonged weight reduction in our prospective study, we were not overly optimistic that this would be the case. To better understand the aspects of metabolism promoting regain, we performed a large cross sectional study in male rats examining the weight reduced state at 0, 8, and 16 weeks of weight maintenance, before and after 8 weeks of relapse (34). The time in weight maintenance was equivocal to a weight-reduced human keeping the weight off for ~10 years (57). A portion of these data is shown in **Figure 5**. We observed that the rate of regain increased with time in weight maintenance (**Figure 5A**), but the level of defended body weight and adiposity was drifting higher (obese rats switched to LF diet, dotted line). The animals were defending a target weight that was drifting upwards while they were weight reduced, an effect we attribute to age. Regardless, the increased rate of regain indicated that the metabolic pressures driving regain were greater as the time in weight maintenance increased. This effect on the rate of regain was most profound in the first week of the relapse period (**Figure 5B**). In addition, we observed that the decreased TEE and enhanced metabolic efficiency observed in our previous study remained unchanged when the length of time in weight maintenance increased. These metabolic adaptations that are thought to be contributing to the biological drive to regain weight did not resolve, even with long-term weight loss maintenance. While some similar mechanisms are likely to underlie the biological drive to regain weight in females, we know that there are many sex-specific differences that also likely exist. Our lab is currently performing these same studies in females to fully characterize the nature and extent of these differences, particularly with respect to (a) the extent and kinetics of the weight regain process and (b) the effects of menopause/loss of ovarian hormones on this process.

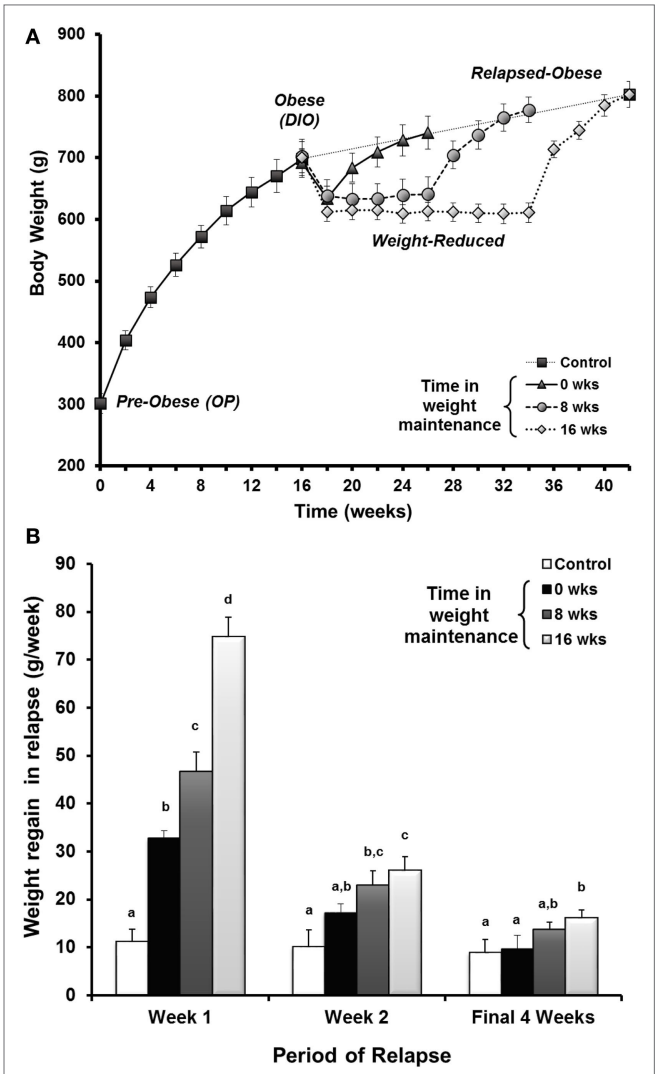

FIGURE 5 | Drive to regain weight increases with time and is higher early in the relapse period. **(A)** Rats in the paradigm examining the length of time in weight maintenance vs. the drive to regain weight. Obesity-prone (OP) rats were maintained on a HF diet for 16 weeks to induce obesity. Rats were calorically restricted on an LF diet to induce 10–15% loss, maintained at this reduced weight for 0, 8, or 16 weeks, then allowed to relapse with free access to LF diet. **(B)** The rate of weight regain shown by time in maintenance at various times during the relapse period. The rate of weight regain for relapsed-obese rats is represented as the average for the first week, the second week, and the final 4 weeks of the relapse period. Data are expressed as means ± SE. With each time period, groups with the same letter designation are not significantly different. Modified from Ref. (34).

Prospective Analysis of *Early* Relapse

To examine this critical early period, energy balance, fuel utilization, and regain were monitored prospectively through the first 2 weeks of relapse. During this time, almost half of the lost weight had been regained (34). We observed that both an increase in drive to eat and a decrease in expenditure contributed to the large energy gap, and neither side of the energy balance equation had normalized by the end of 2 weeks. Enhanced metabolic efficiency persisted throughout this early period of relapse and contributed to the suppressed TEE. In other studies, we utilized nutrient tracers in combination with metabolic phenotyping to examine fuel trafficking during the early stages of weight regain (58). During weight regain, we observed that the oxidation of dietary fat was

substantially suppressed and that ingested fat was preferentially trafficked to adipose tissue for storage. Accompanying this shift in metabolism early in relapse was an increased number of small adipocytes, which would presumably provide an ideal receptacle for the excess ingested energy. When taken together, observation of DIO rats in this paradigm of weight regain suggest that adaptive changes in muscle and adipose tissue establish a metabolic context for rapid, energetically efficient weight regain. In subsequent studies, we have observed how regular exercise counters this metabolic drive to regain weight early in relapse; exercise decreases the energy imbalance or energy gap (**Figure 6**) both by reducing appetite and by increasing the level of expended energy during weight regain (55). Using nutrient tracers, we provided evidence suggesting that regular exercise increases the oxidation of dietary fat and traffics excess energy through more expensive pathways of deposition (59, 60). We have examined the tissue-specific mechanisms of these beneficial effects of exercise in both skeletal muscle (59) and adipose (60), and our analysis of the effects in liver will be forthcoming.

Prospective Analysis of *Complete* Relapse

Our studies of DIO rats in this weight regain paradigm have also examined the later stages of the relapse process to provide a more complete biological picture of weight regain after weight loss. We were interested in following the resolution of the energy gap, non-protein RQ, fuel utilization, and the energetic efficiency of weight regain. The pattern of regain in our rat paradigm reflected the pattern of regain in a meta-analysis of a large number of human regain studies (61), which some have suggested reflects a first-order relationship in the resolution of this biological drive (**Figure 7**). Our observations extended our previous findings by showing that the enhanced metabolic efficiency and suppressed TEE persist throughout the process of relapse. Both increased intake and suppressed expenditure led to a large energy imbalance, or energy gap, which resolves gradually as the weight returns (**Figure 7**). While intake returns to levels observed before weight loss, TEE and REE never completely resolved even after the rats had returned to their previous weight. Both the reduction in feed efficiency and the elevation in non-protein RQ declined after week 2, suggesting that this efficient weight gain and shift in fuel use was most profound early in relapse when much of the lost weight returns (56). As expected, we and others have observed, at least in males, that exercise and physical activity attenuates the biological drive to regain weight early in relapse and leads to a lower body weight and fat mass (55, 59, 60). These studies support the notion that in males, exercise attenuates the drive to eat and increases expended energy above and beyond the additional energetic cost of the exercise bout, and that these effects persist throughout the entire relapse process. However, we have also observed that these beneficial effects of exercise may be greatly diminished if weight regain occurs on an obesogenic diet (62).

Use of DIO/DR Rats for Studying Obesity Therapeutics

To date, we have used the DIO/DR rats to develop a broad picture of the biological drive to regain weight after weight loss, and we have used the experimental paradigm of weight regain in DIO rats to examine the impact of one of the most effective strategies for weight loss maintenance: regular physical activity. Given the importance of the biological adaptations in driving the weight regain process (2), we assert that the use of this model in this paradigm may provide valuable information about specific

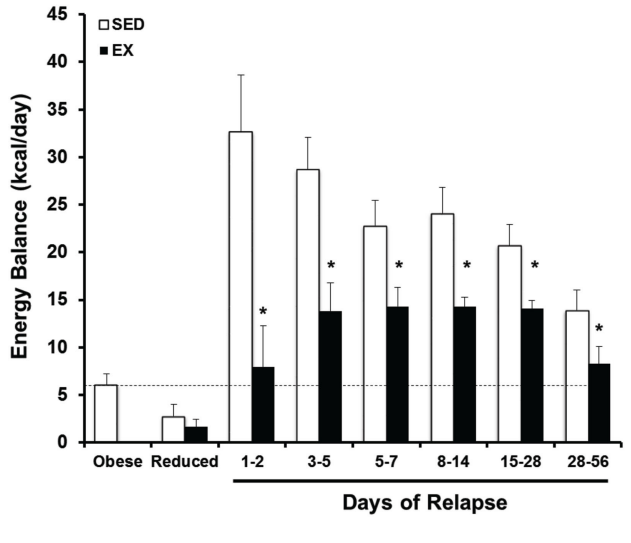

FIGURE 6 | Prospective analysis of the energy gap during weight regain with and without exercise. Energy balance [energy intake – total energy expenditure (EI-TEE)] is shown for obese, weight reduced, and relapsing rats either with (EX) or without (SED) treadmill exercise for several time periods during relapse. During weight regain, the energy gap (energy imbalance) resolves gradually as body weight is gained. Further, exercise reduces the energy gap both by suppressing EI and increasing TEE. *Significant difference between SED and EX rats during that time period, $P < 0.05$. Modified from Ref. (55).

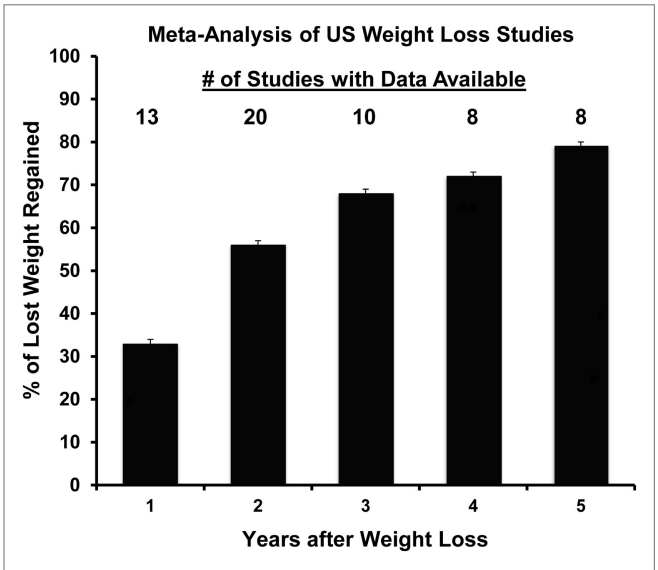

FIGURE 7 | Propensity to regain weight following weight loss: summary of data from a meta-analysis of US weight loss studies. The percentage of lost weight that is regained over a 5-year period according to a meta-analysis of 29 human weight loss studies. Adapted from Ref. (61).

therapeutic strategies or combinations of strategies that are designed to attenuate the biological drive to regain weight after weight loss. In addition, our data suggest that it is critical to assess the sex-specific differences in the biological drive to regain weight, as well as the sex-specific differences in the efficacy of therapeutics and strategies targeting these biological adaptations for weight loss maintenance. The development of a more effective screening process for females discussed earlier will greatly facilitate this important work moving forward.

DIO/DR Studies in Females

Obesity and the Loss of Ovarian Function

As previously stated, we have applied the DIO/DR model to study the effects of obesity on energy homeostasis in females (40). By merging the DIO/DR model with other common approaches used in preclinical research, we have extended the utility of the DIO model for studies of menopause and breast cancer. Menopause is a very complex transition accompanied by a wide array of metabolic derangements in numerous tissues of the body. While there are several groups that have performed elaborate clinical studies either across the natural menopause transition (63) or in studies of ovarian hormone suppression (64–66), it is generally difficult to study the menopause transition in women because of the wide variations in the length of time for this transition, the variable age of onset, and the fact that it is generally only identified retrospectively. To overcome these logistical challenges in preclinical studies, we have utilized surgical OVX to mimic the loss of ovarian function (38, 40, 67, 68). This approach reflects some of the metabolic consequences of the menopausal transition, but it has the added advantage of a clear demarcation of the loss of ovarian hormone production for precise timing for follow-up analyses. We have specifically merged the DIO/DR model with this surgical intervention to study the impact of preexisting obesity on the loss of ovarian hormone production. Following OVX, we have shown that both DIO (obese) and DR (lean) rats exhibit a 3- to 4-week period of rapid weight gain that is accompanied by increased energy intake and reduced SPA levels (38, 40). While DIO and DR rats generally gain the same amount of weight, the weight gain appears to be somewhat slower or delayed in the DIO females (38). While activity levels are reduced after OVX in our model, our data suggest that in this paradigm, weight gain is primarily a result of increased food intake, rather than changes in energy expenditure (40).

Obesity and Postmenopausal Breast Cancer

One of the many comorbidities associated with overweight and obesity is an increased risk for, and mortality from, many cancers including breast cancer (69–72). Surprisingly, obesity's impact on breast cancer prior to menopause is relatively modest and in some cases has even been shown to be protective (73). After menopause, however, obesity increases the incidence, progression, and eventual mortality from breast cancer by up to 40% compared to women at a healthy weight (74). The risk is highest in women with a history of weight gain throughout life, suggesting that a crisis in obesity-driven breast cancer is likely to occur with the current generation of youth, where obesity rates are approaching an unprecedented 20% (75). Despite the known link between obesity and postmenopausal breast cancer, the mechanisms underlying this association are not fully understood. This represents a significant gap in our knowledge, and identifying mechanisms of risk and targets for intervention is critical.

To pursue a deeper understanding of the biological aspects underlying this relationship, we merged the DIO–DR/OVX model with a common preclinical approach to studying mammary tumor biology. Prior to the OP/OR screening, a chemical carcinogen (MNU) is delivered during mammary gland development. The animals are then matured under obesogenic conditions into DIO and DR rats, after which they are subjected to surgical OVX. Merging the models in this fashion has generated an experimental paradigm that can further our understanding of the impact of obesity on postmenopausal breast cancer.

Mammary tumors generated in these animals are reflective of the human condition with respect to the histological characteristics and estrogen receptor (ER) status of the tumors (38, 39). Further reflective of humans, the effect of obesity on mammary tumor incidence is minimal prior to OVX, and the emergence of an obesity effect occurs only after the OVX surgery. Specifically, in response to OVX, obese rats exhibit fewer tumors that regress, more tumors that progress, and more tumors that newly emerge (38). Of all the characteristics of the obese phenotype that were examined, the strongest relationship with tumor promotion was observed with the energetics of weight gain during the ~3- to 4-week period of rapid weight gain that followed OVX. During this time, both groups experienced a dramatic increase in the rate of weight gain. However, despite the two groups eating similar amounts of food, DIO rats gained less weight ($p < 0.01$). Consequently, feed efficiency during this transient period of rapid gain was lower in DIO rats ($p < 0.001$). Feed efficiency during this 3-week period of rapid weight gain was inversely associated with the change in tumor multiplicity ($r = -0.64$, $p < 0.001$) and burden ($r = -0.60$, $p < 0.001$) over the entire post-OVX period. Rats that experienced a lower rate of weight gain and a lower efficiency of storing ingested fuels during this distinct time period after OVX also had a higher level of tumor progression. These observations suggest that the impact of OVX on energy balance and fuel utilization is different for DIO and DR rats in this paradigm and that this difference may affect the latency, survival, and growth of mammary tumors.

OVX-Induced Overfeeding – An Example of Metabolic Inflexibility

We subsequently performed a nutrient tracer study in DIO/DR rats during the early stages of OVX-induced weight gain. Tumor bearing DIO and DR rats were studied after OVX both while in energy balance, and while experiencing their natural OVX-induced positive energy imbalance (and subsequent weight gain) (39). These studies were performed in a metabolic phenotyping system in which energy intake, expenditure, and tissue-specific nutrient trafficking could be carefully measured. In DR rats, the OVX-induced energy imbalance was accompanied by a higher

level of glucose uptake (^{3}H-2-deoxyglucose) in the mammary gland adipose depot, and similar trends were seen in the liver, retroperitoneal adipose, and skeletal muscle. Overfeeding in this context had no effect on the glucose uptake in the tumors of DR rats. Our observations in DIO rats were in direct contrast to those in DR rats. Glucose uptake was unaffected by the OVX-induced positive energy imbalance in all tissues of the DIO rats, with the exception of the tumors, where glucose uptake was increased. Changes in whole body fuel utilization (RER) and dietary fat oxidation in response to this caloric excess also tended to be blunted in the obese. Taken together, our studies of this critical period of OVX-induced overfeeding indicate that DIO rats have an inability to clear and store nutrients in peripheral tissues, but excess nutrients are readily taken-up by mammary tumors.

We have interpreted these observations from the perspective of metabolic flexibility, which we broadly define as the ability to change or adjust nutrient metabolism in response to a metabolic challenge. The challenge in this context is OVX-induced overfeeding. In the insulin-resistant DIO rats, their peripheral tissues exhibit a blunted or impaired response to the excess energy, while their tumors readily take up the excess energy. In the DR rats, their peripheral tissues exhibit greater flexibility in their response to the challenge and are more capable of clearing and metabolizing the excess energy. We would assert that this impaired ability to respond to metabolic stress may underlie many of the metabolic derangements and accompanying pathologies associated with obesity. Even so, metabolic flexibility is a concept that is difficult to specifically define and even harder to study. The DIO/DR model may prove a useful tool to study this important concept in well-defined metabolic contexts.

CONCLUSION

Over the past two decades, the impact of obesity on overall health and wellness has emerged as a major crisis. The study of OP/OR and the DIO/DR rat model has proven to be a valuable tool in translating basic science studies of energy balance and body weight regulation to the human condition and in furthering our understanding of the physiologically relevant condition of obesity. The strengths of this approach are the polygenic nature of the adipose disposition, and that it has been shown to reflect the human condition in a number of ways. The Levin model of inbred lines has been particularly useful in that they have been used to study OP and OR phenotypes prior to and during the development of a particular adiposity phenotype. While the commercial availability of Levin's valuable model continues to be questionable, researchers may continue to pursue the OP/OR selection of outbred strains to produce the DIO/DR phenotypes to further our understanding of obesity and its metabolic complications. Both the inbred and outbred strains have proven extremely valuable for obesity studies of female physiology and have elucidated critical sex-specific differences of obesity and its metabolic complications.

AUTHOR CONTRIBUTIONS

All the authors contributed to the writing of this review, and they have all read and approved the final manuscript.

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REVIEW
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Animal Models for the Study of the Relationships between Diet and Obesity: A Focus on Dietary Protein and Estrogen Deficiency

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Obesity is an increasing major public health concern asking for dietary strategies to limit weight gain and associated comorbidities. In this review, we present animal models, particularly rats and mice, which have been extensively used by scientists to understand the consequences of diet quality on weight gain and health. Notably, modulation of dietary protein quantity and/or quality has been shown to exert huge effects on body composition homeostasis through the modulation of food intake, energy expenditure, and metabolic pathways. Interestingly, the perinatal window appears to represent a critical period during which the protein intake of the dam can impact the offspring's weight gain and feeding behavior. Animal models are also widely used to understand the processes and mechanisms that contribute to obesity at different physiological and pathophysiological stages. An interesting example of such aspect is the situation of decreased estrogen level occurring at menopause, which is linked to weight gain and decreased energy expenditure. To study metabolic disorders associated with such situation, estrogen withdrawal in ovariectomized animal models to mimic menopause are frequently used. According to many studies, clear species-specific differences exist between rats and mice that need to be taken into account when results are extrapolated to humans.

Keywords: animal models, obesity, body composition, dietary protein, food intake, energy expenditure, estrogen deficiency

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INTRODUCTION

Obesity is a worldwide epidemic affecting over 400 million adults with serious comorbidities (1). Obesity develops when energy consumption exceeds energy expenditure and is defined as the accumulation of excess body fat to the extent that its results in health complications and reduces life expectancy (2). As obesity prevalence is rising, the quest to find new treatments to diminish its negative consequences is also increasing. Experimental research needs to determine the mechanisms by which obesity increase the risk of diseases. To investigate the interactions between the components of the diet and the biological processes, epidemiological, experimental, and clinical studies are necessary.

Regarding experimental studies, animal models are essential for *in vivo* and *ex vivo* experimental design. Nutrient and non-nutrient components of food interact with many metabolic pathways at different levels including gene expression regulation. Experimental models, from cells to organoids and animals, are also essential to elucidate mechanisms by which food components can modulate metabolic pathways. To be able to translate, at least partly, the information obtained from an animal model to humans, the choice of the appropriate animal model is a crucial step to avoid as much as possible misinterpretations.

Dietary interventions studies in animals are thus essential to understand the biological roles of specific nutrients before validation in human. In the last century, rats were the most used in biochemical research, but in the last two decades, its popularity decayed due to the limitation to perform reverse genetics in rats. *Mus musculus* is probably the most popular model used to identify the mechanisms of food intake and energy regulation. Even if some extrapolation from mice to humans is hazardous, the mice model has helped us to develop some therapies for obesity, metabolic syndrome, and insulin resistance (3). If mouse models obviously do not mimic all aspects of human diseases, they are, however, the most commonly used models. No other animal model offers such large possibilities of phenotyping in response to metabolic, genetic, and behavioral manipulations. Depending on the target, the most widely used mouse models are (i) spontaneously occurring obese mouse strains that are well characterized, (ii) high-fat diet that rapidly induce weight gain in mice, and (iii) transgenic or gene knockouts mice to determine the influence of a given gene in the development of obesity.

Animal models have thus been used extensively by the scientific community to understand the role of diet quality on health. A better understanding of the relation between diet quality, and also physical activity and progression of chronic disease, such as obesity as presented in this review article, is increasingly important with regard to the increase in the number of obese individuals worldwide.

This review will focus specifically on two typical situations in the rodent models: (i) the impact of protein quality/quantity and (ii) the impact of estrogen deficiency on body weight and composition. A short section on the pig model will conclude this article to summarize the advantages and limitation of this model versus the rodent models, in general terms and in terms of studies on obesity.

PROTEIN QUANTITY/QUALITY

Dietary intervention studies in animal models are essential to understand the biological roles of specific nutrients before validation in humans.

Dietary Protein Intake

Protein is an essential dietary component in which recommended level is defined as the minimum intake required to maintain nitrogen balance; and as the amount of protein sufficient to prevent the catabolism of body protein stores. The recommended daily minimum intake of protein and amino acids (AAs) in adults is 0.8 g/kg of body weight (4). However, recent studies using stable isotope suggest that current dietary protein recommendation may not be sufficient to promote optimal muscle physiology in all populations (5). Epidemiological studies support the notion that especially in the older population, a greater protein intake, up to 19% of the energy, better preserves lean body mass (LBM) (6). In industrialized countries, the main sources of protein are milk, eggs, and meat. The nutritional value of protein is influenced by several factors, especially the AA composition, protein digestibility, protein digestion kinetics, and the ability to transfer AA for protein synthesis. Diets based on either animal or vegetable products supply proteins of different quality in different quantities. Plant proteins are often lower in some specific indispensable AAs when compared to animal proteins. For instance, soy protein is reported as a "complete" protein, but its overall indispensable AA content is lower than the one measured in milk proteins (7). Thus, protein quality, which is defined as the capacity of dietary protein sources to satisfy the metabolic needs for protein, and as the content in essential AAs, is important when considering protein requirements. Correlations between protein nutrition and human health are becoming a highlighted research topic.

Low-Protein (LP) and High-Protein (HP) Diets

Studies have suggested that when rats are placed in food choice position, they regulate their protein intake, so that it corresponds to their nutritional needs (8). Consistent with these results, experiments have shown an increase in food intake when the diet protein content is decreased at the expense of carbohydrates (9). The "protein leverage hypothesis" issued by Simpson and Raubenheimer proposes that paradoxically, proteins, which only represent between 10 and 15% of the average energy intake in adults represent the key factor in body weight and composition regulation (10). These authors have observed that the ratio between the protein and other nutrients (carbohydrates and lipids) has dropped in the last years. Thus, people, according to their hypothesis, tend to consume more dietary proteins to cover their protein needs. This excessive consumption of HP and LP density food, may partly explain the weight gain and obesity measured in these individuals. This observation is in line with numerous animals studies showing that substitution of carbohydrates by proteins in HP diet reduce adiposity and food intake (11, 12), while LP diets are associated with an increase in food intake and fat mass (13–15).

Since LP and HP diets are commonly consumed, it is particularly interesting to study consequences of those diets on human health. However, it should be underlined that the average amount of dietary protein consumed is generally above the recommended dietary intake in Western countries. For instance, in France, the average dietary consumption is 1.7-fold the recommended dietary intake (16). The consumption of HP diets, which can lead to the consumption of dietary protein up to four times the recommended dietary protein intake, are frequently used by individuals who wish to decrease their body weight. Although body weight diminution in overweight and obese individuals is

obviously associated with beneficial outcomes, some deleterious effects of HP diet have been suggested in several studies. Indeed, HP diets are contraindicated for individuals who are suffering or predisposed to kidney diseases (17). Regarding the intestinal physiology, in case of HP consumption, a part of dietary and endogenous proteins escapes full digestion in the small intestine and is transferred to the large intestine, where they are metabolized by the intestinal microbiota that produce, from AAs, various metabolites, some being beneficial, while most of them being deleterious when present in excess (18). An epidemiological analysis performed by Shoda et al. (19) in Japan showed a correlation between incidence of Crohn's disease (an intestinal chronic inflammatory bowel disease) and increase intake of animal protein over a period of 20 years. A prospective cohort study carried out in women also reported a positive association between the level of dietary intake and risk of inflammatory bowel diseases (20). However, Spooren et al. have recently performed a systematic review of the epidemiological studies that have examined the links between protein intake and the risk of developing inflammatory bowel diseases and have reported that most studies performed found no significant association between these two parameters (21). When interpreting the results of these different studies, it is worth taking into consideration that, due to the high complexity of diet, it may appear difficult to collect robust dietary data. Then, it remains possible that the effects of the protein intake on the risk of developing inflammatory bowel diseases may have been biased in some studies by confounding factors.

Regarding the possible links between HP diet and the risk of colorectal cancer, the results obtained from epidemiological and experimental studies do not allow to reach any robust conclusion, the results obtained being rather heterogeneous (22). Observational studies have reported that HP diet is linked with higher mortality due to cardiovascular disease (23, 24). However, as discussed earlier, increased protein consumption is commonly associated with high intake of other alimentary compounds like meat that contains notably heme, *N*-nitroso compounds, and heterocyclic amines, which have been reported to exert negative effects on various health aspects when present in excess. Therefore, these confounding factors do not allow to determine clearly the role of protein *per se* on various health parameters.

Concerning LP diets, there is no clear definition for this type of diet. LP diets are often recommended for patients with anomalies of the AA metabolism including phenylketonuria and those with kidney or liver diseases (25, 26). Furthermore, in developing countries, children during fetal development, lactation, and after weaning are often fed with diets including high carbohydrate but LP level (27, 28). It is therefore important to determine the effects of LP diets on weight and body composition.

The consequences of HP and LP diets on body composition can be studied in animal models with no difference in energy content. For example, experimental HP diets usually contain less digestible carbohydrates but had exactly the same composition regarding lipids, undigestible carbohydrates, minerals, and vitamins (29). The use of animal models is suitable to reveal the underlying mechanisms.

Dietary Protein Intake, Body Weight, and Composition

Numerous studies have reported that HP diets allow reduction of adiposity while maintaining LBM in animals (30–32). In rats, it has been shown that HP diets, in which 50% of the energy is provided by proteins, drastically reduced after 6 months the white adipose tissue compared to a normal protein diet (30). Consistent with these results, Pichon et al. found that increasing protein level in the diet reduced weight gain more strongly than the reduction of carbohydrates/lipids ratio (32). Moreover, this decrease in weight gain was associated with decreased adipocyte size (11).

It has been shown that protein restriction can replicate the effects of calorie restriction over a short period of 8 weeks in mice, with a decrease in circulating insulin, glucose tolerance, and weight gain (15). However, consumption of LP diet over longer periods in mice is generally associated with increased weight, adiposity, and intrahepatic fat (14, 15). On the contrary, growing rats fed for 15 days with a LP diet exhibited a lower body weight but a greater adiposity (13), enlightening the importance of the dietary intervention duration.

Dietary Protein Level and Food Intake

Mellinkoff, according to his aminostatic theory (33), was the first to hypothesize that the fluctuations of plasma AA concentrations could act on the control of food intake. Noting that «a rise in the serum amino acid concentration appears to be accompanied by a waning of appetite», he hypothesized that when plasma AA concentrations reach a threshold, satiety occurs. Furthermore, it is well known since several years that the AA content in the cerebrospinal fluid reflects circulating AA levels, itself linked to the dietary protein composition (34). Some of these AAs can also serve as precursor of neuropeptides that are directly involved in food intake regulation. This is the case for tryptophan, which is a precursor for serotonin, this latter neurotransmitter repressing food intake (35). Similarly, histamine is synthesized from histidine, and high levels of histidine are thought to have a negative effect on food intake through histaminergic neurons activation (36). AA composition could therefore explain why some proteins have been reported to be more satiating than lipids and carbohydrates (37, 38).

Taken together, these observations support the concept that a HP diet decreases food intake, while a LP diet increases it. On the contrary, very LP diet generates an aversive phenomenon (39) that allows the individuals to direct its choice toward balanced food to maintain essential AAs homeostasis (40).

The effects of HP diets on food intake are especially observed during the first days after the introduction of the diet. Once the animals are accustomed to the HP diet, they tend to return to a food intake similar to the one observed in animals fed a control diet.

The effect of both HP and LP diet on food intake is mediated by gastrointestinal peptides. Thus, Batterham and colleagues observed an increase in the plasma levels of the anorectic peptide PYY in mice following ingestion of an HP diet (41). In the same study, they have shown that mice deleted for the PYY gene no longer exhibit a decrease in food intake under a HP diet. In

humans, studies have reported that HP diet is also associated with increases in the concentrations of glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK), and a decrease in ghrelin concentration (42). Interestingly, LP diets are associated with small changes in CCK or ghrelin levels relative to control diets. Morrison and Laeger hypothesized that this blunted response may contribute to the hyperphagia observed in case of LP diet consumption (42).

