THE NEUROENDOCRINE AND PHYSIOLOGICAL IMPACT OF ORGANOPHOSPHATE FLAME RETARDANTS ON ENERGY HOMEOSTASIS IN ADULT MICE

by

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ABSTRACT OF THE DISSERTATION

The neuroendocrine and physiological impact of organophosphate flame retardants on energy homeostasis in adult mice

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The maintenance of energy homeostasis is an integral endocrine-mediated function and is centrally coordinated through hypothalamic melanocortin circuitry. Two nuclear receptors that regulate energy homeostasis are estrogen receptor (ER) α and peroxisome proliferator-activated receptor (PPAR) γ. Disruption of these pathways can cause metabolic disturbances that may lead to serious disorders such as diabetes and metabolic syndrome. This gives cause for concern regarding chemicals that can interfere with endogenous endocrine signaling such as ERα and PPARγ. One such chemical class are organophosphate flame retardants (OPFRs). OPFRs demonstrate widespread human exposure and have been implicated in disruption of energy homeostasis. In this dissertation, I will be examining the toxicological impact of adult exposure to OPFRs on neuroendocrine and physiological endpoints of metabolic disruption. First, I characterize the diet- and sex-dependent physiological effects of adult exposure to OPFRs in wildtype (WT) mice, examining parameters such as weight gain, adiposity, metabolism, activity, ingestive behaviors, glucose tolerance and insulin sensitivity, and plasma peptide hormone levels. I found that OPFR exposure alters circulating peptide hormone levels, feeding behavior, disrupts diurnal fluid intake patterns, and decreases female activity and

energy expenditure while promotes weight gain and adiposity in male mice fed a high-fat diet (HFD). Next, I characterized similar parameters within global ERα knockout (ERαKO) and brain-specific PPARγ knockout (PPARγKO) mice to assess the responsibility of the respective receptors in OPFR-induced disruption of energy homeostasis. I found that the weight gain and adiposity associated with OPFR exposure in WT males can, in part, be attributed to both ERα and PPARγ action. Additionally, I observed numerous novel effects of OPFR in KO genotypes, which may be a result of the absence of ERα and PPARγ targets making alternative OPFR targets more vulnerable to disruption. Lastly, I characterize the neuroendocrine impact of OPFR exposure on hypothalamic neurons governing energy homeostasis. Overall, OPFR exposure augmented neuronal excitability, concluding in a net increase of neuronal output from arcuate neuropeptide Y (NPY) and proopiomelanocortin (POMC) neurons. Collectively, these data represent significant disruptions to energy homeostasis and demonstrate that the capacity for OPFRs to cause adverse health effects extends to adult exposures.

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Vail, G., Walley, S., Yasrebi, A., Maeng, A., Conde, K., Roepke, TA. 2020. The interactions of diet-induced obesity and organophosphate flame retardant exposure on energy homeostasis in adult male and female mice. *Journal of Toxicology and Environmental Health*. *In press*.

Additional Publications

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CHAPTER 1: LITERATURE REVIEW

1. Literature review

1.1 EDCs and the Replacement Game

In developed countries, man-made chemicals have become an invisible aspect of everyday human life. We do not think twice about the plasticizers that prevent the soda bottle from exploding when it falls out of the vending machine, or the extra solvent chemicals in quick-dry formulas that make painting nails that much easier. Developments in chemical production have greatly facilitated human consumerism, and concerningly, a majority of man-made chemicals are not regulated for effects beyond their intended use. This has resulted in unprecedented human and environmental toxicological potential.

One such toxicological category is endocrine disrupting chemicals (EDCs). EDCs are chemicals that interact with and disrupt typical function of the endocrine system governing bodily homeostasis (Zoeller et al. 2012; Bergman et al. 2015). The rise in chemical usage has led to the introduction of a vast array of EDCs to the environment. A few examples of well-known EDCs are pesticides DDT and chlorpyrifos, bisphenol A (BPA) and phthalate plasticizers, polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs). Only after extensive toxicological research have some of these endocrine disrupting chemicals seen regulatory action enacted to safeguard against human exposure. The relatively unregulated state of the chemical industry has generated a concerning “innocent until proven guilty” mentality concerning chemical safety. BPA exemplifies this ideology. BPA is now well-documented as an estrogenic EDC capable of extensive developmental and reproductive toxicity (Matuszczak et al. 2019). However, roughly 60 years of relevant human exposure occurred before BPA usage was banned, and even still, only within certain children’s products such as toys and water bottles

(Matuszczak et al. 2019). Widespread public alarm eventually pressured the chemical industry to search for alternatives to BPA, which has resulted in the development and subsequent unregulated integrations of bisphenols F and S (BPF, BPS) into consumer products (Rochester and Bolden 2015). The alternate use of BPF and BPS effectively resets the “innocent until proven guilty” timeline, placing the burden of human safety evaluation once again on non-industry toxicologists. And, perhaps as expected, both compounds are increasingly implicated in having similar EDC endpoints to BPA (Rochester and Bolden 2015). Unfortunately, BPA is only a singular example of the common “whack-a-mole” regulatory narrative that plagues the chemical industry. Toxic perfluorooctanoic acid (PFOA) used in Teflon production was phased out in favor of indicated toxicant GenX (Gomis et al. 2018; Coperchini et al. 2020); harmful insecticide chlorpyrifos was invented as an alternative to DDT, which itself was a previous substitute for lead arsenate (Eaton et al. 2008; Rahman 2013). There exists an extensive history of regrettable substitution for replacements unsubstantially tested for human safety.

This dissertation will focus on one particular story – flame retardants. Flame retardants (FRs) are a class of chemicals used for their ability to reduce flammability of the objects they are embedded within. The commercial use of FRs greatly expanded following the implementation of a flammability standard in the state of California in 1975. This standard required furniture and upholstery to withstand an open flame test for 12 full seconds (Susan 2010; Technical Bulletin 117 2000). Despite no other state regulations, production companies adopted a global use of FRs to meet Californian standards due to its large share of the home furnishing market. Since then, FR use has become commonplace. There are a small variety of different FRs, but the earliest were known as polychlorinated biphenyls (PCBs). However, these progenitor FRs were found to have serious toxic effects including reproductive, hepatic, immunological, and endocrine toxicity, as well as an identified probable carcinogen (Letz 1983). Following the typical trend, PCBs were then

replaced with another insufficiently studied FR – polybrominated diphenyl ethers (PBDEs). PBDE production became widespread and just before the turn of the millennium, global annual production of PBDEs was estimated to be around 67,125 metric tons, with the vast majority used within the United States (Siddiqi et al. 2003). These replacement products were eventually shown to share many toxic endpoints to PCBs, and were finally phased out of production in 2004 after over two decades of accumulated toxicological data (Zota et al. 2013).

Presently, FR usage is greater than ever. Despite the history of toxicity, FR global demand is projected to expand by 4.6% annually, having reached an estimated production of 2.8 million tons in 2018 (Israel Chemicals ltd. 2015). Bolstering this surging market is a new class of FRs called organophosphate flame retardants (OPFRs). These chemicals have quickly become the leading FR replacement for PBDEs (van der Veen and de Boer 2012; Yang et al. 2019), resulting in widespread human exposure to these lesser known FRs. OPFRs were initially thought to be a safer alternative to PBDEs due to their presumed lesser environmental persistence than PBDEs (Zhang et al. 2016), however, due to their widespread usage, OPFRs have become a ubiquitous presence in modern environments (Yang et al. 2019). OPFRs are commonly detected in outdoor environments such as soil (Mihajlović et al. 2011), surface water (Gao et al. 2014; Staaf and Ostman 2005; Kim et al. 2011a; Regnery and Püttmann 2010), sediments (Cao et al. 2012), and air samples (Li et al. 2015; Möller et al. 2012), though the greatest levels of OPFR accumulate within indoor environments such as office spaces, schools, and family residences (Cristale et al. 2016; Ali et al. 2012; He et al. 2016; Brommer et al. 2012). OPFR presence in modern spaces is accounted for by its overwhelming usage in household and workplace products such as furniture, toys, electronics, foodstuffs, and plastics and resins (van der Veen and de Boer 2012; Li et al. 2019; Peng et al. 2020; Yang et al. 2019; Young et al. 2018). OPFRs are often not chemically bound to their polymer

materials and will escape and accumulate in household and workplace dust (Wang et al. 2015; Brommer et al. 2012; van der Veen and de Boer 2012; Staaf and Ostman 2005). Human exposure occurs primarily through either inhalation or ingestion of household dust (Lehmler et al. 2018), and has resulted in concerning levels of OPFRs and their metabolites within human serum (680-709 ng/ml), urine (1-10 ng/ml), and breast milk (1-10 ng/ml) (Butt et al. 2014; Hoffman et al. 2017; Ma et al. 2019; Ma et al. 2017; Meeker et al. 2013). The rise in human exposure to OPFRs calls for concern for their long-term toxicological impact. Perhaps unsurprisingly, emerging research over the past decade increasingly indicates OPFRs as toxic EDCs, thus restarting the “whack-a-mole” game that toxicologists must play.

Research has revealed OPFRs to have neurotoxic, reprotoxic, osteotoxic, immunotoxic, obesogenic and endocrine disruptive actions (Dishaw et al. 2011; Patisaul et al. 2013; Kylie et al. 2018; Belcher et al. 2014; Pillai et al. 2014; Liu et al. 2013b; Liu et al. 2012a; Hu et al. 2019; Steves et al. 2018; Macari et al. 2020). These varied endpoints are likely due, in part, to action on endocrine functions that govern multiple homeostatic functions. Additionally, the varied OPFRS used in the experimentational research and their different mixtures and exposure windows contribute further complications. For the purpose of this thesis, we will be focusing on OPFR action as an EDC to disrupt energy homeostasis. Energy homeostasis is the complex balancing act the body maintains to ensure efficient intake and usage of energy. Energy balance is largely coordinated by hormonal signaling, thusly, it is a prime target for endocrine disruption (Röder et al. 2016; Richard et al. 2000; Varela and Horvath 2012; Mauvais-Jarvis et al. 2013). It is also regulated neurologically (Timper and Bruning 2017), and disruptions to energy balance, such as diet-induced obesity, comorbid with dysregulated immune function (Ellulu et al. 2017; Lee et al. 2013). Collectively, these encompass the majority of reported OPFR

toxicity endpoints, making energy homeostasis a rational endpoint for examining OPFR action.

1.2 Energy Homeostasis

Energy homeostasis integrates peripheral signals of energy state, such as blood-glucose levels, with external stimuli such as the presence or lack of available food, to initiate bodily responses to maintain optimal energy status. Oftentimes, this is mediated through peripheral actions such as insulin secretin when blood-glucose is high, or, stimulation of lipid metabolism when glucose is low. Energy homeostasis is also a highly centrally regulated mechanism, particularly through actions of hormonally-responsive neurons within the hypothalamus (Timper and Brüning 2017). This segment will briefly touch upon key peripheral and central mechanisms of energy balance.

1.2.1 Peripheral Signals of Energy Homeostasis

Glucose, Insulin, and Glucagon

Glucose is an essential sugar and a key marker for energy state within the body. The liver plays a major role in glucose homeostasis through regulation of glucose production through de novo synthesis and glycogen breakdown (Sharabi et al. 2015). The pancreas is another vital organ for glucose regulation. The pancreas produces both insulin and glucagon which act to decrease and increase blood-glucose levels, respectively (Röder et al. 2016). High levels of insulin are seen as a marker of a sated energy state, in which the pancreas has released insulin to signal the uptake of excess glucose post-feeding. Inversely, glucagon is a marker of low glucose, whereas the pancreas responds by releasing glucagon to stimulate hepatic glucose production (Cherrington et al. 1978).

Ghrelin

Ghrelin is gastrointestinal hormone that potently stimulates feeding, and as is often referred to as “the hunger hormone”. Produced from the stomach, ghrelin primarily acts on the brain to regulate food intake, body weight, adiposity, and glucose homeostasis (Müller et al. 2015; Kojima et al. 1999). However, it is also involved in stimulation gastric motility (Masuda et al. 2000; Asakawa et al. 2001), reducing energy expenditure (Yasuda et al. 2003; Tsubone et al. 2005; Asakawa et al. 2001), and is implicated in feeding motivation and reward feedback (Overduin et al. 2012; Jerlhag et al. 2007). Ghrelin dysregulation is also implicated in eating disorders anorexia nervosa, bulimia nervosa, and binge eating disorder (Atalayer et al. 2013).

Leptin

Leptin is a body weight control hormone mainly produced by adipose tissue. Leptin levels primarily reflect current the amount of energy contained within fat, making leptin a marker of chronic energy state (Kelesidis et al. 2010). However, circulating leptin rapidly decreases during fasting and plays a secondary role in short-term regulation of energy homeostasis as well (Boden et al. 1996; Chan et al. 2003). Leptin functions to reduce appetite and increase energy expenditure (Zhou and Rui 2013), and patients with congenital leptin deficiency are obese due to significant hyperphagia (Farooqi et al. 2007; Strobel et al. 1998). In these cases, leptin replacement is remarkably effective in normalizing food intake and bodyweight (Paz-Filho et al. 2010). Conversely, obese patients without leptin mutations have relatively high circulating leptin due to excess adipose tissue and exhibit leptin receptor signaling impairments and do not respond to additional leptin administration (Heymsfield et al. 1999; Roth et al. 2008).

Lipid homeostasis

Lipids are fatty acid molecules used by the body as an energy source, and as vital components of cellular membranes, bile acids, and certain hormones and vitamins. They are synthesized by the liver or obtained through consumption of biomass and processed into functional lipids such as triglycerides (Jo et al. 2016). Triglycerides act as an important energy supply for cardiac and skeletal muscle (Pascual and Coleman 2016); however, their overproduction and dysregulation is closely associated with metabolic disorders such as diabetes (Ginsberg et al. 2005; Pang et al. 2014).

1.2.2 Central Regulation of Energy Homeostasis

The primary central regulator of energy homeostasis is the hypothalamus. The hypothalamus sits at the base of the brain, just above the pituitary, to which it sends signals stimulating release of hormones to regulate bodily homeostasis. In the case of energy homeostasis, the arcuate (ARC) nucleus of the hypothalamus is of particular importance. The ARC is located adjacent a portion of the blood-brain-barrier permeable to circulating peptide hormones such as insulin, ghrelin, and leptin (Saper et al. 2002; Schwartz et al. 2000). This placement allows ARC neurons to sense and respond to peripheral markers of energy state through their respective receptors.

The Arcuate Melanocortin Circuit

ARC regulation of energy homeostasis is controlled through two neuronal subpopulations: POMC neurons that co-express proopiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) and NPY neurons that co-express neuropeptide Y (NPY) and agouti-related peptide (AgRP). These neurons project to the paraventricular nucleus (PVN) and the lateral hypothalamus (LH), where their combined

inputs influence food intake and energy expenditure (Nahon 2006; Arora and Anubhuti 2006).

POMC/CART neurons

POMC neurons signal to reduce food intake and increase energy expenditure. They exert their anorexigenic actions via two major signaling molecules: CART and α–melanocyte stimulating hormone (α-MSH). α-MSH is a cleavage product of the protein POMC and is an endogenous ligand for melanocortin 4 receptors (MC4R) expressed in neurons in the PVN. MC4R is expressed in PVN neurons containing thyrotropin-releasing hormone (TRH) and neurons containing corticotropin-releasing hormone (CRH) (Siljee et al. 2013). Activation of these neurons via α-MSH stimulates release of TRH and CRH, both of which are involved in the regulation of energy balance (Li et al. 2013). Collectively, α-MSH released from ARC POMC neurons acts to stimulate PVN neurons to diminish energy intake and increase energy expenditure (Simpson et al. 2008).

CART appears to act via a Gi-protein coupled receptor (Lin et al. 2011; Somalwar et al. 2018), and although the specific receptor is yet to be identified, it is clear that hypothalamic CART is another potent inhibitor of food intake. Intracerebroventricular (ICV) injection of CART decreases food intake (Aja et al. 2001; Tachibana et al. 2003), and conversely, antibody knockdown of CART increases food intake (Lambert et al. 1998; Kristensen et al. 1998). As indicative of its name, cocaine and amphetamine regulated transcript (CART) is also involved in pathways associated with reward (Schroeder and Leinninger 2018; Choudhary et al. 2018). Dysregulation of POMC/CART neurons may therefore alter feeding-reward systems, therefore increasing risk of eating disorder development.

POMC neurons respond to peripheral anorexigenic peptides insulin and leptin through respective receptor binding (Paeger et al. 2017), causing neuronal depolarization and α-MSH release, leading to subsequent reductions in feeding and increased energy expenditure (Brüning et al. 2000; Varela and Horvath 2012).

NPY/AgRP neurons

On the other hand, NPY neurons are the major source of orexigenic output from the ARC. These neurons synthesize AgRP, which is an endogenous antagonist to MC4R receptors (Ollmann et al. 1997). They project to the PVN where AgRP release selectively inhibits the anorexigenic action of α-MSH signaling from POMC neurons (Ollmann et al. 1997). NPY neurons are excited during times of fasting (Roepke et al. 2011), and increased output of AgRP increases food intake and decreases energy expenditure (Korner et al. 2003; Goto et al. 2003; Small et al. 2001), in part through inhibition of TRH release from the PVN (Fekete et al. 2002).

NPY neurons also release neuropeptide Y (NPY). ICV administration of NPY elicits reliable increases in food intake, appearing to be through a behavioral mechanism of reduced latency to eat (Clark et al. 1984; Stanley and Leibowitz 1985; Morley et al. 1987; Corp et al. 1990). NPY regulation of feeding behavior is mediated through receptor subfamilies Y1, Y2, and Y5 (Henry et al. 2005). Importantly, ICV injections of NPY only stimulate food intake when injected into the third or lateral ventricles near the hypothalamus, or when injected into the fourth ventricle (Corp et al. 1990; Steinman et al. 1994), highlighting important connections between the hypothalamus and the hindbrain for regulating food intake.

Furthermore, NPY neurons are GABAergic and regulate food intake in part by releasing inhibitory GABA onto POMC neurons (Krashes et al. 2013; Engström Ruud et

al. 2020). NPY neurons are also reported to inhibit PVN neurons via GABA signaling (Cowley et al. 2003). The importance of NPY GABA signaling is highlighted by Tong et al. (2008), who demonstrated that NPY/AgRP neuron-specific deletion of vesicular GABA transporter generates mice that are lean, resistant to obesity and less responsive to the orexigenic effects of ghrelin (Tong et al. 2008).

Ghrelin is well-established to excite NPY neurons and appears to stimulate transcriptional and direct actions on neuronal excitation (Kohno et al. 2003; Hashiguchi et al. 2017; Cowley et al. 2003). On the other hand, anorexigenic peripheral peptides insulin and leptin are shown to inhibit NPY action (Könner et al. 2007; Baver et al. 2014).

