EFFECTS OF FLAME RETARDANTS ON ARCUATE GENE EXPRESSION AND ENERGY HOMEOSTASIS IN MICE

By

ELIZABETH ANN KRUMM

A thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Master of Science

Graduate Program in Endocrinology and Animal Biosciences

Written under the direction of

Troy Adam Roepke

And approved by

_____

_____

_____

New Brunswick, New Jersey

October, 2015

ABSTRACT OF THE THESIS

EFFECTS OF FLAME RETARDANTS ON ARCUATE GENE EXPRESSION AND ENERGY HOMEOSTASIS IN MICE

By ELIZABETH ANN KRUMM

Thesis Director:

Troy Adam Roepke

Flame retardants (FR), including polybrominated diphenyl ethers (PBDE) congener 2,2',4,4'tetrabromodiphenyl ether (BDE-47) and organophosphate FR (OPFR) are ubiquitous in the environment and interact with multiple target receptors, including estrogen receptors (ERs). Estrogenic endocrine disruptors (EDCs) such as bisphenol A (BPA) affect reproduction and energy homeostasis and modulate hypothalamic functions including gene expression. Developmental exposures to EDCs also alter offspring energy homeostasis, although little is known about the effects of FR, especially OPFR. Therefore, we investigated if exposure to FR alters genes in arcuate nucleus (ARC) that are known to be regulated by 17-β estradiol (E2) through classical ER in adults and if developmental exposures to FR elicit negative energy balance in adulthood. In Experiment 1, adult male and female mice were orally dosed daily vehicle (oil), 17-α-ethinyl estradiol (2.5 μg/kg) as a positive control, BDE-47 low or high dose (1 mg/kg or 10 mg/kg), and OPFR mixture low or high dose (1 mg/kg or 10 mg/kg of tris (1,3-dichloro-2-propyl) phosphate (TDCPP), triphenyl phosphate (TPP), and tricresyl phosphate (TCP) each) for 28 days. ARC mRNA expression, weekly cumulative body weight gain, and female uteri were measured. In Experiment 2, pregnant female mice

were fed vehicle, BDE-47 (1mg/kg), and OPFR mixture (1mg/kg) from gestational day 7 (GD7) to postnatal day (PND) 14. Neonatal pup body weight, anogenital distance (AGD), and sex ratio were measured. Weanlings were fed normal or high-fat diet (ND or HFD) and body weights and food intake were measured weekly until PND140. Adults were tested for body composition, metabolic parameters, and glucose homeostasis. While FR altered E2-regulated ARC gene expression in both sexes, there were more striking effects of FR on males. FR amplify effects of HFD, but also promote negative energy balance when given ND in males. In females, FR increased effects of HFD on body weight gain. These data suggest that these FR alter ARC homeostatic gene expression and energy balance in sex-dependent manner.

Acknowledgement

I would like to thank my mentor Dr. Roepke for his guidance and support and, my brother and parents for their encouragement throughout the process. Special thanks to my committee members, Drs. Nicholas Bello and Mehmet Uzumcu for their time and valuable research input. Furthermore, I would like to thank Roepke members – Ali Yasrebi, Jennifer Yang, Kyle Mamounis, and Vipa Patel for their contribution to my thesis. I would also like to thank Dr. Storch for the use of EchoMRI and Oxymax CLAMS system for the project.

TABLE OF CONTENTS

List of Tables

List of Figures

CHAPTER 1:

INTRODUCTION AND BACKGROUND

I. INTRODUCTION

Development and production of synthetic chemicals increases the variety and concentrations of substances in the environment that could potentially act as endocrine disrupting compounds (EDCs). EDCs are substances in our environment, food, and consumer products that interfere with hormone biosynthesis, metabolism, or action that results in the alteration of normal homeostatic control or reproduction [1]. These compounds can bind to receptors, and/or alter enzyme action, metabolism, hormone availability, and gene expression. These effects may accumulate and become more prevalent across the lifespan.

Estrogenic EDCs such as diethylstilbestrol (DES) and bisphenol A (BPA) can lead to transient and permanent structural and functional abnormalities in rodents and humans [2-5]. These abnormalities are dependent on concentration, route of administration, exposure duration, and the developmental period when exposed to the EDC. Furthermore, estrogenic EDCs can alter energy homeostatic parameters. With elevated incidences of obesity and associated medical conditions such as type II diabetes and cardiovascular disease, the interactions of different diets is crucial for the exploring exposure consequences [1,6,7].

Flame retardants (FR) such as polybrominated diphenyl ethers (PBDE) and organophosphate phosphate FR (OPFR) are compounds found in furniture, toys, electronics, clothing, and some plastics that have become ubiquitous in the environment, especially house dust, and have been shown to bind to estrogen receptors (ERs) *in vitro* [8-14]. PBDE congeners with lower molecular weight and bromination (1-5 bromine atoms) such as 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) and 2,2',4,4',5-pentabromodiphenyl ether (BDE-99) are completely absorbed, slowly eliminated, and longer half-lives [15]. Since the early 2000's, European countries and select U.S. states have enforced policies for phasing out PBDEs [16,17]. However, they continue to persist in the

environment from old furniture and electronic products and collect in house dust and as waste. In U.S. and British houses, concentrations were detected from 520-29,000 ng/g of dust and 1-330ng/day consumed [18]. OPFR are a common replacement in products that formerly contained PBDEs, sharing many of their persistent characteristics [16,19-21]. OPFR levels in Chinese house dust ranged from 0.2-1798 μg/g of house dust [22,23]. Continuing levels of PBDEs and rising levels of OPFR raise concerns for potential neurotoxicity and developmental effects induced by various durations of exposure at different stages of life [24,25].

The fact that these FR are at high environmental concentrations lead to speculation of these compounds potentially affect the physiology of the general population in a negative manner. If these compounds interact with ERs, they can affect many systems, as demonstrated from past studies that analyzed consequences of estrogenic EDC exposure. ERs are distributed ubiquitously throughout central and peripheral tissue and are interact with many signaling pathways involved in energy balance [26-37]. Therefore, in the present study, we investigated effects of BDE-47 and OPFR ~ tris(1,3-dichloro-2-propyl)phosphate (TDCPP), triphenyl phosphate (TPP), and tricresyl phosphate (TCP) ~ on 17β-estradiol (E2)-responsive gene expression in the brain and on energy homeostasis in mice.

II. E2-REGULATED PATHWAYS

General Mechanism

By targeting ERs, environmental EDCs have the ability to alter neuroendocrine signaling pathways responsible for neuronal activation, gene expression, and cellular functions. Estradiol (E2), the predominant circulating estrogen, acts on either classical (ERα/β) or nonclassical (Gq-mER or GPR30) receptors that can induce nuclear-initiated or membrane-initiated signaling and gene regulation [38,39]. When E2 acts through

classical ERs, it diffuses through the membrane and binds to an ER dimerized complex. From there, the complex enters the nucleus and binds either to the estrogen response element (ERE) on the genome, controlling gene expression regulation or induces protein-protein interactions with transcriptional factors (i.e. AP-1, SP-1, NF-κB) and initiating transcription through non-ERE promoters [40,41]. Alternatively, E2 can also bind to membrane receptors such as Gq-mER and GPR30, activating intracellular signaling pathways (i.e. EGFR, IGF-1, PI3K, MAPK, PLC-PKC-PKA pathways) [42-48]. Due to ERs distribution, changes in ER signaling can have varied and substantial physiological effects [49,50].

E2-Regulation of Reproduction and Energy Balance

E2-responsive signaling in the hypothalamus regulates reproductive and energy homeostatic functions. Hypothalamic nuclei involved include the arcuate nucleus (ARC), paraventricular nucleus (PVN), dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), lateral hypothalamic area (LHA), and medial preoptic area (mPOA) [51,52]. Specifically, ARC axon terminals are located in an area where the blood barrier is incomplete, allowing direct access to potential peripheral circulating signals such as sex steroid hormones, glucose, leptin, insulin, and ghrelin and to EDCs [53,54]. Therefore, the ARC has the unique role in integrating central and peripheral inputs.

E2 control of the HPG-axis

Traditionally, E2 controls reproductive functions through the hypothalamic-pituitary-gonadal (HPG)-axis. In the ARC and mPOA of the hypothalamus, gonadotropin-releasing hormone (GnRH) neurons are localized. GnRH secretion is controlled by neighboring kisspeptin (KISS-1) neurosn localized in the ARC or in the anteroventral periventricular (AVPV) nucleus [55]. In the ARC, E2 suppresses *Kiss-1* gene expression by

nonclassical ERα signaling (Yang, In Review). If KISS-1 is released, GnRH secretion is promoted [56-58]. Gonadotropin-inhibiting hormone (GnIH) from the DMH or PVN can also inhibit GnRH secretion [59,60]. When GnRH is secreted into the median eminence, it travels via the hypophyseal portal system to the anterior pituitary and binds to its receptors. From the anterior pituitary, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are produced, released into circulation, and travel to the gonads. Depending on the sex, LH and FSH elicit proper gonadal and germ cell development (oogenesis, folliculogenesis, spermatogenesis), estrous cycles, and secretion of sex hormones such as E2 and testosterone (T). E2 and T can inhibit GnRH, LH, and FSH through a negative feedback mechanism. E2 and progesterone also work together to regulate the estrous cycle in females. [61-64] Improper fluctuations of these hormones can lead to abnormal sex differentiation or lower fecundity if given during critical developmental windows [65,66]. Therefore, ER signaling in the hypothalamus regulates appropriate development and function of the reproductive system.

Central melanocortin circuitry

More recently, the concept of E2 regulating energy homeostasis has emerged. Energy balance is controlled through the central melanocortin circuit [54,67]. In the integrative ARC, there are two main populations of neurons known to be involved in energy balance: 1) anorectic neurons that express proopiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) and 2) orexigenic neurons that express neuropeptide Y (NPY) and agouti-related peptide (AgRP). Generally while feeding, the *Pomc* gene is transcribed in POMC/CART neurons and post-translationally cleaved into α-melanocyte-stimulating hormone (α-MSH) and other neuropeptides. α-MSH binds to its melanocortin receptors (MC3/4R) in the PVN to decrease food intake [68]. During fasting, peripheral peptide hormones such as ghrelin travel through the blood brain barrier to bind to its receptor, growth hormone secretagogue receptor (GHSR), in

ARC NPY/AgRP neurons to activate gamma-aminobutyric acid (GABA) release, which inhibits POMC/CART neurons [39,69-73]. Subsequently, NPY and AgRP are released in other hypothalamic nuclei (PVN). Neurons in these nuclei respond to NPY through its Y1/5 receptors and to AgRP as an antagonist to M3/4Rs [74]. Consequently, food intake is increased [39,54].

E2 control of energy balance

E2 modulates food intake and negative energy balance through the hypothalamus, hindbrain, and other areas of the brain, although the mechanism is not completely understood [51,52,75,76]. For example, ovariectomy (ovx) in females leads to increased adiposity, which can be prevented by E2 replacement [77]. Primarily, E2's energy balance effects are mediated through ERα, based on studies with ERα knockout (KO) mice. Phenotypically, ERα KO mice are obese, have decreased energy expenditure and activity, and altered glucose homeostasis [78-85]. POMC and NPY neurons in ARC of mice guinea pigs express *Esr1* [86-89]. Short-term, E2 augments *Pomc* expression or one of its cleaved products, β-endorphin, which is associated with an attenuation of food intake [78,90-93]. Global mouse ERα KO prevents POMC upregulation an anorectic effects by leptin and insulin to promote feeding [94]. Different species exhibit varied E2 long-term effects on hypothalamic POMC expression [95-98]. With long-term E2 exposure, rodent NPY expression in the hypothalamus decreases, which also lowers food intake [99-102].

Although Gq-mER not fully characterized since it has not yet been cloned, this receptor has been functionally identified thus far in the ARC of the hypothalamic POMC, dopamine (DA), and gamma-amino butyric acid (GABA) neurons [39,103-105]. STX, a selective Gq-mER agonist, decreases food intake and meal frequency and controls gene

expression in female guinea pigs [46,106,107]. Therefore, by targeting multipe ERs, E2 has a major role in reproduction and energy balance. Not only does E2 exert its effects on ERs, but it also modulates other homeostatic receptors and cation channels in the ARC

E2 interactions with other energy homeostatic receptors found in the ARC

In the ARC, E2 increases *Ghsr* gene expression in female mice [89]. GHSR is a G-protein-coupled receptor (GPCR) that is activated by ghrelin, a hormone secreted from the gut that promotes hunger [108-110]. GHSR is involved in many intracellular signaling pathways with intracellular calcium homeostasis as the most characterized pathway [111-113]. While ubiquitously distributed in the body, in the ARC, GHSR is found in NPY/AgRP, POMC, Kiss-1/NeurokininB/Dynorphin (KNDy), and tyrosine hydroxylase (TH) neurons [89,114-116]. Primarily, ghrelin binding to GHSR in ARC NPY/AgRP neurons is directly stimulatory to NPY neurons and indirectly inhibitory to POMC/CART neurons [70,72,111,117,118]. Downstream, these actions increase food intake [70,73,74,119]. Peripheral ghrelin administration increases body weights and carbohydrate utilization while decreasing fat oxidation [120]. Additionally, global GHSR knockout (KO) mice are resistant to diet-induced obesity [121]. Thus, by stimulating GHSR, E2 can alter energy homeostasis throughout the body.

E2 also induces insulin receptor (INSR) signaling in rodents [122,123]. INSR is a tyrosine kinase receptor activated by insulin, which is a peptide hormone secreted from the pancreas that promotes the shuttling of glucose from circulation into muscle and fat tissue, inhibits hepatic glucose production, and typically functions as an appetite suppressant [124-130]. Brain-specific INSR KO promotes diet-induced obesity, infertility, elevated body fat, and increased plasma leptin levels [124,125,128,131]. ARC INSR has been shown to act functionally through insulin-like growth factor-1 (IGF-1), phosphoinositide-3 kinase (PI3K), and rapamycin (mTOR) signaling pathways and is located on NPY,

POMC, and KISS-1 neurons [122,123,126,127,129,131-136]. In the ARC, insulin signaling inhibits *Npy* expression and activates POMC neurons to decrease food intake [127,129,135,136]. Peripherally, INSR is widely distributed in tissues such as fat, muscle, and the liver [137,138]. Furthermore, global INSR KO is lethal shortly after birth in rodents, induced diabetic ketoacidosis, and retards growth [138-140]. Through activating INSR, E2 has the ability to influence energy balance centrally and peripherally.

E2 potentiates leptin receptor (LEPR) signaling through PI3K pathways in the ARC of rodents [78,141-143]. LEPRs are cytokine receptors that are activated by leptin, commonly an anorectic signal that is correlated to and secreted from fat [144-146]. Neuron-specific LEPR KO increases plasma leptin glucose, insulin, corticosterone while increasing hypothalamic AgRP and NPY [147]. These receptors have been localized in POMC, NPY, and KISS-1 neurons as well [105,135,136]. In the ARC, leptin activates KISS-1 and POMC neurons while inhibiting NPY neurons, overall lowering food intake [141,142]. Other than the brain, these receptors can be found in many peripheral tissues, including white and brown fat, muscle, kidney, stomach, and bone marrow [148]. LEPR KO mice (*db/db*) exhibit early-onset obesity and insulin resistance [149,150]. Thus, E2 has an additional route, by inducing ARC LEPR, to affect energy balance.

Comparable to E2, peroxisome proliferator-activated receptor gamma (PPARγ) is a receptor has a modulatory role in energy homeostasis that is also expressed in the ARC of rodents [151]. In positive energy balance, PPARγ is known to increase adipocyte differentiation, fat accumulation, insulin sensitivity, and macrophage differentiation while lowering hepatic glucose production [152-156]. PPARγ is a member of the PPAR family that has many natural ligands such as fatty acids, and metabolites of lipoproteins and alkylated phospholipids and is found in peripheral tissue, including liver, fat, and kidney [153,155,157,158]. Centrally, this receptor is highly expressed in the hypothalamus of rodents

[151,159,160]. Neural PPARγ activation increases food intake and body weight gain and brain-specific PPARγ KO lowers feeding behavior with PPARγ agonist treatment [161]. POMC-specific PPARγ KO elevates energy expenditure while decreasing body weight and food intake [160]. In the ARC, PPARγ activation by agonists also augmented *Npy* and *Agrp* expression in rodents [159]. E2 suppresses PPARγ actions in cancer cells and adipocytes through crosstalk, but potential E2-induced effects on neural PPARγ require elucidation [27-29,34,35,162].

Similar to E2, while androgens are more established as sex steroids that control proper reproductive parameters, they also regulate energy balance. Androgen receptors (ARs) are activated by androgens such as T and dihydrotestosterone (DHT). ARs are distributed throughout the body, including in the ARC [163-166]. Furthermore, T decreases ARC POMC neuron activation in rodents [167,168]. In male mice, global AR KO decreases leptin signaling in the ARC [169]. Conversely, the androgen, DHT, increased POMC neuron activity in females [170]. Brain-specific AR KO suppresses hypothalamic nuclear factor-κB-mediated induction of protein tyrosine phosphatase 1B, reduces insulin sensitivity, and impairs, glucose homeostasis, [171]. Peripherally, AR KO promotes adiposity, body weight gain, and insulin and leptin resistance while decreasing activity and food intake in male mice [172-175]. AR KO female mice have increased leptin and adiponectin levels [174]. However, there is controversy over the effects of AR on insulin sensitivity in female rodents [172,176].

Furthermore, there is also a well-studied link between androgen antagonism or insensitivity and elevated E2 levels [26,177-179]. AR antagonism during development can lead to alternative sex differentiation in males such as decreased AGD while increasing instances of cryptorchidism [180]. With complete androgen insensitivity, serum androgens levels are elevated, which increases aromatase activity. Due to the fact that aromatase

converts T to E2, AR antagonism or insensitivity can increase ER stimulation [179]. For example, adipocyte-specific AR KO increased leptin expression in fat by increasing E2 activation in fat [180]. Therefore, it is plausible for E2 to exert its negative energy balance effects when there is AR antagonism or insensitivity.

E2-responsive ARC Cation Channels

In addition to hormone channels, E2 also regulates cation channels in the ARC such as transient (T)-type calcium channels [104,181], Kv.7 family potassium channels [86,182], and transient receptor potential channels (TRPC) [46,136,141,142,183]. While these channels are also distributed peripherally, centrally, they are involved in hypothalamic control of energy homeostasis [44,45,184-187].

Isoforms of T-type calcium channels are associated with an a_1 subunit are $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$ with *Cacna1g*, *Cancna1h*, and *Cacna1i* as their gene names, respectively. These channels are low-voltage-activated and are responsible for neuronal burst firing and neurotransmitter release and found in ARC POMC and KISS-1 neurons [181,188-190]. ERα and Gq-mER activation increases *Cacna1g* expression in female guinea pig ARC [46,181,188,190]. In female mice, E2 augments *Cacna1g* in the ARC in an ERE-dependent manner (Yang et al., In Review). E2 also increases *Cacna1h* expression in the ARC of female guinea pigs by ERα and ERβ activation [181]. Furthermore, E2 has been shown to upregulate *Cacna1i* in female guinea pig pituitary, ARC KISS-1 neurons, and POA GnRH neurons[190,191]. While these channels have been characterized in females, there are no studies of these channels in the ARC of males.

When stimulated by depolarization, KCNQ (Kv.7) channels at the neuron's membrane facilitate a non-inactivating outward potassium current (M-current) to stabilize membrane potential and reduce action potential frequency [86,182,185,192-195]. Particularly, KCNQ channel subunits *Kcnq2, Kcnq3*, and *Kcnq5* are expressed in the ARC with

KCNQ3 being necessary for M-current function and colocalizing with KCNQ2 and KCNQ5 [86,182,192,195]. E2 increases M-current in ARC NPY, but not POMC neurons to decrease NPY activity and subsequently food intake in female rodents [182]. Additionally, fasting in male and female rodents inhibit M-current activity by suppressing *Kcnq2* and *Kcnq3* expression in NPY neurons [182]. Furthermore, ghrelin also inhibits the M-current in NPY neurons (Yasrebi et al., In Review). In POMC neurons, regulation of food intake is mediated through serotonin receptors ($5HT_{2c}R$) [103,104,142,183,196]. M-current is decreased by $5HT_{2c}R$ through phospholipase C (PLC)-mediated phosphatidylinositol 4,5-biphospahte (PIP_2) hydrolysis to increase firing in POMC neurons, which reduces food intake [196,197].

TRPC channel subunits 1, 3, 4, 5, 6, and 7 are non-selective cation channels that increase depolarization in neurosecretory neurons [136,141,142,183,186,189,198]. In particular, *Trpc5* is not only expressed in ARC POMC and KISS-1 neurons in female rodents, but it is also a target of E2, insulin, and leptin [136,141,142,183,198,199]. E2 inhibits ARC KISS-1 neurons through ERα activation, reducing TRPC channel activity [189,198,200]. In POMC neurons, TRPC5 is activated by insulin and leptin receptor signaling pathways, potentially decreasing food intake [138,143,144,185].

Overall, E2 has wide-ranging effects on reproduction and energy balance. E2 and ERs interact with various hormone receptors and cation channels centrally and peripherally, which influence each other in a complex manner. Thus, EDCs mimicking the effects of E2 either through ERs or receptors that alter ER signaling can lead to expansive disruption of homeostatic functions throughout the body.

III. ESTROGENIC EDCS

Estrogenic EDCs Reproductive and Metabolic Effects

A variety of EDCs partially bind to ERs and are both present and widely distributed in the environment at potentially harmful concentrations. Many estrogenic

EDCs have the ability to affect membrane-initiated signaling pathways in different tissues through classical and nonclassical ERs [6,201,202]. *In vitro*, these compounds also bind to GPR30, a neural estrogen-responsive GPCR, and activate adenylate cyclase [203]. During critical developmental periods, exposure to estrogenic compounds such as DES, BPA, and methoxychlor (MXC) can change brain structures through developmental programming, neurogenesis, and alter neural circuitry in adulthood [204-213]. Estrogenic compound exposure at critical developmental windows can induce alternative sexual differentiation such as changing the anogenital distance (AGD) in males and females [204,209,214-216]. Conversely, EDC exposure during adulthood can alter gene expression and/or neuronal activity [217-222].

Furthermore, growing evidence suggests the relevance of maternal EDC exposure on offspring energy homeostasis in rodents and humans. In *in vitro*, select EDCs functionally activate mERs in peripheral tissue such as the pancreas [5,6,201,202,223]. Estrogenic EDCs such as BPA, polychlorinated biphenyl ethers (PCBs), methoxychlor (MXC) and DES alter adipogenesis, lean mass ratios, energy expenditure, body weight, glucose homeostasis, and cholesterol levels in rodents [2,7,224-226]. While the majority of these studies support these estrogenic EDCs acting as obesogens, a few studies find opposite effects where hyperactivity, lean body mass, and even weight loss in rodents [227,228]. Depending on concentration, route of administration, sex, and duration of exposure, estrogenic EDCs can either promote or protect against obesity [2,225-236].

EE2

17-α-ethinyl estradiol (EE2) has been extensively studied and is considered a model EDC. EE2 is orally bioactive derivative of E2 that is used in many oral contraceptives approved in the U.S. since1960 [237]. Recently, EE2 concentrations in sewage water effluent have increased, especially in urban areas [237-239]. This compound

has a high binding affinity for ERα and has a longer half-life than E2 due to a slower breakdown rate. Since EE2's ERα binding affinity is close to E2 in rodents, EE2 is often used as a positive control for E2 for estrogenic EDC studies [240-242].

EE2 treatment in adulthood has been studied for effects later in life. Use of EE2 as a contraceptive is associated with higher incidences of benign liver tumors, liver toxicity, and increased risk of breast cancer with higher concentrations [243,244]. However, EE2 may protect against ovarian cancer at relatively low doses [245]. In energy balance, there have been studies that support change altered fat metabolism in rodents and increased weight gain in young adult women [246-248]. Furthermore, 28-day exposure to high doses of EE2 in adult male rats exacerbates high-fat diet effects including, increased total cholesterol and glycolytic/gluconeogenic and fatty acid synthesis/oxidation processes in the liver [247]. In the brain, EE2 exposure during adulthood lowers noradrenaline turnover rate in the POA and medial basal hypothalamus (MBH) and is protective against quinolinic acid toxicity in the hippocampus of rodents [249-251].

IV. FR STUDIES

Epidemiology of FR

PBDE and OPFR are manufactured in products such as plastics, furniture, and electronics. Recently, FR levels have been increasing in the environment due to the wide range of products and the extensive product distribution. While PBDEs have been decreasing in Sweden, the U.S. China, and England maintain high concentrations [18,22,252-254]. PBDEs such as BDE-47 have been shown to bioaccumulate in marine mammals, fish, birds and humans with half-lives that last for approximately 96 hours [15,24,255,256]. The EPA has designated daily PBDE levels 0.1-7 ng/g of body weight as safe for humans[15,16]. However, in the U.S., the geometric mean of BDE-47 in maternal serum

is 20.1 ng/g of lipid [253]. In California alone, levels in adult human serum range between 20.8-73.0 ng/g of lipid while children serum contain 61.8 ng/g of lipid [257,258]. Furthermore, in Chinese populations, PBDE concentrations in placenta (15.8ng/g lipid), breast milk (13.2ng/g lipid), and fetal cord blood (1.8ng/g lipid) and neonatal urine (1.99ng/mL) with BDE-47 as one of the predominant congeners [253].

Since the early 2000's, OPFR have been replacing PBDEs in the various products policy-induced concentration limitations of 1g/kg in electronics by the European Union and few U.S. states allowing different amounts to continue being manufactured [15-17]. OPFR concentrations continue to rise in women's breast milk (29.0ng/g), urine (0.08-68.7ng/mL), drinking water (2-15ng/L), and air contamination in offices and aircrafts [22,259-263]. Alarmingly, Butt and colleagues found higher levels of OPFR metabolites in the urine of toddlers paired with their mothers [264]. With their high levels in the environment and few *in vivo* studies testing the compounds in mammals, there are pressing concerns about potential toxicity, developmental abnormalities, and other physiological alterations.

PBDE Toxicity

Due to the vast assortment of PBDE congeners, studies elucidate how FR as mixtures, active metabolites, and individually can be toxic in different tissue [257,265-269]. Maternal exposure at low levels (0.06 mg/kg/day) induced fetal anomalies such as decreased ossification and malformed digits on the paws of rats [270]. Therefore, having relatively high concentrations of these compounds may negatively affect the general population in the future.

Particularly, neurotoxicity induced by PBDEs and the commercial, pentabromodiphenyl ether mixture, DE-71, have been examined for mechanism in neural cell lines and mice and through differences in neurocognitive behavior [265,266]. DE-71, 2,2',4,4',5-pentabromodiphenyl ether (BDE-99), and BDE-47 promote oxidative

stress and apoptosis in human neural stem cells [271,272], neuroblastoma cells [273-275], hippocampal neurons [199,276], cerebellar granule cells [276-278], and astrocytes [279,280] by increasing reactive oxygen species (ROS). In rodents, maternal exposure to BDE-47 led to lower spatial learning and memory, neural development and nerve pulse transmission [281-283]. In humans, prenatal exposure is associated with lower IQ scores and higher hyperactive behaviors [284]. DE-71 treatment during adulthood alters frontal cortex circuitry through GABAergic and glutatmatergic systems and damages nigrostriatal dopaminergic system in mice [285,286]. Overall, PBDEs have the potential to impact the central nervous system.

In peripheral tissue, PBDEs also promote hepatotoxicity and nephrotoxicity. In human embryonic kidney cells (HEK293), BDE-47 also induced apoptosis by increasing ROS levels, which is suggested with elevated lactate/alanine ratios [267]. Additionally, relatively low levels of dietary BDE-47 reduced survival rates in Chinook salmon and increased ROS levels in the kidney as well [268]. In rats given DE-71 maternally, offspring increased hepatocyte cytoplasmic vacuolization, suggesting liver toxicity [269]. Gestational BDE-99 induces hepatotoxicity in rats by upregulating hepatic cytochrome P450 isoforms (CYP1A1, CYP1A2, CYP3A2) and elevated ROS production in the liver [287]. Therefore, PBDEs can be harmful throughout the body.

OPFR Toxicity

Currently, the there are few OPFR toxicity studies and most examine toxicity in cell cultures or *in vivo* working with zebrafish and chickens. In PC12 cells, TDCPP elicits concentration-dependent neurotoxicity, inhibits DNA synthesis, and alters neurodifferentiation [288]. For example, also in PC12 cells, TDCPP decreases cell growth and gene expression of GAP43, NF-H, and tubulins (α/β) while increasing apoptosis, abnormal morphology, and CAMKII gene expression [289]. In human embryonic, hepatic

cells, TPP activates p53 to induce apoptosis [290]. Tri-o-cresyl phosphate (TOCP) is an isomer of TCP that has been shown to be toxic in adult hen spinal cord neurons, mice sperm cell culture, PC12 cell line, and mouse neuroblastoma cells to induce organophosphate-induced delayed neuropathy [288,291-294]. In zebrafish larvae, TDCPP decreases body weight, survival, heartbeat rates, and increases incidences of malformation [295]. TPP promotes cardiotoxicity in zebrafish and reduced retinoic acid receptor gene expression [296-298].

FR Thyroid Disruption

The majority of PBDE studies analyze their role in disrupting thyroid function, which is involved in many growth and metabolic-related parameters. PBDE exposure is associated with reduced thyroxin (T4) in animal studies [299-301] and heightened T4 or decreased thyroid-stimulating hormone (TSH) in humans [302,303]. BDE-47 inhibits thyroid hormone (TH) sulfotransferase activity (Butt, 2013). Additionally, BDE-47 changes in an assortment of gene expressions participating TH pathway enzymes [304]. However, there are discrepancies of BDE-47 binding to thyroid receptors (TRs) [304-308]. Thyroid responsive gene expression is affected by early BDE-47 exposure, but BDE-47 alone or in a PBDE mixture does not compete with radioactive triiodothyronine (T3) for TRβ binding and did not show agonistic nor antagonistic characteristics in some studies [304,305]. Conversely, hydroxylated PBDEs (OH-BDEs) have been shown to activate TRβ more effectively than their parent PBDEs [309]. OH-BDE metabolites also bind to thyroid receptors in rat pituitary cell nuclear extracts and transthyretin, a thyroid hormone transport protein [306,310]. Thus, OH-BDEs may be the active compounds eliciting thyroid dysfunction.

Similar to PBDEs, OPFR also have effects on thyroid function. TDCPP upregulates TR gene expression while TPP augments TR protein and gene expression

in zebrafish [311,312]. Zebrafish embryos exposed to TDCPP also have lower whole body T4, higher T3, and upregulation of expression in genes involved in TH metabolism, synthesis, and thyroid gland development [295]. Additionally, TDCPP in chickens decrease free T4 in plasma [313]. Firemaster550, an OPFR mixture that contains TPP, increased serum T4 in pregnant mice [314]. Recently, emerging studies analyze selective PBDEs, PBDE metabolites, and OPFR having binding affinity and targeting ERs. With TR and ER-signaling pathways displaying intricate crosstalk, FR can disrupt complex interactions involved in growth, development, reproduction and energy homeostasis [32,33].

FR ER Binding Affinity

FR affinity studies on ER elucidate more potential mechanisms of action. Low-MW brominated PBDEs such as BDE-47 and certain OPFR (TDCPP, TPP, TCP) bind directly to ERs, functioning as endocrine disrupting compounds [8-11]. Effects can differ depending on the compound (parent or active metabolite) and dose. Select hydroxylated low-brominated PBDEs such as 6-OH-BDE-47 and 6'OH-BDE-099 bind to classical ERs *in vitro* and have a longer half-life than their parents BDE-47 and BDE-99 [8]. 3'-OH-BDE-47 elicits agonistic estrogenic activity while 6'-OH-BDE-47 induces ER antagonistic action [8,9,11]. Additionally, 6-methoxylated-BDE-47 is highly antiandrogenic at 1 μM, estrogenic at 10 μM, and antiestrogenic at 10 and 50 μM in cell lines [10]. TDCPP, TPP, and TCP all bind to ERs *in vitro*, but there are discrepancies whether they act ER antagonist-like or agonist-like [12-14]. However, FR have been shown to interact not only with ERs, but with multiple target receptors *in vitro* as well such as prename X receptor (PXR), TRs, AR, PPARs (α/γ), mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) [13,14,304,311,315-322]. Therefore, potential FR mechanisms of action may affect the body in an intricate manner through multiple receptors.

Effects of FR on Reproduction

FR can elicit many alterations of reproduction. In zebrafish, DE-71 enhances gonadal development, but reduces spawning, fertilization success, hatching success, and larval survival rates [323]. BDE-47 disrupts spermatogenesis, by inducing apoptosis of early leptotene spermatocytes in male rats [324]. Concurrently, maternal exposure to BDE-47 in rats lowers serum E2 and folliculogenesis in female offspring [325]. BDE-47 metabolites 5 and 6-OH-BDE-47 increase E2 secretion, ER gene and protein expression, and aromatase gene and protein expression in porcine ovary and granulosa cells [326,327]. Additionally, gestational exposure to BDE-99 alters preproenkephalin, *Esr1*, *Esr2*, and progesterone receptor mRNA expression in the VMH and mPOA and decrease female sex behavior in rats [328]. In the serum of young girls, elevated PBDEs correlate with delayed puberty while women of reproductive age with higher blood PBDE levels are associated with less successful pregnancies [329-331].

Regarding OPFR, TDCPP, TPP, and TCP are analyzed alone and in a FR mixture Firemaster550 (FM550), which is in many polyurethane components of furniture [264,332]. TPP inhibits AR in human cells [320]. Tri-ortho-cresyl phosphate, a TCP isomer, lowers rat spermatogonial stem cells in male rats [293]. In zebrafish embryos, OPFR treatment increases T and E2 in circulation in males and females while downregulating vitellogenin (*vtg*) in females and upregulates *vtg* gene expression in males, which is a biomarker of estrogenic activity in male fish [321]. Furthermore, TDCPP also heightens plasma-free T in male chicken eggs while reducing spermiation in zebrafish [313,333]. In adult male zebrafish, TDCPP and TPP lower fecundity, elevated plasma E2, and upregulates *gnrh2, gnrh3, fshβ, lhβ, cyp19b, erα, erβ,* and *ar* in the brain. Conversely, female adult zebrafish *gnrhr2* and *gnrhr3* mRNA expression are downregulated in the brain [12,311,333]. In female rats, maternal Firemaster550 exposure promotes earlier vaginal opening [314]. At high doses, butylated forms of TPP and TCP increase albumin, serum

E2, cholesteryl lipidosis in adrenal cortical and ovarian interstitial, and irregular estrous cycles in female adult rats [334,335]. Collectively, FR alter reproductive parameters, but additional studies are required to further characterize effects and elucidate mechanism.

FR and Metabolism

FR exhibit substantial effects on metabolism through actions on peripheral organs. In male liver microsomes, OH-PBDEs inhibit E2 glucuronidation for phase II metabolism to promote E2 bioavailability and activate PPARγ [315,336]. DE-71 decreases phosphoenolpyruvate carboxykinase (PEPCK) activity while increasing liver lipid and CYP1A, CYP2B, and CYP3A in male rats [337]. In human hepatic cells, BDE-47 enhances PXR and constitutive androstane receptor (CAR) activation [304,322]. In fact, *in vivo*, BDE-47 heightens *Cyp3a11* and *Cyp2b1* gene expression while binding and activating PXR, steroid X receptor, but not AhR in the liver of rats [338]. Analysis of global gene expression when rats are maternally exposed to BDE-47 elevates groups of genes involved in carbohydrate metabolism, electron transport, lipid/fatty acid/steroid metabolism, and drug metabolizing enzymes [269,319]. Additionally, treatment with BDE-47 in rats at relatively higher doses for 28 days increases the number of adipocytes, lipolysis, and lowers insulin-stimulated glucose oxidation [301,339]. Furthermore, maternal exposure at relevant doses in the environment the same model augments plasma IGF-1 in males only while promoting higher body weight and length in males and females [305,319].

With OPFR *in vitro*, FM550 components bind to PPARγ in human liver cells [317,318]. TPP activates mouse and human CAR and PXR, but inhibits glucocorticoid receptor (GR) in human liver only [320]. TDCPP, TPP, and TCP all act agonist-like to PXR and antagonistic to GR in human cells [13,296]. High concentrations of TPP treatment on mice liver inhibit liver carboxylesterase1g, which is associated with dyslipidemia [340]. In

zebrafish embryo and larvae, TDCPP upregulates gene expression of aryl hydrocarbon receptors (*ahrs*), PPAR α (*pparα*), *gr,* and mineralocorticoid receptor (*mr)* genes while TPP upregulates *ahr, pparα, gr*, and *mr* genes [311]. Gene expression of *cyp26a1* in liver is also promoted by perinatal TPP exposure in adult zebrafish [13,296]. In chickens, TDCPP injected into the eggs induces cholestatic liver, biliary fibrosis, disrupts lipid and steroid metabolism. Consequences include lowering gallbladder size and plasma cholesterol while elevating circulating bile acid, gene expression *Cyp3a37* and *Cyp2h1*. Furthermore, global gene analysis of these subjects demonstrates alterations in apolipoprotein E, hepatocyte nuclear factor 4α, and *Ppar*α expression. [313,341] Additionally, butylated TCP in female adult rats induces higher total serum cholesterol and low-density lipoprotein while butylated TPP promotes alanine transaminase [335]. At PND 120, maternal exposure to FM550 in rats lowers hepatic carboxylesterase activity and increases body weight in males, male fasting blood glucose, and glucose intolerance 30 min after glucose injection in female offspring [314]. Thus, FR can affect energy balance in an expansive and diverse manner.

V. RATIONALE FOR CURRENT STUDY

Due to BDE-47, TDCPP, TPP, and TCP (or their metabolites) having the ability to bind to ERs and having neurological, reproductive, and metabolic effects, these compounds can potentially alter estrogen-responsive pathways in the regulatory ARC of the hypothalamus during adult exposures. While there is growing body of PBDE research regarding toxicity of different tissue, reproduction, and energy homeostasis in rodents, OPFR studies looking at *in vivo* mammalian models are scarce [314,334,335,340]. Thus far, there are no *in vivo* studies of OPFR treatment on adult male rodents examining alterations in energy balance. Additional studies examining FR not only individually, but in mixtures as well can further elucidate potential pharmacodynamics

and pharmacokinetic effects. Concurrently, many studies analyzing effects of FR do not administer the treatments through daily consumption, which is the primary route of entry for most of these compounds, especially in children [1]. Therefore, in the present study, we investigated if oral dosing of BDE-47 alone or a mixture of the selected OPFR (TDCPP, TPP, TCP) affects ARC gene expression and offspring energy homeostasis in mice. The objectives of the present study were to determine 1) if the selected FR alter ARC gene expression of known E2-regulated genes in a sex-dependent manner and 2) if exposure during critical developmental periods affects sexual differentiation and growth; and 3) if that early exposure negatively affects offspring energy balance and glucose homeostasis.

VI. HYPOTHESIS

1) Exposure to BDE-47 and OPFR mixture (TDCPP, TPP, and TCP) in adult mice will alter ARC E2-regulated gene expression in a sex-dependent manner.

2) Perinatal exposure to BDE-47 and OPFR mixture will increase body weight, adiposity, and altering glucose homeostasis in adult offspring in a sex-dependent manner.

CHAPTER 2:

EFFECT OF FLAME RETARDANTS ON ARCUATE NUCLEUS GENE EXPRESSION

ABSTRACT

Flame retardants (FR) such as polybrominated diphenyl ether (PBDE) congener 2,2',4,4'tetrabromodiphenyl ether (BDE-47) and organophosphate FRs (OPFR) persist in the environment and interact with multiple target receptors involved in energy homeostasis, including estrogen receptors (ERs). Estrogenic EDCs such as bisphenol A (BPA) disrupt homeostatic gene expression in the arcuate nucleus (ARC) of the hypothalamus, which centrally controls energy balance. However, little is known about the effects of FRs, especially OPFR. Therefore, we investigated if exposure to these FR alters adult 17-β estradiol (E2)-regulated gene expression in the ARC. Adult male and female mice were orally dosed daily vehicle (oil), 17α- ethinyl estradiol (2.5 μg/kg), BDE-47 low or high dose (1 mg/kg or 10 mg/kg), and OPFR mixture low or high dose (1 mg/kg or 10 mg/kg of tris (1,3-dichloro-2-propyl) phosphate (TDCPP), triphenyl phosphate (TPP), and tricresyl phosphate (TCP) each) for 28 days. EE2 was used as a positive estrogenic EDC control. ARC mRNA expression, weekly cumulative body weight gain, and uteri weights were measured. In male ARC, FR upregulated *GhsR, Insr, Lepr, Cacna1g, -h, -i, Kcnq2,-3,-5,* mRNA expression while downregulating *Esr1, Cart*, and *Npy.* OPFR increased *Agrp* and *Pomc* ARC expression in males. In female ARC, BDE-47 suppressed ARC *Ghsr* and *Cart*, but augmented *Insr* and *Cacna1g*. OPFR also decreased *Ghsr, Pomc, Cart*, while increasing *Insr* and *Cacna1g* in females. Overall, FR more drastically influenced ARC gene expression in males than in females. These results suggest that these FR alter ARC gene expression in a sex-dependent manner in mice.

Glossary: flame retardants (FR) polybrominated diphenyl ether (PBDE), 2,2',4,4'tetrabromodiphenyl ether (BDE-47), organophosphate FR (OPFR), tris (1,3-dichloro-2-propyl) phosphate (TDCPP), triphenyl phosphate (TPP), and tricresyl phosphate (TCP), estrogen receptor (ER), endocrine-disrupting compound (EDC), bisphenol A (BPA), 17-β estradiol (E2), arcuate nucleus (ARC), gestational day (GD), postnatal day (PND), anogenital distance (AGD), normal diet (ND), high-fat (HFD), neuropeptide Y (NPY), agouti-related peptide (AgRP), proopiomelanocortin (POMC), cocaine- and amphetamine-regulated transcript (CART), growth hormone secretagogue receptor (GHSR), insulin receptor (INSR), leptin receptor (LEPR)

INTRODUCTION

Recently, flame retardants (FR) such as polybrominated diphenyl ethers (PBDE) and organophosphate FR (OPFR) have become ubiquitous in the environment at high concentrations, due to their manufacturing and distribution in electronics, furniture, and plastics and their long half-lives [15,16,19-21]. PBDE levels in adult human sera ranges between 20.8-73.0 ng/g of lipid [257,258] while OPFR in urine are approximately 0.08-68.7ng/mL [22,264]. Production of select PBDE have been limited since the early 2000's, however, they persist in house dust and electronic waste sites and continue to bioaccumulate in the environment [16,24,253]. BDE-47 has been found in house dust at concentration ranges of 0.5-29 μg/g of dust and 0.2-1798 μg/g of dust for OPFR [18,22,23]. Furthermore, OPFR have been replacing the PBDE in products, but few *in vivo* studies have been carried out in mammals.

In particular, PBDE congers such as 2,2',4,4'tetrabromodiphenyl ether (BDE-47) and OPFR tris(1,3-dichoro-2-propyl)phosphate (TDCPP), triphenyl phosphate (TPP), and tricresyl phosphate (TCP) are in commercial FR including Firemaster550 (FM550) have the ability to bind to estrogen receptors (ERs) [8-14]. Therefore, there is potential for these FR to act as estrogenic endocrine disrupting compounds (EDCs). Exposure to certain levels of estrogenic EDCs such as bisphenol A (BPA) and diethylstilbestrol (DES) can lead to transient and permanent changes, including altering aspects of sexual differentiation, adiposity, and insulin sensitivity [2,209,214-216,226].

Due to extensive E2-regulation in the brain, disruption from exposure could potentiate a wide array of problems. Particularly, in the arcuate nucleus (ARC) of the hypothalamus, classical and nonclassical ERs control reproduction and energy homeostasis [44,45,51,86-88,182,187]. The ARC of the hypothalamus is crucial for integration of central and peripheral signaling such as ghrelin, insulin, leptin, and sex hormones due to its location where the blood brain barrier is incomplete [39,54]. POMC- and NPY-expressing

neurons in the ARC promote or inhibit food intake, respectively [51,54]. Estrogenic EDCs such as BPA has been shown previously to change gene expression in the ARC in male and female mice [342].

PBDE have been shown to affect reproductive, metabolic, and neurocognitive parameters. PBDE alter classical E2-regulated gene expression and function in neural and gonadal tissue [326-328], increase adiposity in male rats [337], promote rodent neurotoxicity [285,286], and lower neurocognitive performance in rodents and children [282,284]. Thus far, there are a number studies exhibiting PBDE inducing neurotoxicity and decreased neurocognitive behaviors in rodents, but there are no studies specifically examine PBDE, 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) changing gene expression in the ARC.

Within the last few years, evidence of OPFR augmenting similar effects is growing in tissue and nonmammalian models. For example, BDE-47 and OPFR act as agonists to thyroid receptors (TRs), pregnane X receptor (PXR), androgen receptor (AR) and peroxisome proliferator-activated receptors (PPARα and PPARγ) in human liver cells, which promote adipocyte differentiation and affects glucose homeostasis [304,315-318,320,340,341]. Furthermore, there are interactions between ER, AR, PPARγ, PPARα, and TR signaling pathways in the brain [26-35,37,162]. However, only ERs, PPARγ, and AR are found in the ARC [86-88,151,163,164,182]. Therefore, there is potential for FR to disrupt multiple target pathways in the ARC involved in energy homeostasis, including ERs.

When phasing out one compound and replacing it with another widely distributed products, it is crucial to identify effects that the former compound may have already inflicted and determine if the novel compound is harmful as well [15-17]. These FR interact with ERs and disrupt neural, reproductive, and energy homeostatic gene expression and function. However, there are no known effects these particular FR on the rodent

hypothalamus *in vivo*, especially integrative ARC. Additionally, daily ingestion is the primary route of exposure for many EDCs [1,15,16]. Therefore, we designed a study to investigate the effects of daily orally dosing BDE-47 and a mixture of OPFR (TDCPP, TPP, and TCP) on the ARC of male and female mice at relevant environmental doses. Our hypothesis is that these FR will alter the gene expression of homeostatic genes known to be 17β-estradiol (E2)-regulated in the ARC in a sex-specific manner.