The effect of LP diet on food intake is also mediated through FGF21 secretion. Indeed, the increase in food intake induced under a LP diet is suppressed in FGF21-KO mice (43).

Moreover, the supplementation of some AAs (histidine, phenylalanine, tryptophan, alanine, glutamine, and arginine) can partially mimic the satiating effect of HP diet on food intake and/or gastrointestinal peptides secretion (44–51). For example, oral glutamine or arginine increases the secretion of GLP-1 and improves glucose tolerance in rodents (50, 51). Branched AAs, particularly leucine, can reproduce the effects of a HP diet. Leucine supplementation in the diet or in the drinking water reduces food intake in rats and mice (46, 52). Furthermore, leucine icv injection, but not tryptophan, threonine, methionine, lysine, and serine, reduces food intake and body weight (43, 53), indicating that at least part of the anorectic signal induced by leucine is generated centrally. Leucine and HP diet exert their effect *via* an increase of mTOR activity and a decrease of AMPK activity in the hypothalamus, which leads to an increase in the anorectic pro-opiomelanocortin and a decrease of the orexigenic NPY and AgRP in the arcuate nucleus of the hypothalamus (53–55).

Dietary Protein and Reorientation of Metabolic Pathways

Consumption of a LP diet, providing only 5–6% of the energy as protein, increases food intake, adiposity, and intrahepatic fat content compared to a control diet (14, 15). The increase of the hepatic lipogenesis is correlated to an increase in the SREBP-1c transcription factor and of glycerokinase activity by 30 and 50%, respectively (56). However, using diet in which 10% of the energy was provided by proteins, Henagan et al. found that consumption of a LP diet is associated with a decrease in liver lipogenesis, and in particular of the expression of stearoyl-coenzyme A desaturase (SCD-1), FAS, and SREBP-1c (57). The higher protein level used by Henegan may explain these conflicting results.

The triglyceride (TG) accumulation in adipocytes may also be related to a decrease in lipolysis. Indeed, Buzelle et al. showed that lipogenesis in adipocytes is usually lower under LP diet, and that these cells do not respond to the lipolytic action of noradrenaline (58). Thus, the disability to mobilize fat likely explains the accumulation of TG in white adipose tissue.

As the adiposity reduction related to HP diet is partially mediated by food intake reduction, it is necessary to use a pair-feeding group of animals to adjust the caloric intake of rats fed with a normo-protein diet with the one measured in rats fed with a HP diet (12). However, even if the rats fed with the standard diet are pair fed, they still exhibit a higher adiposity than the HP fed rats. These results support the view that the effects of HP diet involve the reduction of lipogenesis.

Fourteen days after the introduction of a HP diet, gene expression of FAS and ACC in the liver was suppressed (59). Moreover, the expression of FAS and SREBP-1c in the liver of rats fed for 8 weeks with a HP diet compared to rats fed a NP or NP pair-fed diet was also reduced (11).

Studies have shown that under a normo-protein diet, all AAs that are deaminated are oxidized rapidly. By contrast, under a HP diet, only half of the deaminated AAs are oxidized, resulting in the generation of a "carbon skeleton reserve" in the form of α-keto acids (60, 61). Furthermore, high plasmatic AA level increases both insulinemia and glucagonemia, which stimulates gluconeogenesis. Indeed, several AAs, including cationic AAs, are known stimulators of insulin secretion (62, 63). Moreover, Veldhorst and colleagues observed an increase in gluconeogenesis in healthy men fed a HP diet (64). In rats, the increase in dietary protein induces the expression of PEPCK in the fasted and fed rats and of glucose 6-phosphatase, only in the fasted state. These results suggest an increase in hepatic glucose synthesis (65). Ketogenesis is another metabolic pathway by which the carbon skeletons derived from the AA deamination can be managed. In fact, in humans and animals, an increase in circulating ketone body levels (especially β-hydroxybutyrate) was observed in response to HP diet ingestion (64). Finally, HP diets also allow a renewal of glycogen stores and an increase in the conversion of dietary AAs into glycogen (60, 66, 67).

Dietary Protein and Energy Expenditure

Postprandial thermogenesis is defined as the increase in energy expenditure after a meal or after ingestion of a given nutrient. This parameter results from the energy cost corresponding to absorption, digestion, and metabolism of nutrients provided by the meal. In humans, it has been shown that postprandial thermogenesis is in the range of 15–30% of the ingested energy for protein, while for carbohydrates and lipids this value is, respectively, between 5 and 10% and 0 and 3% (68, 69). Mikkelsen et al. showed that when protein energy contribution in meal is increased from 11 to 29%, the energy expenditure is also increased of about 10% per day (70). The increase in energy expenditure associated with the consumption of protein may partly explain their satiating effect. Indeed, several authors have suggested that increased metabolism had an inhibitory effect on food intake (71, 72).

Recent studies report that in mice fed with LP or HP diets, the postprandial thermogenesis is increased compared to normoprotein diet (73). Similar results are reported for total energy expenditure (14, 74, 75). In line with these results, it has been shown that the basal temperature is increased by 1.1°C in animals fed a LP diet, and that administration of norepinephrine is more efficient to increase the basal temperature (+0.2°C) in rats fed a LP diet when compared to rats fed a control diet (76).

The effect of LP and HP diets on total energy expenditure is mediated notably by a modulation of genes encoding uncoupling proteins (UCP). UCP are proton carriers that uncouple their return into the mitochondrial matrix for ATP production, thus decreasing energy production efficiency. The energy from substrates oxidation is then dissipated as heat. Studies have reported an increase in UCP1 expression under LP (76) and HP diets (55). Another study found similar results in rats, showing that

an increase in dietary protein intake is able to upregulate UCP2 expression in the liver. These changes that are associated with increased abundance in UCP are positively correlated to energy expenditure (75).

Both the effects of LP and HP diet on UCP expressions could be modulated by the restriction or supplementation of specific AAs, for instance, histidine supplementation increases the content of UCP1 in brown adipose tissue (44, 45).

On the other hand, Malloy et al. have shown that the energy expenditure in rats fed with a methionine-deficient diet was greater than that of rats fed *ad libitum* or in animals pair-feed to a control diet (77). Consistent with these findings, other studies have shown that methionine restriction was accompanied by an increase in energy expenditure, including thermogenesis and body temperature increase (78, 79).

Interestingly, the consumption of both LP and HP diets results in an increase in energy expenditure. The effects of the two types of diets are summarized in **Figure 1** below.

LP Diet and Protein Quality

Recent studies, using a new scoring system to qualify dietary protein quality, namely the digestible indispensable amino acid score (DIAAS), allowed a better comparison of protein quality. Indeed, this score is not truncated compared to usual systems used to evaluate protein quality like the nitrogen balance measurement. DIAAS classification is based on the relative digestible content of the essential AAs. This classification shows that dairy proteins have the highest quality (80). In accordance with this classification, a study in human volunteers has shown that milk proteins are more efficient to stimulate muscle protein synthesis than soy protein. The best effectiveness of milk protein for such an effect was correlated to the higher proportion of leucine (81).

Evaluation of the so-called ideal protein level to better maintain health is complex. Most animal studies designed to evaluate the impact of LP diet on health barely took into account protein quality, and this may explain discrepancy between different studies. A recent review of Le Couteur et al. (82) suggests that LP diets generate longer lifespans in *ad libitum*-fed mice. Interestingly, the same results were obtained using the *ad libitum* insect model, suggesting that these effects of LP diets apply to very different animal models. However, the protein leverage induced by LP diet, which increases food intake and fat deposition, is not always integrated. Restriction of particular AAs, such as methionine, has been shown to extend life duration in mice (83) and rat (84), and lower serum level of IGF-1, insulin, glucose, and thyroid hormone in serum (85).

A recent study using Balb/C mice under moderate protein restriction shown that protein quality is an important factor for biological effects. Ingestion of low quality protein that reduces IGF-1 serum level is related to decreased LBM and bone quality (86). This study included a group of control mice feed with a control soy-based normal protein diet including 20% of the total energy as soy protein (NP-SOY) and two other groups receiving LP diets. The first one was a soy-based protein restricted diet, with 6% of the total energy as soy protein (LP-SOY), while the second one was a casein-based protein restricted diet with 6% of the total energy as casein (LP-CAS). To avoid the protein leverage effect, a pair-feeding group corresponding to the LP groups was used. As all the diets were isocaloric, the pair feeding allowed to ensure that energy intake was similar in all the groups.

Over the duration of the experiment (60 days), total body weight of LP-SOY mice remained at the baseline value, while NP-SOY and LP-CAS mice gained weight. The difference in total body weight was related to a lower lean mass gain in LP-SOY when compared to LP-CAS and NP-SOY mice. Reduction of IGF-1 plasma level and bone quality related to reduce bone formation was observed in the LP-SOY group (**Table 1**) (86).

The comparison of the effects of LP-SOY and LP-CAS diets on various parameters thus indicates that protein quality is of prime importance in the case of moderate protein restriction. The observed effects on body composition and blood plasma parameters could be partly related to a difference in AA profile, as casein is richer than soy primarily in methionine, and also in proline, serine, threonine, glutamine, valine, tyrosine, isoleucine, and leucine (87–89). Previous studies suggested that reduction of particular AAs in the diet can extend lifespan in mice and rats. However, this study shows that reduced IGF-1 level, which is correlated with reduction of bone formation and LBM including uterus weight, do have adverse consequences on health parameters (86). This latter study shows that LBM response to nutritional interventions, particularly dietary protein quality,

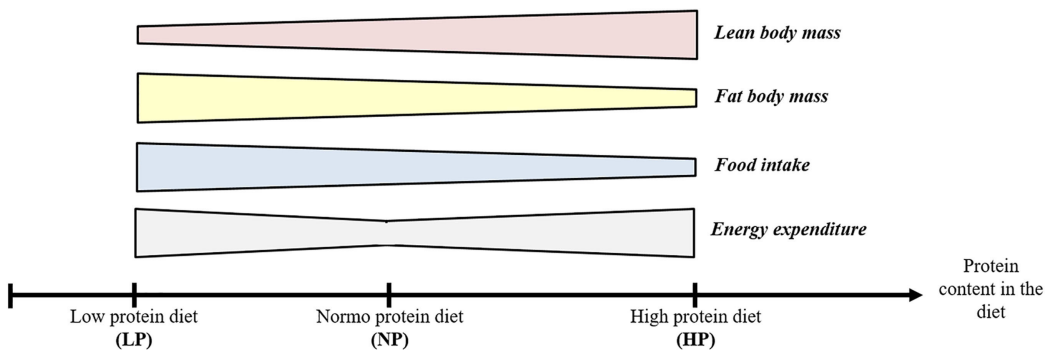

FIGURE 1 | Effects of low protein (LP) and high protein (HP) diet on body composition, food intake, and energy expenditure.

TABLE 1 | Effect of a soy- or casein-based protein restriction on body composition, bone quality, and bone turnover markers after 60 days.

	Diets		
	NP-soy	**Low-protein (LP)-CAS 6%**	**LP-soy 6%**
Weight gain (g)	3.41 ± 0.48^{a}	3.81 ± 0.36^{a}	0.55 ± 0.40^{b}
Lean mass gain (g)	2.05 ± 0.32^{a}	1.57 ± 0.18^{a}	-0.63 ± 0.26^{b}
Uterus (mg)	81 ± 5^{a}	52 ± 3^{b}	24 ± 1^{c}
Femur cortical thickness (mm)	0.232 ± 0.003^{a}	0.226 ± 0.002^{a}	0.205 ± 0.002^{b}
Femur length (mm)	15.72 ± 0.12^{a}	15.62 ± 0.13^{a}	15.00 ± 0.11^{a}
Femoral BMD change (delta%)	14.5 ± 0.5^{a}	13.8 ± 0.9^{a}	6.7 ± 1.5^{b}
IGF-1	325 ± 30^{a}	$302 \pm 18^{a,b}$	247 ± 15^{b}
PINP (bone formation marker)	1.76 ± 0.15^{a}	1.30 ± 0.13^{a}	0.65 ± 0.06^{b}

Data are means ± SEM (n = 15). A one-way ANOVA followed by Bonferroni's post hoc was performed. Means with different superscript letters (a, b, c) are significantly different ($p < 0.05$) according to a Bonferroni multiple comparison test (86).

is a good marker of the minimal dietary protein needed in this experimental model. This endpoint may be especially important for the aging population, because reduced protein intake which is often observed in the elderly, and which reduces LBM and bone mass are associated with fracture, reduction of life quality, and lifespan. However, data in humans also indicate that reduced protein intake may become an important component of anticancer and antiaging dietary interventions (90, 91), indicating that LP intake may induce heterogeneous biological effects. As discussed earlier, the evaluation of the ideal protein level for optimal effect on the health maintenance is complex and needs thus the evaluation of many health outcomes (80).

LP and HP Diet during Gestation, Lactation, and Perinatal Periods

The early-life period, starting even before birth, is a key determinant of adult health. Environmental exposure, particularly nutrition, has a programming effect on later metabolic health.

Low protein and HP intakes during gestation and lactation are commonly viewed as stressors that can lead to changes in the body composition of the offspring.

Thus, it has been shown that feeding LP maternal diet during both gestation and lactation, or only during lactation, decreases the body weight and adiposity in both males and females (92, 93). By contrast, protein restriction only during gestation has no effect on males but leads to a lower LBM and higher body fat mass in females (93).

Interestingly, feeding pregnant rats with a LP diet results in a preference for high-fat foods in the offspring at the age of 12 weeks (94). In the same study, the authors reported that females, but not males, failed to adjust their energy intake and exhibited a higher adiposity. In line with these results, it was shown that a maternal LP diet results in low birth weight and subsequent adipose tissue catch-up growth when the offspring is fed a high-fat diet in male rats (95). Taken together, these results suggest that the exposition to a LP diet result in low birth weight and predispose to obesity when exposed to a high fat diet during the postnatal period.

Infant formulas have a higher protein content than breast milk, and the subsequent increase in protein intake of infants consuming formulas has been associated with increased risk of obesity (96). Thus, the impact of HP intake during the perinatal period needs further studies.

The effects of HP diet during gestation and lactation in animal models are controversial, leading to either no change or birth weight decrease (97–99). Likewise, HP diets can induce increase or decrease of body weight and adiposity (97, 100), depending on the experimental design.

High protein diets during gestation have been associated with higher adiposity and decreased energy expenditure in young male rats (101). Sex-specific effects of HP diets during both gestation and lactation predispose females, but not males, to higher body weight and adiposity (99, 102).

The protein source ingested by the mother during gestation and lactation can also influence body composition. Thus, it has been reported that when the maternal diet include soy protein, the offspring exhibit a higher body weight and adiposity compared to the offspring of dams fed with a casein-based diet. This is probably due to an alteration of food intake regulation in the offspring of dams fed a soy protein-based diet (103). Further experiments, including epigenetic modification measurement, are needed in order to decipher the underlying mechanisms explaining these latter results.

IMPACT OF ESTROGEN DEFICIENCY ON BODY WEIGHT AND PHYSIOLOGICAL/ METABOLIC PARAMETERS: IMPACT OF DIETARY PROTEIN

The prevalence of metabolic syndrome, a constellation of abnormalities that includes obesity, hypertension, glucose intolerance, and dyslipidemia is higher in men than in women, but according to some epidemiological studies, this gender difference disappears after menopause (104). Animal studies have also reported protection of female mice from development of diet-induced obesity compared to age-matched males (105). However, as mentioned earlier, this advantage is lost in women at menopause, and the estrogen level decline is associated with central adiposity, insulin resistance, decreased energy expenditure, and greater risk of cardiovascular diseases (106, 107). Estrogen withdrawal during menopause is also associated with increased production of pro-inflammatory cytokines that are involved in many different diseases including osteoporosis, rheumatoid arthritis, and multiple myeloma (108). **Figure 2** summarizes estrogen actions in the brain, adipose tissue, pancreatic islets, skeletal muscles, bone, liver, and macrophages, indicating the impact of estrogen on many tissues. These latter are known to act in synergy to promote glucose and lipid homeostasis.

As it is difficult, for obvious ethical reasons, to study in depth physiological and metabolic consequences of menopause in women, notably in a mechanistic perspective, surgical ovariectomy in animal models has been used to mimic estrogen deficiency. Ovariectomized (OVX) animal models have been widely used to study metabolic disorders associated with decrease

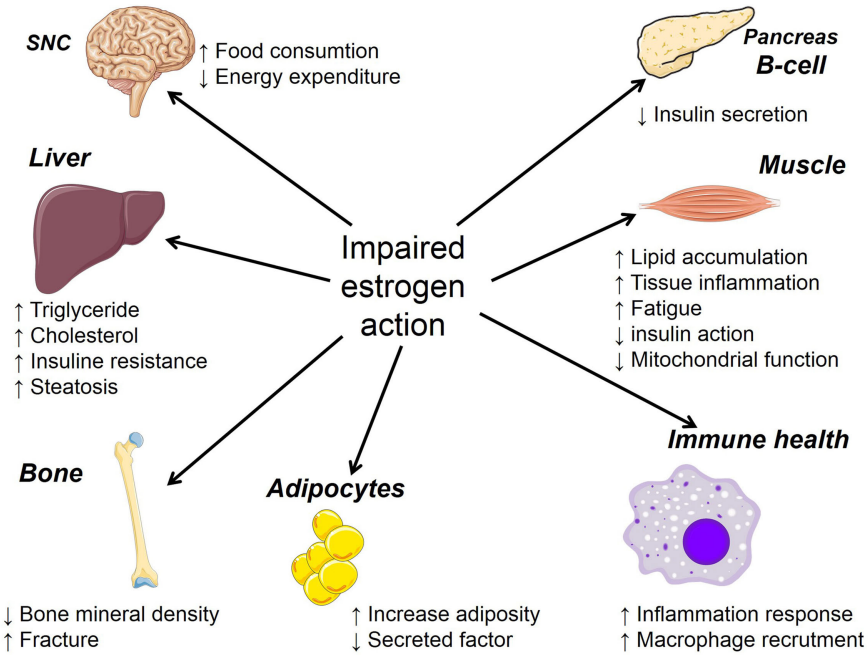

FIGURE 2 | Summary of the consequence of impaired estrogen action on the physiology of many target organs.

of estradiol secretion in order to evaluate pharmacological and nutritional treatments that can safely reduce the consequences of menopause. Indeed, the use of OVX rats or mice to simulate the postmenopausal conditions is well established and represents a reproducible model. Notably, OVX animal models mimic metabolic modifications related to estrogen deficiency over a relatively short period of time. Witte et al. (109) showed that female rats and female mice do not have similar metabolic and behavioral responses after ovariectomy, demonstrating species differences in this experimental model. The OVX-induced weight gain in rats is mediated both by hyperphagia and reduced locomotor activity, while in mice the OVX procedure reduced locomotor activity and metabolic rate. Such species differences in response to OVX need to be taken into account when results are tentatively extrapolated to humans.

The most commonly used mice strain used to mimic consequences of estrogen withdrawal are C57BL/6J mice and C3H/Hen mice. One of the main consequences of the OVX procedure is an increase in the body weight. Incidentally, those models have been setup to study not only consequences of estrogen deficiency on obesity but also on various diseases including cancer, osteoporosis, cardiovascular diseases, and inflammation. Using the OVX C3H/Hen mice model to induce bone loss, studies (88, 89) have shown that even when the surgery was performed at 3 or 6 months, the procedure induced an increase in body weight related to an increase in the visceral and subcutaneous fat mass. However, when the OVX procedure was performed at 6 months, reduction of uterus weight was not observed compared to Sham mice. The spontaneous uterine atrophy observed in older Sham mice explains the absence of measurable difference. In the same study, the effect of raloxifene, a drug commonly indicated for osteoporosis which activates estrogen receptor (ER), prevented bone loss and the increase in body weight and fat mass observed following the OVX procedure (**Table 2**). However, raloxifene supplementation was not able to inhibit uterus weight loss. The preventive effect of raloxifene on weight gain in OVX C3H/Hen mice is in agreement with a recent study showing, in another mouse model, that selective activation of ER positively regulates mice metabolism (110).

Mutations of ER are correlated to different aspects of the metabolic syndrome. Reduced ERα levels in the adipose tissue of obese individuals compared to the non-obese counterparts support a role of estrogen signaling in the control of body weight (112). The impact of ER activation on metabolic dysfunction related to menopause has also been studied using mice strain with a specific deletion of the ERα. Those mice become obese, glucose intolerant, hyperinsulinemeic and have decreased energy expenditure, decreased locomotion, and increased secretion of pro-inflammatory factors. The fat mass increase is associated not only with a decrease of the energy expenditure and of fat oxidation but also with an elevation of the circulating inflammatory markers (113, 114). Consequences of ERα deletion or ovariectomy are similar, supporting that ERα regulates mice energy metabolism (113). Moreover, loss of ERα in the central nervous system has been shown to induce hyperphagia and to decrease energy expenditure (115). Estradiol (E2), the major biologically active form of estrogen, is also known to positively influence insulin action in mice (113). Moreover, estrogen protection of female mice from development of diet-induced obesity and insulin resistance compared to age-matched males has also been demonstrated (105). A recent study using the ovariectomized mice model has shown that stimulation of estradiol receptor

TABLE 2 | Effect of ovariectomy, hormone replacement, or lactoferrin supplementation in OXV mice on body composition and bone mineral density after 12 weeks.

	Groups			
	Sham	**OVX**	**OVX + raloxifene**	**OVX + LF**
Initial body weight (g)	25.14 ± 1.18	24.20 ± 1.43	24.32 ± 1.06	24.70 ± 2.06
Final body weight (g)	35.50 ± 2.34^a	41.74 ± 2.49^b	34.61 ± 2.86^a	42.68 ± 3.39^b
Weight gain (g)	10.36 ± 1.84^a	17.54 ± 2.21^b	10.29 ± 1.43^a	17.98 ± 2.48^b
Uterus (mg)	253 ± 41^a	131 ± 29^b	127 ± 28^b	160 ± 28^b
Fat mass (g)	7.23 ± 1.63^a	12.32 ± 2.03^b	8.25 ± 1.35^a	12.54 ± 2.15^b
Carcass (g)	11.37 ± 0.59	12.87 ± 1.17	11.84 ± 0.72	12.54 ± 1.57
BMD gain (mg/cm^2)	13 ± 2	8 ± 3^a	13 ± 2	13 ± 2

The surgery was performed on 12-week-old C3H mice. Data are means ± SEM (n = 10). A one-way ANOVA followed by Bonferroni's post hoc was performed. Means with different superscript letters (a, b) are significantly different (p < 0.05) according to a Bonferroni multiple comparison test [adapted from Ref. (88, 89, 111)].

prevents weight gain, insulin resistance, and improved systemic metabolism.

Food intake and spontaneous physical activity have been measured in OVX C3H/Hen mice, and both parameters were reduced compared to Sham mice (**Figure 3**). As the spontaneous physical activity reduction of the OVX mice is able to explain only a small part of the reduced ingestion, it is likely that the increased body weight and adiposity is related to a 15% decrease of the resting metabolism in the OVX mice. The absence of increased food intake and the reduced metabolic rate have also been reported in C57BL/6J mice (109). However, in mice lacking ERα in the central nervous system, a hyperphagia and decreased energy expenditure have been reported (115).

Moreover, using OVX C3H/Hen mice, it has been also possible to demonstrate that the OVX procedure is associated with immunological dysregulation. Indeed, Malet et al. (111) shown that estrogen deficiency induces heightened immune response sensitivity and an inflammatory status that were correlated to bone loss. Estrogen withdrawal is associated with T cell activation that produces essential osteoclastic factor such as RANKL and TNFα (108, 116). Lactoferrin (LF) ingestion has been shown to reduce T cell activation, pro-inflammatory cytokines, and consequently bone loss (111). Interestingly, LF is as efficient as raloxifene for the maintenance of bone mineral density in OVX mice, but did not reduce weight gain. However, neither compounds were able to preserve uterus weight (**Table 2**).

The menopause transition is associated not only with an increase in total body fat mass, visceral fat mass, and decreased energy expenditure but also with the increase of many inflammatory markers. Such an increase has indeed consequences on the incidence of many other pathologies including osteoporosis. However, obesity has been considered to have some beneficial effects for bone health in humans by some authors (117). The increase body weight and the ability of adipose tissue to synthesize estrogen support this proposition (118, 119). Since it has been proposed that estrogen synthesized by adipose tissue may have some antiresorptive effect on bone, Cao and Gregoire (120) studied the effect of high-fat diet on bone quality in OVX mice. This study shows that OVX mice fed with a high-fat diet, gain more weight, and had a higher estradiol level than mice feed with a standard diet, raising the question of the origin of estradiol production. However, the high-fat diet was not able to mitigate the OVX-induced bone loss in mice. Then, authors proposed that estrogen, likely synthesized by adipose tissue, does not have the same antiresorptive effect on bone as estrogen secreted by ovaries.

Those studies indicate that many non-elucidated mechanisms are involved in energy homeostasis in OVX mice. However, as OVX mice have a reduced energy expenditure similar to the one observed in estrogen-deficient women, it appears that mice is a useful model in that topic. Regarding the effect of dietary protein on dysfunctions related to ovariectomy, there is a relative paucity of available data. It has been shown that supplementation with water-insoluble fish protein has a cholesterol lowering effect in ovariectomized rats (121). Another dietary protein source, that is soybean extract, has been found to modulate the level of serum TGs in ovariectomized rats fed a cholesterolemic diet (122).

THE PIG MODEL FOR RESEARCH ON OBESITY

Regarding extrapolation to human situations, it is worth noting that the pig model is often considered as a model closer to humans than rodents for several aspects of physiological and metabolic studies. Indeed, the pig model has emerged as a relevant non-primate experimental animal for extrapolation to humans because of numerous similarities regarding anatomy, development, nutrition, and physiology (123–126). Pigs are also an animal model that is truly omnivorous, which make spontaneously individual meals, and which display striking similarities with humans in terms of nutritional requirements (127). It is also worth noting that the gut in the newborn pigs, although more mature that in newborn rodents, is, however, less mature than in infants (128). Another advantage of the pig model is that it is possible to recover a large number of cells (for instance, absorptive intestinal cells from both the small and large intestine after dietary intervention), even in young animals, in order to measure the impact of such intervention on cell metabolism and physiology (129, 130). In the pig model, preterm delivery at 90% gestation is comparable to preterm infant born at approximately 75% gestation (30 weeks) (131), making the pig neonate an interesting model for pediatric studies. In contrast to the rodent models, the size of newborn pig easily allows for tissue sampling and experimental manipulations of nutritional,

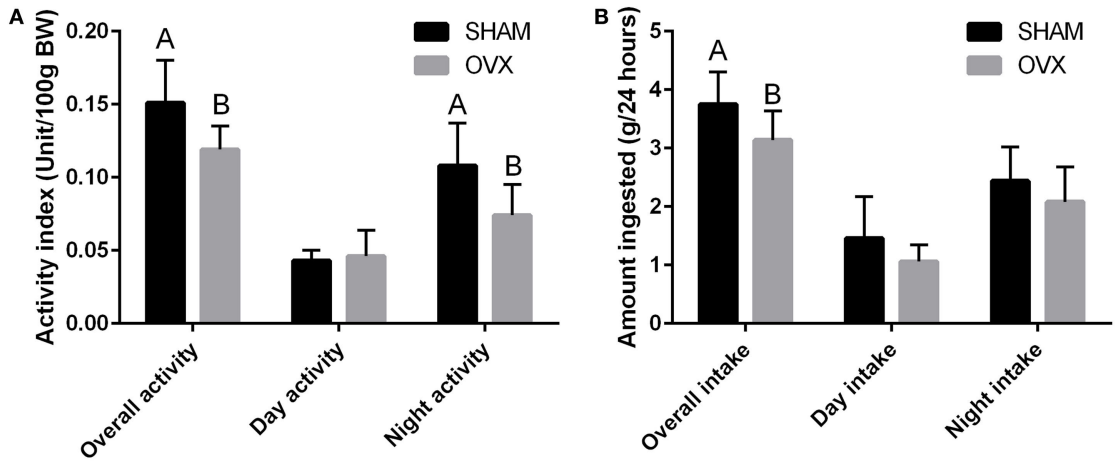

FIGURE 3 | Daily food intake analysis (A) and spontaneous physical activity (B) of Sham and OVX performed 10 weeks after the OVX procedure. Data are means ± SEM ($n = 8$). Groups with different letters are significantly different ($p < 0.05$).

physiological, and metabolic conditions. Finally, the pig model allows multi-catheterization and blood sampling without anemia thus allowing kinetics experiments (132).

However, even if the mini pig models, although relatively expensive, are increasingly used for research projects notably due to their limited size compared to regular farm pigs, the pig model present some drawbacks since it requires extensive areas for breeding, and is a source of abundant polluting compounds in biological fluids (fecal matter and urine), a situation incompatible with the use of the pig model in urban areas. As a matter of fact, in PubMed, the number of articles related to pig and obesity (798 articles) represents only about 2% of the number of articles related to rats/mice and obesity (35,318 articles). The readers are invited to refer to recent reviews regarding the pig model used for studies regarding the genetics of adiposity (133), the obese type 2 diabetes (134), the dietary modulation of gut microbiota and possible impact on obesity (135), the gastrointestinal hormones for eating control (136), and finally the establishment of food preferences and aversions (123) since these aspects will not be developed in this review. Regarding the specific aspect of the impact of the quantity and quality of dietary proteins on the gastrointestinal health in pigs, it has been shown that intestinal fermentation of the proteins results in the production of various potentially deleterious luminal products, which is often associated with growth of potential pathogens. In fact, excessive dietary protein intake (that mimic HP slimming diet) has been shown to stimulate in the pig model the growth of *Clostridium perfringens* and to reduce fecal counts of beneficial *Bifidobacteria* (137).