Other Hypothalamic Neurons

There exist other neuronal subpopulations outside of ARC POMC and NPY neurons that help coordinate energy homeostasis. Aforementioned corticotropin releasing hormone (CRH), and thyrotropin releasing hormone (TRH) neurons are regulated by ARC POMC and NPY neurons and are critical in increasing energy expenditure and decrease intake (Hill 2012). CRH and TRH neurons both project to the brain stem and spinal cord (Sawchenko and Swanson 1982a, 1982b; Sawchenko 1987), to which they relay catabolic signals through sympathetic activation of brown adipose tissue thermogenesis and lipolysis (Buwalda et al. 1998; Arase et al. 1988). TRH also signals for pituitary release of thyroid-stimulating hormone (TSH), which stimulates production of thyroid hormones integral in maintaining metabolic rate (Kim 2008; Bianco et al. 2005), and excessive energy expenditure is a hallmark of hyperthyroidism (De Leo et al. 2016). CRH is responsible in stimulating pituitary release of adrenocorticotropic hormone (ACTH), which is a critical regulator of the adrenal production of cortisol involved in the physiological response to stressful stimuli (Seasholtz 2000). Modern life typically coincides with chronic stress, which intersects with dysregulated energy expenditure, predisposing individuals to

obesity and cardiac diseases (Rosmond 2005; von Känel 2012). Furthermore, CRH activates the sympathetic nervous system and augments catecholamine release, resulting in reduced feeding and increased catabolic actions mediated through action on adipose tissue (Richard et al. 2000; Rabasa and Dickson 2016).

Also important in hypothalamic regulation of energy homeostasis is the lateral hypothalamus (LH). The LH is home for melanin-concentrating hormone (MCH) neurons and orexin neurons, which are most well-known for their reciprocal regulation of sleep and wakefulness (Konadhode et al. 2014). However, these neurons also play a role in regulating feeding behavior (Tsujino and Sakurai 2013; Naufahu et al. 2013). Genetic deletion of MCH or its receptor, MCHR1, confers resistance to diet-induced obesity (Chen et al. 2002; Kokkotou et al. 2005; Mashiko et al. 2005), and impairs food seeking behavior (Adams et al. 2011). Conversely, IVC infusion of MCH augments sucrose intake and promotes obesity in rodents (Sakamaki et al. 2005; Gomori et al. 2003). LH orexin neurons are innervated by ARC NPY neurons (Broberger et al. 1998), suggesting orexin neurons as downstream targets of NPY-induced feeding. This relationship appears bidirectional, as NPY neurons are also excited by orexin, resulting in increased feeding (Muroya et al. 2004; van den Top et al. 2004). Similar to NPY and AgRP, ICV injections of orexin rapidly induces food intake (Lubkin and Stricker-Krongrad 1998; Haynes et al. 1999). Orexin neurons are also glucose- and leptin-inhibited (Goforth et al. 2014; Routh et al. 2014), and may regulate reward-based feeding through connections with the ventral tegmental area (Teegala et al. 2020).

The ventromedial hypothalamus (VMH) represents another hypothalamic nuclei involved in central regulation of energy homeostasis. One VMH neuronal subpopulation are steroidogenic factor 1 (SF-1) expressing neurons. SF-1 neurons are increasingly implicated as essential for the maintenance of energy homeostasis (Choi et al. 2013; Coutinho et al. 2017), and SF-1 knockout within the VMH induced obesity and impaired

adipose thermogenesis (Kim et al. 2011b). SF-1 neurons are glutamatergic and their excitatory actions appear to be important in regulating insulin-induced hypoglycemia (Tong et al. 2007). Additionally, SF-1 neurons are leptin-sensitive (Sohn et al. 2016) and deletion of leptin receptors in SF-1 neurons promotes diet-induced obesity (Dhillon et al. 2006; Bingham et al. 2008).

1.3 Importance of the Problem

The implications of OPFRs as EDC capable of interfering with typical endocrine system function make OPFRs a concern for dysregulated energy homeostasis. The disruption of energy homeostasis increases risk of developing metabolic disorders such as type 2 diabetes and metabolic syndrome. As defined by the World Health Organization (WHO) in 1999, metabolic syndrome is a collection of metabolic symptoms, characterized by insulin resistance, elevated triglycerides, blood pressure, body-mass index, elevated waist-to-hip ratio, and a lowered concentration of high-density “good” cholesterol (Parikh and Mohan 2012). Metabolic syndrome is considered a symptomatic pre-disposition for serious health risk disorders such as heart disease and diabetes, and its prevalence is now estimated to be roughly one quarter of the world population (Saklayen 2018).

Briefly, type 2 diabetes is categorized by the inability to properly respond to circulating glucose levels due to insulin insufficiency or insensitivity, primarily in liver, muscle, and adipose tissue (Olokoba et al. 2012). The onset of diabetes is highly correlated to obesity and may require daily injection of insulin to maintain safe glycemic indices (Pharmacologic Approaches to Glycemic Treatment, 2019). If left untreated, diabetes is a life-threatening condition that affects approximately 400 million individuals worldwide and was the seventh leading cause of death in the U.S. in 2010 (Olokoba et al. 2012; Stokes and Preston 2017).

The prevalence of both diabetes and metabolic syndrome has been steadily increasing worldwide, and are no longer viewed as diseases of the affluent (Moore et al. 2017; Rowley et al. 2017). Increased consumption of a diet high in fat, termed “the western diet”, is often attributed to the increasing rates of obesity and other disruptions of energy homeostasis (Kopp 2019; Naja et al. 2015; Gutiérrez-Fisac et al. 2006). However, concurrent with this mounting global health concern is the rise in human exposure to man-made EDCs. EDCs, like OPFRs, that are capable of disrupting endogenous endocrine function therefore may play a role in sensitizing the global population to the adverse health effects conferred by consumption of a western diet.

1.4 OPFRs and Energy Homeostasis

The three OPFRs that will be the focus of this dissertation are triphenyl phosphate (TPP), tricresyl phosphate (TCP), and tris(1,3-dichloro-2-propyl)phosphate (TDCPP). These OPFRs were selected for their widespread use within commercial FR mixtures, such as Firemaster® 550, and for their EDC capacity through interaction with nuclear receptors governing energy homeostasis. Refer to Figure 1 for the chemical structure of the selected OPFRs.

1.4.1 Firemaster® 550

Most scientific explorations of FR toxicity use doses above the environmental daily exposure of the respective chemical, and one particular study highlighted this issue in a study using collected house dust and assessing endocrine disruption of thyroid hormone (TH) signaling (Kollitz et al. 2018). They found that house dust containing a mixture of FRs significantly antagonized TH signaling, whereas individually isolated FRs from the dust had no marked effect on their own. This study supports the idea of what is called “the

cocktail effect" (Barouki 2017; Balaguer et al. 2017; Sargis 2015), which hypothesizes that mixtures of EDCs convey additive and synergistic effects to their toxicity. We live in a world where man-made chemicals are integrated into everyday life, exposing humans to a multiplicity of diverse EDCs, and the chronic accumulation of many sub-threshold exposures may easily translate into significant disruptions of endocrine function later in life.

In particular, FRs are ubiquitously used as a mixture, and their combined EDC actions may contribute to their disruptive effects. One common commercial FR mixture, Firemaster® 550 (550), is increasingly indicated as a disruptor of energy homeostasis. FM550 contains a mixture of brominated and organophosphate FRs, including TPP. In rats, FM550 accumulates within exposed dams and offspring inducing phenotypic hallmarks of metabolic syndrome (Patisaul et al. 2013). FM550 is also shown to induce adipogenesis, supposedly exerting its effects through PPARγ (Tung et al. 2017a). It is also implicated in behavioral effects on activity, conferring a "reversal of sex differences" whereas FM550 exposure induced hyperactivity in females and heightened anxiety-associated behaviors in males (Baldwin et al. 2017). Behavioral effects are also seen in zebrafish, where developmental FM550 exposure reduced social behavior and caused hypoactivity (Bailey and Levin 2015).

1.4.2 Triphenyl Phosphate (TPP)

Wang et al. (2019) developmentally exposed mice to TPP and reported increased body weight and fat mass, greater hepatic steatosis, and impaired glucose homeostasis and insulin resistance. Furthermore, gut microbial genes showed altered expression of genes involved in lipid metabolism, in particular lipogenesis and fat accumulation (Wang et al. 2019). These results indicate TPP as a developmental obesogen capable of initiating metabolic dysregulation in adult mice. Adipocyte function is implicated by a number of

studies, and TPP is shown to induce cultured adipocyte differentiation, lipolysis, and glucose uptake, coinciding with increased transcription of peroxisome proliferator-activated receptor (PPAR) γ, a genomic regulator of adipose function and metabolism (Cano-Sancho et al. 2017). Furthermore, in a rat model of type 2 diabetes, maternal TPP exposure is shown to accelerate the onset of diabetes and increase food intake and augment obesity and fat mass, coinciding with increased leptin levels (Green et al. 2017). In a recent study, Wang et al. (2020) demonstrated a sexual dimorphic action of TPP to induce glucose intolerance via inhibition of insulin-sensitizing hormone adiponectin in female mice only, following five weeks of oral dosing (Wang et al. 2020). The same group also previously demonstrated sex-dependent dysregulation of metabolic profiles in pubertal mice (Wang et al. 2018a). Metabolic disruption is also indicated in larval zebrafish models, where TPP is found to upregulate thyroid hormones T3 and T4 and associated genes regulating their synthesis (Kim et al. 2015). Interestingly, in adult zebrafish, circulating T3 and T4 was lowered by TPP and TDCPP exposure in males, but elevated within females (Liu et al. 2019). This study also observed sex-dependent associated alterations of brain CRH and TSH expression, indicating the potential for OPFR exposure in disrupting the entire hypothalamus-pituitary-thyroid axis.

Overall, the collective knowledge of TPP disruption of energy homeostasis is overwhelmingly based in developmental studies. This mindset makes sense, as development is a critically sensitive window where disruptions of endocrine function can severely alter the process of maturation and the establishment of functional homeostatic regulation in adulthood. However, human exposure to EDCs such as OPFRs is not restricted to development, and the chronic accumulation of EDC exposure throughout the lifetime poses risk for significant alterations to homeostatic function. Therefore, it is important that future research address the gap in knowledge of the toxicological impact of adult exposure to OPFRs.

1.4.3 Tricresyl Phosphate (TCP) and Tris(1,3-dichloro-2-propyl)phosphate (TDCPP)

TCP and TDCPP are vastly lesser studied OPFRs than TPP, and their toxicological impact on energy homeostasis is therefore less defined. Tri-ortho-cresyl phosphate (TOCP), a specific isomer of TCP, is a documented neurotoxicant (Aldridge 1954; Reichert and Abou-Donia 1980; Song et al. 2012) and is shown to impact placental development (Yang et al. 2020), and has since been phased out of commercial TCP mixtures. Current TCP mixtures contain less than 5% TOCP and these mixtures are not shown to exhibit neurotoxicity. TCP have not yet been examined for effects on energy homeostasis. There exists minimal literature on TDCPP as well; however, human exposure data correlates with altered thyroxine (Meeker and Stapleton 2010) and in zebrafish, it is observed to increase circulating T3 and T4 and alter expression of thyroid-associated genes in a dose-dependent manner (Liu et al. 2019). These results are preceded by another study, which initially found that TDCPP altered expression of many genes important in regulation of the hypothalamus-pituitary-gonad axis, such as aryl-hydrocarbon receptor, thyroid hormone receptor, glucocorticoid receptor, melanocortin receptor, estrogen receptors, and PPARγ (Liu et al. 2013a). Despite the lack in toxicological data for TCP and TDCPP, they still pose a credible risk for disruption of endocrine function. This risk is attributed to 1) the abundance of human exposure and 2) their ability to interact with nuclear receptors such as listed in the 2013 study by Liu et al.

1.4.4 OPFRs interact with ERα and PPARγ

OPFRs were first identified as likely EDC through their ability to interact with nuclear receptors such as estrogen receptor alpha (ERα) and peroxisome proliferator-activated receptor gamma (PPARγ). Both ERα and PPARγ are key regulators of energy

homeostasis, and disruption of their endogenous signaling by OPFRs may lead to metabolic disorders such as diabetes and obesity (Hevener et al. 2015; Botta et al. 2018). A number of *in vitro* assays have determined agonistic activity of TPP upon both ERα and PPARγ (Tung et al. 2017b; Hu et al. 2017; Kojima et al. 2016; Kojima et al. 2013) and was further shown to induce adipogenesis through PPARγ activation (Pillai et al. 2014). TPP also augments transcription of PPARγ, resulting in increased adipocyte differentiation and glucose uptake (Cano-Sancho et al. 2017). Additionally, the commercial OPFR mixture FM550 containing TPP demonstrates significant agonistic activity on PPARγ and resulting adipocyte proliferation (Belcher et al. 2014; Tung et al. 2017a). Furthermore, long-term exposure to TPP in zebrafish elevated plasma 17β-estradiol (E2), as well as differentially altered expression of TRH, thyroxin, POMC, and mineralocorticoid receptor transcripts depending on sex (Liu et al. 2016).

TDCPP and TCP have also been shown to activate and alter expression of both ERα and PPARγ (Liu et al. 2013a; Kojima et al. 2013). Recently, TCP has also been demonstrated to activate the membrane-bound G protein-coupled estrogen receptor (GPER) (Ji et al. 2020). While most studies demonstrated OPFRs as having agonistic activity on nuclear receptors, in another study, TPP, TCP, and TDCPP all demonstrated antagonistic capacity through inhibition of E2 binding to estrogen receptors (ERs), leading to increased plasma E2 and testosterone (Liu et al. 2012b). One possible explanation for mixed agonistic/antagonistic literature could be that OPFRs are capable of weak agonistic action upon ERs, and their ER binding competes with endogenous E2, conferring an antagonistic observation of decreased E2 signaling.

Together, these data make for compelling evidence for OPFR EDC capacity in disrupting ERα and PPARγ signaling pathways important in the regulation of energy homeostasis. Figure 1 visually represents the overall hypothesis that OPFRs are acting

on ERα and PPARγ both peripherally and centrally through hypothalamic neurons that direct feeding behavior and energy expenditure.

1.5 Estrogen and Energy Homeostasis

Estrogen (E2) is most known for its role in reproduction; however, it is also an essential regulator of energy homeostasis. The process of reproduction requires a great deal of energy and estrogen signaling helps coordinate the two homeostatic functions to ensure proper energy availability during pregnancy, and to inhibit unnecessary reproductive function during times of starvation.

In females, the majority of E2 is produced from the ovaries. The onset of menopause and its associated decreased ovary function causes a drop in circulating E2, which is has well-documented effects on core temperature, i.e. energy expenditure, and weight gain. In animals, ovariectomy (OVX) leads to increased body weight gain and fat tissue accumulation (Litwak et al. 2014; Blaustein and Wade 1976). OVX does result in moderate increases in food intake (Iwasa et al. 2018), however, it appears that OVX-induced weight gain is more attributable to decreased activity and energy expenditure (Messina and Overton 2006; Rogers et al. 2009). Regardless, reductions in estrogenic signaling are attributable to disturbances of energy homeostasis, as is clearly demonstrated in ERα deficient mouse models which exhibit an obese phenotype and marked reductions in activity and energy expenditure (Heine et al. 2000; Ribas et al. 2010). ERα KO mice exhibit reduced activity and metabolic rates ($V.O_2$, energy expenditure) compared to wild-type littermates leading to greater adiposity and are resistant to the anorexigenic effects of E2 (Mamounis et al. 2014). Additionally, the symptomatic onset of metabolic syndrome is

accelerated during menopause when circulating estrogen levels drastically decline (Carr 2003).

Estrogenic signaling occurs through a variety of ERs, with ERα being the most common regulator of energy homeostasis. Briefly, E2 binds to ERα, initiating a conformational change to allow subsequent translocation to the nucleus where it binds to the Estrogen Response Element (ERE) to stimulate transcriptional regulation of energy homeostasis (Fuentes and Silveyra 2019). ERα also exists within the cellular membrane, and its receptor binding initiates a separate mechanism of transcriptional activation, as well as non-genomic alterations such as membrane ion channel modification (Fuentes and Silveyra 2019). Importantly, activation of ERE-independent signaling is shown to protect against diet-induced obesity (Yasrebi et al. 2017). Outside of ERα, there is also ERβ, another nuclear hormone receptor, as well as alternative membrane G protein-coupled receptors. Both G protein-coupled estrogen receptor (GPER) and the putative Gq-coupled membrane estrogen receptor (Gq-mER) are capable of eliciting rapid cellular responses to E2 and aid in the regulation of energy homeostasis (Shi et al. 2013; Prossnitz and Hathaway 2015; Roepke et al. 2010; Mauvais-Jarvis et al. 2013).

Estrogenic regulation of energy balance is largely controlled via central actions on neurons governing feeding and energy expenditure. Primarily, this consists of POMC and NPY neurons within the hypothalamic ARC nucleus. E2 is considered an overall anorexigenic hormone, and it excites anorexigenic POMC neurons (Stincic et al. 2018) through their expressed ERα (Xu et al. 2011; Santollo et al. 2012). Conversely, while NPY neurons are not known to express ERα, E2 suppress NPY expression and release (Frank et al. 2014; Crowley et al. 1985), and inhibits NPY neuronal excitability through augmentation of a hyperpolarizing potassium current known as the M-current (Roepke et al. 2011).

Other hypothalamic neurons that express ERα are ARC KNDy (Kisspeptin, Neurokinin B, and Dynorphin expressing) neurons (Wang et al. 2018b). These neurons help regulate ARC control of energy homeostasis through direct glutamatergic excitation of POMC neurons and indirect GABAergic inhibition of NPY neurons (Fu and van den Pol 2010; Nestor et al. 2016). Importantly, these actions are augmented by E2, demonstrating an estrogenic mechanism for KNDy regulation of energy homeostasis (Qiu et al. 2018). In addition, steroidogenic factor 1 (SF-1) neurons in the VMH also express ERα, and its selective knockout results in abdominal obesity (Xu et al. 2011). Orexin and MCH neurons in the LH do not express ERα, but their actions do appear to be influenced by estrogen (Muschamp and Hull 2007).

Estrogen is also important in peripheral regulations of energy homeostasis. ERα is expressed in adipose tissue and E2 helps direct fat accumulation and distribution (Kim et al. 2014), and plays important roles in maintaining glucose homeostasis, as exemplified by global ERα knockout (Bryzgalova et al. 2006). Estrogen also intersects with central leptin signaling, and its deficiency is indicated in leptin-insensitivity (Ainslie et al. 2001). The interaction of estrogen and ghrelin levels is less clear. Some studies report that estrogen replacement therapy increases circulating ghrelin, whereas others report no such effect (Kellokoski et al. 2005; Dafopoulos et al. 2010; Sakata et al. 2006). However, ghrelin and E2 signaling do appear to coincide in the regulation of KNDy neurons. Ghrelin excites KNDy neurons and this excitation is mediated by E2 (Frazao et al. 2014; Yang et al. 2016; Qiu et al. 2018). Lastly, ERα has reported interactions with mitochondrial DNA to direct cellular metabolism and energy expenditure (Gupte et al. 2015; Chen et al. 2004).