MATERIALS AND METHODS

Chemicals

2,2',4,4'-tetrabromodiphenyl ether (BDE-47) was purchased from ChemService (West Chester, PA) while Tris(1,3-dichloro-2-propyl)phosphate (TDCPP), triphenyl phosphate (TPP), and 17α-ethinyl estradiol (EE2) were purchased from Sigma-Aldrich (St. Louis, MO). Tricresyl phosphate (TCP) was purchased from AccuStandard (New Haven, CT). Each compound was dissolved in acetone before dissolution in sesame oil (Sigma-Aldrich, New Haven, CT; Spectrum, Boulder, CO). Ketamine was purchased from Henry Schein (Melville, NY, USA) and used for sedation prior to killing.

Animals.

Animal treatments are in accordance with institutional guidelines based on National Institutes of Health standards and were performed with Institutional animal Care and Use Committee approval at Rutgers University. Wild-type (WT) mice with a C57BL/6 background were bred in-house and maintained under controlled temperature (25°C) and 12/12h light/dark cycle with water and food ad libitum.

Due to the daily ingestion being the primary route of exposure for many EDCs, animals were treated with the compounds orally on a daily schedule [1]. EE2 was used as a positive control as it is a model EDC that binds to classical ERs [240-242,343,344]. Male and

female WT mice (n=8) were orally dosed with either sesame oil, EE2 (2.5 µg/kg), low dose BDE-47 (BDE-1; 1mg/kg), high dose BDE-47 (BDE-10; 10mg/kg), low dose of a mixture of OPFR (OP-1; TDCPP, TPP, TCP each 1mg/kg), or a high dose of the OPFR mixture (OPFR-10; each at 10mg/kg) in peanut butter (0.5-0.6 g) for 4 weeks. The mixtures of OPFR have equal concentrations of the respective compounds. Mice were tested in adulthood and matched by weight and age. Ages for male and mice ranged from postnatal day (PND) 45 – 133 and from PND 52 – 236, respectively.

Ovariectomy

Females between 8 and 22 weeks old were bilaterally ovariectomized (ovx) using isoflurane anesthesia and no-touch technique according to NIH guidelines for Survival Rodent Surgery. Analgesic (4mg/kg caprofen (Rimadyl®)) was given immediately after surgery and one day after surgery. Animals were monitored for recovery for three days postsurgery.

Tissue Dissection and Blood Preparation

Animals were sacrificed within an hour of the final dose. After sedation with ketamine (100uL of 100mg/ml stock, i.p.) and decapitation, brains were removed and rinsed in ice-cold Sorenson's Phosphate Buffer (0.2 M sodium phosphate, dibasic and 0.2 M sodium phosphate, monobasic) for 30-60 sec. Basal hypothalamus (BH) was cut into 1-mm thick coronal rostral and caudal blocks using a brain slice matrix (Ted Pella, Redding, CA, USA) corresponding to Plates 42-47 and Plates 48-53 from The Mouse Brain in Stereotaxic Coordinates [345]. These slices were transferred to a 50/50 RNAlater®/Pyrogard water solution and stored until microdissection at 4°C (overnight). ARC was microdissected from the BH using a dissecting microscope. Until RNA

extraction, ARC was stored at -80°C. Trunk blood collected for serum compound metabolites, and uteri weighed.

After sacrifice, whole trunk blood was stored at 4°C for 1 hr, it was centrifuged twice, once at 13000 rpm at 4°C for 30 min to remove large blood clots and finally at 13000 rpm at 4°C for 15 min to collect serum samples. Serum samples were stored at -80 °C until LC/MS analysis. Serum levels of BDE-47 and OPFR metabolites at their high dose treatments were measured using LC/MS at EOHSI (Sample analysis was not completed at the time of the writing of the thesis).

RNA Extraction

RNA was extracted from ARC using Ambion RNAqueous® Micro Kits (Life Technologies, Inc., Carlsbad, CA, USA) according to manufacturer protocol. DNase-I treatment was used to remove genomic DNA contamination. RNA samples were tested for concentration using NanoDrop™ ND-2000 spectrophotometer (ThermoFisher, Inc., Waltham, MA, USA). RNA quality was assessed using Agilent 2100 Bioanalyzer using RNA 6000 Nano Kit (Agilent Technologies, Inc., Santa Clara, CA, USA) with samples with a RNA integrity number (RIN)>6 were used for quantitative real-time PCR (qPCR).

Reverse Transcription

ARC cDNA was produced from 200ng total RNA using Superscript III reverse transcriptase (Life Technologies, Inc.), 4 uL 5X Buffer, 25 mM MgCl2, 10mM dNTP (Clontech Laboratories, INc., Mountain View, CA, USA), 100 ng random hexamer primers (Promega Corporation, Madison, WI, USA), 40U/uL Rnasin (Promega) and 100 mM DTT in DEPC-treated water (Gene Mate, Bioexpress, Inc., Kaysville, UT, USA) in a total volume of 20 uL. Reverse transcription protocol was set to 5 min at 25°C, 60 min at 50°C, 15 min at 70°C. cDNA was diluted 1:20 using nuclease-free water (Gene Mate) to

produce a final cDNA concentration of 0.5 ng/uL and was stored at -20°C. RNA from BH tissue containing ARC from male and female mice were used as positive controls while BH with no reverse transcriptase was used as a negative control.

Quantitative real-time PCR

ARC was analyzed by qPCR for mRNA expression of genes found to be E2-regulated in our preliminary investigations or from the literature [39,46,47,54,77,86,87,103,104,106,107,136,141,142,181,182,188-190,196,198]: *Pomc, Cart, Npy, Agrp, Esr1, LepR, InsR, GhsR, Trpc5, Cacna1g, -h, -i, Kcnq2, Kcnq3*, and *Kcnq5*. *Gapdh, Actb*, and *Hprt* were used as reference genes using the geomean of the three reference genes in our calculation of relative mRNA expression. Primers were designed to span exon-exon junctions and synthesized by Life Technologies using Clone Manager 5 software (Sci Ed Software, Cary, NC, USA) (Table 1). We used 4 ug cDNA (equivalent to 2 ng total RNA) and amplified with either PowerSYBR Green master mix (Life Technologies) or Sso Advanced SYBR Green (BioRad, Inc., Hercules, CA, USA) on CFX-Connect Real-time PCR instrument (BioRad). Standard curves for each primer pair was generated using serial dilutions of BH cDNA in triplicate to determine percent efficiencies (acceptable efficiency: 90% - 110%) (Table 1). Amplification protocol for all genes was as follows: initial denaturing 95°C for 10 min (PowerSYBR®) or 3 min (Sso Advanced™) followed by 40 cycles if amplification at 94°C for 10 sec (denaturing), 60°C for 45 sec (annealing), and a dissociation step for melting point analysis with 60 cycles of 95°C for 10 sec, 65°C to 95°C (in increments of 0.5°C) for 5 sec and 95°C for 5 sec. Positive and negative controls were included in each qPCR plate design including a nuclease-free water blank. qPCR data was analyzed using comparative $\Delta\Delta C_q$ method utilizing a calibrator of cDNA diluted 1:20 from intact male BH. Values were expressed as relative mRNA expression in comparison to oil controls. We maintained a consistent threshold level that was set to

the lowest but steepest slope of the exponential curve in all plates. Linear quantity of target genes was calculated using the $2^{-\Delta\Delta Cq}$ formula [346,347]. Data were expressed as n-fold difference normalized to oil control.

Statistical Analysis

All data were analyzed using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). Cumulative weekly body weight measurements were analyzed by repeated measures two-way ANOVA followed (treatment X time) by *post hoc* Bonferroni's multiple comparison tests between Oil and EDC groups within each time point. One-way ANOVA was used for analyzing uterine weights and qPCR data for EDC treatments in comparison to oil. All data were expressed as mean ± SEM and significance was set at $\alpha \leq 0.5$.

RESULTS

1. Cumulative Weekly Body Weight Gain

It is well documented that E2 replacement attenuates female post-ovx body weight gain [77,81]. Therefore, we examined if EE2 and the selected FR elicit the same effect. Due to the treatments being introduced in adulthood with subjects having already obtained a stable weight into adulthood, we observed cumulative weekly body weight gain, comparing all subsequent body weights with initial body weights. EE2, used as positive estrogenic EDC control, decreased cumulative body weight gain in males (2.7 ± 0.4 g, $p < 0.05$) compared to oil-treated males (4.3 ± 0.7 g) at week 4 (ANOVA: time: $F(4,56) = 71.47$, $p < 0.0001$; treatment x time: $F(4,56) = 4.54$, $p < 0.01$; Figure 1A). Additionally, OP-1-treated females had higher cumulative body weight gain (4.7 ± 0.7 g, $p < 0.05$) at week 4 in comparison to females treated with oil (2.9 ± 0.5 g) (ANOVA: $F(2,21) = 3.53$, $p < 0.05$; $F(4,84) = 80.37$, $p < 0.0001$; $F(8,84) = 3.46$, $p < 0.01$,

respectively; Figure 1F). However, EE2 and BDE-47 treatment did not alter cumulative body weight gain in females or males compared to their oil-treated controls (Figure 1B, C, D, & E).

2. Uterine Weights

E2 replacement is known to amplify uterine weights through ERα-mediated mechanism [81,348]. Thus, we investigated if these EDCs also altered uterine weights. EE2 increased uterine weight (2.2 ± 0.5 g, $p < 0.01$) compared to oil (0.6 ± 0.5 g). There were no significant effects of FR treatment on uterine weight at any dose (ANOVA: $F(5,38) = 5.6$, $p < 0.001$; data not shown).

3. Flame-retardants regulate ARC gene expression

Selected flame-retardants altered ARC gene mRNA expression in a sex-specific manner. These genes selected are primarily involved in reproduction, energy homeostasis, and neuronal excitability or are E2-responsive [46,86,87,89,136,141,142,181,182,188-190]. Genes analyzed were grouped based on function as receptors, ion channels, and neuropeptides. Oil was used as a negative control and EE2 as a positive estrogenic control**.** For each gene, all EDC treatments (EE2, BDE-1, BDE-10, OP-1, OP-10) were compared to oil in which -1 and -10 represent the dose (1 or 10 mg/kg/day).

A. Receptors: Esr1, Ghsr, Insr, Lepr

Gene expression of ARC hormone receptors for E2 (ERα), ghrelin (GHSR), insulin (INSR), and leptin (LEPR) modulate energy balance and were examined as potential targets for the selected EDCs [86,88,89,127,129,136,141,142,182,349]. *Esr1* mRNA expression was decreased by ~70 % in males treated with EDCs (ANOVA: $F(5,41) =$

31.9, $p < 0.0001$; Figure 2A). However, in females, there was no significant effect of EDC treatment on ARC *Esr1* mRNA expression compared to oil-treated females (Figure 2B). All EDC treatments augmented *Ghsr* gene expression in males by ~4-6-fold (ANOVA: $F(5,41) = 25.2$, $p < 0.0001$; Figure 2C). Conversely, in females, BDE-10, OP-1, and OP-10 treatments suppressed *Ghsr* expression by ~20% (BDE-10: $p < 0.01$; OP-1 and OP-10: $p < 0.05$), but there was no effect of BDE-1-and EE2-treated in females (ANOVA: $F(5,41) = 4.2$, $p < 0.01$; Figure 2D). All EDC treatments upregulated *Insr* gene expression by ~2-7-fold in males and females (ANOVA: males: $F(5,40) = 11.3$, $p < 0.0001$; females: $F(5,41) = 11.2$, $p < 0.0001$; Figure 3A & B). *Lepr* gene expression was increased by ~3-4-fold in males with all EDC treatments (ANOVA: $F(5,41) = 11.9$, $p < 0.0001$; Figure 3C). However, there was no significant difference in *Lepr* gene expression between any EDC treatment and females orally dosed with oil (Figure 3D).

B. Cation channel subunits: Cacna1g, -h, -i, Kcnq2,-3,-5, Trpc5

We also examined cation channel subunits that are involved in neuroendocrine functions [46,86,136,141,142,181,182,188,189]. These include calcium channel subunits ($Ca_v3.1$, $Ca_v3.2$, $Ca_v3.3$) that produce the T-type calcium current [181,188,189]; the KCNQ subunits (KCNQ2, KCNQ3, KCNQ5) that produce the M-current, a potassium current [86,182,196], and the transient receptor potential 5 (TRPC5) subunit that produces non-selective cation current under the control of leptin and insulin in the ARC [136,141,142,181]. *Cacna1g* mRNA expression was increased in males by ~3-5-fold with all EDC treatments (ANOVA: $F(5,41) = 6.3$, $p < 0.001$; Figure 4A). Furthermore, all FR treatments augmented *Cacna1g* mRNA expression 3-5-fold in females with all FR treatments ($p < 0.0001$), but EE2 did not significantly affect *Cacna1g* expression (ANOVA: $F(5,39) = 25.1$, $p < 0.001$; Figure 4B). Except for BDE-1, all EDC treatments upregulated *Cacna1h* and *Cacna1i* gene expression ~2-4-fold (BDE-10 and OP-1: $p < 0.01$; EE2 and OP-10: $p < 0.0001$)

and ~2-3-fold ($p < 0.0001$), respectively, in males (ANOVA: *Cacna1h*: $F(5,41) = 8.2$, $p < 0.001$; *Cacna1i*: $F(5,41) = 11.5$, $p < 0.0001$; Figure 4C & E). Conversely, in females, only EE2 increased *Cacna1h* and *Cacna1i* mRNA expression by ~30-50% ($p < 0.05$) (ANOVA: *Cacna1h*: $F(5,41) = 4.9$, $p < 0.01$; *Cacna1i*: $F(5,42) = 4.8$, $p < 0.01$; Figure 4D & F).

In males, EE2, BDE-10, OP-1, and OP-10 increased *Kcnq2* (BDE-10: $p < 0.01$; EE2, OP-1, and OP-10: $p < 0.0001$) and *Kcnq5* (Kcnq2: BDE-10: $p < 0.01$; EE2: $p < 0.001$; OP-1 and OP-10: $p < 0.0001$) gene expression by ~2-3-fold for both genes while there was no significant difference for BDE-1 treatment (ANOVA: *Kcnq2*: $F(5,40) = 11.9$, $p < 0.0001$; *Kcnq5*: $F(5,39) = 8.5$, $p < 0.0001$; Figure 5A & C). However, all EDC treatments elevated *Kcnq3* mRNA expression in males (ANOVA: $F(5,41) = 13.2$, $p < 0.0001$; Figure 5B). Gene expression for *Kcnq2, Kcnq3*, and *Kcnq5* were increased in females only by EE2-treatment by ~60% for *Kcnq2 and Kcnq3* (Kcnq2 and Kcnq3: $p < 0.0001$) and ~2-fold for *Kcnq5* expression ($p < 0.001$)(ANOVA: *Kcnq2*: $F(5,42) = 5.6$, $p < 0.001$; *Kcnq3*: $F(5,42) = 12.0$, $p < 0.0001$; *Kcnq5*: $F(5,39) = 8.5$, $p < 0.0001$; Figure 5D, E, & F). In males, all EDC treatments increased *Trpc5* gene expression by ~3-5-fold (ANOVA: $F(5,41) = 17.7$, $p < 0.0001$; Figure 6 A). However, in females, EE2 treatment only suppressed *Trpc5* mRNA expression by 30% ($p < 0.05$) (ANOVA: $F(5,42) = 3.0$, $p < 0.05$; Figure 6B).

C. Neuropeptides Npy, Agrp, Pomc, Cart

Finally, we examined ARC neuropeptides involved in energy homeostasis and reproduction, including neuropeptide Y (NPY), agouti-related peptide (AgRP), proopiomelanocortin (POMC), and cocaine- and amphetamine-regulated transcript (CART) [51,54]. While all EDC treatments decreased *Npy* mRNA expression by ~30-70% in males, there was no significant change for females in any treatment compared to oil

(ANOVA: males: F(5,40) = 32.2, $p < 0.0001$; Figure 7A & B). Only OP-10 increased *Agrp* gene expression by ~2-fold in males ($p < 0.01$) (ANOVA: F(5,40) = 6.2, $p < 0.001$; Figure 7C). In females, there were no significant effects of any EDC treatment on *Agrp* mRNA expression (Figure 7D). OP-10-treatment increased *Pomc* mRNA expression by ~2-fold in males ($p < 0.001$) (ANOVA: F(5,40) = 4.92, $p < 0.01$; Figure 8A). Interestingly, while not having a significant overall effect, OP-10-treated females had suppressed *Pomc* gene expression by ~30% in *post-hoc* analysis (ANOVA: ns, $p < 0.05$; Figure 8B). Finally, males orally dosed with all EDC treatments decreased *Cart* mRNA expression by ~50-70% (ANOVA: F(5,39) = 16.8, $p < 0.0001$; Figure 8C). Females treated with BDE-1 and OP-10 had suppressed *Cart* mRNA expression by ~30% (BDE-1 and OP-10: $p < 0.05$), but EE2, BDE-10, and OP-1 treatment in females had no significant differences compared to oil-treated females (ANOVA: F(5,41) = 2.7, $p < 0.05$; Figure 8D).

DISCUSSION

FR such as PBDE and OPFR are an emerging group of EDCs that are found at detectable levels in the environment with BDE-47, TDCPP, TPP, and TCP amongst the predominant FR detected. In the current study, we found that FR, BDE-47 and the selected OPFR (TDCPP, TPP, and TCP), had striking effects on homeostatic gene expression in the ARC, including hormone receptors, cation channels, and neuropeptides in a sex-specific manner. Specifically, these FR influenced male ARC mRNA expression more drastically than in females. Many of these genes are regulated by E2 in the ARC through multiple receptor-mediated pathways and modulate central energy homeostasis [44-46,51,86,87,103,182,187,188].

In *in vitro* studies, the selected FR bind to classical ERs (ERα/β), although there are discrepancies whether or not they act as agonists or antagonists [8-14]. From this

study, these FR modulate ARC genes are known to be regulated by E2. However, in addition to ERs, BDE-47 and the OPFR examined also bind to other nuclear receptors involved energy homeostasis such as AR, PPARs (α/γ), PXR, TRs, GRs and MRs in fat, liver, and kidney cells [10,13,14,304,311,315-320]. Similar to ERs, AR and PPARγ are also located in the ARC [151,163,166,169]. ERs can participate in crosstalk between PPARγ and AR in cancer cell lines in which E2 suppresses PPARγ actions in cancer and fat cells while AR activation upregulates ERβ in breast cancer cells [26-29,34,35,162,350-352]. However, it is currently unknown whether E2 affects PPARγ in the central nervous system and ERβ was not examined in the present study [27-29,34,35,162]. In future studies, FR influences on the expression of *Ar, Esr2,* and genes involved in various signaling pathways in the ARC could also be analyzed. Overall, there is potential for FR to affect ER signaling either directly or indirectly from crosstalk of other ARC receptors, including AR and PPARγ.

In the integrative ARC, classical and nonclassical ERs can control central energy balance [44-46,51,86,87,182,187]. Therefore, we analyzed known E2-regulated homeostatic genes. In the ARC, nuclear-initiated (classical) ERα/β signaling can act through estrogen response element (ERE)-dependent and ERE-independent mechanisms to control gene expression while membrane-initiated ERα/β pathways must first activated signal transduction pathways prior to controlling gene expression [44,45,187]. Membrane-associated ERs (mERs) also activate or inhibit secondary messenger cascades that can affect central energy homeostasis and neuronal activity [44,45,48,64,187]. Therefore, there are multiple-receptor-mediated ER mechanisms that FR can activate to control gene expression.

Past studies have investigated EDCs such as BPA and EE2 and their abilities to bind to ERα/β and mERs such as GPR30 or ER-X *in vitro* and alter hypothalamic gene

expression in adult mammalian models [5,6,201,221,223,224,353-358]. Centrally, BPA exposure during adulthood suppresses *Esr1* gene expression in the hypothalamus of male rodents [357]. BPA treatment also increased progesterone receptor activation and gene expression in the female rodent preoptic area (POA) and ventromedial nucleus of the hypothalamus (VMH) [354-357]. Additionally, BPA exposure also augmented kisspeptin mRNA and protein expression in anteroventral periventricular nucleus of adult female mice [221]. Specifically in the ARC, EE2 and BPA treatment during early puberty increased ER-labeled neurons [3]. Peripherally, adult exposure to BPA in males also inhibited protein kinase B phosphorylation in skeletal muscle [224]. However, little is known about effects of EDC exposure during adulthood on central homeostatic genes, specifically in the ARC. In the present study, we found that exposure to the selected FR during adulthood altered ARC gene expression of genes that are E2-responsive and involved in energy homeostasis, but it is uncertain whether they act specifically at ERs or other ARC receptors [204,205,207,220,221,234,235].

Cumulative body weight gain and neuropeptide ARC gene expression

FR subtly disrupted normal cumulative body weight gain in females and changed ARC neuropeptide gene expression in both sexes. At week 4, EE2 lowered cumulative body weight gain in males and OP-1-treated females gained more body weight. The fact EE2 has well-known anorectic effects coincides with decreased male body weight gain. However, OPFR affected female cumulative body weight gain in a dose-dependent manner in which only the low dose was effective. While Patisaul and colleagues (2013) have shown that perinatal OPFR treatment in females increases body weight, this finding that OPFR exposure during adulthood similarly promotes body weight gain in females is novel [314]. Since these treatment effects on cumulative body weight gain are observed toward the end of the treatment paradigm (week 4), it is possible that a longer

duration of orally dosing FR would elicit a more pronounced effect. Furthermore, anorectic neurons co-expressing POMC and CART (POMC/CART) and orexigenic neurons that express both NPY and AgRP (NPY/AgRP) in the ARC are incorporated into the central melanocortin system, which promotes or inhibits food intake downstream in other hypothalamic nuclei such as the paraventricular nucleus (PVN) [54,68]. Thereby, ARC neuropeptide gene expression can be linked to cumulative body weight gain.

In females, FR decreasing ARC *Cart* gene expression correlates with the higher cumulative body weight gain in OPFR-treated females since *Cart* is considered anorectic. Regarding BDE-47 treatment, it is possible that mRNA expression changes did not necessarily lead to alterations in CART protein expression due factors such as posttranslational modification. By pooling ARC from different animals within the same treatment (BDE-1) and then applying western blotting specifically for CART we could examine this possibility.

Surprisingly, in males, FR induced opposite effects in genes that are typically co-expressed in the same ARC neurons and have similar effects on food intake (POMC/CART; NPY/AgRP). Specifically, FR treatments upregulated *Pomc* and *Agrp* gene expression while *Npy* and *Cart* were downregulated in the ARC. However, the ARC has a heterogeneous population of these neurons and additional single-cell studies are required to elucidate FR effects on the activation of these particular ARC neurons [72,359]. Moreover, compensatory responses involved in satiety and reward signaling from neuronal projections between the ARC and other hypothalamic nuclei such as the PVN can modulate the gene expression of these neuropeptides and food intake differently [360-362]. Due to the fact that FR did not affect cumulative body weight gain in males, it is possible for that compensatory actions are more active in males. Additional investigation can examine the plausibility of this effect by double-labeling for vesicular glutamate transporter 1, glutamic acid decarboxylase 67, POMC, and NPY in the PVN after FR

treatment, which would determine if FR exposure altered the density of particular innervated projections [342].

Esr1 gene expression and FR receptor-mediated mechanism implications

In a sex-dependent manner, FR exposure decreases *Esr1* gene expression in male ARC. *Esr1* is expressed in rodent ARC POMC and NPY neurons [86,87,182]. Thus, E2 can exert its anorectic effects by activating POMC neurons and inhibiting NPY neurons [39,47,78,97,103]. Additionally, chronic E2 administration downregulates *Esr1*, which is the effect that we observed in the males exposed to EE2 and FR [363]. Therefore, the present study supports implications for FR inducing changes in reproductive parameters such as spermatogenesis in addition to energy balance in male mice [292,293,324]. However, female *Esr1* gene expression was unaffected even though *Esr1* is more highly expressed in the ARC and OPFR exposure increased cumulative body weight gain in females [364]. Whereas, it is well established that ovx promotes hyperphagia, which can be prevented by E2 replacement [77]. Therefore, the selected FR may not be acting directly on ERs.

Due to males having a higher sensitivity to the selected FR, these compounds may directly act on other receptors that are highly expressed in the male ARC such as PPARγ and AR [151,159,163-166,169]. Recently, we found that OPFR exposure upregulates ARC *Pparγ* expression in adult male mice, which is consistent with *in vitro* studies that demonstrate that the selected FR can act as PPARγ agonists (unpublished data) [315-318,339]. Conversely, past studies that examine the effects of hypothalamic PPARγ activation contradict the effects that we observed in ARC neuropeptide gene expression from FR exposure. Neural PPARγ activation by agonists also augmented *Npy* and *Agrp* expression in the ARC of rodents. Additionally, a study by Long and colleagues (2015) suggests that PPARγ activation in POMC neurons results in higher body weights. While

OPFR exposure upregulated *Pomc* expression in male ARC, body weight was not increased in this study [159,160]. However, since the ARC is a heterogeneous population and changes in *Pomc* gene expression does not necessarily equate to correlated alteration in protein expression these FR acting as PPARγ agonists is still plausible [72,359].

While we had not examined the potential effects of FR on AR gene expression in the ARC, past *in vitro* studies support that these FR act as AR antagonists [10,13,320]. Androgen insensitivity (AIS) or antagonism is typically associated high levels of circulating of testosterone (T), which indirectly promotes aromatase activity in males and female rodents to produce more E2 [365-372]. Thus, FR could potentially affect hypothalamic *Esr1* gene expression indirectly by acting directly on ARs [368-370]. Moreover, POMC neurons can be affected depending on the androgen and the sex of the animal in which T inhibits ARC POMC neuron activation in rodents of both sexes while dihydrotestosterone (DHT) stimulated POMC neurons in females [167,168,170]. However, there are also other androgens that ARs can bind to and may affect POMC activation such as dehydroepiandrosterone (DHEA). Additionally, body weight alterations are dependent on the type of global AR KO model, which could still apply to the findings in our study [174]. Further investigation is required to determine which receptors these FR are directly acting upon to control these alterations in ARC gene expression. These studies could include utilizing global or brain-specific PPARγ, ER, or AR KO models or known receptor agonists and antagonists.

Hormone peptide receptor ARC expression

While there is no previous evidence of the selected FR directly binding to the examined peptide hormone receptors, they altered ARC *Ghsr, Insr*, and *Lepr* gene expression in a sex-dependent manner. *Ghsr* is mostly expressed on NPY neurons and

is orexigenic while both *Insr* and *Lepr* are expressed in NPY and POMC neurons and are anorectic [89,111,113,115,126,127,129,136,141,142,295]. Additionally, these hormone receptors are E2-regulated [78,89,90,122,136,141]. While not necessarily acting through ERs, FR upregulated *Ghsr*, *Insr*, and *Lepr* expression in male ARC, similar to EE2 treatment in the present study and E2 in past studies, but *Lepr* was unaffected in females [78,89,373]. This sex-dependent effect could be due differential leptin blood-brain barrier permeability, in which leptin permeability is lower in females than in males [374-379]. FR suppressed *Ghsr* while increasing *Insr* ARC expression in females. The difference in ARC *Ghsr* expression amongst males and females may be due to females having more E2 than males, which can inhibit GHSR signaling [380].

If FR are acting directly on PPARγ and/or AR, ARC *Ghsr, Insr*, and *Lepr* gene expression can be altered depending on the sex of the animal because of their high distribution in male ARC [10,13,320]. PPARγ regulates an array of genes involved in lipid metabolism, adipogenesis, glucose homeostasis, and insulin sensitivity and neural PPARγ signaling is necessary for the full effect of thiazolidinediones on hepatic insulin-sensitization [153,155,158,381]. Interestingly, global AR KO also decreases leptin signaling in the ARC and promotes insulin and leptin resistance [169,172,174,175]. Additionally, brain-specific AR KO suppresses hypothalamic nuclear factor-κB-mediated induction of protein tyrosine phosphatase 1B, reduces insulin sensitivity, and impairs glucose homeostasis [171]. Therefore, additional studies examining FR binding affinity in neural tissue may be required to evaluate if these compounds centrally act as AR agonist or antagonists. Furthermore, previous studies indicate that both PPARγ and AR crosstalk with GHSR peripherally, but there are no studies that examine if PPARγ or AR activation affects GHSR signaling, especially in the brain [175,382-386]. Overall, FR-induced PPARγ or

AR activation could possibly increase ARC *Ghsr, Insr*, and *Lepr* gene expression in males and augment ARC *Insr* gene expression in females.

Electrophysiology experiments can evaluate if ARC NPY and POMC neurons in males treated with FR are more sensitive to ghrelin, insulin, or leptin, which would correlate with the upregulation of *Ghsr*, *Insr*, and *Lepr* gene expression in males and increased *Insr* gene expression in females. However, FR-treated females may be more insensitive to ghrelin as *Ghsr* gene expression was suppressed. If circulating concentrations of these hormone peptides were also elevated, NPY and POMC neurons could be activated or inhibited, which could modulate many energy homeostatic parameters [387-392]. To examine the physiological implication FR exposure upregulated *Insr* gene expression in the ARC, we could utilize glucose and insulin tolerance tests or a euglycemic clamp and determine if FR treatment alters whole-body glucose homeostasis by promoting glucose clearance and/or insulin sensitivity [387,389-391]. Furthermore, additional studies would benefit from measuring food intake after FR treatment since NPY, AgRP, and ghrelin are considered orexigenic and POMC, CART, insulin, and leptin anorectic [54,78,124,393].

Cation Channel expression in the ARC

The selected estrogen-responsive cation channel subunits are involved in the neuronal activation or inhibition [86,136,141,142,181-183,189,198]. These cation channels are found throughout the brain in different neurons, including ARC POMC and NPY neurons [86,182,192,193,196,394,395]. While AR and PPARγ influences these cation channels in peripheral tissue such as muscle, little is known about their effects in neural tissue [396-401]. *Cacna1g*, *-h*, and *–i* are the gene names for T-type calcium channel subunits ($Ca_v3.1$, $Ca_v3.2$, $Ca_v3.3$ respectively), which induce neuronal firing and neurotransmitter release [181,188,189]. In addition to E2, DHEA also targets *Cacna1g, -i*, and *–h*, expression in brain tissue, but

it is unknown whether it is also effective in the ARC [181,188,189,191,397]. Transient receptor potential cation channel 5 (TRPC5) is a non-selective cation channel that induces depolarization in ARC neurosecretory neurons of rodents [136,141,142]. In ARC POMC neurons, E2, insulin, and leptin signaling target *Trpc5* [136,141,142]. The KCNQ channel subunits, KCNQ2, -3, and -5 are responsible for the M-current in the brain, which is a non-inactivating outward potassium current that stabilizes membrane potential and reduces action potential frequency [86,182]. Recently, we found that ghrelin also inhibits the M-current in ARC NPY neurons in mice (Yasrebi et al., In Review). Therefore, peripheral signals such as ghrelin, insulin, and leptin differentially affect these cation channels and modulate the activation of NPY and POMC neurons, which controls energy and glucose homeostasis (Yasrebi et al., In Review) [86,104,136,142,181,182,189,196,387,390].

In males, FR augmented all cation channel subunits examined, which indicates that FR could potentially alter neuron excitability, although the mechanism of action is still unknown. If ARC neurons, including NPY and POMC neurons, are found to be more sensitive to ghrelin, insulin, or leptin after FR exposure by using electrophysiology, correlating with increased *Ghsr, Insr*, and *Lepr* gene expression, cation channel activation may also be influenced. In the present study, *Kcnq2, -3*, and *-5* are all upregulated by FR treatment in male ARC. If NPY neurons of FR-treated males were more sensitive to ghrelin, these neurons could downregulate *Kcnq2, -3,* and *-5* gene expression, potentially inhibiting the M-current, and more effectively and promote NPY neuron excitability (Yasrebi et al., In Review). Thereby, this effect would indicate that hunger and potentially food intake could be more readily induced in FR-treated males if there are also higher levels of ghrelin in the brain [70,73,253]. In ARC POMC neurons of FR-treated males, presumably, these neurons may be more sensitive to insulin and leptin, which could promote TRPC5 gene expression or activation [136,142]. Overall, these actions would increase POMC neuron activity and possibly decrease food intake, depending on

neural insulin and leptin concentrations [54]. However, additional studies would confirm if FR-induced alterations in cation channels and NPY and POMC neuron activity would result in changes in aspects such as hunger and food intake [39,72,359].

Conclusion

Collectively, FR alter homeostatic gene expression in the ARC of adult male and female mice, impacting males more greatly than females. Results from the current study provide insight into the effects of FR in a part of the brain that controls energy homeostasis in a mammalian *in vivo* model. Since these ARC genes regulate energy homeostasis such as food intake, energy expenditure, body composition, and glucose homeostasis, studies analyzing these parameters elucidate additional physiological effects [54,99,134,197,402]. However, future studies that analyze FR mechanisms that promote these effects and their physiological ramifications will greatly enhance our understanding of the impacts of FR exposure.

Table1. Primer list. Product and primer length are represented in number of base pairs.

Gene	Accession #	Product Length	Primer Sequence	Primer Length	Efficiency (%)
Actβ	NM_007393.3	63	F:GCCCTGAGGCTCTTTTCCA	849-867	100.7
			R:TAGTTTCATGGATGCCACAGGA	890-911	
Gapdh	NM_008084.2	98	F:TGACGTGCCGCCTGGAGAAA	778-797	93.1
			R:AGTGTAGCCCAAGATGCCCTTCAG	852-875	
Hprt	NM_013556	117	F:GCTTGCTGGTGAAAAGGACCTCTCGAAG	631-658	107
			R:CCCTGAAGTACTCATTATAGTCAAGGGCAT	718-747	
ERα *(Esr1)*	NM_007956	107	F:GCGCAAGTGTTACGAAGTG	919-937	96.4
			R:TTCGGCCTTCCAAGTCATC	1007-1025	
Ghsr	NM_177330	122	F:CAGGGACCAGAACCACAAAC	1003-1022	123
			R:AGCCAGGCTCGAAAGACT	1107-1124	
Insr	NM_010568	89	F: GTGTTCGGAACCTGATGAC	1215-1233	114
			R: GTGATACCAGAGCATAGGAG	1686-1706	
Lepr	NM_146146.2	149	F:AGAATGACGCAGGGCTGTAT	3056-3075	104.8
			R:TCCTTGTGCCCAGGAACAAT	3185-3204	
Ca_v 3.1 (*Cacna1g*)	NM_009783	87	F: ACACTGGAACCGGCTTGAC	2935–2954	100.6
			R: CTGCGGAGAAGCTGACATTCTG	3059 –3078	
Ca_v 3.2 (*Cacna1h*)	NM_001163691	284	F: CTCTGGGCTTCCTTTAGTAG	2640 –2659	95.6
			R: ATCTCCCAGACGCTTATG	2906 –2923	
Ca_v 3.3 (*Cacna1i)*	NM_001044308	128	F: TGGGCATTTTTGGCAAGAA	965–973	104.2
			R: CAGTGCGGATGGCTGACA	1093–1110	
Kcnq2	NM_133322	171	F:GGTGCTGATTGCCTCCATTG	644-663	105
			R:TCCTTGCTGTGAGCGTAGAC	795-814	
Kcnq3	NM_152923.1	94	F:GCTGCTGGAAACCTTTGC	474-491	105
			R:ACGCCAGCCTTTGTATCG	550-567	
Kcnq5	NM_023872.2	99	F:GGGCACAATCACACTGACAAC	915-935	101
			R:GAAATGCCAAGGAGTGCGAAG	993-1013	
Trpc5	NM_009428	195	F: TGGTAGTGCTGCTGAATATG	2241-2260	103.3
			R: TGAACCAGTTGCCAAGATAG	2416-2435	
Npy	NM_023456	182	F:ACTGACCCTCGCTCTATCTC	106-125	100
			R:TCTCAGGGCTGGATCTCTTG	268-287	
Agrp	NM_007427.2	146	F:CTCCACTGAAGGGCATCAGAA	287-307	105
			R:ATCTAGCACCTCCGCCAAA	414-432	
Pomc	NM_008895	200	F:GGAAGATGCCGAGATTCTGC	145-164	103
			R:TCCGTTGCCAGGAAACAC	327-344	
Cart	NM_013732	169	F:GCTCAAGAGTAAACGCATTCC	277-297	95
			R:GTCCCTTCACAAGCACTTCAA	425-445	

Figure 1. Male and female average cumulative body weight gain. (**A, C, E**) Male cumulative body weight gain. (**B, D, F**) Female cumulative body weight gain. All data were analyzed using repeated measures two-way ANOVA (treatment X time) with Bonferroni multiple comparison test (*$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$) and compared to oil unless otherwise noted. Bars represent mean ± SEM. For all treatment and oil groups for each sex, n = 8. FR doses (mg/kg/day) are represented as -1 or -10.

Figure 2. Male and female ARC average relative *Esr1* and *Ghsr* average relative mRNA expression. (**A & C**) Male *Esr1* and *Ghsr* average relative mRNA expression, respectively. (**B** & **D**) Female *Esr1* and *Ghsr* average relative mRNA expression, respectively. All data were analyzed using one-way ANOVA with Bonferroni multiple comparison test of treatments compared to oil (*$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$). All qPCR data were normalized to oil. Bars represent mean ± SEM. For all treatment and oil groups for each sex, n = 8. FR doses (mg/kg/day) are represented as -1 or -10.

Figure 3. Male and female ARC average relative *Insr* and *Lepr* average relative mRNA expression. (**A & C**) Male *Insr* and *Lepr* average relative mRNA expression, respectively. (**B** & **D**) Female *Insr* and *Lepr* average relative mRNA expression, respectively. All data were analyzed using one-way ANOVA with Bonferroni multiple comparison test of treatments compared to oil (*$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$). All qPCR data were normalized to oil. Bars represent mean ± SEM. For all treatment and oil groups for each sex, n = 8. FR doses (mg/kg/day) are represented as -1 or -10.

Figure 4. Male and female ARC average relative *Cacna1g*, *Cacna1h*, and *Cacna1i* average relative mRNA expression. (**A**, **C**, **E**) Male *Cacna1g*, *Cacna1h*, and *Cacna1i* average relative mRNA expression, respectively. (**B**, **D**, **F**) Female *Cacna1g*, *Cacna1h*, and *Cacna1i* average relative mRNA expression, respectively. All data were analyzed using one-way ANOVA with Bonferroni multiple comparison test of treatments compared to oil (*$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$). All qPCR data were normalized to oil. Bars represent mean ± SEM. For all treatment and oil groups for each sex, n = 8. FR doses (mg/kg/day) are represented as -1 or -10.

Figure 5. Male and female ARC average relative *Kcnq2*, *Kcnq3*, and *Kcnq5* mRNA expression. (**A**, **C**, **E**) Male *Kcnq2*, *Kcnq3*, and *Kcnq5* average relative mRNA expression, respectively. (**B**, **D**, **F**) Female *Kcnq2*, *Kcnq3*, and *Kcnq5* average relative mRNA expression, respectively. All data were analyzed using one-way ANOVA with Bonferroni multiple comparison test of treatments compared to oil (*$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$). All qPCR data were normalized to oil. Bars represent mean ± SEM. For all treatment and oil groups for each sex, n = 8. FR doses (mg/kg/day) are represented as -1 or -10.

Figure 6. Male and female ARC average relative *Trpc5* mRNA expression. (**A**) Male *Trpc5* average relative mRNA expression. (**B**) Female *Trpc5* average relative mRNA expression. All data were analyzed using one-way ANOVA with Bonferroni multiple comparison test of treatments compared to oil (*$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$). All qPCR data were normalized to oil. Bars represent mean ± SEM. For all treatment and oil groups for each sex, n = 8. FR doses (mg/kg/day) are represented as -1 or -10.

Figure 7. Male and female ARC average relative *Npy* and *Agrp* mRNA expression. (**A & C**) Male *Npy* and *Agrp* average relative mRNA expression. (B & D) Female *Npy* and *Agrp* average relative mRNA expression. All data were analyzed using one-way ANOVA with Bonferroni multiple comparison test of treatments compared to oil (**p* < 0.05, ** *p* < 0.01, ****p* < 0.001, **** *p* < 0.0001). All qPCR data were normalized to oil. Bars represent mean ± SEM. For all treatment and oil groups for each sex, n = 8. FR doses (mg/kg/day) are represented as -1 or -10.

Figure 8. Male and female ARC average relative *Pomc* and *Cart* mRNA expression. (**A & C**) Male *Pomc* and *Cart* average relative mRNA expression. (B & D) Female *Pomc* and *Cart* average relative mRNA expression. All data were analyzed using one-way ANOVA with Bonferroni multiple comparison test of treatments compared to oil (**p* < 0.05, ** *p* < 0.01, ****p* < 0.001, **** *p* < 0.0001). All qPCR data were normalized to oil. Bars represent mean ± SEM. For all treatment and oil groups for each sex, n = 8. FR doses (mg/kg/day) are represented as -1 or -10.

Figure 1.

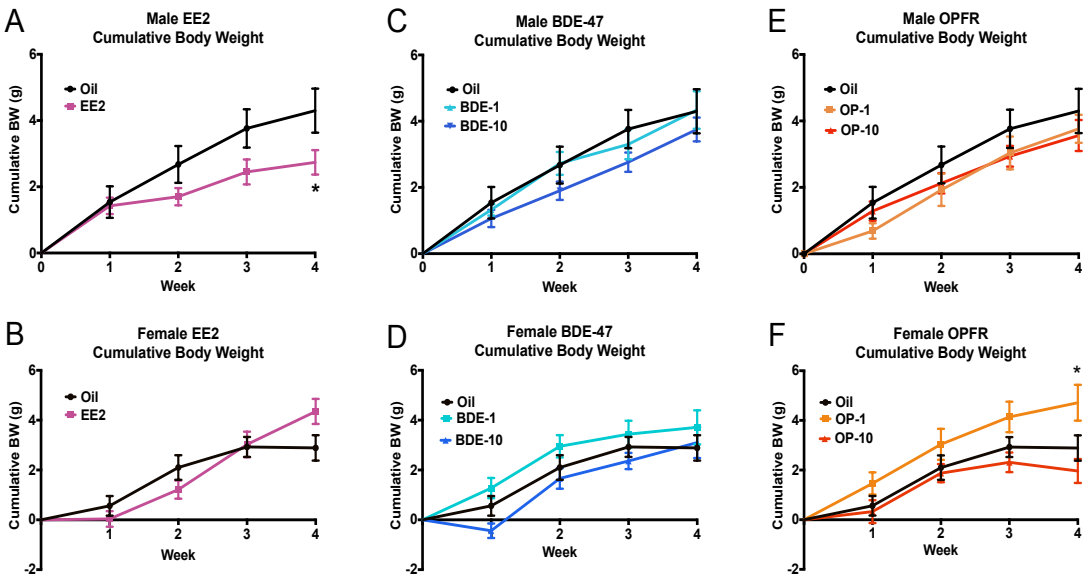

A
Male EE2
Cumulative Body Weight
Oil
EE2
Cumulative BW (g)
Week
*
C
Male BDE-47
Cumulative Body Weight
Oil
BDE-1
BDE-10
Cumulative BW (g)
Week
E
Male OPFR
Cumulative Body Weight
Oil
OP-1
OP-10
Cumulative BW (g)
Week
B
Female EE2
Cumulative Body Weight
Oil
EE2
Cumulative BW (g)
Week
D
Female BDE-47
Cumulative Body Weight
Oil
BDE-1
BDE-10
Cumulative BW (g)
Week
F
Female OPFR
Cumulative Body Weight
Oil
OP-1
OP-10
Cumulative BW (g)
Week
*

Figure 2.

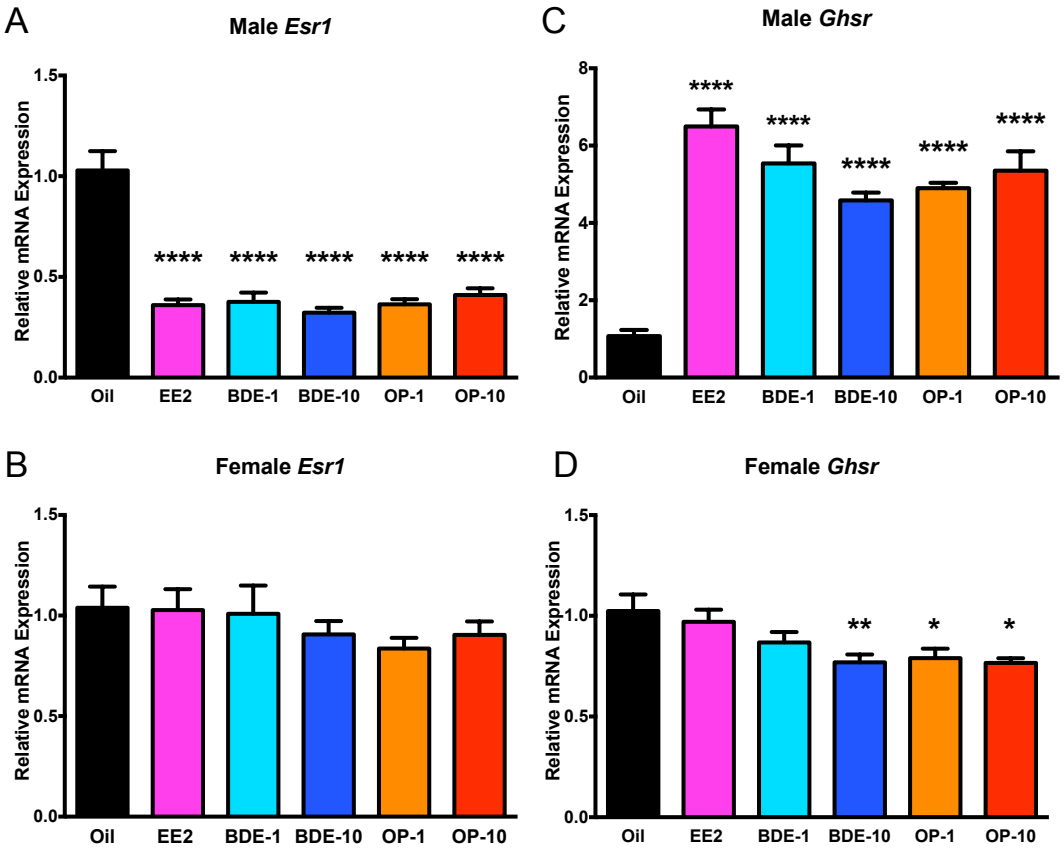

A
Male *Esr1*
Relative mRNA Expression
1.5
1.0
0.5
0.0
****
Oil
EE2
BDE-1
BDE-10
OP-1
OP-10
C
Male *Ghsr*
8
6
4
2
0
B
Female *Esr1*
D
Female *Ghsr*
**
*

Figure 3.