An increasing number of studies in pigs indicates that the gastrointestinal health is influenced by both the composition of the intestinal microbiota and its metabolic activity (138), this latter being impacted by the dietary composition, notably in terms of quantity and quality of dietary proteins (139). Indeed, the protein digestibility and protein AA composition are parameters that impact the profile of AA-derived bacterial metabolites in the large intestine. The use of fermentable carbohydrates to reduce deleterious protein-derived bacterial metabolites in pigs is well established (140), and for instance, soybean oligosaccharides have been shown to increase the presumably beneficial short-chain fatty acids while decreasing the protein-derived catabolites in the intestinal luminal content in weaned piglets (141). Last, interesting data have been recently obtained regarding the impact of the amount of dietary protein consumed by pigs on parameters like expression of AA and peptide transporters (125), or signaling pathways related to protein synthesis in muscles (142). Then, from these examples, it appears that using pig models for confirmation of data obtained in rodents represents a useful experimental strategy before further development of clinical studies implying dietary intervention with human volunteers, notably in overweight and obese individuals.

CONCLUSION AND PERSPECTIVES

Animal models are necessary in order to understand the mechanisms underlying the various biological parameters involved in the risk of obesity. Even if it is recognized that obesity results primarily from higher long-term energy consumption than energy expenditure, notably in case of low level of physical activity, we present here two situations in which animal models have been useful to understand how dietary (quantity and quality of dietary proteins) and physiological (menopause) modifications can impact parameters closely related to the development of obesity including body composition, food intake, energy expenditure, and tissue metabolism and physiology. Future research, notably in terms of mechanisms of action, using relevant animal models on the impact of dietary modifications at the different periods of age (notably during gestation, lactation, and perinatal periods of life) should allow to better enlighten on the best strategy for limiting the risk of obesity in young and aging adults.

AUTHOR CONTRIBUTIONS

C-DT and BA wrote the manuscript that was improved by BF and TD.

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REVIEW
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Consumption of Substances of Abuse during Pregnancy Increases Consumption in Offspring: Possible Underlying Mechanisms

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Correlative human observational studies on substances of abuse have been highly dependent on the use of rodent models to determine the neuronal and molecular mechanisms that control behavioral outcomes. This is particularly true for gestational exposure to non-illicit substances of abuse, such as excessive dietary fat, ethanol, and nicotine, which are commonly consumed in our society. Exposure to these substances during the prenatal period has been shown in offspring to increase their intake of these substances, induce other behavioral changes, and affect neurochemical systems in several brain areas that are known to control behavior. More importantly, emerging studies are linking the function of the immune system to these neurochemicals and ingestion of these abused substances. This review article will summarize the prenatal rodent models used to study developmental changes in offspring caused by prenatal exposure to dietary fat, ethanol, or nicotine. We will discuss the various techniques used for the administration of these substances into rodents and summarize the published outcomes induced by prenatal exposure to these substances. Finally, this review will cover some of the recent evidence for the role of immune factors in causing these behavioral and neuronal changes.

Keywords: prenatal fat, prenatal ethanol, prenatal nicotine, inflammation, ingestive behavior

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INTRODUCTION

Scientific research has relied heavily on the use of animal models to identify various characteristics of diseases and disorders found in humans. These animal models serve an important purpose when there is limited ability to ethically evaluate such disorders in humans. Most limiting in human research are studies of embryonic development and the effects produced by exposure to substances of abuse, such as alcohol, nicotine, and dietary fat, which occur as a result of voluntary maternal consumption. In humans, ingestion of alcohol during pregnancy triggers neurological disorders and increases the risk of fetal alcohol syndrome in the offspring (1, 2), effects subsequently confirmed and characterized using animal models (3–5). Also, smoking during pregnancy increases the risk of a decrease in birth weight (6, 7) and multiple behavioral problems (8), including attention deficit disorders (9) and increased propensity to abuse drugs (10, 11). In human observational studies, increased intake of dietary fats and obesity during pregnancy are found to increase the risk for dietary obesity in offspring (12–14).

Further testing of these physiological and behavioral changes using animal models exposed to substances of abuse have revealed disturbances in the development of neuronal circuits that modulate

both homeostatic and reward pathways (15–17). The main players involved include a variety of neuropeptides that are found in various regions of the hypothalamus and forebrain and are shown to modulate neuronal function that may ultimately contribute to the behavioral changes in offspring. These behavioral changes include an increased propensity to ingest these substances of abuse (15–18), with a significant crossover effect from one substance to another (19). Although great strides have been taken to characterize these changes in neurochemical systems that control behavior, the molecular mechanisms involved in producing these disturbances in the brain have yet to be determined.

In addition to these neurochemicals, the field of ingestive behavior has recently focused attention on immunology. These new studies build on prior research of neurological disorders and neurodegenerative diseases, which show the immune system to play a large part in the health, function, and development of neurons and other cell types in the central nervous system (20–24), along with the development of embryos (25, 26). Recently, both human and animal observational studies have demonstrated that exposure to these substances of abuse, in addition to altering classical neuropeptide and neurotransmitter systems, also disturbs inflammatory systems in key regions of the brain that control ingestion and related behaviors (17, 27, 28). Prenatal inflammation itself has been shown to increase the risk of developing neurological disorders and diseases, such as autism (29, 30) and other psychiatric disorders (31), which the offspring are at a higher risk of developing when exposed during gestation to substances of abuse (32–35).

To understand these neurochemical and immune systems affected by prenatal substance exposure and their possible role in promoting consummatory and other behaviors in the offspring, the use of animal models involving prenatal manipulations is clearly essential. This review will cover the current techniques used to perform prenatal studies using rodent models and their general conclusions about the neuronal changes induced by embryonic exposure to environmental substances. It will also summarize the current research linking these neuronal and neurochemical changes to inflammatory systems, focusing on the three most commonly abused substances, dietary fat, alcohol, and nicotine.

EXPERIMENTAL METHODS USED TO INTRODUCE ENVIRONMENTAL FACTORS INTO PREGNANT RATS

There are a few factors that must be taken into consideration when designing an experiment using a rodent model. The first is to choose the appropriate rodent strain to use in your model, with various strains having different preferences for different substances. Rat strains with a preference for dietary fats include Sprague-Dawley (36), Brattleboro (37, 38), and Zucker (39), with the latter having an obese phenotype. In alcohol studies, several different strains are used, including outbred rats such as Wistar (40) and Long-Evans (41), and also genetically modified rats that have increased alcohol intake, such as ALKO alcohol (42), high alcohol drinking or HAD (40), and Sardinian preferring (sP) (43) rats. In nicotine research, rats have been found to show differences in behavioral effects between different strains (44, 45) that are attributed to genetic variability. Some of the rat strains used in nicotine studies include Sprague-Dawley (46), Long-Evans (47), Lewis (48), Holtzman (49, 50), and Wistar (51).

The second factor is choosing the method for administering the substance of abuse. This can be broken down into two main paradigms, forced or choice. The forced paradigm does not give the animal a choice in intake, with the substance being the only option or its administration being forced. This is in contrast to a choice paradigm, whereby the animal has one or more competing substances to choose from, with one of the options generally being a control substance, such as chow or water. Studies on dietary fat have used both choice and forced paradigms, with some reports using a combination of the two. Generally, a high-fat and a low-fat diet are made available to the rat, with intake measured daily (52, 53). In combination paradigms, rats are exposed to the high-fat diet in conjunction with their usual diet for a period of several days until acclimation to the new diet is achieved, after which the high-fat diet is given as the only choice (52, 53). Under forced conditions, rats may be given an oil emulsion *via* oral gavage (54). Studies of ethanol and nicotine, in choice paradigms have used both methods of self-administration (55, 56) and two bottle conditions (57–59). Generally, the concentration of ethanol or nicotine is given in intervals, ranging from a low to high concentration, until the desired concentration is reached (60, 61), with some groups combining palatable sucrose with ethanol or nicotine until voluntary drinking of the drug is established (62). Forced exposure methods, in contrast, include oral gavage, direct injection into the peritoneal cavity (61, 63), intravenous infusion (64), a liquid diet (65), or having the substance as a sole liquid source (66–69).

In studies relating inflammation to ingestive behavior, a specific inflammatory mediator or an agent that induces inflammation, such as lipopolysaccharide, can be administered to any area of the rat through injection. This includes systemic infusion (70), intraperitoneal injection (71, 72), or use of an osmotic minipump (73–75).

These methods are only a brief summary, with a wide range of models and rodents used to study the effects of prenatal exposure. Once a particular model is well established, further measurements of behavior in tissues and cells of different type can be extensively performed. The sections below will focus on our current understanding of how prenatal exposure to substances of abuse affect neuronal systems that control behavior in offspring and how the inflammatory response may be a factor in promoting those changes (**Figure 1**).

PRENATAL HFD EXPOSURE

Animal models investigating the effects of excessive HFD intake during pregnancy have revealed several changes in both the physiology and behavior of offspring. Prenatal exposure to a HFD has been shown to induce several effects in offspring. These include increased body weight, faster weight gain, and larger fat pads (15, 76–78), as well as behavioral changes that include increased ingestion (15, 52, 76), autism spectrum disorders (32,

33), depression (79), and attention hyperactive disorders (80) along with a decrease in spatial memory acquisition (78, 81). Increased understanding of the neuronal systems involved in invoking these behavioral changes is made possible by the numerous animal models used to study these phenomena. These behavioral changes have been attributed to changes in the neurochemistry of various brain regions involved in homeostatic, reward, emotional, and memory processes (**Figure 2**) and, more recently, to changes in inflammatory processes.

Prenatal HFD Exposure Alters Hypothalamic Neurocircuitry

Changes in specific brain areas caused by prenatal HFD exposure seem to control different aspects of HFD intake. The change in homeostatic processes occurs in the hypothalamus, a region important in controlling ingestive behavior. Several lines of evidence show prenatal exposure to a HFD to produce changes in both the developing embryo and in adolescent and adult offspring. These include an increase in the neurogenesis of hypothalamic orexigenic peptide neurons (15, 82), with increased synthesis of the peptides that further induce HFD intake (15). These neuropeptides include galanin and enkephalin in the medial paraventricular nucleus (15, 83), orexin and melanin-concentrating hormone in the perifornical lateral hypothalamus (15), and also ghrelin in the midbrain (84).

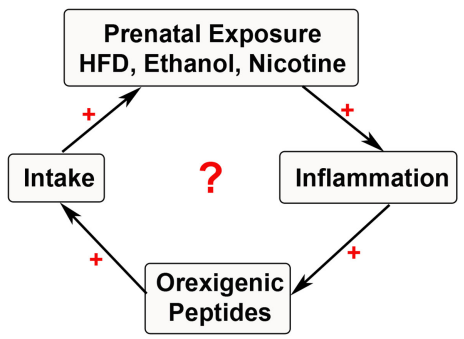

FIGURE 1 | Cycle of substance exposure. The schematic depicts the current hypothesis of a simplified positive feedback loop involving prenatal exposure to substances of abuse that stimulate inflammatory systems. This inflammation may be involved in stimulating neuropeptides that further increase ingestive behavior, thus leading to a cyclical increase in exposure during the prenatal period with negative outcomes in the offspring.

Prenatal HFD Exposure Alters VTA–NA System in Offspring

The centers controlling the rewarding aspects of intake consist of the ventral tegmental area (VTA) and the nucleus accumbens (NA) core and shell, which contain the dopaminergic signaling system, μ-opioid receptors, and glutamatergic inputs that are activated by rewarding substances (85, 86). Similar to drug addiction (85), prolonged intake of a HFD has been shown to block dopamine reuptake and enhance dopaminergic function (87). Similarly, exposure to this diet during the prenatal period has been found in adult offspring to increase the levels and expression of dopamine in the NA core and decrease the expression of tyrosine hydroxylase in the VTA, thus decreasing the formation of dopamine (88, 89). Reduced expression of the μ-opioid receptor (89) and increased levels of enkephalin are also found in the VTA and NA regions, with injection of an enkephalin analog into the NA shown to increase HFD intake (90, 91). Similar changes in dopamine, dopamine transporter, and μ-opioid receptor have been found in other studies using maternal junk food or obesity-prone offspring (92, 93), in addition to a reduction in dopamine release in the nucleus accumbens and other terminal sites of dopamine release (92). These studies suggest that prenatal HFD exposure markedly alters the reward pathway, inducing a compensatory mechanism that leads the offspring to ingest excessive amounts of dietary fat to obtain a rewarding feeling caused by the reduced dopamine function (88, 92). Epigenetic changes involving hypomethylation are also found for the dopamine transporter, μ-opioid receptor, and enkephalin, suggesting long-term changes and consequences in offspring (89). While studies in the VTA–NA system have mostly focused on dopamine and agonists of the μ-opioid receptor, other targets are also involved. These include ghrelin, a neuropeptide, known to stimulate the

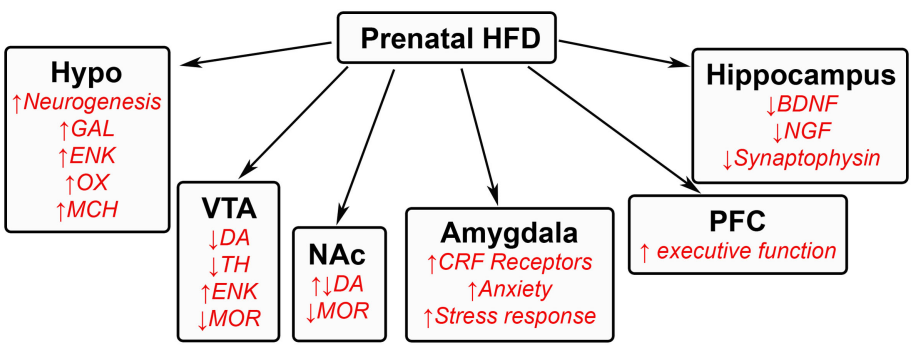

FIGURE 2 | Effects of prenatal HFD exposure on offspring brain. A schematic summarizing some of the changes that occur in the brains of offspring after being exposed to a HFD during gestation. GAL, galanin; ENK, enkephalin; OX, orexin; MCH, melanin-concentrating hormone; DA, dopamine; TH, tyrosine hydroxylase; MOR, μ-opioid receptor; CRF, corticotrophin releasing factor; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor.

rewarding effects of food intake (94) and promote the rewarding feeling of food intake (95), which is also abundantly expressed in the VTA and found to increase HFD feeding after injection into the VTA (84).

Prenatal HFD Has Global Effects on Other Areas of the Brain in Offspring

Other brain regions also show permanent changes that affect behavior. In the hippocampus, prenatal HFD exposure in offspring decreases expression and levels of proteins that are involved in memory function, such as brain-derived neurotrophic factor, nerve growth factor, and synaptophysin, suggesting a delay in memory acquisition (78, 81). The transcription of genes controlling executive function in the prefrontal cortex is also markedly increased by dietary fat in offspring (96). The emotional aspect of feeding, controlled by the amygdala, has been found in adult rats to evoke several changes in neurochemical pathways (97, 98) that in turn may induce changes in anxiety as well as feeding. Although there are only a few studies of prenatal HFD exposure that have examined the amygdala, there is some evidence that altered functioning of this brain region is involved in emotional changes in offspring that may further promote consumption. Exposure to a fat-rich diet during the prenatal period causes in offspring an increase in corticosteroid receptors in the amygdala (99). This exposure also increases anxiety in an open field, an elevated plus maze, and during light–dark transition tasks, while increasing corticosteroid levels in response to stress (99), suggesting an overall increase in the stress response and thus anxiety. These responses have been reported to increase ingestive behavior in attempt to reduce stress (100–102). These global brain changes affecting decision-making may be involved in both the impulsive and rational choice to overeat.

Prenatal HFD Induces Epigenetic Changes in Offspring

The effect of a HFD during the prenatal period on gene expression in developing neurons is thought to be attributed to epigenetic changes. In human adults, several studies in peripheral tissue reveal alterations in histone modification at promoters of proteins that are affected by dietary fat (103) and in methylation in specific tissue such as skeletal muscle (104). Prenatal exposure to a HFD has also been shown to alter methylation or microRNA expression in placental tissue (105) and adipose tissue (106, 107). That epigenetic changes may be transmitted to offspring is indicated by studies showing a generational effect on specific genes during dietary protein restriction (108, 109). While there is little evidence on the epigenetic effects of prenatal HFD exposure in neurons of embryos and postnatal offspring, several reviews exist that describe global metabolic epigenetic changes in the periphery (110, 111), indicating the need for more such studies in the brain.

Relationship between Dietary Fat and Inflammation

While several studies examining the effects of acute and chronic inflammatory mediators in adult obese animals have revealed an increase in fat intake and weight gain (112), evidence from prenatal inflammatory studies is more limited. Early findings show chronic HFD intake to induce a systemic low-grade inflammation characterized by an increase in cytokines and chemokines (113, 114). This HFD intake also increases the activation of several inflammatory signaling pathways, such as jun amino-terminal kinases, nuclear factor kappa light-chain enhancer, inhibitor of nuclear factor kappa-B kinase subunit beta, peroxisome proliferator-activator receptor, and toll-like receptors (115–118). Chronic treatment with an agent, such as lipopolysaccharide, that induces inflammation can increase body fat mass and caloric intake, and these effects are exacerbated by a HFD (119), suggesting a strong link between HFD and inflammation. More recent studies have uncovered a major role for chemokines, specifically CCL2, which is affected by a HFD and may also mediate neuronal function. This chemokine has been found early on to be increased in obese animals and during HFD intake (120) and, along with its receptor CCR2, is found in all of the key brain areas involved in HFD ingestion (121, 122). Furthermore, blocking the CCR2 receptor with an antagonist is shown to improve symptoms of obesity and decrease food intake (123, 124). In limited studies, prenatal HFD exposure has been found to increase CCL2 in peripheral organs, such as the liver, in offspring (125).

Recent studies from our lab have found a positive relationship between CCL2 and both the migration and expression of orexigenic peptide neurons in primary hypothalamic neurons (126). Exposing cultured embryonic hypothalamic neurons to increasing levels of CCL2 revealed a dose-dependent increase in migration as well as expression of the orexigenic peptides, enkephalin, and galanin in neurons (126). These hypothalamic enkephalin-expressing neurons are found to co-express the receptor, CCR2 (**Figure 3**), with CCL2 treatment increasing the number of colocalized neurons (126), suggesting an important role for this chemokine in neuronal growth during the prenatal

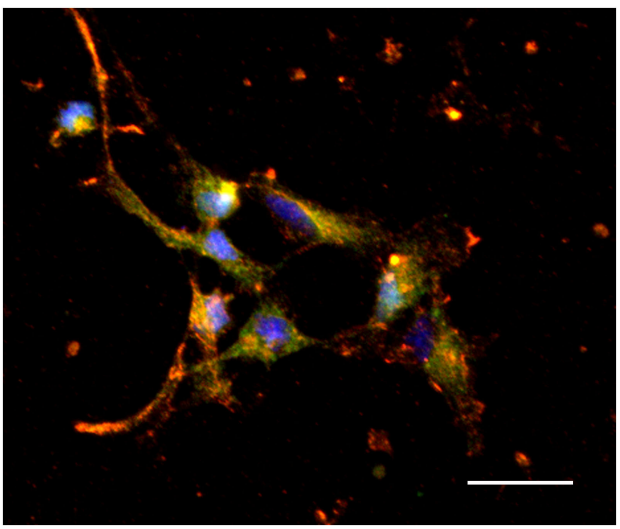

FIGURE 3 | Colocalization of CCR2 and enkephalin in hypothalamic neurons. Hypothalamic neurons extracted from chow-exposed embryos showing CCR2 to colocalize with the orexigenic peptide, enkephalin (orange). Scale: 25 μm. Green: enkephalin, red: CCR2, and blue: dapi.

period. In addition, in rats exposed to a HFD during gestation, this chemokine system is found to be greatly altered (127). Prenatal HFD exposure decreases expression of CCL2 while increasing the expression of its receptors, CCR2 and CCR4, in the hypothalamus, and these HFD-exposed neurons are found to exhibit markedly reduced sensitivity to the actions of CCL2 on neuronal migration and peptide expression. With this limited evidence raising new but interesting questions, future studies using the prenatal HFD and prenatal inflammation models should shed light on the molecular mechanisms leading to the neuronal changes and, in turn, altering ingestive behavior in the offspring.

PRENATAL ETHANOL EXPOSURE

Original ethanol studies have shown that exposure during the prenatal period to high levels of alcohol is associated with developing fetal alcohol spectrum disorder in offspring (1–5), with many negative developmental, behavioral, and physiological outcomes (128, 129). These high levels of alcohol, within 20–30% or 6 g/kg/day range, decrease the development of neurons in several brain areas (40, 130–132) and additionally induce epigenetic changes in fetal DNA (133–135). Currently unknown are the effects of low levels of alcohol consumption, within 5% or 1–2 g/kg/day range (16, 136–138), on fetal development and ultimately on offspring behavior. Recent studies have demonstrated that low levels of ethanol exposure during gestation induce several behavioral, neurochemical, and developmental effects, similar to prenatal HFD exposure that are caused by changes in brain regions involved in homeostasis, reward, emotional, memory, and inflammatory processes. These changes are thought to induce excessive drinking (16, 137–139), increased preference (17, 139, 140), and reinforcement (141, 142) for ethanol in offspring during the adolescent period to adulthood. These low levels have also been linked to other behavioral changes, such as hyperactivity (74). While ethanol has several targets in many brain regions, studies of low ethanol levels are lacking. This review will summarize some of the current findings in the field (**Figure 4**).

Low Levels of Ethanol Exposure Alters Hypothalamic Neurocircuitry in Offspring

In the hypothalamus, the same orexigenic neuropeptides known to stimulate HFD intake, namely enkephalin, galanin, orexin, and melanin-concentrating hormone, are also found to stimulate ethanol intake [for review, see Ref. (143); (17)]. While different neurochemical systems in the brain are known to be altered by prenatal exposure to ethanol (144–146), the stimulatory effects of prenatal ethanol on these specific neuropeptides are particularly notable, given the potency of their effects on behavior and the sensitivity of the peptide neurons to low doses of ethanol (16, 147). A study from our group has also found low levels of ethanol to increase the genesis of hypothalamic neurons containing enkephalin, orexin, galanin, and melanin-concentrating hormone (16, 17). Additionally, prenatal ethanol exposure is shown to affect stress hormones in the hypothalamus, causing in adolescent and adult offspring an increase in the expression of corticotropin-releasing factor (CRF) in the hypothalamic paraventricular nucleus (144, 148–149) along with levels of corticosterone (144) and also adrenocorticotropic hormone in this same region (144, 150). Prenatal ethanol also increases the levels of these peptides and hormones in response to stress (151–154), with increased stress linked to further consummatory behavior (155–157). Not surprisingly, the CRF system has been linked to addiction of other substances of abuse (158), including dietary fat.

Low Levels of Ethanol Exposure Alters VTA–NA Center in Offspring

Several studies have linked low levels of ethanol during the prenatal period to changes in the mesolimbic area. Increased neurogenesis of enkephalin neurons is found in the NA shell (16), with overall increased levels of enkephalin in both the VTA (159) and NA core (147, 160). These changes may significantly increase ethanol intake in offspring, as high levels of enkephalin have been shown to activate dopamine terminals in the NA (161, 162). The effects

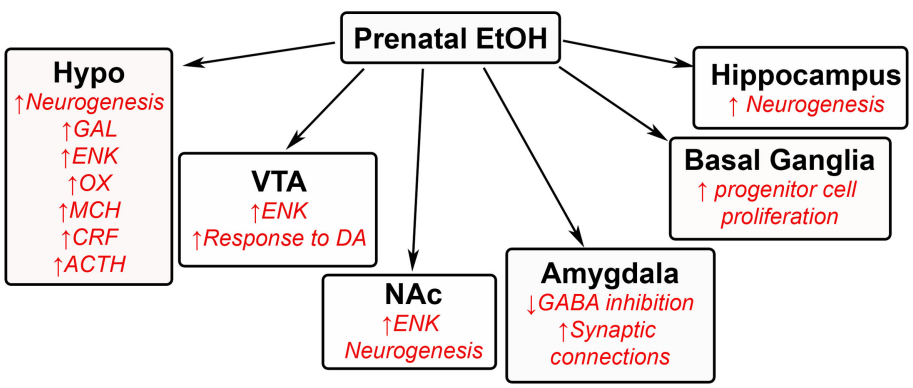

FIGURE 4 | Effects of prenatal ethanol exposure on offspring brain. A schematic summarizing some of the changes that occur in the brains of offspring after being exposed to low levels of ethanol during gestation. GAL, galanin; ENK, enkephalin; OX, orexin; MCH, melanin-concentrating hormone; CRF, corticotrophin releasing factor; ACTH, adrenocorticotropic hormone; DA, dopamine; GABA, γ-aminobutyric acid.

of prenatal ethanol on the dopaminergic system in these brain regions are also significant, with the VTA having an increased response to dopaminergic agonists and the NA having increased sensitivity to the stimulatory effects of alcohol in offspring (137, 163, 164). Although ghrelin has been found to be involved in the rewarding feeling of alcohol (165), there are currently no studies on how low ethanol levels during the prenatal period affects this peptide and other neurochemical systems in these brain regions of offspring.

Prenatal Ethanol Has Global Effects on Other Areas of the Brain in Offspring

While there exists plenty of research describing the effects of high gestational ethanol exposure on the developing brain, there are only a few studies measuring the effects of low ethanol exposure on other brain areas not discussed above. Some of the findings include an ethanol-induced increase in progenitor cell proliferation in the basal ganglia (166) and a decrease in neural activity in the infralimbic cortex (164). They also include increased neurogenesis in regions of the hippocampus (167). The amygdala has been suggested to be affected by low levels of ethanol during the prenatal period. Offspring exposed to low ethanol display anxiety-like behavior when exposed to stressful conditions, and this behavior has been related to both an increase in synaptic connectivity in the basolateral amygdala (168) and a decrease in GABA inhibition (169), both of which stimulate the excitability of the amygdala (168, 169). Further studies on the effects of low ethanol concentrations in these other brain regions are needed to determine the extent of ethanol's action on neuronal development throughout the brain.

Prenatal Ethanol Induces Epigenetic Changes in Offspring

There are several studies that reveal high ethanol exposure during the prenatal period to induce dramatic epigenetic changes in offspring. The adult liver provides a clear example, with high levels of ethanol exposure found to alter DNA methylation related to alcoholic liver disease (170–172). Also, chronic maternal ethanol exposure is shown to decrease methylation at a gene called agouti viable yellow, which affects the color of their coat, that is passed down to offspring (173), while acute prenatal exposure to high levels of ethanol globally causes hypomethylation of DNA in embryos (133). Long-term prenatal exposure to high ethanol levels also induces changes in methylation and microRNA in hippocampal neurons (174). In light of these studies of high ethanol exposure, further investigations of epigenetic effects are clearly needed involving low concentrations of ethanol, which as described above have strong, stimulatory effects on neuronal development in the brain.

Relationship between Ethanol and Inflammation

Although only a few studies exist, ethanol intake has also been linked to inflammatory systems. The most commonly studied peripheral organ is the liver, with excessive drinking linked to alcoholic liver disease that increases inflammatory mediators (175, 176). More recent studies in adult animals have also shown ethanol exposure to stimulate inflammatory systems in the central nervous system. Endotoxin treatment after ethanol exposure has been found to induce a long-term inflammatory state in the brain (177) and increase nitric oxide synthase and cyclooxygenase, which lead to inflammation (178). This increase in inflammation has also been detected in offspring after prenatal exposure. Similar to prenatal HFD exposure, our lab recently found prenatal ethanol to induce several changes in the CCL2 chemokine system. We found low levels of ethanol during gestation to increase in the offspring the genesis of neurons that co-express CCR2 and melanin-concentrating hormone in the lateral hypothalamus (17), a neuropeptide implicated in excessive ethanol drinking (179). With current research showing low levels of ethanol exposure to increase drinking in offspring and produce changes in the immune system that ultimately affects neuronal function, future research on inflammatory systems could be very informative and important.

PRENATAL NICOTINE EXPOSURE

The effects of prenatal nicotine exposure are broad in nature, affecting both behavioral and neuronal development in several regions of offspring brain. Human studies show that children exposed to tobacco during gestation exhibit an increased risk for tobacco use, craving, and withdrawal (180), as well as dependence (181). Animal studies similarly reveal increased nicotine self-administration and consumption in adolescent and adult offspring (182–185), along with increased ingestion of other substances including fat and ethanol (18). Additional behavioral problems include an increased risk of hyperactivity (186), impulsivity (185), and anxiety (34, 35). High levels of nicotine exposure are also associated with detrimental effects, such as growth retardation (187). While these nicotine studies lead one to question whether these changes are attributed to certain chemicals from the tobacco (188, 189) rather than to nicotine itself and result from social smoking as well as chronic smoking, the overall evidence clearly demonstrates that prenatal nicotine exposure negatively affects offspring. Similar to prenatal HFD and ethanol exposure, these changes in physiology and behavior induced by nicotine or smoking may be attributed to neuronal changes in the offspring brain (**Figure 5**).

Prenatal Nicotine Exposure Alters Hypothalamic Neurocircuitry

Similar to dietary fat and ethanol, prenatal nicotine exposure has been found to affect the neuronal architecture and function of the hypothalamus. Several neuropeptides have been found to be altered in offspring during exposure to both low and high levels of nicotine. Some of the findings include a decrease in CRF and an increase in glucocorticoid receptors in the hypothalamus (190). They also show an increase in several orexigenic peptides, including neuropeptide Y, agouti-related peptide, and proopiomelanocortin in the arcuate nucleus (191), enkephalin in the medial hypothalamic paraventricular nucleus, and orexin and melanin-concentrating hormone in the perifornical lateral hypothalamus

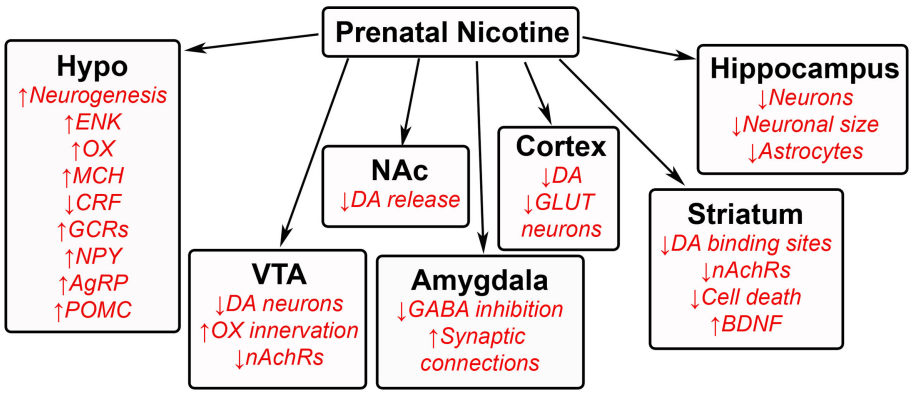

FIGURE 5 | Effects of prenatal nicotine exposure on offspring brain. A schematic summarizing some of the changes that occur in the brains of offspring after being exposed to nicotine during gestation. GAL, galanin; ENK, enkephalin; OX, orexin; MCH, melanin-concentrating hormone; CRF, corticotrophin releasing factor; GCRs, glucocorticoid receptors; NPY, neuropeptide Y; AgRP, agouti-related protein; POMC, proopiomelanocortin; DA, dopamine; nAchRs, nicotinic acetylcholine receptors; GABA, γ-aminobutyric acid; GLUT, glutamate; BDNF, brain-derived neurotrophic factor.