1.6 PPARγ and Energy Homeostasis

PPARγ is often referred to as the “master regulator of adipogenesis,” and regulates energy homeostasis primarily through metabolic pathways within adipose cells (Shao et al. 2016). As reviewed by Lamichane et al. (2018), these receptors were first identified and named for their induction of peroxisome proliferation (Lamichane et al. 2018). Peroxisomes are small organelles within cells that contain oxidizing enzymes primarily used in the breakdown of fatty acid chains through beta oxidation. In addition to directing fatty acid metabolism, PPARγ can also sense and respond to systemic lipid state through agonistic binding of endogenous lipid ligands (Grygiel-Górniak 2014). This confers a regulatory mechanism for its actions, and also demonstrates how diet-induced obesity and its associated heightened lipid-load may influence PPARγ activity. PPARγ is also activated by eicosanoids, which are important signaling molecules in inflammation pathways (Grygiel-Górniak 2014). Obesity is increasingly described as a chronic state of inflammation (Lee et al. 2013; Ellulu et al. 2017), providing another link for the intersection of PPARγ and dysregulated energy homeostasis.

PPARγ binding of endogenous ligands such as essential fatty acids and eicosanoids induces transcriptional activation of genes involved in systemic energy homeostasis. Briefly, ligand binding causes PPARγ to heterodimerize with the retinoid X receptor, and the resulting conformational change allows the heterodimer to bind to PPAR response elements in both cellular and mitochondrial DNA. Response element binding facilitates transcription of genes regulating fatty acid uptake, lipid partitioning, adipogenesis, as well as stimulating expression of uncoupling proteins (UCPs) involved in adipocyte mitochondrial production of heat, important for thermoregulation and also implicated in energy expenditure (Lamichane et al. 2018; Ricquier and Bouillaud 2000).

While peripheral PPARγ is predominantly expressed in adipose tissue, it is also present within the liver, where it appears to play a role in the development of steatosis. Upregulated *Pparg* is associated with a high-fat diet induction of steatosis (Inoue et al. 2005), and knockout of hepatic *Pparg* ameliorates diet-induced steatosis (Lee et al. 2018). PPARγ also plays an essential role in the development and subsequent treatment of type 2 diabetes mellitus (Wang 2010). Insulin resistance is thought to be instigated, in part, by dysregulated storage of triglycerides within skeletal muscle (Ginsberg et al. 2005; Pang et al. 2014). Overproduction of triglycerides can be brought upon by disordered regulation of the metabolic processing of fatty acids, which is largely controlled by PPARγ. Consequently, PPARγ is now a major therapeutic target via synthetic agonists called thiazolidinediones (TZDs) for the treatment of insulin resistance (Auwerx et al. 1996). An associated side effect of TZDs is weight gain, which is largely attributed to PPARγ stimulation of adipogenesis (Wilding 2006). However, modest TZD-induced hyperphagia does appear to contribute to the reported weight gain (Shimizu et al. 1998), and these actions appear to be regulated by interaction with hypothalamic feeding pathways (Lu et al. 2011).

Both NPY and POMC neurons express PPARγ (Sarruf et al. 2009), and third ventricle injection of PPARγ agonist rosiglitazone triggers feeding behavior in mice and hamsters (Garretson et al. 2015). Conversely, brain-specific deletion of the receptor confers resistance to diet-induced obesity and abolishes the effects of TZD rosiglitazone in mice (Lu et al. 2011). A specific TZD route is further confirmed by Long et al. (2014) in which POMC-specific PPARγ knockout mice failed to respond to rosiglitazone (Long et al. 2014). When fed a high-fat diet, these mice also exhibited improved leptin sensitivity and increased energy expenditure and activity, leading to reduced food intake and less weight gain and fat mass. Collectively, these data demonstrate a specific role for PPARγ in

regulating the anorexigenic actions of POMC neurons. In this case, PPARγ appears to play an inhibitory function in POMC neurons, restricting their excitability, perhaps through limiting their responsiveness to leptin activation.

Additionally, Li et al. (2018) reported that adenoviral-directed overexpression of hypothalamic PPARγ reduced food intake as well as diminished hypothalamic and plasma ghrelin levels. PPARγ suppression of ghrelin was also replicated in cultured embryonic hypothalamic mHypoE-42 cells, through enhanced mTORC1 signaling (Li et al. 2018). Ghrelin is also implicated in hepatic mTOR-PPARγ pathways, where its receptor binding influences adipogenesis associated with liver steatosis (Li et al. 2014; Rasineni et al. 2020).

1.7 The Hypothesis

Figure 1 visually represents the general hypothesis of this dissertation. We hypothesize that OPFR interaction with ERα and PPARγ sex-dependently disrupts endocrine regulation of energy balance through peripheral and central means. Specifically, we hypothesize that OPFR is dysregulating hypothalamic melanocortin circuitry regulating feeding behavior and energy expenditure. Further, while it is understandable that majority of EDC research focuses on developmentally sensitive exposures, the ubiquitous presence of OPFRs within the modern environment calls for a need to explore the lifetime adverse effects of these chemicals.

The modern environment exposes humans to a multiplicity of man-made chemicals, and it is becoming increasingly clear that exposure safety cannot wholly be determined by the no observable adverse effects levels (NOAELs) of individual chemicals. This is especially true for EDCs, which exhibit a propensity for additive and synergistic

interactions. The “cocktail effect” theorizes that chronic, sub-threshold exposures to EDCs will accumulate into significant adverse effects later in life. In 2012, the World Health Organization (WHO) released a report on the current state of EDC science, specifically calling for “more comprehensive assessments of human and wildlife exposures to diverse mixtures of EDCs”. For these reasons, this dissertation will examine the effects of subchronic exposures to a mixture of common OPFRs TPP, TCP, and TDCPP in adult male and female mice.

1.8 Preliminary Data

In 2018, our lab published an exploratory study of OPFR effects in adult mice using the same mixture of TPP, TCP, and TDCPP (Krumm et al. 2018). This study used two exposure doses, 1 and 10 mg/kg bw/day of each TPP, TCP, and TDCPP in a mixture. These doses were initially chosen based off a 2013 study by Patisaul et al., which reported dose-dependent adverse effects of maternal FM550 exposure ranging from 0.03 to 3 mg/kg bw/day. Since then, our lab has adopted a 1 mg/kg bw/day approach to study the EDC effects of OPFRs. Data from dust collection samples identify OPFR concentrations ranging from 100 ng/g to 100μg/g (Hoffman et al. 2017; Betts 2013; Dishaw et al. 2014; Meeker and Stapleton 2010), which places typical human daily exposure approximately 10-1,000 fold less than 1 mg/kg bw/day. Our scientific choice to use greater than human environmental exposures is rationalized by the need for a mechanistic understanding of how OPFRs interact with and disrupt endogenous endocrine function. However, human exposure data reports serum OPFRs and their metabolites at 680-709 ng/ml (Ma et al. 2017). Interestingly, in our preliminary study, Krumm et al. observed serum concentrations of only 2-5 ng/ml within adult mice, ~100x lower than in human samples, perhaps a result of higher metabolic rates in mice, than within humans. This supports a justified use of 1

mg/kg bw/day as a scientifically appropriate dosage to examine human-relative exposure. And importantly, this dose is shown to be effective in eliciting sex-dependent alterations to adult energy homeostasis (Krumm 2018).

Our previous study used adult, intact male and ovariectomized (OVX) female mice fed a standard chow diet and orally exposed to a 1 mg/kg bw/day each of TPP, TCP, and TDCPP (Krumm 2018). This study observed a reduction in food intake by male mice, and a corresponding decreased weight gain over the four-week exposure. Conversely, OVX female mice displayed increased weight gain, but only when exposed to the high dose of 10 mg/kg. Interestingly, male mice also demonstrated elevated plasma ghrelin, and reduced insulin and leptin levels, whereas ghrelin was diminished in OVX females. Additional sex-specific results were observed in hypothalamic expression of genes involved in energy homeostasis, to which male mice exhibited more alterations than OVX females. Adult male mice exhibited decreased hypothalamic expression of *Npy* and increased expression of *Pomc*. Interestingly, *Agrp* was increased, while *Cart* was decreased, suggesting an unbalanced dysregulation of NPY and POMC neuronal production of neuropeptides. Furthermore, male mice showed striking upregulation of hormone receptors for insulin, leptin, and ghrelin, indicating potential increased hypothalamic sensitivity to circulating markers of energy state. *Kiss1* expression was augmented in both sexes, implying potential increased KNDy neuronal activity. Also examined were hypothalamic expression of neuronal cation channels important in regulating ARC neuronal excitability. Male mice saw upregulation of potassium channel KCNQ subunit genes, T-type calcium channel subunits, and nonselective cation channel TRPC5. These findings indicated the potential for OPFR to excite neurons governing hypothalamic regulation of energy homeostasis. Lastly, male mice exhibited reduced expression of *Esr1* (ERα) transcripts, whereas both sexes experienced increased expression of *Pppag*. These data indicate that OPFR exposure is capable of eliciting

striking sex-dependent alterations to energy homeostatic pathways, with a marked potential mechanism being through ERα and/or PPARγ interaction.

Overall, this study provided a mechanistic foundation and compelling incentive for future detailed examination of the EDC impact of adult OPFR exposure on energy homeostasis. While there exist numerous publications of OPFR developmental toxicity, to date, this study represents the only published mammalian exploration of OPFR impact on adult regulation of energy balance. The focus of this dissertation will help address this knowledge gap through the combined utilization of rigorous physiological, behavioral, and metabolic experimentation with targeted neuronal examination of the arcuate circuitry governing energy homeostasis.

1.9 Summary and objectives of the dissertation

This dissertation aims to determine the neuroendocrine and physiological impacts of OPFRs on energy homeostasis in adult mice. The objectives of the dissertation are summarized below:

Objective 1: To determine the impact of adult exposure to OPFRs on physiological and metabolic parameters in a model of diet-induced obesity using intact male and female mice. Mice were treated for 4 weeks with OPFR mixture and then assessed for effects on weight gain, adiposity, metabolism, activity, ingestive behaviors, glucose and insulin tolerance, and plasma hormone levels. We found sex-dependent alterations of energy homeostasis, in particular weight gain, adiposity, activity, and fluid and food intake patterns. (Chapter 2)

Objective 2: To assess the interactions of nuclear receptors ERα and PPARγ with OPFR exposure and diet-induced obesity utilizing both global ERα, and brain-specific PPARγ knockout models. Adult male and female mice were treated for 4 weeks with OPFR mixture and then assessed for disruption of energy homeostasis by examining OPFR effects on weight gain, adiposity, ingestive behaviors, and glucose and insulin tolerance. We characterized the role ERα and PPARγ may play in mediating OPFR-induced dysregulation of energy homeostasis. (Chapter 3)

Objective 3: To determine the neuroendocrine impact of adult OPFR exposure on hypothalamic neurons governing energy balance. Mice were exposed to OPFRs for 4 weeks and then electrophysiologically tested for altered neuronal function using GFP-NPY and EGFP-POMC transgenic mice for targeted examination. We found that OPFR exposure increased neuronal output from the melanocortin circuit. (Chapter 4)

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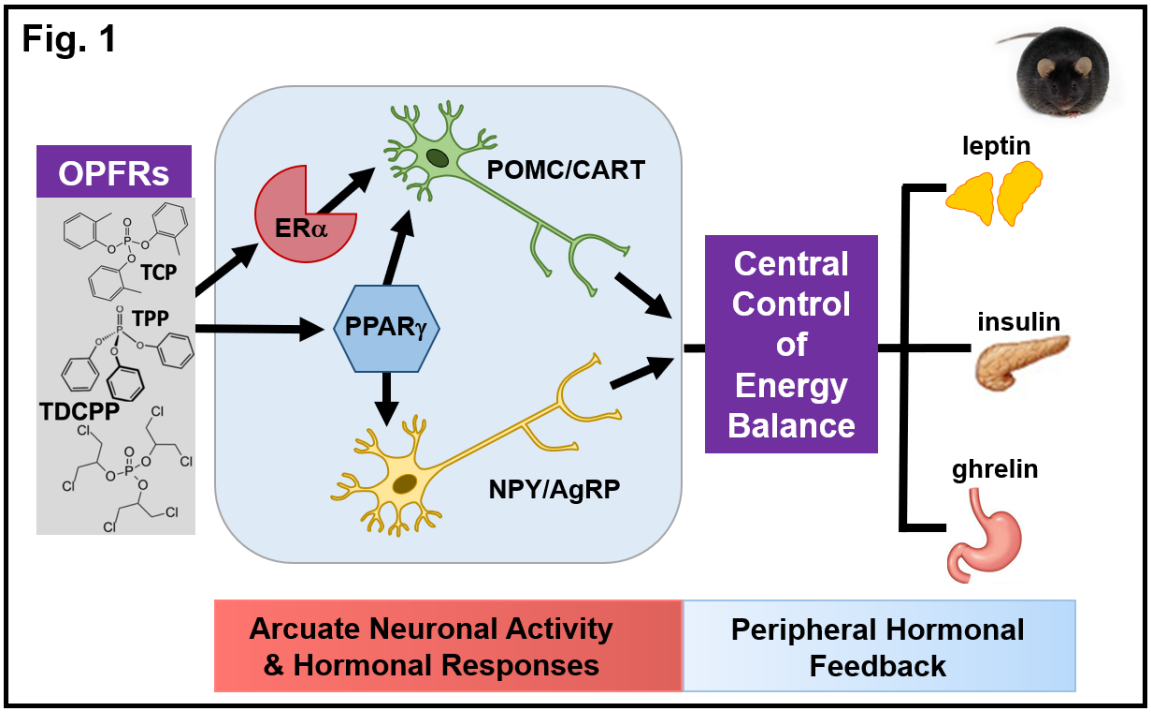
Figures

Figure 1



Schematic representation of the dissertation hypothesis. OPFRs tricresyl phosphate, triphenyl phosphate, and tris(1,3-dichloro-2-propyl)phosphate (TCP, TPP, and TDCPP, respectively) interact with hormone receptors estrogen receptor α (ERα) and peroxisome proliferator-activated receptor γ (PPARγ) within proopiomelanocortin/cocaine and amphetamine-regulated transcript (POMC/CART) and neuropeptide Y/Agouti-related peptide (NPY/AgRP) neurons, disrupting the central control of energy homeostasis that is informed and influenced by peripheral hormones leptin, insulin, and ghrelin.

CHAPTER 2: THE INTERACTIONS OF DIET-INDUCED OBESITY AND ORGANOPHOSPHATE FLAME RETARDANT EXPOSURE ON ENERGY HOMEOSTASIS IN ADULT MALE AND FEMALE MICE

2. The interactions of diet-induced obesity and organophosphate flame retardant exposure on energy homeostasis in adult male and female mice

2.1 Abstract

Endocrine disrupting chemicals (EDC) are becoming increasingly prevalent in the environment and many are shown to accumulate within human tissues and interact with endogenous hormone receptors. One such EDC is organophosphate flame-retardants (OPFR). OPFR interact with multiple hormone receptors involved in homeostasis, including estrogen receptor (ER) α and peroxisome proliferator-activated receptor (PPAR) γ. We have previously reported sex-dependent alterations of energy homeostasis attributed to OPFR exposure via ERα in adult mice fed a standard chow diet. Dysregulation of energy homeostasis deters adaptability to metabolic perturbations, which may increase susceptibility to disorders such as metabolic syndrome. In the current study, we repeated the previous adult exposure experiment with a common mixture of 1 mg/kg bw each of tricresyl phosphate (TCP), triphenyl phosphate (TPP), and tris(1-3-dichloro-2propyl)phosphate (TDCPP) for 7 weeks while adding a metabolic variable of diet. Mice were fed either a low-fat diet (LFD, 10% kcal fat) or a high fat (HFD, 45% kcal fat) to generate a diet-induced obesity model. We recorded body weight, crude food intake, body composition, metabolic rate, locomotor activity, meal patterns, glucose and insulin tolerance, and plasma peptide hormone levels. Consistent with our previous observations, OPFR altered weight gain in LFD-fed males, differentially with diet, while females remained unaffected. OPFR treatment also revealed subtle sex-dependent perturbations

in metabolic activity. During the night (~0100-0400 h), males exhibited elevated oxygen consumption, indicating higher metabolic activity, while in females these parameters were decreased, irrespective of diet. In females, OPFR disrupted feeding behavior and abolished diurnal drinking patters, whereas nighttime fluid consumption was increased in male mice. Despite no marked effect of OPFR on glucose or insulin tolerance, OPFR treatment altered circulating levels of insulin and leptin in females, and ghrelin within males. In summary, these data indicate that adult OPFR exposure can influence, and perhaps exacerbate, the effects of diet-induced obesity in adult mice by altering activity, ingestive behavior, and metabolism.

2.2 Introduction

Metabolic syndrome, a constellation of conditions including obesity, hypertension, dyslipidemia, and pre-diabetes, has emerged as a national health crisis affecting over 90 million adults and costing over $100 billion each year (Boudreau et al. 2009). A major factor underlying the alarming rise in metabolic syndrome is the consumption of western diets high in fat and sugar (Drake et al. 2018; Rodriguez-Monforte et al. 2017; Moreno-Fernandez et al. 2018). However, it is also clear that diet is not the only factor. Environmental exposure to endocrine-disrupting compounds (EDCs) that perturb nutrient and hormone metabolism through central and peripheral actions has been postulated to promote metabolic syndrome (Decherf and Demeneix 2011). Indeed, investigators demonstrated that exposure to EDCs or metabolic disruptors increase sensitivity to adverse health outcomes induced by a western diet (Brulport et al. 2017; Strakovsky et al. 2015; Grun and Blumberg 2009; Mackay et al. 2013). One group of ubiquitous EDCs are flame retardants used in the production of electronics, furniture, toys, and foodstuffs (Yang et al. 2019; Peng et al. 2020; Young et al. 2018; Li et al. 2019). The flame retardant

market used to be dominated by polybrominated diphenyl ethers (PBDE), before being phased out of American and European production in 2004 due to neurological and metabolic health concerns (Zota et al. 2011; Herbstman et al. 2010; Gilbert et al. 2012; Shaw et al. 2010).