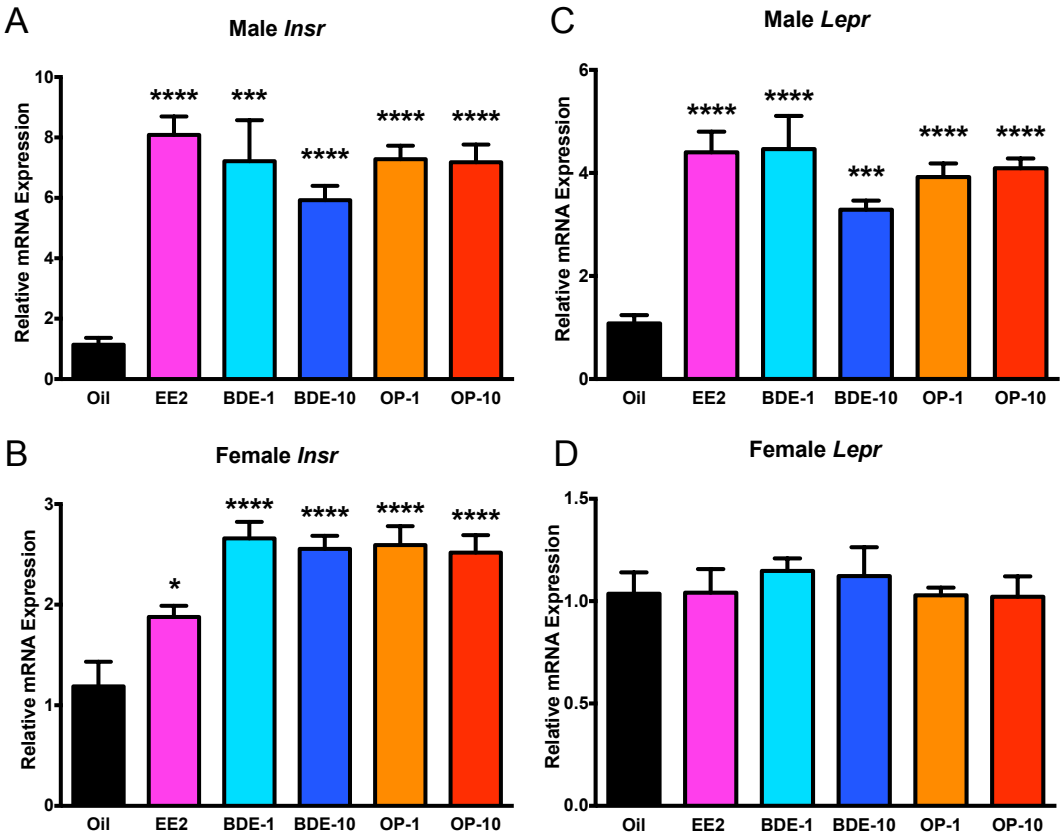

A
Male *Insr*
Relative mRNA Expression
10
8
6
4
2
0
****
***
****
****
****
Oil
EE2
BDE-1
BDE-10
OP-1
OP-10
C
Male *Lepr*
Relative mRNA Expression
6
4
2
0
****
****
***
****
****
Oil
EE2
BDE-1
BDE-10
OP-1
OP-10
B
Female *Insr*
Relative mRNA Expression
3
2
1
0
*
****
****
****
****
Oil
EE2
BDE-1
BDE-10
OP-1
OP-10
D
Female *Lepr*
Relative mRNA Expression
1.5
1.0
0.5
0.0
Oil
EE2
BDE-1
BDE-10
OP-1
OP-10

Figure 4.

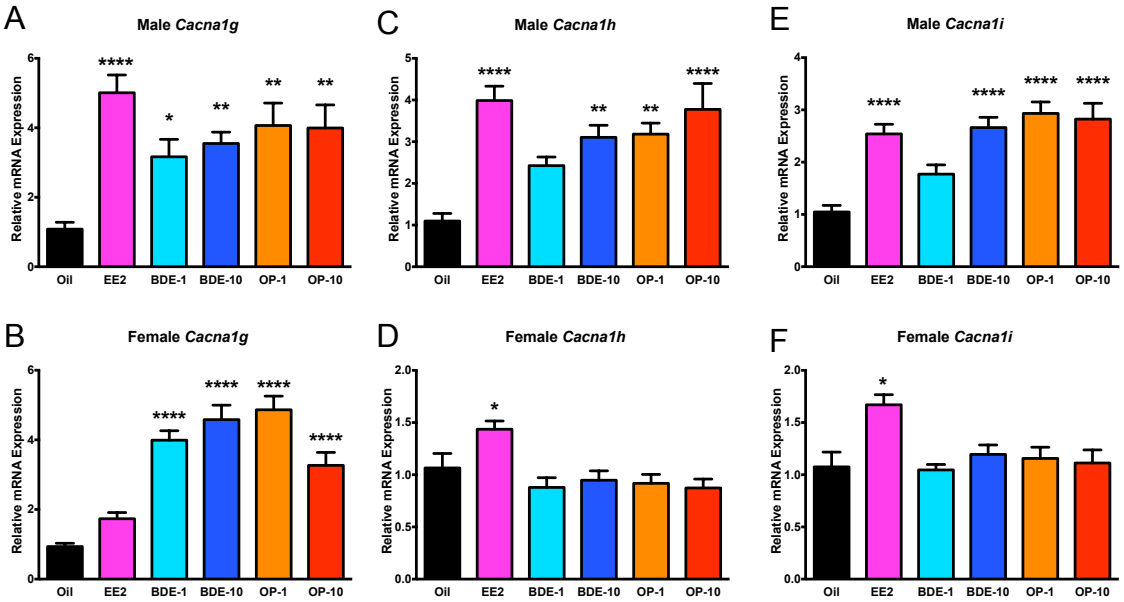

A
Male Cacna1g
Relative mRNA Expression
Oil
EE2
BDE-1
BDE-10
OP-1
OP-10
C
Male Cacna1h
E
Male Cacna1i
B
Female Cacna1g
D
Female Cacna1h
F
Female Cacna1i

Figure 5.

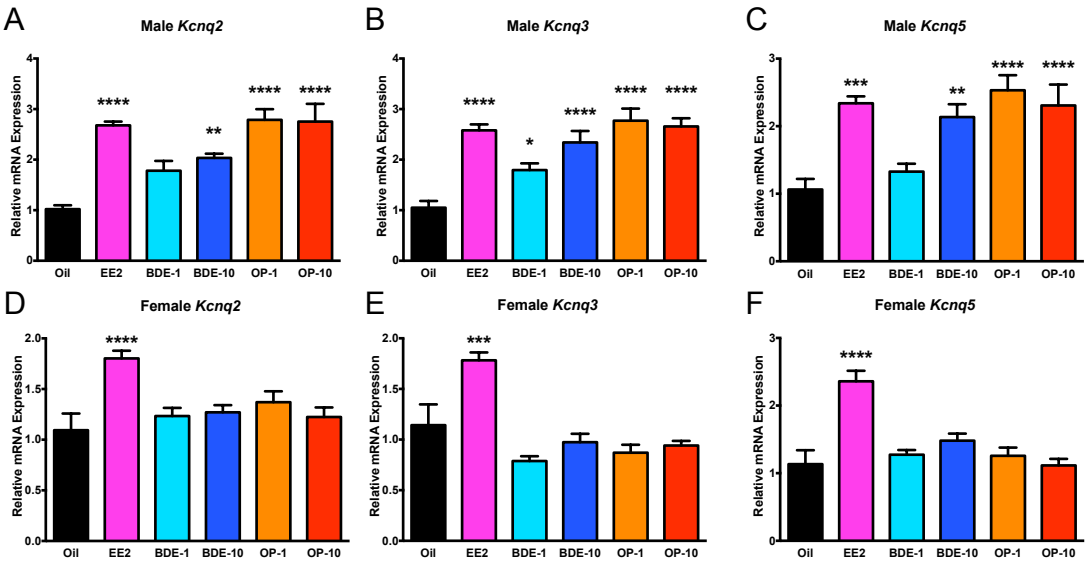

A
Male Kcnq2
B
Male Kcnq3
C
Male Kcnq5
D
Female Kcnq2
E
Female Kcnq3
F
Female Kcnq5
Relative mRNA Expression
Oil
EE2
BDE-1
BDE-10
OP-1
OP-10

Figure 6.

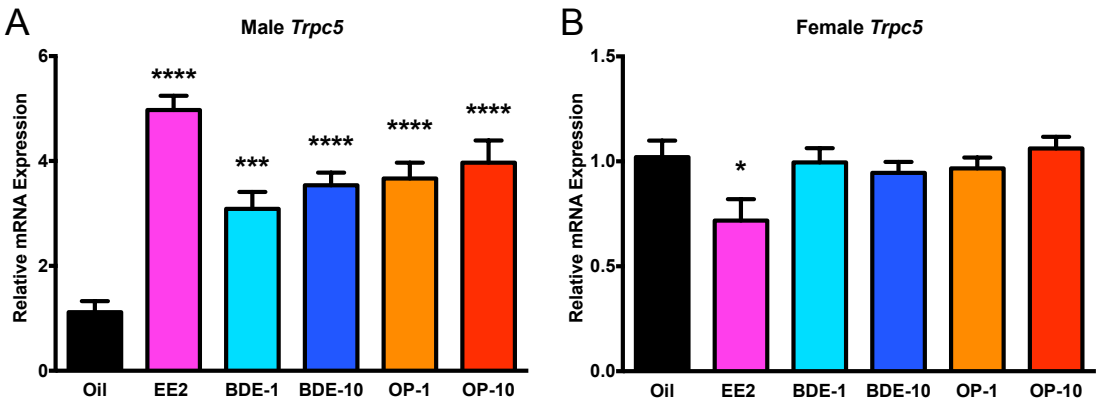

A
Male *Trpc5*
Relative mRNA Expression
6
4
2
0
****
***
****
****
****
Oil
EE2
BDE-1
BDE-10
OP-1
OP-10
B
Female *Trpc5*
Relative mRNA Expression
1.5
1.0
0.5
0.0
*
Oil
EE2
BDE-1
BDE-10
OP-1
OP-10

Figure 7.

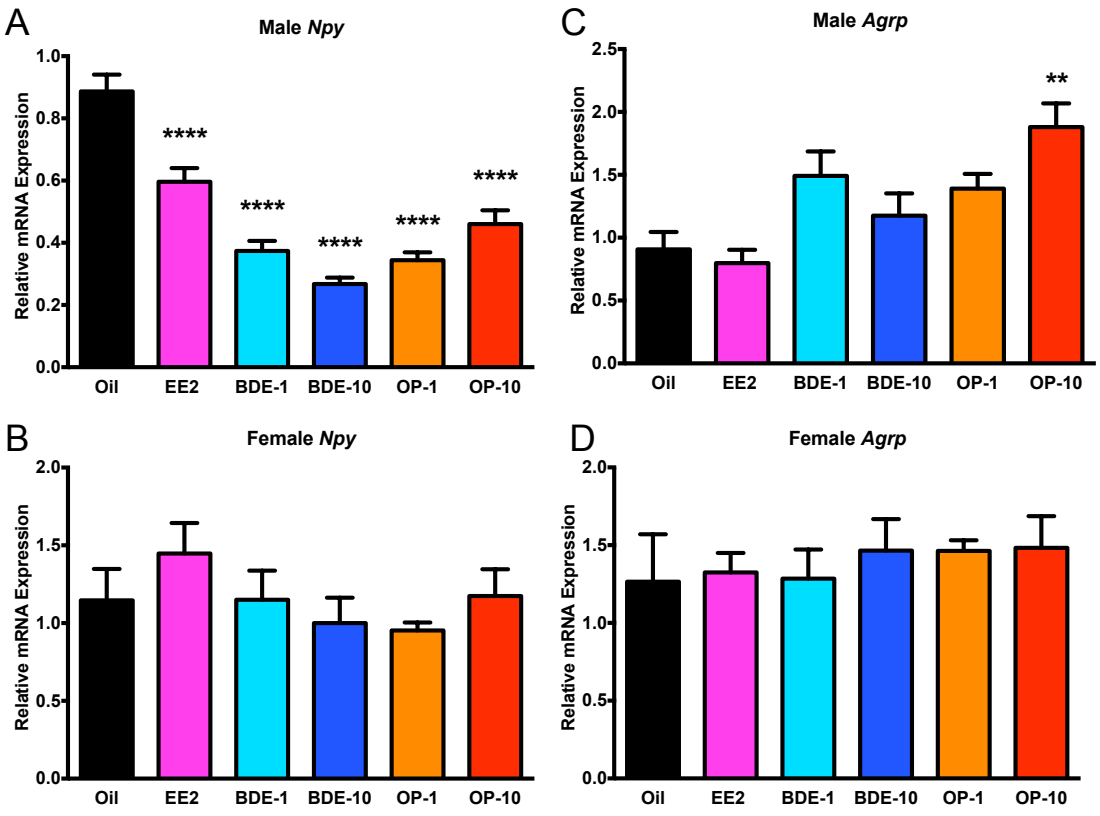
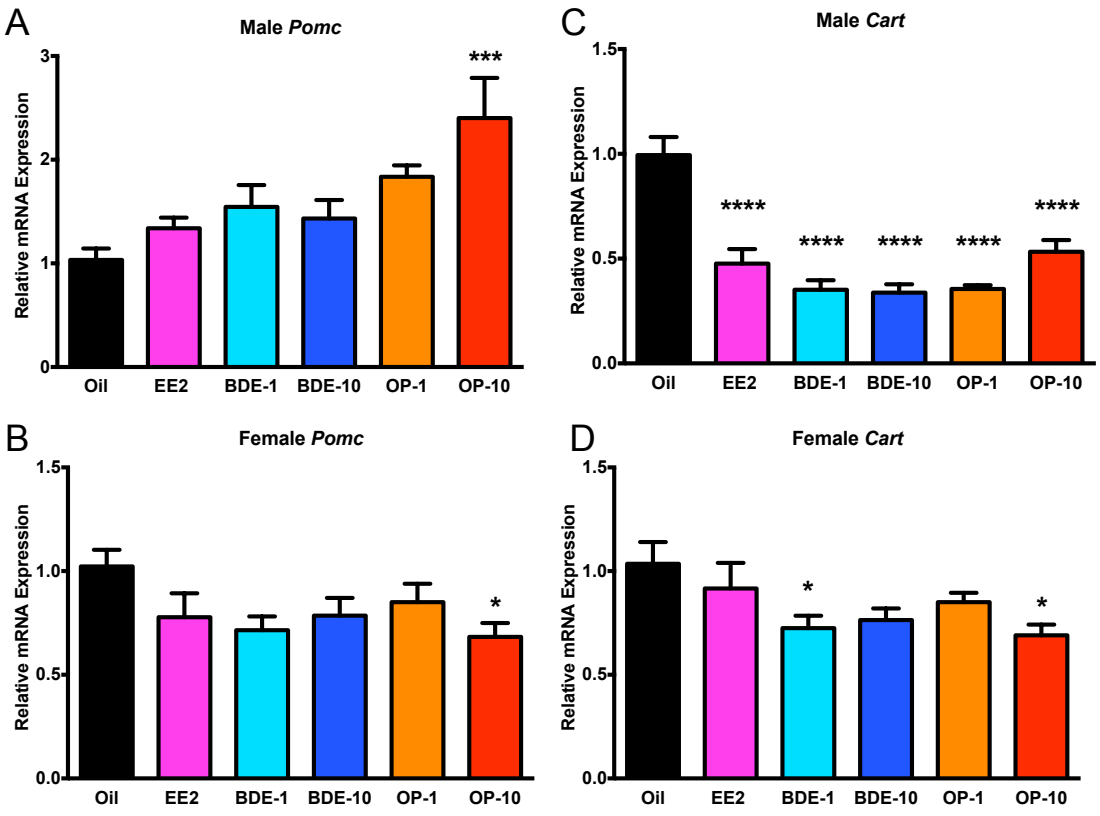

A
Male Npy
Relative mRNA Expression
1.0
0.8
0.6
0.4
0.2
0.0
****
****
****
****
Oil
EE2
BDE-1
BDE-10
OP-1
OP-10
C
Male Agrp
Relative mRNA Expression
2.5
2.0
1.5
1.0
0.5
0.0
**
Oil
EE2
BDE-1
BDE-10
OP-1
OP-10
B
Female Npy
Relative mRNA Expression
2.0
1.5
1.0
0.5
0.0
Oil
EE2
BDE-1
BDE-10
OP-1
OP-10
D
Female Agrp
Relative mRNA Expression
2.0
1.5
1.0
0.5
0.0
Oil
EE2
BDE-1
BDE-10
OP-1
OP-10

Figure 8.


A
Male *Pomc*
Relative mRNA Expression
3
2
1
0
***
Oil
EE2
BDE-1
BDE-10
OP-1
OP-10
C
Male *Cart*
Relative mRNA Expression
1.5
1.0
0.5
0.0
****
****
****
****
****
Oil
EE2
BDE-1
BDE-10
OP-1
OP-10
B
Female *Pomc*
Relative mRNA Expression
1.5
1.0
0.5
0.0
*
Oil
EE2
BDE-1
BDE-10
OP-1
OP-10
D
Female *Cart*
Relative mRNA Expression
1.5
1.0
0.5
0.0
*
*
Oil
EE2
BDE-1
BDE-10
OP-1
OP-10

CHAPTER 3:

EFFECTS OF FLAME RETARDANTS ON ENERGY HOMEOSTASIS

ABSTRACT

Flame retardants (FR) polybrominated diphenyl ether (PBDE) congener 2,2',4,4'tetrabromodiphenyl ether (BDE-47) and organophosphate FR (OPFR) persist in the environment and interact with multiple target receptors in peripheral tissue. Developmental exposures to EDCs such as bisphenol A (BPA) alter offspring energy homeostasis, but few studies examine effects of FR. Therefore, we investigated if perinatal FR exposure disrupts energy balance in adulthood. In this experiment, pregnant mice were orally dosed daily vehicle, BDE-47 (1mg/kg), and OPFR mixture (1mg/kg of tris (1,3-dichloro-2-propyl) phosphate (TDCPP), triphenyl phosphate (TPP), and tricresyl phosphate (TCP) each) from gestational day 7 (GD7) to postnatal day (PND) 14. Pup body weights, anogenital distance (AGD), and sex ratio were measured. Weanlings were fed normal or high-fat diet (ND or HFD) and body weights and food intake were measured weekly until PND140. Adult offspring were tested for body composition, energy expenditure, and glucose homeostasis. OPFR-treated males had lower AGDs. In adulthood, FR-treated males fed a ND decreased percent fat and activity while increasing energy expenditure, and promoted glucose tolerance. When fed a HFD, FR-treated males suppressed the effects of HFD on insulin tolerance. OPFR-treated males fed HFD produced more heat and promoted glucose intolerance. BDE-47-treated males fed a HFD increased body weight gain and reduced percent lean mass. OPFR-treated females fed a ND gained more body weight and decreased blood triglycerides. OPFR-treated females fed a HFD consumed more energy and decreased uterine weight. Therefore, these data suggest that these FR alter sex differentiation during development and energy balance in adulthood in sex-dependent manner.

INTRODUCTION

Within the last few years, detectable concentrations of flame retardants (FR) in particular polybrominated diphenyl ethers (PBDE) and organophosphate FR (OPFR) have been reported in breast milk, placenta, and maternal, fetal, and toddler sera [253,257-261]. More concerning is the fact that levels of these compounds are elevated in toddlers when paired with their mothers [264]. PBDE congener, 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), and OPFR tris(1,3-dichloro-2-propyl)phosphate (TDCPP), triphenyl phosphate (TPP), and tricresyl phosphate (TCP) are FR that are predominantly in the environment. BDE-47 has been found in house dust at concentration ranges of 0.5-29 μg/g of dust and 0.2-1798 μg/g of dust for OPFR [18,22,23].

Interestingly, BDE-47 and OPFR (TDCPP, TPP, and TCP) have binding affinities to nuclear receptors such as classical estrogen receptors (ERα/β), androgen receptors (AR), peroxisome proliferator-activated receptors (PPARα/γ), pregnane X receptor (PXR), and thyroid receptors (TRs) *in vitro*, potentially acting as endocrine-disrupting compounds (EDCs) [8,13,296,306,311,315,316,320,339]. Therefore, additional studies for maternal exposure to BDE-47 in mammals are crucial for evaluating potential health conditions later in life [269,305,319,403]. Additionally, OPFR have hastily replaced PBDE in many respects, but few toxicological studies are analyzing long-term ramifications of early exposure to OPFR in mammalian models [314].

The developmental origins of health and disease (DOHaD) hypothesis proposes that disruptions in the environment at early age can elicit alternative developmental changes, affecting a cohort later in life [404]. These alterations during development can lead to increased risk to an assortment of diseases in adulthood. Maternal exposure to EDCs through gestation and lactation can lead to disruption of reproductive and energy homeostasis [210,213,234]. Studies examining bisphenol A (BPA) and diethylstilbestrol

demonstrate how EDCs can sex differentiation, neural circuitry, increased adipogenesis, and other developmental programming effects when exposed at critical developmental periods [2,3,5,6,221,342].

Regarding BDE-47 perinatal exposure, high BDE-47 serum levels have been associated with altered thyroid hormone levels in pregnant women and pubertal onset in young girls [329,331,405]. Maternal BDE-47 treatment in rats elevates higher body weight and length in males and females [305,319]. Global gene analysis of rat livers also given BDE-47 to dams exhibits changes in carbohydrate and lipid metabolism [319]. In rats, perinatal exposure to BDE-47 increases body weight in both sexes and elevates plasma insulin growth factor-1 and glucose uptake in male rats [403]. However, various aspects such as dose, route of administration, species, sex, duration of exposure, and multiple receptor targets add to the complexity of how PBDE affect an experimental model.

The selected OPFR affect metabolism, gene expression, and homeostasis in non-mammalian models, but few studies exist in mammalian models. In *in vitro*, TDCPP, TPP, and TCP activate PXR, TRs, PPARα, PPARγ, and AR in rodent and human liver, fat, and kidney tissue [10,13,14,304,311,315-320]. However, the only maternal exposure rodent study examined the effects of a commercial OPFR mixture Firemaster550 (FM550), which contains TPP, in rats. When given maternally in rats, FM550 increased body weight in both sexes. In female mice, FM550 advanced puberty, altered glucose tolerance, and reduced exploratory behavior in females. Male rats increased fasting blood glucose. [403]. Currently, no studies in mice have been reported.

The effect of these FR on energy homeostasis and their potential effects centrally and peripherally are still being studied. While there are numerous studies on the effect of PBDE, few studies analyze effects of OPFR exposure mammals *in vivo* and only one study treated the subjects perinatally [301,305,314,334,335,340,406,407]. Additionally, these potential estrogenic EDCs are commonly found in mixtures and the primary route of entry of is

through ingestion. Therefore, we examined if perinatal exposure through orally dosing select estrogenic BDE-47 and OPFR mixture (TDCPP, TPP, TCP) from GD7-PND14 at relevant environmental concentrations negatively impacts developmental and metabolic parameters in mouse offspring. Our hypothesis is that perinatal exposure of these FR will increase body weight, adiposity, and altering glucose homeostasis in adult offspring in a sex-dependent manner.

MATERIALS AND METHODS

Animals.

Animal treatments are in accordance with institutional guidelines based on National Institutes of Health standards and were performed with Institutional animal Care and Use Committee approval at Rutgers University. Wild-type C57Bl/6J female and male mice were bred in-house, maintained under controlled temperature (25°C) and 12/12h light/dark cycle with water and food ad libitum. Males and females were mated where males remained housed with the females for a week. Females were acclimated to peanut butter at least 3 days prior to male removal. Afterwards, female mice were orally dosed daily with either sesame oil, BDE-47 (1mg/kg), OPFR mixture (1mg/kg of each (TDCPP, TPP, TCP) in peanut butter from approximately gestational day 7 to postnatal day 14 (GD7-PND14). Dams were weighed every 3 days to ensure accurate dosing based on body weight. If dams did not produce offspring, they were untreated for a week to clear compound levels. At PND2, average litter weights were measured and the number of offspring noted. If the litter exceeded 8 pups, the excess pups were randomly culled. The sex ratio of each litter was determined between PND5-7 by comparing anogenital distances (AGDs). Litters with fewer than 4 pups were culled and not used for

the experiment. Unsexed offspring were weighed on the day of dosing termination (PND14).

On PND21, offspring were weaned, separated by sex, have AGDs measured, and given either a control normal diet (ND) (Research Diets, D12450B) or high-fat diet (HFD) (Research Diets, D12451) *ad libitum*. The dam's first litter received ND while the second litter was given HFD. Overall, 3-4 litters for each treatment and diet were bred. Weekly body weights and food intake was measured until PND140 (20 weeks).

Food Intake

From food intake (g), energy intake was calculated by the kcal/g food dietary information provided by Research Diets. Average weekly energy intake was calculated per tub per animal. From energy intake, juvenile feeding efficiency (cumulative bodyweight weeks 3-8/Energy Intake weeks 3-8) and adult feeding efficiency (cumulative body weight weeks 9-20/energy intake weeks 9-20) were calculated.

Body Composition and Energy Expenditure

At the end of the 20 weeks, EchoMRI (Echo Medical Systems, Houston, TX, USA) assessed body mass composition. Comprehensive Lab Animal Monitoring System (CLAMS) analyzed VO2, VCO2, RER, heat, and locomotor activity (Columbus Instruments, Inc., Columbus, OH, USA) for 24 hrs.

Glucose and Insulin Tolerance Tests

GTT and ITT were conducted to gauge glucose and insulin tolerance within 2-3 days in between the tests. Animals were fasted for 4 hr for both glucose tolerance tests (GTT) and insulin tolerance tests (ITT). For GTT, animals were injected of 2 g/kg glucose and for the ITT, animals were injected with 0.75 U/kg insulin, after initial tail

blood sample were collected. Blood samples were collected from the tail vein at 0, 15, 30, 60, 90, 120, and 180 minutes after administration of glucose for GTT. For ITT, blood samples were collected from the tail vein at 0, 15, 30, 60, 90, and 120 minutes. AlphaTrak glucometer (Abbott Laboratories, Abbott Park, IL, USA) was used to measure blood glucose concentration. 4% Lidocaine anesthetic cream (L.M.X.4, Cincinnati, OH) was applied to the tail 15 minutes before the incision. After sufficient recovery from the ITT (at least 2 days after), all animals were sacrificed.

Triglyceride analysis and Tissue Dissection

After subject sacrifice, trunk blood was collected in BD Vacutainer tubes coated with K2 EDTA (Becton, Dickinson & Co., Franklin Lakes, NJ) and analyzed for triglyceride levels using a CardioChek (Polymer Technology Systems, Inc., Indianapolis, IN, USA). 1uL 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF; 0.1mg/mL sterile saline; Sigmal-Aldrich, New Haven, CT) for every 100uL of blood was used to inhibit blood serine protease activity. In females, uteri were dissected and weighed.

Statistical Analysis

All data were analyzed using GraphPad Prism 6c software (GraphPad Software, Inc., La Jolla, CA, USA). Cumulative weekly body weight, GTT/ITT data, and hourly VO2 consumption for each type of EDC in comparison to oil were analyzed by repeated measures multivariate ANOVA (treatment X diet X time) followed by *post hoc* analysis with Fisher's Least Significant Difference (LSD) tests between treatment group (diet and EDC) within time points. One-way ANOVA was used for analyzing PND2 litter weight, PND5 sex ratio, PND14 unsexed litter weights, PND21 AGDs, and PND21 sexed body weights for EDC treatments in comparison to oil with Fisher's LSD test *post-hoc*

analysis. Body composition, night and day CLAMS measurements, energy intake, feeding efficiency, GTT and ITT AUC data, fasting glucose, and uterine weights were analyzed using two-way ANOVA (treatment X diet) followed by *post hoc* Fisher's LSD tests across diets within perinatal treatments or across diets and perinatal treatments compared to ND of the same perinatal treatment. All data were expressed as mean ± SEM and significance was set at $\alpha \leq 0.05$.

RESULTS

1. Developmental Growth and Sex Differentiation Parameters

Growth during early development (PND2-PND21) and biomarkers of sex differentiation (AGDs) were measured. In analyzing PND2 and PND14 litter weights, PND21 body weights, PND5 sex ratios, and PND21 AGDs, all data were compared to oil. For PND2 and PND14, the animals were unsexed when measured. Samples sizes for PND2 and PND14 unsexed litter weights and litter sex ratio were as follows: oil (n = 7 litters), BDE-47 (n = 6 litters), OPFR (n = 7 litters). PND21 body weights and AGDs were measured in males and females designated. PND21 is also the day when mice were weaned and fed either a ND or HFD. Samples sizes for male and female PND21 groups were as follows: oil males (n = 24), BDE-47 males (n = 29), OPFR males (n = 28), oil females (n = 18), BDE-47 females (n = 19), and OPFR females (n = 18).

There was no effect of FR treatment in developmental growth, but OPFR-treated males exhibited modified sex differentiation. FR treatment had no effect on litter weights of unsexed pups at either PND2 or PND14 or body weights in sexed males or females at PND21 (Figure 9). There was also no effect of perinatal FR treatment on sex ratio (number of males/pups in litter) (Figure 10A). However, at PND21, males perinatally treated with OPFR had lower AGDs (6.61 ± 0.15 mm, $p < 0.05$) than males given oil

(7.174 ± 0.18 mm) (ANOVA: $F(2,78) = 4.19$, $p < 0.05$; Figure 10B). There was also no significant effect of perinatal treatment on female AGDs or male mice given BDE-47 compared to oil-treated males (Figure 10B &C).

2. Average cumulative body weight gain and body weights

All average cumulative body weight gain (weekly body weight – PND21 body weight) was compared between FR treatments (FR ND and FR HFD) and oil (oil ND and oil HFD) and within each weekly time point. These measurements were taken from PND21-140. In addition to cumulative body weight gain, actual body weights at week 20 were evaluated to examine potential body weight alterations at the end of the overall study. Sample sizes for each group for males is as follows: oil ND (n=11), oil HFD (n=13), BDE-47 ND (n=16), BDE-47 HFD (n=13), OPFR mixture ND (n=15), OPFR mixture HFD (n=13). Sample sizes for each group of females is as follows: oil ND (n=9), oil HFD (n=9), BDE-47 ND (n=8), BDE-47 HFD (n=11), OPFR mixture ND (n=8), OPFR mixture HFD (n=10).

As a treatment effect, BDE-47-treated males fed a HFD gained more body weight compared to oil-treated males also fed a HFD on weeks 14-16, 19, and 20 (BDE-47 HFD vs. oil HFD, $p < 0.05$). In males perinatally treated with BDE-47 compared to oil-treated males, there were significant effects of time (ANOVA: $F(16, 784) = 765.4$, $p < 0.0001$), diet (ANOVA: $F(1,49) = 85.3$, $p < 0.0001$), and time x diet (ANOVA: $F(16,784) = 57.2$, $p < 0.0001$) and time x treatment (ANOVA: $F(16,784) = 3.0$, $p < 0.0001$) in cumulative body weight gain. As expected, males treated with oil and on a HFD had gained more body weight at weeks 7-20 than oil-treated males on a ND ($p < 0.05$). At weeks 8-20, BDE-47-exposed males fed a HFD gained more body weight (BDE-47 HFD vs. BDE-47 ND, $p < 0.01$). There was no significant treatment effect of BDE-47 on cumulative body weight gain in males when on a ND (BDE-47 ND vs. oil ND). (Figure 11A)

However, there was no effect of BDE-47 treatment in females on cumulative body weight gain (BDE-47 vs. oil). In females perinatally treated with BDE-47 in comparison to oil-treated females there were effects of time (ANOVA: $F(16,528) = 223.7$, $p < 0.0001$), diet (ANOVA: $F(1,33) = 16.2$, $p < 0.001$), and time x diet (ANOVA: $F(16,528) = 13.0$, $p < 0.0001$) in cumulative body weight gain. Oil-treated females fed a HFD exhibited increased body weight gain at weeks 11-20 ($p < 0.05$) while BDE-47-treated females fed a HFD gained more body weight at weeks 12-20 compared to their ND-fed counterparts (BDE-47 HFD vs. BDE-47 ND, $p < 0.05$). (Figure 11B)

There was also no effect of OPFR treatment in males regarding cumulative body weight gain (OPFR vs. oil). Males perinatally treated with OPFR compared to oil-treated males, had significant effects of time (ANOVA: $F(16,768) = 735.9$, $p < 0.0001$), diet (ANOVA: $F(1,48) = 82.6$, $p < 0.0001$), and time x diet (ANOVA: $F(16, 784) = 47.6$, $p < 0.0001$) in cumulative body weight gain. At weeks 6-20, males perinatally treated with oil or OPFR gained more body weight when fed a HFD than their treatment counterparts given a ND (OPFR HFD vs. OPFR ND, $p < 0.05$). (Figure 11C)

Conversely, OPFR-treated females exhibited higher body weight gain compared to oil-treated females when both groups were given a ND at weeks 12, 16, and 19 ($p < 0.05$). Females perinatally treated with OPFR in comparison to oil-treated females had effects of diet (ANOVA: $F(1,32) = 19.83$, $p < 0.001$), time (ANOVA: $F(16,512) = 253.7$, $p < 0.0001$), treatment (ANOVA: $F(1,32) = 5.71$, $p < 0.05$), and time x diet (ANOVA: $F(16,512) = 13.64$, $p < 0.0001$) in cumulative body weight gain. When fed a HFD, OPFR-treated females gained more body weight at weeks 10-20 than oil-treated females on a ND (OPFR HFD vs. OPFR ND, $p < 0.05$). Oil-treated females also on a HFD gained more body weight at weeks 11-20 than their ND-fed counterparts (oil HFD vs. oil ND, $p < 0.05$). There was no effect of OPFR treatment on females given HFD (OPFR HFD vs. oil HFD). (Figure 11D)

There was no effect of FR treatment on body weights at week 20 in males or females. At week 20, as expected, there was a significant effect of diet (ANOVA: $F(1,75) = 170.1$, $p < 0.0001$), but not for treatment or treatment x diet in male body weights. When fed a HFD, males in all treatment groups had higher body weights at week 20 (oil: 44.17 ± 1.2 g; BDE-47: 47.4 ± 0.81 g; OPFR: 44.1 ± 1.3 g; oil, BDE-47 and OPFR, $p < 0.0001$) compared to their ND-fed counterparts (oil: 32.5 ± 1.0 g; BDE-47: 33.3 ± 1.4 g; OPFR: 32.3 ± 1.0 g). Similar to male body weights at week 20, there was also a significant effect of diet only in female body weights (ANOVA: $F(1,49) = 28.2$, $p < 0.0001$). At week 20, females given any perinatal treatment and fed a HFD had higher body weights (oil: 26.1 ± 1.2 g; BDE-47: 26.9 ± 1.4 g; OPFR: 26.7 ± 1.3; oil and OPFR, $p < 0.01$; BDE-47, $p < 0.001$) compared to ND-fed females (oil: 21.8 ± 0.5 g; BDE-47: 21.1 ± 0.5 g; OPFR: 22.3 ± 0.8 g). (Figure 12)

4. Energy Intake and Feeding Efficiency

Energy intake and feeding efficiency (cumulative body weight/energy intake) were noted to evaluate links between energy entering the body and the efficiency of that energy being converted to gained body weight. Females treated with OPFR consumed more energy on a HFD (87.7 ± 7.6 kcal, $p < 0.05$) compared to controls on a HFD (70.8 ± 4.8 kcal) and to OPFR-treated females fed a ND (ANOVA: treatment or treatment x diet: ns; 66.6 ± 5.2 kcal) **(**Figure 13D). There was overall a significant diet effect (ANOVA: $F(1,14) = 7.32$, $p < 0.05$), but no effects for treatment or treatment x diet in females regarding energy intake. There were no significant effects for female juvenile or adult feeding efficiency regarding treatment or diet (Figure 13E & F). There were also no significant treatment or diet effects in energy intake or feeding efficiency in males (Figure 13A-C).

5. Body Composition

When fed ND, percent fat in OPFR-treated males was attenuated (17.6 ± 2.0%, $p < 0.05$) compared to oil-treated males also given a ND (24.7 ± 3.8%) (ANOVA: treatment or treatment x diet: ns; Figure 14A). There were significant effects of diet (ANOVA: males: $F(1,75) = 70.58$, $p < 0.0001$; females: $F(1,49) = 32.38$, $p < 0.0001$), but no significant effects of treatment or treatment x diet in average male and female percent fat of body weight. HFD-fed males had increased percent fat when treated with oil (32.9 ± 1.2%, $p < 0.01$), BDE-47, (37.2 ± 0.7%, $p < 0.0001$), and OPFR (32.3 ± 1.7%, $p < 0.0001$) compared to ND-fed males (oil ND: 24.7 ± 3.8%; BDE-47 ND: 20.2 ± 2.6%; OPFR ND: 17.6 ± 2.0%). Percent fat was also increased in HFD-fed females compared to ND-fed females (oil: 15.7 ± 0.8%; BDE-47: 15.9 ± 1.4%; OPFR: 15.0 ± 1.2%) in oil- (28.7 ± 2.6 %, $p < 0.001$), BDE-47- (25.9 ± 3.4%, $p < 0.01$), and OPFR-treated females (23.8 ± 1.7%, $p < 0.01$) (Figure 14B).

Percent lean mass of body weight was altered in FR-treated mice on a HFD. HFD-fed males, regardless of treatment, had decreased percent lean mass (oil: 57.8 ± 0.8%; BDE-47: 53.3 ± 0.5%; OPFR: 59.2 ± 1.9%; $p < 0.0001$) in comparison to males fed a ND (oil: 68.9 ± 1.3%; BDE-47: 68.7 ± 1.5%; OPFR: 70.5 ± 1.9%). There were overall effects of treatment (ANOVA: $F(2,75) = 3.65$, $p < 0.05$) and diet (ANOVA: $F(1,75) = 107.5$, $p < 0.0001$), but not for treatment x diet in percent lean mass of body weight in males. When fed a HFD, males treated with BDE-47 had higher percent lean mass (BDE-47: 53.3 ± 0.5%, $p < 0.05$) compared to oil-treated males also fed a HFD (oil: 57.8 ± 0.8%). However, there were no significant effects of OPFR treatment in male percent lean mass of body weight compared to oil-treated males. In females, there was an effect of diet only (ANOVA: $F(1,49) = 26.14$, $p < 0.0001$) and not treatment or treatment x diet regarding percent lean mass of body weight (Figure 14C). Similar to the males, HFD-fed females in all perinatal treatment groups had lower percent lean mass (oil: 65.3 ± 2.4%,

$p < 0.05$; BDE-47: 65.3 ± 3.2%, $p < 0.001$; OPFR: 67.6 ± 1.3%, $p < 0.05$) compared to ND-fed females (oil: 73.1 ± 0.8%; BDE-47: 76.4 ± 1.1%, OPFR: 74.9 ± 1.5%) (Figure 14D).

6. Energy Expenditure

Energy expenditure was analyzed amongst the parameters of oxygen consumption (VO2), carbon dioxide production (VCO2), respiratory exchange ratio (RER = VCO2/VO2), heat, and activity (X-axis and Z-axis) using two-way ANOVA. While VO2 was analyzed hourly and by average nighttime (7pm-7am) and daytime (7am-7pm) for 24 hours, all other changes in energy expenditure were reported as only average nighttime and daytime values. Hourly VO2 was analyzed with two-way ANOVA (treatment x time or diet x time). In *post-hoc* analysis, data were compared either between overall treatment or diet within each time point (hours 0-23). Average nighttime and daytime VO2 were compared between either perinatal treatment (oil, BDE-47, OPFR) or diet (ND or HFD) when using two-way ANOVA. For the hourly, nighttime and daytime measurements, data were compared between treatments (EDC vs. oil) within diet and between diets (ND vs. HFD) within EDC treatment in *post-hoc* analysis.

6a. Oxygen Consumption

FR-treated males on a HFD exhibited more striking effects in oxygen consumption than oil-treated males. Oxygen consumption (VO2) is a biomarker that reflects the amount of adenosine triphosphate (ATP) utilized in aerobic metabolism [408-410]. Therefore, if oxygen consumption increases, theoretically, so does energy expenditure. In oil-treated mice fed a HFD, males and females consumed more oxygen compared to their ND-fed controls (oil ND vs. oil HFD) at hours 2, 4, 11, 13, 14, 21, and 22 in males and at hours 7, 14, and 17 in females (Figure 15A & B). There were effects

of time (ANOVA: males: $F(23,506) = 10.12$, $p < 0.0001$; females: $F(23,368) = 6.649$, $p < 0.0001$) and diet x time (ANOVA: males: $F(23,506) = 2.669$, $p < 0.0001$; females: $F(23,368) = 2.13$, $p < 0.01$) in hourly oxygen consumption of oil-treated males on a HFD compared to oil-treated males on a ND and females perinatally treated with oil fed a HFD compared to oil-treated females on a ND. BDE-47-treated males fed a HFD consumed less oxygen at hours 1, 2, 14, 17, and 20-23 compared to ND-fed males also perinatally treated with BDE-47 (BDE-47 ND vs. BDE-47 HFD; Figure 15C & D). In BDE-47-treated mice, there were effects of time in males and females (ANOVA: males: $F(23,621) = 11.42$, $p < 0.0001$; females: $F(23, 391) = 6.688$, $p < 0.0001$) and effects of diet (ANOVA: $F(1,27) = 5.04$, $p < 0.05$) and diet x time in males only (ANOVA: $F(23,621) = 1.81$, $p < 0.05$). In OPFR-treated mice on a HFD, mice consumed less oxygen compared to ND at hours 0-5, 9, 10, 13, 14, 17, 19, and 23 for males and at hour 0 (12:00 am) for females (OPFR ND vs. OPFR HFD; Figure 15E & F). In mice perinatally treated with OPFR, there were effects of time (ANOVA: males: $F(23,598) = 9.199$, $p < 0.0001$; females: $F(23,368) = 5.471$, $p < 0.0001$) in males and females and effects of diet in males only (ANOVA: $F(1,26) = 5.00$, $p < 0.05$) in hourly oxygen consumption, but there were no significant effects of diet x time in males or females.

Mice perinatally treated with BDE-47 had modified hourly oxygen consumption on a HFD. Overall, there were effects of time in mice fed a ND (ANOVA: males: $F(23,575) = 13.78$, $p < 0.0001$; females: $F(23,345) = 5.802$, $p < 0.0001$) and a HFD (ANOVA: males: $F(23,552) = 8.457$, $p < 0.0001$; females: $F(23,414) = 8.141$, $p < 0.0001$), and treatment x time in mice fed a HFD (ANOVA: males: $F(23,552) = 2.311$, $p < 0.001$; females: $F(23,414) = 1.70$, $p < 0.05$) regarding hourly oxygen consumption. BDE-47-treated females on a ND consumed more oxygen compared to oil-treated females at hour 10 only ($p < 0.05$). When fed a HFD, mice perinatally treated with BDE-47 consumed more oxygen at hour 5 in males ($p < 0.05$) and at hour 15 in females (p

<0.01) compared to their oil-treated counterparts that were given a HFD. However, at hours 11 and 15 in males, HFD-fed males with BDE-47 perinatal treatment consumed less oxygen in comparison to oil-treated males also given HFD (hour 11, $p < 0.01$; hour 25, $p < 0.05$). (Figure 16)

Hourly oxygen consumption was altered in OPFR-treated mice. When comparing OPFR and oil perinatal treatment, there were effects of time in ND-fed mice (ANOVA: males: $F(23,552) = 8.857$, $p < 0.0001$; females: $F(23,345) = 4.358$, $p < 0.0001$) and mice on a HFD (ANOVA: males: $F(23,552) = 12.42$, $p < 0.0001$; females: $F(23,391) = 7.101$, $p < 0.0001$) and treatment x time in ND-fed mice (ANOVA: males: $F(23,552) = 1.58$, $p < 0.01$; females: $F(23,414) = 1.92$, $p < 0.01$) and in mice on a HFD (ANOVA: females: $F(23,391) = 2.320$, $p < 0.001$). However, there was no effect of treatment x time in HFD-fed males. Additionally, there were also no overall treatment effects for males or females. When fed a ND, OPFR-treated mice consumed more oxygen at hours 4 and 5 in males ($p < 0.05$) and hours 7, and 13 in females ($p < 0.01$). Conversely, OPFR-treated females consumed less oxygen when on a HFD than females perinatally treated with oil also fed HFD at hours 16 and 17 ($p < 0.05$). (Figure 17)

Average daytime and nighttime oxygen consumption was changed in FR-treated males. There was an effect of diet (ANOVA: $F(1,75) = 14.3$, $p < 0.001$) in nighttime male oxygen consumption, but not treatment or treatment x diet while daytime oxygen consumption in males had no significant effects of diet or treatment x diet. Males fed a HFD and perinatally treated with BDE-47 or OPFR consumed less oxygen at nighttime compared to their treatment controls on a ND (BDE-47 ND vs. BDE-47 HFD: 2537 ± 81.60 mL/kg/hr, $p < 0.05$; OPFR ND vs. OPFR HFD: 2510 ± 153.1 mL/kg/hr, $p < 0.01$). However, oil-treated males did not have any significant diet effects during daytime or nighttime (Figure 18A & B). OPFR-treated males on a ND consumed more oxygen during the daytime than oil-treated males also fed a ND (OPFR ND: 2730 ± 96.16

mL/kg/hr; oil ND: 2355 ± 202.6 mL/kg/hr, $p < 0.05$) however, there was no overall effect of treatment on daytime oxygen consumption (ANOVA: treatment and treatment x diet: ns). Males treated with OPFR and fed a HFD consumed less oxygen during the daytime in comparison to OPFR-treated males on a ND (OPFR HFD: 2510 ± 153.1 mL/kg/hr; OPFR ND: 2937 ± 107.9 mL/kg/hr, $p < 0.01$) without an overall effect of diet (ANOVA: diet: ns). There were no significant effects of treatment or diet regarding night and day VO2 in females (Figure 18C & D).