(18, 192). One of the more important findings from our lab shows that exposure to nicotine actually stimulates the genesis of neurons that express enkephalin, orexin, and melanin-concentrating hormone in the offspring hypothalamus (18), with these peptides positively related to the intake of nicotine (193). A small number of epigenetic studies also show changes in DNA methylation of the gene encoding brain-derived neurotrophic factor in human studies (194, 195), revealing the need for further epigenetic studies of specific cell types.

Prenatal Nicotine Exposure Alters VTA–NA Center in Offspring

Prenatal nicotine exposure has been found to varying degrees to change neurons in the VTA and NA in offspring. With regards to the mesolimbic dopamine system, prenatal nicotine exposure decreases the number of dopaminergic neurons in the VTA (196), dopamine release from the NA (197, 198), and the number of dopamine binding sites in the striatum (199), altering the rewarding effects of nicotine in offspring. Neuronal connections to the VTA are also affected, with orexin innervation from the lateral hypothalamus to the VTA found to be increased (192). Additionally, prenatal nicotine reduces the number of nicotinic cholinergic receptor expression in both the VTA and NA core (196). Similar to the neurogenesis effect in the hypothalamus, prenatal nicotine increases cell survival in the NA and inhibits cell death related pathways (200), with this increase in cell survival consistent with the finding that prenatal nicotine exposure increases the nerve growth factor, BDNF (201). Further studies on this reward region in offspring will shed more light on how prenatal exposure reprograms offspring to become more prone to abusing nicotine.

Prenatal Nicotine Has Global Effects on Other Areas of the Brain in Offspring

Prenatal nicotine exposure has been found to affect several other brain regions in offspring. In the hippocampus, this exposure decreases the number of neurons (202) while increasing the number of astrocytes (202), and it also decreases the neuronal area and cell size (203, 204), suggesting decreased hippocampal function. Similar effects are also found in the cortex of early postnatal rats (205), pre-weaned rats (204), and embryos (206), with studies revealing fewer glutamatergic neurons (207). These changes in the cortex induced by prenatal exposure have been linked to cognitive deficits and impaired executive control, causing rats to be more impulsive (208). Similar to the VTA and NA, dopamine levels are also decreased in the cortex of postnatal offspring (209). In the amygdala, one study found nicotine exposure to reduce the size of the amygdala in adolescent offspring (210), while a recent study from our lab has described an increase in neurogenesis and expression of enkephalin neurons in the central amygdala (18). With nicotine intake shown to generally reduce anxiety, future studies with prenatal exposure that relate behavior to amygdaloid function in offspring, as well as to other brain regions involved in decision making, would be interesting.

Prenatal Nicotine Induces Epigenetic Changes in Offspring

Several studies show prenatal nicotine exposure to have epigenetic effects on peripheral organs in offspring. Prenatal nicotine has been found to decrease methylation on the promoter-expressing angiotensin receptor type 1a (211) and increase histone acetylation of the protein and fatty acid synthase in liver (212). Human studies have also reveal global changes in DNA methylation in offspring (213, 214). Evidence of a generational effect has also been shown in rat models, with maternal nicotine use and exposure during the prenatal period found to induce asthma and epigenetic changes in lungs of offspring that are two generations past the original exposure (215). This evidence suggests that the changes induced by prenatal nicotine exposure on brain neurochemical systems may be related to epigenetic changes occurring during development.

Relationship between Nicotine and Inflammation

While reports of pure nicotine on adult systems generally reveal a reduction in inflammation (216, 217), several studies in humans show cigarette smoking to cause an increase in inflammation (218). Also, in a rat model, exposure to pure nicotine during the gestational period is found to increase the inflammatory mediators, IL-6 and TNF-alpha, in newborn blood serum (219). While this evidence is limited, it suggests the possibility that prenatal nicotine may have similar effects to HFD and ethanol exposure on inflammatory mediators, including CCL2.

GENERAL CONCLUSION

The current knowledge of the neural control of ingestive behavior in offspring that are prenatally exposed to substances of abuse has come a long way from observational human studies. We are now only beginning to piece together how these changes in specific brain regions affect the overall neuronal communication within the brain. In addition, other systems of the central nervous system, such as glial cells, astrocytes, and oligodendrocytes, may also play a major role in this disturbed communication. More importantly is the emerging function of the immune system in the development of these neuronal systems in offspring and how substances of abuse disturb its actions. Future studies using these prenatal animal models will provide much insight in both the molecular and neuronal network changes as well as the mechanisms leading to these changes.

AUTHOR CONTRIBUTIONS

KP and SL prepared and revised the manuscript.

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Hedonics Act in Unison with the Homeostatic System to Unconsciously Control Body Weight

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INTRODUCTION

With the global obesity crisis continuing to take its toll, the demand for solutions has increased. The discussion about nature vs. nurture and biology vs. psychology has culminated in declaring obesity as a disease by some medical organizations. Environmental factors and genetic predisposition, rather than personal responsibility are to blame, as for any other disease. This view implies that the biological processes regulating body weight are essentially operating at the unconscious realm. Although this has long been accepted for the so-called homeostatic regulation of energy balance, it is less clear for the hedonic controls. Here, we critically evaluate the important question how rodent models can help understand the contribution of hedonic neural processes to body weight regulation. When looking at the concepts of reward, reinforcement, motivation, pleasure addiction, and their neural mechanisms, in the context of eating and exercise, the new view emerges that homeostatic and hedonic controls are closely interrelated and often act in unison at the unconscious level to achieve biologically adaptive responses. Although the discussion of a body weight set point has been neglected in recent years, this topic becomes more pressing as an important aspect for effective treatment of obesity.

HEDONIC MECHANISMS OVERPOWER HOMEOSTATIC REGULATION

When the body weight of animals and humans is disturbed by periods of either under- or overfeeding, it promptly returns to pre-perturbation levels through a process termed homeostatic regulation that involves the controls of both energy intake and energy expenditure (1, 2). The basic hypothalamic circuitry underlying this regulation has long been known (3) and was much refined, particularly over the last 20 years in the wake of the discovery of leptin. In brief, two distinct neural populations in the mediobasal hypothalamus act as primary energy sensors and engage a complex network of effector circuits controlling both energy-in and energy-out in a biologically adaptive fashion [for review, see Ref. (4–7)].

However, while most agree with such basic homeostatic regulation, there has been much discussion regarding the exact level of defended body weight and the mechanisms involved (8–13). Clearly, there is no fixed set point around which mammalian species regulate their body weight. Rather, it is flexible, depending on both internal and external conditions including genetic and epigenetic predisposition, food availability, food palatability, and other

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environmental factors (10). This is best illustrated by the seasonably variable and homeostatically defended body weight set point of hibernators (14).

One factor that is widely believed to be very important for influencing the individual body weight set point is food hedonics, particularly the shift toward higher body weight by highly palatable, calorie-dense foods (**Figure 1A**). The clearest example of this shift in defended body weight is the cafeteria diet-induced obese rat and mouse (15). Although it is suspected that the increased availability of highly palatable, energy-dense foods is also mostly responsible for the current obesity epidemic, it is much harder to prove, because of difficulties to strictly control energy balance and environmental conditions in humans over extended periods of time as it is possible in animal models. A widely accepted view is that in genetically and/or epigenetically susceptible individuals, the obesogenic food environment is able to establish a new, higher body weight set point that is similarly defended against forced fasting and overfeeding as in normal weight individuals (11). Therefore, one of the key issues in understanding body weight regulation is the neurological explanation for this shift in defended body weight. What are the neural mechanisms that allow availability and palatability of energy-dense foods to overpower the basic homeostatic defense system? Understanding these mechanisms could lead to the development of more specific drugs or behavioral interventions in the fight against obesity.

HEDONIC PROCESSING IS AN INTEGRAL PART OF THE HOMEOSTATIC REGULATORY SYSTEM

The view that the hedonic and homeostatic neural circuitries are not separate entities but are part of the same regulatory system is rapidly gaining traction. This is based on evidence for bidirectional modulation of corticolimbic brain areas by interoceptive signals, and of the hypothalamus by exteroceptive signals and their cognitive and emotional correlates (**Figure 1B**).

Bottom-up Modulation of Corticolimbic Circuits of Cognition and Motivation by Interoceptive Signals of Nutrient Availability

The bottom-up control of hedonic and cognitive processes by internal signals is not a new insight. Given the crucial importance of nutrients for survival, it is a fundamental attribute of the expression of hunger and goes back to the beginning of evolution of the nervous system. Specifically, the hungry state is characterized by increased incentive salience attribution (the mechanism by which a goal object such as food is becoming highly desired and wanted – a behavioral magnet), which is neurologically manifested by heightened activity of the mesolimbic dopamine system (16–18). What is new, are some of the messengers and neural mechanisms shown to be involved. For example, it is now clear that one of the most eminent homeostatic regulators of body weight – leptin – modulates appetite by acting not only on the hypothalamus but also on components of the mesolimbic dopamine system (19–22) and on olfactory and taste sensory processing (23–25). Similarly, many other internal signals of nutrient availability, such as ghrelin, intestinal GLP-1 and PYY, and insulin, as well as glucose and fat, also partly act on corticolimbic structures involved in the cognitive and rewarding aspects of food intake control (26–36). Effects on cognitive functions by these hormones are interesting in the context of human studies showing impairments of both cognitive and metabolic functions in obese patients (37–39). Although the common link is not yet known, a leading hypothesis suggest that intestinal dysbiosis resulting from an interaction between sub-optimal nutrition, gut microbiota, and the innate immune system with subsequent changes in gut-to-brain signaling and blood–brain barrier integrity are important (40–43).

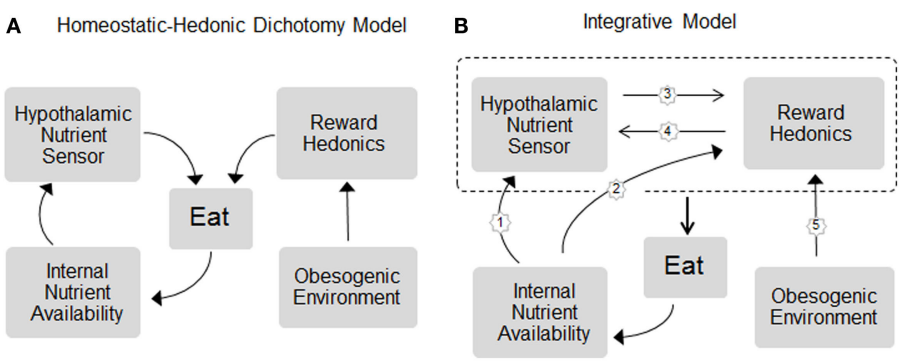

FIGURE 1 | Schematic representation of the (A) dichotomy and (B) integrative models of homeostatic and hedonic control of food intake and regulation of body weight. In the dichotomy model, homeostatic and hedonic mechanisms are largely independent. In the integrative model, the neural circuitries for internal nutrient sensing and hedonic processing act in concert to control eating and body weight and the hedonic system becomes part of the homeostatic system. Signals of both short- and long-term internal nutrient availability, such as leptin, gut hormones, and metabolites, are sensed by both, the hypothalamic nutrient sensor (pathway 1) and hedonic/reward processing centers (2), and these circuits communicate bidirectionally with each other (3 and 4) to unconsciously affect eating. For simplicity, energy expenditure as effector mechanism in the regulation of body weight is not included. For details, see text.

Top-down Modulation of the Classical Hypothalamic Regulator by Sensory, Cognitive, and Motivational Signals

The other driver of this integrated view is new insight into the top-down modulation of classical homeostatic circuitries by cognitive and emotional processing in corticolimbic systems (44). Cue-induced, conditioned food intake is thought to be an important mechanism in overeating by humans in an obesogenic environment (45, 46) and has been studied in rodents for quite some time (47). Some of the relevant pathways involved in this cognition-dependent food intake have been identified in the rat by demonstrating dependence on amygdala and prefrontal cortex-to-lateral hypothalamus projections (48, 49). Most recently, evidence for top-down modulation of AGRP neurons in the mediobasal hypothalamus, the epicenter of classical homeostatic regulation, was presented. These powerful neurons have been thought to be mainly controlled by circulating hormones and metabolites in a relatively slow waxing and waning fashion commensurate with the fasted and fed states. Using modern, genetically based neuron-specific technology, it was demonstrated that activity of AGRP neurons is also controlled on a second-by-second basis by the conditioned expectation of imminent food ingestion (50, 51). This acute external sensory and cognitive control over AGRP neuron firing rate is likely accomplished by direct or indirect inputs from a number of cortical and subcortical areas as demonstrated by neuron-specific retrograde viral tracing (52).

CONTROL OF FOOD INTAKE AND REGULATION OF ENERGY BALANCE IS PREDOMINANTLY SUBCONSCIOUS

It is clear that the classical hypothalamic neural circuitry responsible for the homeostatic regulation of energy balance and body weight, similar to homeostatic regulation of other bodily functions, such as blood glucose or blood pressure, is operating largely beyond awareness, at the unconscious level. In addition and as discussed above, the incentive sensitization mechanism by which interoceptive signals of energy depletion such as low leptin drive "wanting" through the mesolimbic dopamine system (16, 18, 53) is also largely operating outside awareness as demonstrated in human neuroimaging studies (54–56). Even in the absence of metabolic hunger and associated interoceptive sensitization signals, conscious awareness of the cue does not seem necessary. This has been shown in rats with cue-induced conditioned food intake (47, 48). Furthermore, the human brain can learn the value of monetary rewards and use it for decision-making without conscious processing of contextual cues (57). Although optimal decision-making requires self-control, represented in the dorsolateral prefrontal cortex (58, 59), the transformation of reward-driven behavioral action is not under obligatory control of this brain area and often constrains the free will to act (60). Finally, neural activity in certain brain areas can be going on for quite some times before humans become aware of their own decision (61, 62), suggesting that much of the processes leading to a decision are taking place at the unconscious level.

Ingestive behavior in both humans and rodents appears to become particularly resistant to cognitive controls when it is highly habitual (63, 64). Under normal conditions, information about possible outcomes is important for cue-induced goal-directed actions making such actions sensitive to devaluation. However, habitual behavior no longer depends on learned reward expectations and is thus largely insensitive to mechanisms of reward devaluation (64, 65). The neural circuits governing non-habitual behaviors are differently organized than those for habitual or automatic behaviors. Non-habitual behaviors heavily depend on the ventral striatum (nucleus accumbens) and the ventromedial prefrontal cortex, whereas habitual behaviors depend more on the dorsolateral striatum (65, 66). The memory storage and recall mechanisms are also different for habitual vs. non-habitual actions and behaviors. In distinction to declarative memories which require a conscious mind, procedural memories operate largely below the level of conscious awareness and storage is more distributed (67–69). As a consequence, procedural memories and the habitual ingestive behaviors they guide are relatively resistant to inhibitory cognitive control and executive functions.

CONCLUSION

Animal models have been crucial for dissecting the complex mechanisms underlying predisposition to obesity. Given that the overwhelming majority of genetic loci linked to human obesity are associated with neural functions (70), it is not surprising that the neural controls of food intake and regulation of energy balance are a main component of these mechanisms. Although functional neuroimaging in humans is also starting to make important contributions, only the more invasive approaches in rodents have been able to provide mechanistic explanations. As a result, the traditional dichotomy between homeostatic and non-homeostatic/hedonic systems responsible for the control of appetite and regulation of body weight, although heuristically still useful, no longer adequately describes the extensive anatomical and functional interactions between the two systems. In addition, much of the output of this larger interactive system is bypassing awareness. The implications of these new insights are far reaching as they will guide not only future research but also the design of pharmacological and behavioral therapies for obesity and eating disorders.

AUTHOR CONTRIBUTIONS

HM and CM helped conceive the opinion, reviewed the literature, wrote parts of the manuscript, and edited the pre-final version of the manuscript. EQ-C and SY were involved in discussions of the original idea, reviewed parts of the literature, wrote parts of the manuscript, and edited the pre-final manuscript. H-RB conceived the original idea for the opinion, discussed several draft versions of the manuscript with all coauthors, researched the literature, and wrote the final manuscript.

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REVIEW
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The Use of Rat and Mouse Models in Bariatric Surgery Experiments

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Animal models have been proven to be a crucial tool for investigating the physiological mechanisms underlying bariatric surgery in general and individual techniques in particular. By using a translational approach, most of these studies have been performed in rodents and have helped to understand how bariatric surgery may or may not work. However, data from studies using animal models should always be critically evaluated for their transferability to the human physiology. It is, therefore, the aim of this review to summarize both advantages and limitations of data generated by animal based experiments designed to investigate and understand the physiological mechanisms at the root of bariatric surgery.

Keywords: RYGB, VSG, food intake, energy expenditure, rat, mouse, human

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INTRODUCTION

Obesity and its related comorbidities have detrimental effects for the affected individual and pose a major challenge on public health systems worldwide. Despite the availability of a number of pharmacotherapies, the best treatment option leading to clinically relevant and maintained body weight loss is bariatric surgery (1–6). Bariatric surgery leads to a long-term reduction in body weight and in obesity-related morbidity and is currently the only treatment modality with a proven mortality benefit (4). Several techniques are currently employed. The gold-standard since many years is the Roux-en-Y gastric bypass (RYGB), followed by vertical sleeve gastrectomy (VSG) and, with decreasing numbers, adjustable gastric banding (AGB); the so-called mini-gastric bypass (MGB) has gained some popularity recently.

The treatment success of RYGB appears to be associated at least in part with changes in gastrointestinal hormones and bile acids that have been found to exert some role in the control of eating (7, 8). Despite the very different surgical approach, RYGB and VSG are associated with some extent with similar hormonal changes (9, 10). By contrast, reduced eating and weight loss after AGB is generally thought to result rather from mechanical restriction due to the reduced filling capacity of the stomach, although recent animal data suggest some role for gastrointestinal hormones, too (11). The MGB has been introduced ~20 years ago and has gained some popularity, in particular in non-academic surgical centers. However, surprisingly little research has been performed to study the underlying mechanisms that lead to body weight loss after MGB (12, 13).

Animal models have been proven to be a crucial tool for investigating the physiological mechanisms underlying bariatric surgery in general and individual techniques in particular. By using a translational approach, most of these studies have been performed in rodents and have helped to understand how bariatric surgery may or may not work. However, data from studies using animal models should always be critically evaluated for their transferability to the human physiology. It is, therefore, the aim of this review to summarize both advantages and limitations of data generated by

animal-based experiments designed to investigate and understand the physiological mechanisms at the root of bariatric surgery.

ROLE OF BARIATRIC SURGERY IN ANTI-OBESITY THERAPY

The number of non-surgical options to treat obesity are limited and the long-term success of dietary or life style interventions is minimal (2, 14). New drugs have recently been approved for obesity treatment, but long-term data are not available yet (1, 6). Insights into mechanisms of bariatric surgery, in particular RYGB and VSG, have opened up new treatment avenues against obesity. Among these, gut hormone-based strategies represent the most promising approach and are mainly focused on analogs of glucagon-like peptide-1 (GLP-1), such as liraglutide (Saxenda®) (15, 16). However, combinations of different hormones, such as amylin and leptin analogs, have also delivered remarkable results (17, 18).

However, due to an enormous discrepancy between the number of performed bariatric interventions, on the one hand, and the number of formally eligible patients worldwide, on the other hand [e.g., a number of ~0.5 Mio operations worldwide performed in 2013 (19) compares to the number of obese individuals of about 100 Mio in the USA alone (20–22)], it is obvious that the obesity epidemic cannot be successfully addressed by surgical means alone and that other non-surgical methods with a similar or superior efficacy and safety profile are urgently needed.

In this regard, research with animal models has significantly helped to elucidate some of the potential mechanisms underlying bariatric surgery. In comparison to human studies where investigating food intake is predominantly reliant on verbal report and dietary recall measures of patients, animal experiments allow the assessment of objective and unbiased data regarding postoperative changes of food intake. Furthermore, employment of genetic knockout models or specific antibodies directed against specific gut hormones or their receptors can only be performed in animals and have, thus, helped to differentiate between associative and causative relationships of proposed mechanisms of bariatric surgery. However, it needs to be emphasized that neither qualitative nor quantitative data generated in animals should be extrapolated to the human setting "one-to-one" and that animal studies unfold their additional value first of all in a translational experimental setting. In other words, it seems less relevant that weight loss rates are similar in rats and patients after RYGB, as long as the weight loss is mediated by similar physiological mechanisms in both settings.

Most animal research has been performed with RYGB and VSG interventions in rats or mice. Although the effect sizes of RYGB and VSG operations were found to show quantitative differences, it appeared that qualitative changes were remarkably robust between different studies. In other words, variables, such as surgical technique, pre-, peri-, and postoperative diet, baseline weight, and level of adiposity which all may affect the study outcome, were found to have a surprisingly little effect on the general information gain of these studies [discussed in Ref. (23, 24)].

ANIMAL MODELS OF BARIATRIC SURGERY TECHNIQUES

Although surgical methods to reduce body weight were first introduced more than 50 years ago and have been used in increasing frequency ever since, it is surprising that researchers only recently started to develop greater interest in post-bariatric physiological mechanisms. Koopmans and colleagues were among the first scientists who systematically used animal models to investigate the underlying mechanisms of bariatric surgery. These authors were able to demonstrate that a method called ileal transposition was not only effective in treating both genetic and hypothalamic lesion-induced obesity in rats, but also that the loss of body weight and body fat was associated with a reduction in eating. They further observed that ileal transposition caused hypertrophy of the small intestine and concluded that the early contact of the distal small intestine with undigested food and digestive enzymes may lead to an increased release of gastrointestinal hormones as one mediating factor (25–30).

Two other groups also made early contributions to the current literature of rodent models of bariatric surgery. Atkinson and Brent demonstrated that blood circulating factors seem to be critical for the reduction in eating after intestinal bypass operations in rats (31), and Meguid et al. were among the first to study altered brain signaling post-RYGB (32, 33). More recent studies built on these seminal experiments and the explanations how bariatric surgery and in particular RYGB reduce eating and body weight are still manifold. Finally, only a few groups published research with models of the AGB [e.g., Ref. (11)], and even less animal research has been done using the MGB technique (12).

RYGB AND VSG IN RATS AND MICE

The interest of the scientific community in post-bariatric physiology has grown exponentially over the last decade and many groups contributed significantly to the growing knowledge regarding the underlying mechanisms of bariatric surgery by using animal models of RYGB and VSG [e.g., Ref. (9, 10, 24, 34–55)]. Many of the reported effects showed striking similarities to what has been observed in RYGB or VSG patients.

For example, RYGB and VSG in rats and mice typically induced a rapid and sustained body weight loss which was mainly due to a reduction in body fat mass (40, 51, 56, 57). Post-surgical weight loss correlated to a large extent with reduced spontaneous eating, but an increase in energy expenditure may also play a role (51, 57, 58).

In addition, animal models also provided compelling arguments *against* traditional concepts such as intestinal malabsorption and mechanical restriction. There is nowadays a large body of evidence indicating that neither malabsorption nor restriction are the only mechanisms that exclusively explain the overall reduction in caloric intake and body weight after bariatric surgery (10, 51). In regard to caloric malabsorption, both animals and patients are able to digest and absorb ingested nutrients to a similar extent after RYGB than their respective controls. However, some reduction in fat digestibility has been described in rats and

mice after RYGB when postoperatively exposed to a high-fat diet (35, 57), and a recent study indicated that intestinal glucose absorption may also be reduced after RYGB as a consequence of reduced sodium delivery and sodium-dependent glucose absorption in the proximal parts of the reconstructed small intestine (59). Thus, some role of maldigestion and malabsorption for body weight loss after RYGB needs to be considered in this scenario.

In regard to mechanical restriction, various studies have shown that RYGB- or VSG-operated rats and mice are indeed able to ingest larger quantities of food if they are metabolically challenged, e.g., subsequent to temporary food restriction after surgery (10, 24) or during lactation in female reproducing animals (60). Other arguments against a major impact of mechanical restriction on reduced eating after RYGB surgery include the observation that RYGB rats do not increase prandial drinking that might suggest an attempt to overcome mechanical constraint through food dilution with water. Furthermore, food intake after RYGB can be increased in rats and humans by somatostatin analogs that block the release of gastrointestinal hormones but which do not alter the mechanical situation post-RYGB (61, 62). In addition, differences in food intake and body weight are not related to the size of the gastro-jejunostomy in RYGB rats (63).

It needs to be emphasized that it was certainly wrong to explain the observed changes in eating behavior after bariatric surgery entirely and exclusively with mechanistic concepts, such as restriction and malabsorption, but it might be equally incorrect to neglect their impact. A typical finding after both RYGB and VSG operations is a change in meal pattern. Similar to RYGB patients, rats typically eat smaller meals after RYGB, which is partly compensated by an increase in meal frequency. In more detail, the size of nocturnal meals has been found to be markedly reduced post-RYGB, while the size of diurnal meals was actually increased compared to sham-operated control animals (23, 42, 51, 64). Furthermore, even if RYGB or VSG rats are metabolically challenged leading to high levels of total food intake (e.g., temporary food restriction; pregnancy and lactation), the rats do not seem to increase their meal size (10, 24, 60). Finally, a recent study showed that increased intake induced by antagonizing melanocortin-4 (MC4) receptors in the hypothalamus was entirely dependent on an increase in meal number, but not meal size (65). In summary, the available data indicate that RYGB (or VSG) rats may be mechanically restricted in a sense that the amount of food that can be eaten in a single meal is limited, but that rats are able to adapt to specific metabolic situations by increasing meal frequency.

MECHANISTIC STUDIES TO EXPLAIN REDUCED EATING AND WEIGHT LOSS AFTER RYGB AND VSG

Animal experiments provide valuable insight into the mechanisms that are at play after bariatric surgery and that lead not only to a reduction of body weight but also to long-term maintenance of the lower body weight. The available data indicate that RYGB- or VSG-operated individuals develop a new set point of their body weight that is defended even if challenged by certain experimental conditions (e.g., temporary food restriction or forced overfeeding).

Leptin may play an important role in the control of this set point defense because leptin-deficient ob/ob mice do not exhibit the same benefits in body weight and metabolic control compared to control animals unless leptin is replaced (66). Thus, the bariatric surgery procedure itself may be most critical for the extent of weight loss until the newly defended body weight may be reached. This may also indicate that further temporary manipulations (e.g., additional calorie restriction, alterations in food composition) may not necessarily result in additional long-term benefits.

The latter point is also important in a different context. Various studies showed that RYGB and VSG may lead to an alteration in food preference with rats and patients choosing to eat less high-fat and sugary foods in favor of less energy dense alternatives when offered a choice (42, 53, 57, 67–69). However, a recent review of the human literature found that reported changes in dietary macronutrients after RYGB were modest and only transient in nature (70). Although alterations in dietary selection could conceivably contribute to improved glycemia and body weight after RYGB and VSG surgery, it remains unclear whether they represent an essential contributor to these beneficial effects after surgery or not. Based on the findings reported in the previous paragraph, this may actually not be the case, with the respective consequences for dietary counseling.

Roux-en-Y gastric bypass and VSG lead to characteristic changes in the concentration of gut hormones and bile acids (8, 41, 71–73), which is a robust phenomenon consistently reported in basically all published studies. Nevertheless it needs to be stated that most attempts to establish a causal role of gut hormones and bile acids for the post-bariatric outcome have failed so far. For example, data obtained from GLP-1 receptor knockout animals or using GLP-1 receptor antagonists are negative in that RYGB- or VSG-induced effects did not differ from wildtype control animals (43, 74–76). Thus, while changes in gut hormones alone cannot explain the RYGB- or VSG-induced effects, it appears that rather a combination of multiple physiological alterations and interactions are at play. These include elevations in basal or postprandial concentrations of many gut hormones [GLP-1, cholecystokinin (CCK), amylin, peptide YY (PYY), etc.], increased levels, and altered composition of bile acids, as well as alterations in the diversity and composition of gut microbiota after bariatric surgery (7, 35, 41, 71, 76–78).

The large number of studies describing changes in the periphery after bariatric surgery contrasts with the remarkable paucity of data addressing changes in central nervous system function that may explain the effects of bariatric surgery. The most in-depth studies described the potential contribution of MC4 receptor signaling that is an important center point for the control of energy balance in general. The published data indicate that there may be a species difference in the relevance of MC4 signaling, because VSG effects were still present in MC4-deficient rats (46). By contrast, RYGB-induced changes differed between MC4-deficient mice and respective controls, and there appeared to be a gene dosage effect (34, 44, 79). The latter may also explain why RYGB or VSG patients with mutations in the MC4 gene typically still respond to bariatric surgery because they do not

correspond to a full receptor knockout. Interestingly, some rare mutations in the MC4 were associated with a bigger weight loss and a faster resolution of diabetes post-surgery (80) [but see Ref. (81)]. Recent data indicated that RYGB also changes signaling in feeding areas of the caudal hindbrain in RYGB rats (82) but no equivalent human data are currently available.

Future experiments need to be designed to mimic specific aspects of bariatric surgery and to define the causal role of specific mechanisms for the beneficial effects of bariatric surgery. Manipulations may, e.g., include the local infusion of nutrients in specific gut segments, manipulations of nutrient contact with the gut mucosa, diversion of pancreatic juices and bile acids, and perhaps also the transposition of specific gut segments, similar to the procedure that Koopmans and colleagues had used more than 30 years ago (25, 29, 30).