Recently, an alternative class of retardants, organophosphate flame retardants (OPFRs) have emerged as a leading replacement for PBDEs. Despite already existing toxicity data on these flame retardants, OPFRs quickly became widely used among home furnishing manufacturers resulting in widespread human exposure. OPFRs are embedded in household products and released into domestic and workplace dust through which humans are exposed, primarily via inhalation and ingestion, resulting in detectable levels in human serum (680-709 ng/g lipid), urine (1-10 ng/ml), and breast milk samples (1-10 ng/ml) (Ma et al. 2017; Butt et al. 2014; Meeker et al. 2013; Hoffman et al. 2017; Ma et al. 2019). While OPFRs are not yet shown to accumulate within adipose tissue to the same degree as do PBDEs, aryl OPFRs are hydrophobic (Yang et al. 2019) and do demonstrate accumulation in other biological tissues (Hou et al. 2017; Ma et al. 2013). Further, data also demonstrated OPFR ability to interact with nuclear receptors important in the pathogenesis of metabolic syndrome (Gray et al. 2005; Pap et al. 2016; Belcher et al. 2014; Pillai et al. 2014), leading to concern over potential long-term adverse health effects.

Homeostatic regulation of feeding behaviors and energy balance is a complex system but predominantly controlled via neuroendocrine pathways originating in the hypothalamus (Waye and Trudeau 2011). Briefly, the hypothalamus consists of multiple nuclei in which discrete neuronal subgroups communicate with each other to integrate peripheral indicators of energy state (Williams et al. 2001). With inputs from the limbic forebrain, the hypothalamus synthesizes both emotional and reward aspects of feeding drive and communicates with the hindbrain for execution (Grill and Hayes 2012; Berthoud 2002). Within the hypothalamus lies the arcuate nucleus (ARC) which sits adjacent to a

leaky portion of the blood-brain-barrier, and thus its neurons are in a unique position to directly sense energy state through peripheral signals such as glucose, insulin, leptin, and ghrelin (Schwartz et al. 2000; Saper et al. 2002). ARC neurons express receptors for these molecules, and their combined inputs to the paraventricular nucleus (PVN) and lateral hypothalamus (LH) to help dictate food intake (Arora and Anubhuti 2006; Nahon 2006). Because hypothalamic control of energy homeostasis is highly regulated through hormone signaling pathways including estrogen receptor (ER) α and peroxisome proliferator-activated receptor (PPAR) γ (Sarruf et al. 2009; Garretson et al. 2015; Roepke et al. 2011), any EDC, such as OPFR, that interact with these receptors may alter the complex balance of these pathways, increasing susceptibility to metabolic disorders such as obesity and diabetes.

While there has been increasing focus of epidemiological and toxicological data surrounding OPFR exposure, physiological influences of OPFRs on energy homeostasis in adult mammals is underexplored. While most endocrine disruption studies focused on developmental exposure, it is important to understand the mechanistic role of EDC exposures throughout the lifespan, including adulthood. Little is yet known on how adult exposure to OPFR may interact with neuroendocrine control over energy homeostasis; however, in our previously published exploratory study, adult, sub-chronic OPFR exposure decreased body weight gain and energy intake in intact male mice (Krumm et al. 2018). Further, expression of genes central to hypothalamic control of energy homeostasis were markedly altered by OPFR exposure, differentially in intact males and ovariectomized female mice (Krumm et al. 2018). These data indicate a crucial need for further investigation before OPFRs should be presumed any safer than their PBDE predecessors.

Since ERα and PPARγ receptors are highly expressed in the ARC and hypothalamus as a whole, and because OPFR are known to interact with these receptors, OPFR may

be disrupting energy homeostasis as a consequence of these interactions. Previously Krumm et al (2018) used triphenyl phosphate (TPP), tricresyl phosphate (TCP), and tris(1-3-dichloro-2propyl)phosphate (TDCPP) in a mixture of 1 mg/kg of each OPFR. This mixture was selected because of the prevalent human exposure to these OPFRs, simultaneously, at similar levels (Yang 2019). This was considered particularly important as there may be competing or potentiating actions of either parent compounds or their metabolites on ERα and PPARγ, as the proposed route of pathogenesis. Since Krumm et al (2018) reported that exposure of adults to OPFR produced sex-specific changes in body weight, peripheral peptide hormone expression, and gene expression, it was of interest to determine whether this may translate to an increased sensitivity to diet-induced obesity attributed to effects on feeding behavior, fat accumulation, metabolism, and activity patterns. Thus, the aim of this study was to investigate these parameters in intact adult male and female mice with or without a high-fat diet (HFD) challenge for 7 weeks with continuous daily oral dosing of the same 1mg/kg bw OPFR mixture. This dose was selected to be consistent with previous literature (Krumm et al. 2018; Patisaul et al. 2013; Wang et al. 2019b), and because Krumm et al (2018) reported murine serum concentrations of TPP, TCP, and TDCPP similar to that found within human serum samples.

2.3 Methods

Animals

All animal experiments were approved by the Rutgers University Institutional Animal Care and Use Committee and followed guidelines based on National Institutions of Health standards. Female and male wild-type (WT C57BL/6J; Taconic) mice bred in-house and provided food and water *ad libitum* under controlled temperature (23 °C) and light cycle

(12/12 h light/dark cycle). At weaning, animals were ear-tagged for identification and fed a standard low-phytoestrogen chow diet (Lab Diets 5V75) until the start of the experiment.

Diets

To examine the effects of adult OPFR exposure on a mouse model of diet-induced obesity, mice were fed either a low-fat diet (LFD, 3.85 kcal/g, 10% fat, 20% protein, 70% carbohydrate; D12450H) or high-fat diet (HFD, 4.73 kcal/g, 45% fat, 20% protein, 35% carbohydrate; D12451; Research Diets). Mice were fed LFD or HFD concurrently with OPFR treatment starting at 10 weeks of age and separated into weight-matched groups (male: CONTROL – 27.4 ± 0.6 g, OPFR – 27.1 ± 0.4 g; female: CONTROL – 20.6 ± 0.3 g, OPFR – 20.9 ± 0.2 g) and dosed for the duration of the study.

Organophosphate flame retardants (OPFRs) Dosing

The OPFR mixture consisted of 1 mg/kg bw each of tricresyl phosphate (TCP, CAS no. 1330-78-5; purity = 99%) which was purchased from AccuStandard (New Haven, CT), and purchased from Sigma-Aldrich (St. Louis, MO) were triphenyl phosphate (TPP, CAS no. 115-86-6; purity 99%) and tris(1,3-dichloro-2-propyl)phosphate (TDCPP, CAS no. 13674-87-8; purity 95.6%). One hundred (100) mg of each OPFR were dissolved together in the same 1 ml of acetone (Sigma-Aldrich) for long term storage. One hundred (100) μl of this stock was then transferred to 10 ml of sesame oil (Sigma-Aldrich) to create a 1 mg/ml mixture of OPFR-oil. Vehicle control-oil was produced by adding 100 ml acetone to 10 ml sesame oil. Each mixture was stirred for 48-72 h to evaporate the acetone from the mixture. For daily dosing, 1 ml/g bw of OPFR mixture or vehicle control-oil was mixed with powdered peanut butter (~50 mg) and provided to mice for oral consumption of total exposure of 1 mg/kg of each OPFR/day of OPFR-oil or equivalent vehicle control-oil. Weekly body weights were taken and used for dosage calculation. Starting at 10 weeks

of age, all mice were dosed at 900-1100 h every day for an approximate 7 total weeks in a sub-chronic paradigm.

Experimental Design

Adult male and female mice (*n* = 16 males, *n* = 14 females) were pair-housed and weight-matched per group, fed LFD or HFD, and dosed with either vehicle control-oil or OPFR-oil for 4 weeks in two sequential batches of mice (8 males/batch; 6-8 females/batch) to ensure sufficient sample size for metabolic and feeding behavior investigations. Body composition (fat and lean mass) was assessed by EchoMRI^{TM} Body Composition (Houston, TX) on the day of first dose. Body weight and food intake were measured weekly. After the 4 weeks exposure, body composition was determined followed by Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH) to measure oxygen consumption ($V.O_2$), carbon-dioxide production ($V.CO_2$), respiratory exchange ratio (RER), energy expenditure, and general locomotor activity in a 72 h trial under constant 25 ^{0}C and 12:12 h light/dark cycle. Mice were single-housed for the duration. The respiratory exchange ratio (RER) is a measurement of substrate utilization (ratio of carbohydrates vs. lipids). General metabolic rate is also determined through mouse heat expenditure. Food and water intake and activity (X, Y, and Z plane and running wheel) are also recorded. After CLAMS, mice were transferred to the Biological Data Acquisition (BioDAQ, Research Diets, New Brunswick, NJ) chambers for 1 week with 72 h of habituation and 96 h measurement of feeding behaviors (meal size, frequency, duration). LFD or HFD chow were contained in a touch-sensitive hopper and food consumption was measured by decreases in hopper food weight. Whenever the mouse touched the hopper for food, the system denoted that as a “bout.” When the interval between bouts was greater than 300 sec, the food ingested was determined to be a “meal.” A meal could consist of any number of bouts, until the inter-

bout interval exceeded 300 sec. Next, all mice were tested for glucose and insulin tolerance. For the glucose tolerance test (GTT), mice were fasted for 5hr and then intraperitoneally (IP) injected with a bolus of 2 g/kg glucose. Blood-glucose was measured from tail bleeds using an AlphaTrak glucometer (Zoetis, Parsippany, NJ). Glucose measurements were taken at 0, 15, 30, 60, 90, and 120 min post-injection. Four days later, insulin tolerance tests (ITT) were performed using an IP injection of 0.75 U/kg insulin after a 4 h fast. After insulin injection, glucose was measured in tail-blood at 0, 15, 30, 60, 90, and 120 min. With 1-week recovery from ITT, mice were dosed at 0900 h, fasted at 1000 h, and euthanized at 1100hr by decapitation after sedation with ketamine (100 mg/ml). Female mice were euthanized during diestrus to control for cycling steroid hormone levels. Trunk blood was collected in K^+-EDTA coated tubes with the addition of proteinase inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (1 mg/ml, Sigma-Aldrich) to protect against peptide degradation. Samples were maintained on ice until centrifugation at 1,000g for 15 min at 4 °C. Plasma supernatant was collected and stored at -80 °C until analysis for insulin, leptin, and ghrelin levels, using a multiplex assay (MMHMAG-44 K, EMD Millipore, Billerica, MA).

Data Analysis

All data are depicted as mean ± SEM. Data were analyzed using GraphPad Prisim software (GraphPad Software, LA Jolla, CA) by a two-way ANOVA (OPFR and Diet) with a *post-hoc* Newman-Keuls multiple comparisons test, or with Statistica 7.1 software (StatSoft, Tulsa, OK, USA) by multi-factorial ANOVA or with repeated-measures, three-way ANOVA (Diet, OPFR, Time), followed with *post-hoc* Newman-Keuls multiple comparisons test. Effects were considered significant at $P \leq 0.05$.

2.4 Results

Note: the collective results of this study are visually summarized in Table 1

Physiological Parameters

Body weight gain and crude food intake of mice fed vehicle control-oil and OPFR-oil mixture (1 mg/kg each of TCP, TPP, and TDCPP) were taken over the course of 4 weeks and followed by body composition assessment by EchoMRI^{TM} (Fig. 1 and Fig. 2). Feeding efficiency was calculated using the ratio of body weight gain to crude food intake and depicted as grams gained to kcal consumed. As expected, bodyweight gain and feeding efficiency were increased in all HFD fed animals compared to the LFD animals. However, OPFR-exposed male mice fed HFD exhibited significantly greater weight gain (Fig. 1A, $F(1,60)_{OPFR} = .20$, $P < 0.05$; $F_{Diet} = 120.6$ $P < 0.0001$, $F_{OPFR*Diet} = 8.24$, $P < 0.01$) after 4 weeks over their oil-control counterparts. HFD-fed male mice also displayed elevated fat mass and decreased lean mass from OPFR exposure (Fig. 1C, $F(1,60)_{OPFR} = 6.27$, $P < 0.05$, $F_{Diet} = 103.68$, $P < 0.0001$; Fig. 1D, $F(1,60)_{OPFR} = 6.27$, $P < .05$, $F_{Diet} = 103.68$, $P < .0001$). While no marked main effect of OPFRs or interactions were observed in female mice, HFD-fed females gained more weight and more fat mass than LFD-fed counterparts. OPFR-treated mice given HFD resulted in reduced lean mass (Fig. 2D, $F(1,60)_{OPFR} = .26$, $P < 0.05$, $F_{Diet} = 12.92$, $P < 0.001$), whereas in oil-control mice no marked difference in diet was noted, indicating a potential influence of OPFR exposure on HFD effects.

Metabolic Parameters

Metabolic parameters such as $V.O_2$, $V.CO_2$, RER, and heat were measured in a 72 h run in the CLAMS system (Fig. 3 and Fig. 4). While diet-induced patterns were distinct in both males and females, the OPFR influence appeared only in LFD groups, and during peak feeding time around 200-300 h for both sexes. In LFD-fed males, OPFR significantly

augmented $V.O_2$ and $V.CO_2$ during 200-300 h (Fig. 3A, $P < 0.01$, $P < 0.01$), while LFD-fed females responded with diminished $V.O_2$ during 200-300 h (Fig. 3A 3, $P < 0.01$) and decreased $V.CO_2$ during 200-400 h (Fig. 3B, $P < 0.01$, $P < 0.01$). It is noteworthy that females fed LFD also significantly exhibited elevated RER from 300-500 h (Fig. 4C, $P < 0.05$, $P < 0.05$), as well as reduced RER from 1700-1800 h, right before lights off (Fig. 4C, $P < 0.01$). There were no marked main effects in RER for males. Finally, OPFR decreased energy expenditure in females fed both LFD (Fig. 4D, 200-300 h: $P < 0.01$, 300-400 h: $P < 0.001$), and females fed HFD (Fig. 4D, 100-200 h: $P < 0.05$). Interestingly, male mice fed LFD displayed significantly elevated energy expenditure during 200-300 h (Fig. 3D, $p < 0.0001$), indicating contrasting differences in activity dependent upon sex. Diet did not exert a marked effect on energy expenditure, save for an elevated energy expenditure in LFD-fed males following OPFR treatment at a single time point: 200-300 h (Fig. 3D, $P < 0.0001$).

Activity

In the same CLAMS system, activity levels were also measured over 72 h, including locomotor activity, wheel running, and water intake (Fig. 5). As mice are a nocturnal species, movement and use of the exercise wheel were increased during nighttime in all groups. However, in female mice, OPFR significantly decreased nighttime activity and wheel use in both LFD (Fig. 5E, $P < .05$; Fig. 5F, $P < .01$) and HFD groups (Fig. 5E, $P < .05$; Fig. 5F, $P < .001$). There was a main effect of OPFR on both activity and wheel use (Fig. 5E, $F(1,104)_{OPFR} = 9.87$, $P < 0.01$; Fig. 5F, $F(1,38) = 17.31$, $P < 0.001$), as well as an interaction between OPFR and time (Fig. 5E, $F(1,104)_{OPFR*Time} = 6.33$ $P < 0.05$; Fig. 5F, $F(1,38)_{OPFR*Time} = 12.51$, $P < 0.001$). Male mice did not exhibit this OPFR-induced pattern but did respond to HFD with a reduction in nighttime activity (Fig. 5C, $P < .05$).

Perhaps more interestingly, water intake was augmented in males during the nighttime in both LFD- and HFD-fed groups (Fig. 5A, $P < 0.001$, $p < 0.05$). OPFR induced an overall significant effect (Fig. 5A, $F(1,118)_{OPFR} = 7.12$, $P < 0.01$) as well as OPFR interactions with both time and diet (Fig. 5A, $F(1,118)_{OPFR*Time} = 4.85$, $P < 0.05$, $F(1,118)_{OPFR*Diet} = 4.58$, $P < 0.05$). Furthermore, OPFR exposure induced marked differences between diets and time (Fig. 5A, $P < 0.05$; day: $P < 0.0001$, night: $P < 0.05$), while differences were not observed in oil-control male mice. Females exhibited the opposite effect, wherein oil-control groups exhibited typical elevated water intake during the night (Fig. 5D, $P < 0.01$, $P < 0.05$), but within OPFR treatment, the differences between daytime and nighttime drinking were abrogated, indicating potential dysregulation of diurnal fluid intake behaviors.

Feeding Behaviors

The BioDAQ apparatus was utilized for reliable analysis of total and hourly food intake, as well as meal size, duration, and frequency over a 96-h trial period (Fig. 6). Overall, OPFR-treated female mice ate less HFD (Fig. 6A, $F(1,35)_{OPFR} = 6.15$, $P < 0.05$, $F(1,35)_{OPFR*Diet} = 13.17$, $P < 0.001$) and consumed fewer meals per day (Fig. 6B, $F(1,42)_{OPFR} = 6.56$, $P < 0.05$, $F(1,42)_{Diet} = 57.63$, $p < 0.0001$, $F(1,42)_{OPFR*Diet} = 12.23$, $P < 0.001$) than their oil-control counterparts. When hourly feeding patterns were analyzed, the difference between HFD-fed groups was also evident with OPFR-treated females who consumed less HFD than oil-control during two periods during the dark cycle. Control HFD mice displayed a spike in food intake at 0300 h, whereas food intake for OPFR-treated HFD animals was significantly less during this time (Fig. 6E, $P < 0.01$). In addition, OPFR exposure decreased consumption of HFD at 2000 h respective of either treatment and diet (Figure 6E, $P < .05$, $P < .01$). These time-specific perturbations suggest OPFR may be dysregulating diurnal feeding patters in female mice. Surprisingly, no significant effects

of OPFRs were observed on meal patters in males; however, in the analysis of hourly consumption, OPFR treatment significantly altered HFD consumption at time points 0400 h and 2100, similar to females (data not shown).

Glucose and Insulin Tolerance

Glucose and insulin tolerance tests (Fig. 7 and Fig. 8) were performed to determine whether endocrine disruption by OPFRs might compromise the body's ability to tolerate sudden changes in glucose homeostasis. Overall, there were minimal to no alterations in glucose or insulin tolerance attributed to OPFR exposure. As expected, HFD elevated circulating glucose levels and increased the latency time to return to baseline after glucose or insulin injection, but did not display significantly differences between treatments.

Peptide Hormones

Terminal plasma hormone levels of insulin, leptin, and ghrelin were measured. Alterations in these hormones indicate perturbed energy homeostatic control in peripheral endocrine organs. Insulin levels were elevated in HFD-fed males as compared to LFD-fed males in the OPFR group (Fig. 9A, $P < 0.05$), where there was no significant difference in controls. Interestingly, in female mice OPFR exposure increased insulin in LFD-fed females compared to oil-control counterparts ($P < 0.01$), producing HFD-induced fall in insulin (Fig. 9D, $P < 0.01$, $F(1,28)_{OPFR} = 7.97$, $P < 0.01$). In males, HFD elevated plasma leptin concentrations in both treatment groups (Fig. 9B). In female mice, OPFR induced a rise in leptin in the HFD-fed group compared to oil-control (Fig. 9E, $F(1,28)_{OPFR} = 4.41$, $P < 0.01$, $F(1,28)_{OPFR*Diet} = 5.87$, $P < 0.05$). OPFR treatment suppressed ghrelin in males (Fig. 9C, $F(1,28)_{OPFR} = 5.66$, $P < .05$) and eliminated the HFD-induced reduction in ghrelin. In females, HFD induced ghrelin in in both treatments (Fig. 9F, $P < .01$, $P < .001$).