6b. VCO2

Daytime carbon dioxide production was altered in FR-treated males. There were effects of diet only during the nighttime and daytime (ANOVA: night: $F(1,75) = 58.44$, $p < 0.0001$; day: $F(1,75) = 23.07$, $p < 0.0001$) in males while there were no effects of treatment or treatment x diet. At nighttime, males in all perinatal treatment groups produced less carbon dioxide when fed a HFD (oil: 2085 ± 36.2 mL/kg/hr, $p < 0.001$; BDE-47: 2099 ± 72.49 mL/kg/hr, $p < 0.0001$; OPFR: 2123 ± 142.8 mL/kg/hr, $p < 0.0001$) in comparison to ND-fed males (oil: 2812 ± 134.1 mL/kg/hr; BDE-47: 2887 ± 159.4 mL/kg/hr; OPFR: 3002 ± 138.5 mL/kg/hr). During the daytime, males perinatally treated with OPFR reduced carbon dioxide production during the daytime when on a ND compared to oil-treated males also on a ND (OPFR: 2709 ± 125.2 mL/kg/hr, $p < 0.05$; oil: 2271 ± 222.6), however, there was no overall treatment effect (ANOVA: treatment or treatment x diet: ns). FR-treated males fed a HFD produced less carbon dioxide during the daytime (BDE-47: 2011 ± 44.04 mL/kg/hr, $p < 0.01$; OPFR: 1990 ± 128.6 mL/kg/hr, $p < 0.001$) compared to ND-fed in BDE-47- and OPFR-treated males (BDE-47: 2560 ± 146.3 mL/kg/hr; OPFR: 2709 ± 125.2 mL/kg/hr), but there was no diet effect in oil males. (Figure 19A & B)

There was only an effect of diet during the nighttime and daytime (ANOVA: night: $F(1,49) = 25.94$, $p < 0.0001$; day: $F(1,49) = 25.8$, $p < 0.0001$) in female carbon dioxide production and no significant effects of treatment or treatment x diet. Females in all perinatal treatment groups on a HFD reduced nighttime (oil: 2747 ± 108.3 mL/kg/hr, $p < 0.05$; BDE-47: 2833 ± 156.2 mL/kg/hr, $p < 0.01$; OPFR: 2839 ± 175.2 mL/kg/hr, $p < 0.001$) and daytime (oil: 2539 ± 93.87 mL/kg/hr, $p < 0.05$; BDE-47: 2661 ± 198.3 mL/kg/hr, $p < 0.01$; OPFR: 2590 ± 233.8 mL/kg/hr, $p < 0.01$) carbon dioxide production compared to their ND-fed controls (nighttime: oil: 3249 ± 233.4 mL/kg/hr; BDE-47: 3571 ± 138.5 mL/kg/hr; OPFR: 3749 ± 183.5 mL/kg/hr; daytime: oil: 3028 ± 210.5 mL/kg/hr; BDE-47: 3264 ± 106.3 mL/kg/hr; OPFR: 3288 ± 138.7 mL/kg/hr), but there was no significant effect of diet in oil-treated females. (Figure 19C & D)

6c. RER

Daytime substrate utilization was modified in FR-treated males. Respiratory exchange ratio (RER = VCO2/VO2) is a measurement of substrate utilization (carbohydrates vs. fat) for metabolism. If the RER is closer to 0.7, fat is primarily oxidized and if it is closer to 1.0, carbohydrates are mostly used. There was a significant effect on diet only (ANOVA: night: $F(1,75) = 235.7$, $p < 0.0001$; day: $F(1,75) = 22.93$, $p < 0.0001$) in male substrate utilization during the nighttime and daytime. During the nighttime, males in all treatment groups fed a HFD primarily oxidized fat (oil: 0.81 ± 0.01, $p < 0.05$; BDE-47: 0.82 ± 0.01, $p < 0.01$; OPFR: 0.84 ± 0.01, $p < 0.001$) while males on a ND mostly utilized carbohydrates (oil: 1.00 ± 0.02; BDE-47: 0.98 ± 0.02; OPFR: 1.01 ± 0.01). OPFR-treated males fed a ND utilized carbohydrates during the daytime more than oil-treated males also fed a ND (OPFR: 0.99 ± 0.02; oil: 0.89 ± 0.08, $p < 0.05$), although, there was no overall effect of treatment (ANOVA: treatment or treatment x diet: ns). HFD-fed BDE-47- and OPFR-treated males utilized fat as a fuel during the daytime

(BDE-47: 0.825 ± 0.004, $p < 0.05$; OPFR: 0.84 ± 0.01, $p < 0.01$) more than their ND-fed counterparts (BDE-47: 0.95 ± 0.02; OPFR: 0.99 ± 0.02), but there was no effect of diet in oil-treated males. (Figure 20A & B)

Females in all perinatal treatment groups had an effect of diet (ANOVA: night: $F(1,49) = 144.9$, $p < 0.0001$; day: $F(1,49) = 91.41$, $p < 0.0001$) in nighttime and daytime substrate utilization, but not an effect for treatment or for treatment x diet. In all perinatal treatment groups, females fed a HFD treatments exhibited increased RER compared to ND-fed females and used fats as their main fuel during the nighttime (oil: 0.84 ± 0.01, $p < 0.05$; BDE-47: 0.84 ± 0.01, $p < 0.01$; OPFR: 0.86 ± 0.02, $p < 0.01$) and daytime (oil: 0.84 ± 0.01 $p < 0.0001$; BDE-47: 0.84 ± 0.01, $p < 0.0001$; OPFR: 0.846 ± 0.02, $p < 0.0001$). Conversely, females in all treatment groups on a ND mostly utilized carbohydrates during the night (oil: 1.00 ± 0.04, BDE-47: 1.04 ± 0.01, OPFR: 1.07 ± 0.01) and day (oil: 0.98 ± 0.04, BDE-47: 1.1 ± 0.02; OPFR: 1.0 ± 0.02). (Figure 20C & D)

6d. Heat

Heat production was changed in FR-treated males and females during the nighttime and daytime. Heat production was measured to determine energy output in which heat production usually increases due to carbohydrate utilization. There was an effect of diet in heat production for males during the nighttime (ANOVA: $F(1,75) = 31.26$, $p < 0.0001$), but no treatment or treatment x diet effects. At nighttime, oil- and BDE-47 treated males on a HFD, produced more heat (oil: 0.59 ± 0.02 kcal/hr, $p < 0.0001$; BDE-47: 0.59 ± 0.02 kcal/hr, $p < 0.001$) compared to their ND-fed controls (oil: 0.48 ± 0.02 kcal/hr; BDE-47: 0.51 ± 0.01 kcal/hr), but had no significant effect of diet on OPFR-treated males (OPFR HFD vs. OPFR ND). Males treated perinatally with OPFR and fed a HFD produced less heat during the nighttime compared to oil-treated males also given a HFD (OPFR: 0.53 ± 0.03 kcal/hr, $p < 0.05$; oil: 0.59 ± 0.02 kcal/hr), but there was no

overall effect of treatment (ANOVA: treatment or treatment x diet: ns). During the daytime, there was an effect of diet (ANOVA: $F(2,75) = 4.14$, $p < 0.05$) and treatment x diet (ANOVA: $F(1,75) = 48.66$, $p < 0.0001$) in the heat production of males. BDE-47- and oil-treated males fed a HFD produced more heat (oil: 0.56 ± 0.02 kcal/hr, 0.0001; BDE-47: 0.568 ± 0.001 kcal/hr, $p < 0.0001$) in comparison to their ND-fed controls during the daytime (oil: 0.40 ± 0.04 kcal/hr; BDE-47: 0.46 ± 0.01 kcal/hr), but not in OPFR-treated males. Furthermore, BDE-47-treated males on a HFD produced more heat during the daytime when fed ND (BDE-47: 0.45 ± 0.01 kcal/hr, $p < 0.05$) compared to oil-treated males also given a ND (oil: 0.40 ± 0.01 kcal/hr). When fed a HFD, OPFR-treated males produced less heat during the daytime (OPFR: 0.50 ± 0.02 kcal/hr, $p < 0.05$) than oil-treated males on a HFD (oil: 0.56 ± 0.02 kcal/hr). (Figure 21A & B)

There were effects of diet in nighttime and daytime heat production measurements for females, but no effects of treatment or treatment x diet (ANOVA: night: $F(1,49) = 5.00$, $p < 0.05$; day: $F(1,49) = 6.68$, $p < 0.01$) in females within all perinatal treatment groups. During the nighttime and daytime, oil-treated females fed a HFD produced more heat (night: 0.45 ± 0.02 kcal/hr, $p < 0.05$; day: 0.41 ± 0.02 kcal/hr, $p < 0.05$) than oil-treated females on a ND (night: 0.37 ± 0.02 kcal/hr; day: 0.34 ± 0.02 kcal/hr). However, there were no significant treatment effects in oil-treated females or effects of diet or treatment in BDE-47- or OPFR-treated females. (Figure 21C & D)

7. Activity

During the nighttime, X total activity was altered in OPFR-treated males. X total activity was determined by the number of times that a subject crossed the X-axis of the CLAMS cage. There was a significant effect of diet (ANOVA: $F(1,75) = 5.73$, $p < 0.05$) in males regarding total activity on the X-axis of the CLAMS cage (X total activity) during the nighttime, but not the daytime. For nighttime and daytime X total activity in males,

there were no significant effects of treatment or treatment x diet. When fed HFD, males perinatally treated with oil or BDE-47 had decreased X total activity during the nighttime (oil: 299 ± 44.1 counts, $p < 0.05$; BDE-47: 307 ± 16.2 counts, $p < 0.05$) compared to their treatment counterparts fed a ND (oil: 454 ± 79.0 counts; BDE-47: 443 ± 46.2 counts) while there was no effect of diet in OPFR-treated males. OPFR-treated males on a ND had decreased X total activity during the nighttime compared to oil-treated males also on a ND (OPFR: 280 ± 36.0 counts, $p < 0.01$; oil: 454 ± 79.0 counts), but there was no overall effect of treatment (ANOVA: treatment and treatment x diet: ns) (Figure 22A & B). There were no significant effects of diet, treatment, or treatment x diet on X total activity in females during the nighttime or daytime (Figure 22C & D).

Nighttime Z total activity was modified in FR-treated females. Z total activity was determined by the number of times that a subject crossed the Z-axis of the CLAMS cage. When perinatally treated with oil, females fed a HFD had increased Z total activity during the nighttime (HFD: 174 ± 31.3 counts, $p < 0.05$) in comparison to oil-treated females fed a ND (ND: 50.3 ± 22.2 counts), but there was no overall effect of diet in females (ANOVA: diet or treatment x diet: ns; Figure 23C & D). There were also no other significant diet effects in female mice perinatally treated with BDE-47 or OPFR during the nighttime. There were no significant effects of diet, treatment, or treatment x diet in Z total activity at nighttime in males. Furthermore, there were no effects of diet, treatment, or treatment x diet during the daytime in Z total activity of females. Interestingly, there was a significant effect of treatment (ANOVA: $F(2,75) = 4.21$, $p < 0.05$) in males during the daytime, without an effect in *post-hoc* analysis. There were also no significant effects of diet or treatment x diet in Z total activity during the daytime in males. (Figure 23A & B)

8. Glucose and Insulin Tolerance

Glucose homeostasis was altered in FR-treated males. GTT and ITT were used to determine glucose and insulin tolerance. Data for these tests were analyzed regarding fasting glucose, blood glucose levels throughout the tests for each time point (15, 30, 90, 120, 180 min), and average area under the curve (AUC) blood glucose for the overall test. There was a significant effect of diet (ANOVA: $F(1,75) = 17.86$, $p < 0.0001$) on 4-hour fasting blood glucose in males, no effects of treatment or treatment x diet. While there was no overall effect involving treatment, OPFR-treated males on a HFD exhibited increased blood glucose when fasted (227.2 ± 14.2 mg/dL, $p < 0.05$) compared to oil-treated males also given a HFD (193.8 ± 10.3 mg/dL) (ANOVA: treatment or treatment x diet: ns). However, there were no overall effects involving treatment or in *post-hoc* analysis in females. When fed a HFD, males and females perinatally treated with oil or OPFR had higher fasting blood glucose (males: oil: 194 ± 10.3 mg/dL, $p < 0.05$; OPFR: 227 ± 14.2 mg/dL, $p < 0.001$; females: oil: 168.0 ± 5.7 mg/dL, $p < 0.01$; OPFR: 186.0 ± 6.3 mg/dL, $p < 0.01$) than ND-fed oil-treated males or females (male: oil: 158 ± 10.1 mg/dL; OPFR: 168.8 ± 8.3 mg/dL; female: oil: 130.0 ± 17.8 mg/dL; OPFR: 156.9 ± 6.0 mg/dL), but no effect of diet in BDE-47-treated males or females. (Figure 24)

In the GTT, there was significant effects of diet (ANOVA: $F(1,49) = 9.08$, $p < 0.05$), time (ANOVA: $F(6,294) = 131.0$, $p < 0.0001$), and time x diet (ANOVA: $F(6,294) = 14.94$, $p < 0.0001$) in oil- and BDE-47-treated males, but there were no effects of treatment. Oil-treated males fed a HFD had a decrease in glucose clearance at 60-120min during the GTT (60 min, $p < 0.05$; 90 min, $p < 0.001$; 120 min, $p < 0.01$). In BDE-47-treated males on a HFD, glucose clearance was attenuated from 60-180min (60 min, $p < 0.001$; 90 and 120 min, $p < 0.001$; 180 min, $p < 0.01$). There were no effects of treatment or any interactions involving treatment for BDE-47- and oil-treated males. (Figure 25A)

OPFR-treated males were more glucose tolerant on a ND and glucose intolerant when fed a HFD in comparison to their oil-treated counterparts. There were significant effects of diet (ANOVA: $F(1,48) = 23.62$, $p < 0.0001$), time (ANOVA: $F(6,288) = 137.2$, $p < 0.0001$), time x diet (ANOVA: $F(6,288) = 20.70$, $p < 0.0001$), and treatment x diet (ANOVA: $F(1,48) = 8.00$, $p < 0.01$) in the GTT of OPFR- and oil-treated males. In males perinatally treated with oil, glucose clearance was attenuated from 60-120 min (60 min, $p < 0.05$; 90 min, $p < 0.001$; 120 min, $p < 0.01$). OPFR-treated males on a HFD had lower glucose clearance at 30, 120, and 180 min time points (30 min, $p < 0.05$; 120 and 180 min, $p < 0.01$) compared to oil-treated males also given a HFD. Furthermore, males treated with OPFR and given a ND demonstrated higher glucose clearance compared to oil-treated males on a ND at 60 and 90 min time points (60 min, $p < 0.01$; 90 min, $p < 0.05$). OPFR-treated males fed a HFD exhibited a decrease in glucose clearance from 30-180 min compared to their ND-fed counterparts (30 and 180 min, $p < 0.001$; 60, 90, and 120 min, $p < 0.0001$). (Figure 25B)

Glucose tolerance was modified in males perinatally treated with FR. There were effects of diet (ANOVA: $F(1,75) = 3.824$, $p < 0.0001$) and treatment x diet (ANOVA: $F(2,75) = 3.82$, $p < 0.05$) in average glucose clearance (AUC) over the course of the GTT in males. OPFR-treated males had lower glucose clearance compared to oil-treated males when both groups were on a HFD (OPFR: 105071 ± 6289.3 mg/dL• min, $p < 0.05$; oil: 86735 ± 4772.1 mg/dL• min). BDE-47- and OPFR-treated males fed a HFD demonstrated a decrease in glucose clearance (BDE-47 HFD: 91198 ± 7928.1 mg/dL• min, $p < 0.001$; OPFR HFD: 105071 ± 6289.3 mg/dL• min, $p < 0.0001$) compared to ND-fed males given the same perinatal treatment (BDE-47 ND: 62297 ± 4324.5 mg/dL• min; OPFR ND: 56801 ± 4860.7mg/dL• min), but there was no significant effect of diet in oil-treated males. (Figure 25C)

BDE-47-treated females exhibited a subtle change in glucose tolerance when fed a HFD. When analyzing glucose tolerance at each time point, there were overall effects of diet, but not in *post-hoc* analysis. Throughout the GTT, females perinatally treated with BDE-47 in comparison to oil-treated females had effects of diet (ANVOA: $F(1,33) = 11.53$, $p < 0.01$) and time only (ANOVA: $F(6,198) = 102.26$, $p < 0.0001$) (Figure 25D). In comparison, the glucose clearance between groups perinatally treated with OPFR and oil in females, there were only effects of diet (ANVOA: $F(1,32) = 6.03$, $p < 0.01$), and time (ANOVA: $F(6,192)$, $p < 0.0001$) (Figure 25E). However, females exhibited an effect of diet only ($F(1,49) = 14.37$, $p = 0.0004$) in average glucose clearance (AUC) for the entire GTT. Females treated with BDE-47 and fed HFD had higher average glucose clearance (62223 ± 4886.1 mg/dL• min, $p < 0.01$) than BDE-47-treated females given a ND (42193 ± 3552.4 mg/dL• min), but there were no effects of diet for oil- or OPFR-treated females (Figure 25F).

BDE-47-treated males fed a HFD had improved insulin tolerance in comparison to oil-treated males given the same diet. In ITT, there were significant effects of diet (ANOVA: $F(1,49) = 24.33$, $p < 0.0001$), time (ANOVA: $F(5,245) = 20.70$, $p < 0.0001$), and treatment x diet (ANOVA: $F(1,49) = 4.4$, $p < 0.05$) when comparing oil- and BDE-treated males. As a treatment effect, BDE-47-treated males had higher insulin-induced glucose clearance at 15, 30, and 90 min time points (90 min, $p < 0.05$; 15 and 30 min, $p < 0.01$) compared HFD-fed males perinatally treated with oil. BDE-47-treated males on a HFD had increased insulin-induced glucose clearance at 0 and 120 min time points (0 min, $p < 0.05$; 120 min, $p < 0.01$). Additionally, males perinatally treated with oil and fed a HFD exhibited decreased insulin-induced glucose clearance from 0-120 min (0 min, $p < 0.01$; 15, 30, and 90 min, $p < 0.0001$; 60 min, $p < 0.001$) compared to their ND-fed counterparts. (Figure 26A)

Similar to the BDE-47 males, insulin tolerance was ameliorated in OPFR-treated males on a HFD. In ITT, there was an effect of diet (ANOVA: $F(1,48) = 26.18$, $p < 0.0001$), time (ANOVA: $F(5,240) = 17.31$, $p < 0.0001$), and treatment x diet (ANOVA: $F(1,48) = 7.58$, $p < 0.01$), and treatment x diet x time (ANOVA: $F(5,240) = 3.83$), $p < 0.01$) when comparing OPFR- and oil-treated males. OPFR-treated males given HFD exhibited increased insulin-induced glucose clearance compared to oil-treated males also given HFD from 15-120 min (15, 30, 60 and 120 min, $p < 0.05$; 90 min, $p < 0.01$). At the 120 min time point of the ITT, OPFR-treated males fed ND had recovered from the insulin challenge more quickly than oil-treated males given ND ($p < 0.05$). Males treated with OPFR and fed a HFD had decreased insulin-induced glucose clearance from 0-30 min (0 min, $p < 0.001$; 15 and 30 min, $p < 0.05$) compared to OPFR-treated males on a ND. In oil-treated males also fed a HFD, insulin-induced glucose clearance was attenuated from 0-120 min (0 min, $p < 0.05$; 60 min, $p < 0.01$; 15, 30, 90, 120 min, $p < 0.0001$). (Figure 26B)

Overall, insulin tolerance was promoted in FR-treated males on a HFD. In ITT, there were effects of diet ($F(2,71) = 3.79$, $p < 0.01$) and treatment x diet ($F(1,71) = 18.35$, $p < 0.0001$) on average insulin-induced glucose clearance (AUC) in males. However, while HFD-fed males perinatally treated with oil had decreased insulin-induced glucose clearance (25587 mg/dL• min ± 1520.8, $p < 0.0001$) compared to oil-treated males fed a ND (14578 ± 1598.3 mg/dL• min), there were no effects of diet in BDE-47- or OPFR-treated males. When given HFD, BDE-47- and OPFR-treated males had increased insulin-induced glucose clearance (BDE-47: 20123 ± 1749.2 mg/dL• min, $p < 0.05$; OPFR: 18775 ± 1644.8 mg/dL• min, $p < 0.01$) compared to oil-treated males fed a HFD (oil: 25587 ± 1520.8 mg/dL• min). (Figure 26C)

Conversely, there were no treatment or diet effects in in insulin tolerance upon *post-hoc* analysis in females given any perinatal treatment. In ITT, there were significant

effects of time (ANOVA: $F(5,165) = 53.20$, $p < 0.0001$) and time x diet only (ANOVA: $F(5,165) = 2.91$, $p < 0.05$) when comparing oil- and BDE-47-treated females (Figure 26D). There was an effect of time only (ANOVA: $F(5,160) = 57.36$, $p < 0.0001$) in ITT when comparing OPFR-treated and oil-treated females (Figure 26E). There were also no significant effects for diet, treatment, or treatment x diet in average glucose clearance (AUC) for the ITT with any perinatal treatment in females (Figure 26F).

8. Triglycerides and terminal uterine weights

Circulating triglycerides were measured at the time of sacrifice as a biomarker for alterations in fat metabolism [149,411,412]. There were significant effects of treatment and treatment x diet ($F(2,49) = 4.13$, $p < 0.05$; $F(2.46) = 5.50$, $p < 0.05$, respectively) in terminal female triglyceride levels (Figure 27B). OPFR-treated female blood triglyceride levels were attenuated (42.6 ± 3.9 mg/dL, $p < 0.05$) in comparison to oil-treated females (53.1 ± 1.4 mg/dL) when both groups were fed a HFD. Additionally, OPFR-treated females on a HFD had suppressed blood triglyceride levels (42.6 ± 3.9 mg/dL, $p < 0.05$) compared to females also treated with OPFR and given a ND (55.6 ± 1.8 mg/dL). Conversely, there were no significant effects of diet or treatment in BDE-47-treated females. There were also no significant effects of diet or treatment in terminal blood triglyceride levels of males (Figure 27A).

Terminal uterine wet weights were measured in females. In past studies, E2 promotes uterine growth while HFD reduces uterine contractility [77,81,413]. Therefore, we examined if either one of these factors of an interaction between them altered uterine weights, but terminal uterine weights (uterine weight/body weight) did not exhibit overall effects of treatment, diet, or treatment x diet. However, OPFR-treated females on a HFD had reduced uterine weights ratios (3.2 ± 0.2, $p < 0.05$) in comparison to oil-treated

females also given a HFD (4.3 ± 0.6) (ANOVA: treatment or treatment x diet: ns). There were no other significant effects of treatment in BDE-47-treated females. (Figure 27C)

DISCUSSION

PBDE are found in baby products such as baby bottles and strollers, but levels have recently been limited in products [16,17]. Primarily, OPFR have replaced PBDE in these widely distributed products, but few studies have examined these compounds at relevant levels, in mammalian models during critical developmental periods [269,281,305,314,319,403]. BDE-47 and the selected OPFR (TDCPP, TPP, and TCP) are amongst the predominant FR in the environment [18,22,23]. Additionally, EDC mixtures are useful to evaluate potential synergistic or additive effects of these compounds and previous studies have analyzed OPFR mixtures such as Firemaster550 (FM550), which contains TPP. Past studies analyzed EDCs such as DES, BPA, and polychlorinated biphenyls (PCBs) acting obesogenic when exposed during critical developmental windows to change development, feeding behavior, body composition, energy expenditure, and fat metabolism in adulthood [7,204,205,207,220,221,235,342,414]. However, there are few studies that analyze energy homeostatic effects of perinatal exposure to these FR, especially OPFR, in mammalian models [269,305,314,319,403].

In the present study, perinatal exposure to the OPFR mixture decreased male AGDs during development. In adulthood, these FR amplified effects of HFD in both sexes, but elicited more striking effects on male energy balance and glucose homeostasis. Furthermore, FR promoted negative energy balance in males and positive energy balance in females when given ND. Therefore, FR alter development and energy homeostasis in a sex-dependent manner.

Developmental Growth and Cumulative Body Weight Gain in adulthood

Despite past studies demonstrating perinatal treatment of BDE-47 and FM550 increased body weight and length in male and female offspring during development, FR had no effect on developmental growth [314,403]. However, in the case of BDE-47, the concentrations were lower than the ones tested in the current study and the route and frequency of administration varied. Additionally, while FM550 was administered in a similar fashion, the OPFR mixture in this study is composed of different compounds and was given for a longer period of time, which could have produced alternative results.

Regarding cumulative body weight gain, there were diet effects that were apparent in male offspring earlier (week 6) than in females (weeks 10-11), wherein mice fed a HFD gained more body weight. As a treatment effect, females exposed to OPFR on a ND and BDE-47-treatment males fed a HFD gained more body weight. However, there were no treatment effects in body weight at week 20 for males or females compared to their same-sex controls. Thus, although body weights were relatively normal during early development, FR altered cumulative body weight gain, but not actual body weight in adulthood. These FR interact with multiple receptors involved in energy balance centrally and peripherally, including classical ERs (ERα/β), but also with AR, PPARs (α/γ), PXR, and TRs in liver, kidney, and fat cells. [10,13,14,304,311,315-320]. Therefore, the selected FR may modulate energy homeostasis as a gross anatomical result of these receptor interactions and their physiological mechanism [8-11,10,13,14,304,311,315-320]. Between the potential for multiple receptor targets and crosstalk amongst receptor signaling, these compounds may affect growth, development, and metabolism in a complex manner [26-33,35,37].

Biomarkers of sex hormone action: AGD and uterine weight

While there were no differences in FR litter sex ratios, males treated with OPFR exhibited lower AGDs at PND21. Past studies of OPFR exposure during pregnancy exhibit changes in reproductive function such as average pubertal onset in offspring rats [314]. Furthermore, OPFR have been shown to interact with classical ERs and AR *in vitro* and alterations in sex hormone levels or actions could lead to disruption in sex differentiation [8,12-14,311,415]. Despite discrepancies whether the examined OPFR act as classical ER agonists or antagonists [12-14,274], TPP consistently act as an AR antagonist in human cells [13,320]. Therefore, it is plausible that OPFR treatment during development could produce lower AGDs in males as a result of AR antagonism [178,416].

However, in HFD-fed females, perinatal OPFR treatment decreased terminal uterine weight in comparison to oil-treated females given the same diet. Hypertrophic growth of the uterus is a well-known marker of indirect ER-signaling and was examined at the time of sacrifice [81,348]. In adult female rats, Latendresse and colleagues (1993, 1995) found that high doses of butylated TPP and TCP increase serum E2. Conversely, TDCPP potentially acts as a classical ER antagonist, which may attenuate endogenous uterine hypertrophy [12,13,334,335]. Additionally, females perinatally treated with OPFR on a HFD had lower uterine weights compared to oil-treated controls on the same diet, independent of changes in whole body weights. Previously, food restriction alters uterine growth with an association to obesity in adult offspring [417]. While not restricting food intake, there is a link between uterine weight and diet, but additional studies would need to clarify effects of HFD on uterine growth. Currently, studies have only analyzed HFD impacting uterine activity, although, lack of uterine activity could potentially result of reduced uterine growth [413]. Therefore, further investigation is required to understand this interaction with the chosen OPFR, sex hormones, and HFD.

Energy balance alterations in adult offspring

The known FR-sensitive nuclear receptors, have primary roles in energy homeostasis, which can be evaluated by measuring their effects on energy intake, energy expenditure, body composition, glucose homeostasis, and body weight [30,79,82,85,153,155,158,161,172-176,393,409,418-425]. Regarding negative energy balance effects, PPARα is known to directly induce fat metabolism and TR is known to increase catabolism while ERα and AR typically elicit these effects indirectly to promote weight loss, fat oxidation, and energy expenditure [30,79,82,85,155,172-176,418-422]. Conversely, PPARγ and PXR favor positive energy balance by increasing body weight, fat mass and uptake, and food intake [153,155,158,161,423-425]. PPARγ directly promotes fat uptake and accumulation while PXR more indirectly impacts energy balance. All of these receptors can be found centrally and in peripheral tissue such as liver, fat, and kidney [49,50,153,155,422,426,427]. Therefore, FR could potentially affect energy homeostasis in a multitude of ways by targeting these receptors.

In the present study, males were more susceptible to changes in energy balance from perinatal FR exposure by altering body composition, energy expenditure, and glucose homeostasis. However, due to FR being an emerging EDC group, there are few studies that analyze perinatal FR treatment effects on energy homeostasis and there are currently no studies that analyze the effects of FR on energy expenditure [269,305,314,319,403]. Negative energy balance was induced in OPFR-treated males by demonstrating increased oxygen consumption (VO2), carbon dioxide production (VCO2), heat production, and carbohydrate utilization (RER) during the day, overall leading reduced fat accumulation. In particular, decreased percent body fat in OPFR-treated males was unexpected because OPFR treatment *in vitro* increases adipocyte differentiation and hepatic PPARγ activation [317,318]. Thus, these findings emphasizes the importance of *in vivo* adult and perinatal exposure models, which account for compensatory mechanisms

throughout the body to maintain homeostasis [393]. Additionally, these OPFR were administered in a mixture, which complicates the elucidation of the receptor-mediated mechanism. Therefore, these effects of this OPFR mixture on energy balance, especially concerning energy expenditure, are novel.

Furthermore, these FR also promote positive energy balance in males. Regarding energy expenditure, when fed a HFD, males given both FR treatments had decreased oxygen consumption and OPFR-treated males on a ND reduced nighttime activity in comparison to oil-treated males given the same diet. At least in BDE-47-treated males on a HFD, these alterations in energy expenditure lead to decreased percent lean mass. These effects of FR promoting positive energy balance are consistent with our finding that BDE-47-treated males fed a HFD also gained more body weight. A previous study by Suvorov and Takser (2010) also supports the promotion of positive energy balance from perinatal BDE-47 exposure in which this treatment modifies hepatic carbohydrate and lipid metabolism in rat offspring [319]. Since there are currently no reports of FR-induced changes in energy expenditure, especially in combination with different diets, further investigation of FR effects peripherally and centrally, especially in the hypothalamus, are required.

Centrally and peripherally, food intake, energy, and glucose homeostasis is regulated, which can lead to alterations body weight [68,428]. The hypothalamus controls glucose homeostasis, insulin sensitivity, and hepatic glucose production, containing glucose-sensing neuron populations in the ventromedial hypothalamus (VMH), lateral hypothalamus (LH), and arcuate nucleus (ARC) [68,388,389,429]. In particular, the ARC is positioned in which its axon terminals are where the blood-brain barrier is incomplete, able to receive input from peripheral satiety signals such as glucose, insulin, leptin, and EDCs [54]. The ARC has at least two populations of neurons that mediate feeding and participate in the melanocortin system, including neurons that express neuropeptide Y

(NPY) and agouti-related peptide (AgRP) and neurons that express proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). POMC/CART neurons are considered anorectic and glucose-excited while NPY/AgRP neurons are typically orexigenic and glucose-inhibited [54,390,430-432]. Through the inhibition of closure of ATP-sensitive potassium (KATP) channels in POMC neurons, whole-body glucose was impaired when challenged with a systemic glucose load, exhibiting the importance of POMC as a glucose-sensing neuron [390]. Peripheral hormones such as insulin and leptin can activate POMC neurons and suppress activity in NPY neurons through the mediation of these KATP channels to control energy expenditure, locomotor activity, glucose homeostasis, insulin sensitivity, and hepatic glucose production, but little is known about the effects of perinatal FR treatment on these parameters [136,142,269,305,314,319,391,392,403,433-437].

Additionally, FR-treated males had altered glucose homeostasis. Males treated with OPFR and on a HFD had reduced glucose clearance when systemically challenged with a glucose load. In male rats, perinatal FM550 increases fasting blood glucose, which coincides with higher glucose intolerance [314]. When challenged with an insulin load, FR-treated males fed a HFD and OPFR-treated males on a ND improved glucose clearance. In past studies, perinatal BDE-47 elevates plasma IGF-1 and glucose uptake in male rats, which support that insulin-induced glucose clearance amelioration in males, but this parameter has not been directly tested in OPFR [403]. Improved insulin-induced glucose clearance in perinatally FR-treated males could signify increased insulin sensitivity, but future studies using a euglycermic clamp can confirm this speculation. If perinatal FR treatment in males improves insulin sensitivity, FR may have also affected hepatic glucose production in these males [387,389,391,392]. Further investigation could determine if FR induces its potential effect on hepatic glucose centrally and/or peripherally and if hepatic glucose production is increased or decreased [387,389,391,392].

In OPFR-treated females fat metabolism may be altered. OPFR lowered terminal blood triglycerides in females when a HFD, indicating a treatment effect and effect of the interaction of treatment and HFD. Decreased blood triglycerides could suggest an alteration in fat metabolism, which is supported by the fact that these females mostly utilized fat [411,412]. Females given both HFD and OPFR treatment also consumed more energy than their oil counterparts given the same diet, which would normally promote positive energy balance. However, there was not a significant effect of cumulative body weight gain, body weight at week 20, or percent body fat compared to females fed HFD and perinatally treated with oil. Therefore, it is plausible that both fat oxidation and adipogenesis are both augmented and homeostasis in body weight and body composition are consequently maintained [317,318].

In previous studies, OPFR have been shown to alter fat metabolism [13,296,311,316-318,320,338,340,341]. In *in vivo*, TPP elevates liver triglycerides in male mice and dyslipidemia in chicken embryos [340,341]. In *in vitro* studies, TDCPP, TPP, and TCP affect PPARγ, PPARα, and TR signaling in liver and fat tissue of rodents, pigs, and humans [13,296,311,316-318,320]. Specifically, PPARγ is known to facilitate fat synthesis and uptake while PPARα and TR induces fat catabolism [438,439]. Since PPARγ, PPARα, and TR all interact with OPFR, possibly HFD and OPFR are simultaneously stimulating fat accumulation through PPARγ and fat oxidation through PPARα and TR. Furthermore, PPARγ and PPARα agonists are found to participate in HFD-induced inflammation through macrophages [440,441]. Therefore, OPFR treatment and HFD may overall promote energy homeostasis in females through fat metabolism and the immune system, but further investigation of these genes, proteins, and pathways after OPFR treatment would have to be examined to confirm this concept.

Implications for future studies

Overall, while we have observed sex-dependent effects of perinatal FR exposure on energy homeostasis, the next step would be to test for central and peripheral mechanisms of action that elicit these effects. Interestingly, FR interact with ER, PPARγ, and AR are receptors that are distributed centrally in the hypothalamus and peripherally in tissue such as fat, muscle, and liver [86,88,153,155,158,166,182,240]. Previous studies demonstrate that long-term E2 treatment decreases *Npy* expression in the hypothalamus, lowering food intake [99-101]. Global ERα knockout (KO) prevents POMC upregulation and anorectic effects of leptin and insulin to induce feeding in female mice [94]. Peripherally, ERα KO mice are hyperphagic and have decreased energy expenditure and altered glucose homeostasis [79-85]. However, males were more greatly affected by FR treatment, diet, or the interaction between the two factors and ER are more highly expressed in females [364]. Therefore, it is possible that FR are acting directly on PPARγ and AR due to their high distribution in male ARC [151,159,163-166,169].

Past *in vitro* studies in peripheral tissue suggest that the selected FR act as PPARγ agonists [315-318,339]. In previous studies, PPARγ activation by agonists also augmented *Npy* and *Agrp* gene expression in the ARC of rodents [159]. Central PPARγ signaling is also necessary for the full effect of systemic thiazolidinediones on hepatic insulin-sensitization [381]. Additionally, PPARγ deletion in POMC neurons elevates energy expenditure while decreasing body weight and food intake [160]. While these studies support the FR- and diet-induced positive energy balance effects in this study, it does not account for the effects the promoted negative energy balance.

Conversely, androgens can differentially influence POMC neuronal activation depending on the sex and androgen, indicating that potential FR interaction with AR could elicit effects that induce both positive and negative balance effects. Testosterone

(T) inhibits ARC POMC neuron activation in rodents of both sexes while dihydrotestosterone (DHT) activates POMC neurons in females [167,168,170]. Brain-specific AR KO suppresses hypothalamic nuclear factor-κB-mediated induction of protein tyrosine phosphatase 1B, reduces insulin sensitivity, and impairs glucose homeostasis in mice [171] Furthermore, global AR KO also decreases leptin signaling in the ARC and promotes insulin and leptin resistance [169,172,174,175]. In previous work, these FR are consistently characterized as AR antagonists in *in vitro* peripheral tissue [10,13,320]. Androgen insensitivity (AIS) is linked to abnormal sex differentiation and elevated E2 levels due to the fact that there is an increased concentration of circulating T, which can be converted to E2 by aromatase [365-372,442]. Du and colleagues (2009) also showed that T inhibits PPARγ in a transcriptional transaction assay, therefore, (AIS) may indirectly promote PPARγ activation [443]. Presumably, FR-induced AIS could affect energy and glucose homeostasis directly, indirectly, centrally, and peripherally. However, to determine FR mechanism receptor, global and tissue-specific KO models in males and females, are required to elucidate potential receptor-mediated mechanisms in each sex in different tissues.

In addition to receptor-mediated mechanisms, potential FR effects in hypothalamic control on energy and glucose homeostasis should be considered. Recently, we found that FR treatment alter homeostatic ARC gene expression also in a sex-dependent manner in adult mice (Chapter 2). In future studies, we can examine if perinatal treatment of FR impacts of FR on central and peripheral gene networks by using RNA sequencing or quantitative real-time PCR on genes in the liver, fat, and hypothalamus. By utilizing electrophysiology on glucose-sensing neurons FR-induced effects on glucose uptake, insulin activity, energy expenditure, and hepatic glucose production in peripheral tissues can be examined. To determine how perinatal FR

treatment affects different hypothalamic nuclei that comprise the melanocortin neurocircuitry, we could double-label POMC, NPY, glutamate transporter 1, or glutamic acid decarboxylase 67 in the hypothalamus to visualize the density of innervated neuronal projections. FR effects upstream of POMC and NPY neurons could be analyzed by testing electrophysiology in these particular neurons for their sensitivity to ghrelin, insulin, and leptin. Furthermore, by using green fluorescent tagging for melanocortin in the neurons of FR-treated subjects, these neurons can be examined for their response to α-MSH as a downstream effect. Therefore, while we did not examine FR mechanism in this study, the results elucidate their effects to perpetuate additional research.

Conclusion

From this study, we have extensively tested parameters of development and energy homeostasis in male and female offspring that orally treated daily to FR (GD7-PND14). Previously effects of perinatal BDE-47 and TPP in a FM550 mixture have been observed using the same concentration as this study. However, this is the first study to examine effects this particular OPFR in a mixture and to combine FR treatments with HFD [403] [281,305,314,319,403]. While OPFR altered developmental sex differentiation in males, both FR treatments diversely impacted adult energy and glucose homeostasis. Therefore, perinatal exposure to these compounds produced sex-dependent effects in energy expenditure, glucose homeostasis, and fat metabolism in adulthood.

Figure 9. Average unsexed pup litter weights for PND2 and PND14 and sexed male and female PND21 body weights. (**A**) PND2 average unsexed litter weights (**B**) PND14 average unsexed litter weights (**C**) PND21 average body weights of sexed males. (**D**) PND21 average body weights of sex females. All data were analyzed using one-way ANOVA with Fisher's LSD tests with BDE-47 and OPFR groups compared to oil. Data are represented as mean ± SEM.

Figure 10. PND5 average litter sex ratio and PND21 average AGD for males and females**.** (**A**) PND5 average litter sex ratio (fraction of males/ # pups in litter) (**B**) PND21 male average AGD (**C**) PND21 female average AGD. All data were analyzed using one-way ANOVA with Fisher's LSD tests with BDE-47 and OPFR groups compared to oil (*p < 0.05). Data are represented as mean ± SEM.

Figure 11. Weekly average cumulative body weights for males and females. (**A**) Male BDE-47 ND, BDE-47 HFD, oil ND, and oil HFD cumulative body weight gain. (**B**) Male OPFR ND, OPFR HFD, oil ND, and oil HFD cumulative body weight gain. (**C**) Female BDE-47 ND vs. oil ND cumulative body weight gain. (**D**) Female OPFR ND vs. oil ND cumulative body weight gain. All data were analyzed using two-way ANOVA (time X treatment) with Fisher's LSD tests. For significant effects between treatment (EDC vs. oil) *p < 0.05 and significant effects between diets (ND vs. HFD) a = p < 0.05, b = p < 0.01. Letter colors represent which data points are compared (oil diet effect (black); EDC diet effect (BDE-47 = blue or OPFR = orange). Data are represented as mean ± SEM.

Figure 12. Male and female week 20 average actual body weights. (**A**) Male week 20 average actual body weights. (**B**) Female week 20 average actual body weights. All data were analyzed using two-way ANOVA (diet X treatment) with Fisher's LSD tests. For significant diet effects, *p < 0.05, ** p < 0.01, ***p < 0.001, **** p < 0.0001. Data are represented as mean ± SEM.

Figure 13. Male and female weekly average energy intake and juvenile and adult feeding efficiencies. (**A**) Male energy intake (**B**) Male juvenile feeding efficiency (**C**) Male adult feeding efficiency (**D**) Female energy intake (**E**) Female juvenile feeding efficiency (**F**) Female adult feeding efficiency. All data were analyzed using two-way ANOVA (diet X treatment) with. For significant effects, *p < 0.05, ** p < 0.01, ***p < 0.001, **** p < 0.0001. Bracketed bars represent the comparisons between treatments within diet. Diet effects do not have bars. Data are represented as mean ± SEM.

Figure 14. Average male and female percent fat and percent lean mass of body weight. (**A**) Male average percent fat of body weight (**B**) Female average percent fat of body weight (**C**) Male average percent lean mass of body weight (**E**) Female average percent lean mass of body weight. All data were analyzed using two-way ANOVA (diet X treatment) with Fisher's LSD tests. For significant effects, *p < 0.05, ** p < 0.01, ***p < 0.001, **** p < 0.0001. Bracketed bars represent the comparisons between treatments within diet. Diet effects do not have bars. Data are represented as mean ± SEM.

Figure 15. Male and female average hourly VO2 for 24 hours diet effects. (**A**) Hourly VO2 male oil ND mice compared to oil HFD males (**B**) Hourly VO2 female oil ND mice compared to oil HFD females (**C**) Hourly VO2 male BDE-47 ND subjects vs. male BDE-47 HFD (**D**) Hourly VO2 female BDE-47 ND mice vs. female BDE-47 HFD mice (**E**) Hourly VO2 OPFR ND in comparison to OPFR HFD for males (**F**) Hourly VO2 OPFR ND

in comparison to OPFR HFD for females. All data were analyzed using two-way ANOVA (treatment X time) with Fisher's LSD tests between diets (ND vs. HFD) within treatments (a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$, d = $p < 0.0001$). Data are represented as mean ± SEM. Black bars across x-axis represent day and night cycle.

Figure 16. Male and female average hourly VO2 for 24 hours (BDE-47 vs. oil). (**A**) Hourly average VO2 in males between BDE-47 and oil within ND (**B**) Female hourly average VO2 between BDE-47 and oil within ND (**C**) Hourly VO2 for males between BDE-47 and oil within HFD (**D**) Average hourly VO2 in females between BDE-47 and oil within HFD. All data were analyzed using two-way ANOVA (treatment X time) with Fisher's LSD tests between treatments (BDE-47 vs. oil) within diet (a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$, d = $p < 0.0001$). Data are represented as mean ± SEM. Black bars across x-axis represent day and night cycle.

Figure 17. Male and female average hourly VO2 for 24 hours (OPFR vs. oil). (**A**) Hourly average VO2 in males between OPFR and oil within ND (**B**) Female hourly average VO2 between OPFR and oil within ND (**C**) Hourly VO2 for males between OPFR and oil within HFD (**D**) Average hourly VO2 in females between OPFR and oil within HFD. All data were analyzed using two-way ANOVA (treatment X time) with Fisher's LSD tests between treatments (OPFR vs. oil) within diets (a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$, d = $p < 0.0001$). Data are represented as mean ± SEM. Black bars across x-axis represent day and night cycle.

Figure 18. Night and day average VO2 for males and females. (**A**) Male nightly average VO2. (**B**) Day average VO2 in males (**C**) Female nightly average VO2 (**D**) Day average VO2 in females. All data were analyzed using two-way ANOVA (treatment X diet) with Fisher's LSD tests between and within treatments and diets. For significant effects, *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$. Bracketed bars represent the comparisons for treatment effects and diet effects do not have bars. Data are represented as mean ± SEM.

Figure 19. Night and day average CO2 for males and females. (**A**) Male nightly average VCO2. (**B**) Day average VCO2 in males (**C**) Female nightly average VCO2 (**D**) Day average VCO2 in females. All data were analyzed using two-way ANOVA (treatment X diet) with Fisher's LSD tests between and within treatments and diets. For significant effects, *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$. Bracketed bars represent the comparisons between treatments within diet. Diet effects do not have bars. Data are represented as mean ± SEM.

Figure 20. Night and day average RER for males and females. (**A**) Male nightly average RER. (**B**) Day average RER in males (**C**) Female nightly average RER (**D**) Day average RER in females. All data were analyzed using two-way ANOVA (treatment X diet) with Fisher's LSD tests between and within treatments and diets. For significant effects, *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$. Bracketed bars represent the comparisons between treatments within diet. Diet effects do not have bars. Data are represented as mean ± SEM.

Figure 21. Night and day average heat for males and females. (**A**) Male nightly average heat. (**B**) Day average heat in males (**C**) Female nightly average heat (**D**) Day average heat in females. All data were analyzed using two-way ANOVA (treatment X diet) with Fisher's LSD tests between and within treatments and diets. For significant effects, *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$. Bracketed bars represent the comparisons

between treatments within diet. Diet effects do not have bars. Data are represented as mean ± SEM.