COMPARISON OF ANIMAL MODELS IN BARIATRIC SURGERY RESEARCH WITH THE CLINICAL SITUATION IN HUMANS

The validity of animal model data depends on the similarities in phenomena and mechanisms between humans and animals. For the most part, it seems safe to say that the similarities largely outnumber potential differences that may be more quantitative than qualitative.

The key effect of bariatric surgery seems to be a loss of excessive fat mass by resetting the system of body weight control in both animals and humans (10, 56, 66). In humans, loss of excessive body weight is typically more pronounced in more obese patients (83). On the other hand, diabetic patients with a body mass index between 22 and 35 lose on average ~20% of their total weight after RYGB (84), which is markedly less than the typical weight loss in heavier patients. Interestingly, a similar phenomenon is seen in animals where post-RYGB body weight loss also seems to correlate with the degree of preoperative obesity. Obese OLETF rats, i.e., rats that are obese because they overeat due to the lack of functional CCK1 receptors, lost markedly more weight compared to their lean LETO controls after RYGB (49), and recent studies in mice with different degrees of obesity corroborated these findings (56).

One aspect that differs between rodents and humans is the difference in weight growth curves; i.e., in contrast to obese humans, where the "control condition" typically refers to a stable body weight, control groups of rats or mice often gain weight over the observation period of a study. Here, the effect of bariatric surgery may be a prevention of this weight gain rather than an absolute weight loss. However, animal models allow the detailed study of major components contributing to the body weight loss in standardized and reproducible conditions, i.e., reduced caloric intake, increased energy expenditure, or reduced energy availability from ingested nutrients. By contrast, data on food selection and intake in humans rely in most cases on self-reported food intake that is vulnerable to inaccuracy for several reasons (70).

A further advantage of animal models is the use of specific control groups for reduced caloric intake or weight loss in respect to the metabolic consequences of bariatric surgery; in other words, pair fed or body weight matched controls, or controls for specific metabolic situations allow to distinguish bariatric surgery effects that are specific to the surgical manipulations, such as the anatomical re-arrangement of the small bowel anatomy versus effects that are rather a consequence of the induced weight loss (23, 24, 50, 51, 71, 85).

As discussed, body weight loss after bariatric surgery is mainly due to reduced energy intake and changes in meal patterns (42, 51, 64), and alterations in food choice and taste preference may also play a role (38, 42, 47, 53, 67, 69, 86). Further important similarities between animal models and humans undergoing bariatric surgery include changes in the postoperative profile of gut hormones and bile acids, but also the metabolic beneficial effects of bariatric surgery. The latter comprise, e.g., rapid improvements of insulin sensitivity, insulin secretory capacity, and cardiovascular function [e.g., Ref. (36, 45, 50, 71, 87–99)]. Similar to the effects on energy balance, a large number of follow-up experiments were performed to study the potential mechanisms underlying the metabolic effects of bariatric surgery. This included the manipulation of hormone systems or signaling cascades, and some studies clearly indicated that changes induced by bariatric surgery, e.g., elevated GLP-1 levels, do contribute to post-surgery metabolic effects in rats and humans (71, 93). However, other studies revealed rather disappointing results in a sense that blockade of GLP-1 signaling was often not able to offset the effects of bariatric surgery (75, 76).

Another contributing factor to weight loss after bariatric in humans and animal models may be the change in complexity and diversity in the gut microbiota. RYGB and VSG alter the composition of the gut microbiota and transplantation studies indicate that these alterations may also play a causal role in the improved metabolic status after bariatric surgery (35, 73, 100). For example, by colonizing germ-free mice with stools from the patients, Tremaroli et al. demonstrated that the surgically altered microbiota promoted reduced fat deposition in recipient mice. Mice also had a lower respiratory quotient, indicating decreased utilization of carbohydrates as fuel, suggesting that the gut microbiota may play a direct role in the reduction of adiposity observed after bariatric surgery (101).

Finally, not only the beneficial but also the negative consequences of bariatric surgery seem to be recapitulated in animal models, similar to what is seen in human patients. To give just three examples, RYGB causes a demineralization of the skeletal system potentially leading to an increased risk in bone fractures (85, 102–104). The underlying reasons for this effect are not clear, but own results indicate that a more acidotic status post-RYGB leading to increased calcium release from the bone may play a role. Second, RYGB may increase the risk for excessive alcohol intake in patients (105, 106) – a behavior which was also found in rats that did not prefer alcohol before the surgical intervention. Third, even though the metabolic status improves markedly in most diabetic patients after RYGB, some patients were found to have large fluctuations of their blood glucose concentration after RYGB surgery, especially in the periprandial phase paralleled by prolonged episodes of hypoglycemia. Similar findings have been reported in rodent RYGB models. The reason

for these larger than normal glycemic fluctuations is not entirely clear, but may be linked to increased secretion of GLP-1 leading to a strong increase of insulin release which then may require the compensatory release of counterregulatory hormones. In other words, despite the markedly improved general metabolic status, the fine tuning of glucose control may not be achieved by RYGB (50, 107–111).

NOTES OF CAUTION ON THE USE OF ANIMAL MODELS IN BARIATRIC SURGERY RESEARCH

Not all findings reported in rodent bariatric surgery models find their direct equivalent in the clinical situation, or vice versa, but some of the differences among species may be more quantitative than qualitative. Four examples will be discussed here.

First, the weight loss of RYGB and VSG in rats and mice is due to a reduction in eating and an increase of energy expenditure (respectively, the prevention of its decrease in weight-reduced animals). The relative importance of the energy expenditure component seems to be bigger in mice than rats; in fact, in some mouse studies, increased energy expenditure appeared to explain most of the surgery induced weight loss because food intake in RYGB-operated mice was higher than that in sham-operated controls (40, 51). By contrast, only some studies in humans report an increase in total energy expenditure. However, similar to the increased diet-induced thermogenesis that has been reported in rats, postprandial energy expenditure also seems to increase in some RYGB patients (55, 112–115).

The reason for the real or apparent species differences in respect to energy expenditure after RYGB is not clear but it may be more a general phenomenon of biology and physiology rather than a specific finding after bariatric surgery. The control of energy balance via energy expenditure may be much more efficient in mice with their large body surface to body mass ratio; in humans, this ratio is opposite, and this may be reflected in the more important control of energy balance via energy intake. Rats may be between both extremes, and this may explain why both energy intake and energy expenditure are typically affected by bariatric surgery in rats.

Second, bariatric surgery and in particular RYGB and VSG also lead to changes in food selection, and some reports claim that consumption of high-fat and sweet foods decreases post-RYGB (70, 116, 117). Similar findings have been reported in rats and mice because they chose to ingest lower amounts of high-fat or high-sugar diets than sham-operated controls. This decrease in intake is progressive and is reminiscent of a learning process (conditioned avoidance) (40, 42, 53, 57, 68). Interestingly, the decrease in sugar intake may also be due to an altered taste sensitivity because RYGB has been shown to lower the sucrose detection threshold in patients after RYGB (67) [see also Ref. (117, 118)]. Furthermore, brief access tests in rodents often did not indicate reduced avidity for sucrose or high fat and rats' voluntary work for sucrose or lipid solutions is not decreased (119–122). Nonetheless, whether the findings in animal models can be directly translated into the human situation is not clear, and only few objectively assessed data in humans are available. Changes in macronutrient intake in rats seem to be a long-term effect, while lasting changes in relative macronutrient intake in humans have typically not been observed. In other words, it is not clear whether the proportion of fat in the diet of post-RYGB individuals is decreased over extended periods of time, and it is also not clear if and for how long changes in diet composition contribute to reduced energy intake and weight loss in RYGB-operated patients.

Third, a typical finding in RYGB-operated rats is a massive hypertrophy of the intestinal wall in the Roux limb and to a lesser extent in the common channel of the RYGB reconstruction (51, 77, 123–125). The hypertrophic small intestinal epithelium may contribute to the increase in total energy expenditure in rats, and it may contribute to sufficient nutrient digestibility and absorption despite the altered gut anatomy. Whether the human gut hypertrophies to a similar extent in RYGB patients is still a matter of debate and only few well-controlled studies have been performed. One recent study, however, clearly indicated that the small intestine in RYGB patients showed a clear hypertrophic response (126). Furthermore, anecdotal evidence also reports that gut hypertrophy may also occur in RYGB patients; in a rather dramatic recent case, a short common channel was associated with massive mucosal hypertrophy eventually leading to a functional ileus (personal observation, Marco Bueter). The general extent and underlying mechanisms of gut hypertrophy post-bariatric surgery will need to be defined in further well-controlled clinical studies.

Finally, potential influences of anatomical differences between rodents (or other animal models) and humans need to be considered even though evidence for an important impact of these differences on study outcome is limited. The gastrointestinal anatomy differs between humans and rodents in some aspects. Mice, e.g., have an extensive portion of their stomach covered by cutaneous mucosa (called "forestomach" by some), and the proventriculus in rats also has no human equivalent. Furthermore, mice but not rats have a gall bladder. Despite that, delivery of bile into the proximal small intestine is also dependent on CCK in rats and no principal difference seems to exist between mice, rats, or humans in respect to the elevation of circulating bile acids after RYGB and VSG (8, 59, 71, 73, 88, 127–130). Of note, there are also some differences in the bile acid profile post-RYGB in humans compared to rat or pig RYGB models. Some bile acid species, such as the free bile acids cholic acid, chenodeoxycholic acid, and deoxycholic acid were similarly increased, but glyco-conjugated bile acid species concentrations depended on the animal model, and no global increase in tauro-conjugated species was observed. These differences may be relevant because different bile acid species have different affinity and efficacy at the various bile acid receptors (130).

MODELS OF BARIATRIC OPERATIONS OTHER THAN RYGB AND SG

Mini-Gastric Bypass

Knowledge from previous experiments can now be used for the optimized design to study mechanisms of more recently introduced bariatric surgery procedures. The so-called MGB

has gained some interest because it has the reputation to be an easier version of the classical RYGB. However, very little information about possible mechanisms of action is available. More importantly, data about the long-term effects and potential negative consequences are not available so far. Interestingly, this technique has been reported to lead to an increase rather than a decrease in eating. Energy expenditure has not been studied in detail after MGB, but it is generally assumed that maldigestion and malabsorption may be important components in the weight lowering effect of MGB (12, 13, 131, 132). If the latter were the case, the mechanisms of action would clearly differ between RYGB and MGB and would put a note of caution on the use of the MGB due to the potential of developing deficiencies in essential nutrients.

Biliopancreatic Diversion

The biliopancreatic diversion (BPD) introduced and developed by Scopinaro consists of a partial gastrectomy with a Roux-en-Y gastro-jejunostomy forming an alimentary limb and a duodeo-jejunal biliopancretic limb anastomosed to the distal ileum. The operation leads to significant weight loss with normal absorption of bile salts, water and electrolytes (133). This operation is generally performed in much lower numbers than RYGB and SG and has its main indication in severely and mega-obese patients (19). Rat models of BPD revealed that serum protein, cholesterol, and triglycerides fell by 25–40% postoperatively (134), while the procedure was associated with intestinal hypertrophy and with increased GLP-1, GLP-2, and PYY levels (135).

Biliopancreatic Diversion with Duodenal Switch

To preserve physiologic gastric emptying and to prevent anastomotic ulcer after BPD by decreasing the effects of alkaline biliary reflux, Hess developed a modified BPD procedure with the alimentary limb being directly anastomosed to the post-pyloric duodenum (136). This operation is today known as BPD with duodenal switch (BPD-DS) and also includes a VSG, before the use of VSG as a stand-alone procedure (137). The BPD-DS is considered by some as the most efficient surgery in treating obesity and T2DM, but the rate of early complications is higher and it might also be associated with a higher perioperative mortality (138); for this reason, the BPD-DS is not extensively performed worldwide (19). BPD-DS operations in rats showed that the procedure is associated with an increased fecal energy loss as well as a (compensatory) intestinal hypertrophy with elevated levels of fasting and postprandial plasma GLP-1 and PYY (139), while there is a reduced expression of thermogenic genes in the interscapular brown adipose tissue (140).

OUTLOOK AND CONCLUSION

Overall, rat and mouse experiments in bariatric surgery have been proven to be an important and relevant research tool that has led and will lead to important findings translatable into the clinical situation. Some differences have been identified, but careful experimental designs still allow clinically relevant conclusions. More studies are needed that directly compare effects and consequences of bariatric surgery procedures across species. This includes the assessment of similar parameters pre- and post-bariatric surgery in human patients and animal models, but also similarly designed experiments that yield mechanistic information in all relevant species. Only few examples are available in the literature [e.g., Ref. (67, 70, 121, 122, 129, 130)]. Nonetheless, without animal models, our knowledge on how bariatric surgery works (or may not work!) would be very limited and the vast literature that is available indicates that most animal models seem to recapitulate remarkably well the findings in humans. Future research in animal models of bariatric surgery will most likely include the more frequent use of larger animal models, e.g., minipigs or dogs (129, 141, 142). Larger animals offer significant advantages compared to rats and mice; e.g., larger blood volumes can be collected over extended periods of time, and specific interventions in defined parts of the gastrointestinal tract may be easier to perform in larger animal models. Furthermore, specific aspects of energy expenditure may be more similar to humans in larger animals compared to small animals, in particular the mouse.

AUTHOR CONTRIBUTIONS

TL wrote paper, performed literature research, and produced final version. MB revised paper, performed literature research, and edited text.

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MINI REVIEW
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Increased Hydration Can Be Associated with Weight Loss

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This mini-review develops the hypothesis that increased hydration leads to body weight loss, mainly through a decrease in feeding, and a loss of fat, through increased lipolysis. The publications cited come from animal, mainly rodent, studies where manipulations of the central and/or the peripheral renin–angiotensin system lead to an increased drinking response and a decrease in body weight. This hypothesis derives from a broader association between chronic hypohydration (extracellular dehydration) and raised levels of the hormone angiotensin II (AngII) associated with many chronic diseases, such as obesity, diabetes, cancer, and cardiovascular disease. Proposed mechanisms to explain these effects involve an increase in metabolism due to hydration expanding cell volume. The results of these animal studies often can be applied to the humans. Human studies are consistent with this hypothesis for weight loss and for reducing the risk factors in the development of obesity and type 2 diabetes.

Keywords: drinking, water, angiotensin, lipolysis, hypovolemia, hypohydration

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INTRODUCTION

Increased water intake is associated with loss of body weight produced *via* two mechanisms, decreased feeding and increased lipolysis. The obverse also appears to be true. Mild, but chronic, hypohydration is correlated with increased body weight and its attendant dysfunctions (1). The common denominator likely is angiotensin II (AngII), the principal hormone of body fluid regulation. In what follows, this hypothesis will be tested against the available evidence (2).

AngII acts on two, seven transmembrane domain peptide receptors, AT1 and AT2. Working through the AT1 receptor AngII stimulates thirst (the act of seeking out and drinking fluids, mainly water), an appetite for sodium, the release of anti-diuretic hormone (ADH or vasopressin) to conserve water *via* the kidneys, and vasoconstriction (conserving perfusion pressure to all organs and cells). The principal physiological signal for an increase in plasma AngII is extracellular dehydration (hypovolemia) (3). The responses listed above enable the rapid return of plasma volume to normal levels, thus reducing the signal for AngII generation. This is the physiological response to hypovolemia displayed by rodents.

However, chronically elevated AngII appears to be involved in several chronic human diseases (2). Antagonists of the renin–angiotensin system (RAS) are prescribed in 85% of cases to treat cardiovascular disease (4, 5). The same antagonists are used to treat obesity (6), diabetes (7, 8), cancer (9), and Alzheimer's disease (10). These effects could result if a subsection of the population was chronically, but mildly, hypohydrated [e.g., Ref. (11)], i.e., chronically, but mildly, hypovolemic.

These chronic diseases also involve metabolic dysfunctions (12, 13). This has been observed for cardiovascular disease (14, 15), obesity (16), diabetes (17–19), cancer (20), and Alzheimer's disease (21). In other words, chronic hypohydration may be driving the continuous release of AngII and the metabolic dysfunction found in the chronic human diseases.

Given that in animals AngII stimulates appropriate drinking responses, why is that some humans appear not to respond appropriately to the same AngII signal? The influence of other, perhaps

cognitive, factors on appropriate drinking responses has been noted in kidney stone formation, where increased water intake is recommended as a preventative measure, but compliance is difficult (22, 23). The authors noted that "not knowing the benefits of water drinking," "not liking the taste," and "the need to urinate frequently" influenced patient's behavior.

METHODS

This mini-review concentrates on angiotensin and metabolic function by looking at the effect of central and peripheral manipulations of the RAS that increase drinking, reduce food intake, decrease body weight, and produce fat loss through increased lipolysis. Literature searches used keywords: angiotensin, drinking, water intake, body weight loss, obesity, diabetes, RAS antagonists, metabolism, hydration, atrial peptides, UCP1, insulin resistance, and mitochondria. Research and clinical articles are cited where there is an associated increase in water intake, a decrease in body weight, a decrease in body fat, and/or a decrease in the markers of the risk of developing obesity and type 2 diabetes. There is a large literature on the RAS and body weight regulation as well as metabolism but not all articles measured water intake and thus are not cited.

CENTRAL AngII, DRINKING, AND WEIGHT LOSS

Administration of AngII into the brain of behaving animals increases drinking. Rats can consume over 2 h up to 15 ml of water in response after a single injection of AngII, depending on the dose and the site of injection (24–30). A decrease in feeding following drinking stimulated by intracranial AngII was noted early on, but this appeared to fade as the drinking response waned (31). Furthermore, in rats, chronically administered AngII over several days or weeks increased drinking (at least a doubling in daily intake), which was associated with a small decrease in food intake and a decrease in body weight, mainly through loss of fat (32–35). The decrease in body weight following the AngII infusion was greater than that in pair-fed rats.

Several mechanisms not necessarily related to the increased drinking have been suggested for this, AngII produces an increase in uncoupling protein I (33, 35). Others have suggested an increased thermic effect of food, an increased feeding hormone effect, or even an increased in stress hormone release (35). Both mechanisms imply a change in metabolic activity.

RAS ANTAGONISTS DRINKING AND WEIGHT LOSS

In other rodent models of obesity, using either angiotensin-converting enzyme (ACE) inhibitors or AT1-specific antagonists increased drinking significantly with an associated decrease in food intake and body weight mainly through loss of fat. In some cases, the fat loss was specifically linked to increased lipolysis (36–41). The drinking responses ranged from a 30% increase to up to a doubling of normal intake in both rats and mice. With two AT1-specific antagonists, candesartan and losartan, this effect is observed in obese, rather than lean, rats (42, 43). Use of the renin inhibitor aliskiren in mice on both low-fat and high-fat diets demonstrated a significantly increased drinking response with a lower body weight gain and loss of body fat over a 43-day treatment period (44).

Increased drinking to RAS blockade may appear paradoxical, but it could be in response to blockade-induced increased urine flow (45, 46) or to peripheral blockade-induced increase in AI passing through the blood–brain barrier, converting to AngII in the brain, and activating hypothalamic AT1 receptors (47–49). It could also be in response to the hypovolemia produced by the RAS blockade, but no data were found to support this.

The same RAS inhibitors have been reported to be renoprotective, reduce obesity, and improve insulin sensitivity in rodents, but without recording water intakes (50–53). Similar results occurred in one human study (54), yet not in another (55), both without recorded fluid intakes. Hypohydration has been shown to lead to hyperglycemia (56), which is linked with the major problems of obesity and type 2 diabetes.

RAS "KNOCKOUT" MICE DRINKING AND WEIGHT LOSS

Similar "paradoxical" results are found when the renin gene is knocked out, mice drink copiously (2.4 ± 0.1 compared with 9.2 ± 0.7 ml/day), are hyperactive, thin, have low body fat, and do not develop obesity (57). A decrease in body weight and % fat with an increase in activity was observed in renin-deficient mice on a high-fat diet, but no water intakes were given (58). Similar results occur in mice lacking the AT1 receptor (59, 60); however, no decrease in body weight was observed, despite a nearly three-fold increase in drinking in these AT1-receptor KO mice (61). Furthermore, angiotensinogen-deficient mice exhibit a decrease in body weight and % body fat with an increase in activity. Water intakes were not reported in this study (62), but have been noted by others (63, 64). Similarly, in ACE gene knockout mice, water intake was doubled (from 4.2 ± 0.2 to 9.8 ± 0.5 ml/day), food intake was slightly decreased, whereas body weight and body fat were significantly decreased (fat by 10%) compared with intact controls (65).

Further details on studies on the role of the RAS in food intake and metabolic parameters are in the excellent reviews by Mathai et al. (37) and by de Kloet et al. (66). In nearly all human and animal studies, pharmacological blockade of the RAS decreases body weight, food intake, and body fat. Unfortunately, most, if not all, studies did not report measurements of water intake. This argues for clinical studies on the effects of hydration on body weight regulation.

MECHANISMS

What Physiological Link Exists between Increased Drinking and Lipolysis?

Work in humans with administration of hypoosmotic solutions showed that there was an increase in lipolysis (67–69). The

studies also show an increase in lipolysis with increased drinking indicating and, by inference, an increase in metabolism. This produced the hypothesis that increased hydration leads to an increase in cell volume and from that to increased insulin sensitivity (70–72). Furthermore, the RAS has been linked also with mitochondrial dysfunction (73–75), and treatments with RAS antagonists improved mitochondrial function (76–80). Because the same treatment induces increased water intake, this suggests that an increased hydration may enhance mitochondrial function and thus metabolism. These mechanisms are illustrated in **Figures 1** and **2**.

Some studies report an increase in activity with increased hydration, but the authors did not look at activity alone in the overall effects on body weight decrease.

Another plausible mechanism is that increased water intake drives thermogenesis (83–87) that would lead also to a decrease in weight gain.

Physiologically, increased water intake leads to an increase in blood volume with an attendant increase in right atrium pressure. This would release atrial natriuretic peptide (ANP), which was one of the first identified natriuretic peptides (88). This family of cardiac natriuretic peptides activates uncoupling protein 1 (UCP1) that increases fat metabolism and leads to a loss of body weight (89–95). A significant increase in UCP1 was observed in renin knockout mice fed a high-fat diet (58), and these animals drink copious amounts of water (57). Furthermore, receptors for atrial peptides have been demonstrated in brown adipose tissue (96, 97).

Physiologically, the presence of AngII is linked almost exclusively to extracellular dehydration (or extracellular thirst). The physiological stimuli for thirst are known (3) and can be broken down to intracellular and extracellular deficits. *Intracellular dehydration* involves an increase in plasma osmolality (normal levels between 295 and 300 mosmol/kg water), leading to activation of hypothalamic osmoreceptors that stimulate drinking and the release of ADH that in turn conserves hydration by increasing renal water reabsorption. This action should return plasma osmolality to normal levels, reduce the motivation to drink, and stop the release of ADH. *Extracellular dehydration*, or a decrease in blood (plasma) volume (hypovolemia), leads to renin release from the kidney, which acts enzymatically on angiotensinogen in the blood making angiotensin I (AngI). AngI is transformed by ACE into AngII. As mentioned in Section "Introduction," AngII stimulates the seeking out and drinking of fluids (mainly water), an appetite for sodium, the release of ADH, and vasoconstriction. These actions should return plasma volume to normal levels while reducing blood AngII levels, the motivation to drink, to eat salt (mainly sodium), and the release of ADH. Most hypohydration leads to a mixture of intracellular and extracellular stimuli that should stimulate the behavioral acts of drinking and sodium intake, as well as the release of ADH, thus allowing correct regulation of body (and cellular) hydration.

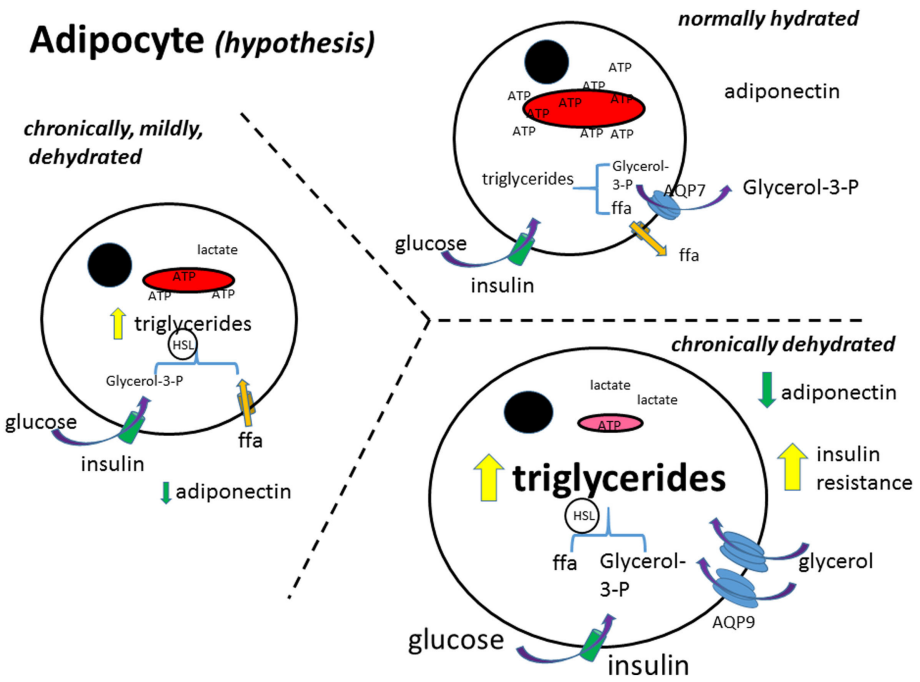

FIGURE 1 | **Adipocyte metabolism (hypothesis)**. In the normally hydrated (euhydrated) adipocyte, triglycerides are formed from glucose and free fatty acid uptake, and as well broken down (lipolysis); the rate depending on the needs of the cell for ATP. Glycerol in excess is exported out of the cell. Free fatty acids (ffa) are either metabolized or exported (81). As the adipocyte gets more and more dehydrated, formation of triglycerides increases and the ffa are not able to be transformed into pyruvate and thence metabolized in the mitochondria. The glycerol transporter, aquaporin 9 (AQP9), increases, bringing in more glycerol to make more triglycerides. Glucose uptake is further stimulated by insulin, increasing also triglyceride synthesis. Black circle, cell nucleus; red structure, mitochondria; ffa, free fatty acid; aa, amino acid; AQP7 + 9, aquaporin 7 and 9; HSL, hormone-sensitive lipase.

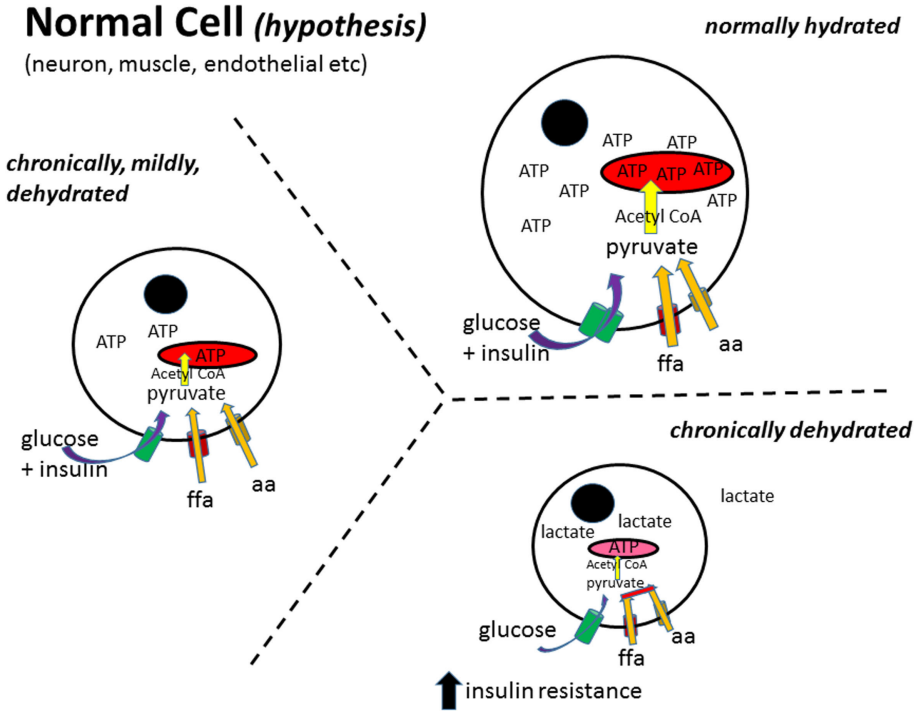

FIGURE 2 | **Normal cell metabolism (hypothesis)**. In a normally hydrated (euhydrated) cell, all substrates are taken up by their appropriate transporter mechanisms and enzymatically converted to pyruvate, transported into the mitochondria, converted to acetyl-CoA, which then enters the tricarboxylic acid cycle to generate ATP (82). As the cell gets more and more dehydrated, the metabolism of free fatty acids (ffa) and amino acids (aa) to pyruvate and/or acetyl-CoA decreases producing a dependence on glucose as the main fuel source [as has been reported for obesity (16)]. Furthermore, as the cell decreases in size, the ability of insulin to stimulate glucose uptake decreases, leading to insulin resistance. Black circle, cell nucleus; red structure, mitochondria; ffa, free fatty acid; aa, amino acid.

Although thirst is an effective motivation in most animal studies, it may not be a sufficient or adequate stimulus for drinking in many humans, including the ill, the elderly, and infants (98). The increased blood levels of AngII indicate that part of the human population may be chronically, but mildly, hypohydrated. As suggested earlier, chronic hypohydration is driving continuous release of AngII and, by extension, the metabolic dysfunction found in cardiovascular disease, obesity, diabetes, cancer, and Alzheimer's disease.

RODENT AND HUMAN HYDRATION

In its homozygous form, the Brattleboro rat figures prominently in studies of metabolism. This animal does not produce ADH and thus urinates copiously and consequently drinks considerably, up to 200 ml/day. These animals grow more slowly than their littermate controls with ADH for the same amount of food ingested (99–101). In the Brattleboro rat, this could be due to a significantly increased metabolism as observed in neurons when measuring fluorine 18-labeled fluorodeoxyglucose uptake with a PET scanner (102).