2.5 Discussion

Recently, endocrine disruption has been in the scientific spotlight as its capacity for inducing a multiplicity of metabolic outcomes is well-documented. Through extensive study, safety concerns over one group of flame retardants such as PBDEs resulted in their decline in use in products for personal and professional purposes (Shaw et al. 2010). However, one of the replacement compounds for PBDEs, OPFRs, have not been as thoroughly investigated despite posing a potential risk to exposed populations. Understandably, most OPFR research, so far, has focused on sensitive developmental time periods (*in utero*, neonatal, juvenile), however, exposure is not limited to children, and the accumulated exposure of these compounds throughout a lifetime may pose a risk for developing endocrine and metabolic disorders in adulthood. The prevalence of OPFR exposure combined with the current gap in our understanding of the mechanisms underlying OPFR-mediated toxicity led us to investigate OPFR-induced metabolic toxicity in an adult exposure mouse model. Our investigation utilized a mixture of three common OPFRs (TPP, TCP, TDCPP) because their concurrent human exposure and their known interactions with nuclear receptors including ERα and PPARγ (Gray et al. 2005; Pap et al. 2016; Belcher et al. 2014; Pillai et al. 2014; Tung et al. 2017). Previously, Krumm et al., (2018) demonstrated that adult exposure to a mixture of these three OPFRs on a standard chow diet altered hypothalamic and hepatic gene expression and body weight gain and energy intake in a sex-dependent manner. The current study utilized the same OPFR mixture in adult mice fed either a low-fat diet (LFD) or high-fat diet (HFD) to examine the toxicological intersection of OPFR exposure and diet-induced obesity. We evaluated the effects of OPFR treatment on a range of physiological measures pertaining to metabolic homeostasis, including weight gain, body composition, metabolism, activity, ingestive behaviors, glucose homeostasis, and circulating peptide hormones.

In the current study, we observed weight gain in male mice was detected when exposed to OPFR and fed HFD compared to HFD-fed control males. The rise in body weight was due to enhanced fat accumulation, suggesting OPFR augments fat accumulation when food intake is high in fat. Such an effect is consistent with an interaction with PPARγ, a receptor often referred to as the "master regulator of adipogenesis" (Shao et al. 2016; Lefterova et al. 2014). Conversely, OPFR-treated males fed LFD gained less weight compared to control males, which is in agreement with our previous findings (Krumm et al. 2018). In OPFR-treated females, regardless of diet, there was no marked effect on body weight. This is dissimilar to findings of our earlier study where OPFR exposure was associated with weight gain in ovariectomized, adult mice fed a standard chow diet (Krumm et al. 2018). The difference may be related to effects of ovariectomy, which by itself is known to lead to increased weight gain (Mamounis et al. 2014), or due to the use of a standard chow vs. the semi-purified LFD used in the current study. Regardless, there does appear to be a disruptive capacity of OPFR on weight gain, perhaps differentially dependent upon sex, diet, or ovarian status. Aside from weight gain, a decrease in food intake and meals per day was observed in HFD-fed male mice. An augmented weight gain despite decreased HFD intake suggests that in addition to altered feeding behavior, OPFR treatment is also disrupting metabolism of fatty foods. While there was no effect on body weight n OPFR-treated females in this study, both leptin and insulin levels were elevated by OPFR treatment. Such effects could explain the lack of increased weight gain. In keeping with this theory, no marked increases in either leptin or insulin were observed in male OPFR-treated mice. Interestingly, OPFR-treated males also exhibited lowered ghrelin levels. Taken together, these alterations demonstrate OPFR's ability to disrupt energy homeostasis and support its capacity as an EDC with sex-specific actions.

Adult exposures to other EDC previously reported metabolic effects including and not limited to glucose homeostasis, thyroid hormone levels, and fat metabolism (Ding et al. 2014; Marmugi et al. 2014; Moghaddam et al. 2015; Brulport et al. 2017; Bertuloso et al. 2015; Sharan et al. 2014). Data indicate that OPFRs disrupt metabolism (Fernie et al. 2015; Wang et al. 2019a; Du et al. 2016), supporting our examination of metabolic parameters including $V.O_2$, $V.CO_2$, RER, and energy expenditure. In males, significant perturbations in metabolism were observed during the nighttime. However, in female mice, OPFR treatment consistently decreased $V.O_2$, $V.CO_2$, and energy expenditure on LFD during the night. In addition, RER was significant elevated on LFD. The shift in RER may indicate an enhanced carbohydrate utilization by OPFR on LFD, as opposed to lipid utilization. However, this rise occurred concomitantly with a fall in carbohydrate usage during the later afternoon/early evening, prior to nighttime when the mice are more active. More notable than the indicated substrate utilization alterations, the observed decrease in $V.O_2$, $V.CO_2$, and energy expenditure indicates that mice are simply using less energy overall, i.e., they are less active, when dosed with OPFRs. This response may be due to a variety of mechanisms, but the simplest may be an effect on mitochondria. Mitochondria are responsible for respiration and energy production at the cellular level, and if OPFRs are impinging on mitochondrial function, it may result in basal perturbations of metabolic mitochondrial activity. This hypothesis is supported by a recent study in which both TPP and TDCPP were found to decrease basal mitochondrial respiration in zebrafish embryos. These investigators also reported a reduction in maximal mitochondrial respiration attributed to TDCPP exposure (Lee et al. 2019). Further, in the nematode *C. elegans*, TPP and TDCPP both disrupted mitochondrial membrane permeability in a similar manner as brominated FR predecessors, indicating mitochondrial toxicity (Behl et al. 2016). Both TPP and TCP also increased mitochondrial activity and superoxide production in a immortalized murine cell line (Schang et al. 2016). Finally, both estrogen receptors and

PPARγ will bind to mitochondrial DNA response elements, establishing a direct route for their respective regulation of mitochondrial function (Chen et al. 2004; Yang et al. 2004; Irwin et al. 2012; Chang and Ha 2018; Corona and Duchen 2016; Rettberg et al. 2014). These studies establish a precedent to support a hypothesis that the respiratory effects noted may in part be due to a mitochondrial effect of OPFR. However, regardless of the cellular mechanism, our data suggest that even sub-chronic exposures to OPFRs altered respiration and suppressed metabolism, which, over a lifespan, may influence energy homeostasis and result in increased risk of excess weight gain and adiposity.

Mice are generally nocturnal animals, and thus their activity is greatest during the night. In our experiment on mouse locomotor activity and wheel running, female mice were more susceptible to OPFR exposure than males and displayed a marked reduction in both X-plane movement (general locomotor activity) and wheel running during the night. These data indicate that in female, but not male, mice, OPFR ingestion is interfering with neurological pathways that control activity, producing reduced locomotor motivation. Many of these pathways express ERα, a steroid receptor that is known to increase activity upon activation (Ogawa et al. 2003; Shughrue et al. 1997; Hatcher et al. 2018; Ogawa et al. 1998), or PPARγ, a nuclear receptor that is also involved in exploratory behaviors (Domi et al. 2016; Moreno et al. 2004). Interestingly, developmental exposure to the commercial OPFR mixture, FM550, in rats induced hyperactivity in female rats (Baldwin et al. 2017). The conflicting data between our findings and the FM550 study may be a result of differences in species, exposure window, and chemical content. Regardless, OPFR produced a striking effect on activity in adult females. Importantly, in regards to energy homeostasis, a reduction of activity (or increased sedentary behavior) is a well-known contributing factor to an obese phenotype and related sequelae such as metabolic

syndrome and type 2 diabetes, both in rodent models and humans (de Rezende et al. 2014; Hamilton et al. 2014).

Locomotor motivation is a complex and multifaceted behavioral characteristic which is influenced by more than just the search for food. Through the lens of energy homeostasis, activity is associated with energy expenditure, a process tightly under hormonal control and particularly through 17β-estradiol (Rettberg et al. 2014; Lopez and Tena-Sempere 2015). Energy expenditure is also controlled, in part, by actions of hypothalamic neurons in the arcuate nucleus (Nahon 2006), and activity of these neurons may be modulated by 17β-estradiol in an energy state-sensitive manner (Roepke et al. 2011). Because OPFR interact with ERs, their exposure may be impinging on estrogenic mediation of energy homeostasis, and increasing the risk of metabolic disruption. Further, activity is not only dictated by energy status. Motivation and mood may also be involved, and lack of motivation to move is symptomatic and causative for a variety of mood disorders (Zhai et al. 2015; Schwartz et al. 2000). Future studies may benefit from exploring OPFR action on brain regions involved in motivation such as the ventral tegmental area, ventral striatum, prefrontal cortex, amygdala, and dorsal media habenula (Hsu et al. 2014; Kim 2013).

In addition to effects on energy expenditure, water intake was also dysregulated. OPFR-treated males, regardless of diet, exhibited an increase in nighttime water intake, while OPFR-treated females displayed an ablation of typical diurnal drinking patterns. A possible explanation for female mice is that dysregulation of water intake is a direct result of OPFR actions decreasing activity and energy expenditure. If animals are moving less, then the motivation to eat as well as to drink will also diminish. However, there are likely to be additional complexities to this simple explanation. Although female mice are moving less, these animals were still more active during the night than they were during the daytime. Therefore, the apparent increase in daytime fluid consumption is not necessarily

contributable to solely mouse activity. The eliminated difference between day and nighttime fluid intake may in fact, be a direct effect on fluid homeostasis to either increase daytime, or decrease nighttime consumption. Further, despite no marked activity alterations in males, OPFR treatment elevated nighttime fluid consumption that may indicate an impact of OPFR on control of fluid balance, either centrally, or peripherally in the kidneys. Indeed, TDCPP produced cytotoxic effects on cultured renal cells (Killilea et al. 2017), and in a human population study of approximately 1,500 patients, TDCPP exposure correlated with markers of kidney damage and chronic renal disease (Kang et al. 2019).

There are also many areas of the brain that control fluid balance including the paraventricular hypothalamus, supraoptic nucleus, median preoptic area, organum vasculosum laminae terminalis, and subfornical organ (Curtis 2009). Many of these nuclei express ERs and are involved in the control of fluid balance in response to 17β-estradiol (Shughrue et al. 1997; Santollo and Daniels 2015b; Santollo et al. 2013; Curtis 2009; Santollo and Daniels 2015a). In hormone replacement therapies, E2 produced a direct effect on water intake (Krause et al. 2003; Santollo et al. 2013), its actions mediated in part through dampening of angiotensin II (AngII) signaling (Kisley et al. 1999; Findlay et al. 1979; Danielsen and Buggy 1980; Jonklaas and Buggy 1984). Thus, OPFR interfere with this estrogen-sensitive balance is another possible pathway for the observed effect of OPFRs on fluid intake. However, like any homeostatic function, thirst is regulated through a multitude of pathways, allowing for alternate avenues of OPFR actions. Thirst is closely related to energy homeostasis, and the powerful “hunger” hormone ghrelin is also known to exert effects on fluid intake, reducing water consumption by inhibiting AngII (Mietlicki et al. 2009; Hashimoto et al. 2010; Plyler and Daniels 2017), which as previously indicated, is also under the influence of E2. Conversely, intracerebroventricular infusions of AngII diminishes food and enhances energy expenditure, establishing an AngII link

between food and fluid intake mediated by ghrelin (Porter and Potratz 2004). In our current study, OPFR decreased circulating ghrelin in male mice on LFD, supporting a ghrelin-mediated hypothesis for the dipsogenic effect of OPFR on male mice. Finally, somatostatin, produced both centrally in the ventromedial nucleus of the hypothalamus, and peripherally by delta cells in the digestive system, is involved in thirst generation and may be a target for OPFR dysregulation. The central effect of somatostatin to increase food and water intake (Karasawa et al. 2014; Stengel et al. 2010) was shown to be altered by exposure to bisphenol A, another well-known estrogenic EDC (Facciolo et al. 2002; Facciolo et al. 2005). Taken together, these data offer a precedented route for OPFR EDC action on fluid regulation.

The collective observations of this study demonstrate a clear sex-dependent effect of OPFR exposure within adult mice. Body composition was only altered in males, while feeding behavior and activity were largely only modified within females. And circulating ghrelin was diminished in males, while leptin and insulin were elevated within female mice. These differences are likely attributed to the innate biological differences between male and female mice. Energy homeostasis is well-documented to be a sexually-dimorphic function (Shi et al. 2009; Woods et al. 2003; Wu and O'Sullivan 2011), through which estrogen plays a substantial role (Rettberg et al. 2014; Nestor et al. 2014; Lopez and Tena-Sempere 2015). The decline in circulating estrogen following menopause is associated with adverse health effects such as weight gain, hot flashes, and increased risk of diabetes and cardiovascular disease (Cignarella and Bolego 2010; Clegg et al. 2017), and estrogen replacement therapies show marked protection against these effects (Warren et al. 2015). Estrogen is typically present at roughly ten-fold higher serum concentration within females than within males, therefore OPFR interference with estrogenic signaling is likely to have differing impacts, depending on sex. Further, Krumm et al., (2018) reported decreased expression of ERα in males, but not in ovariectomized

females, highlighting a sex-specific interaction of OPFR with estrogenic signaling. Expression of other OPFR-target PPARγ was found to be upregulated, but not dependent on sex. PPARγ is not well-known for sex-differences, but a few studies implicate differential PPARγ expression and regulation of adipose tissue and immune function (Kadowaki et al. 2007). One study using brain-specific knockout (KO) of PPARγ found that KO females gained more weight than their littermate KO male counterparts (Fernandez et al. 2017). Together, these implicate that OPFR interactions with ERα and PPARγ may contribute to the observed sex-dependent effects of the current study.

Part of the reason for the growing concern over EDCs is what is termed the “cocktail effect,” wherein exposures to multiple different EDCs might induce additive or synergistic effects, depending upon the period of exposure, developmental or throughout the lifespan. Since most EDCs are lipophilic and have potential to bioaccumulate, adults may be even more sensitive to their effects. What might be minor perturbations within short-term experiments, given prolonged exposure, combined EDC exposures may culminate in significant disruptions (Lauretta et al. 2019). Thus, consistent with the multiplicity of EDC exposures in human, our experimental protocols used a mixture of organophosphate EDCs. While this represents a more accurate-to-life model than studying singular effects of one specific OPFR, different OPFR can have differing (agonistic or antagonistic) effects on ERs and PPARs (Kojima et al. 2013; Liu et al. 2013). To help address this, future studies will need to examine the effects of OPFR exposures in tissue- or cell-specific ERα and PPARγ knockout mouse models. While more mechanistic data will be useful, the current studies help elucidate the importance of more research into the safety of OPFRs, which appear to be interfering with estrogenic and/or PPARγ control of energy expenditure through receptor-mediated actions.

2.6 Conclusion

In summary, our findings indicate that adult exposure to a common mixture of OPFRs, has disruptive actions on energy homeostasis that potentially exacerbates diet-induced obesity. We observed a multitude of sex-dependent effects on metabolism, energy expenditure, weight-gain, activity, water intake and circulating hormone concentrations. Most notable were OPFR-induced alterations in water intake and behavioral activity. These results add to other findings that suggest OPFR may exert adverse effects via interaction with endocrine receptors. Our findings highlight the need for further research into the safety of OPFRs, particularly with prolonged exposures as are occurring within the adult human population. The observed effects on physiological measurements of energy homeostasis also suggest that prolonged OPFR exposure may play a role in developing metabolic disorders, such as diet-induced obesity, diabetes, and metabolic syndrome. Despite the apparent risk, OPFR still continue to be a leading FR in the United States. This highlights the need for continued research into their safety. The current research shows that OPFRs may affect health through interactions with ERs and PPARγ, and other mechanisms remain possible. However, it would be interesting to investigate the mechanistic roots of the dysregulated fluid homeostasis noted herein, as well as behavioral studies to tease out whether the sedentary behavior of OPFR-treated females is a mood, or motivation effect. Further, while this study focused primarily on peripheral and behavioral outcomes, energy homeostasis is tightly regulated through central processes in the hypothalamus. Thus, it will also be important to investigate potential OPFR actions on neuronal subpopulations that regulate feeding and reward pathways in the brain.

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Tables

Endpoint	Males		Females	
	LFD	HFD	LFD	HFD
Bodyweight Gain	n.s.	↑↑	n.s.	n.s.
Feeding Efficiency	n.s.	n.s.	n.s.	n.s.
Fat Mass	n.s.	↑↑	n.s.	n.s.
Lean Mass	n.s.	↓↓	n.s.	n.s.
V.O2	↑	n.s.	↓	n.s.
V.CO2	↑	n.s.	↓	n.s.
RER	n.s.	n.s.	↓↑	n.s.
Energy Expenditure	↑	n.s.	↓	↓
Activity	n.s.	n.s.	↓↓	↓↓
Water Intake	↑↑	↑↑	↓↑	↓↑
96 h Food Intake	n.s.	n.s.	n.s.	↓↓
Hourly Food Intake	n.s.	↓↑	n.s.	↓↑
Meal Frequency	n.s.	n.s.	n.s.	↓↓
Meal Duration	n.s.	n.s.	n.s.	n.s.
Meal Size	n.s.	n.s.	n.s.	n.s.
Fasting Glucose	n.s.	n.s.	n.s.	n.s.
Glucose Tolerance	n.s.	n.s.	n.s.	n.s.
Insulin Tolerance	n.s.	n.s.	n.s.	n.s.
Insulin	n.s.	n.s.	↑	n.s.
Leptin	n.s.	n.s.	n.s.	↑
Ghrelin	↓	n.s.	n.s.	n.s.

Table 1. Summary of data comparing control- and OPFR-treated groups.

Up arrows denote an OPFR-induced increase and down arrows denote an OPFR-induced decrease. One arrow indicates a modest effect and two arrows indicate a strong effect. One up and one down arrow indicates a mixed effect dependent on time of day. N.S. = not significant.

Figures

Figure 1. Physiological parameters in WT males orally dosed with an OPFR mixture (1 mg/kg) for ~7 weeks. (A) % Body Weight Gain over 4 weeks; (B) Feeding Efficiency; (C) Body composition % Fat Mass; (D) Body composition % Lean Mass. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparisons test. Uppercase letters denote diet effects within EDC group. Lowercase letters denote OPFR effects within diet (a=*P*<.05; =*P*<.01; c=*P*<.001; d=*P*<.0001). Data (B n=8; A, C, D n=16) are presented as mean ± SEM.