Figure 22. Night and day average total X-axis activity for males and females. (**A**) Male nightly average total X activity. (**B**) Day average total X activity in males (**C**) Female nightly average total X activity (**D**) Day average total X activity in females. All data were analyzed using two-way ANOVA (treatment X diet) with Fisher's LSD tests between and within treatments and diets. For significant effects, *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$. Bracketed bars represent the comparisons between treatments within diet. Diet effects do not have bars. Data are represented as mean ± SEM.

Figure 23. Night and day average total Z-axis activity for males and females. (**A**) Male nightly average total Z activity. (**B**) Day average total Z activity in males (**C**) Female nightly average total Z activity (**D**) Day average total Z activity in females. All data were analyzed using two-way ANOVA (treatment X diet) with Fisher's LSD tests between and within treatments and diets. For significant differences between diets and within treatments, *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$. Data are represented as mean ± SEM.

Figure 24. Male and female 4-hour fasting glucose (**A**) Male fasting blood glucose (**B**) Female fasting blood glucose. All data were analyzed using two-way ANOVA (diet X treatment) with Fisher's LSD tests. For significant effects, *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$. Bracketed bars represent the comparisons between treatments within diet. Diet effects do not have bars. Data are represented as mean ± SEM.

Figure 25. Male and female blood glucose over time points for glucose tolerance tests (GTTs) and glucose average AUC for the entire test. (**A**) Male blood glucose time points for GTT (BDE-47 vs. oil). (**B**) Male blood glucose for GTT time points (OPFR vs. oil). **C**) Male average glucose AUC for GTTs. (**D**) Female blood glucose for GTT time points (BDE-47 vs. oil). (**E**) Female blood glucose for GTT time points (OPFR vs. oil). (**F**) Female average glucose AUC for GTTs. All data were analyzed using two-way ANOVA (time x diet x treatment) for GTT time points; (diet x treatment) for glucose average AUC with Fisher's LSD tests between and across diets and treatments. For significant difference between diets within diets (a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$, d = $p < 0.0001$) and letter colors indicate which data points are compared. Significant differences between treatments (OPFR vs. oil), *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$. For blood glucose AUC, bracketed bars represent the comparisons for treatment effects. Data are represented as mean ± SEM.

Figure 26. Male and female average blood glucose over time points for insulin tolerance tests (ITTs) and glucose average AUC for the entire test. (**A**) Male blood glucose time points for ITT (BDE-47 vs. oil). (**B**) Male blood glucose for ITT time points (OPFR vs. oil). **C**) Male average glucose AUC for ITTs. (**D**) Female blood glucose for ITT time points (BDE-47 vs. oil). (**E**) Female blood glucose for ITT time points (OPFR vs. oil). (**F**) Female average glucose AUC for ITTs. All data were analyzed using two-way ANOVA (time x diet x treatment) for ITT time points; (diet x treatment) for glucose average AUC with Fisher's LSD tests between and across diets and treatments. For significant difference between diets within diets (a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$, d = $p < 0.0001$) and letter colors indicate which data points are compared. Significant differences between treatments (OPFR vs. oil), *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$. For

blood glucose AUC, bracketed bars represent the comparisons for treatment effects. Data are represented as mean ± SEM.

Figure 27. Terminal female and male blood triglycerides and female uterine weights. (**A**) Male terminal blood triglycerides (**B**) Female terminal blood triglycerides **(C**) Average terminal female uterine weights (uterine weight/body weight). All data were analyzed using two-way ANOVA (treatment X diet) with Fisher's LSD tests between and within treatments. For significant effects, *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$. Bracketed bars represent the comparisons between treatments within diet. Diet effects do not have bars. Data are represented as mean ± SEM.

Figure 9.

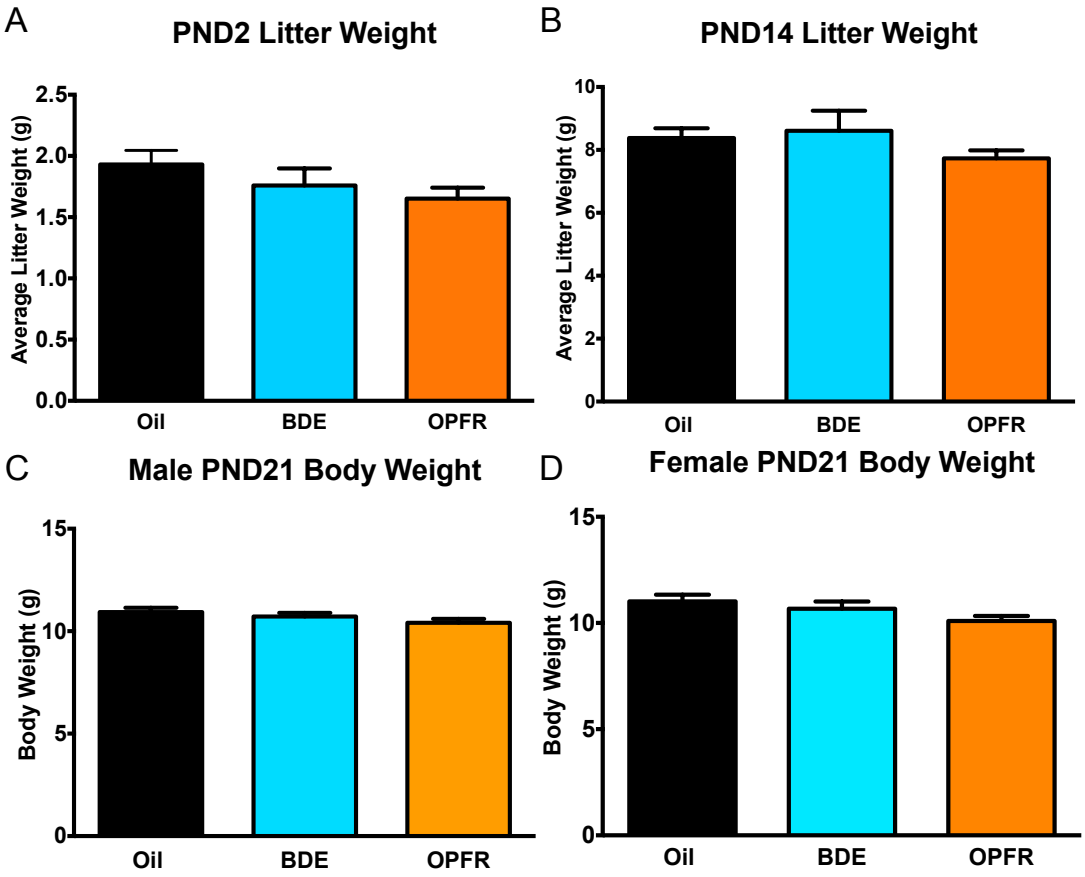

A
PND2 Litter Weight
Average Litter Weight (g)
2.5
2.0
1.5
1.0
0.5
0.0
Oil
BDE
OPFR
B
PND14 Litter Weight
Average Litter Weight (g)
10
8
6
4
2
0
Oil
BDE
OPFR
C
Male PND21 Body Weight
Body Weight (g)
15
10
5
0
Oil
BDE
OPFR
D
Female PND21 Body Weight
Body Weight (g)
15
10
5
0
Oil
BDE
OPFR

Figure 10.

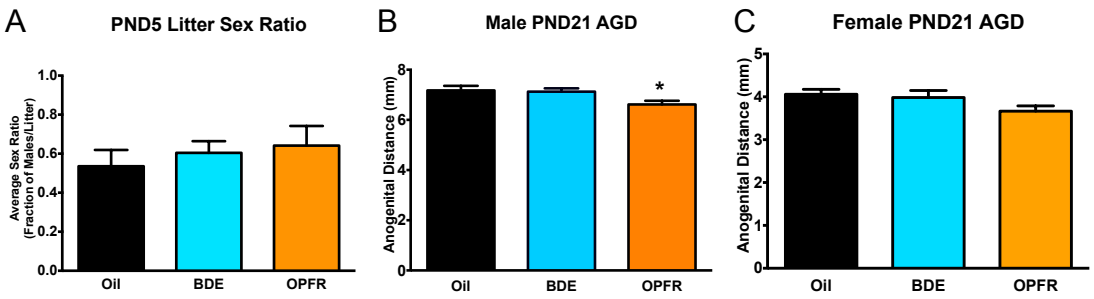

A
PND5 Litter Sex Ratio
Average Sex Ratio (Fraction of Males/Litter)
1.0
0.8
0.6
0.4
0.2
0.0
Oil
BDE
OPFR
B
Male PND21 AGD
Anogenital Distance (mm)
8
6
4
2
0
*
Oil
BDE
OPFR
C
Female PND21 AGD
Anogenital Distance (mm)
5
4
3
2
1
0
Oil
BDE
OPFR

Figure 11.

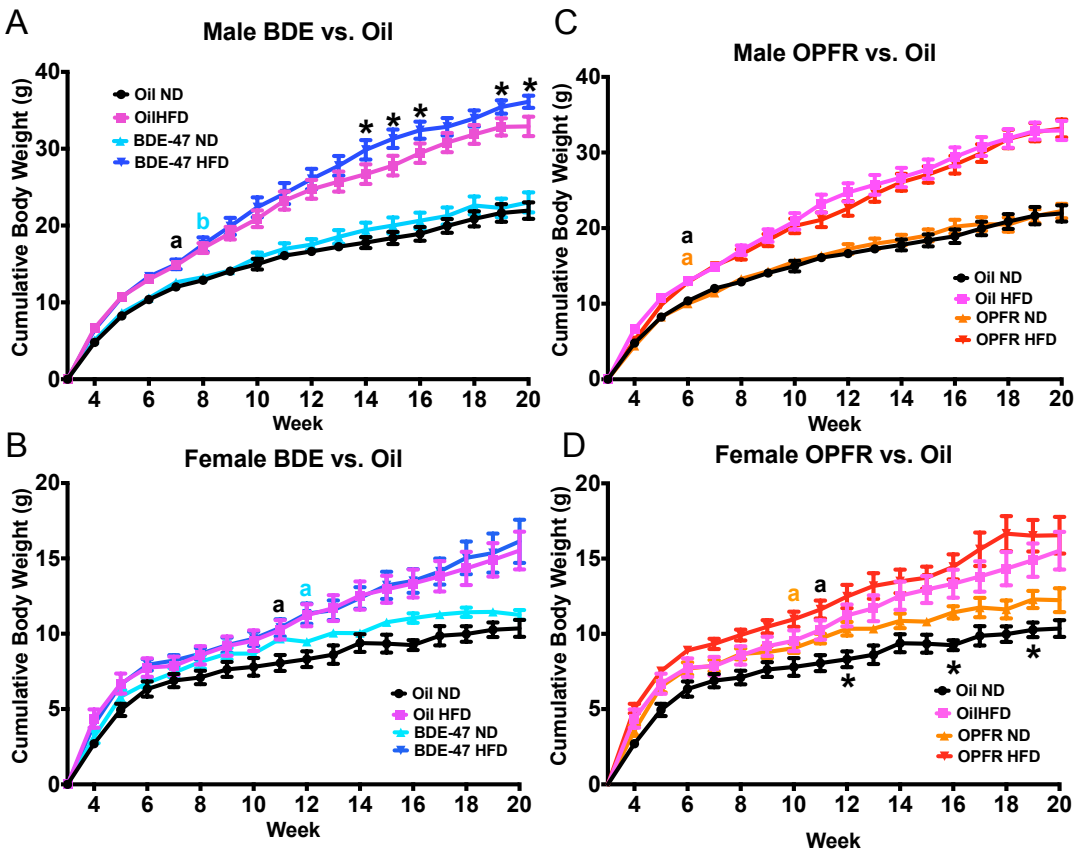

A
Male BDE vs. Oil
Cumulative Body Weight (g)
Week
Oil ND
OilHFD
BDE-47 ND
BDE-47 HFD
C
Male OPFR vs. Oil
Oil ND
Oil HFD
OPFR ND
OPFR HFD
B
Female BDE vs. Oil
D
Female OPFR vs. Oil

Figure 12.

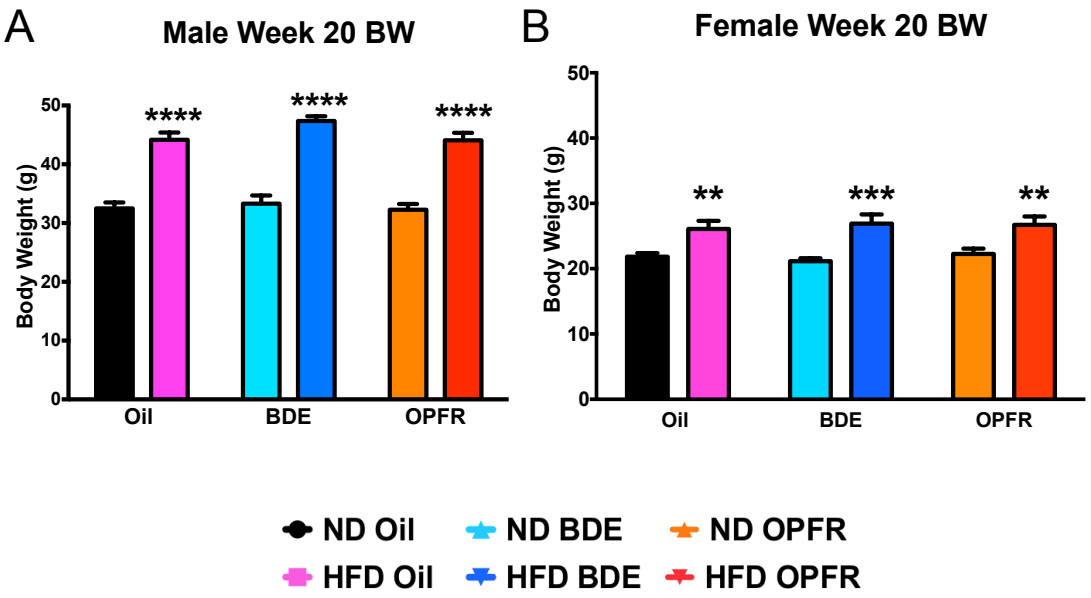

A
Male Week 20 BW
Body Weight (g)
50
40
30
20
10
0
****
****
****
Oil
BDE
OPFR
B
Female Week 20 BW
Body Weight (g)
50
40
30
20
10
0
**
***
**
Oil
BDE
OPFR
ND Oil
ND BDE
ND OPFR
HFD Oil
HFD BDE
HFD OPFR

Figure 13.

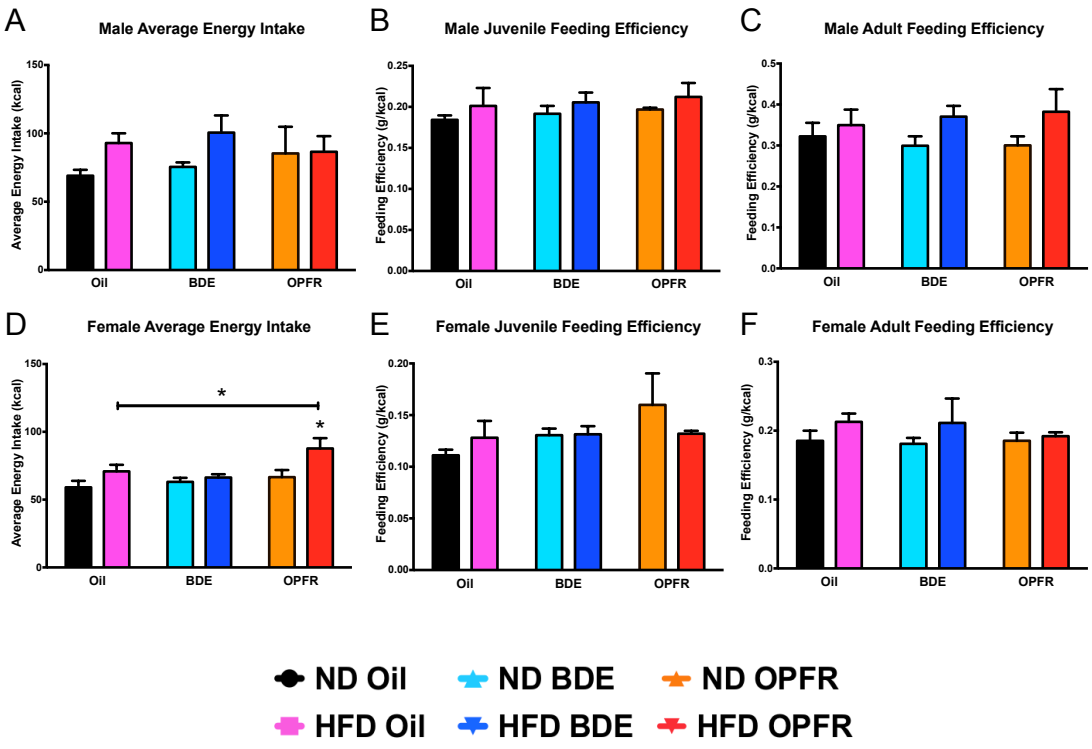

A
Male Average Energy Intake
Average Energy Intake (kcal)
150
100
50
0
Oil
BDE
OPFR
B
Male Juvenile Feeding Efficiency
Feeding Efficiency (g/kcal)
0.25
0.20
0.15
0.10
0.05
0.00
Oil
BDE
OPFR
C
Male Adult Feeding Efficiency
Feeding Efficiency (g/kcal)
0.5
0.4
0.3
0.2
0.1
0.0
Oil
BDE
OPFR
D
Female Average Energy Intake
Average Energy Intake (kcal)
150
100
50
0
*
*
Oil
BDE
OPFR
E
Female Juvenile Feeding Efficiency
Feeding Efficiency (g/kcal)
0.20
0.15
0.10
0.05
0.00
Oil
BDE
OPFR
F
Female Adult Feeding Efficiency
Feeding Efficiency (g/kcal)
0.3
0.2
0.1
0.0
Oil
BDE
OPFR
ND Oil
ND BDE
ND OPFR
HFD Oil
HFD BDE
HFD OPFR

Figure 14.

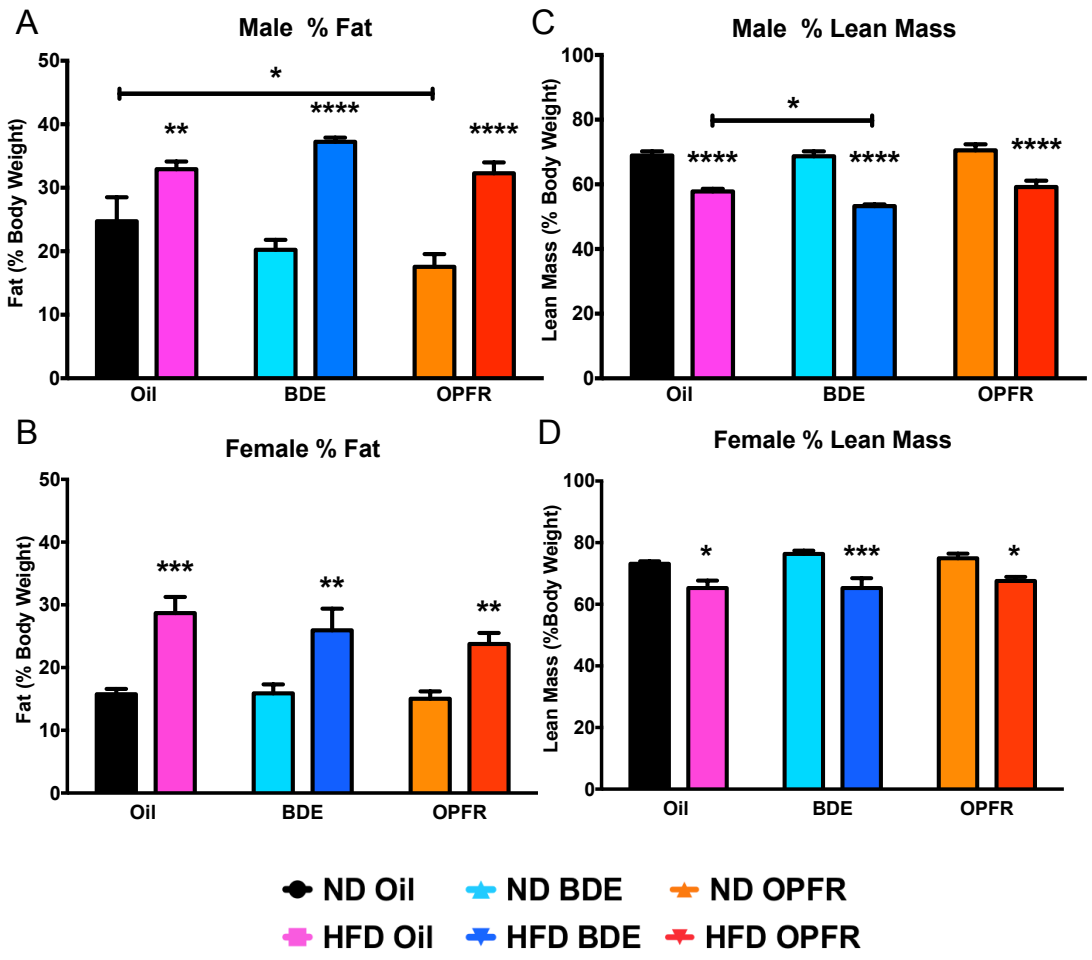

A
Male % Fat
Fat (% Body Weight)
50
40
30
20
10
0
*
**
****
****
Oil
BDE
OPFR
C
Male % Lean Mass
Lean Mass (% Body Weight)
100
80
60
40
20
0
*
****
****
****
Oil
BDE
OPFR
B
Female % Fat
Fat (% Body Weight)
50
40
30
20
10
0
***
**
**
Oil
BDE
OPFR
D
Female % Lean Mass
Lean Mass (%Body Weight)
100
80
60
40
20
0
*
***
*
Oil
BDE
OPFR
ND Oil
ND BDE
ND OPFR
HFD Oil
HFD BDE
HFD OPFR

Figure 15.

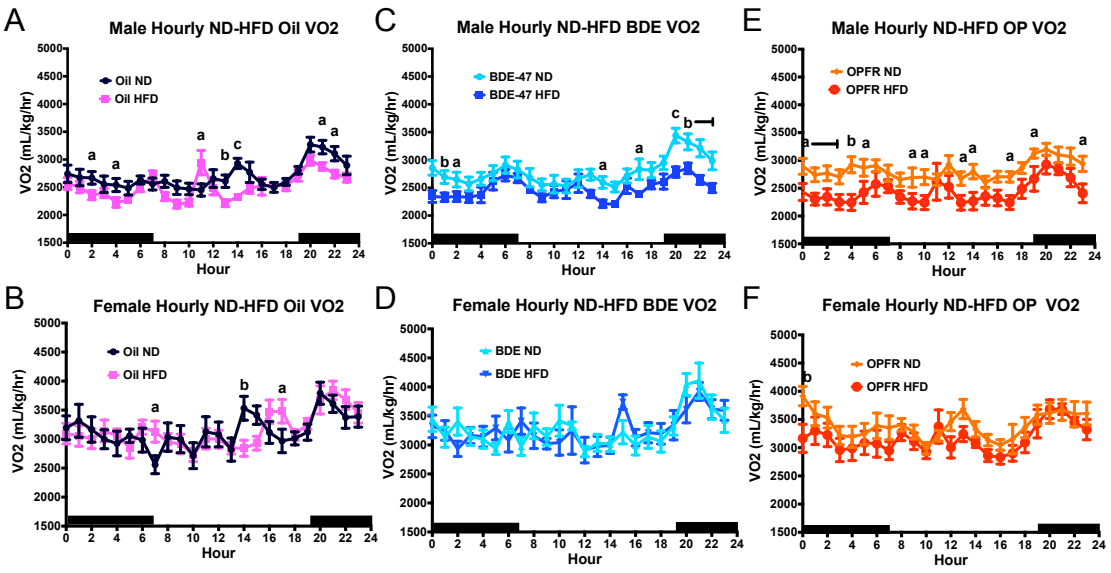

A
Male Hourly ND-HFD Oil VO2
Oil ND
Oil HFD
VO2 (mL/kg/hr)
Hour
C
Male Hourly ND-HFD BDE VO2
BDE-47 ND
BDE-47 HFD
VO2 (mL/kg/hr)
Hour
E
Male Hourly ND-HFD OP VO2
OPFR ND
OPFR HFD
VO2 (mL/kg/hr)
Hour
B
Female Hourly ND-HFD Oil VO2
Oil ND
Oil HFD
VO2 (mL/kg/hr)
Hour
D
Female Hourly ND-HFD BDE VO2
BDE ND
BDE HFD
VO2 (mL/kg/hr)
Hour
F
Female Hourly ND-HFD OP VO2
OPFR ND
OPFR HFD
VO2 (mL/kg/hr)
Hour

Figure 16.

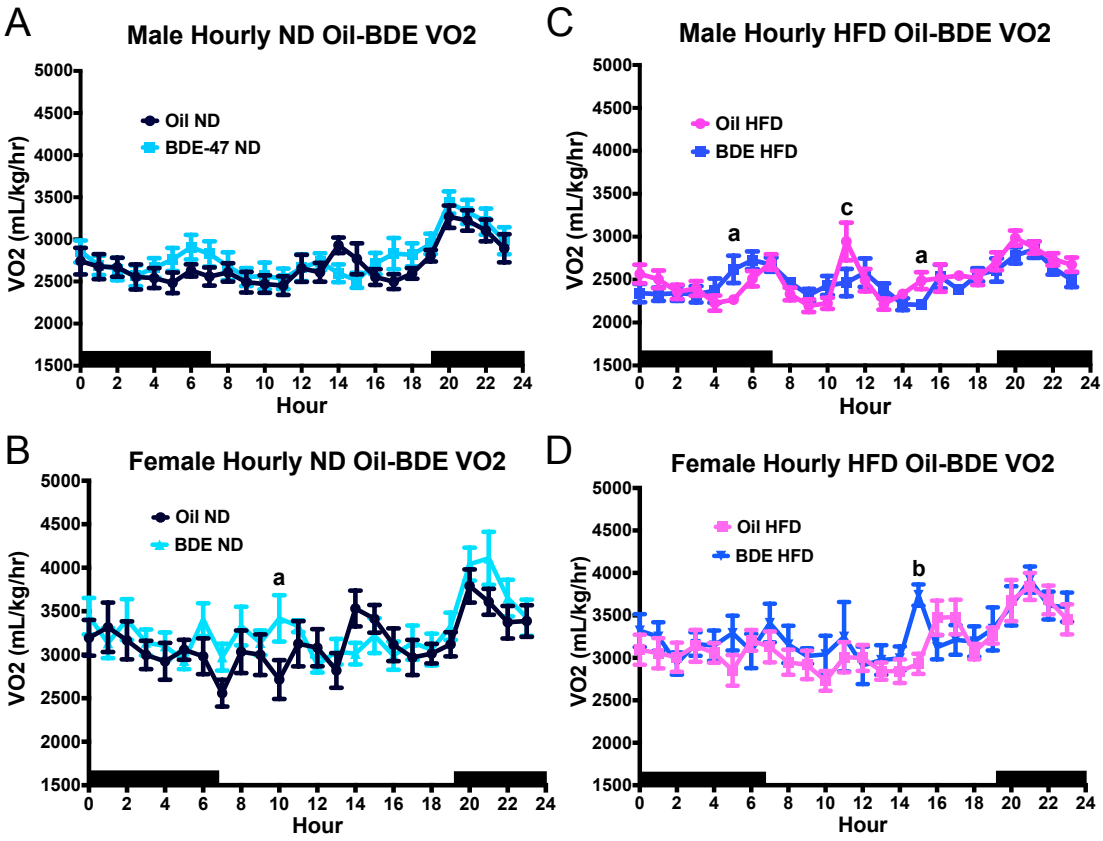

A
Male Hourly ND Oil-BDE VO2
Oil ND
BDE-47 ND
VO2 (mL/kg/hr)
Hour
C
Male Hourly HFD Oil-BDE VO2
Oil HFD
BDE HFD
a
c
a
VO2 (mL/kg/hr)
Hour
B
Female Hourly ND Oil-BDE VO2
Oil ND
BDE ND
a
VO2 (mL/kg/hr)
Hour
D
Female Hourly HFD Oil-BDE VO2
Oil HFD
BDE HFD
b
VO2 (mL/kg/hr)
Hour

Figure 17.

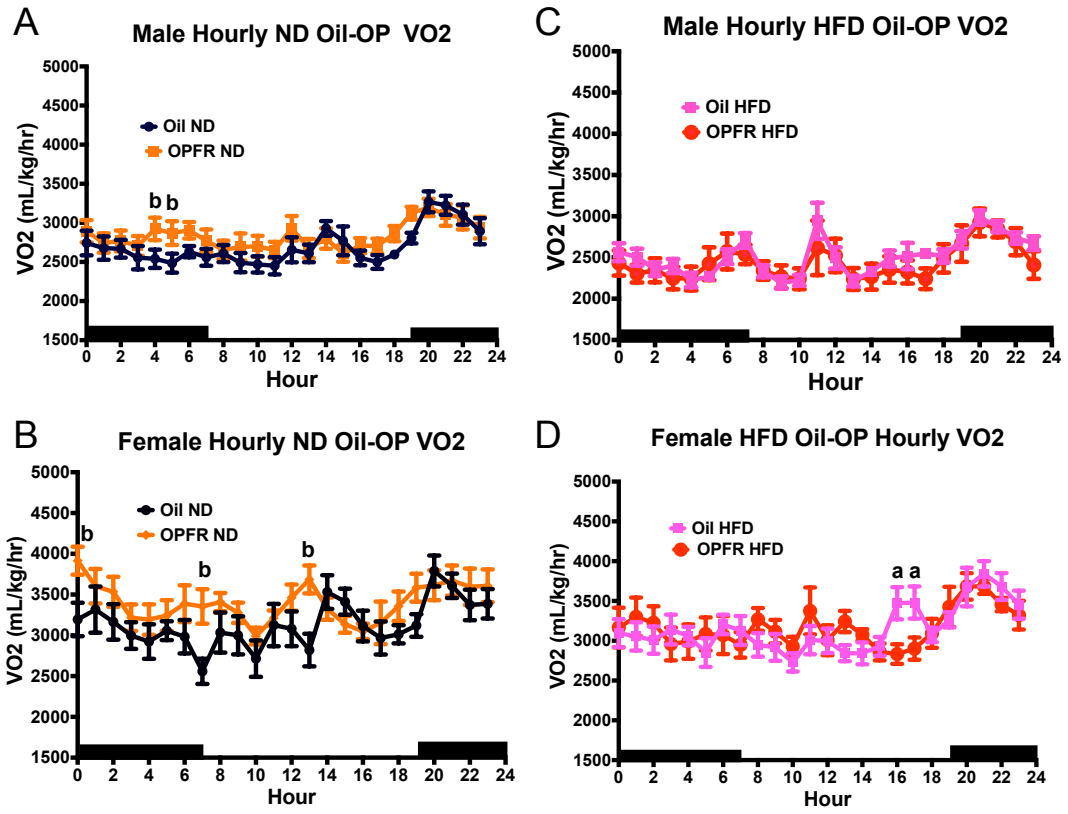

A
Male Hourly ND Oil-OP VO2
Oil ND
OPFR ND
b b
VO2 (mL/kg/hr)
Hour
C
Male Hourly HFD Oil-OP VO2
Oil HFD
OPFR HFD
VO2 (mL/kg/hr)
Hour
B
Female Hourly ND Oil-OP VO2
Oil ND
OPFR ND
b
b
b
VO2 (mL/kg/hr)
Hour
D
Female HFD Oil-OP Hourly VO2
Oil HFD
OPFR HFD
a a
VO2 (mL/kg/hr)
Hour

Figure 18.

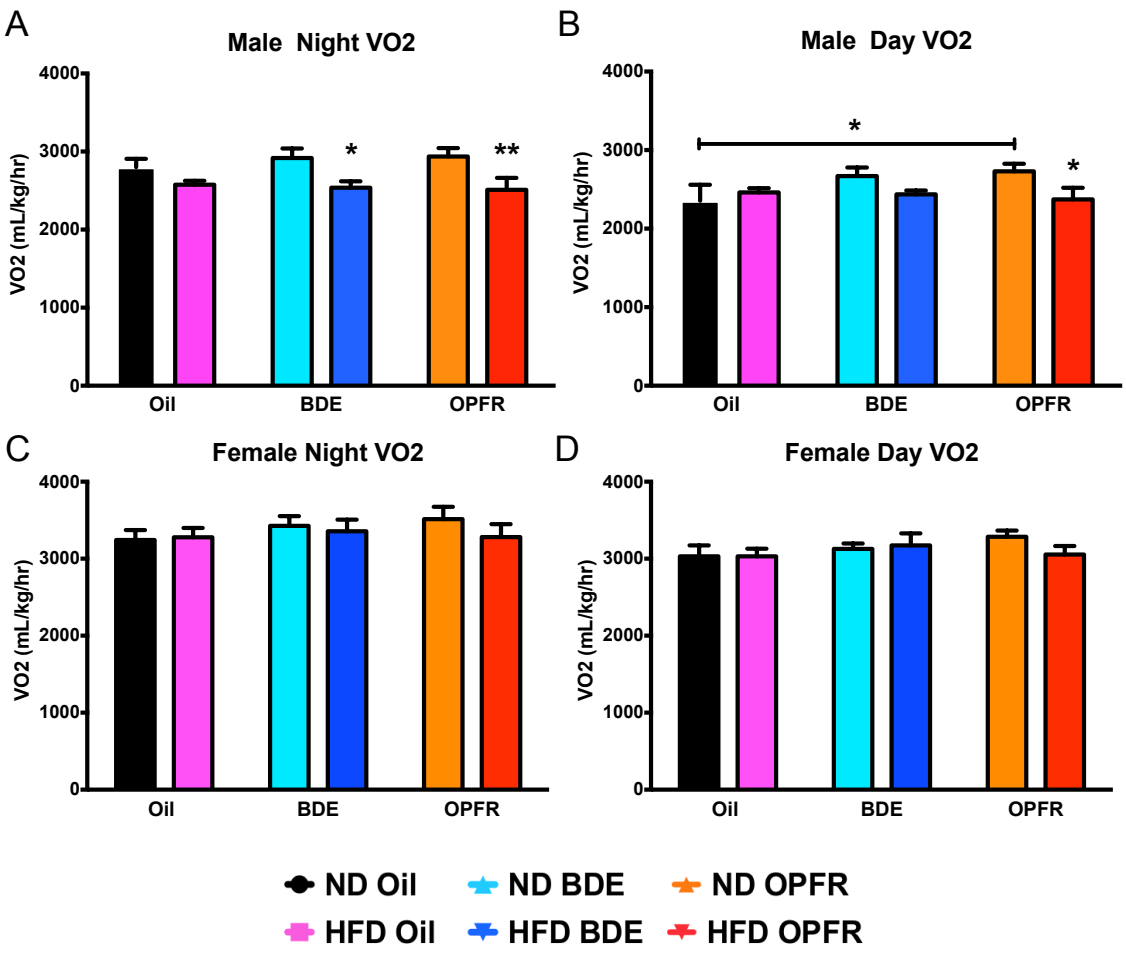

A
Male Night VO2
VO2 (mL/kg/hr)
4000
3000
2000
1000
0
Oil
BDE
OPFR
*
**
B
Male Day VO2
*
*
C
Female Night VO2
D
Female Day VO2
ND Oil
ND BDE
ND OPFR
HFD Oil
HFD BDE
HFD OPFR

Figure 19.

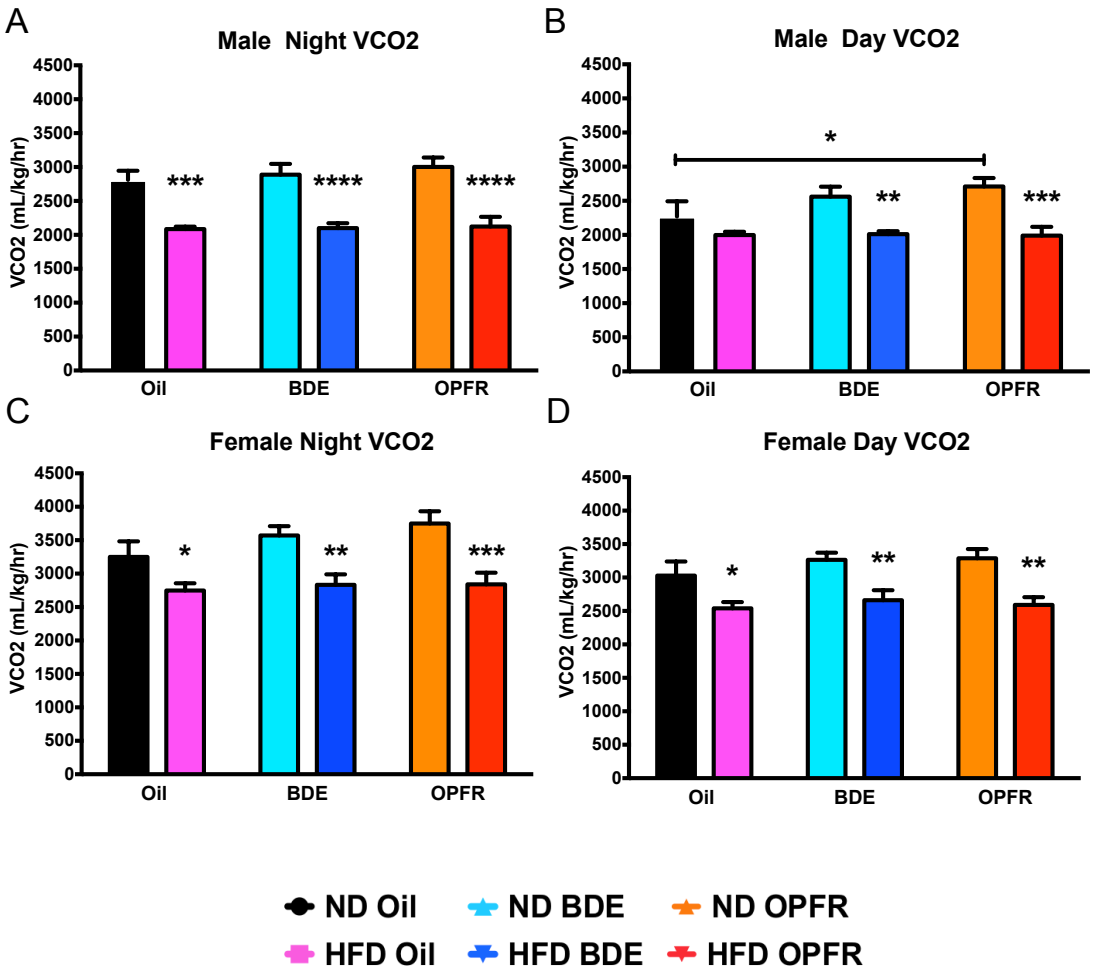

A
Male Night VCO2
VCO2 (mL/kg/hr)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
***
****
****
Oil
BDE
OPFR
B
Male Day VCO2
*
**
***
C
Female Night VCO2
*
**
***
D
Female Day VCO2
*
**
**
ND Oil
ND BDE
ND OPFR
HFD Oil
HFD BDE
HFD OPFR

Figure 20.

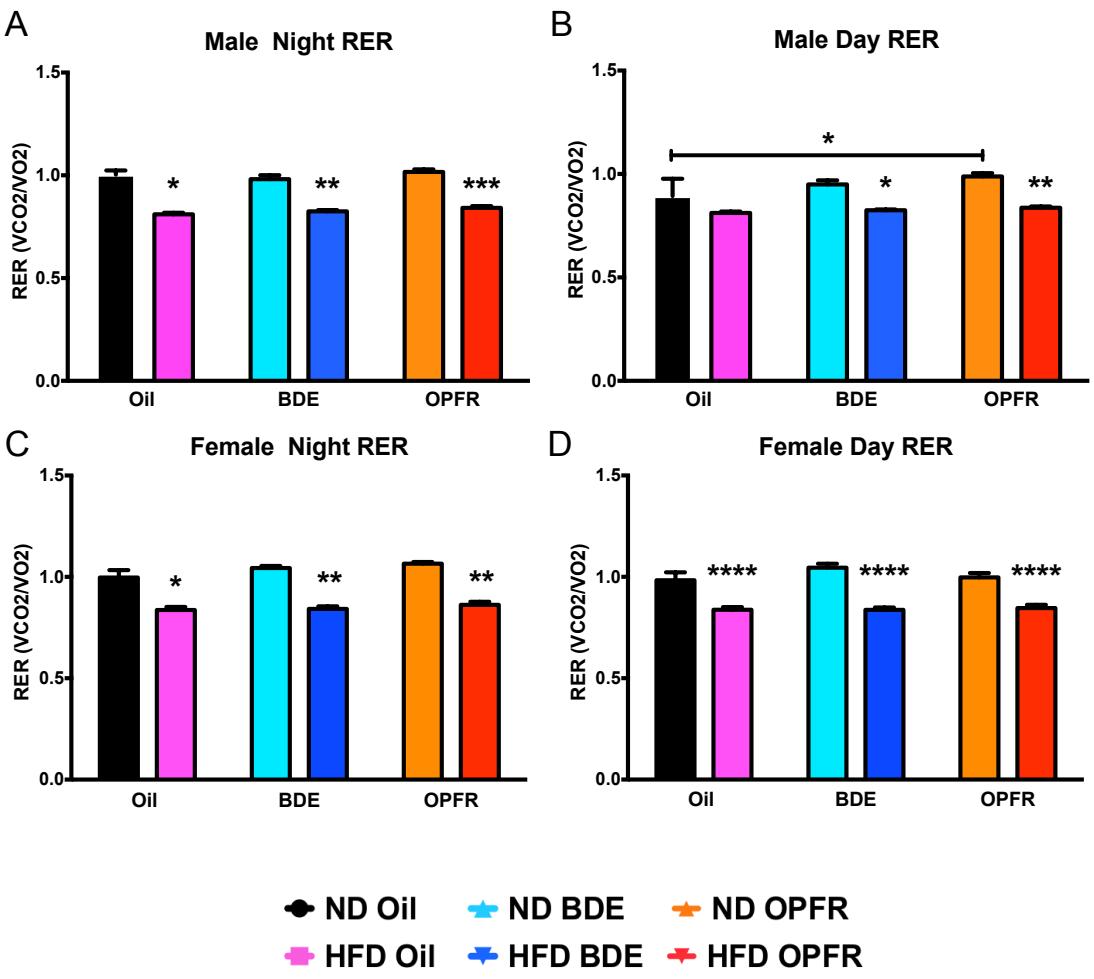

A
Male Night RER
RER (VCO2/VO2)
1.5
1.0
0.5
0.0
*
**
***
Oil
BDE
OPFR
B
Male Day RER
RER (VCO2/VO2)
*
*
**
Oil
BDE
OPFR
C
Female Night RER
RER (VCO2/VO2)
*
**
**
Oil
BDE
OPFR
D
Female Day RER
RER (VCO2/VO2)
****
****
****
Oil
BDE
OPFR
ND Oil
ND BDE
ND OPFR
HFD Oil
HFD BDE
HFD OPFR

Figure 21.

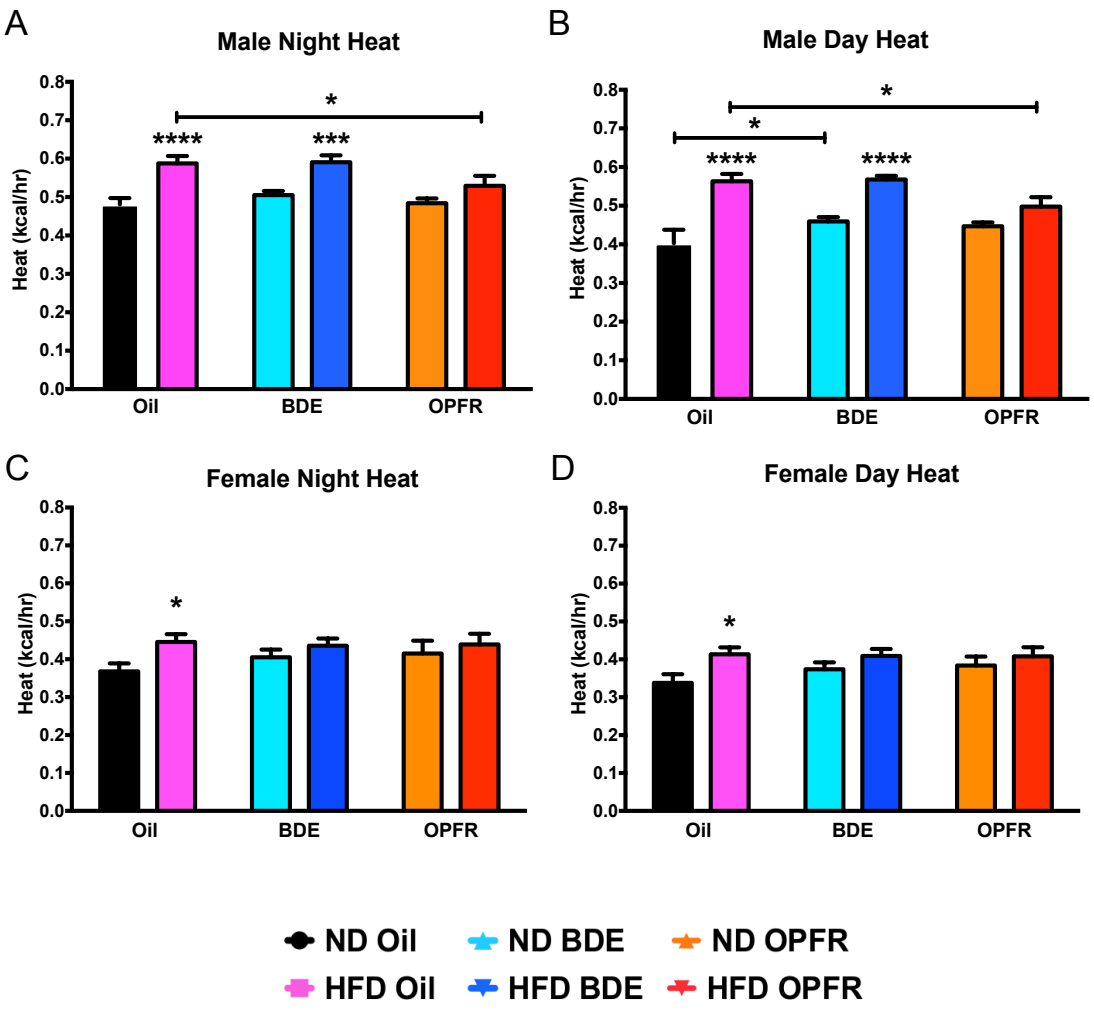

A
Male Night Heat
Heat (kcal/hr)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
****
***
*
Oil
BDE
OPFR
B
Male Day Heat
Heat (kcal/hr)
****
****
*
*
Oil
BDE
OPFR
C
Female Night Heat
Heat (kcal/hr)
*
Oil
BDE
OPFR
D
Female Day Heat
Heat (kcal/hr)
*
Oil
BDE
OPFR
ND Oil
ND BDE
ND OPFR
HFD Oil
HFD BDE
HFD OPFR

Figure 22.