Human studies suggest a similar effect as an increase in water intake has been associated with a decrease in body weight in obese, overweight, and normal children, and adults (103–111). Furthermore, addition of 500 ml of water before eating breakfast or a hypocaloric meal reduces energy intake (112) or increases weight loss (113). In a recent random controlled trial, there was a significant weight loss between a group eating meals with a pre-meal water load compared with the controls without a pre-meal water load (114).

DIETS, DRINKING, AND WEIGHT LOSS

To take this further, in rodents, a high-protein diet is associated with weight loss (115, 116) and with increased drinking (117, 118). This increased drinking may reflect the increased urine output (119, 120) needed to excrete the added urea resulting from the additional dietary protein metabolism (121). Nevertheless, based on the evidence reviewed above, the weight loss observed while on not in a high-protein diet also could be a direct result of the increased water intake. Furthermore, an increased protein diet is also associated with an increase in size and number of functionally normal liver cell mitochondria (122, 123). This would correlate with an increase in cell size following an increase in hydration as mentioned above. Finally, weight loss produced using a hypocaloric diet induces a significant (30%) increase in water intake in both young (4 months old) and old (9 months old) female mice (124).

DISCUSSION AND CONCLUSION

This brief review highlights the considerable evidence that an increase in water intake, i.e., increased hydration, leads to loss of body weight. In rodent studies, the effect is clear and consistent.

At the least, this requires that measurement of water intake must be included in an experiment concerning rodents and all aspects of body weight regulation, from ingestive behavior to metabolic function. An increase in metabolism is one likely mechanism for the weight loss effect (125) because this can lead to increased mitochondrial function. In adipocytes, ramping up mitochondrial activity increases lipolysis. Human studies should also address the question of hydration with the increased use of RAS antagonists in the treatment of insulin resistance (126). Body weight regulation is a complex process, and increased water intake should be part of the measures required to reduce the overall risk factors.

As mentioned in Section "Introduction," the effects of chronic mild hypohydration extend beyond fostering obesity. Extracellular dehydration-induced AngII, and the attendant possible mitochondrial dysfunction, may contribute not only to obesity and diabetes but also to cardiovascular disease, cancer, and Alzheimer's disease. Furthermore, there could be other "symptoms" linking these major health problems to hypohydration such as a decrease in brain volume that is also associated with Alzheimer's disease, obesity, and diabetes and could be (127). A simple solution for reducing these modern chronic diseases would be to increase water intake across the general population. Given that hypohydration is a chronic circumstance, the effects of increased water intake would likely appear as younger groups age, as seen in schools to ameliorate childhood obesity (107, 110) and where dehydration is an issue at the start of the day (128, 129). Hypohydration occurs in France in that water intake is less than the National Nutrition Program recommendation of at least 1.5 l/day (130). The precise amounts of additional water needed and the relative importance of the different possible pathways and mechanisms remain to be specified. The implementation of such a policy would then require a public health initiative.

A limitation of this mini-review is that it concentrates mainly on papers dealing with the hypovolemia (or hypohydration)-related hormone AngII and the stimulated water intake that has effects on body weight, lipolysis, and food intake. There are a large number of studies in both animals and humans looking at the effects of RAS antagonist treatments for reducing the risk of cardiovascular disease, obesity, diabetes, cancer, and even Alzheimer's disease where water intake, or even thirst responses, is not reported.

AUTHOR CONTRIBUTIONS

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Increased Cost of Motor Activity and Heat Transfer between Non-Shivering Thermogenesis, Motor Activity, and Thermic Effect of Feeding in Mice Housed at Room Temperature – Implications in Pre-Clinical Studies

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The components of energy expenditure, total metabolic rate (TMR), resting metabolic rate (RMR), thermogenic response to feeding (TEF), activity, and cost of activity were measured in fed and fasted mice housed at 22 and 30°C. Mice housed at 22°C had more than two times larger TMR and RMR. Mice at 22°C were less active when fasted but more active when fed. Cost of activity was nearly doubled in the fasted and in the fed state. Analysis of the short-term relation between TMR, RMR, and bouts of activity showed that, at 22°C, the bouts of activity induced a decrease in the intensity of RMR that reflected the reduced need for thermal regulation induced by the heat released from muscular contraction. This phenomenon induced a considerable underestimation of TEF and prevented its reliable measurement when mice were housed at 22°C. Correlation between TMR and activity measured across time in individual mice was very strong at both 22 and 30°C, but the correlation measured across mice was much weaker at 30°C and no longer significant at 22°C. We suspect that this phenomenon was due to the fact that RMR is a much more reliable predictor of TMR than activity. RMR is more variable at 22°C than at 30°C because of heat transfers between thermal regulation and heat released by other discontinuous processes, such as activity and TEF. Therefore, more noise is introduced into the correlations performed across multiple mice between TMR and activity at 22°C. On the other hand, it should be kept in mind that the doubling of TMR and RMR at 22°C is fueled by an increased non-shivering thermogenesis that can obviously modify how the mouse responds to pharmacological and nutritional challenges. Taken together, these results suggest that in pre-clinical studies, mice should be housed in conditions where thermal regulation is limited as is generally the case in humans. However, the increased sensitivity of mice to small changes in ambient temperature can also be used as a versatile tool to investigate the role of thermal regulation on the energy balance equation in humans.

Keywords: mouse, indirect calorimetry, spontaneous motor activity, cost of activity, thermal regulation

Abbreviations: AMR, activity metabolic rate; RMR, resting metabolic rate; RQ, respiratory quotient; TEF, thermic effect of feeding; TMR, total metabolic rate.

INTRODUCTION

Obesity research to get a mechanistic understanding and to provide guidelines for clinical investigations has used mainly mouse models for experiments that are not ethical in humans. No other animal model offers such large possibilities of phenotyping in response to metabolic, genetic, and behavioral manipulations (1). However, it is important that mouse and human biology are similar in order to get reliable predictive values from mouse experiments.

Energy balance is determined by the equilibrium between energy intake and energy expenditure from basal metabolic rate, thermic effect of feeding, cost of activity, and thermoregulation. In obesity research, it appears that the cost of thermoregulation was until recently an underestimated component despite the fact that it is widely acknowledged that in small endothermic rodents energy demand to maintain body temperature can become an important component of the energy Budget (2). In contrast, humans who have a mass about 3000-fold larger than mice live predominantly close to thermo-neutrality. Humans maintain their body temperature without thermoregulatory effort, the heat generated by ongoing metabolism is the only process needed. The mouse is very sensitive to ambient temperatures that decrease below thermal neutrality (28–31°C) because of its small size, and therefore very large body surface area to mass ratio. To maintain their body temperature, mice rely heavily on thermogenic processes specifically devoted to heat production, which are mainly uncoupled respiration in brown adipose tissue (3, 4), but also depend on shivering thermogenesis and heat generated by muscular activity (5, 6).

In most cases, mice studies have been conducted at temperatures of 20–22°C, which is far below their thermal neutrality (30–32°C). This condition increases the cost of thermoregulation that can double energy requirements (7) and subsequently increases food intake, sympathetic activity, blood pressure, and heart rate. Therefore, the question is raised whether this large amount of energy produced to maintain body temperature can affect not only resting metabolism but also the amount and the cost of locomotor activity, the thermogenic response to feeding (TEF), and more generally the responses to various metabolic challenges. Indeed, the extra heat produced by the activity cost (physical work is only ~20% efficient) and TEF (enzymatic reactions are ~60% efficient) can potentially reduce the energy required for thermoregulation. Therefore, it is possible to consider that heat released by activity or feeding will reduce the cost of thermoregulation and can induce an underestimation of activity or feeding costs. This phenomenon was suggested in a previous paper which showed that TEE was correlated with activity when mice were housed at 30°C but not when they were housed at 20°C. At this lower temperature, energy expended from activity was masked by the reduction of the energy expended for thermoregulation (8). Moreover, evaluation of drug effects on energy expenditure may be altered when mice are housed at room temperature because the compensatory reduction in cold-induced thermogenesis can offset the drug-induced increase. It has been suggested also that when mice are housed below thermal neutrality, BAT thermogenesis may play an important role in food intake control and energy balance regulation (9). An inadequate response to cold was reported also in Lep^{ob}/Lep^{ob} mice (10), which may explain why it is only at temperatures below thermal neutrality that they have a lower energy expenditure than wild-type mice (11, 12).

According to these results, analysis of the preliminary results of a current study lets us suspect that ambient temperature could have profound effects on the mechanisms of adaptation of mice to low-protein diets. We extracted the control mice of this study to focus on the evolution of energy metabolism components when mice are acclimatized to the vivarium temperature (22°C) or at thermal neutrality (30°C). In this article, we report changes induced on resting and total metabolic rate (TMR), spontaneous motor activity, cost of activity, and the TEF.

MATERIALS AND METHODS

Animals and Housing

Twenty-one female Balb/cOlaHsd mice were singly housed in a conventional facility with a reversed 12:12-h dark–light cycle (lights on at 20:00 hours). All experimental procedures complied with institutional guidelines and policies to prevent pain and distress under license from the French Veterinary Service (Ethics committee agreement number 12-095 and 13-012). The mice were provided by Harlan Laboratories (France) at 7 week of age and were allowed 2 weeks adaptation to the laboratory conditions before any experimental manipulation.

The mice used in this study were the control mice of two different experiments performed in 2014 and 2016. Water and food were provided *ad libitum* during the two studies unless otherwise stated. Mice were fed either a soy protein or a casein diet [by energy: soy protein or casein 24%, carbohydrate 66% (56.4% corn starch, 9.6% sucrose), fat (soy oil) 10%]. Food quotient of the two diets was 0.93. The results of the casein and soy-protein fed mice were pooled after we controlled for the similar reactivity of the two groups to the differences in ambient temperature.

In the first study ($n = 11$, 6 soy and 5 casein), the mice were housed continuously at 22°C. In the second study ($n = 10$, 5 soy and 5 casein), the mice were first housed at 22°C during 5 weeks, then the room temperature was increased to 30°C, and the mice maintained under these housing conditions for four more weeks (**Figure 1**).

Indirect Calorimetry

The indirect calorimetry system used in this study was a custom designed system working in pull mode and described in detail in several previous publications (13–15). Respiratory quotient (RQ) was calculated as the ratio of CO_2 production (VCO_2) over O_2 consumption (VO_2). Metabolic rate was calculated in watts (W) using the Weir equation (14). Spontaneous activity was measured by force transducers located under the floor of the cage. Data acquisition and data processing were performed by computer programs developed in the laboratory and written in the LabVIEW®.

In a first study, TMR and spontaneous activity (Act) were measured in *ad libitum*-fed mice housed at 22°C, and the TEF was measured at 30°C. In a second study, mice followed the reverse

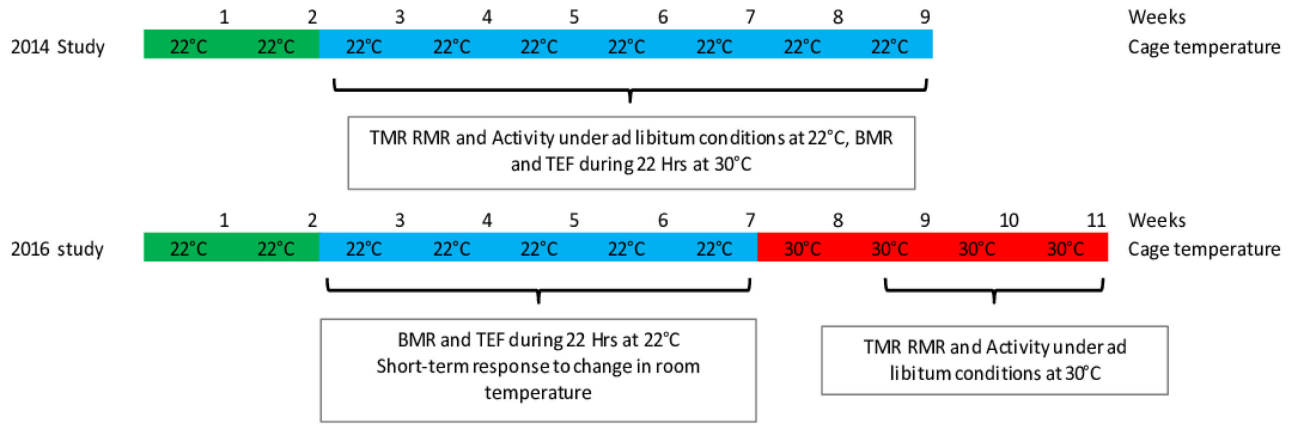

FIGURE 1 | **Experimental design of the two studies**.

procedure, i.e., TEF was measured in mice housed at 22°C, and TMR and activity were measured under *ad libitum* conditions at 30°C after the mice were accustomed for at least 10 days to this temperature (**Figure 1**).

Measurement of TMR, RMR, and Activity under *Ad Libitum* Conditions

Mice were previously accustomed for 3–4 days to the calorimetry procedure by being housed in the same cages as used for the calorimetry recording. For the recording, they were kept in the same cage, and the cages were connected to the calorimetry system.

VO_2, VCO_2, and intensity of spontaneous motor activity were recorded from five chambers (2.5 L volume, constant flow rate of 600 mL/min). Each chamber, then room air (to correct for background VO_2–VCO_2) was sampled during 100 s, so that each cage was sampled every 10 min. In the cages, a sheet of blotting paper was used as bedding. Water and food were freely available in small boxes fixed on the side of the chambers. Data acquisition was performed without interruption during 2 days. Day 2 was used for data analysis.

Analysis of the components of energy expenditure provided TMR and resting metabolic rate (RMR), RQ, and intensity of activity. The relation between TMR and activity was computed across time for each mouse. To improve the correlation between changes in the intensity of TMR and changes in the intensity of activity, a slight convolution of the activity signal was performed in order to reproduce the smoothing of the respiratory response induced by the dead space of the chambers (**Figure 2A**). RMR was obtained as the *Y*-axis intercept of the correlation between TMR and activity, and the cost of activity was computed as the slope of the correlation between TMR and activity (**Figure 2B**). The metabolic rate of activity (AMR) was computed as the difference between TMR and RMR.

The relations between TMR, AMR, and activity and TMR and RMR were also computed across multiple mice by using mean daily TMR, AMR, RMR, and activity values obtained in each mouse.

During experiment 1, temperature in the experimental room was regulated at 20°C in order to maintain a temperature of 21–22°C in the metabolic chambers. During experiment 2, mice were previously acclimatized for at least 1 week at a room temperature of 30°C, and temperature in the experimental room was maintained at 29°C in order to maintain a temperature of 30–31°C in the metabolic cages.

Measurement of the Thermic Response to Feeding and of the Short-Term Changes Induced by Activity on TMR, RMR, and RQ during a Cycle of Fasting and Refeeding

These measurements were performed by measuring VO_2, VCO_2, and spontaneous motor activity continuously on one single cage at 2 s intervals. The uninterrupted acquisition on one cage and the high frequency of data sampling were required to perform a detailed analysis of the short-term relation between changes in VO_2 and VCO_2 and intensity of activity in order to be able to precisely compute the energy cost of activity and subsequently to compute RMR and TEF without artifacts due to variability in spontaneous activity. This process is based on a filtering procedure according to the method of Kalman and has been described in detail in several previous publications from our laboratory (14, 15) and more recently in one by Van Klinken and colleagues (16). Examples of results on individual mice are given **Figure 3**. Temperature in the cage was adjusted by decreasing room temperature below the required value in the cage and heating the wall of the cage with a heating coil controlled by a temperature gage. This system allowed the temperature (±0.2°C) to be maintained stable in the cage.

Mice were housed in the cage between 17:00 and 18:00 hours with water but no food and were kept overnight (i.e., mostly during their light period) in the metabolic cage (**Figure 3**). The next morning, a calibrated meal of 1 g (16 kJ) was introduced into the cage without interrupting data acquisition, and data recording was continued during 6–7 h. Average RMR and RQ during the 2 h that preceded the meal were used as baseline RMR and RQ values

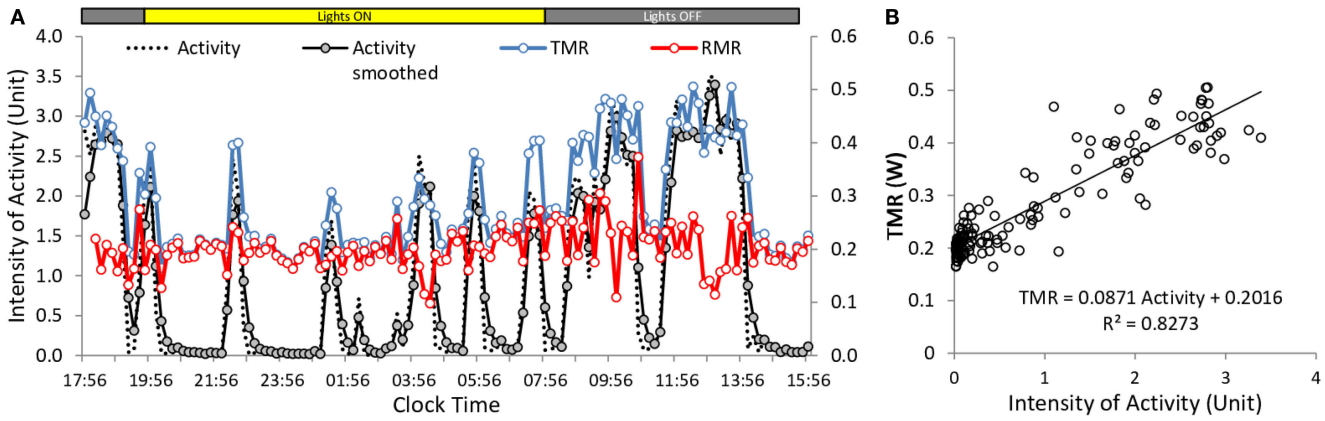

FIGURE 2 | Example of calculation of activity cost and RMR in mice under *ad libitum* conditions. Data were obtained from the multiplexed device. Acquisition was at 10 min intervals. **(A)** Changes in activity, TMR and RMR measured along the time. The original activity trace was slightly smoothed to better correlate with TMR. **(B)** Correlation between intensity of activity and TMR. Slope of the regression gives the cost of activity and origin gives RMR. RMR in **(A)** was computed as TMR (activity × cost).

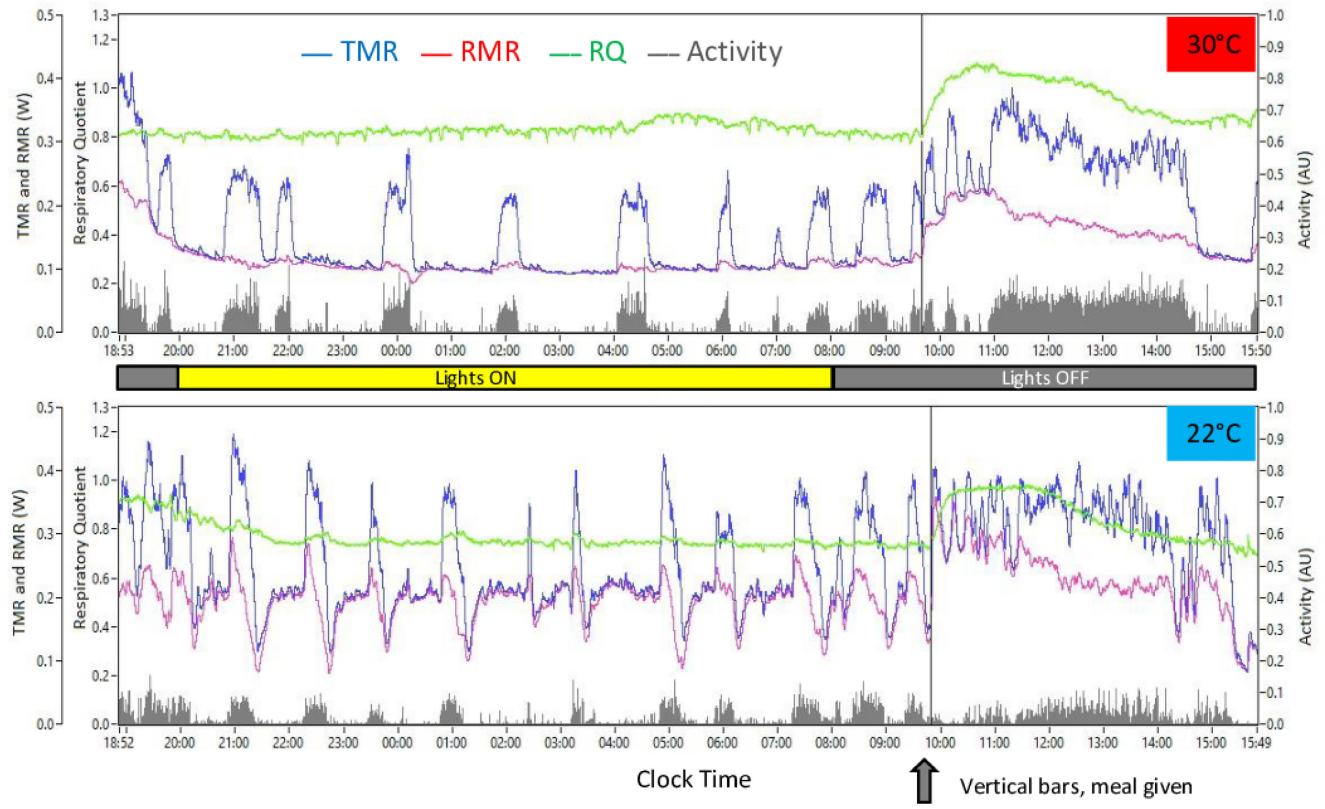

FIGURE 3 | Example of results observed in response to ingestion of a meal-test after overnight (mainly during the light period for the mouse) food restriction (screen copies of the computer program developed in the laboratory to edit the calorimetry data). Top, mouse housed at 30°C (2014 study); bottom, mouse housed at 22°C (2016 study). VO_2, VCO_2, and activity data were initially recorded at 2 s intervals then processed by the method of Kalman to compute TMR and RMR. RQ was computed as VCO_2/VO_2. Vertical bars crossing the figures indicate time when the meal was introduced into the cage.

to calculate the changes induced by ingestion of the meal. TEF was computed in kJ as the cumulative increase above baseline RMR during 6 h after meal delivery. Short-term changes in TMR, RMR, and RQ in relation to the bouts of activity were studied by pooling activity periods extending from one hour before to one after well differentiated bouts of spontaneous activity that

occurred between 23:00 and 08:00 hours, i.e., in fasted mice in the post-absorptive state and after non-shivering thermogenesis (NST) had time to switch off in mice housed at 30°C (see **Figure 3**). Hourly changes in the intensity of activity were also computed to compare intensity of spontaneous activity during fasting and refeeding.

All experiments were performed in mice usually housed at 22°C. During experiment 1, temperature in the metabolic cage was regulated at 30°C; during experiment 2, temperature was regulated at 22°C.

Statistical Analysis

Data are presented as mean ± SEM. Values at 22 and 30°C were compared using a Student's *t*-test in Excel, or by two-way ANOVA in R® when means were compared in relation to a third parameter (time for TEF, distribution classes for TMR activity, and RQ). Significant ANOVA results were followed using *post hoc* Tukey tests. Significance was set at $P < 0.05$.

RESULTS

RMR, RQ, Activity, and TEF in Fasted-Refed Mice

Analysis of the changes in RMR and RQ during a cycle of fasting and refeeding showed large differences between mice housed at 22°C compared to those at 30°C. RMR measured after an overnight fast during the last 3 h before refeeding (~06:30–09:30) was nearly twice as large in mice housed at 22°C (**Table 1**; **Figure 4A**). Conversely, RQ was significantly lower attesting a greater reliance on fat derived substrates (**Table 1**; **Figure 4D**). During the fasting period (~23:00–10:00), activity was low and not significantly different at 30 and 22°C (**Figures 3** and **5**).

Meal-induced increase in RMR was greatly reduced in mice housed at 22°C (**Table 1**; **Figure 4B**), and therefore, TEF computed by extrapolating pre-meal RMR appeared three times smaller [**Table 1**; **Figure 4C**; 22°C(1)]. However, it appeared that post-meal RMR was lower than pre-meal RMR 4 h after the meal and onward, suggesting that during the post-prandial period, the extra heat released by activity and TEF decreased the extra-energy expended for thermal regulation. Therefore, computing TEF by extrapolating pre-meal RMR probably underestimated TEF at 22°C. If RMR measured 6 h after meal onset was used as baseline, then TEF at 22°C was similar to TEF at 30°C [**Figure 4C**, 22°C(2)].

The meal-induced increase in RQ was of similar amplitude at 22 and 30°C but was of significantly shorter duration in mice housed at 22°C (**Table 1**; **Figures 4E,F**). After ingestion of the test-meal, and until the end of the experiment, spontaneous activity was significantly higher in mice housed at 30°C (**Figures 3** and **5**).

TABLE 1 | Components of energy expenditure in fasted-refed mice.

	30°C (n = 11)		22°C (n = 10)				
	Mean ± SEM		Mean ± SEM		°C	Time	°C × T
Fasting RMR (W)	0.213	0.006	0.397	0.011	$<10^{-9}$	–	–
Fasting TMR (W)	0.273	0.015	0.492	0.015	$<10^{-12}$	–	–
TEF (kJ)	3.149	0.196	1.009	0.295	$<10^{-15}$	$<10^{-15}$	$<10^{-12}$
TEF (% ingested)	19.68	1.22	6.309	1.845	$<10^{-15}$	$<10^{-15}$	$<10^{-12}$
RQ	0.823	0.008	0.748	0.005	$<10^{-6}$	–	–
AUC RQ	4.374	0.205	3.644	0.276	$<10^{-12}$	$<10^{-2}$	0.99

TMR and RMR values are adjusted to 20 g BW. AUC, area under curve.

Relation between TMR, RMR, RQ, and Activity Measured across Time in Fasted and Fed Mice

Fasted Mice

In mice housed at 30°C, the mean peak intensity of the bouts of activity was ~40 U and occurred 15 min after the onset of activity (**Figure 6A**). Mean duration of the activity periods was 30–40 min. The bouts of activity-induced parallel changes in the intensity of TMR, reflecting the metabolic cost of activity but only marginally modified the intensity of RMR. A very small but significant increase was however observed during the first min of activity [0.0347 ± 0.0037W, $P < 10^{-5}$ (+17% vs. baseline)].

At 22°C, the mean peak intensity of the bouts of activity was very significantly reduced down to one half of the intensity observed at 30°C (~20 U) (**Figure 6B**). Activity increased TMR but also profoundly affected the evolution of RMR: RMR increased very significantly during the first 5 min of activity [0.146 ± 0.013 W, $P < 10^{-16}$ (+ 37% vs. baseline)] then decreased progressively down to a value lower than before the onset of activity. The decrease lasted as long as the activity duration. After activity stopped, RMR increased progressively again and returned to pre-activity values in 30 min.

As we observed that the increase in TMR appeared of similar amplitude at 22 and 30°C despite the very significant decrease in the intensity of the bouts of activity, we calculated more precisely the cost of activity by processing the differences between RMR and TMR (δMR) in relation to activity (**Figures 6C,D**). This data processing confirmed that the cost of activity was higher at 22°C than at 30°C. The correlation between δMR and activity computed during the first 15 min of activity where the correlation was the best indicated a doubling of the metabolic cost of activity (**Figure 6E**).

As already quoted, RQ was significantly lower in mice housed at 22°C (**Figure 6F**) indicative of a greater reliance of fat derived energy. Activity induced transient changes of small amplitude that were similar at 22 and 30°C indicating that muscle contraction was fueled by the available substrate mix as used by the other tissues of the body.

Fed Mice

In fed mice, despite the fact that data acquisition was performed at a lower frequency and that the mice were fed, the correlations measured across time in each mouse between TMR and activity remained high ($0.85 < R < 0.87$) (**Table 2**, **Figure 2B**). This allowed the recording of fairly precise and reproducible values for RMR and cost of activity from the origin and from the slope of the correlations, respectively. TMR, RMR, and daily activity were significantly higher in mice at 22°C, and the cost of activity was approximately two times larger at 22°C than at 30°C (**Table 2**) as

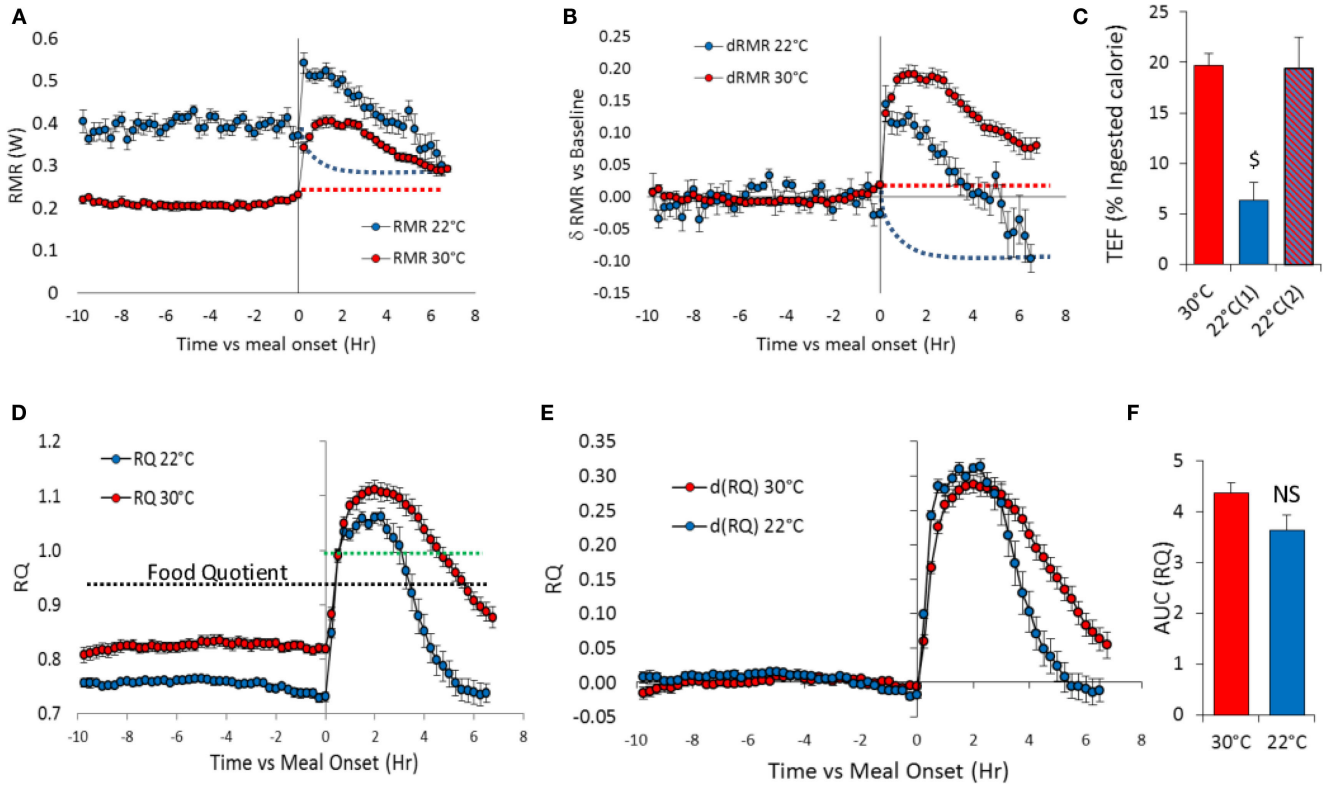

FIGURE 4 | **Meal induced changes in TMR, RMR (watts), and RQ in overnight fasted mice at 30°C (n = 11) and 22°C (n = 10)**. Test-meal (1 g, 16 kJ) given at t = 0. **(A)** Absolute TMR and RMR values, **(B)** TMR and RMR values relative to pre-meal values, **(C)** TEF computed as the cumulative increase in RMR over pre-meal values [30 and 22°C(1)] or after taking into account the possible decrease in RMR at 22°C [22°C(2)] [$: $P < 10^{-5}$ vs. 30 and 22°C(2)]. See TEF in kJ **Table 1**. Red dashed lines – extrapolation of fasting RMR at 30°C. Blue dashed lines – estimated decrease in RMR induced by activity and TEF used to adjust the calculation of TEF at 22°C [22°C(2)] **(D)** Absolute RQ values. Black dashed lines – food quotient (0.93). Green dashed lines – figures the RQ value of 1. **(E)** RQ values above 1 imply that part of the ingested carbohydrates is converted to lipids (lipogenesis). RQ changes relative to pre-meal RQ values, **(F)** Area under curve (AUC) computed as the cumulative changes in RQ over pre-meal values.