Figure 2. Physiological parameters in WT females orally dosed with an OPFR mixture (1 mg/kg) for ~7 weeks. (A) % Body Weight Gain over 4 weeks; (B) Feeding Efficiency; (C) Body composition % Fat Mass; (D) Body composition % Lean Mass. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparisons test. Uppercase letters denote diet effects within EDC group. Lowercase letters denote OPFR effects within diet (a=*P*<.05; =*P*<.01; c=*P*<.001; d=*P*<.0001). Data (B n=8; A, C, D n=14) are presented as mean ± SEM.

Figure 3. Analysis of metabolism in WT male mice orally dosed with an OPFR mixture (1 mg/kg) for ~5 weeks. (A) V.O2; (B) V.CO2; (C) Respiratory Exchange Ratio; (D) Energy Expenditure. Dark line above X-axis represents dark/light hours. Data were analyzed with a repeated measures three-way ANOVA with post-hoc Newman-Keuls multiple comparisons test. Lowercase letters denote OPFR effect within diet group; uppercase letters denote diet effects within EDC group, or when barred, denote comparisons between day and night (a=*P*<.05; =*P*<.01; c=*P*<.001; d=*P*<.0001). Data (n=14) are presented as mean ± SEM.

Figure 4. Analysis of metabolism in WT female mice orally dosed with an OPFR mixture (1 mg/kg) for ~5 weeks. (A) V.O2; (B) V.CO2; (C) Respiratory Exchange Ratio; (D) Energy Expenditure. Dark line above X-axis represents dark/light hours. Data were analyzed with a repeated measures three-way ANOVA with post-hoc Newman-Keuls multiple comparisons test. Lowercase letters denote OPFR effect within diet group; uppercase letters denote diet effects within EDC group, or when barred, denote comparisons between day and night (a=*P*<.05; =*P*<.01; c=*P*<.001; d=*P*<.0001). Data (n=14) are presented as mean ± SEM.

Figure 5. Analysis of daytime vs. nighttime activity in WT mice orally dosed with an OPFR mixture (1 mg/kg) for ~5 weeks. (A,D) Water Intake; (B,E) Activity; and (C,F) Wheel Running. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparisons test. Lowercase letters denote OPFR effect within diet group; uppercase letters denote diet effects within EDC group, or when barred, denote comparisons between day and night (a=*P*<.05; =*P*<.01; c=*P*<.001; d=*P*<.0001). Data (n=14) are presented as mean ± SEM.

Figure 6. Feeding behaviors in WT females orally dosed with an OPFR mixture (1 mg/kg) for ~5 weeks. (A) Total Food Ingested; (B) Meals/Day; (C) Meal Duration; (D) Meal Size; and (E) Average Hourly Food Intake. Data were analyzed by a two-way ANOVA (A, B, C, D) and a repeated-measures three-way ANOVA (E) with post-hoc Newman-Keuls multiple comparisons test. Capped and lowercase letters denote diet effects within EDC group; uppercase letters denote OPFR effects within diet (a=*P*<.05; =*P*<.01; c=*P*<.001; d=*P*<.0001). Data (n=8-14 for all groups) are presented as mean ± SEM.

Figure 7. Glucose tolerance tests in WT mice orally dosed with an OPFR mixture (1 mg/kg) for ~6 weeks. (A) Male GTT; (B) Area under the curve (AUC) of Male GTT (C) Female GTT; (D) Area under the curve (AUC) of Female GTT. Data were analyzed by a two-way ANOVA (B, C, E, F) or a repeated-measures, three-way ANOVA (A, D) with post-hoc Newman-Keuls multiple comparisons test. Capped letters denote diet effects within EDC group and lowercase letters denote EDC effect within diet (a=*P*<.05; =*P*<.01; c=*P*<.001; d=*P*<.0001). Data (n=8 for all groups) are presented as mean ± SEM.

Figure 8. Insulin tolerance tests in WT mice orally dosed with an OPFR mixture (1 mg/kg) for ~6 weeks. (A) Male ITT; (B) Area under the curve (AUC) of Male ITT (C) Female ITT; (D) Area under the curve (AUC) of Female ITT. Data were analyzed by a two-way ANOVA (B, D) or a repeated-measures, three-way ANOVA (A, C) with post-hoc Newman-Keuls multiple comparisons test. Capped letters denote diet effects within EDC group and lowercase letters denote EDC effect within diet (a=*P*<.05; =*P*<.01; c=*P*<.001; d=*P*<.0001). Data (n=8 for all groups) are presented as mean ± SEM

Figure 9. Terminal Plasma peptide hormone concentrations in WT mice orally dosed with an OPFR mixture (1 mg/kg) for ~7 weeks. (A,D) Insulin; (B,E) Leptin; and (C,F) Ghrelin. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparisons test. Uppercase letters denote diet effects within EDC group; lowercase letters denote OPFR effects within diet (a=*P*<.05; =*P*<.01; c=*P*<.001; d=*P*<.0001). Data (n=8) are presented as mean ± SEM.

Figure 1

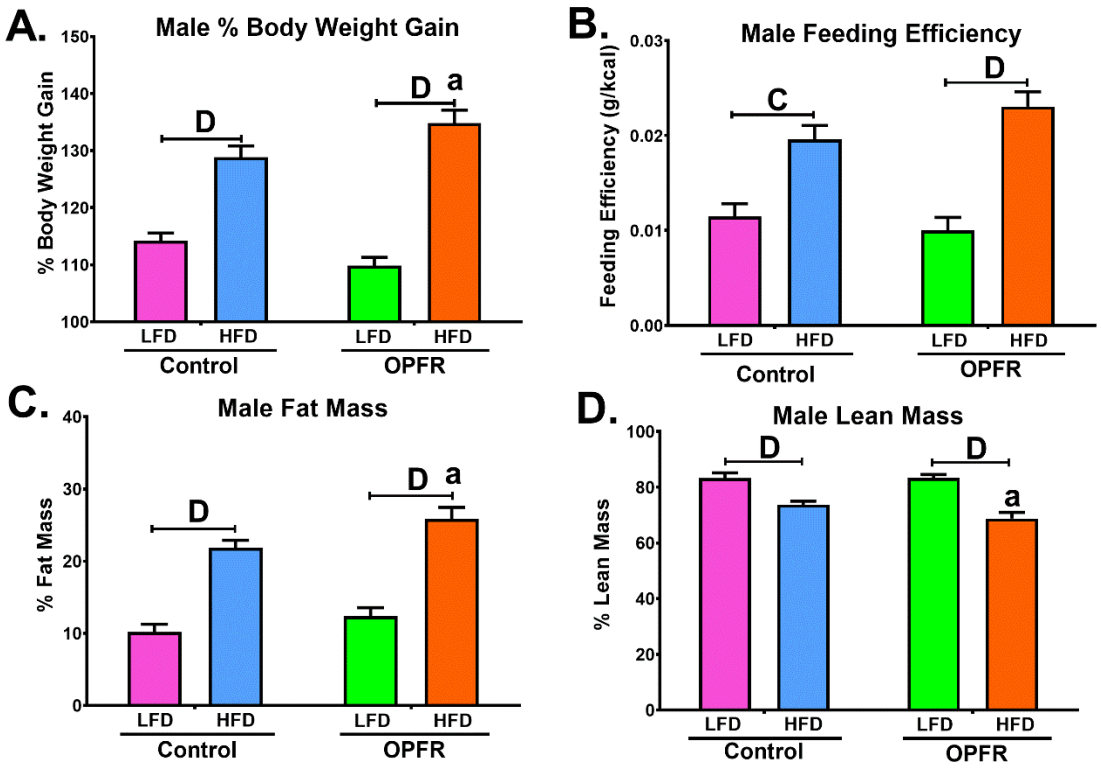

A.
Male % Body Weight Gain
% Body Weight Gain
150
140
130
120
110
100
D
D a
LFD
HFD
LFD
HFD
Control
OPFR
B.
Male Feeding Efficiency
Feeding Efficiency (g/kcal)
0.03
0.02
0.01
0.00
C
D
LFD
HFD
LFD
HFD
Control
OPFR
C.
Male Fat Mass
% Fat Mass
40
30
20
10
0
D
D a
LFD
HFD
LFD
HFD
Control
OPFR
D.
Male Lean Mass
% Lean Mass
100
80
60
40
20
0
D
D
a
LFD
HFD
LFD
HFD
Control
OPFR

Figure 2

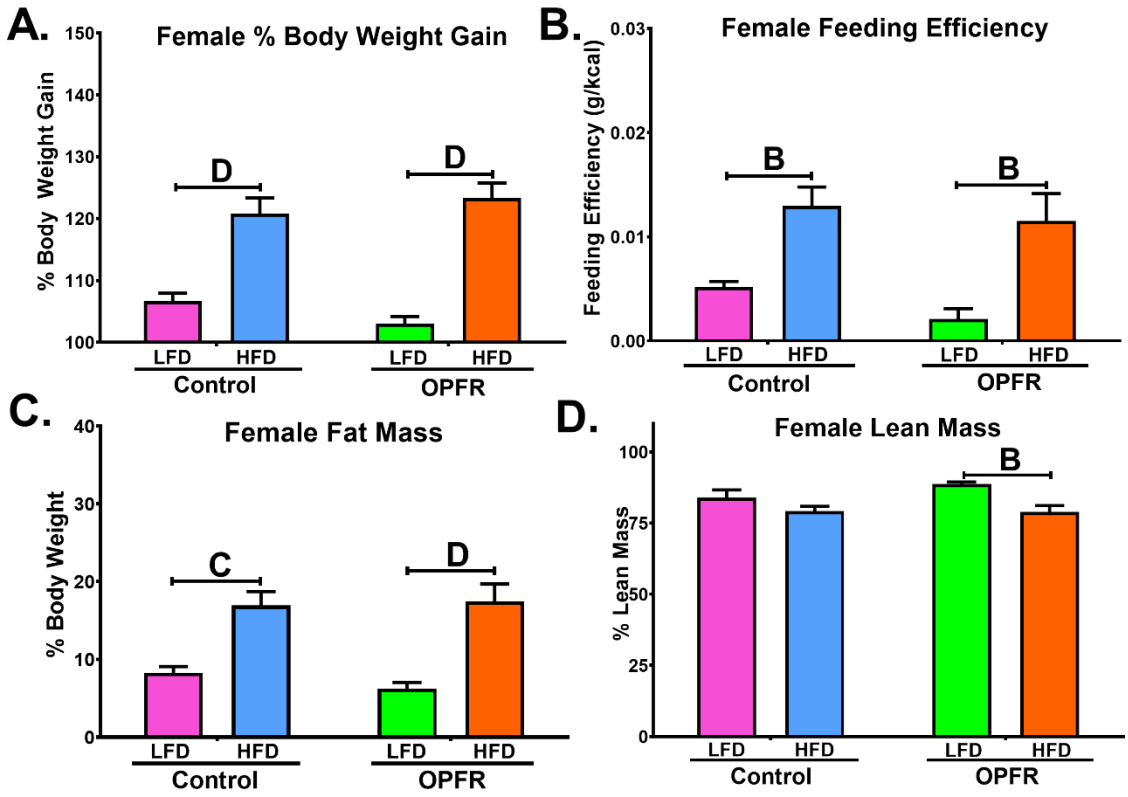

A.
Female % Body Weight Gain
% Body Weight Gain
150
140
130
120
110
100
D
D
LFD
HFD
LFD
HFD
Control
OPFR
B.
Female Feeding Efficiency
Feeding Efficiency (g/kcal)
0.03
0.02
0.01
0.00
B
B
LFD
HFD
LFD
HFD
Control
OPFR
C.
Female Fat Mass
% Body Weight
40
30
20
10
0
C
D
LFD
HFD
LFD
HFD
Control
OPFR
D.
Female Lean Mass
% Lean Mass
100
75
50
25
0
B
LFD
HFD
LFD
HFD
Control
OPFR

Figure 3

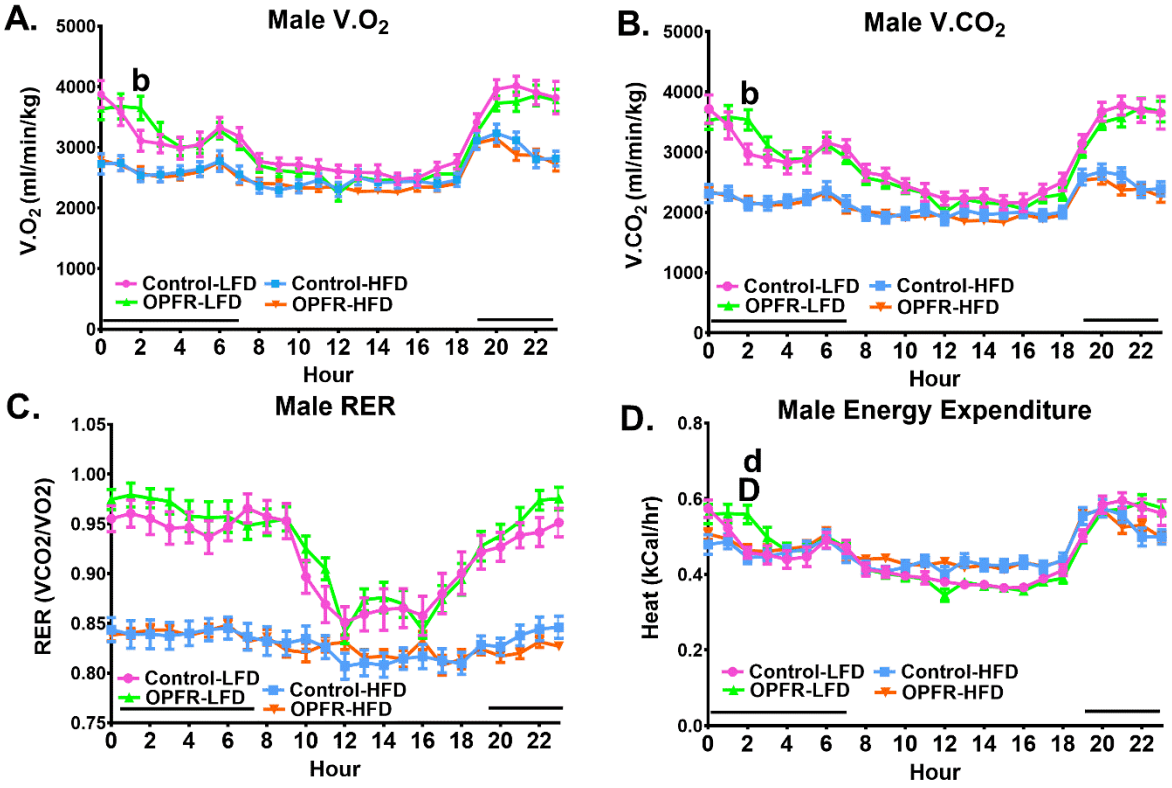

A.
Male V.O2
V.O2 (ml/min/kg)
b
Control-LFD
Control-HFD
OPFR-LFD
OPFR-HFD
Hour
B.
Male V.CO2
V.CO2 (ml/min/kg)
b
Control-LFD
Control-HFD
OPFR-LFD
OPFR-HFD
Hour
C.
Male RER
RER (VCO2/VO2)
Control-LFD
OPFR-LFD
Control-HFD
OPFR-HFD
Hour
D.
Male Energy Expenditure
Heat (kCal/hr)
d
D
Control-LFD
Control-HFD
OPFR-LFD
OPFR-HFD
Hour

Figure 4

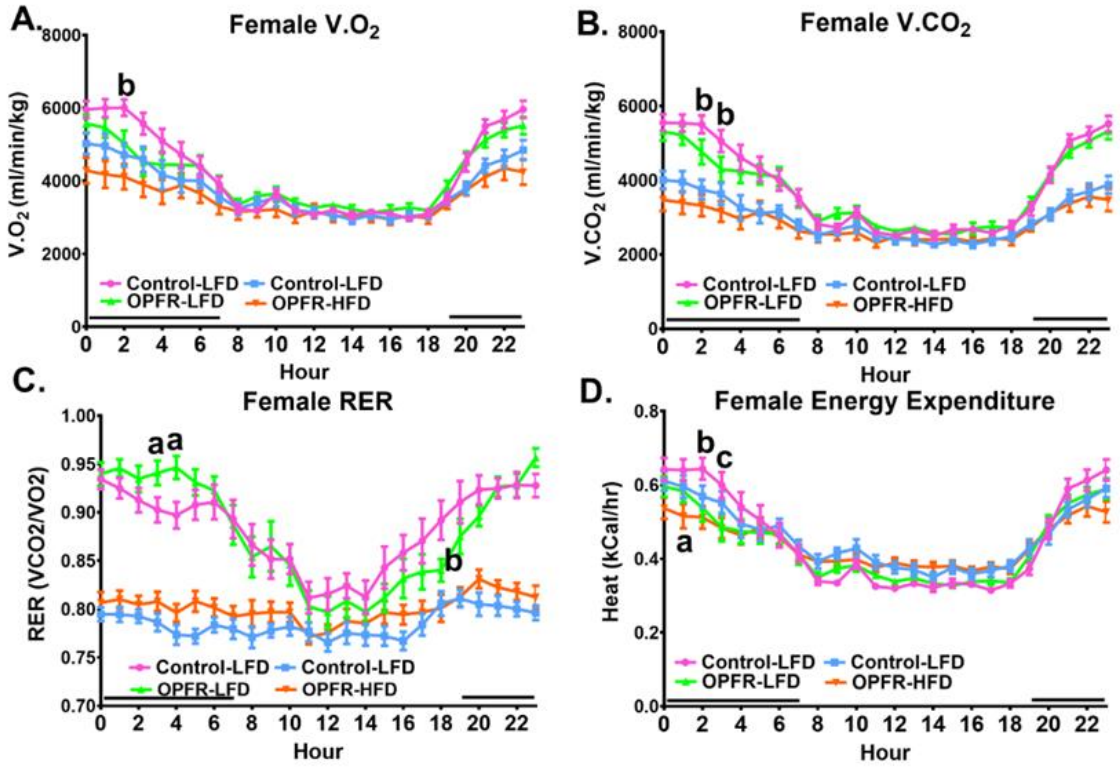

A.
Female V.O2
V.O2 (ml/min/kg)
b
Control-LFD
Control-LFD
OPFR-LFD
OPFR-HFD
Hour
B.
Female V.CO2
V.CO2 (ml/min/kg)
b b
Control-LFD
Control-LFD
OPFR-LFD
OPFR-HFD
Hour
C.
Female RER
RER (VCO2/VO2)
a a
b
Control-LFD
Control-LFD
OPFR-LFD
OPFR-HFD
Hour
D.
Female Energy Expenditure
Heat (kCal/hr)
b c
a
Control-LFD
Control-LFD
OPFR-LFD
OPFR-HFD
Hour