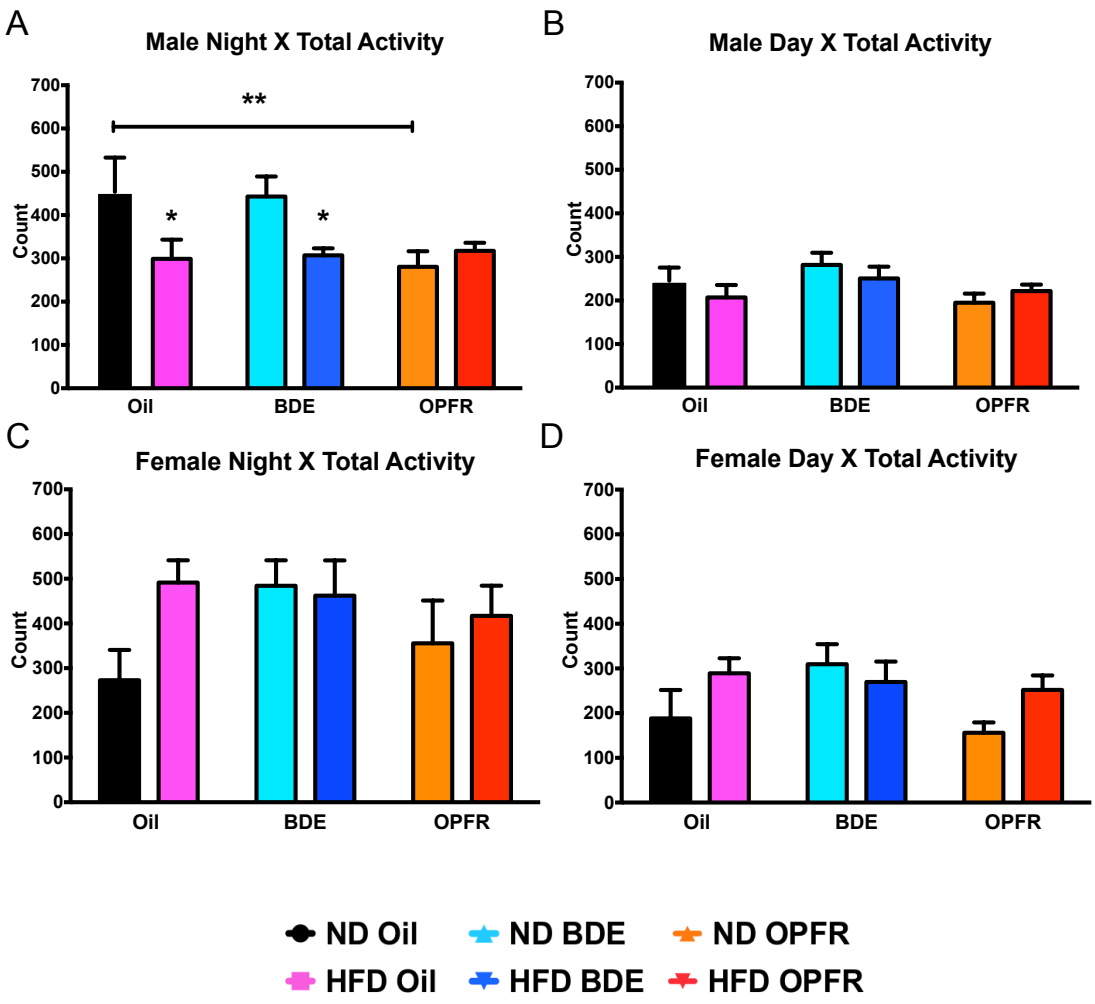

A
Male Night X Total Activity
B
Male Day X Total Activity
C
Female Night X Total Activity
D
Female Day X Total Activity
Count
0
100
200
300
400
500
600
700
Oil
BDE
OPFR
**
*
*
ND Oil
ND BDE
ND OPFR
HFD Oil
HFD BDE
HFD OPFR

Figure 23.

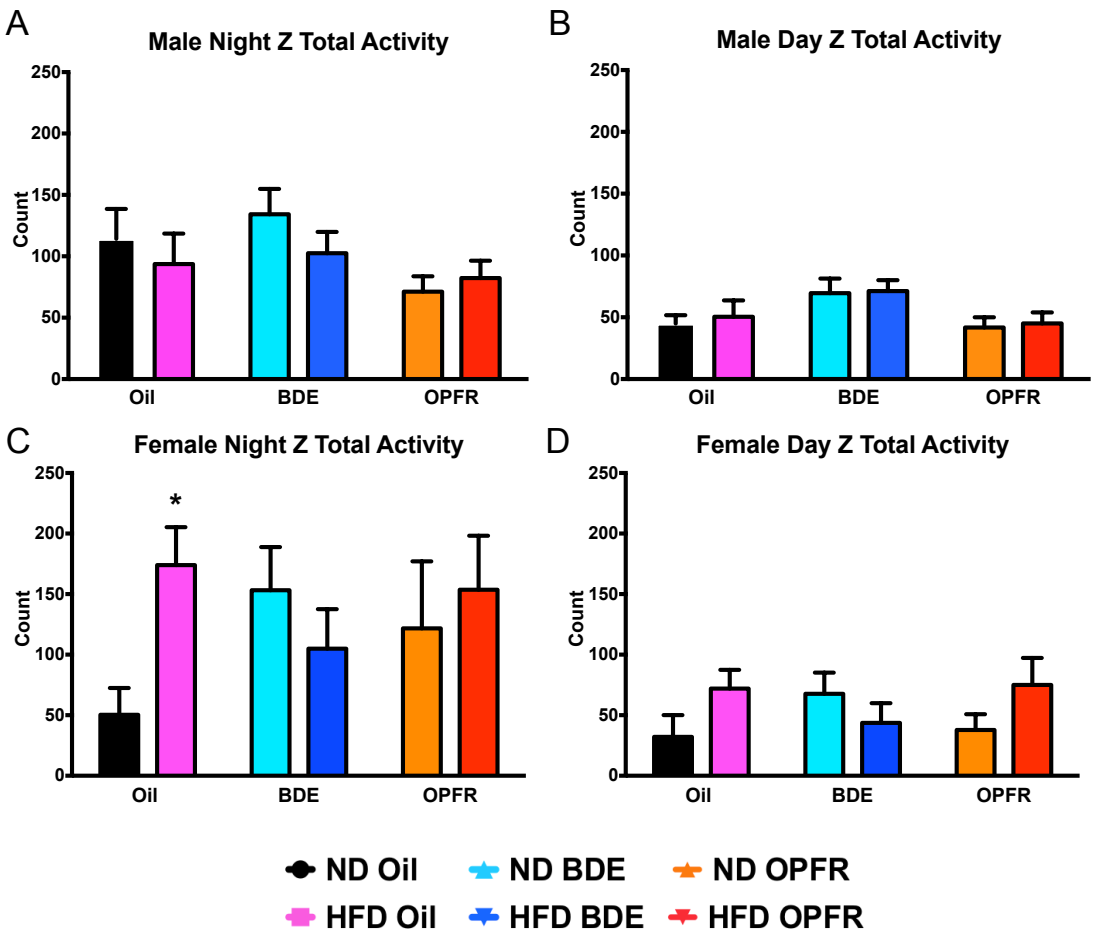

A
Male Night Z Total Activity
Count
250
200
150
100
50
0
Oil
BDE
OPFR
B
Male Day Z Total Activity
Count
250
200
150
100
50
0
Oil
BDE
OPFR
C
Female Night Z Total Activity
*
Count
250
200
150
100
50
0
Oil
BDE
OPFR
D
Female Day Z Total Activity
Count
250
200
150
100
50
0
Oil
BDE
OPFR
ND Oil
ND BDE
ND OPFR
HFD Oil
HFD BDE
HFD OPFR

Figure 24.

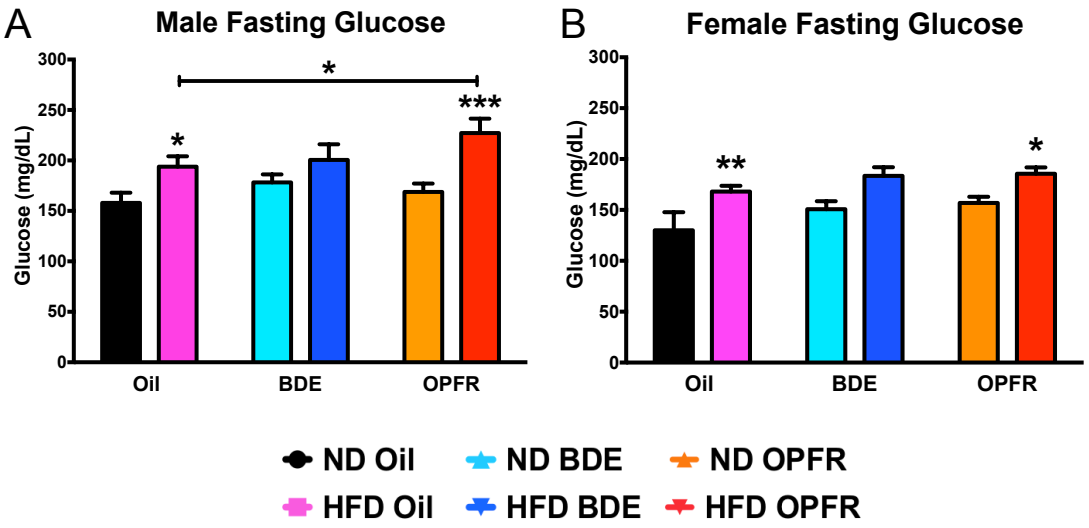

A
Male Fasting Glucose
Glucose (mg/dL)
300
250
200
150
100
50
0
*
*
***
Oil
BDE
OPFR
B
Female Fasting Glucose
Glucose (mg/dL)
300
250
200
150
100
50
0
**
*
Oil
BDE
OPFR
ND Oil
ND BDE
ND OPFR
HFD Oil
HFD BDE
HFD OPFR

Figure 25.

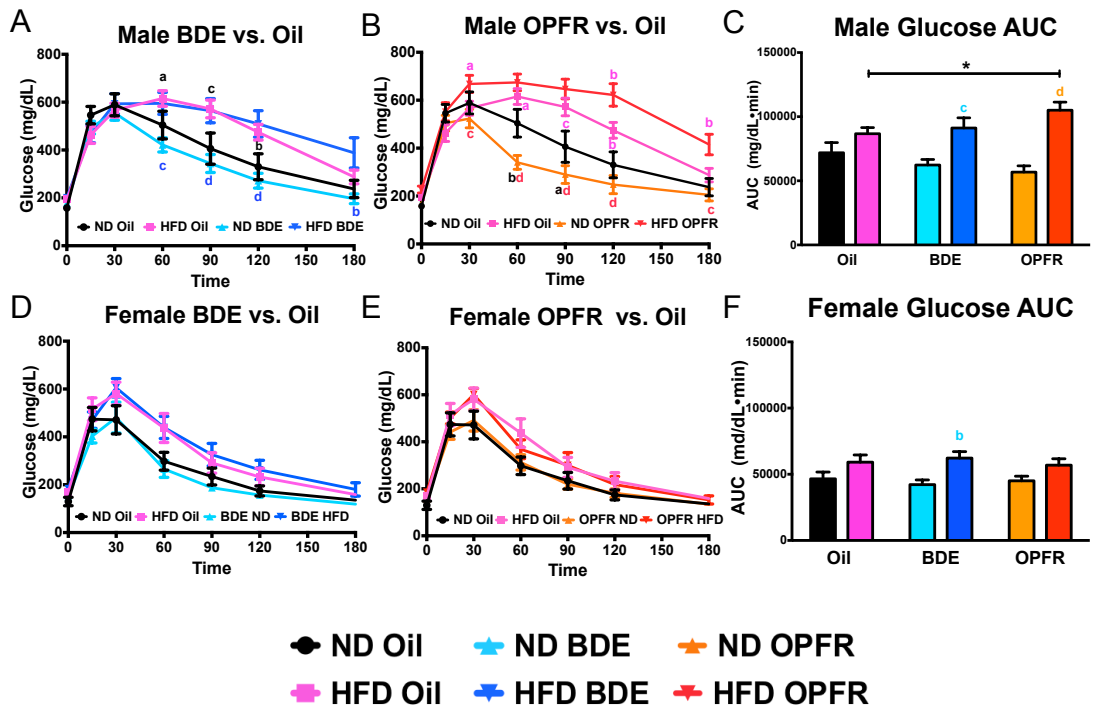

A
Male BDE vs. Oil
Glucose (mg/dL)
Time
ND Oil
HFD Oil
ND BDE
HFD BDE
B
Male OPFR vs. Oil
Glucose (mg/dL)
Time
ND Oil
HFD Oil
ND OPFR
HFD OPFR
C
Male Glucose AUC
AUC (mg/dL*min)
Oil
BDE
OPFR
D
Female BDE vs. Oil
Glucose (mg/dL)
Time
ND Oil
HFD Oil
BDE ND
BDE HFD
E
Female OPFR vs. Oil
Glucose (mg/dL)
Time
ND Oil
HFD Oil
OPFR ND
OPFR HFD
F
Female Glucose AUC
AUC (md/dL*min)
Oil
BDE
OPFR
ND Oil
ND BDE
ND OPFR
HFD Oil
HFD BDE
HFD OPFR

Figure 26.

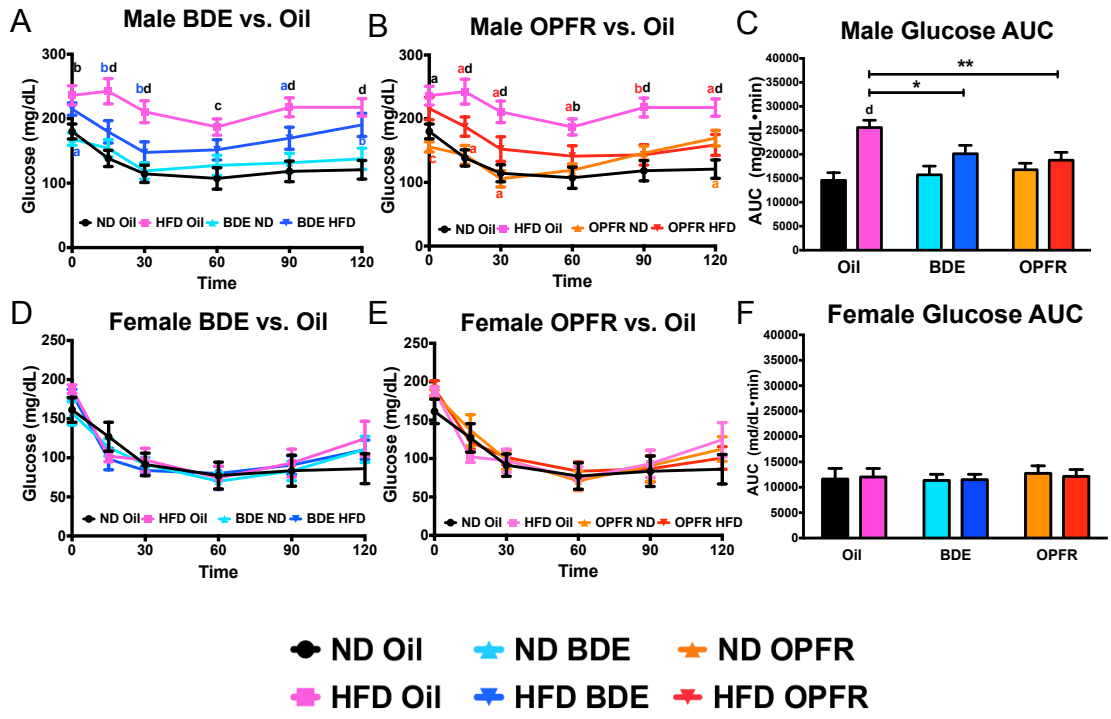
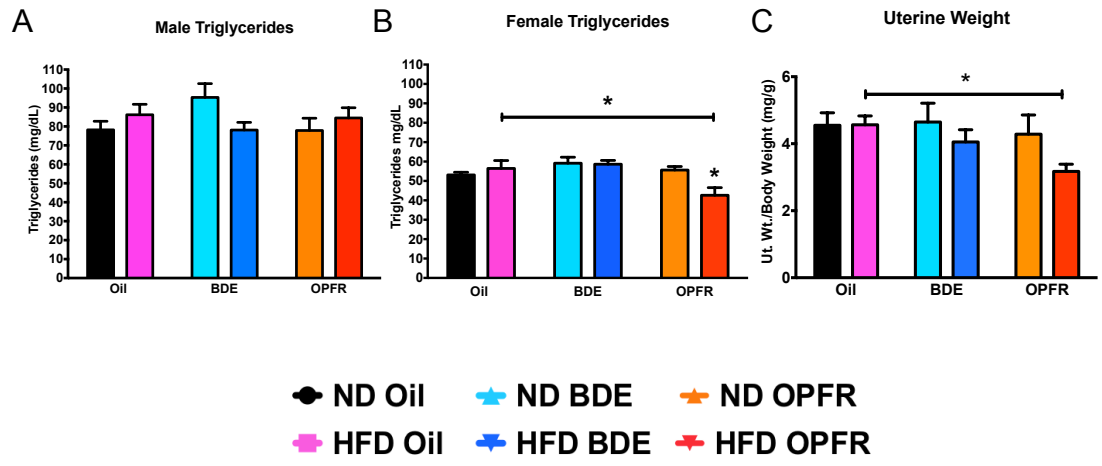

A
Male BDE vs. Oil
Glucose (mg/dL)
Time
ND Oil
HFD Oil
BDE ND
BDE HFD
B
Male OPFR vs. Oil
Glucose (mg/dL)
Time
ND Oil
HFD Oil
OPFR ND
OPFR HFD
C
Male Glucose AUC
AUC (mg/dL*min)
Oil
BDE
OPFR
D
Female BDE vs. Oil
Glucose (mg/dL)
Time
ND Oil
HFD Oil
BDE ND
BDE HFD
E
Female OPFR vs. Oil
Glucose (mg/dL)
Time
ND Oil
HFD Oil
OPFR ND
OPFR HFD
F
Female Glucose AUC
AUC (md/dL*min)
Oil
BDE
OPFR
ND Oil
ND BDE
ND OPFR
HFD Oil
HFD BDE
HFD OPFR

Figure 27.


A
Male Triglycerides
Triglycerides (mg/dL)
Oil
BDE
OPFR
B
Female Triglycerides
Triglycerides mg/dL
*
*
Oil
BDE
OPFR
C
Uterine Weight
Ut. Wt./Body Weight (mg/g)
*
Oil
BDE
OPFR
ND Oil
ND BDE
ND OPFR
HFD Oil
HFD BDE
HFD OPFR

CHAPTER 4:

SUMMARY

SUMMARY

Flame retardants (FR), including polybrominated diphenyl ethers (PBDEs) and organophosphate FR (OPFR), that we examined in the present studies (2,2',4,4'-tetrabromodiphenyl ether (BDE-47), tris(1,3-dichloro-2-propyl)phosphate (TDCPP), triphenyl phosphate (TPP), and tricresyl phosphate (TCP)) are detected at high concentrations in the environment, but little is known about their effects on energy homeostasis in *in vivo* mammalian models [15,16,22-24]. In the present studies, we found FR exposure during adulthood altered hypothalamic homeostatic gene expression and developmental FR exposure modified energy expenditure, glucose homeostasis, and fat metabolism. These FR-induced effects in central and peripheral parameters of energy balance were sex-dependent and more striking in males. While we have observed outcomes of flame retardant exposure on energy balance, the next step would be to examine the central and peripheral FR-induced mechanisms of action that elicit these effects.

Unexpectedly, male mice were more susceptible to FR-induced alteration in energy homeostasis, but it is unclear how FR is primarily exerting its effects. FR interact with many receptors involved in metabolism centrally and peripherally *in vitro* such as androgen receptor (AR), estrogen receptors (ERs), peroxisome proliferator-activated receptors (PPARα/γ), and thyroid receptor (TR), which increases the difficulty in isolating receptor-mediated mechanisms [9-11,13,271,304,311,315-317,319,320]. Additionally, these receptors also participate in crosstalk with each other, which further complicates this investigation [26-35,37,443-445]. Therefore, we could utilize global and tissue-specific knockout (KO) models in males and females to determine potential receptor-mediated mechanisms in each sex and in different tissues.

Future studies that investigate FR mechanism in central and peripheral tissue would be beneficial to clarify the cause of the effects that we observed in the current

studies. Global gene network analysis of central and peripheral gene networks by using RNA sequencing or quantitative real-time PCR on genes in tissues such as the liver, fat, and hypothalamus can be examined to elucidate FR-induced effects throughout the body. In the present studies (Chapter 1), we have examined that FR exposure alters gene expression in the ARC, which has an integrative role in the hypothalamic melanocortin neurocircuitry to control energy homeostasis [54]. However, future studies could involve examining if FR exposure also alters gene expression in other hypothalamic nuclei that also have roles in energy balance, including the paraventricular nucleus, dorsomedial hypothalamus, lateral hypothalamus, and ventromedial hypothalamus by using real-time quantitative PCR (qPCR). To determine the components of the melanocortin neurocircuitry that are activated from FR exposure, immunohistochemistry can be used to double-label for proopiomelanocortin (POMC), neuropeptide Y (NPY), glutamate transporter 1, or glutamic acid decarboxylase 67 in the hypothalamus to visualize the density of innervated neuronal projections.

Furthermore, neuronal cell type should be considered when studying the expression of genes and proteins that are ubiquitously expressed throughout the hypothalamus, including the ARC [72,359]. After FR treatment, green fluorescent tagging for POMC, NPY, or kisspeptin can be used to visualize individual neurons in the heterogeneous ARC [72,359]. Implementing qPCR on these single cells can determine if the FR affects the gene expression we observed in this study are similarly influenced in individual neurons [72,359]. By utilizing electrophysiology on glucose-sensing neurons, effects of FR exposure on insulin, leptin, and ghrelin sensitivity and cation channel activity can be examined. Additionally hormone assays could be used to test if FR exposure elevates the levels of these peptide hormones in the blood. Depending on if these neurons are sensitive to ghrelin, insulin, or leptin, and if the concentrations of these hormone peptides are also increased in circulation, energy balance parameters

such as glucose homeostatic parameters such as food intake, glucose uptake, energy expenditure, and hepatic glucose production in peripheral tissues may also be altered [387,388,390,392].

Further investigation can characterize additional toxicological ramifications of FR exposure. A more accurate dose-response curve for these compounds should be established due to recent studies that revealed harmful effects at or below the no-observed-adverse-effect-level (NOAEL) designated by the U.S. Environmental Protection Agency [15,16,281,305,314,319,403]. Since we experienced difficulty with breeding successful pregnancies and litter in dams exposed to OPFR in the present study, maternal toxicity and care studies will enhance our understanding of effects of OPFR exposure during pregnancy. In the past, studies demonstrate that EDCs such as bisphenol A decreases maternal care, but these behaviors have not been tested in subjects for these compounds [206,446]. In the present study, perinatal FR exposure clearly altered energy balance, but studying maternal-fetal and lactational transfer of flame retardants and their metabolites by using liquid chromatography/mass spectrometry would elucidate the concentrations that are affecting offspring.

Although the current studies were exploratory, they elucidate central and peripheral consequences of FR exposure on energy homeostasis. Interestingly, these compounds were examined at relevant levels in the environment and we observed diverse effects, especially in male mice [18,22,24]. Therefore, these studies potentiate further investigation to determine if the FR exposure currently in the environment could impact the general public and by what mechanism.

REFERENCES

1 Diamanti-Kandarakis, E. *et al.* Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocrine reviews* **30**, 293-342, doi:10.1210/er.2009-0002 (2009).

2 Newbold, R. R., Jefferson, W. N., Padilla-Banks, E. & Haseman, J. Developmental exposure to diethylstilbestrol (DES) alters uterine response to estrogens in prepubescent mice: low versus high dose effects. *Reproductive toxicology (Elmsford, N.Y.)* **18**, 399-406, doi:10.1016/j.reprotox.2004.01.007 (2004).

3 Ceccarelli, I., Della Seta, D., Fiorenzani, P., Farabollini, F. & Aloisi, A. M. Estrogenic chemicals at puberty change ERalpha in the hypothalamus of male and female rats. *Neurotoxicology and teratology* **29**, 108-115, doi:10.1016/j.ntt.2006.10.011 (2007).

4 Golden, R. J. *et al.* Environmental endocrine modulators and human health: an assessment of the biological evidence. *Critical reviews in toxicology* **28**, 109-227, doi:10.1080/10408449891344191 (1998).

5 Nadal, A., Ropero, A. B., Fuentes, E., Soria, B. & Ripoll, C. Estrogen and xenoestrogen actions on endocrine pancreas: from ion channel modulation to activation of nuclear function. *Steroids* **69**, 531-536, doi:10.1016/j.steroids.2004.05.010 (2004).

6 Ropero, A. B., Alonso-Magdalena, P., Ripoll, C., Fuentes, E. & Nadal, A. Rapid endocrine disruption: environmental estrogen actions triggered outside the nucleus. *The Journal of steroid biochemistry and molecular biology* **102**, 163-169, doi:10.1016/j.jsbmb.2006.09.019 (2006).

7 Alonso-Magdalena, P., Garcia-Arevalo, M., Quesada, I. & Nadal, A. Bisphenol-A treatment during pregnancy in mice: a new window of susceptibility for the development of diabetes in mothers later in life. *Endocrinology* **156**, 1659-1670, doi:10.1210/en.2014-1952 (2015).

8 Li, X., Gao, Y., Guo, L. H. & Jiang, G. Structure-dependent activities of hydroxylated polybrominated diphenyl ethers on human estrogen receptor. *Toxicology* **309**, 15-22, doi:10.1016/j.tox.2013.04.001 (2013).

9 Meerts, I. A. *et al.* In vitro estrogenicity of polybrominated diphenyl ethers, hydroxylated PDBEs, and polybrominated bisphenol A compounds. *Environmental health perspectives* **109**, 399-407 (2001).

10 Hu, W. *et al.* Endocrine effects of methoxylated brominated diphenyl ethers in three in vitro models. *Marine pollution bulletin* **62**, 2356-2361, doi:10.1016/j.marpolbul.2011.08.037 (2011).

11 Gosavi, R. A., Knudsen, G. A., Birnbaum, L. S. & Pedersen, L. C. Mimicking of estradiol binding by flame retardants and their metabolites: a crystallographic analysis. *Environmental health perspectives* **121**, 1194-1199, doi:10.1289/ehp.1306902 (2013).

12 Liu, X., Ji, K. & Choi, K. Endocrine disruption potentials of organophosphate flame retardants and related mechanisms in H295R and MVLN cell lines and in zebrafish. *Aquatic toxicology (Amsterdam, Netherlands)* **114-115**, 173-181, doi:10.1016/j.aquatox.2012.02.019 (2012).

13 Kojima, H. *et al.* In vitro endocrine disruption potential of organophosphate flame retardants via human nuclear receptors. *Toxicology* **314**, 76-83, doi:10.1016/j.tox.2013.09.004 (2013).

14 Bittner, G. D., Denison, M. S., Yang, C. Z., Stoner, M. A. & He, G. Chemicals having estrogenic activity can be released from some bisphenol A-free, hard and

clear, thermoplastic resins. *Environmental health : a global access science source* **14**, 103, doi:10.1186/1476-069x-13-103 (2015).

15 EPA, U. S. IRIS Health Assessment of 2,2',4,4'-Tetrabromodiphenyl Ether (BDE-47) CASRN 5436-43-12,2',4,4',5-Pentabromodiphenyl Ether (BDE-99) CASRN 60348-60-92,2',4,4',5,5'-Hexabromodiphenyl Ether (BDE-153) CASRN 68631-49-22,2',3,3',4,4',5,5',6,6'-Decabromodiphenyl Ether (BDE-209) CASRN 1163-19-5. (2008).

16 EPA, U. S. Furniture flame retardancy partnership: environmental profiles of chemical flame retardant alternatives. doi:EPA 742-R-05-002A (2005).

17 UNEP. in *Stockholm convention on persistent organic pollutants.* (United Nations Environmental Programme).

18 Sjodin, A. *et al.* Concentration of polybrominated diphenyl ethers (PBDEs) in household dust from various countries. *Chemosphere* **73**, S131-136, doi:10.1016/j.chemosphere.2007.08.075 (2008).

19 Abdallah, M. A. *et al.* High-resolution mass spectrometry provides novel insights into products of human metabolism of organophosphate and brominated flame retardants. *Analytical and bioanalytical chemistry* **407**, 1871-1883, doi:10.1007/s00216-015-8466-z (2015).

20 van der Veen, I. & de Boer, J. Phosphorus flame retardants: properties, production, environmental occurrence, toxicity and analysis. *Chemosphere* **88**, 1119-1153, doi:10.1016/j.chemosphere.2012.03.067 (2012).

21 Hughes, M. F., Edwards, B. C., Mitchell, C. T. & Bhooshan, B. In vitro dermal absorption of flame retardant chemicals. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* **39**, 1263-1270 (2001).

22 Dishaw, L. V., L, J. M., Roberts, S. C. & Stapleton, H. M. Exposures, mechanisms, and impacts of endocrine-active flame retardants. *Current opinion in pharmacology* **19**, 125-133, doi:10.1016/j.coph.2014.09.018 (2014).

23 He, C. T. *et al.* Occurrence of organophosphorus flame retardants in indoor dust in multiple microenvironments of southern China and implications for human exposure. *Chemosphere* **133**, 47-52, doi:10.1016/j.chemosphere.2015.03.043 (2015).

24 Stapleton, H. M. *et al.* Novel and high volume use flame retardants in US couches reflective of the 2005 PentaBDE phase out. *Environmental science & technology* **46**, 13432-13439, doi:10.1021/es303471d (2012).

25 Birnbaum, L. S. & Staskal, D. F. Brominated flame retardants: cause for concern? *Environmental health perspectives* **112**, 9-17 (2004).

26 Naderi, A. & Hughes-Davies, L. A functionally significant cross-talk between androgen receptor and ErbB2 pathways in estrogen receptor negative breast cancer. *Neoplasia (New York, N.Y.)* **10**, 542-548 (2008).

27 Chu, R. *et al.* The cross-talk between estrogen receptor and peroxisome proliferator-activated receptor gamma in thyroid cancer. *Cancer* **120**, 142-153, doi:10.1002/cncr.28383 (2014).

28 Surazynski, A., Jarzabek, K., Miltyk, W., Wolczynski, S. & Palka, J. Estrogen-dependent regulation of PPAR-gamma signaling on collagen biosynthesis in adenocarcinoma endometrial cells. *Neoplasma* **56**, 448-454 (2009).

29 Bonofiglio, D. *et al.* Estrogen receptor alpha binds to peroxisome proliferator-activated receptor response element and negatively interferes with peroxisome proliferator-activated receptor gamma signaling in breast cancer cells. *Clinical cancer research : an official journal of the American Association for Cancer Research* **11**, 6139-6147, doi:10.1158/1078-0432.ccr-04-2453 (2005).

30 Yoon, M. PPARalpha in Obesity: Sex Difference and Estrogen Involvement. *PPAR research* **2010**, doi:10.1155/2010/584296 (2010).
31 Crisafulli, C. *et al.* PPAR-alpha contributes to the anti-inflammatory activity of 17beta-estradiol. *The Journal of pharmacology and experimental therapeutics* **331**, 796-807, doi:10.1124/jpet.109.156646 (2009).
32 Vasudevan, N., Koibuchi, N., Chin, W. W. & Pfaff, D. W. Differential crosstalk between estrogen receptor (ER)alpha and ERbeta and the thyroid hormone receptor isoforms results in flexible regulation of the consensus ERE. *Brain research. Molecular brain research* **95**, 9-17 (2001).
33 Vasudevan, N., Ogawa, S. & Pfaff, D. Estrogen and thyroid hormone receptor interactions: physiological flexibility by molecular specificity. *Physiological reviews* **82**, 923-944, doi:10.1152/physrev.00014.2002 (2002).
34 Foryst-Ludwig, A. *et al.* Metabolic actions of estrogen receptor beta (ERbeta) are mediated by a negative cross-talk with PPARgamma. *PLoS genetics* **4**, e1000108, doi:10.1371/journal.pgen.1000108 (2008).
35 Wang, X. & Kilgore, M. W. Signal cross-talk between estrogen receptor alpha and beta and the peroxisome proliferator-activated receptor gamma1 in MDA-MB-231 and MCF-7 breast cancer cells. *Molecular and cellular endocrinology* **194**, 123-133 (2002).
36 Grimaldi, M. *et al.* Reporter cell lines to evaluate the selectivity of chemicals for human and zebrafish estrogen and peroxysome proliferator activated gamma receptors. *Frontiers in neuroscience* **9**, 212, doi:10.3389/fnins.2015.00212 (2015).
37 Duarte-Guterman, P., Navarro-Martin, L. & Trudeau, V. L. Mechanisms of crosstalk between endocrine systems: regulation of sex steroid hormone synthesis and action by thyroid hormones. *General and comparative endocrinology* **203**, 69-85, doi:10.1016/j.ygcen.2014.03.015 (2014).
38 Filardo, E. J. & Thomas, P. GPR30: a seven-transmembrane-spanning estrogen receptor that triggers EGF release. *Trends in endocrinology and metabolism: TEM* **16**, 362-367, doi:10.1016/j.tem.2005.08.005 (2005).
39 Smith, A. W., Ronnekleiv, O. K. & Kelly, M. J. Gq-mER signaling has opposite effects on hypothalamic orexigenic and anorexigenic neurons. *Steroids* **81**, 31-35, doi:10.1016/j.steroids.2013.11.007 (2014).
40 McDevitt, M. A. *et al.* New insights into the classical and non-classical actions of estrogen: evidence from estrogen receptor knock-out and knock-in mice. *Molecular and cellular endocrinology* **290**, 24-30, doi:10.1016/j.mce.2008.04.003 (2008).
41 Bjornstrom, L. & Sjoberg, M. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. *Molecular endocrinology (Baltimore, Md.)* **19**, 833-842, doi:10.1210/me.2004-0486 (2005).
42 Micevych, P. E. & Kelly, M. J. Membrane estrogen receptor regulation of hypothalamic function. *Neuroendocrinology* **96**, 103-110, doi:10.1159/000338400 000338400 (2012).
43 Levin, E. R. Integration of the extranuclear and nuclear actions of estrogen. *Molecular endocrinology (Baltimore, Md.)* **19**, 1951-1959, doi:10.1210/me.2004-0390 (2005).
44 Kelly, M. J., Qiu, J., Wagner, E. J. & Ronnekleiv, O. K. Rapid effects of estrogen on G protein-coupled receptor activation of potassium channels in the central nervous system (CNS). *The Journal of steroid biochemistry and molecular biology* **83**, 187-193 (2002).

45 Ronnekleiv, O. K., Malyala, A. & Kelly, M. J. Membrane-initiated signaling of estrogen in the brain. *Seminars in reproductive medicine* **25**, 165-177, doi:10.1055/s-2007-973429 (2007).
46 Roepke, T. A. *et al.* Genes associated with membrane-initiated signaling of estrogen and energy homeostasis. *Endocrinology* **149**, 6113-6124, doi:10.1210/en.2008-0769 (2008).
47 Malyala, A., Kelly, M. J. & Ronnekleiv, O. K. Estrogen modulation of hypothalamic neurons: activation of multiple signaling pathways and gene expression changes. *Steroids* **70**, 397-406, doi:10.1016/j.steroids.2005.03.004 (2005).
48 Vasudevan, N. & Pfaff, D. W. Membrane-initiated actions of estrogens in neuroendocrinology: emerging principles. *Endocrine reviews* **28**, 1-19, doi:10.1210/er.2005-0021 (2007).
49 Brandenberger, A. W., Tee, M. K., Lee, J. Y., Chao, V. & Jaffe, R. B. Tissue distribution of estrogen receptors alpha (ER-alpha) and beta (ER-beta) mRNA in the midgestational human fetus. *The Journal of clinical endocrinology and metabolism* **82**, 3509-3512, doi:10.1210/jcem.82.10.4400 (1997).
50 Bottner, M., Thelen, P. & Jarry, H. Estrogen receptor beta: tissue distribution and the still largely enigmatic physiological function. *The Journal of steroid biochemistry and molecular biology* **139**, 245-251, doi:10.1016/j.jsbmb.2013.03.003 (2014).
51 Schneider, J. E., Wise, J. D., Benton, N. A., Brozek, J. M. & Keen-Rhinehart, E. When do we eat? Ingestive behavior, survival, and reproductive success. *Hormones and behavior* **64**, 702-728, doi:10.1016/j.yhbeh.2013.07.005 (2013).
52 Della Torre, S., Benedusi, V., Fontana, R. & Maggi, A. Energy metabolism and fertility: a balance preserved for female health. *Nature reviews. Endocrinology* **10**, 13-23, doi:10.1038/nrendo.2013.203 (2014).
53 Barros, R. P. & Gustafsson, J. A. Estrogen receptors and the metabolic network. *Cell metabolism* **14**, 289-299, doi:10.1016/j.cmet.2011.08.005 (2011).
54 Schwartz, M. W., Woods, S. C., Porte, D., Jr., Seeley, R. J. & Baskin, D. G. Central nervous system control of food intake. *Nature* **404**, 661-671, doi:10.1038/35007534 (2000).
55 Skorupskaite, K., George, J. T. & Anderson, R. A. The kisspeptin-GnRH pathway in human reproductive health and disease. *Human reproduction update* **20**, 485-500, doi:10.1093/humupd/dmu009 (2014).
56 Navarro, V. M. Interactions between kisspeptins and neurokinin B. *Advances in experimental medicine and biology* **784**, 325-347, doi:10.1007/978-1-4614-6199-9_15 (2013).
57 Eghlidi, D. H., Haley, G. E., Noriega, N. C., Kohama, S. G. & Urbanski, H. F. Influence of age and 17beta-estradiol on kisspeptin, neurokinin B, and prodynorphin gene expression in the arcuate-median eminence of female rhesus macaques. *Endocrinology* **151**, 3783-3794, doi:10.1210/en.2010-0198 (2010).
58 Overgaard, A., Ruiz-Pino, F., Castellano, J. M., Tena-Sempere, M. & Mikkelsen, J. D. Disparate changes in kisspeptin and neurokinin B expression in the arcuate nucleus after sex steroid manipulation reveal differential regulation of the two KNDy peptides in rats. *Endocrinology* **155**, 3945-3955, doi:10.1210/en.2014-1200 (2014).
59 Xiang, W. *et al.* The Inhibitory Effects of RFamide-Related Peptide 3 on Luteinizing Hormone Release Involves an Estradiol-Dependent Manner in Prepubertal but Not in Adult Female Mice. *Biology of reproduction*, doi:10.1095/biolreprod.115.128777 (2015).

60 Iwasa, T. *et al.* Developmental changes in the mammalian gonadotropin-inhibitory hormone (GnIH) ortholog RFamide-related peptide (RFRP) and its cognate receptor GPR147 in the rat hypothalamus. *International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience* **30**, 31-37, doi:10.1016/j.ijdevneu.2011.10.003 (2012).

61 Meethal, S. V. *et al.* Identification of a regulatory loop for the synthesis of neurosteroids: a steroidogenic acute regulatory protein-dependent mechanism involving hypothalamic-pituitary-gonadal axis receptors. *Journal of neurochemistry* **110**, 1014-1027, doi:10.1111/j.1471-4159.2009.06192.x (2009).

62 Herbison, A. E. Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons. *Endocrine reviews* **19**, 302-330, doi:10.1210/edrv.19.3.0332 (1998).

63 Wennink, J. M., Delemarre-van de Waal, H. A., Schoemaker, R., Schoemaker, H. & Schoemaker, J. Luteinizing hormone and follicle stimulating hormone secretion patterns in girls throughout puberty measured using highly sensitive immunoradiometric assays. *Clinical endocrinology* **33**, 333-344 (1990).

64 Glidewell-Kenney, C. *et al.* Nonclassical estrogen receptor alpha signaling mediates negative feedback in the female mouse reproductive axis. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 8173-8177, doi:10.1073/pnas.0611514104 (2007).

65 Addison, M. L. & Rissman, E. F. Sexual dimorphism of growth hormone in the hypothalamus: regulation by estradiol. *Endocrinology* **153**, 1898-1907, doi:10.1210/en.2011-1982 (2012).

66 Matsuda, K. I., Mori, H. & Kawata, M. Epigenetic mechanisms are involved in sexual differentiation of the brain. *Reviews in endocrine & metabolic disorders* **13**, 163-171, doi:10.1007/s11154-012-9202-z (2012).

67 Cone, R. D. Anatomy and regulation of the central melanocortin system. *Nature neuroscience* **8**, 571-578, doi:10.1038/nn1455 (2005).

68 Saper, C. B., Chou, T. C. & Elmquist, J. K. The need to feed: homeostatic and hedonic control of eating. *Neuron* **36**, 199-211 (2002).

69 Tong, Q., Ye, C. P., Jones, J. E., Elmquist, J. K. & Lowell, B. B. Synaptic release of GABA by AgRP neurons is required for normal regulation of energy balance. *Nature neuroscience* **11**, 998-1000, doi:10.1038/nn.2167 (2008).

70 Briggs, D. I., Enriori, P. J., Lemus, M. B., Cowley, M. A. & Andrews, Z. B. Diet-induced obesity causes ghrelin resistance in arcuate NPY/AgRP neurons. *Endocrinology* **151**, 4745-4755, doi:10.1210/en.2010-0556 (2010).

71 Wang, Q. *et al.* Arcuate AgRP neurons mediate orexigenic and glucoregulatory actions of ghrelin. *Molecular metabolism* **3**, 64-72, doi:10.1016/j.molmet.2013.10.001 (2014).

72 Horvath, T. L., Bechmann, I., Naftolin, F., Kalra, S. P. & Leranth, C. Heterogeneity in the neuropeptide Y-containing neurons of the rat arcuate nucleus: GABAergic and non-GABAergic subpopulations. *Brain research* **756**, 283-286 (1997).

73 Pu, S. *et al.* Interactions between neuropeptide Y and gamma-aminobutyric acid in stimulation of feeding: a morphological and pharmacological analysis. *Endocrinology* **140**, 933-940, doi:10.1210/endo.140.2.6495 (1999).

74 Rediger, A. *et al.* MC4R dimerization in the paraventricular nucleus and GHSR/MC3R heterodimerization in the arcuate nucleus: is there relevance for body weight regulation? *Neuroendocrinology* **95**, 277-288, doi:10.1159/000334903 (2012).

75 Mauvais-Jarvis, F. Estrogen and androgen receptors: regulators of fuel homeostasis and emerging targets for diabetes and obesity. *Trends in endocrinology and metabolism: TEM* **22**, 24-33, doi:10.1016/j.tem.2010.10.002 (2011).

76 Mauvais-Jarvis, F., Clegg, D. J. & Hevener, A. L. The role of estrogens in control of energy balance and glucose homeostasis. *Endocrine reviews* **34**, 309-338, doi:10.1210/er.2012-1055 (2013).

77 Asarian, L. & Geary, N. Cyclic estradiol treatment normalizes body weight and restores physiological patterns of spontaneous feeding and sexual receptivity in ovariectomized rats. *Hormones and behavior* **42**, 461-471 (2002).

78 Gao, Q. *et al.* Anorectic estrogen mimics leptin's effect on the rewiring of melanocortin cells and Stat3 signaling in obese animals. *Nature medicine* **13**, 89-94, doi:10.1038/nm1525 (2007).

79 Heine, P. A., Taylor, J. A., Iwamoto, G. A., Lubahn, D. B. & Cooke, P. S. Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 12729-12734, doi:10.1073/pnas.97.23.12729 (2000).

80 Hevener, A. L., Clegg, D. J. & Mauvais-Jarvis, F. Impaired estrogen receptor action in the pathogenesis of the metabolic syndrome. *Molecular and cellular endocrinology*, doi:10.1016/j.mce.2015.05.020 (2015).

81 Mamounis, K. J., Yang, J. A., Yasrebi, A. & Roepke, T. A. Estrogen response element-independent signaling partially restores post-ovariectomy body weight gain but is not sufficient for 17beta-estradiol's control of energy homeostasis. *Steroids* **81**, 88-98, doi:10.1016/j.steroids.2013.10.018 (2014).

82 Rogers, N. H., Perfield, J. W., 2nd, Strissel, K. J., Obin, M. S. & Greenberg, A. S. Reduced energy expenditure and increased inflammation are early events in the development of ovariectomy-induced obesity. *Endocrinology* **150**, 2161-2168, doi:10.1210/en.2008-1405 (2009).

83 Handgraaf, S. *et al.* Prevention of obesity and insulin resistance by estrogens requires ERalpha activation function-2 (ERalphaAF-2), whereas ERalphaAF-1 is dispensable. *Diabetes* **62**, 4098-4108, doi:10.2337/db13-0282 (2013).

84 Stubbins, R. E., Holcomb, V. B., Hong, J. & Nunez, N. P. Estrogen modulates abdominal adiposity and protects female mice from obesity and impaired glucose tolerance. *European journal of nutrition* **51**, 861-870, doi:10.1007/s00394-011-0266-4 (2012).

85 Musatov, S. *et al.* Silencing of estrogen receptor alpha in the ventromedial nucleus of hypothalamus leads to metabolic syndrome. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 2501-2506, doi:10.1073/pnas.0610787104 (2007).