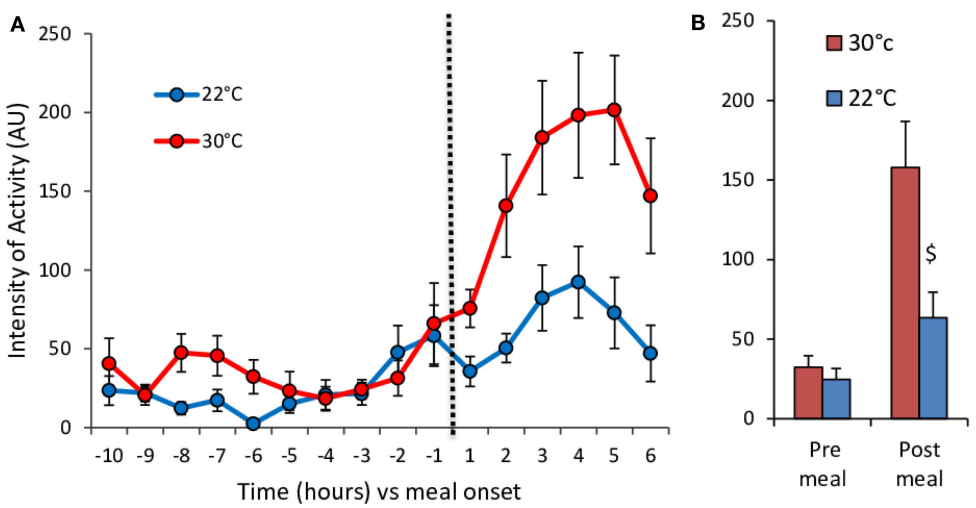

FIGURE 5 | **Hourly changes in the level of spontaneous activity (A) and mean activity values (B) before (pre-meal) and after (post-meal) delivery of the test-meal at 30°C (n = 11) and 22°C (n = 10)**. **(A)** Time effect, $P < 10^{-15}$; temperature effect, $P < 10^{-9}$; time × temperature effect, $P < 10^{-4}$. **(B)** $, $P < 0.01$.

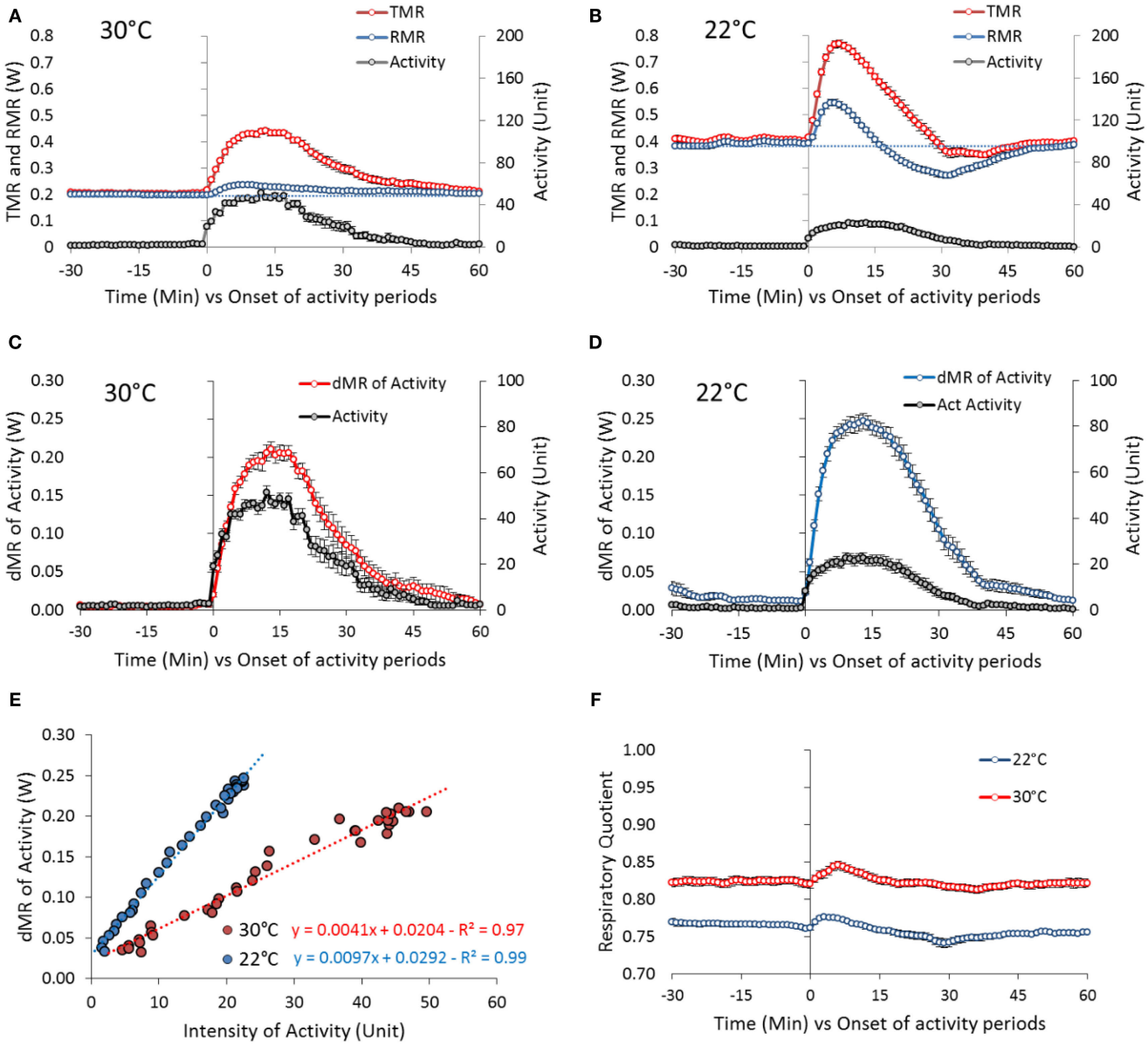

FIGURE 6 | Activity-induced changes in TMR and RMR in mice housed at 30°C (31 periods of activity from 11 mice) and 22°C (42 periods of activity from 10 mice). The periods of activity have been chosen as periods of well differentiated bouts of activity, preceded and followed by at least 1 h of quite complete rest that occurred during the overnight fast and the morning before the test-meal. The first 5 h of recording after the mice were housed in the metabolic chamber (18:00–23:00 hours) were discarded to focus on the response of mice in the post-absorptive state and adapted to the temperature in the cage (see **Figure 3**). **(A,B)** Absolute changes in TMR and RMR at 30 and 22°C. Blue dashed line: extrapolation of pre-activity RMR values. **(C,D)** Changes in δMR (δMR = TMR − RMR and reflects the true direct activity cost). **(E)** Correlation between activity and δMR changes at 30 and 22°C. **(F)** Absolute changes in RQ.

already observed in fasted mice. The AMR computed as TMR minus RMR was also significantly increased.

Relation between TMR, RMR, AMR, and Activity Measured across Multiple Mice in *Ad Libitum* Fed Mice

We observed a significant effect of activity on AMR at both 22 and 30°C (**Figure 7A**). However, activity did not significantly affect TMR at 22°C while the effect at 30°C was reduced and remained only borderline significant (**Figure 7B**). On the other hand, we observed a very strong correlation between TMR and RMR (**Figure 7C**), which may be related to the fact that RMR accounted for 68–74% of TMR (**Table 2**).

Distribution of TMR and RMR Values in *Ad Libitum* Fed Mice

Total metabolic rate and RMR values of *ad libitum* fed mice were more than doubled at 22°C (**Table 2**; **Figure 8**). This result is

TABLE 2 | Components of energy expenditure in *ad libitum* fed mice.

	30°C (n = 9)		22°C (n = 11)		
	Mean ± SEM		Mean ± SEM		P
TMR (W)	0.244	0.024	0.686	0.020	$<10^{-9}$
RMR (W)	0.167	0.018	0.510	0.016	$<10^{-10}$
RMR (% TMR)	68.02	1.56	74.43	0.72	$<10^{-2}$
Activity (AU)	0.945	0.095	1.463	0.102	$<10^{-2}$
AMR (W)	0.077	0.007	0.175	0.006	$<10^{-8}$
AMR (% TMR)	31.98	1.56	25.57	0.72	$<10^{-2}$
Cost of act (W/AU)	0.083	0.008	0.123	0.007	$<10^{-3}$
Cor. Coef. between TMR and activity	0.869	0.015	0.856	0.008	NS

TMR and RMR are adjusted to 20 g BW. N = 9 instead of 10 at 30°C because recording of the activity signal failed on one cage.

related to a strong shift to the right of the distribution frequency at 22°C. The distributions of TMR values are also less Gaussian than the distribution of RMR values, with a shift to the right that reflects the energy expended with activity, more pronounced in mice housed at 22°C. This shift induced a clear separation between mean and median activity values that was hardly visible on RMR. Unexpectedly, the distribution of RMR values at 30°C showed a peak at 0.15 W, i.e., at a value lower than RMR values measured after an overnight fast (**Table 1**), which suggests that at 30°C, under close to usual living conditions and despite continuous access to food, mice had possibly periods of very low metabolism.

Distribution of Activity Values in *Ad Libitum* Fed Mice

Contrary to what was observed in food restricted mice, spontaneous activity was larger at 22°C when mice were fed *ad libitum* (**Figure 9C**). This increase relies on both more activity of high intensity (above 3 U, **Figure 9A**) and on the fact that, at 22°C, the mice were never quite completely restless and therefore had a much smaller peak of low activity values than mice at 30°C. The strong shift to the left, down to 0.4 AU, of the median activity intensity when mice were housed at 30°C shows that they were completely inactive half of the time. Accordingly, **Figure 9B** shows that the occurrence of activities of very low intensities (between 0 and 0.1) amounted to 38% of the time in mice housed at 30°C, while it was only 12% in mice housed at 22°C. In contrast, mice at 22°C exhibited increased occurrence of activities of intensities between 0.1 and 0.4 (27% of time vs. 12%, **Figure 9B**) testifying to a form of restlessness.

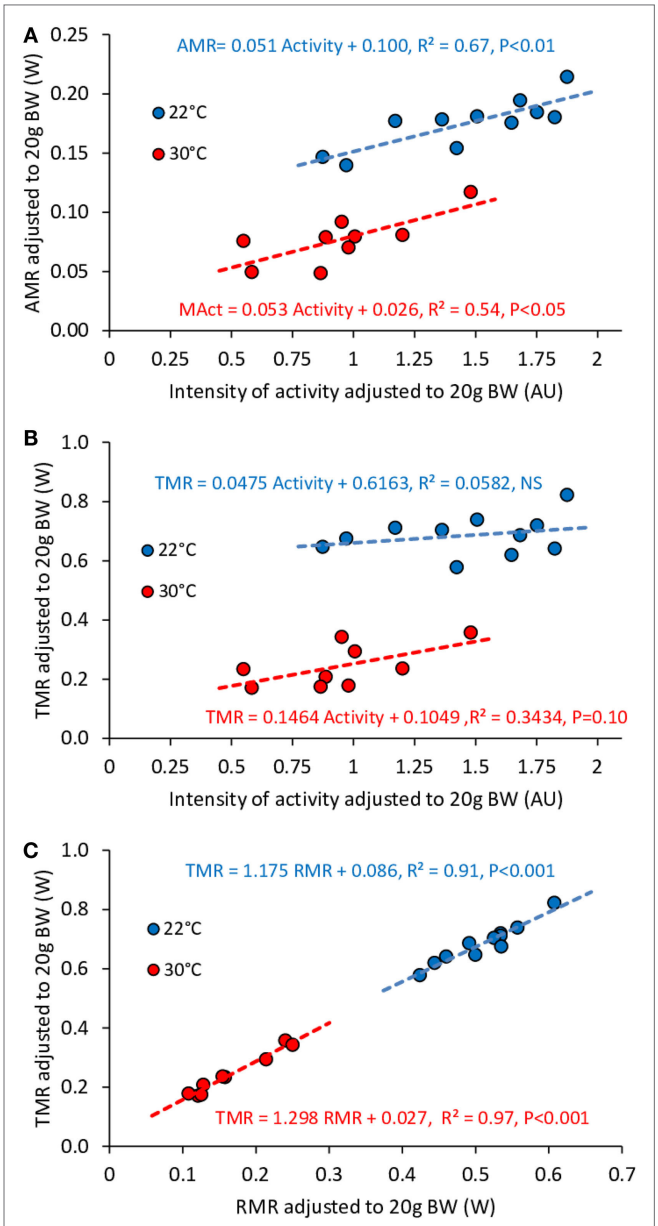

FIGURE 7 | Correlation between AMR and activity (A), TMR and activity (B), TMR and RMR (C) in *ad libitum* fed mice housed at 30°C (n = 9) and 22°C (n = 11). Data are adjusted to 20 g BW. Despite the fact that intensity of activity affects significantly AMR, the consequences on TMR are not significant. The strongest predictor of TMR appears to be RMR.

DISCUSSION

This study confirms that energy expenditure is approximately doubled in mice housed singly at room temperature (22°C) vs. mice housed at thermal neutrality (30°C) (2, 17). The most significant results of this study are that (1) spontaneous activity in mice at 22°C is reduced when the mice have no access to food but increased when they are fed, (2) the energy cost of activity is doubled when the mice are housed at 22°C, (3) RMR is decreased during activity at 22°C, and (4) TEF is probably largely underestimated when measured at 22°C. Taken together, these results shed more light on how the energy expended with NST affects the components of energy expenditure and can undermine the use of mice housed below thermal neutrality as a model of human physiology.

Spontaneous Physical Activity

We observed that activity was reduced at 22°C when the mice were fasted, but increased when they were under *ad libitum* conditions. Activity reduction in fasted mice at 22°C was primarily

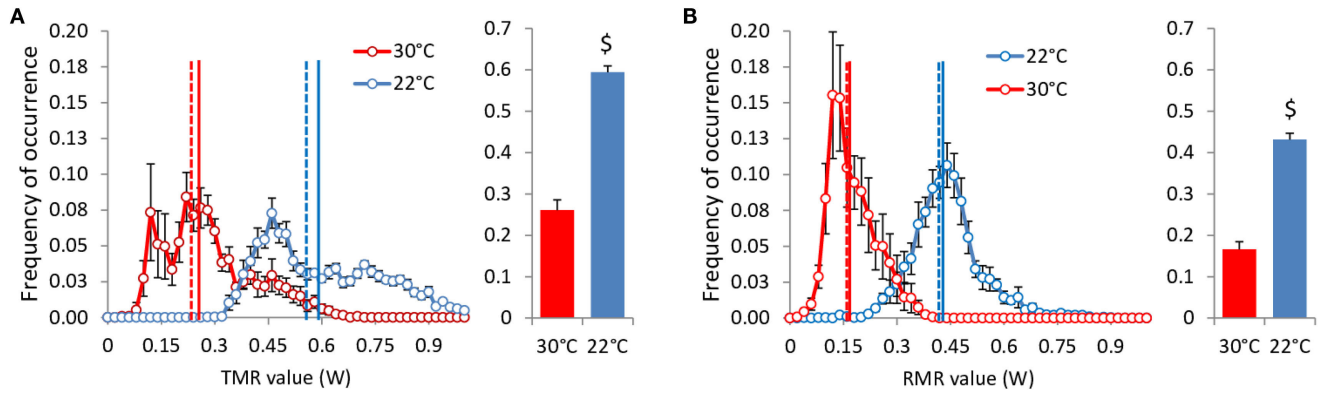

FIGURE 8 | TMR (A) and RMR (B) values distribution adjusted to 20 g BW in *ad libitum* fed mice housed at 30°C (n = 9) and 22°C (n = 11). Vertical lines: mean (solid) and median (dashed). $: $P < 10^{-5}$.

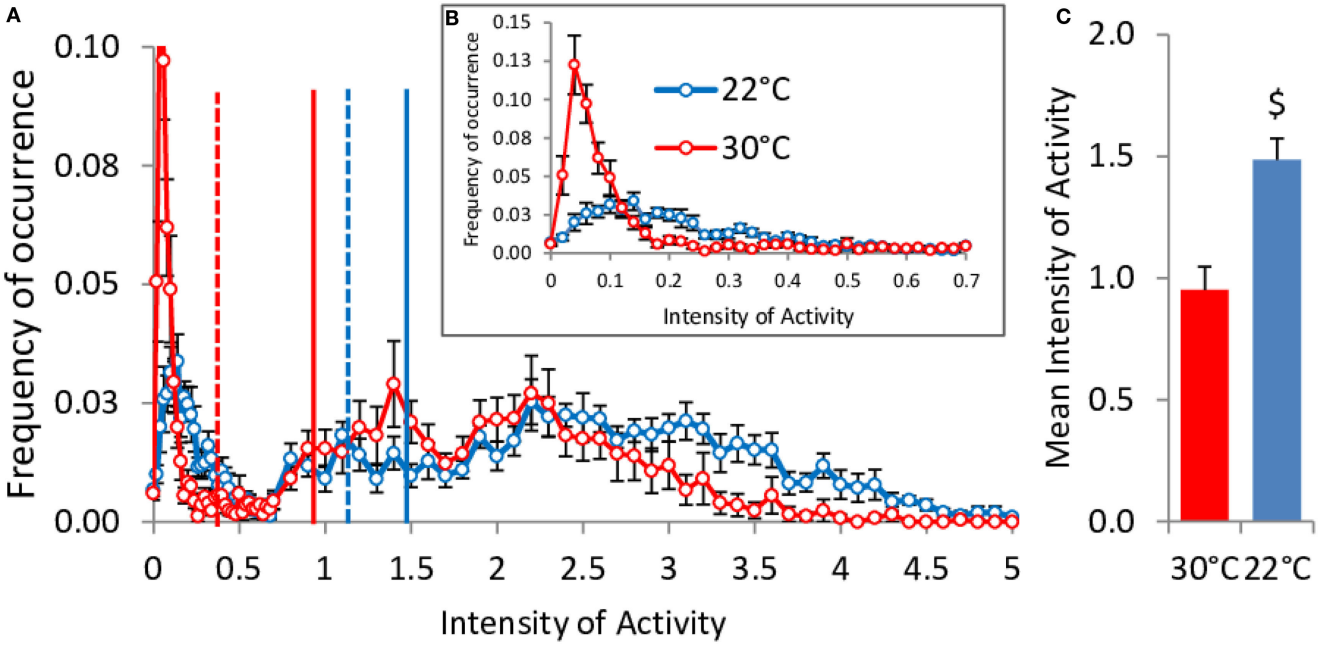

FIGURE 9 | Activity values distribution in *ad libitum* fed mice housed at 30°C (n = 9) and 22°C (n = 11). Vertical lines: mean (solid) and median (dashed). **(A)** Overall distribution of the activity values ($P < 10^{-6}$ 22°C vs. 30°C). Inbox **(B)**, zoom of the distribution on the lower intensities of activity values. **(C)** Mean activity values ($, $P < 0.001$).

due to a strong decrease in the intensity of the bursts of activity and not in a reduction in the number of activity periods (**Figure 3** gives a typical example of this phenomenon). It was not possible to perform such a detailed analysis of the amplitude of the bursts of activity in *ad libitum* fed mice because data acquisition was performed in a multiplexed design and measurements were performed at 10 min instead of 2 s intervals. However, we observed that the average activity intensity in mice housed at 22°C was 30% higher than in mice housed at 30°C. In addition, a percent cumulative frequency analysis of the activity data (18) indicated that fed mice housed at 22°C spent less time fully inactive and more time restless or highly active. It is probable that the low ambient temperature made the mice fell less comfortable and induced fidgeting that decreased the time spent fully inactive. On the other side of the distribution, the increased occurrence of high intensities of activity was probably related to the larger food intake induced by the increased energy requirements.

Brown and colleagues previously reported that, in fasted rats, activity was reduced at room temperature (21°C) vs. thermal neutrality (28°C) (19). They reported that video recordings indicated

a cold-defensive posture at 21°C in order to decrease convective and radiant heat transfer. In our study, we did not perform video recordings but it was visually obvious that fasted mice housed at 22°C were huddled up on themselves and were rather reluctant to move. The decrease in activity in fasted mice at 22°C can therefore be the result of a behavioral adaptation to the cold to reduce heat losses in conditions where they had no opportunity to access food and refill their energy stores (20). In contrast, it seems that when under *ad libitum* feeding conditions, mice react to the decrease in room temperature by resting less, moving, and eating more. We were not able to analyze properly the food intake recordings in this study, but we observed no decrease in BW in the mice housed at 22°C during the calorimetric studies (mean δBW 22°C = 0.19 ± 0.44), which implies that mice housed at 22°C ate approximately two times more than those housed at 30°C. In contrast, under *ad libitum* conditions, Kaiyala and colleagues (21) did not report any significant effect of temperature on spontaneous physical activity. The reason for this difference remains unclear. One possible explanation may be related to the fact that, because the difference in activity under *ad libitum* conditions relies on the lowest and largest activity levels, the less precise measurement of activity with red light-beams as used by Kaiyala and colleagues, vs. force transducers used here, may indicate that they missed these differences. However, the differences in TMR at 21 and 30°C was also smaller in the Kaiyala study than in this one, and therefore, the difference may also be due to the mouse strain or sex.

Relation between Activity, RQ, TMR, and RMR in Fasted Mice

Respiratory quotient was poorly affected by the occurrence of the bursts of activity at 22°C as well as at 30°C showing that muscle contraction was fueled by the available mixture of circulating glucose and fatty acids. Such lack of a specific increase in glucose oxidation and the strong correlation between activity and TMR probably reflects the fact that the bursts of activity were of low intensity and therefore that the work load on the muscles was low enough to be fueled by the current circulating mix of carbohydrates and lipids.

The bursts of spontaneous activity induced a rapid doubling of TMR at both 30 and 22°C. This increase reflects the energy required to fuel muscular effort. In the experiments performed to measure TEF, the high rate of data acquisition combined with the data processing by the Kalman filtering (13–15) allowed us to perform a very detailed analysis of the short-term changes between activity, TMR, and RMR in fasted mice. This analysis showed that activity marginally increased RMR at 30°C but induced curvilinear changes in RMR when the mice were housed at 22°C. At this temperature, RMR increased during the first 5 min then declined rapidly below the level measured before the onset of activity and finally reached a nadir at the end of the activity period. During the rest periods following the activity bursts, RMR returned to pre-activity values within ~30 min. The increase in RMR during the first 5 min of activity surprised us but was already described by Brown and colleagues (19), although not as precisely as here, which supports the idea that this increase was not a computational artifact. It has been suggested that the temperature set-point may be increased during activity (7) possibly to heat muscles and to improve muscular work, which may explain why this phenomenon is observed with more intensity in mice housed at 22°C than in those housed at 30°C. The following decrease in RMR reflects obviously that the heat released by the working muscles reduced the cost of thermoregulation. Accordingly, when the mice stopped moving, the heat released from the muscles progressively decreased, therefore thermal regulation was progressively restored and RMR increased back to pre-activity values. This process was also suggested by Brown and colleagues in the rat (19). According to their calculations, the decrease in what they called "supplementary thermogenesis" lasted 1–1.5 h after the end of activity. The difference may be due to the inferred timing adjustment of their equations or because the measurement were done on rats in conditions where the cold stress induced a smaller response than what we report for mice in this study (heat production was increased by only 25% instead of 100% here). On the other hand, Kaiyala and colleagues (21) reported that the thermoregulatory effort of mice housed at 21°C was reduced during the light period when activity and feeding were the highest.

The relation between the TMR increase above RMR (δMR) and the activity signal intensity computed from data acquired at a high rate showed a strong linear correlation between δMR and activity and indicated a doubling of the activity cost in fasted mice housed at 22°C. This result was confirmed in mice fed *ad libitum* where the correlation between TMR and activity, despite a less precise fit, unambiguously pointed to a significant increase of the activity cost at 22°C. Again, this phenomenon was already observed in rats by Brown and colleagues (19) who reported that the increase in heat production induced by activity was of 0.040 vs. 0.068 J/min/$g^{0.67}$ at 28 and 21°C, respectively. Another study in which the cost of activity was investigated at different temperatures (7) also reported higher energy expended in the cold. The authors did not report directly the slope of the correlation between TMR and activity but in their discussion quoted that the energy cost per unit of activity was increased when mice were housed at low temperature (4°C). Therefore, the results of this study line up with previous reports showing that the cost of activity is increased when rats or mice are housed below thermal neutrality.

Abreu-Vieira and colleagues (7) suggested that this increase was likely due to increased heat loss from the less compact body position and disruption of the unstirred air layer around the body. However, from our data and in particular from the analysis of the very short-term changes between activity and TMR, we could observe that the extra cost of activity remained strongly correlated to the intensity of the activity signal, and therefore was produced in line with the ATP production for muscular contraction. In this context, the most plausible mechanism is an increased uncoupling between respiration and ATP production, possibly sustained by an increased expression of UCP2 and/or UCP3 in muscles to assist thermal regulation (22). It has been suggested already that variations in gene expression of UCP2 and UCP3 in muscles may affect the energy cost of exercise (23, 24). Abreu-Vieira and colleagues (7) also suggested that mice defend a higher body temperature during physical activity and that such increased uncoupling at 22°C may be a way to help increase

muscle temperature. The reason for this uncoupling may be to warm the muscles and increase muscle performance at lower ambient temperatures (25). Another parameter in favor of this interpretation is, as discussed at the beginning of this section, the transient increase in RMR observed during the first 5 min of activity periods at 22°C but not at 30°C. This could be interpreted as an increased heat production by NST to warm-up the muscles at the onset of muscular effort.

Relation between Activity, and TMR in Fed Mice

Despite the fact that, in free-feeding mice, the relation between TMR and activity measured across time in individual mice remained very strong at 22°C as well as at 30°C, and that the cost of activity at 22°C was approximately two times higher than at 30°C (as observed in fasted mice), we observed that when measured across multiple mice, the level of activity did not affect any more TMR in mice housed at 22°C and strongly weakened the relation between activity and TMR at 30°C. However, both at 22 and 30°C, AMR still strongly correlated with the amount of activity. These results are fully in line with those previously reported by Virtue et al. (8) who observed in larger groups of mice ($n = 27$) that at 30°C both total-EE and activity-EE correlated with activity while, at 24°C, only activity-EE correlated with activity. To explain the fact that the relation between TMR and activity decreases (but generally remains significant) at 30°C and is no longer observed at 22°C, one must take into account that the main determinant of TMR is RMR. In this study RMR accounted for more than 70% of TMR and R^2 between TMR and RMR was above 0.90 at both temperatures. Therefore, it is not surprising that on a daily basis the activity effect on TMR be reduced by the variability in RMR, and finally vanishes below thermal neutrality where RMR fluctuates more as a result of heat transfer between thermal regulation and heat released by other discontinuous processes, such as activity and TEF. In addition, as seen when comparing **Figures 2**, **6** and **7**, the range of TMR values available to fit the correlation with activity is much larger when measured across time in a single mouse than when measured between mice, which further weakens the correlation [see also Ref. (8)].

Note that we performed this same analysis in fasted mice between 23:00 and 08:30 hours, i.e., when mice were in the post-absorptive state and adapted to the temperature in the metabolic cage. However, in these mice the level of activity was very low (**Figure 5**) and consequently individual TMR clustered around the mean group value (30°C, Mean 0.232, CV 8.93%, 22°C, mean 0.449, CV 7.48%). This prevented us from performing a precise analysis of the relation between TMR and activity across multiple mice and to reveal any effect of the activity level on TMR at 30°C as well as at 22°C (see **Figure 10**).