Figure 5

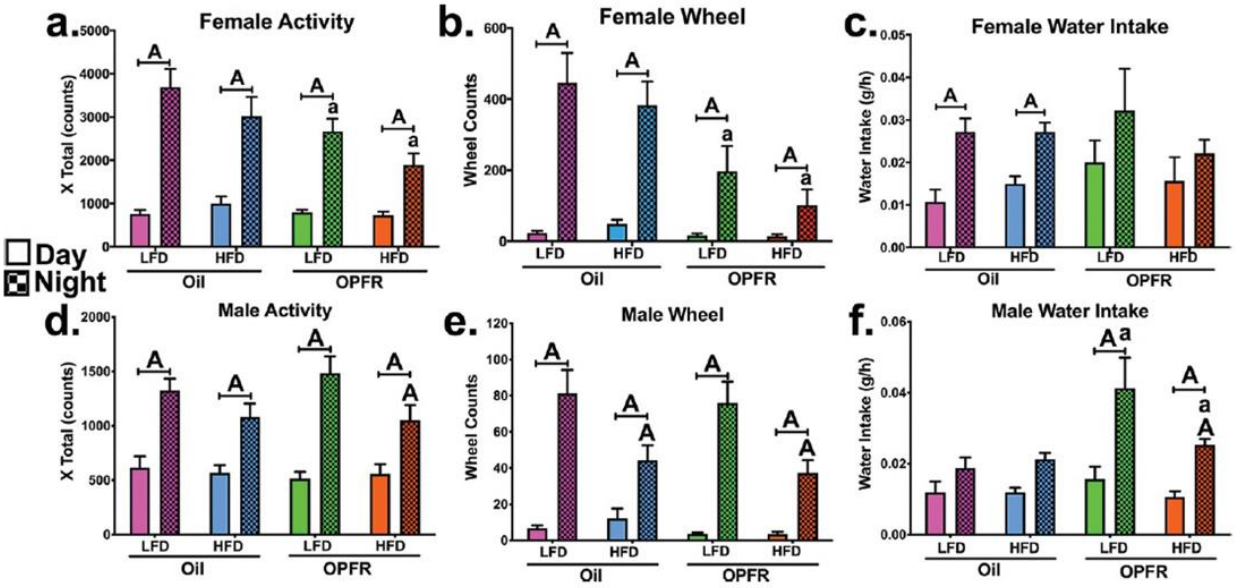

a.
Female Activity
X Total (counts)
b.
Female Wheel
Wheel Counts
c.
Female Water Intake
Water Intake (g/h)
Day
Night
d.
Male Activity
X Total (counts)
e.
Male Wheel
Wheel Counts
f.
Male Water Intake
Water Intake (g/h)
LFD
HFD
Oil
OPFR

Figure 6

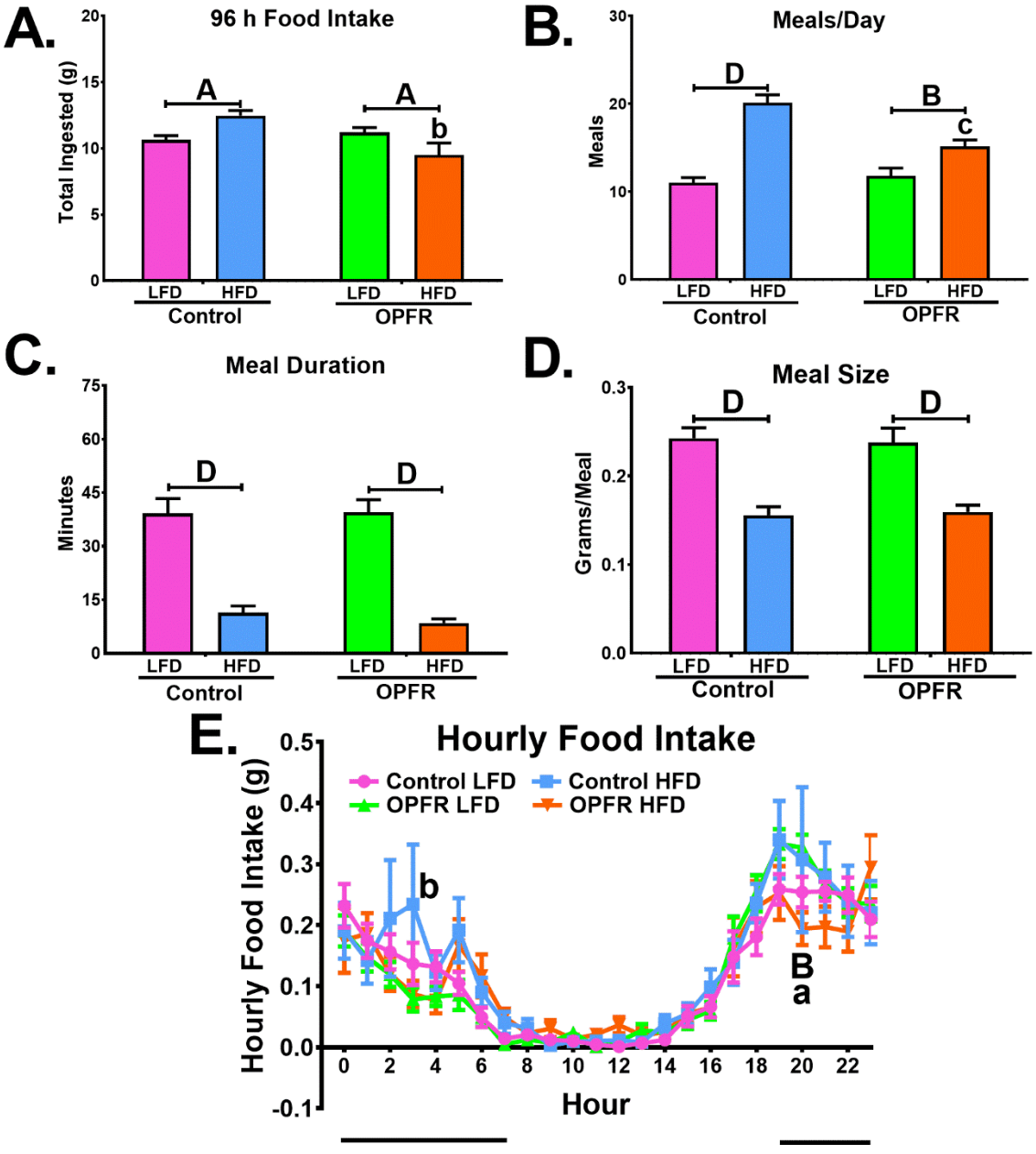

A.
96 h Food Intake
Total Ingested (g)
A
A
b
LFD
HFD
LFD
HFD
Control
OPFR
B.
Meals/Day
Meals
D
B
c
C.
Meal Duration
Minutes
D
D
D.
Meal Size
Grams/Meal
D
D
E.
Hourly Food Intake
Hourly Food Intake (g)
Control LFD
Control HFD
OPFR LFD
OPFR HFD
b
B
a
Hour

Figure 7

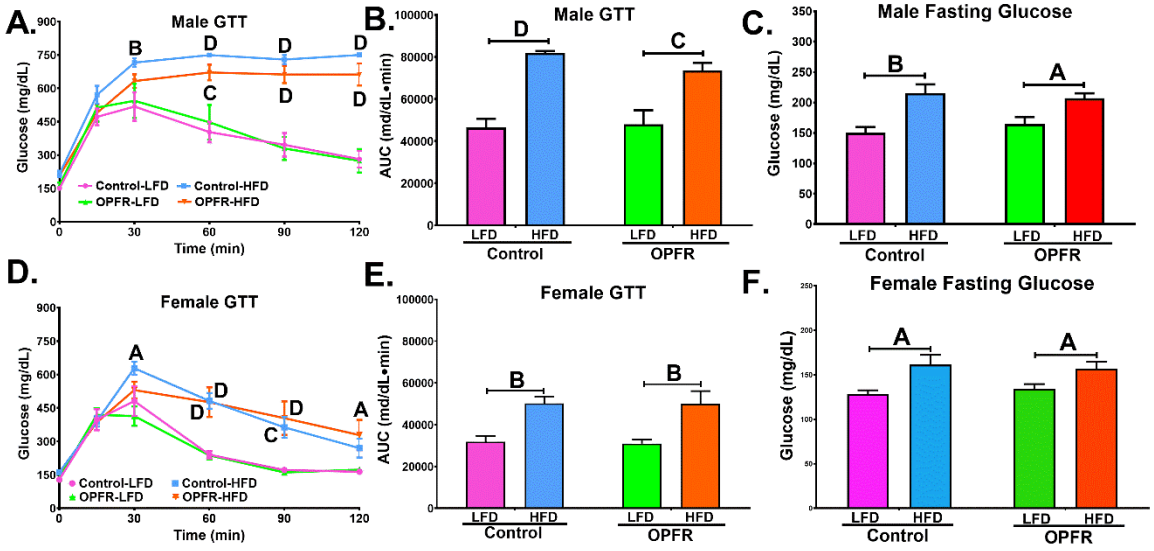

A.
Male GTT
Glucose (mg/dL)
Time (min)
Control-LFD
Control-HFD
OPFR-LFD
OPFR-HFD
B.
Male GTT
AUC (md/dL•min)
LFD
HFD
Control
OPFR
C.
Male Fasting Glucose
Glucose (mg/dL)
D.
Female GTT
E.
Female GTT
F.
Female Fasting Glucose

Figure 8

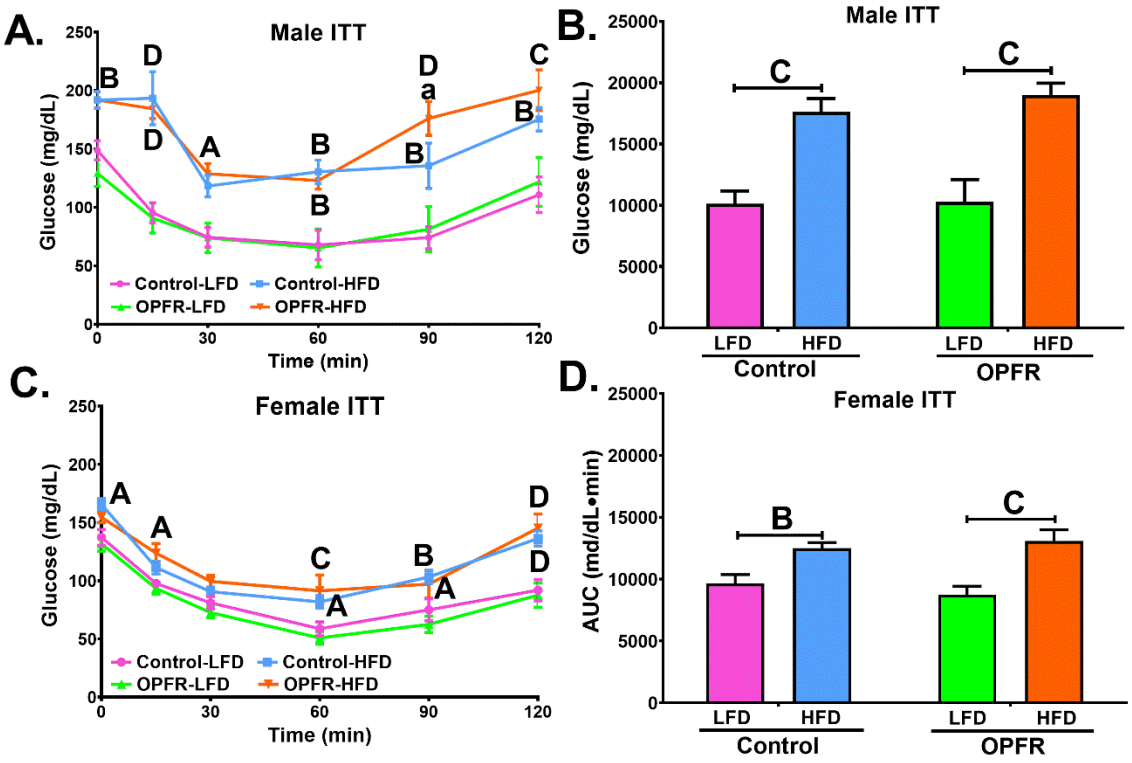
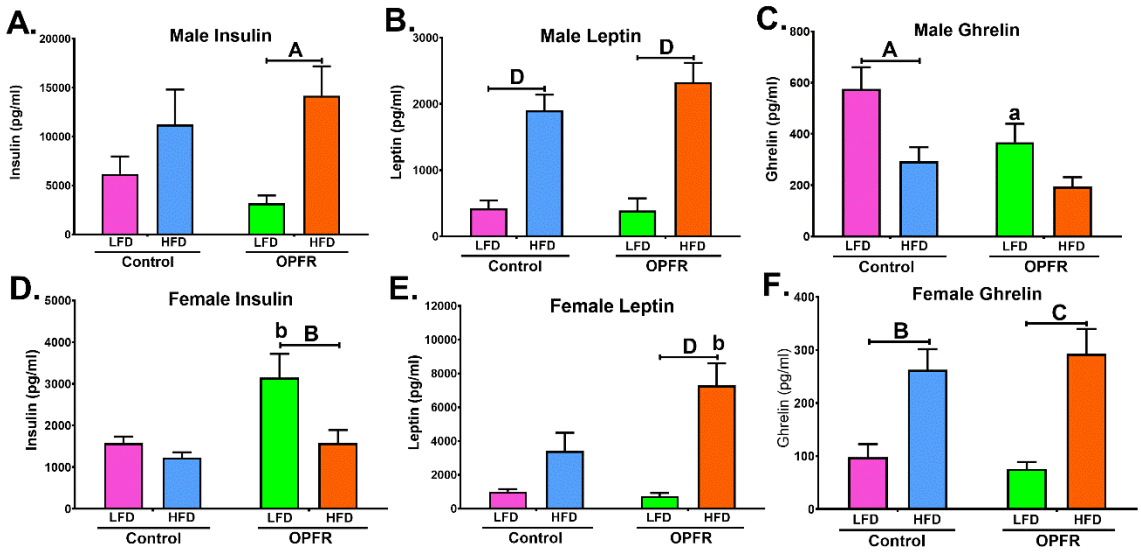

A.
Male ITT
Glucose (mg/dL)
Time (min)
Control-LFD
Control-HFD
OPFR-LFD
OPFR-HFD
B.
Male ITT
Glucose (mg/dL)
LFD
HFD
Control
OPFR
C.
Female ITT
Glucose (mg/dL)
Time (min)
Control-LFD
Control-HFD
OPFR-LFD
OPFR-HFD
D.
Female ITT
AUC (md/dL•min)
LFD
HFD
Control
OPFR

Figure 9


A.
Male Insulin
Insulin (pg/ml)
20000
15000
10000
5000
0
A
LFD
HFD
LFD
HFD
Control
OPFR
B.
Male Leptin
Leptin (pg/ml)
3000
2000
1000
0
D
D
LFD
HFD
LFD
HFD
Control
OPFR
C.
Male Ghrelin
Ghrelin (pg/ml)
800
600
400
200
0
A
a
LFD
HFD
LFD
HFD
Control
OPFR
D.
Female Insulin
Insulin (pg/ml)
5000
4000
3000
2000
1000
0
b
B
LFD
HFD
LFD
HFD
Control
OPFR
E.
Female Leptin
Leptin (pg/ml)
12000
10000
8000
6000
4000
2000
0
D
b
LFD
HFD
LFD
HFD
Control
OPFR
F.
Female Ghrelin
Ghrelin (pg/ml)
400
300
200
100
0
B
C
LFD
HFD
LFD
HFD
Control
OPFR

CHAPTER 3: IMPLICATIONS FOR ERα AND BRAIN-SPECIFIC PPARγ KNOCKOUT MODELS IN THE INTERACTION OF ORGANOPHOSPHATE FLAME RETARDANTS AND DIET-INDUCED OBESITY IN ADULT MICE

3. Implications for ERa and brain-specific PPARγ knockout models in the interaction of organophosphate flame retardants and diet-induced obesity in adult mice

3.1 Abstract

The expanding integration of synthetic chemicals into modern life exposes human populations to increased human health hazards such as endocrine disrupting chemicals (EDCs). EDCs interact with and disrupt endogenous signaling pathways governing biological homeostasis. A growing concern for EDCs is their capacity to dysregulate homeostatic maintenance of food consumption and metabolism, leading to serious health concerns such as diabetes and metabolic syndrome. One emerging class of EDCs is organophosphate flame retardants (OPFRs). OPFRs interact with nuclear receptors peroxisome proliferator-activated receptor (PPAR) γ and estrogen receptor (ER) α, both of which are important regulators of energy homeostasis. Previously, we reported sex-dependent interactions of a mixture of OPFRs with energy homeostasis and a model of diet-induced obesity within adult mice. Using the same mixture {1 mg/kg bw/day of each triphenyl phosphate, tricresyl phosphate, and tris(1,3-dichloro-2-propyl)phosphate}, we exposed male and global ERα knockout (ERαKO), and brain-specific PPARγ knockout (PPARγKO) mice concurrently with either a low-fat diet (LFD) or high-fat diet (HFD). We measured body weight and composition, crude food intake, meal patterns, and glucose and insulin tolerance. Comparatively to our previous study, OPFR increased male body weight and fat mass. These effects were not present within either ERαKO or PPARγKO mice, suggesting that these receptors are important for OPFR dysregulation of body composition. Interestingly, we also observed numerous novel effects of OPFR exposure

within knockout mice. Males experienced alterations to glucose and insulin tolerance, more so in PPARγKO mice. Additionally, feeding efficiency was reduced when fed LFD. On the other hand, females of both knockout genotypes exhibited decreased energy intake and diminished fat mass and demonstrated perturbed insulin tolerance. Novel effects of OPFR in knockout models are intriguing, and perhaps are due to the loss of receptor making the animal more vulnerable to homeostatic disruption. Collectively, these data demonstrate both direct and indirect actions of OPFR on PPARγ- and ERα-mediated pathways governing energy homeostasis.