86 Roepke, T. A., Malyala, A., Bosch, M. A., Kelly, M. J. & Ronnekleiv, O. K. Estrogen regulation of genes important for K+ channel signaling in the arcuate nucleus. *Endocrinology* **148**, 4937-4951, doi:10.1210/en.2007-0605 (2007).

87 Roepke, T. A., Ronnekleiv, O. K. & Kelly, M. J. Physiological consequences of membrane-initiated estrogen signaling in the brain. *Frontiers in bioscience (Landmark edition)* **16**, 1560-1573 (2011).

88 Xu, Y. *et al.* Distinct hypothalamic neurons mediate estrogenic effects on energy homeostasis and reproduction. *Cell metabolism* **14**, 453-465, doi:10.1016/j.cmet.2011.08.009 (2011).

89 Frazao, R. *et al.* Estradiol modulates Kiss1 neuronal response to ghrelin. *American journal of physiology. Endocrinology and metabolism* **306**, E606-614, doi:10.1152/ajpendo.00211.2013 (2014).

90 Thornton, J. E., Loose, M. D., Kelly, M. J. & Ronnekleiv, O. K. Effects of estrogen on the number of neurons expressing beta-endorphin in the medial basal hypothalamus of the female guinea pig. *The Journal of comparative neurology* **341**, 68-77, doi:10.1002/cne.903410107 (1994).

91 Priest, C. A. & Roberts, J. L. Estrogen and tamoxifen differentially regulate beta-endorphin and cFos expression and neuronal colocalization in the arcuate nucleus of the rat. *Neuroendocrinology* **72**, 293-305, doi:54598 (2000).

92 Taylor, J. A., Goubillon, M. L., Broad, K. D. & Robinson, J. E. Steroid control of gonadotropin-releasing hormone secretion: associated changes in pro-opiomelanocortin and preproenkephalin messenger RNA expression in the ovine hypothalamus. *Biology of reproduction* **76**, 524-531, doi:10.1095/biolreprod.106.055533 (2007).

93 Broad, K. D., Kendrick, K. M., Sirinathsinghji, D. J. & Keverne, E. B. Changes in pro-opiomelanocortin and pre-proenkephalin mRNA levels in the ovine brain during pregnancy, parturition and lactation and in response to oestrogen and progesterone. *Journal of neuroendocrinology* **5**, 711-719 (1993).

94 Hirosawa, M. *et al.* Ablation of estrogen receptor alpha (ERalpha) prevents upregulation of POMC by leptin and insulin. *Biochemical and biophysical research communications* **371**, 320-323, doi:10.1016/j.bbrc.2008.04.073 (2008).

95 Morton, G. J., Mystkowski, P., Matsumoto, A. M. & Schwartz, M. W. Increased hypothalamic melanin concentrating hormone gene expression during energy restriction involves a melanocortin-independent, estrogen-sensitive mechanism. *Peptides* **25**, 667-674, doi:10.1016/j.peptides.2004.02.007 (2004).

96 Mystkowski, P. *et al.* Hypothalamic melanin-concentrating hormone and estrogen-induced weight loss. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **20**, 8637-8642 (2000).

97 Treiser, S. L. & Wardlaw, S. L. Estradiol regulation of proopiomelanocortin gene expression and peptide content in the hypothalamus. *Neuroendocrinology* **55**, 167-173 (1992).

98 Bethea, C. L., Hess, D. L., Widmann, A. A. & Henningfeld, J. M. Effects of progesterone on prolactin, hypothalamic beta-endorphin, hypothalamic substance P, and midbrain serotonin in guinea pigs. *Neuroendocrinology* **61**, 695-703 (1995).

99 Baskin, D. G., Norwood, B. J., Schwartz, M. W. & Koerker, D. J. Estradiol inhibits the increase of hypothalamic neuropeptide Y messenger ribonucleic acid expression induced by weight loss in ovariectomized rats. *Endocrinology* **136**, 5547-5554, doi:10.1210/endo.136.12.7588307 (1995).

100 Ainslie, D. A. *et al.* Estrogen deficiency causes central leptin insensitivity and increased hypothalamic neuropeptide Y. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity* **25**, 1680-1688, doi:10.1038/sj.ijo.0801806 (2001).

101 Shimizu, H., Ohtani, K., Kato, Y., Tanaka, Y. & Mori, M. Withdrawal of [corrected] estrogen increases hypothalamic neuropeptide Y (NPY) mRNA expression in ovariectomized obese rat. *Neuroscience letters* **204**, 81-84 (1996).

102 Bonavera, J. J., Dube, M. G., Kalra, P. S. & Kalra, S. P. Anorectic effects of estrogen may be mediated by decreased neuropeptide-Y release in the hypothalamic paraventricular nucleus. *Endocrinology* **134**, 2367-2370, doi:10.1210/endo.134.6.8194462 (1994).

103 Qiu, J. *et al.* Rapid signaling of estrogen in hypothalamic neurons involves a novel G-protein-coupled estrogen receptor that activates protein kinase C. *The*

Journal of neuroscience : the official journal of the Society for Neuroscience **23**, 9529-9540 (2003).

104 Qiu, J. *et al.* A G-protein-coupled estrogen receptor is involved in hypothalamic control of energy homeostasis. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **26**, 5649-5655, doi:10.1523/jneurosci.0327-06.2006 (2006).

105 Smith, J. T., Acohido, B. V., Clifton, D. K. & Steiner, R. A. KiSS-1 neurones are direct targets for leptin in the ob/ob mouse. *Journal of neuroendocrinology* **18**, 298-303, doi:10.1111/j.1365-2826.2006.01417.x (2006).

106 Roepke, T. A. *et al.* Contribution of a membrane estrogen receptor to the estrogenic regulation of body temperature and energy homeostasis. *Endocrinology* **151**, 4926-4937, doi:10.1210/en.2010-0573 (2010).

107 Roepke, T. A., Qiu, J., Bosch, M. A., Ronnekleiv, O. K. & Kelly, M. J. Cross-talk between membrane-initiated and nuclear-initiated oestrogen signalling in the hypothalamus. *Journal of neuroendocrinology* **21**, 263-270, doi:10.1111/j.1365-2826.2009.01846.x (2009).

108 Egecioglu, E. *et al.* Growth hormone receptor deficiency results in blunted ghrelin feeding response, obesity, and hypolipidemia in mice. *American journal of physiology. Endocrinology and metabolism* **290**, E317-325, doi:10.1152/ajpendo.00181.2005 (2006).

109 Burger, K. S. & Berner, L. A. A functional neuroimaging review of obesity, appetitive hormones and ingestive behavior. *Physiology & behavior* **136**, 121-127, doi:10.1016/j.physbeh.2014.04.025 (2014).

110 Blum, I. D. *et al.* Reduced anticipatory locomotor responses to scheduled meals in ghrelin receptor deficient mice. *Neuroscience* **164**, 351-359, doi:10.1016/j.neuroscience.2009.08.009 (2009).

111 Cowley, M. A. *et al.* The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron* **37**, 649-661 (2003).

112 Yin, Y., Li, Y. & Zhang, W. The growth hormone secretagogue receptor: its intracellular signaling and regulation. *International journal of molecular sciences* **15**, 4837-4855, doi:10.3390/ijms15034837 (2014).

113 Kohno, D., Gao, H. Z., Muroya, S., Kikuyama, S. & Yada, T. Ghrelin directly interacts with neuropeptide-Y-containing neurons in the rat arcuate nucleus: Ca2+ signaling via protein kinase A and N-type channel-dependent mechanisms and cross-talk with leptin and orexin. *Diabetes* **52**, 948-956 (2003).

114 Gnanapavan, S. *et al.* The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. *The Journal of clinical endocrinology and metabolism* **87**, 2988, doi:10.1210/jcem.87.6.8739 (2002).

115 Willesen, M. G., Kristensen, P. & Romer, J. Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. *Neuroendocrinology* **70**, 306-316 (1999).

116 Pirnik, Z. *et al.* Effect of ghrelin receptor agonist and antagonist on the activity of arcuate nucleus tyrosine hydroxylase containing neurons in C57BL/6 male mice exposed to normal or high fat diet. *Journal of physiology and pharmacology : an official journal of the Polish Physiological Society* **65**, 477-486 (2014).

117 Goto, M. *et al.* Ghrelin increases neuropeptide Y and agouti-related peptide gene expression in the arcuate nucleus in rat hypothalamic organotypic cultures. *Endocrinology* **147**, 5102-5109, doi:10.1210/en.2006-0104 (2006).

118 Kamegai, J. *et al.* Central effect of ghrelin, an endogenous growth hormone secretagogue, on hypothalamic peptide gene expression. *Endocrinology* **141**, 4797-4800, doi:10.1210/endo.141.12.7920 (2000).

119 Chen, H. Y. *et al.* Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein. *Endocrinology* **145**, 2607-2612, doi:10.1210/en.2003-1596 (2004).

120 Tschop, M., Smiley, D. L. & Heiman, M. L. Ghrelin induces adiposity in rodents. *Nature* **407**, 908-913, doi:10.1038/35038090 (2000).

121 Zigman, J. M. *et al.* Mice lacking ghrelin receptors resist the development of diet-induced obesity. *The Journal of clinical investigation* **115**, 3564-3572, doi:10.1172/jci26002 (2005).

122 Panno, M. L. *et al.* Evidence that the mouse insulin receptor substrate-1 belongs to the gene family on which the promoter is activated by estrogen receptor alpha through its interaction with Sp1. *Journal of molecular endocrinology* **36**, 91-105, doi:10.1677/jme.1.01848 (2006).

123 Quesada, A. & Etgen, A. M. Functional interactions between estrogen and insulin-like growth factor-I in the regulation of alpha 1B-adrenoceptors and female reproductive function. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **22**, 2401-2408 (2002).

124 Bruning, J. C. *et al.* Role of brain insulin receptor in control of body weight and reproduction. *Science (New York, N.Y.)* **289**, 2122-2125 (2000).

125 Klockener, T. *et al.* High-fat feeding promotes obesity via insulin receptor/PI3K-dependent inhibition of SF-1 VMH neurons. *Nature neuroscience* **14**, 911-918, doi:10.1038/nn.2847 (2011).

126 Niswender, K. D. *et al.* Insulin activation of phosphatidylinositol 3-kinase in the hypothalamic arcuate nucleus: a key mediator of insulin-induced anorexia. *Diabetes* **52**, 227-231 (2003).

127 Maejima, Y., Kohno, D., Iwasaki, Y. & Yada, T. Insulin suppresses ghrelin-induced calcium signaling in neuropeptide Y neurons of the hypothalamic arcuate nucleus. *Aging* **3**, 1092-1097 (2011).

128 Kleinridders, A., Ferris, H. A., Cai, W. & Kahn, C. R. Insulin action in brain regulates systemic metabolism and brain function. *Diabetes* **63**, 2232-2243, doi:10.2337/db14-0568 (2014).

129 Schwartz, M. W. *et al.* Central insulin administration reduces neuropeptide Y mRNA expression in the arcuate nucleus of food-deprived lean (Fa/Fa) but not obese (fa/fa) Zucker rats. *Endocrinology* **128**, 2645-2647, doi:10.1210/endo-128-5-2645 (1991).

130 Obici, S., Zhang, B. B., Karkanias, G. & Rossetti, L. Hypothalamic insulin signaling is required for inhibition of glucose production. *Nature medicine* **8**, 1376-1382, doi:10.1038/nm798 (2002).

131 Plum, L., Schubert, M. & Bruning, J. C. The role of insulin receptor signaling in the brain. *Trends in endocrinology and metabolism: TEM* **16**, 59-65, doi:10.1016/j.tem.2005.01.008 (2005).

132 Schubert, M. *et al.* Role for neuronal insulin resistance in neurodegenerative diseases. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 3100-3105, doi:10.1073/pnas.0308724101 (2004).

133 Pardini, A. W. *et al.* Distribution of insulin receptor substrate-2 in brain areas involved in energy homeostasis. *Brain research* **1112**, 169-178, doi:10.1016/j.brainres.2006.06.109 (2006).

134 Baskin, D. G., Breininger, J. F. & Schwartz, M. W. Leptin receptor mRNA identifies a subpopulation of neuropeptide Y neurons activated by fasting in rat hypothalamus. *Diabetes* **48**, 828-833 (1999).
135 Williams, K. W. *et al.* Segregation of acute leptin and insulin effects in distinct populations of arcuate proopiomelanocortin neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **30**, 2472-2479, doi:10.1523/jneurosci.3118-09.2010 (2010).
136 Qiu, J. *et al.* Insulin excites anorexigenic proopiomelanocortin neurons via activation of canonical transient receptor potential channels. *Cell metabolism* **19**, 682-693, doi:10.1016/j.cmet.2014.03.004 (2014).
137 Watanabe, M., Hayasaki, H., Tamayama, T. & Shimada, M. Histologic distribution of insulin and glucagon receptors. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica ... [et al.]* **31**, 243-256 (1998).
138 Kido, Y. *et al.* Tissue-specific insulin resistance in mice with mutations in the insulin receptor, IRS-1, and IRS-2. *The Journal of clinical investigation* **105**, 199-205, doi:10.1172/jci7917 (2000).
139 Kitamura, T., Kahn, C. R. & Accili, D. Insulin receptor knockout mice. *Annual review of physiology* **65**, 313-332, doi:10.1146/annurev.physiol.65.092101.142540 (2003).
140 Joshi, R. L. *et al.* Targeted disruption of the insulin receptor gene in the mouse results in neonatal lethality. *The EMBO journal* **15**, 1542-1547 (1996).
141 Qiu, J., Fang, Y., Bosch, M. A., Ronnekleiv, O. K. & Kelly, M. J. Guinea pig kisspeptin neurons are depolarized by leptin via activation of TRPC channels. *Endocrinology* **152**, 1503-1514, doi:10.1210/en.2010-1285 (2011).
142 Qiu, J., Fang, Y., Ronnekleiv, O. K. & Kelly, M. J. Leptin excites proopiomelanocortin neurons via activation of TRPC channels. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **30**, 1560-1565, doi:10.1523/jneurosci.4816-09.2010 (2010).
143 Bennett, P. A. *et al.* Differential expression and regulation of leptin receptor isoforms in the rat brain: effects of fasting and oestrogen. *Neuroendocrinology* **67**, 29-36 (1998).
144 Mela, V., Vargas, A., Meza, C., Kachani, M. & Wagner, E. J. Modulatory influences of estradiol and other anorexigenic hormones on metabotropic, G-coupled receptor function in the hypothalamic control of energy homeostasis. *The Journal of steroid biochemistry and molecular biology*, doi:10.1016/j.jsbmb.2015.07.014 (2015).
145 Klok, M. D., Jakobsdottir, S. & Drent, M. L. The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review. *Obesity reviews : an official journal of the International Association for the Study of Obesity* **8**, 21-34, doi:10.1111/j.1467-789X.2006.00270.x (2007).
146 Pan, H., Guo, J. & Su, Z. Advances in understanding the interrelations between leptin resistance and obesity. *Physiology & behavior* **130**, 157-169, doi:10.1016/j.physbeh.2014.04.003 (2014).
147 de Luca, C. *et al.* Complete rescue of obesity, diabetes, and infertility in db/db mice by neuron-specific LEPR-B transgenes. *The Journal of clinical investigation* **115**, 3484-3493, doi:10.1172/jci24059 (2005).
148 Fei, H. *et al.* Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 7001-7005 (1997).

149 Kobayashi, K. *et al.* The db/db mouse, a model for diabetic dyslipidemia: molecular characterization and effects of Western diet feeding. *Metabolism: clinical and experimental* **49**, 22-31 (2000).
150 Wang, B., Chandrasekera, P. C. & Pippin, J. J. Leptin- and leptin receptor-deficient rodent models: relevance for human type 2 diabetes. *Current diabetes reviews* **10**, 131-145 (2014).
151 Sarruf, D. A. *et al.* Expression of peroxisome proliferator-activated receptor-gamma in key neuronal subsets regulating glucose metabolism and energy homeostasis. *Endocrinology* **150**, 707-712, doi:10.1210/en.2008-0899 (2009).
152 Astapova, O. & Leff, T. Adiponectin and PPARgamma: cooperative and interdependent actions of two key regulators of metabolism. *Vitamins and hormones* **90**, 143-162, doi:10.1016/b978-0-12-398313-8.00006-3 (2012).
153 Ahmadian, M. *et al.* PPARgamma signaling and metabolism: the good, the bad and the future. *Nature medicine* **19**, 557-566, doi:10.1038/nm.3159 (2013).
154 Bernardo, A. & Minghetti, L. PPAR-gamma agonists as regulators of microglial activation and brain inflammation. *Current pharmaceutical design* **12**, 93-109 (2006).
155 Ferre, P. The biology of peroxisome proliferator-activated receptors: relationship with lipid metabolism and insulin sensitivity. *Diabetes* **53 Suppl 1**, S43-50 (2004).
156 Cifani, C. *et al.* Regulation of hypothalamic neuropeptides gene expression in diet induced obesity resistant rats: possible targets for obesity prediction? *Frontiers in neuroscience* **9**, 187, doi:10.3389/fnins.2015.00187 (2015).
157 Lehrke, M. & Lazar, M. A. The many faces of PPARgamma. *Cell* **123**, 993-999, doi:10.1016/j.cell.2005.11.026 (2005).
158 Gray, S. L., Dalla Nora, E. & Vidal-Puig, A. J. Mouse models of PPAR-gamma deficiency: dissecting PPAR-gamma's role in metabolic homoeostasis. *Biochemical Society transactions* **33**, 1053-1058, doi:10.1042/bst20051053 (2005).
159 Garretson, J. T., Teubner, B. J., Grove, K. L., Vazdarjanova, A. & Ryu, V. Peroxisome proliferator-activated receptor gamma controls ingestive behavior, agouti-related protein, and neuropeptide Y mRNA in the arcuate hypothalamus. **35**, 4571-4581, doi:10.1523/jneurosci.2129-14.2015 (2015).
160 Long, L., Toda, C., Jeong, J. K., Horvath, T. L. & Diano, S. PPARgamma ablation sensitizes proopiomelanocortin neurons to leptin during high-fat feeding. *The Journal of clinical investigation* **124**, 4017-4027, doi:10.1172/jci76220 (2014).
161 Ryan, K. K. *et al.* A role for central nervous system PPAR-gamma in the regulation of energy balance. *Nature medicine* **17**, 623-626, doi:10.1038/nm.2349 (2011).
162 Jeong, S. & Yoon, M. 17beta-Estradiol inhibition of PPARgamma-induced adipogenesis and adipocyte-specific gene expression. *Acta pharmacologica Sinica* **32**, 230-238, doi:10.1038/aps.2010.198 (2011).
163 Ciofi, P., Krause, J. E., Prins, G. S. & Mazzuca, M. Presence of nuclear androgen receptor-like immunoreactivity in neurokinin B-containing neurons of the hypothalamic arcuate nucleus of the adult male rat. *Neuroscience letters* **182**, 193-196 (1994).
164 Kashon, M. L., Hayes, M. J., Shek, P. P. & Sisk, C. L. Regulation of brain androgen receptor immunoreactivity by androgen in prepubertal male ferrets. *Biology of reproduction* **52**, 1198-1205 (1995).
165 Simerly, R. B., Chang, C., Muramatsu, M. & Swanson, L. W. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ

hybridization study. *The Journal of comparative neurology* **294**, 76-95, doi:10.1002/cne.902940107 (1990).

166 Brock, O., De Mees, C. & Bakker, J. Hypothalamic expression of oestrogen receptor alpha and androgen receptor is sex-, age- and region-dependent in mice. *Journal of neuroendocrinology* **27**, 264-276, doi:10.1111/jne.12258 (2015).

167 Borgquist, A., Meza, C. & Wagner, E. J. The role of AMP-activated protein kinase in the androgenic potentiation of cannabinoid-induced changes in energy homeostasis. *American journal of physiology. Endocrinology and metabolism* **308**, E482-495, doi:10.1152/ajpendo.00421.2014 (2015).

168 Nohara, K. *et al.* Early-life exposure to testosterone programs the hypothalamic melanocortin system. *Endocrinology* **152**, 1661-1669, doi:10.1210/en.2010-1288 (2011).

169 Fan, W. *et al.* Functional potentiation of leptin-signal transducer and activator of transcription 3 signaling by the androgen receptor. *Endocrinology* **149**, 6028-6036, doi:10.1210/en.2008-0431 (2008).

170 Nohara, K., Laque, A., Allard, C., Munzberg, H. & Mauvais-Jarvis, F. Central mechanisms of adiposity in adult female mice with androgen excess. *Obesity (Silver Spring, Md.)* **22**, 1477-1484, doi:10.1002/oby.20719 (2014).

171 Yu, I. C. *et al.* Neuronal androgen receptor regulates insulin sensitivity via suppression of hypothalamic NF-kappaB-mediated PTP1B expression. *Diabetes* **62**, 411-423, doi:10.2337/db12-0135 (2013).

172 Lin, H. Y. *et al.* Insulin and leptin resistance with hyperleptinemia in mice lacking androgen receptor. *Diabetes* **54**, 1717-1725 (2005).

173 Wilson, C. *et al.* Testosterone increases GLUT4-dependent glucose uptake in cardiomyocytes. *Journal of cellular physiology* **228**, 2399-2407, doi:10.1002/jcp.24413 (2013).

174 Rana, K., Davey, R. A. & Zajac, J. D. Human androgen deficiency: insights gained from androgen receptor knockout mouse models. *Asian journal of andrology* **16**, 169-177, doi:10.4103/1008-682x.122590 (2014).

175 Matsumoto, T. *et al.* The androgen receptor in health and disease. *Annual review of physiology* **75**, 201-224, doi:10.1146/annurev-physiol-030212-183656 (2013).

176 Corbould, A. Effects of androgens on insulin action in women: is androgen excess a component of female metabolic syndrome? *Diabetes/metabolism research and reviews* **24**, 520-532, doi:10.1002/dmrr.872 (2008).

177 Galani, A., Kitsiou-Tzeli, S., Sofokleous, C., Kanavakis, E. & Kalpini-Mavrou, A. Androgen insensitivity syndrome: clinical features and molecular defects. *Hormones (Athens, Greece)* **7**, 217-229 (2008).

178 Pallares, M. E. *et al.* Maternal administration of flutamide during late gestation affects the brain and reproductive organs development in the rat male offspring. *Neuroscience* **278**, 122-135, doi:10.1016/j.neuroscience.2014.07.074 (2014).

179 Noriega, N. C., Ostby, J., Lambright, C., Wilson, V. S. & Gray, L. E., Jr. Late gestational exposure to the fungicide prochloraz delays the onset of parturition and causes reproductive malformations in male but not female rat offspring. *Biology of reproduction* **72**, 1324-1335, doi:10.1095/biolreprod.104.031385 (2005).

180 Yu, I. C. *et al.* Hyperleptinemia without obesity in male mice lacking androgen receptor in adipose tissue. *Endocrinology* **149**, 2361-2368, doi:10.1210/en.2007-0516 (2008).

181 Bosch, M. A., Hou, J., Fang, Y., Kelly, M. J. & Ronnekleiv, O. K. 17Beta-estradiol regulation of the mRNA expression of T-type calcium channel subunits: role of

estrogen receptor alpha and estrogen receptor beta. *The Journal of comparative neurology* **512**, 347-358, doi:10.1002/cne.21901 (2009).

182 Roepke, T. A., Qiu, J., Smith, A. W., Ronnekleiv, O. K. & Kelly, M. J. Fasting and 17beta-estradiol differentially modulate the M-current in neuropeptide Y neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **31**, 11825-11835, doi:10.1523/jneurosci.1395-11.2011 (2011).

183 Sohn, J. W. *et al.* Serotonin 2C receptor activates a distinct population of arcuate pro-opiomelanocortin neurons via TRPC channels. *Neuron* **71**, 488-497, doi:10.1016/j.neuron.2011.06.012 (2011).

184 Iftinca, M. C. Neuronal T-type calcium channels: what's new? Iftinca: T-type channel regulation. *Journal of medicine and life* **4**, 126-138 (2011).

185 Tinel, N. *et al.* M-type KCNQ2-KCNQ3 potassium channels are modulated by the KCNE2 subunit. *FEBS letters* **480**, 137-141 (2000).

186 Riccio, A. *et al.* mRNA distribution analysis of human TRPC family in CNS and peripheral tissues. *Brain research. Molecular brain research* **109**, 95-104 (2002).

187 Ronnekleiv, O. K., Zhang, C., Bosch, M. A. & Kelly, M. J. Kisspeptin and Gonadotropin-Releasing Hormone Neuronal Excitability: Molecular Mechanisms Driven by 17beta-Estradiol. *Neuroendocrinology*, doi:10.1159/000370311 (2014).

188 Qiu, J. *et al.* Estrogen upregulates T-type calcium channels in the hypothalamus and pituitary. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **26**, 11072-11082, doi:10.1523/jneurosci.3229-06.2006 (2006).

189 Bosch, M. A., Tonsfeldt, K. J. & Ronnekleiv, O. K. mRNA expression of ion channels in GnRH neurons: subtype-specific regulation by 17beta-estradiol. *Molecular and cellular endocrinology* **367**, 85-97, doi:10.1016/j.mce.2012.12.021 (2013).

190 Gottsch, M. L. *et al.* Molecular properties of Kiss1 neurons in the arcuate nucleus of the mouse. *Endocrinology* **152**, 4298-4309, doi:10.1210/en.2011-1521 (2011).

191 Zhang, C., Bosch, M. A., Rick, E. A., Kelly, M. J. & Ronnekleiv, O. K. 17Beta-estradiol regulation of T-type calcium channels in gonadotropin-releasing hormone neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29**, 10552-10562, doi:10.1523/jneurosci.2962-09.2009 (2009).

192 Cooper, E. C. & Jan, L. Y. M-channels: neurological diseases, neuromodulation, and drug development. *Archives of neurology* **60**, 496-500, doi:10.1001/archneur.60.4.496 (2003).

193 Hille, B., Dickson, E., Kruse, M. & Falkenburger, B. Dynamic metabolic control of an ion channel. *Progress in molecular biology and translational science* **123**, 219-247, doi:10.1016/b978-0-12-397897-4.00008-5 (2014).

194 Peters, H. C., Hu, H., Pongs, O., Storm, J. F. & Isbrandt, D. Conditional transgenic suppression of M channels in mouse brain reveals functions in neuronal excitability, resonance and behavior. *Nature neuroscience* **8**, 51-60, doi:10.1038/nn1375 (2005).

195 Robbins, J. KCNQ potassium channels: physiology, pathophysiology, and pharmacology. *Pharmacology & therapeutics* **90**, 1-19 (2001).

196 Roepke, T. A., Smith, A. W., Ronnekleiv, O. K. & Kelly, M. J. Serotonin 5-HT2C receptor-mediated inhibition of the M-current in hypothalamic POMC neurons. *American journal of physiology. Endocrinology and metabolism* **302**, E1399-1406, doi:10.1152/ajpendo.00565.2011 (2012).

197 Acuna-Goycolea, C. & van den Pol, A. N. Peptide YY(3-36) inhibits both anorexigenic proopiomelanocortin and orexigenic neuropeptide Y neurons: implications for hypothalamic regulation of energy homeostasis. *The Journal of*

neuroscience : the official journal of the Society for Neuroscience **25**, 10510-10519, doi:10.1523/jneurosci.2552-05.2005 (2005).

198 Zhang, C., Roepke, T. A., Kelly, M. J. & Ronnekleiv, O. K. Kisspeptin depolarizes gonadotropin-releasing hormone neurons through activation of TRPC-like cationic channels. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **28**, 4423-4434, doi:10.1523/jneurosci.5352-07.2008 (2008).

199 He, P. *et al.* PBDE-47-induced oxidative stress, DNA damage and apoptosis in primary cultured rat hippocampal neurons. *Neurotoxicology* **29**, 124-129, doi:10.1016/j.neuro.2007.10.002 (2008).

200 Dubois, S. L. *et al.* Positive, but not negative feedback actions of estradiol in adult female mice require estrogen receptor alpha in kisspeptin neurons. *Endocrinology* **156**, 1111-1120, doi:10.1210/en.2014-1851 (2015).

201 Watson, C. S., Bulayeva, N. N., Wozniak, A. L. & Alyea, R. A. Xenoestrogens are potent activators of nongenomic estrogenic responses. *Steroids* **72**, 124-134, doi:10.1016/j.steroids.2006.11.002 (2007).

202 Jeng, Y. J., Kochukov, M. & Watson, C. S. Combinations of physiologic estrogens with xenoestrogens alter calcium and kinase responses, prolactin release, and membrane estrogen receptor trafficking in rat pituitary cells. *Environmental health : a global access science source* **9**, 61, doi:10.1186/1476-069x-9-61 (2010).

203 Thomas, P. & Dong, J. Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. *The Journal of steroid biochemistry and molecular biology* **102**, 175-179, doi:10.1016/j.jsbmb.2006.09.017 (2006).

204 Dickerson, S. M., Cunningham, S. L., Patisaul, H. B., Woller, M. J. & Gore, A. C. Endocrine disruption of brain sexual differentiation by developmental PCB exposure. *Endocrinology* **152**, 581-594, doi:10.1210/en.2010-1103 (2011).

205 Dickerson, S. M., Cunningham, S. L. & Gore, A. C. Prenatal PCBs disrupt early neuroendocrine development of the rat hypothalamus. *Toxicology and applied pharmacology* **252**, 36-46, doi:10.1016/j.taap.2011.01.012 (2011).

206 Engell, M. D., Godwin, J., Young, L. J. & Vandenbergh, J. G. Perinatal exposure to endocrine disrupting compounds alters behavior and brain in the female pine vole. *Neurotoxicology and teratology* **28**, 103-110, doi:10.1016/j.ntt.2005.10.002 (2006).

207 Gore, A. C., Walker, D. M., Zama, A. M., Armenti, A. E. & Uzumcu, M. Early life exposure to endocrine-disrupting chemicals causes lifelong molecular reprogramming of the hypothalamus and premature reproductive aging. *Molecular endocrinology (Baltimore, Md.)* **25**, 2157-2168, doi:10.1210/me.2011-1210 (2011).

208 Masutomi, N. *et al.* Impact of dietary exposure to methoxychlor, genistein, or diisononyl phthalate during the perinatal period on the development of the rat endocrine/reproductive systems in later life. *Toxicology* **192**, 149-170 (2003).

209 Takagi, H. *et al.* Impact of maternal dietary exposure to endocrine-acting chemicals on progesterone receptor expression in microdissected hypothalamic medial preoptic areas of rat offspring. *Toxicology and applied pharmacology* **208**, 127-136, doi:10.1016/j.taap.2005.02.002 (2005).

210 Rubin, B. S., Murray, M. K., Damassa, D. A., King, J. C. & Soto, A. M. Perinatal exposure to low doses of bisphenol A affects body weight, patterns of estrous cyclicity, and plasma LH levels. *Environmental health perspectives* **109**, 675-680 (2001).

211 Zama, A. M. & Uzumcu, M. Epigenetic effects of endocrine-disrupting chemicals on female reproduction: an ovarian perspective. *Frontiers in neuroendocrinology* **31**, 420-439, doi:10.1016/j.yfrne.2010.06.003 (2010).

212 Zama, A. M. & Uzumcu, M. Targeted genome-wide methylation and gene expression analyses reveal signaling pathways involved in ovarian dysfunction after developmental EDC exposure in rats. *Biology of reproduction* **88**, 52, doi:10.1095/biolreprod.112.104802 (2013).

213 Uzumcu, M., Zama, A. M. & Oruc, E. Epigenetic mechanisms in the actions of endocrine-disrupting chemicals: gonadal effects and role in female reproduction. *Reproduction in domestic animals = Zuchthygiene* **47 Suppl 4**, 338-347, doi:10.1111/j.1439-0531.2012.02096.x (2012).

214 Rosenfeld, C. S. Effects of maternal diet and exposure to bisphenol A on sexually dimorphic responses in conceptuses and offspring. *Reproduction in domestic animals = Zuchthygiene* **47 Suppl 4**, 23-30, doi:10.1111/j.1439-0531.2012.02051.x (2012).

215 Amstislavsky, S. Y., Kizilova, E. A., Golubitsa, A. N., Vasilkova, A. A. & Eroschenko, V. P. Preimplantation exposures of murine embryos to estradiol or methoxychlor change postnatal development. *Reproductive toxicology (Elmsford, N.Y.)* **18**, 103-108, doi:10.1016/j.reprotox.2003.10.008 (2004).

216 Christiansen, S. *et al.* Low-dose effects of bisphenol A on early sexual development in male and female rats. *Reproduction (Cambridge, England)* **147**, 477-487, doi:10.1530/rep-13-0377 (2014).

217 Goldman, J. M., Murr, A. S., Buckalew, A. R., Schmid, J. E. & Abbott, B. D. Methoxychlor-induced alterations in the histological expression of angiogenic factors in pituitary and uterus. *Journal of molecular histology* **35**, 363-375 (2004).

218 Schuh, R. A., Kristian, T., Gupta, R. K., Flaws, J. A. & Fiskum, G. Methoxychlor inhibits brain mitochondrial respiration and increases hydrogen peroxide production and CREB phosphorylation. *Toxicological sciences : an official journal of the Society of Toxicology* **88**, 495-504, doi:10.1093/toxsci/kfi334 (2005).

219 Schuh, R. A., Richardson, J. R., Gupta, R. K., Flaws, J. A. & Fiskum, G. Effects of the organochlorine pesticide methoxychlor on dopamine metabolites and transporters in the mouse brain. *Neurotoxicology* **30**, 274-280 (2009).

220 Lafuente, A., Cabaleiro, T., Caride, A. & Esquifino, A. I. Toxic effects of methoxychlor administered subcutaneously on the hypothalamic-pituitary-testicular axis in adult rats. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* **46**, 1570-1575, doi:10.1016/j.fct.2007.12.017 (2008).

221 Wang, X. *et al.* Bisphenol A enhances kisspeptin neurons in anteroventral periventricular nucleus of female mice. *The Journal of endocrinology* **221**, 201-213, doi:10.1530/joe-13-0475 (2014).

222 Wang, Q. *et al.* [Effects of bisphenol A on voltage-dependent T-type calcium channels in mouse testis and epididymis, and the role of estrogen receptors]. *Wei sheng yan jiu = Journal of hygiene research* **44**, 23-27 (2015).

223 Nadal, A. *et al.* Nongenomic actions of estrogens and xenoestrogens by binding at a plasma membrane receptor unrelated to estrogen receptor alpha and estrogen receptor beta. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 11603-11608, doi:10.1073/pnas.97.21.11603 (2000).

224 Moon, M. K. *et al.* Long-term oral exposure to bisphenol A induces glucose intolerance and insulin resistance. *The Journal of endocrinology* **226**, 35-42, doi:10.1530/joe-14-0714 (2015).

225 Somm, E. *et al.* Perinatal exposure to bisphenol a alters early adipogenesis in the rat. *Environmental health perspectives* **117**, 1549-1555, doi:10.1289/ehp.11342 (2009).
226 Fagnant, H. S. *et al.* Fetal and Neonatal Exposure to the Endocrine Disruptor, Methoxychlor, Reduces Lean Body Mass and Bone Mineral Density and Increases Cortical Porosity. *Calcified tissue international*, doi:10.1007/s00223-014-9916-x (2014).
227 Nunez, A. A., Kannan, K., Giesy, J. P., Fang, J. & Clemens, L. G. Effects of bisphenol A on energy balance and accumulation in brown adipose tissue in rats. *Chemosphere* **42**, 917-922 (2001).
228 Anderson, O. S. *et al.* Perinatal bisphenol A exposure promotes hyperactivity, lean body composition, and hormonal responses across the murine life course. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **27**, 1784-1792, doi:10.1096/fj.12-223545 (2013).
229 vom Saal, F. S. & Myers, J. P. Bisphenol A and risk of metabolic disorders. *Jama* **300**, 1353-1355, doi:10.1001/jama.300.11.1353 (2008).
230 Khalil, N., Chen, A. & Lee, M. Endocrine disruptive compounds and cardio-metabolic risk factors in children. *Current opinion in pharmacology* **19c**, 120-124, doi:10.1016/j.coph.2014.09.015 (2014).
231 Romano, M. E., Savitz, D. A. & Braun, J. M. Challenges and future directions to evaluating the association between prenatal exposure to endocrine disrupting chemicals and childhood obesity. *Current epidemiology reports* **1**, 57-66, doi:10.1007/s40471-014-0007-3 (2014).
232 Miyawaki, J., Sakayama, K., Kato, H., Yamamoto, H. & Masuno, H. Perinatal and postnatal exposure to bisphenol a increases adipose tissue mass and serum cholesterol level in mice. *Journal of atherosclerosis and thrombosis* **14**, 245-252 (2007).
233 Masuno, H., Iwanami, J., Kidani, T., Sakayama, K. & Honda, K. Bisphenol a accelerates terminal differentiation of 3T3-L1 cells into adipocytes through the phosphatidylinositol 3-kinase pathway. *Toxicological sciences : an official journal of the Society of Toxicology* **84**, 319-327, doi:10.1093/toxsci/kfi088 (2005).
234 Newbold, R. R. Developmental exposure to endocrine-disrupting chemicals programs for reproductive tract alterations and obesity later in life. *The American journal of clinical nutrition* **94**, 1939s-1942s, doi:10.3945/ajcn.110.001057 (2011).
235 Ross, M. G. & Desai, M. Developmental programming of offspring obesity, adipogenesis, and appetite. *Clinical obstetrics and gynecology* **56**, 529-536, doi:10.1097/GRF.0b013e318299c39d (2013).
236 Ryan, K. K. *et al.* Perinatal exposure to bisphenol-a and the development of metabolic syndrome in CD-1 mice. *Endocrinology* **151**, 2603-2612, doi:10.1210/en.2009-1218 (2010).
237 Evans, G. & Sutton, E. L. Oral contraception. *The Medical clinics of North America* **99**, 479-503, doi:10.1016/j.mcna.2015.01.004 (2015).
238 Li, Z. *et al.* Occurrence and risk assessment of pharmaceuticals and personal care products and endocrine disrupting chemicals in reclaimed water and receiving groundwater in China. *Ecotoxicology and environmental safety* **119**, 74-80, doi:10.1016/j.ecoenv.2015.04.031 (2015).
239 Fayad, P. B., Prevost, M. & Sauve, S. On-line solid-phase extraction coupled to liquid chromatography tandem mass spectrometry optimized for the analysis of steroid hormones in urban wastewaters. *Talanta* **115**, 349-360, doi:10.1016/j.talanta.2013.05.038 (2013).

240 Kuiper, G. G. *et al.* Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138**, 863-870, doi:10.1210/endo.138.3.4979 (1997).
241 Kwon, J. H., Katz, L. E. & Liljestrand, H. M. Modeling binding equilibrium in a competitive estrogen receptor binding assay. *Chemosphere* **69**, 1025-1031, doi:10.1016/j.chemosphere.2007.04.047 (2007).
242 Mattison, D. R., Karyakina, N., Goodman, M. & LaKind, J. S. Pharmaco- and toxicokinetics of selected exogenous and endogenous estrogens: a review of the data and identification of knowledge gaps. *Critical reviews in toxicology* **44**, 696-724, doi:10.3109/10408444.2014.930813 (2014).
243 Breast cancer and hormonal contraceptives: collaborative reanalysis of individual data on 53 297 women with breast cancer and 100 239 women without breast cancer from 54 epidemiological studies. *Lancet* **347**, 1713-1727 (1996).
244 Yamamoto, Y. *et al.* Estrogen receptor alpha mediates 17alpha-ethynylestradiol causing hepatotoxicity. *The Journal of biological chemistry* **281**, 16625-16631, doi:10.1074/jbc.M602723200 (2006).
245 Koike, E. *et al.* Exposure to ethinyl estradiol prenatally and/or after sexual maturity induces endometriotic and precancerous lesions in uteri and ovaries of mice. *Congenital anomalies* **53**, 9-17, doi:10.1111/j.1741-4520.2012.00383.x (2013).
246 Jin, Y. *et al.* Chronic exposure of mice to environmental endocrine-disrupting chemicals disturbs their energy metabolism. *Toxicology letters* **225**, 392-400, doi:10.1016/j.toxlet.2014.01.006 (2014).
247 Jin, Y. *et al.* Oral exposure of pubertal male mice to endocrine-disrupting chemicals alters fat metabolism in adult livers. *Environmental toxicology*, doi:10.1002/tox.22013 (2014).
248 Reubinoff, B. E. *et al.* Effects of low-dose estrogen oral contraceptives on weight, body composition, and fat distribution in young women. *Fertility and sterility* **63**, 516-521 (1995).
249 Hiemke, C. & Ghraf, R. Effects of short-term exposure to catecholestrogens on catecholamine turnover in the preoptic-hypothalamic brain of ovariectomized rats. *Brain research* **240**, 295-301 (1982).
250 Tejwani, G. A., Vaswani, K. K. & Barbacci, J. C. Effect of oral contraceptives on the rat brain and pituitary opioid peptides. *Peptides* **6**, 555-561 (1985).
251 Picazo, O., Becerril-Montes, A., Huidobro-Perez, D. & Garcia-Segura, L. M. Neuroprotective actions of the synthetic estrogen 17alpha-ethynylestradiol in the hippocampus. *Cellular and molecular neurobiology* **30**, 675-682, doi:10.1007/s10571-009-9490-3 (2010).
252 Meironyte, D., Noren, K. & Bergman, A. Analysis of polybrominated diphenyl ethers in Swedish human milk. A time-related trend study, 1972-1997. *Journal of toxicology and environmental health. Part A* **58**, 329-341 (1999).
253 Chen, Z. J. *et al.* Polybrominated diphenyl ethers (PBDEs) in human samples of mother-newborn pairs in South China and their placental transfer characteristics. *Environment international* **73**, 77-84, doi:10.1016/j.envint.2014.07.002 (2014).
254 Fan, X., Kubwabo, C., Rasmussen, P. E. & Wu, F. Simultaneous determination of thirteen organophosphate esters in settled indoor house dust and a comparison between two sampling techniques. *The Science of the total environment* **491-492**, 80-86, doi:10.1016/j.scitotenv.2013.12.127 (2014).
255 Muirhead, E. K., Skillman, A. D., Hook, S. E. & Schultz, I. R. Oral exposure of PBDE-47 in fish: toxicokinetics and reproductive effects in Japanese Medaka

(Oryzias latipes) and fathead minnows (Pimephales promelas). *Environmental science & technology* **40**, 523-528 (2006).

256 Su, G., Greaves, A. K., Gauthier, L. & Letcher, R. J. Liquid chromatography-electrospray-tandem mass spectrometry method for determination of organophosphate diesters in biotic samples including Great Lakes herring gull plasma. *Journal of chromatography. A* **1374**, 85-92, doi:10.1016/j.chroma.2014.11.022 (2014).

257 Wu, X. M. *et al.* Polybrominated diphenyl ether serum concentrations in a Californian population of children, their parents, and older adults: an exposure assessment study. *Environmental health : a global access science source* **14**, 23, doi:10.1186/s12940-015-0002-2 (2015).

258 Zota, A. R. *et al.* Polybrominated diphenyl ethers, hydroxylated polybrominated diphenyl ethers, and measures of thyroid function in second trimester pregnant women in California. *Environmental science & technology* **45**, 7896-7905, doi:10.1021/es200422b (2011).

259 Li, J. *et al.* Occurrence of organophosphate flame retardants in drinking water from China. *Water research* **54**, 53-61, doi:10.1016/j.watres.2014.01.031 (2014).

260 Sundkvist, A. M., Olofsson, U. & Haglund, P. Organophosphorus flame retardants and plasticizers in marine and fresh water biota and in human milk. *Journal of environmental monitoring : JEM* **12**, 943-951, doi:10.1039/b921910b (2010).

261 Kim, J. W. *et al.* Organophosphorus flame retardants (PFRs) in human breast milk from several Asian countries. *Chemosphere*, doi:10.1016/j.chemosphere.2014.02.033 (2014).

262 Hoffman, K., Daniels, J. L. & Stapleton, H. M. Urinary metabolites of organophosphate flame retardants and their variability in pregnant women. *Environment international* **63**, 169-172, doi:10.1016/j.envint.2013.11.013 (2014).

263 Yang, F., Ding, J., Huang, W., Xie, W. & Liu, W. Particle size-specific distributions and preliminary exposure assessments of organophosphate flame retardants in office air particulate matter. *Environmental science & technology* **48**, 63-70, doi:10.1021/es403186z (2014).

264 Butt, C. M., Congleton, J., Hoffman, K., Fang, M. & Stapleton, H. M. Metabolites of organophosphate flame retardants and 2-ethylhexyl tetrabromobenzoate in urine from paired mothers and toddlers. *Environmental science & technology* **48**, 10432-10438, doi:10.1021/es5025299 (2014).

265 Costa, L. G., de Laat, R., Tagliaferri, S. & Pellacani, C. A mechanistic view of polybrominated diphenyl ether (PBDE) developmental neurotoxicity. *Toxicology letters* **230**, 282-294, doi:10.1016/j.toxlet.2013.11.011 (2014).

266 Costa, L. G., Pellacani, C., Dao, K., Kavanagh, T. J. & Roque, P. J. The brominated flame retardant BDE-47 causes oxidative stress and apoptotic cell death in vitro and in vivo in mice. *Neurotoxicology* **48**, 68-76, doi:10.1016/j.neuro.2015.03.008 (2015).

267 Wu, H., Cao, L., Li, F., Lian, P. & Zhao, J. Multiple biomarkers of the cytotoxicity induced by BDE-47 in human embryonic kidney cells. *Chemosphere* **126**, 32-39, doi:10.1016/j.chemosphere.2015.01.055 (2015).

268 Arkoosh, M. R. *et al.* Dietary Exposure to Individual Polybrominated Diphenyl Ether Congeners BDE-47 and BDE-99 Alters Innate Immunity and Disease Susceptibility in Juvenile Chinook Salmon. *Environmental science & technology*, doi:10.1021/acs.est.5b01076 (2015).

269 Dunnick, J. K., Brix, A., Cunny, H., Vallant, M. & Shockley, K. R. Characterization of polybrominated diphenyl ether toxicity in Wistar Han rats and use of liver

microarray data for predicting disease susceptibilities. *Toxicologic pathology* **40**, 93-106, doi:10.1177/0192623311429973 (2012).