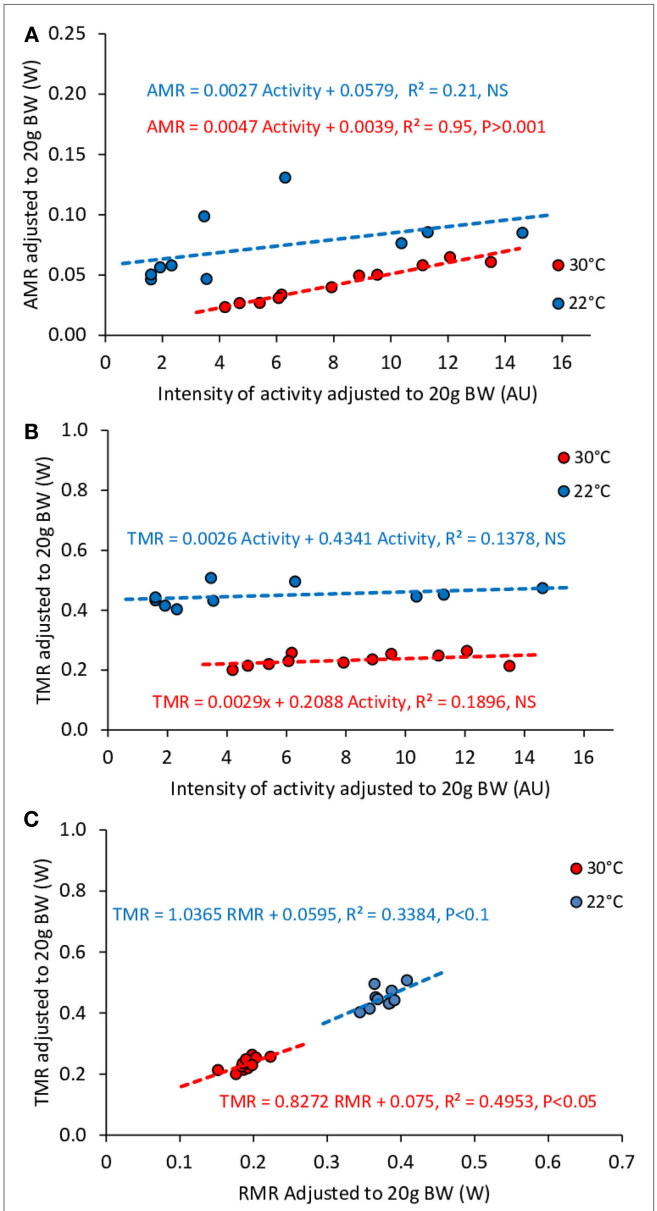

FIGURE 10 | Correlation between AMR and activity (A), TMR and activity (B), TMR and RMR (C) in fasting mice housed at 30°C ($n = 11$) and 22°C ($n = 10$). Data were adjusted to 20 g BW. The small ranges of activity, TMR, and RMR values in fasting mice prevented results interpretation.

RQ, RMR, and TEF

Resting metabolic rate in fasted mice at 22°C was two times that at 30°C, a result in line with the increase reported previously in most studies (17). In contrast, the increase in RMR induced by ingestion of the test meal at 22°C, i.e., TEF, was only one third of the response observed in mice housed at 30°C. At first glance, this could be interpreted as a strong reduction in TEF in mice housed at 22°C but, taking into account the strong interplay between heat generation for thermoregulation and heat released by muscular contraction and TEF, it is highly probable that at 22°C, fasting RMR values decreased rapidly after meal ingestion. This was confirmed by the observation that at 22°C, post-meal RMR was lower than pre-meal RMR 4 h after the meal and onward. It was not possible to measure directly the time course of the decrease in RMR after meal ingestion, but if we refer to the fast pace of changes in RMR observed in response to bursts of activity, it is possible that the cost of thermal regulation decreased within

minutes after the meal was given. Therefore, at 22°C, the RMR value measured after ingestion of the test-meal was the result of the increase in RMR induced by TEF and the decrease in NST induced by the heat released from activity and TEF. In these conditions, pre-meal RMR cannot be extrapolated to compute TEF whereas this extrapolation is possible at thermal neutrality when pre-meal RMR is equal to basal metabolic rate and cannot be further decreased. An argument supporting the hypothesis that in our experimental conditions NST was much reduced by the combined effect of activity and TEF is that, when TEF was computed in reference to RMR measured 6 h after the meal, we obtained TEF values similar to those measured at 30°C. Thus, in mice at 22°C, the standard method to measure TEF cannot be applied. In conditions where it is not possible to measure precisely the decrease in NST induced by activity and TEF, it must be acknowledged that TEF cannot be accurately measured in mice housed at 22°C.

Respiratory quotient was significantly decreased after an overnight fast in mice housed at 22°C confirming that mice exhausted more quickly their glycogen stores and, after a few hours of fast had to rely on their lipid stores. The RQ response to feeding showed also that the overall increase in RQ was similar at 22 and 30°C, but that the increase was of shorter duration, reflecting the fact that mice housed at 22°C used more quickly the carbohydrates brought by the meal. Therefore not only the intensity of TEF but also the metabolic fate on the ingested nutrients is greatly affected by the increased energy demand of mice housed at 22°C.

These significant differences in TEF and RQ responses to ingestion of a test-meal at 22 and 30°C should be considered carefully because when TEF and RQ are measured in humans, great care is taken to avoid any thermal stress.

Limitations of This Study

A main limitation in the interpretation of the results of this study is the lack of measurement of body temperature and caloric intake during calorimetry studies. The absence of caloric intake data was partly compensated for by the fact that we observed no significant changes in BW during the calorimetry studies at 22°C as well as at 30°C, indicating that energy balance was preserved and thus that caloric intake equaled total energy expenditure. In contrast, continuous online measurement of body temperature would have helped to verify that mice did not decrease their temperature set point at 22°C to reduce the cost of thermoregulation. This would have necessarily influenced the response to activity and feeding and would have provided a possible explanation for the increased cost of activity and fluctuations of RMR at 22°C. Comparison of gene expression in muscles and in white and brown adipose tissue of mice acclimatized at 22 and 30°C would have been helpful too, but in the study framework from which these data were extracted, the mice acclimatized to 30°C were reacclimatized to 22°C before organ and tissue collection.

CONCLUSION

In mice housed at 22°C, resting energy expenditure is doubled by NST to maintain thermal regulation, and the cost of activity is also doubled. Intensity of NST is highest at rest and is rapidly tuned down when extra heat is released from muscular contraction and feeding. In this context, the respective roles of basal metabolic rate, NST, activity, and thermic effect of feeding in the energy balance equation are very difficult to decipher. NST in humans is most of the time close to 0. If the mouse is intended to serve as a model of human physiological regulation, it may be reasonable to house them close to thermal neutrality, in particular when they are singly housed without bedding for measurements of metabolic and behavioral parameters. On the other hand, if housing temperature is used as a tool, the mouse can be a very interesting model to study the possible role of NST in the energy balance equation.

AUTHOR CONTRIBUTIONS

PE and AB designed the study, performed the experiment, analyzed the data, and wrote the paper.

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Normocaloric Diet Restores Weight Gain and Insulin Sensitivity in Obese Mice

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An increased incidence of obesity is registered worldwide, and its association with insulin resistance and type 2 diabetes is closely related with increased morbidity and mortality for cardiovascular diseases. A major clinical problem in the management of obesity is the non-adherence or low adherence of patients to a hypocaloric dietetic restriction. In this study, we evaluated in obese mice the effects of shifting from high-calorie foods to normal diet on insulin sensitivity. Male C57BL/6JOlaHsd mice ($n = 20$) were fed with high fat diet (HFD) for a 24-week period. Afterward, body weight, energy, and food intake were measured in all animals, together with parameters of insulin sensitivity by homeostatic model assessment of insulin resistance and plasma glucose levels in response to insulin administration. Moreover, in half of these mice, *Glut4* mRNA levels were measured in muscle at the end of the high fat treatment, whereas the rest of the animals ($n = 10$) were shifted to normocaloric diet (NCD) for 10 weeks, after which the same analyses were carried out. A significant reduction of body weight was found after the transition from high to normal fat diet, and this decrease correlated well with an improvement in insulin sensitivity. In fact, we found a reduction in serum insulin levels and the recovery of insulin responsiveness in terms of glucose disposal measured by insulin tolerance test and *Glut4* mRNA and protein expression. These results indicate that obesity-related insulin resistance may be rescued by shifting from HFD to NCD.

Keywords: insulin resistance, obesity, Glut4, diet, glucose

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INTRODUCTION

Modern lifestyle is often characterized by sedentary activities and overeating. As a consequence, in the last decades, this has been responsible for the increased incidence and prevalence of obesity and obesity-induced comorbidities, such as insulin resistance and metabolic syndrome (1, 2) that may contribute to type 2 diabetes mellitus (T2DM) and cardiovascular disease (3). Several studies have demonstrated that a healthy lifestyle can lead to weight loss and improve insulin sensitivity (4–7). In this regard, a crucial role is played by the nutrient composition of the diet, both in terms of total caloric intake and the variety of its components, with particular attention to the different types of fatty acids (8, 9). Unfortunately, most of anti-obesity interventions are often limited by the difficulty to maintain a low-calorie dietary regimen, especially when long-term treatments are required (10, 11). Thus, few anti-obesity programs have been found to be helpful.

To date, several animal models have been used to evaluate the effects of various dietetic regimens on body weight and metabolic parameters. A validated experimental model is represented by mice fed with a high fat diet (HFD), which develop obesity, insulin resistance, and dyslipidemia (8–13).

In the present study, we evaluated the effects of the transition from HFD to normocaloric diet (NCD) (regular food with no additive agents or nutraceutical compounds) on body weight and insulin responsiveness in C57BL/6JOlaHsd mice, a strain of mice genetically prone to develop obesity and insulin resistance (14).

MATERIALS AND METHODS

Animals and Study Design

Five-week-old male C57BL/6JOlaHsd mice ($n = 20$), NCD and HFD, were purchased from Harlan Laboratories S.r.l (Udine, Italy). Mice were housed in individual cages and maintained on 12-h light/dark cycle at 21 ± 1°C and 50 ± 5% humidity with free access to water and food *ad libitum*. Animals were fed with HFD containing 60.3% kcal fat, 21.3% kcal carbohydrate, and 18.4% kcal protein (HFD group) for 24 weeks. After this period, 10 mice were euthanized by cervical dislocation and the other 10 were fed with NCD only (Teklad Global 18% kcal fat, 58% kcal carbohydrate, and 24% kcal protein) (NCD group) for the subsequent 10 weeks. A schematic representation of the study design is shown in **Figure 1**. Body weight, girth waist, and food intake were recorded at weekly interval for all animals (15). Liver, skeletal muscle, and abdominal fat were excised, weighted, and stored in liquid nitrogen. This study was performed following the Italian (D.M. 116/92) and ECC regulations (O.J. of E.C.L 358/1 12/18/1986), in accordance with the guide for the care and use of laboratory animals and approved by the local ethical committee.

Biochemical Analysis

Blood samples were collected after 12 h of fasting. Serum was separated by centrifugation at 1700 *g* for 10 min at room temperature and stored at −20°C, until use. Total cholesterol and triglycerides were measured using commercial reagents (Siemens Healthcare Diagnostics, Milano, Italy) and an automated biochemistry analyzer (Dimension EXL, Siemens Healthcare Diagnostics). Insulin levels were measured using ELISA kit (Rat/Mouse Insulin ELISA Kit, EMD Millipore Corporation, Darmstadt, Germany), according to the manufacturers' instructions.

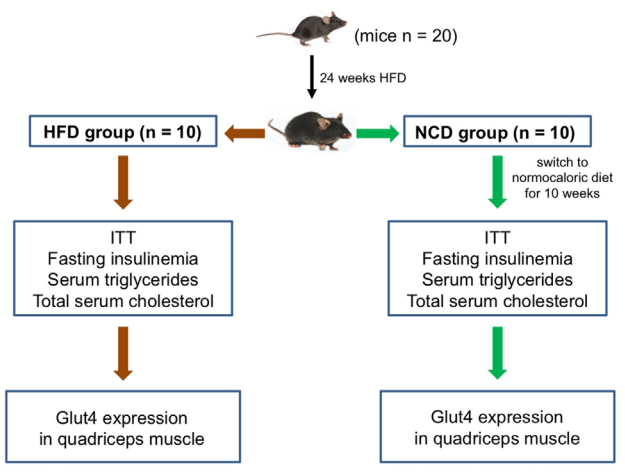

FIGURE 1 | Study design. A schematic representation of the study protocol and experimental plan is shown.

Insulin Tolerance Test

Insulin tolerance test (ITT) was performed in both HFD and NCD groups, as previously described (16). Animals were fasted for 12 h, weighed, and injected intraperitoneally with insulin (1 U/kg body weight Regular®, Novorapid, Novonordisk, Roma, Italy). Blood glucose levels were measured after 0, 15, 30, 60, and 90 min using an automatic glucometer (Glucocard, Menarini Diagnostics, Firenze, Italy).

Expression of Glucose Transporter Type 4

Total RNA was isolated from quadriceps skeletal muscle using TRIzol reagent (Life Technologies, Monza, Italy), following the manufacturer's recommended protocol and quantified with a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA levels were normalized against 18S ribosomal RNA in each sample, and cDNAs were synthesized from 1 μg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Primers for mouse *Glut4* and *ribosomal protein S9* (*RPS9*) were designed according to sequences from the GenBank database. Relative quantification was made using a real-time thermocycler (Eppendorf Mastercycler ep realplex, Milano, Italy). In a 20-μl final volume, 1 μl of cDNA solution was mixed with SYBR Green RealMasterMix (Eppendorf) and 0.2 μM of each sense and antisense primers. SYBR Green fluorescence was measured, and relative quantification was made against either RPS9 or *Gapdh* cDNAs, used as internal standards. All PCR reactions were carried out in triplicates. Glut4 protein expression was measured in quadriceps muscle from six to eight mice of each group, using a rabbit anti-Glut4 polyclonal antibody as previously described (17).

Statistical Analysis

Results are expressed as mean ± SD. The independent *t*-test was used to evaluate intergroup differences. All statistical analyses were performed using GraphPad Prism version 5.0 statistical software (GraphPad Software Inc., San Diego, CA, USA). *p* values lower than 0.05 were considered statistically significant.

RESULTS

Effects of Normocaloric Diet on Body Weight and Biochemical Parameters

Twenty mice were fed with HFD (HFD group) for 24 weeks, reaching a weight of approximately 43 g, with fasting plasma glucose levels between 90.5 and 117.7 mg/dL, which were consistent with a condition of impaired fasting glucose. After the 24-week period, half of the mice were fed with NCD for the following 10 weeks (NCD group). A significant decrease of body weight was observed in the NCD group compared to the HFD group (27%, $p < 0.001$),

as a result of the decrease in energy intake due to the less caloric supply derived from the NCD rather than the different food intake (**Figure 2**). In the NCD group, we also observed a decrease in liver size, fat depots, and girth waist (**Table 1**). Moreover, shifting to NCD resulted in a significant decrease in plasma glucose levels ($p < 0.05$) and serum insulin levels ($p < 0.01$), as well as triglycerides ($p < 0.05$) and total cholesterol ($p < 0.05$) (**Figure 3**).

Effects on Insulin Sensitivity

Next, we evaluated the effects of NCD on insulin sensitivity. ITT performed in mice before and after NCD showed a better response to insulin in terms of changes in blood glucose concentrations in the NCD group than in the HFD group. In fact, the glucose-lowering effect of exogenous insulin was

TABLE 1 | Weight and waist in HFD and NCD mice.

	HFD	NCD	*p* value
Liver (g)	1.24 ± 1.09	1.09 ± 0.09	<0.05
White adipose (g)	1.54 ± 0.31	1.29 ± 0.08	<0.05
Epididymis (g)	0.60 ± 0.16	0.33 ± 0.08	<0.01
Girth waist (cm)	10.55 ± 0.42	9.54 ± 0.17	<0.01

Values are expressed as mean ± SD.

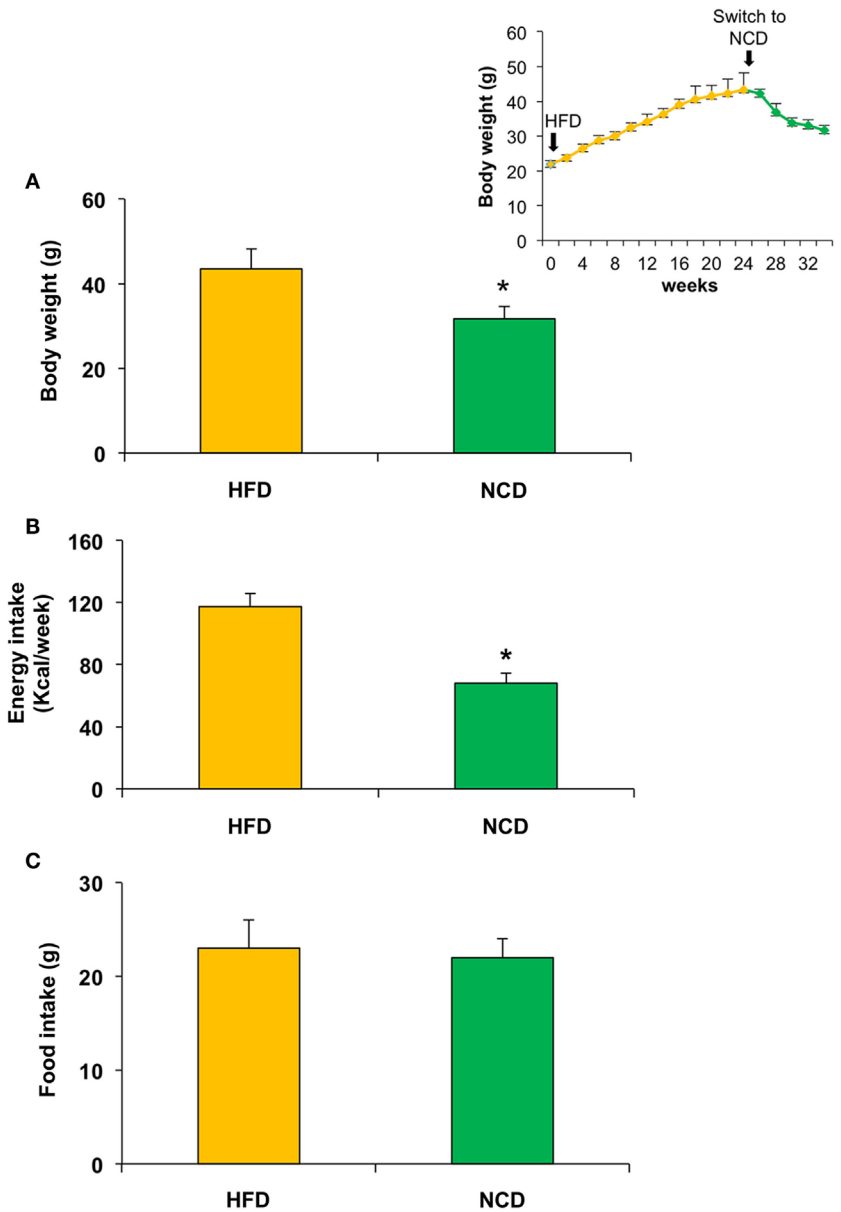

FIGURE 2 | Effects on body weight, food, and energy intake in mice fed with high fat diet (HFD) for 24 weeks and with normocaloric diet (NCD) for other 10 weeks. A significant reduction of body weight and energy intake was observed in NCD mice **(A,B)**, whereas no significant difference was detected in food intake **(C)**. Body weight over the time is shown in the inset. Values are expressed as mean ± SD. *$p < 0.001$.

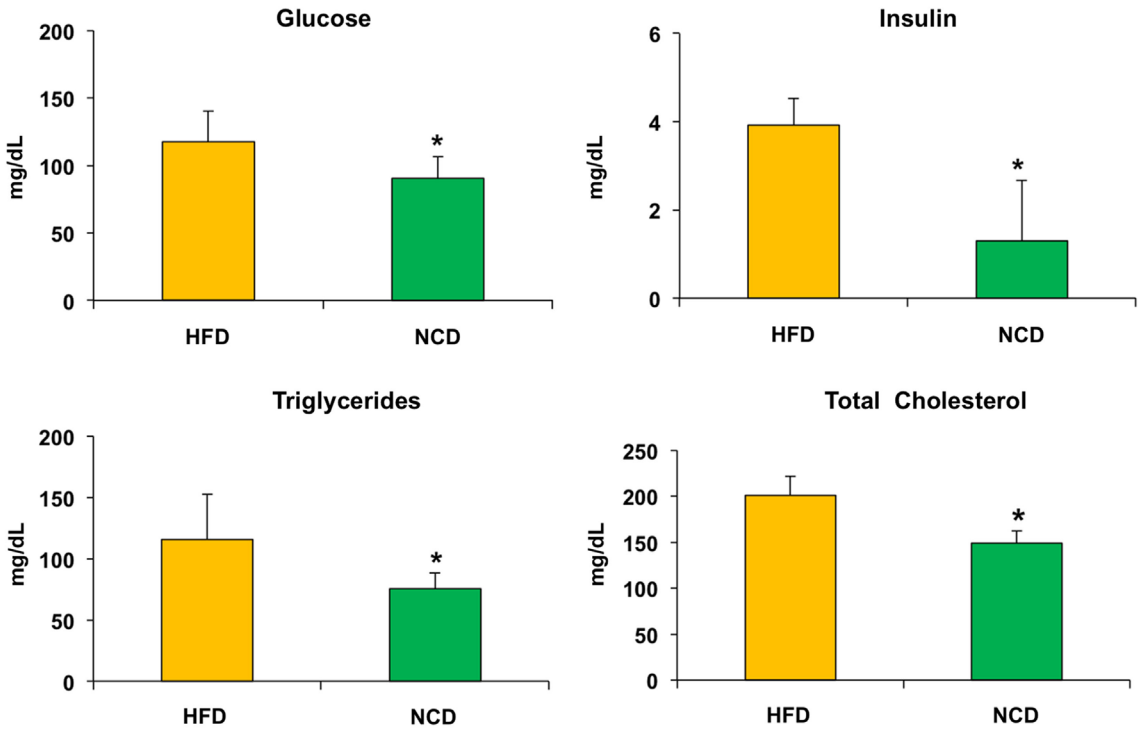

FIGURE 3 | **Biochemical parameters**. Blood samples were collected as indicated in Section "Materials and Methods." After 10 weeks of feeding with a normocaloric diet (NCD), mice showed a significant reduction of plasma glucose levels and serum insulin levels, as well as a reduction in both triglycerides and total cholesterol when compared to the HFD. Values are expressed as mean ± SD. *$p < 0.05$.

enhanced in NCD mice during ITT and was reduced in HFD mice (**Figure 4**). From a mechanistic point of view, the improvement in insulin sensitivity in mice in response to NCD was dependent, at least in part, on an increase in *Glut4* expression induced in skeletal muscle following the transition from HFD to NCD. To show such a molecular link between restoration of insulin sensitivity and NCD, total RNA was extracted from skeletal muscle of animals before and after shifting to NCD, after insulin stimulation, and *Glut4* mRNA and protein levels were measured. As shown in **Figure 5**, both insulin-stimulated Glut4 mRNA and protein expression were significantly increased in skeletal muscle of NCD mice as compared with that of HFD mice ($p < 0.05$).

DISCUSSION

Obesity is a chronic disorder that can cause other health problems, such as diabetes, hypertension, hepatic steatosis, obstructive sleep apnea, and atherosclerosis (18). The association of obesity with T2DM is well established, due to the negative influence of excessive body fat on peripheral insulin action and hepatic function, leading to insulin resistance (19). Treatment of obesity includes hypocaloric diet, exercise, and lifestyle modifications, with dietary manipulation still representing the first-line therapeutic approach for this common disorder (20, 21). However, it is still debated which is the more appropriated dietetic regimen to obtain a weight loss, which may be at the same time rapid, well tolerated, and sustainable for a long period of time. Although the importance of calorie restriction in this condition is well recognized, also for the positive psychological benefit for the patient and the family, there is no doubt that a major problem in treating obesity is still represented by the relatively low level of adherence of affected subjects to low/very low-calorie diets (22–24). Thus, many dietary strategies have been proposed to overcome such obstacles, but the results are not satisfactory enough in most of obese patients (25, 26). In these individuals, we hypothesized that shifting to normocaloric balanced diet, formulated to avoid excess fat, rather than hypocaloric diet – which would obtain a better compliance especially in view of long-term treatment – might be sufficient, in addition to physical exercise and lifestyle change, to get more satisfactory results in terms of weight loss and consequent improvement in obesity-related insulin resistance. This hypothesis is well supported by the present finding in our mouse model of obesity and obesity-induced insulin resistance. In fact, shifting from HFD to NCD for 10 weeks, caused a significant reduction of body weight mainly due to the reduction of visceral fat, together with the overall reduction of triglycerides, total cholesterol, and, most importantly, restoration of insulin sensitivity, as reflected by the decline in fasting insulin levels. A similar approach treating obese mice with NCD has also been

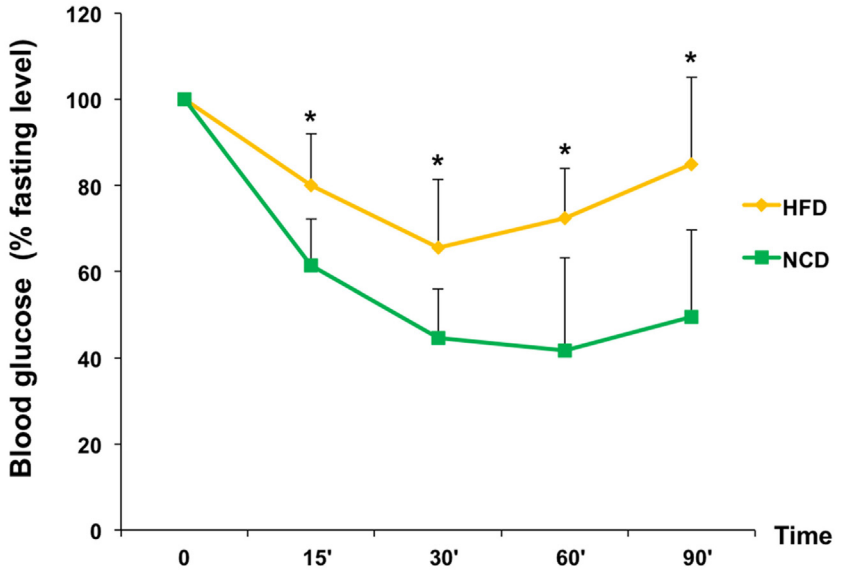

FIGURE 4 | **Insulin sensitivity**. HFD and NCD mice fasted for 12 h were injected intraperitoneally with insulin (1 U/kg). Blood glucose levels were measured with a glucometer, as reported in Section "Materials and Methods." Values are expressed as mean ± SD. *$p < 0.05$.

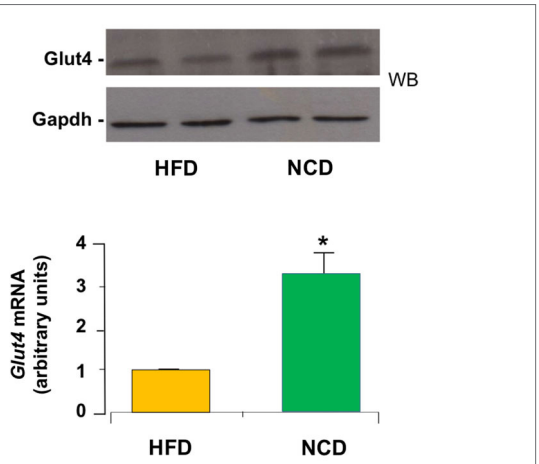

FIGURE 5 | **Expression of Glut4**. Glut4 mRNA levels were measured by qRT-PCR in skeletal muscle from HFD and NCD mice, after insulin stimulation. Results are the mean ± SD for six animals per group. *$p < 0.05$ versus HFD mice. A representative Western blot (WB) of Glut4 in quadriceps muscle from six to eight mice of each group is shown in duplicate in the autoradiogram. Gapdh, control of protein loading.

used in a few other studies where, however, some nutraceutical compounds or other ingredients were added to regular food (27–30). This is slightly different than what we did in our study, in which NCD itself, without any additive agent, was able to improve insulin sensitivity and Glut4 expression.

Glut4 is the major insulin-dependent glucose transporter in muscle. Abnormalities at this level are a hallmark of peripheral insulin resistance (31). In the present study, the improvement in insulin sensitivity associated with increased *Glut4* mRNA expression in NCD mice provides a possible mechanistic explanation as to how the normal calorie diet can improve insulin responsiveness and supports the hypothesis that rescue from insulin resistance and diabetes can be reached without the adoption of a low-calorie diet. If confirmed in obese humans, such an approach, in association with adequate and individualized physical exercise programs, might be able to contribute to counteract the long-term failure of the current therapeutic approaches adopted in these individuals, and this would confirm further the appropriateness of mouse models for studying human obesity. However, on the other hand, it is also known that marked interspecies differences exist between human and mouse with respect to behavioral control of food uptake, tissue energy disposal and storage, weight, and weight loss, which emphasize the influence of non-genetic environmental factors and genetic modifiers in determining the phenotypic variations observed in humans and animal models of obesity. Thus, caution is required in generalizing these findings. As a limitation of the present work, the fact is that mice of different ages were compared in our study.

In conclusion, numerous anti-obesity initiatives have been adopted up to now, which include lifestyle changes, drug treatments, and surgery. However, because of the limited efficacy and the occurrence of adverse events in affected treated patients, alternative and complementary therapies for weight loss have been investigated, including acupuncture, dietary supplements, etc. Our findings in the current work provide valuable information about the efficacy of shifting to NCD in restoring weight and insulin sensitivity in HFD-induced obese mice. Similar studies in obese humans would reveal whether this strategy, probably better accepted by patients, may be successful in correcting weight gain and obesity-related insulin resistance.

AUTHOR CONTRIBUTIONS

GEL contributed to animal testing and drafting of the manuscript; RFDR elaborated figures and tables and contributed to the analysis of the results; SML contributed to animal testing and drafting of the manuscript; BA performed the molecular analysis; NC performed the operation on the animals and supervised the animals' maintenance during the treatment period; TM and GDS reviewed the final version of the manuscript; AB contributed to the conception of the idea and critically reviewed the manuscript; DR contributed to the conception of the idea, drafted the manuscript, and critically reviewed the final manuscript; and MC contributed to animal testing, analysis of the results, and editing of the manuscript. All authors read and approved the submitted version.

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