270 Berger, R. G. *et al.* Exposure to an environmentally relevant mixture of brominated flame retardants affects fetal development in Sprague-Dawley rats. *Toxicology* **320**, 56-66, doi:10.1016/j.tox.2014.03.005 (2014).

271 Li, T., Wang, W., Pan, Y. W., Xu, L. & Xia, Z. A hydroxylated metabolite of flame-retardant PBDE-47 decreases the survival, proliferation, and neuronal differentiation of primary cultured adult neural stem cells and interferes with signaling of ERK5 MAP kinase and neurotrophin 3. *Toxicological sciences : an official journal of the Society of Toxicology* **134**, 111-124, doi:10.1093/toxsci/kft083 (2013).

272 Gassmann, K. *et al.* BDE-47 and 6-OH-BDE-47 modulate calcium homeostasis in primary fetal human neural progenitor cells via ryanodine receptor-independent mechanisms. *Archives of toxicology* **88**, 1537-1548, doi:10.1007/s00204-014-1217-7 (2014).

273 Zhang, M. *et al.* [Effects of PBDE-47 on oxidative stress and apoptosis in SH-SY5Y cell]. *Zhonghua lao dong wei sheng zhi ye bing za zhi = Zhonghua laodong weisheng zhiyebing zazhi = Chinese journal of industrial hygiene and occupational diseases* **25**, 145-147 (2007).

274 Tagliaferri, S. *et al.* Low concentrations of the brominated flame retardants BDE-47 and BDE-99 induce synergistic oxidative stress-mediated neurotoxicity in human neuroblastoma cells. *Toxicology in vitro : an international journal published in association with BIBRA* **24**, 116-122, doi:10.1016/j.tiv.2009.08.020 (2010).

275 He, W. *et al.* Effects of PBDE-47 on cytotoxicity and genotoxicity in human neuroblastoma cells in vitro. *Mutation research* **649**, 62-70, doi:10.1016/j.mrgentox.2007.08.001 (2008).

276 Kodavanti, P. R. *et al.* Developmental Exposure to a Commercial PBDE Mixture: Effects on Protein Networks in the Cerebellum and Hippocampus of Rats. *Environmental health perspectives* **123**, 428-436, doi:10.1289/ehp.1408504 (2015).

277 Blanco, J., Mulero, M., Lopez, M., Domingo, J. L. & Sanchez, D. J. BDE-99 deregulates BDNF, Bcl-2 and the mRNA expression of thyroid receptor isoforms in rat cerebellar granular neurons. *Toxicology* **290**, 305-311, doi:10.1016/j.tox.2011.10.010 (2011).

278 Huang, S. C., Giordano, G. & Costa, L. G. Comparative cytotoxicity and intracellular accumulation of five polybrominated diphenyl ether congeners in mouse cerebellar granule neurons. *Toxicological sciences : an official journal of the Society of Toxicology* **114**, 124-132, doi:10.1093/toxsci/kfp296 (2010).

279 Giordano, G., Kavanagh, T. J. & Costa, L. G. Neurotoxicity of a polybrominated diphenyl ether mixture (DE-71) in mouse neurons and astrocytes is modulated by intracellular glutathione levels. *Toxicology and applied pharmacology* **232**, 161-168, doi:10.1016/j.taap.2008.06.018 (2008).

280 Giordano, G., Kavanagh, T. J. & Costa, L. G. Mouse cerebellar astrocytes protect cerebellar granule neurons against toxicity of the polybrominated diphenyl ether (PBDE) mixture DE-71. *Neurotoxicology* **30**, 326-329, doi:10.1016/j.neuro.2008.12.009 (2009).

281 Suvorov, A. & Takser, L. Delayed response in the rat frontal lobe transcriptome to perinatal exposure to the flame retardant BDE-47. *Journal of applied toxicology : JAT* **31**, 477-483, doi:10.1002/jat.1667 (2011).

282 Koenig, C. M., Lango, J., Pessah, I. N. & Berman, R. F. Maternal transfer of BDE-47 to offspring and neurobehavioral development in C57BL/6J mice. *Neurotoxicology and teratology* **34**, 571-580, doi:10.1016/j.ntt.2012.09.005 (2012).
283 He, P. *et al.* Toxic effect of PBDE-47 on thyroid development, learning, and memory, and the interaction between PBDE-47 and PCB153 that enhances toxicity in rats. *Toxicology and industrial health* **27**, 279-288, doi:10.1177/0748233710387002 (2011).
284 Chen, A. *et al.* Prenatal polybrominated diphenyl ether exposures and neurodevelopment in U.S. children through 5 years of age: the HOME study. *Environmental health perspectives* **122**, 856-862, doi:10.1289/ehp.1307562 (2014).
285 Bradner, J. M., Suragh, T. A. & Caudle, W. M. Alterations to the circuitry of the frontal cortex following exposure to the polybrominated diphenyl ether mixture, DE-71. *Toxicology* **312**, 48-55, doi:10.1016/j.tox.2013.07.015 (2013).
286 Bradner, J. M. *et al.* Exposure to the polybrominated diphenyl ether mixture DE-71 damages the nigrostriatal dopamine system: role of dopamine handling in neurotoxicity. *Experimental neurology* **241**, 138-147, doi:10.1016/j.expneurol.2012.12.013 (2013).
287 Blanco, J., Mulero, M., Domingo, J. L. & Sanchez, D. J. Gestational exposure to BDE-99 produces toxicity through upregulation of CYP isoforms and ROS production in the fetal rat liver. *Toxicological sciences : an official journal of the Society of Toxicology* **127**, 296-302, doi:10.1093/toxsci/kfs082 (2012).
288 Dishaw, L. V. *et al.* Is the PentaBDE replacement, tris (1,3-dichloro-2-propyl) phosphate (TDCPP), a developmental neurotoxicant? Studies in PC12 cells. *Toxicology and applied pharmacology* **256**, 281-289, doi:10.1016/j.taap.2011.01.005 (2011).
289 Ta, N. *et al.* Toxicity of TDCPP and TCEP on PC12 cell: changes in CAMKII, GAP43, tubulin and NF-H gene and protein levels. *Toxicology letters* **227**, 164-171, doi:10.1016/j.toxlet.2014.03.023 (2014).
290 Li, F. *et al.* Affinities of organophosphate flame retardants to tumor suppressor gene p53: an integrated in vitro and in silico study. *Toxicology letters* **232**, 533-541, doi:10.1016/j.toxlet.2014.12.006 (2015).
291 Zou, C., Kou, R., Gao, Y., Xie, K. & Song, F. Activation of mitochondria-mediated apoptotic pathway in tri-ortho-cresyl phosphate-induced delayed neuropathy. *Neurochemistry international* **62**, 965-972, doi:10.1016/j.neuint.2013.03.013 (2013).
292 Chen, J. X. *et al.* Involvement of neuropathy target esterase in tri-ortho-cresyl phosphate-induced testicular spermatogenesis failure and growth inhibition of spermatogonial stem cells in mice. *Toxicology letters* **211**, 54-61, doi:10.1016/j.toxlet.2012.03.004 (2012).
293 Liu, M. L. *et al.* Tri-ortho-cresyl phosphate induces autophagy of rat spermatogonial stem cells. *Reproduction (Cambridge, England)* **149**, 163-170, doi:10.1530/rep-14-0446 (2015).
294 Fowler, M. J., McLean, G., Flaskos, J. & Hargreaves, A. J. The effects of tricresyl phosphate on axon outgrowth and neurofilament levels in mouse N2a neuroblastoma cells. *Biochemical Society transactions* **25**, S574 (1997).
295 Wang, Q. *et al.* Exposure of zebrafish embryos/larvae to TDCPP alters concentrations of thyroid hormones and transcriptions of genes involved in the hypothalamic-pituitary-thyroid axis. *Aquatic toxicology (Amsterdam, Netherlands)* **126**, 207-213, doi:10.1016/j.aquatox.2012.11.009 (2013).

296 Isales, G. M. *et al.* Triphenyl phosphate-induced developmental toxicity in zebrafish: Potential role of the retinoic acid receptor. *Aquatic toxicology (Amsterdam, Netherlands)* **161c**, 221-230, doi:10.1016/j.aquatox.2015.02.009 (2015).

297 McGee, S. P., Konstantinov, A., Stapleton, H. M. & Volz, D. C. Aryl phosphate esters within a major PentaBDE replacement product induce cardiotoxicity in developing zebrafish embryos: potential role of the aryl hydrocarbon receptor. *Toxicological sciences : an official journal of the Society of Toxicology* **133**, 144-156, doi:10.1093/toxsci/kft020 (2013).

298 Du, Z., Wang, G., Gao, S. & Wang, Z. Aryl organophosphate flame retardants induced cardiotoxicity during zebrafish embryogenesis: By disturbing expression of the transcriptional regulators. *Aquatic toxicology (Amsterdam, Netherlands)* **161c**, 25-32, doi:10.1016/j.aquatox.2015.01.027 (2015).

299 Hallgren, S., Sinjari, T., Hakansson, H. & Darnerud, P. O. Effects of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) on thyroid hormone and vitamin A levels in rats and mice. *Archives of toxicology* **75**, 200-208 (2001).

300 Zhou, T., Taylor, M. M., DeVito, M. J. & Crofton, K. M. Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. *Toxicological sciences : an official journal of the Society of Toxicology* **66**, 105-116 (2002).

301 Hoppe, A. A. & Carey, G. B. Polybrominated diphenyl ethers as endocrine disruptors of adipocyte metabolism. *Obesity (Silver Spring, Md.)* **15**, 2942-2950, doi:10.1038/oby.2007.351 (2007).

302 Chevrier, J. *et al.* Polybrominated diphenyl ether (PBDE) flame retardants and thyroid hormone during pregnancy. *Environmental health perspectives* **118**, 1444-1449, doi:10.1289/ehp.1001905 (2010).

303 Turyk, M. E. *et al.* Longitudinal biomonitoring for polybrominated diphenyl ethers (PBDEs) in residents of the Great Lakes basin. *Chemosphere* **81**, 517-522, doi:10.1016/j.chemosphere.2010.07.037 (2010).

304 Hu, X. *et al.* Effect on metabolic enzymes and thyroid receptors induced by BDE-47 by activation the pregnane X receptor in HepG2, a human hepatoma cell line. *Toxicology in vitro : an international journal published in association with BIBRA* **28**, 1377-1385, doi:10.1016/j.tiv.2014.07.004 (2014).

305 Suvorov, A., Bissonnette, C., Takser, L. & Langlois, M. F. Does 2,2',4,4'-tetrabromodiphenyl ether interact directly with thyroid receptor? *Journal of applied toxicology : JAT* **31**, 179-184, doi:10.1002/jat.1580 (2011).

306 Nakamura, N. *et al.* Cell type-dependent agonist/antagonist activities of polybrominated diphenyl ethers. *Toxicology letters* **223**, 192-197, doi:10.1016/j.toxlet.2013.09.007 (2013).

307 Li, X. *et al.* Combined 3D-QSAR, molecular docking and molecular dynamics study on thyroid hormone activity of hydroxylated polybrominated diphenyl ethers to thyroid receptors beta. *Toxicology and applied pharmacology* **265**, 300-307, doi:10.1016/j.taap.2012.08.030 (2012).

308 Yang, J. & Chan, K. M. Evaluation of the toxic effects of brominated compounds (BDE-47, 99, 209, TBBPA) and bisphenol A (BPA) using a zebrafish liver cell line, ZFL. *Aquatic toxicology (Amsterdam, Netherlands)* **159**, 138-147, doi:10.1016/j.aquatox.2014.12.011 (2015).

309 Li, F. *et al.* Hormone activity of hydroxylated polybrominated diphenyl ethers on human thyroid receptor-beta: in vitro and in silico investigations. *Environmental health perspectives* **118**, 602-606, doi:10.1289/ehp.0901457 (2010).

310 Chen, Q., Yu, L., Yang, L. & Zhou, B. Bioconcentration and metabolism of decabromodiphenyl ether (BDE-209) result in thyroid endocrine disruption in zebrafish larvae. *Aquatic toxicology (Amsterdam, Netherlands)* **110-111**, 141-148, doi:10.1016/j.aquatox.2012.01.008 (2012).

311 Liu, C. *et al.* Effects of tris(1,3-dichloro-2-propyl) phosphate and triphenyl phosphate on receptor-associated mRNA expression in zebrafish embryos/larvae. *Aquatic toxicology (Amsterdam, Netherlands)* **128-129**, 147-157, doi:10.1016/j.aquatox.2012.12.010 (2013).

312 Kim, S. *et al.* Thyroid disruption by triphenyl phosphate, an organophosphate flame retardant, in zebrafish (Danio rerio) embryos/larvae, and in GH3 and FRTL-5 cell lines. *Aquatic toxicology (Amsterdam, Netherlands)* **160**, 188-196, doi:10.1016/j.aquatox.2015.01.016 (2015).

313 Farhat, A. *et al.* In Ovo effects of two organophosphate flame retardants--TCPP and TDCPP--on pipping success, development, mRNA expression, and thyroid hormone levels in chicken embryos. *Toxicological sciences : an official journal of the Society of Toxicology* **134**, 92-102, doi:10.1093/toxsci/kft100 (2013).

314 Patisaul, H. B. *et al.* Accumulation and endocrine disrupting effects of the flame retardant mixture Firemaster(R) 550 in rats: an exploratory assessment. *Journal of biochemical and molecular toxicology* **27**, 124-136, doi:10.1002/jbt.21439 (2013).

315 Fang, M., Webster, T. F., Ferguson, P. L. & Stapleton, H. M. Characterizing the peroxisome proliferator-activated receptor (PPARgamma) ligand binding potential of several major flame retardants, their metabolites, and chemical mixtures in house dust. *Environmental health perspectives* **123**, 166-172, doi:10.1289/ehp.1408522 (2015).

316 Kamstra, J. H. *et al.* Transcriptional and epigenetic mechanisms underlying enhanced in vitro adipocyte differentiation by the brominated flame retardant BDE-47. *Environmental science & technology* **48**, 4110-4119, doi:10.1021/es405524b (2014).

317 Belcher, S. M., Cookman, C. J., Patisaul, H. B. & Stapleton, H. M. In vitro assessment of human nuclear hormone receptor activity and cytotoxicity of the flame retardant mixture FM 550 and its triarylphosphate and brominated components. *Toxicology letters* **228**, 93-102, doi:10.1016/j.toxlet.2014.04.017 (2014).

318 Pillai, H. K. *et al.* Ligand binding and activation of PPARgamma by Firemaster(R) 550: effects on adipogenesis and osteogenesis in vitro. *Environmental health perspectives* **122**, 1225-1232, doi:10.1289/ehp.1408111 (2014).

319 Suvorov, A. & Takser, L. Global gene expression analysis in the livers of rat offspring perinatally exposed to low doses of 2,2',4,4'-tetrabromodiphenyl ether. *Environmental health perspectives* **118**, 97-102, doi:10.1289/ehp.0901031 (2010).

320 Honkakoski, P., Palvimo, J. J., Penttila, L., Vepsalainen, J. & Auriola, S. Effects of triaryl phosphates on mouse and human nuclear receptors. *Biochemical pharmacology* **67**, 97-106 (2004).

321 Liu, X., Ji, K., Jo, A., Moon, H. B. & Choi, K. Effects of TDCPP or TPP on gene transcriptions and hormones of HPG axis, and their consequences on reproduction in adult zebrafish (Danio rerio). *Aquatic toxicology (Amsterdam, Netherlands)* **134-135**, 104-111, doi:10.1016/j.aquatox.2013.03.013 (2013).

322 Sueyoshi, T. *et al.* Flame retardant BDE-47 effectively activates nuclear receptor CAR in human primary hepatocytes. *Toxicological sciences : an official journal of the Society of Toxicology* **137**, 292-302, doi:10.1093/toxsci/kft243 (2014).

323 Han, X. B., Yuen, K. W. & Wu, R. S. Polybrominated diphenyl ethers affect the reproduction and development, and alter the sex ratio of zebrafish (Danio rerio). *Environmental pollution (Barking, Essex : 1987)* **182**, 120-126, doi:10.1016/j.envpol.2013.06.045 (2013).

324 Huang, S. *et al.* 2,2',4,4'-Tetrabromodiphenyl ether disrupts spermatogenesis, impairs mitochondrial function and induces apoptosis of early leptotene spermatocytes in rats. *Reproductive toxicology (Elmsford, N.Y.)* **51c**, 114-124, doi:10.1016/j.reprotox.2015.01.009 (2015).

325 Talsness, C. E. *et al.* In utero and lactational exposures to low doses of polybrominated diphenyl ether-47 alter the reproductive system and thyroid gland of female rat offspring. *Environmental health perspectives* **116**, 308-314, doi:10.1289/ehp.10536 (2008).

326 Karpeta, A., Barc, J., Ptak, A. & Gregoraszczuk, E. L. The 2,2',4,4'-tetrabromodiphenyl ether hydroxylated metabolites 5-OH-BDE-47 and 6-OH-BDE-47 stimulate estradiol secretion in the ovary by activating aromatase expression. *Toxicology* **305**, 65-70, doi:10.1016/j.tox.2012.10.021 (2013).

327 Karpeta, A., Ptak, A. & Gregoraszczuk, E. L. Different action of 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) and its hydroxylated metabolites on ERalpha and ERbeta gene and protein expression. *Toxicology letters* **229**, 250-256, doi:10.1016/j.toxlet.2014.05.022 (2014).

328 Faass, O., Ceccatelli, R., Schlumpf, M. & Lichtensteiger, W. Developmental effects of perinatal exposure to PBDE and PCB on gene expression in sexually dimorphic rat brain regions and female sexual behavior. *General and comparative endocrinology* **188**, 232-241, doi:10.1016/j.ygcen.2013.04.008 (2013).

329 Windham, G. C. *et al.* Brominated Flame Retardants and Other Persistent Organohalogenated Compounds in Relation to Timing of Puberty in a Longitudinal Study of Girls. *Environmental health perspectives*, doi:10.1289/ehp.1408778 (2015).

330 Harley, K. G. *et al.* PBDE concentrations in women's serum and fecundability. *Environmental health perspectives* **118**, 699-704, doi:10.1289/ehp.0901450 (2010).

331 Peltier, M. R., Koo, H. C., Getahun, D. & Menon, R. Does exposure to flame retardants increase the risk for preterm birth? *Journal of reproductive immunology* **107**, 20-25, doi:10.1016/j.jri.2014.11.002 (2015).

332 La Guardia, M. J. & Hale, R. C. Halogenated flame-retardant concentrations in settled dust, respirable and inhalable particulates and polyurethane foam at gymnastic training facilities and residences. *Environment international* **79**, 106-114, doi:10.1016/j.envint.2015.02.014 (2015).

333 Wang, Q. *et al.* Developmental exposure to the organophosphorus flame retardant tris(1,3-dichloro-2-propyl) phosphate: Estrogenic activity, endocrine disruption and reproductive effects on zebrafish. *Aquatic toxicology (Amsterdam, Netherlands)* **160**, 163-171, doi:10.1016/j.aquatox.2015.01.014 (2015).

334 Latendresse, J. R., Azhar, S., Brooks, C. L. & Capen, C. C. Pathogenesis of cholesteryl lipidosis of adrenocortical and ovarian interstitial cells in F344 rats caused by tricresyl phosphate and butylated triphenyl phosphate. *Toxicology and applied pharmacology* **122**, 281-289, doi:10.1006/taap.1993.1197 (1993).

335 Latendresse, J. R., Brooks, C. L. & Capen, C. C. Toxic effects of butylated triphenyl phosphate-based hydraulic fluid and tricresyl phosphate in female F344 rats. *Veterinary pathology* **32**, 394-402 (1995).

336 Lai, Y., Lu, M., Lin, S. & Cai, Z. Glucuronidation of hydroxylated polybrominated diphenyl ethers and their modulation of estrogen UDP-glucuronosyltransferases. *Chemosphere* **86**, 727-734, doi:10.1016/j.chemosphere.2011.10.047 (2012).

337 Nash, J. T., Szabo, D. T. & Carey, G. B. Polybrominated diphenyl ethers alter hepatic phosphoenolpyruvate carboxykinase enzyme kinetics in male Wistar rats: implications for lipid and glucose metabolism. *Journal of toxicology and environmental health. Part A* **76**, 142-156, doi:10.1080/15287394.2012.738457 (2013).

338 Pacyniak, E. K. *et al.* The flame retardants, polybrominated diphenyl ethers, are pregnane X receptor activators. *Toxicological sciences : an official journal of the Society of Toxicology* **97**, 94-102, doi:10.1093/toxsci/kfm025 (2007).

339 Tung, E. W., Boudreau, A., Wade, M. G. & Atlas, E. Induction of adipocyte differentiation by polybrominated diphenyl ethers (PBDEs) in 3T3-L1 cells. *PloS one* **9**, e94583, doi:10.1371/journal.pone.0094583 (2014).

340 Morris, P. J. *et al.* Organophosphorus flame retardants inhibit specific liver carboxylesterases and cause serum hypertriglyceridemia. *ACS chemical biology* **9**, 1097-1103, doi:10.1021/cb500014r (2014).

341 Farhat, A. *et al.* Tris(1,3-dichloro-2-propyl) phosphate perturbs the expression of genes involved in immune response and lipid and steroid metabolism in chicken embryos. *Toxicology and applied pharmacology* **275**, 104-112, doi:10.1016/j.taap.2013.12.020 (2014).

342 Mackay, H. *et al.* Organizational effects of perinatal exposure to bisphenol-A and diethylstilbestrol on arcuate nucleus circuitry controlling food intake and energy expenditure in male and female CD-1 mice. *Endocrinology* **154**, 1465-1475, doi:10.1210/en.2012-2044 (2013).

343 Leibl, H. & Spona, J. Differential stimulation by 17 beta-estradiol and synthetic estrogens of progesterone-receptor and of translocation of estrogen-receptor in rat pituitary and uterus. *Endocrinology* **110**, 265-271, doi:10.1210/endo-110-1-265 (1982).

344 Overgaard, A. *et al.* The effect of perinatal exposure to ethinyl oestradiol or a mixture of endocrine disrupting pesticides on kisspeptin neurons in the rat hypothalamus. *Neurotoxicology* **37**, 154-162, doi:10.1016/j.neuro.2013.04.012 (2013).

345 Franklin, K. B. J. a. P., G. . *The mouse brain in stereotaxic coordinates*. 3rd edn, (Academic Press, 2008).

346 Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif.)* **25**, 402-408, doi:10.1006/meth.2001.1262 (2001).

347 Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research* **29**, e45 (2001).

348 Kurita, T. *et al.* Regulation of progesterone receptors and decidualization in uterine stroma of the estrogen receptor-alpha knockout mouse. *Biology of reproduction* **64**, 272-283 (2001).

349 Washburn, N. *et al.* Receptor subtypes and signal transduction mechanisms contributing to the estrogenic attenuation of cannabinoid-induced changes in energy homeostasis. *Neuroendocrinology* **97**, 160-175, doi:10.1159/000338669 (2013).

350 Claessens, F. & Tilley, W. Androgen signalling and steroid receptor crosstalk in endocrine cancers. *Endocrine-related cancer* **21**, E3-5, doi:10.1530/erc-14-0274 (2014).

351 Rizza, P. *et al.* Estrogen receptor beta as a novel target of androgen receptor action in breast cancer cell lines. *Breast cancer research : BCR* **16**, R21, doi:10.1186/bcr3619 (2014).

352 Zhu, M. L. & Kyprianou, N. Androgen receptor and growth factor signaling cross-talk in prostate cancer cells. *Endocrine-related cancer* **15**, 841-849, doi:10.1677/erc-08-0084 (2008).

353 Prossnitz, E. R., Arterburn, J. B. & Sklar, L. A. GPR30: A G protein-coupled receptor for estrogen. *Molecular and cellular endocrinology* **265-266**, 138-142, doi:10.1016/j.mce.2006.12.010 (2007).

354 Funabashi, T., Kawaguchi, M. & Kimura, F. The endocrine disrupters butyl benzyl phthalate and bisphenol A increase the expression of progesterone receptor messenger ribonucleic acid in the preoptic area of adult ovariectomized rats. *Neuroendocrinology* **74**, 77-81, doi:54672 (2001).

355 Funabashi, T., Sano, A., Mitsushima, D. & Kimura, F. Bisphenol A increases progesterone receptor immunoreactivity in the hypothalamus in a dose-dependent manner and affects sexual behaviour in adult ovariectomized rats. *Journal of neuroendocrinology* **15**, 134-140 (2003).

356 Picot, M. *et al.* Vulnerability of the neural circuitry underlying sexual behavior to chronic adult exposure to oral bisphenol a in male mice. *Endocrinology* **155**, 502-512, doi:10.1210/en.2013-1639 (2014).

357 Wisniewski, P. *et al.* Adult exposure to bisphenol A (BPA) in Wistar rats reduces sperm quality with disruption of the hypothalamic-pituitary-testicular axis. *Toxicology* **329**, 1-9, doi:10.1016/j.tox.2015.01.002 (2015).

358 Alonso-Magdalena, P. *et al.* Low doses of bisphenol A and diethylstilbestrol impair Ca2+ signals in pancreatic alpha-cells through a nonclassical membrane estrogen receptor within intact islets of Langerhans. *Environmental health perspectives* **113**, 969-977 (2005).

359 King, C. M. & Hentges, S. T. Relative number and distribution of murine hypothalamic proopiomelanocortin neurons innervating distinct target sites. *PloS one* **6**, e25864, doi:10.1371/journal.pone.0025864 (2011).

360 Bharne, A. P., Borkar, C. D., Subhedar, N. K. & Kokare, D. M. Differential expression of CART in feeding and reward circuits in binge eating rat model. *Behavioural brain research* **291**, 219-231, doi:10.1016/j.bbr.2015.05.030 (2015).

361 Lee, J. S., Lee, E. Y. & Lee, H. S. Hypothalamic, feeding/arousal-related peptidergic projections to the paraventricular thalamic nucleus in the rat. *Brain research* **1598**, 97-113, doi:10.1016/j.brainres.2014.12.029 (2015).

362 Mandelblat-Cerf, Y. *et al.* Arcuate hypothalamic AgRP and putative POMC neurons show opposite changes in spiking across multiple timescales. *eLife* **4**, doi:10.7554/eLife.07122 (2015).

363 Ellison-Zelski, S. J., Solodin, N. M. & Alarid, E. T. Repression of ESR1 through actions of estrogen receptor alpha and Sin3A at the proximal promoter. *Molecular and cellular biology* **29**, 4949-4958, doi:10.1128/mcb.00383-09 (2009).

364 Mozhui, K., Lu, L., Armstrong, W. E. & Williams, R. W. Sex-specific modulation of gene expression networks in murine hypothalamus. *Frontiers in neuroscience* **6**, 63, doi:10.3389/fnins.2012.00063 (2012).

365 Chabab, A. & Sultan, C. Increased peripheral aromatase activity in prepubertal children with partial androgen insensitivity syndrome. *Hormone research* **22**, 83-88 (1985).

366 Stillman, S. C., Evans, B. A. & Hughes, I. A. Aromatase activity in genital skin fibroblasts from normal and androgen-insensitive individuals. *The Journal of endocrinology* **127**, 177-183 (1990).

367 Stillman, S. C., Evans, B. A. & Hughes, I. A. Androgen dependent stimulation of aromatase activity in genital skin fibroblasts from normals and patients with androgen insensitivity. *Clinical endocrinology* **35**, 533-538 (1991).

368 Roselli, C. E. & Resko, J. A. Aromatase activity in the rat brain: hormonal regulation and sex differences. *The Journal of steroid biochemistry and molecular biology* **44**, 499-508 (1993).

369 Roselli, C. E. & Resko, J. A. Androgens regulate brain aromatase activity in adult male rats through a receptor mechanism. *Endocrinology* **114**, 2183-2189, doi:10.1210/endo-114-6-2183 (1984).

370 Roselli, C. E., Salisbury, R. L. & Resko, J. A. Genetic evidence for androgen-dependent and independent control of aromatase activity in the rat brain. *Endocrinology* **121**, 2205-2210, doi:10.1210/endo-121-6-2205 (1987).

371 Boyar, R. M. *et al.* Studies of gonadotropin-gonadal dynamics in patients with androgen insensitivity. *The Journal of clinical endocrinology and metabolism* **47**, 1116-1122, doi:10.1210/jcem-47-5-1116 (1978).

372 Erickson, G. F. & Hsueh, A. J. Stimulation of aromatase activity by follicle stimulating hormone in rat granulosa cells in vivo and in vitro. *Endocrinology* **102**, 1275-1282, doi:10.1210/endo-102-4-1275 (1978).

373 Genabai, N. K. & Briski, K. P. Adaptation of arcuate insulin receptor, estrogen receptor-alpha, estrogen receptor-beta, and type-II glucocorticoid receptor gene profiles to chronic intermediate insulin-induced hypoglycemia in estrogen-treated ovariectomized female rats. *Journal of molecular neuroscience : MN* **41**, 304-309, doi:10.1007/s12031-009-9314-4 (2010).

374 Woods, S. C., Gotoh, K. & Clegg, D. J. Gender differences in the control of energy homeostasis. *Experimental biology and medicine (Maywood, N.J.)* **228**, 1175-1180 (2003).

375 Clegg, D. J., Riedy, C. A., Smith, K. A., Benoit, S. C. & Woods, S. C. Differential sensitivity to central leptin and insulin in male and female rats. *Diabetes* **52**, 682-687 (2003).

376 Wiedenhoft, A., Muller, C., Stenger, R., Blum, W. F. & Fusch, C. Lack of sex difference in cerebrospinal fluid (CSF) leptin levels and contribution of CSF/plasma ratios to variations in body mass index in children. *The Journal of clinical endocrinology and metabolism* **84**, 3021-3024, doi:10.1210/jcem.84.9.5983 (1999).

377 Burguera, B. *et al.* Obesity is associated with a decreased leptin transport across the blood-brain barrier in rats. *Diabetes* **49**, 1219-1223 (2000).

378 Ahima, R. S. & Lazar, M. A. Adipokines and the peripheral and neural control of energy balance. *Molecular endocrinology (Baltimore, Md.)* **22**, 1023-1031, doi:10.1210/me.2007-0529 (2008).

379 Banks, W. A. Role of the blood-brain barrier in the evolution of feeding and cognition. *Annals of the New York Academy of Sciences* **1264**, 13-19, doi:10.1111/j.1749-6632.2012.06568.x (2012).

380 Clegg, D. J. *et al.* Estradiol-dependent decrease in the orexigenic potency of ghrelin in female rats. *Diabetes* **56**, 1051-1058, doi:10.2337/db06-0015 (2007).

381 Lu, M. *et al.* Brain PPAR-gamma promotes obesity and is required for the insulin-sensitizing effect of thiazolidinediones. *Nature medicine* **17**, 618-622, doi:10.1038/nm.2332 (2011).

382 Wang, L. *et al.* Role of ghrelin on testosterone secretion and the mRNA expression of androgen receptors in adult rat testis. *Systems biology in reproductive medicine* **57**, 119-123, doi:10.3109/19396368.2010.529984 (2011).

383 Yeh, A. H., Jeffery, P. L., Duncan, R. P., Herington, A. C. & Chopin, L. K. Ghrelin and a novel preproghrelin isoform are highly expressed in prostate cancer and ghrelin activates mitogen-activated protein kinase in prostate cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **11**, 8295-8303, doi:10.1158/1078-0432.ccr-05-0443 (2005).
384 Buyse, J. *et al.* Ghrelin modulates fatty acid synthase and related transcription factor mRNA levels in a tissue-specific manner in neonatal broiler chicks. *Peptides* **30**, 1342-1347, doi:10.1016/j.peptides.2009.04.015 (2009).
385 Demers, A. *et al.* A concerted kinase interplay identifies PPARgamma as a molecular target of ghrelin signaling in macrophages. *PloS one* **4**, e7728, doi:10.1371/journal.pone.0007728 (2009).
386 Kim, S. W. *et al.* Ghrelin inhibits early osteogenic differentiation of C3H10T1/2 cells by suppressing Runx2 expression and enhancing PPARgamma and C/EBPalpha expression. *Journal of cellular biochemistry* **106**, 626-632, doi:10.1002/jcb.22042 (2009).
387 Girard, J. Insulin's effect on the liver: "direct or indirect?" continues to be the question. *The Journal of clinical investigation* **116**, 302-304, doi:10.1172/jci27743 (2006).
388 Karnani, M. & Burdakov, D. Multiple hypothalamic circuits sense and regulate glucose levels. *American journal of physiology. Regulatory, integrative and comparative physiology* **300**, R47-55, doi:10.1152/ajpregu.00527.2010 (2011).
389 Belgardt, B. F., Okamura, T. & Bruning, J. C. Hormone and glucose signalling in POMC and AgRP neurons. *The Journal of physiology* **587**, 5305-5314, doi:10.1113/jphysiol.2009.179192 (2009).
390 Parton, L. E. *et al.* Glucose sensing by POMC neurons regulates glucose homeostasis and is impaired in obesity. *Nature* **449**, 228-232, doi:10.1038/nature06098 (2007).
391 Sohn, J. W. Ion channels in the central regulation of energy and glucose homeostasis. *Frontiers in neuroscience* **7**, 85, doi:10.3389/fnins.2013.00085 (2013).
392 Lin, H. V. *et al.* Divergent regulation of energy expenditure and hepatic glucose production by insulin receptor in agouti-related protein and POMC neurons. *Diabetes* **59**, 337-346, doi:10.2337/db09-1303 (2010).
393 Hill, J. O., Wyatt, H. R. & Peters, J. C. Energy balance and obesity. *Circulation* **126**, 126-132, doi:10.1161/circulationaha.111.087213 (2012).
394 Jentsch, T. J. Neuronal KCNQ potassium channels: physiology and role in disease. *Nature reviews. Neuroscience* **1**, 21-30, doi:10.1038/35036198 (2000).
395 Shi, L. *et al.* Peptide hormone ghrelin enhances neuronal excitability by inhibition of Kv7/KCNQ channels. *Nature communications* **4**, 1435, doi:10.1038/ncomms2439 (2013).
396 Kimchi, T., Xu, J. & Dulac, C. A functional circuit underlying male sexual behaviour in the female mouse brain. *Nature* **448**, 1009-1014, doi:10.1038/nature06089 (2007).
397 Chevalier, M. *et al.* Dehydroepiandrosterone (DHEA) inhibits voltage-gated T-type calcium channels. *Biochemical pharmacology* **83**, 1530-1539, doi:10.1016/j.bcp.2012.02.025 (2012).
398 Nakajima, T. *et al.* Troglitazone inhibits voltage-dependent calcium currents in guinea pig cardiac myocytes. *Circulation* **99**, 2942-2950 (1999).
399 Svensson, J., Moverare-Skrtic, S., Windahl, S., Swanson, C. & Sjogren, K. Stimulation of both estrogen and androgen receptors maintains skeletal muscle

mass in gonadectomized male mice but mainly via different pathways. *Journal of molecular endocrinology* **45**, 45-57, doi:10.1677/jme-09-0165 (2010).

400 Majeed, Y. *et al.* Rapid and contrasting effects of rosiglitazone on transient receptor potential TRPM3 and TRPC5 channels. *Molecular pharmacology* **79**, 1023-1030, doi:10.1124/mol.110.069922 (2011).

401 Sukumar, P. *et al.* Constitutively active TRPC channels of adipocytes confer a mechanism for sensing dietary fatty acids and regulating adiponectin. *Circulation research* **111**, 191-200, doi:10.1161/circresaha.112.270751 (2012).

402 Balthasar, N. *et al.* Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. *Neuron* **42**, 983-991, doi:10.1016/j.neuron.2004.06.004 (2004).

403 Suvorov, A., Battista, M. C. & Takser, L. Perinatal exposure to low-dose 2,2',4,4'-tetrabromodiphenyl ether affects growth in rat offspring: what is the role of IGF-1? *Toxicology* **260**, 126-131, doi:10.1016/j.tox.2009.03.018 (2009).

404 Barker, D. J., Eriksson, J. G., Forsen, T. & Osmond, C. Fetal origins of adult disease: strength of effects and biological basis. *International journal of epidemiology* **31**, 1235-1239 (2002).

405 Vuong, A. M. *et al.* Maternal Polybrominated Diphenyl Ether (PBDE) Exposure and Thyroid Hormones in Maternal and Cord Sera: The HOME Study, Cincinnati, USA. *Environmental health perspectives*, doi:10.1289/ehp.1408996 (2015).

406 Jugan, M. L., Levi, Y. & Blondeau, J. P. Endocrine disruptors and thyroid hormone physiology. *Biochemical pharmacology* **79**, 939-947, doi:10.1016/j.bcp.2009.11.006 (2010).

407 Hamers, T. *et al.* Biotransformation of brominated flame retardants into potentially endocrine-disrupting metabolites, with special attention to 2,2',4,4'-tetrabromodiphenyl ether (BDE-47). *Molecular nutrition & food research* **52**, 284-298, doi:10.1002/mnfr.200700104 (2008).

408 Ainsworth, B. E. *et al.* Compendium of physical activities: an update of activity codes and MET intensities. *Medicine and science in sports and exercise* **32**, S498-504 (2000).

409 Hopkins, M. *et al.* Modelling the associations between fat-free mass, resting metabolic rate and energy intake in the context of total energy balance. *International journal of obesity (2005)*, doi:10.1038/ijo.2015.155 (2015).

410 Fletcher, J. R., Esau, S. P. & Macintosh, B. R. Economy of running: beyond the measurement of oxygen uptake. *Journal of applied physiology (Bethesda, Md. : 1985)* **107**, 1918-1922, doi:10.1152/japplphysiol.00307.2009 (2009).

411 Chen, H. C. & Farese, R. V., Jr. Fatty acids, triglycerides, and glucose metabolism: recent insights from knockout mice. *Current opinion in clinical nutrition and metabolic care* **5**, 359-363 (2002).

412 Zechner, R., Strauss, J. G., Haemmerle, G., Lass, A. & Zimmermann, R. Lipolysis: pathway under construction. *Current opinion in lipidology* **16**, 333-340 (2005).

413 Elmes, M. J., Tan, D. S., Cheng, Z., Wathes, D. C. & McMullen, S. The effects of a high-fat, high-cholesterol diet on markers of uterine contractility during parturition in the rat. *Reproduction (Cambridge, England)* **141**, 283-290, doi:10.1530/rep-10-0378 (2011).

414 Newbold, R. R., Padilla-Banks, E. & Jefferson, W. N. Environmental estrogens and obesity. *Molecular and cellular endocrinology* **304**, 84-89, doi:10.1016/j.mce.2009.02.024 (2009).

415 Wilson, C. A. & Davies, D. C. The control of sexual differentiation of the reproductive system and brain. *Reproduction (Cambridge, England)* **133**, 331-359, doi:10.1530/rep-06-0078 (2007).

416 Mitchell, R. T. *et al.* Anogenital distance plasticity in adulthood: implications for its use as a biomarker of fetal androgen action. *Endocrinology* **156**, 24-31, doi:10.1210/en.2014-1534 (2015).

417 Breton, C. The hypothalamus-adipose axis is a key target of developmental programming by maternal nutritional manipulation. *The Journal of endocrinology* **216**, R19-31, doi:10.1530/joe-12-0157 (2013).

418 Park, C. J. *et al.* Genetic rescue of nonclassical ERalpha signaling normalizes energy balance in obese Eralpha-null mutant mice. *The Journal of clinical investigation* **121**, 604-612, doi:10.1172/jci41702 (2011).

419 Moreno, M. *et al.* Chronic 17beta-estradiol treatment improves skeletal muscle insulin signaling pathway components in insulin resistance associated with aging. *Age (Dordrecht, Netherlands)* **32**, 1-13, doi:10.1007/s11357-009-9095-2 (2010).

420 Cullingford, T. E., Dolphin, C. T. & Sato, H. The peroxisome proliferator-activated receptor alpha-selective activator ciprofibrate upregulates expression of genes encoding fatty acid oxidation and ketogenesis enzymes in rat brain. *Neuropharmacology* **42**, 724-730 (2002).

421 Knauf, C. *et al.* Peroxisome proliferator-activated receptor-alpha-null mice have increased white adipose tissue glucose utilization, GLUT4, and fat mass: Role in liver and brain. *Endocrinology* **147**, 4067-4078, doi:10.1210/en.2005-1536 (2006).

422 Amin, A., Dhillo, W. S. & Murphy, K. G. The central effects of thyroid hormones on appetite. *Journal of thyroid research* **2011**, 306510, doi:10.4061/2011/306510 (2011).

423 He, J. *et al.* PXR ablation alleviates diet-induced and genetic obesity and insulin resistance in mice. *Diabetes* **62**, 1876-1887, doi:10.2337/db12-1039 (2013).

424 Negri-Cesi, P. *et al.* Sexual differentiation of the rodent hypothalamus: hormonal and environmental influences. *The Journal of steroid biochemistry and molecular biology* **109**, 294-299, doi:10.1016/j.jsbmb.2008.03.003 (2008).

425 Spruiell, K. *et al.* Role of pregnane X receptor in obesity and glucose homeostasis in male mice. *The Journal of biological chemistry* **289**, 3244-3261, doi:10.1074/jbc.M113.494575 (2014).

426 Zhang, H. *et al.* Rat pregnane X receptor: molecular cloning, tissue distribution, and xenobiotic regulation. *Archives of biochemistry and biophysics* **368**, 14-22, doi:10.1006/abbi.1999.1307 (1999).

427 Lamba, V. *et al.* PXR (NR1I2): splice variants in human tissues, including brain, and identification of neurosteroids and nicotine as PXR activators. *Toxicology and applied pharmacology* **199**, 251-265, doi:10.1016/j.taap.2003.12.027 (2004).

428 Berthoud, H. R. Multiple neural systems controlling food intake and body weight. *Neuroscience and biobehavioral reviews* **26**, 393-428 (2002).

429 Rother, E., Konner, A. C. & Bruning, J. C. Neurocircuits integrating hormone and nutrient signaling in control of glucose metabolism. *American journal of physiology. Endocrinology and metabolism* **294**, E810-816, doi:10.1152/ajpendo.00685.2007 (2008).

430 Ibrahim, N. *et al.* Hypothalamic proopiomelanocortin neurons are glucose responsive and express K(ATP) channels. *Endocrinology* **144**, 1331-1340, doi:10.1210/en.2002-221033 (2003).

431 Claret, M. *et al.* AMPK is essential for energy homeostasis regulation and glucose sensing by POMC and AgRP neurons. *The Journal of clinical investigation* **117**, 2325-2336, doi:10.1172/jci31516 (2007).

432 Murphy, B. A. *et al.* Fasting enhances the response of arcuate neuropeptide Y-glucose-inhibited neurons to decreased extracellular glucose. *American journal of physiology. Cell physiology* **296**, C746-756, doi:10.1152/ajpcell.00641.2008 (2009).

433 Baquero, A. F. & de Solis, A. J. Developmental switch of leptin signaling in arcuate nucleus neurons. **34**, 9982-9994, doi:10.1523/jneurosci.0933-14.2014 (2014).

434 Mirshamsi, S. *et al.* Leptin and insulin stimulation of signalling pathways in arcuate nucleus neurones: PI3K dependent actin reorganization and KATP channel activation. *BMC neuroscience* **5**, 54, doi:10.1186/1471-2202-5-54 (2004).

435 Plum, L. *et al.* Enhanced PIP3 signaling in POMC neurons causes KATP channel activation and leads to diet-sensitive obesity. *The Journal of clinical investigation* **116**, 1886-1901, doi:10.1172/jci27123 (2006).

436 Elmquist, J. K., Coppari, R., Balthasar, N., Ichinose, M. & Lowell, B. B. Identifying hypothalamic pathways controlling food intake, body weight, and glucose homeostasis. *The Journal of comparative neurology* **493**, 63-71, doi:10.1002/cne.20786 (2005).

437 McIntyre, R. L. *et al.* Polybrominated diphenyl ether congener, BDE-47, impairs insulin sensitivity in mice with liver-specific Pten deficiency. *BMC obesity* **2**, 3, doi:10.1186/s40608-014-0031-3 (2015).

438 Jones, J. R. *et al.* Deletion of PPARgamma in adipose tissues of mice protects against high fat diet-induced obesity and insulin resistance. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 6207-6212, doi:10.1073/pnas.0306743102 (2005).

439 Michalik, L. *et al.* International Union of Pharmacology. LXI. Peroxisome proliferator-activated receptors. *Pharmacological reviews* **58**, 726-741, doi:10.1124/pr.58.4.5 (2006).

440 Chinetti, G. *et al.* PPAR-alpha and PPAR-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. *Nature medicine* **7**, 53-58, doi:10.1038/83348 (2001).

441 Paukkeri, E. L. *et al.* Anti-inflammatory properties of a dual PPARgamma/alpha agonist muraglitazar in in vitro and in vivo models. *Arthritis research & therapy* **15**, R51, doi:10.1186/ar4211 (2013).

442 Roselli, C. E. The effect of anabolic-androgenic steroids on aromatase activity and androgen receptor binding in the rat preoptic area. *Brain research* **792**, 271-276 (1998).

443 Du, J., Zhang, L. & Wang, Z. Testosterone inhibits the activity of peroxisome proliferator-activated receptor gamma in a transcriptional transaction assay. *Die Pharmazie* **64**, 692-693 (2009).

444 Moss, P. E., Lyles, B. E. & Stewart, L. V. The PPARgamma ligand ciglitazone regulates androgen receptor activation differently in androgen-dependent versus androgen-independent human prostate cancer cells. *Experimental cell research* **316**, 3478-3488, doi:10.1016/j.yexcr.2010.09.015 (2010).

445 Yang, C. C. *et al.* Peroxisome proliferator-activated receptor gamma-independent suppression of androgen receptor expression by troglitazone mechanism and pharmacologic exploitation. *Cancer research* **67**, 3229-3238, doi:10.1158/0008-5472.can-06-2759 (2007).

446 Palanza, P. L., Howdeshell, K. L., Parmigiani, S. & vom Saal, F. S. Exposure to a low dose of bisphenol A during fetal life or in adulthood alters maternal behavior in mice. *Environmental health perspectives* **110 Suppl 3**, 415-422 (2002).