3.2 Introduction

Endocrine disrupting chemicals (EDCs) are substances capable of disrupting typical endocrine system function. The endocrine system plays a vital role in maintaining bodily homeostasis and EDC interference can result in reprotoxic, immunotoxic, neurotoxic, and obesogenic endpoints. Concerningly, EDCs can be found in a variety of household products, such as toys, furniture, most plastics, nail polish, and foodstuffs. The now ubiquitous nature of EDCs within the environment reveals EDCs as a substantial human health risk. In the past, toxicological research has motivated regulatory action to limit human exposure for such EDCs as polybrominated diphenyl ethers (PBDEs) (Zota et al. 2013). PBDEs were a popular variety of flame-retardant (FR) chemicals utilized for their dampening effect on flammability (Cádiz et al. 2011), until research revealed toxicological impact on reproduction, neurodevelopment, and thyroid homeostasis (Linares et al. 2015; Zota et al. 2011; Gilbert et al. 2012; Herbstman et al. 2010). The subsequent decline in PBDE use was matched with the rise of another FR known as organophosphate flame retardants (OPFRs) (Zota et al. 2013; Israel Chemicals ltd. 2015; Yasin et al. 2016; van

der Veen and de Boer 2012). OPFRs were originally thought to be a safer alternative to PBDEs because they were expected to have less persistence in the environment (Zhang et al. 2016). Despite this, their overwhelming usage has allowed for OPFR detection at concerning concentrations in human serum (680-709 ng/m), urine (1-10 ng/ml), and breast milk (1-10 ng/ml) (Butt et al. 2014; Hoffman et al. 2017; Ma et al. 2017; Ma et al. 2019; Meeker et al. 2013). Furthermore, OPFRs are now known to have similar toxicological impacts as their predecessors, demonstrating neurological, reproductive, immune, obesogenic, and endocrine disruptive effects (Dishaw et al. 2011; Patisaul et al. 2013; Kylie et al. 2018; Belcher et al. 2014; Pillai et al. 2014; Liu et al. 2013; Liu et al. 2012; Hu et al. 2019; Steves et al. 2018). In combination with their prevalent human exposure, OPFRs present as a strikingly unaddressed human health risk.

EDC capacity of OPFRs has been identified, in part, through their ability to interact with nuclear hormone receptors such as estrogen receptor alpha (ERα) and peroxisome proliferator-activated receptor gamma (PPARγ). Three commonly used OPFRs that demonstrate these interactions are triphenyl phosphate (TPP), tris(1,3-dichloro-2-proply)phosphate (TDCPP), and tricresyl phosphate (TCP). *In vitro* studies show that TPP activates ERα and PPARγ (Kojima et al. 2013; Pillai et al. 2014; Tung et al. 2017), and TDCPP is known to upregulate ERα and associated genes (Liu et al. 2013). TCP also demonstrates agonistic activity on ERα (Kojima et al. 2013). However, in another study, TPP, TDCPP, and TCP all acted as antagonists to ERα, blocking receptor binding of estrogen (E2), but caused elevated E2 and testosterone levels in developing zebrafish (Liu et al. 2012). The mixed capacity for disruption may be attributed to weak agonistic activity of these compounds competing with endogenous ligand signaling. Regardless, the ability of TPP, TDCPP, and TCP to interact with ERα and PPARγ is why these chemicals

were selected for our current study investigating OPFR EDC action on ERα and PPARγ associated signaling.

ERα is involved in many homeostatic pathways, but in particular, it shares common ground with PPARγ in the maintenance of energy homeostasis. ERα and its endogenous ligand 17β-estradiol demonstrate an overall "catabolic" effect, decreasing energy intake and increasing its expenditure (Mauvais-Jarvis et al. 2013). Disruption of estrogenic regulation of energy balance can result in metabolic syndrome and its symptomatic sequalae obesity, hypertension, and pre-diabetes (Hevener et al. 2015). ERα is densely present within adipose tissue, where it acts to regulate fat distribution and storage (Rettberg et al. 2014). Menopause and the resulting decline in circulating E2 is associated with increased bodyweight gain, altered leptin and adiponectin levels, and increased risk for obesity and type 2 diabetes (Rettberg et al. 2014). The integrated crosstalk between ERα and insulin-like growth factor 1 receptor (IGF-1R) have been extensively studied, indicating the role of E2 in insulin signaling (Song et al. 2004; Mendez and Garcia-Segura 2006; Kahlert et al. 2000; Garcia-Segura et al. 2010). Furthermore, ERα knockout (ERαKO) mice exhibit insulin insensitivity and severe intra-abdominal obesity (Heine et al. 2000), the effects of which were potentiated by a high-fat diet (Ribas et al. 2010). ERα is also expressed within the brain, and particularly concentrated in the arcuate (ARC) nucleus of the hypothalamus (Shughrue et al. 1997). The ARC contains neuropeptide Y (NPY) and proopiomelanocortin (POMC) neurons, both of which are integral in central regulation of feeding behavior and are regulated by E2 actions through multiple types of estrogen receptors (Stincic et al. 2018; Saito et al. 2016; de Souza et al. 2011; Acosta-Martinez et al. 2007).

PPARγ is also expressed by both NPY and POMC neurons, through which it too is capable of influencing feeding behavior (Garretson et al. 2015; Sarruf et al. 2009). Like

ERα, PPARγ is peripherally predominant within adipose tissues, where it directs adipogenesis and lipid metabolism (Wang 2010). Disordered fatty acid metabolism and storage is associated insulin resistance, and one of the leading pharmacological therapy are thiazolidinediones (TZDs), which are potent PPAR agonists (Wang 2010). Adipocyte PPARγ is thought to be the major target for TZD action, however Lu and company (2011) observed that brain-specific knockout of PPARγ (PPARγKO) abolished the effects of TDZ rosiglitazone (Lu et al. 2011). This demonstrates an essential role of neuronal PPARγ in protecting against insulin insensitivity. Neuronal action of rosiglitazone induces ingestive and hoarding behaviors in male mice and hamsters (Garretson et al. 2015) that is abolished with targeted knockout of PPARγ within POMC neurons (Stump et al. 2016). Brain-specific PPARγKO mice also exhibit resistance to diet-induced obesity (Lu et al. 2011).

Previously, we demonstrated that sub-chronic OPFR exposure within adult mice elicits sex-dependent alterations of feeding behavior and energy homeostasis (Chapter 2, Vail et al., 2020 *in press*). These effects intersected with diet-induced obesity, resulting in exposed males gaining more weight and fat mass only when fed a high-fat diet (HFD). In addition, while exposed females saw no body weight effects, they ate less food and consumed fewer HFD meals per day (Chapter 2, Vail et al., 2020 *in press*). Because ERα and PPARγ are integrated in central and peripheral regulation of feeding behavior, and because OPFRs are known to interact with each, we hypothesize that OPFR disruption of energy homeostasis is, in part, due to action through these respective receptor-mediated pathways. To further elucidate the toxicological intersection of OPFR exposure and diet-induced obesity with PPARγ and ERα signaling, we utilized total ERαKO and brain-specific PPARγKO models for adult male and female mice fed either a

low-fat, or high-fat diet. We hypothesize that without its presumed targets, OPFR exposure will be unable of producing the effects seen in wild-type mice.

3.3 Methods

Animals

All animal experiments were conducted with approval by the Rutgers University Institutional Animal Care and Use Committee and followed National Institution of Health standards. Male *Pparg*$^{fl/fl}$ mice and female *Syn1*$^{Cre/+}$ mice were purchased from Jackson Laboratories and bred to generate *Pparg*$^{fl/+}$/*Syn1*$^{Cre/+}$ offspring. Female *Pparg*$^{fl/+}$/*Syn1*$^{Cre/+}$ offspring were then bred with *Pparg*$^{fl/fl}$ male mice to produce *Pparg*$^{fl/fl}$/*Syn1*$^{Cre/+}$ transgenic mice with selective knockout of PPARγ only within the brain (PPARγKO). Importantly, *Syn1-cre* expresses within the testis and male Syn1-cre mice are capable of producing confounding germline recombinants.(Rempe et al. 2006) To avoid this, we maintained the *Syn1-cre* allele within female breeders, which do not experience this effect. Estrogen Receptor alpha knockout transgenic mice (ERαKO) were selectively bred as previously described (Yasrebi et al. 2017) simultaneously with PPARγKO mice, and both strains were a fed food and water *ad libitum* and maintained under controlled temperature (23 °C) and photoperiod conditions (12/12 h light/dark cycle). At weaning, animals were number-tagged and ear-clipped for genotyping and fed a standard low-phytoestrogen chow diet (Lab Diets 5V75) until the start of experimentation.

Genotyping

Ear-clips were taken from all mice for genotyping at weaning, and again upon euthanasia for confirmation. Genotyping for ERαKO mice was determined using

previously published protocols using forward and reverse primers (Ex3a-F: CTGTAGGCTTTGTCTTCGCTTT, Ex3a-R: CAACCAAGGAGAACAGACAGACTTA).(Hewitt et al. 2014; Hewitt et al. 2010) Genotyping for PPARγKO mice required testing for the presence of both *Syn1-cre* and the absence of *Pparg*. To this end, we used primers according to established protocols from Jackson Laboratory (Syn1-Cre+: XXXF: CTCAGCGCTGCCTCAGTCT, XXXR: GCATCGACCGGTAATGCA; and Syn1-Cre-: XXXF: CTAGGCCACAGAATTGAAAGATCT, XXXR: GTAGGTGGAAATTCTAGCATCATCC) to detect for heterozygosity of the *Syn1-cre* gene. In addition, primers (XXXF: TGGCTTCCAGTGCATAAGTT, XXXR: TGTAATGGAAGGGCAAAAGG) were utilized to detect homozygous absence of *Pparg*. Ear-clip DNA was extracted and *Syn1*-cre was amplified in RedTaq mix (Sigma) with 9 cycles of 94 °C for 20 s, 65 °C for 15 s, 68 °C for 10 s, followed by another 9 cycles of 94 °C for 15 s, 60 °C for 15 s, 72 °C for 10s 68 °C for 10 s, and lastly 27 cycles of 94 °C for 15 s, 60 °C for 15 s, 72 °C for 10 s. *Pparg* DNA was amplified using the same temperature paradigm, but extended the first two cycles to have 15 repeats, and the last cycle was repeated 44 times. Amplified DNA was loaded into wells of 3% agarose gel in 1x TBE buffer for DNA electrophoresis separation.

Diets

To examine the intersection of adult OPFR exposure and PPARγ and ERα influence on diet-induced obesity, male and female PPARγKO and ERαKO mice were fed either a low-fat diet (LFD, 3.85 kcal/g, 10% fat, 20% protein, 70% carbohydrate; D12450H) or high-fat diet (HFD, 4.73 kcal/g, 45% fat, 20% protein, 35% carbohydrate; D12451; Research Diets). Starting at 10 weeks of age, mice were continually fed either LFD or HFD concurrently with OPFR treatment up through study completion.

OPFR dosing

A singular mixture of three OPFRs were used in this study to emulate the mixed nature of human exposures. OPFRs utilized were tricresyl phosphate (TCP, CAS no. 1330-78-5; purity 99%; purchased from AccuStandard, New Haven, CT), and triphenyl phosphate (TPP, CAS no. 115-86-6; purity 99%) and tris (1,3-dichloro-2-propyl)phosphate (TDCPP, CAS no. 13674-87-8; purity 95.6%) (both purchased from Sigma-Aldrich, St. Louis, MO). 100 mg of each OPFR were dissolved as a mixture within the same 1 ml acetone (Sigma) for long term storage to generate 1 mg/ml stock mixture of OPFR-acetone. A working solution was generated by transferring 100 μl OPFR-acetone into 10 ml sesame oil (Sigma-Aldrich) to create an oil mixture containing 1 mg/ml OPFR (OPFR-oil). To generate control-oil mixture, 100 μl acetone was added to 10 ml sesame oil (control-oil). OPFR-oil and control-oil mixtures were left stirring for 48-72 h to evaporate the acetone from the mixture. Based on body weight, the resulting mixtures were then added to dehydrated peanut butter vehicle (~50 mg) to create rehydrated peanut butter with a final concentration of 1 mg/kg bw OPFR or equivalent amount of OPFR-free peanut butter. The resulting 1 mg/kg bw doses were placed on weigh paper and supplied to mice daily to be consumed orally. Oral exposure began at 10 weeks of age at 0900-1100 h each day for ~ 5 weeks in a sub-chronic paradigm.

Experimental Design

Adult male and female mice (n = 8 per sex, per genotype, per diet, per treatment) were weight-matched in paired housing and fed either LFD or HFD and dosed daily with OPFR-oil or control-oil for 4 weeks. Mice started treatment in sequential batches of 8 male and 8 female mice to ensure adequate sample size for metabolic and feeding behavior studies. Body composition (fat and lean mass) were quantified by EchoMRI^{TM} Body composition

(Houston, TX) on the first day of dosing (baseline), and again after completion of 4 weeks exposure to OPFR-oil mixture. During this time, body weight and crude food intake per cage were measured weekly. Afterwards, mice were transferred to the Biological Data Acquisition (BioDAQ, Research Diets, New Brunswick, NJ) chambers for 1 week. Mice were continually dosed with OPFR-oil or control-oil during this time. Mice received 94 h habituation and 72 h data acquisition for feeding behaviors (meal size, duration, frequency). LFD or HFD chow were contained in touch-sensitive hoppers and food intake was measured as decreased chow weight within the hoppers. Whenever the mouse touched the hopper for food, the system denoted that as a "bout." When the interval between bouts was greater than 300 s, the food eaten was determined to be a "meal." A meal could consist of any number of bouts, until the inter-bout interval exceeded 300 s. Some mice fed HFD exhibited what we refer to as "food chewing" behavior, where chow was removed from the hopper but was used for enrichment chewing, and not actually consumed. When food chewing behavior was observed, feeding data for that day was excluded from analysis for the respective mouse. This accounts for the variation in *n* within our feeding behavior data. Lastly, all mice were tested for glucose and insulin tolerance. Prior to the glucose tolerance test (GTT), mice were fasted for 5 h and then intraperitoneally (IP) injected with a bolus of 2 g/kg glucose. Blood-glucose was measured from tail bleeds using an AlphaTrak glucometer (Zoetis, Parsippany, NJ) at 0, 15, 30, 60, 90, and 120 min post-injection. Mice were given a 4-day recovery period before then undergoing the insulin tolerance test (ITT). After a 4 h fast, mice were IP injected with 0.75 U/kg insulin and blood-glucose was measured from tail bleeds at 0, 15, 30, 60, 90, and 120 min. 1 week following the ITT, mice were dosed at 0900 h, fasted at 1000 h, and euthanized at 1100 h by decapitation after sedation with 100 mg/ml ketamine. Female mice were euthanized during diestrus to control for circulating ovarian hormone levels. Trunk blood was collected in K^+-EDTA coated tubes with the addition of proteinase

inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (1 mg/ml, Sigma-Aldrich) to protect against peptide degradation. Samples were maintained on ice until centrifugation at 1,100 rcf for 15 min at 4 °C. Plasma supernatant was collected and stored at -80 °C for future analysis of insulin, leptin, and ghrelin levels, using a multiplex assay (MMHMAG-44 K, EMD Millipore, Billerica, MA; these studies are not complete due to the COVID pandemic).

Data Analysis

All data are depicted as mean ± SEM. Data were analyzed using GraphPad Prism software (GraphPad Software, LA Jolla, CA) by a two-way ANOVA (OPFR and Diet) with a *post-hoc* Newman-Keuls multiple comparisons test, or with Statistica 7.1 software (StatSoft, Tulsa, OK, USA) by multi-factorial ANOVA or with repeated-measures, three-way ANOVA (Diet, OPFR, Time), followed with *post-hoc* Newman-Keuls multiple comparisons test. Effects were considered significant at $P \leq 0.05$.

3.4 Results

Physiological Parameters

Body weight and crude food intake were measured over the course of 4 weeks in male and female mice lacking expression of estrogen receptor alpha (ERαKO), or brain-specific peroxisome proliferator-activated receptor gamma (PPARγKO). During this time, mice received either control-oil or OPFR-oil mixture (1 mg/kg each of TCP, TPP, and TDCPP). Feeding efficiency was calculated as the ration of bodyweight gain to crude food intake, and is represented as grams gained to kcal consumed. Afterwards, we assessed body composition of lean and fat mass by EchoMRI^{TM} (Figures 1-4). Baseline bodyweights were

taken at day zero, just prior to treatment and diet initiation (ERαKO males: control – 23.3 ± 0.4 g, OPFR – 24.2 ± 0.4 g; ERαKO females: control – 23.2 ± 0.5 g, OPFR – 22.5 ± 0.5g; PPARγKO males: control – 24.6 ± 0.7 g, OPFR – 24.5 ± 0.3 g; PPARγKO females: control – 19.9 ± 0.4 g, OPFR – 19.4 ± 0.3 g).

ERαKO

Male ERαKO mice exhibited an elevation in energy intake, feeding efficiency, bodyweight gain, as well as increased fat mass and reduced lean mass when fed a high fat diet (HFD), in comparison to a low-fat diet (LFD, Fig. 2). While OPFR treatment decreased the feeding efficiency of ERαKO males fed LFD (Fig. 2C, $F(1,12)_{OPFR}= 7.91$; $P < .05$), this did not result in any changes in body mass and OPFR appears to have minimal impact in ERαKO males. In ERαKO females, HFD significantly augmented bodyweight gain, but only when fed OPFR-oil mixture (Fig. 2A, $F(1,12)_{Diet}= 4.67$; $P < .05$). Interestingly, while OPFR-exposed ERαKO females gained more weight fed HFD compared to LFD, there was no difference in caloric between diets in OPFR-treated females (Fig. 2B). This may be attributed to a decreased HFD intake compared to oil-control in female ERαKO mice (Fig. 2B, $F(1,12)_{OPFR}= 10.28$; $P < .01$) and suggests an altered metabolic processing of HFD. Female ERαKO, OPFR-exposed mice also experienced decreased fat mass when fed LFD (Fig. 2D, $F(1,12)_{OPFR}= 5.58$; $P < .05$).

PPARγKO

PPARγKO males did not appear to be affected by OPFR exposure within in these parameters (Fig. 3), showing the same patterns as ERαKO males (Fig. 1). However, in female PPARγKO mice, HFD significantly augmented bodyweight gain, again, only within OPFR-treated animals (Fig. 4A, $F(1,22)_{Diet}= 8.68$; $P < .01$; $F(1,22)_{OPFR}= 7.37$, $P < .05$).

This appears to be due to a reduction in LFD weight-gain. Supporting these data, we observed that female PPARγKO mice exposed to OPFR exhibit a 3-fold reduction in LFD feeding efficiency (Fig. 4C, $F(1,11)_{OPFR}= 10.31$, $P < .01$). Meaning, that OPFR-exposed females fed LFD are gaining less weight than their oil-fed counterparts, despite equivalent energy intake. Also, *post-hoc* analysis revealed a 5 ± 2% decrease in fat mass by OPFR exposure in PPARγKO females fed HFD (Fig. 4D, $F(1,24)_{Diet}= 4.44$, $P < .05$; $F(1,24)_{OPFR*Diet}= 4.41$, $P < .05$).

Feeding Behaviors

The BioDAQ apparatus was utilized for more in-depth analysis of feeding behaviors, expanding on our physiological parameter findings. Over a 96-h trial period, hourly and total food intake were measured, and the size, duration, and frequency of meals was calculated.

ERαKO

OPFR did not impact either total or hourly patterns of food intake in ERαKO males (Fig. 5A & 5B). Neither did OPFR affect meal duration or size. Meal frequency, while increased by HFD in oil-fed mice (Fig. 5C, $F(1,26)_{Diet}=5.24$, $P < .05$), was unaffected by diet in OPFR-fed mice. On the other hand, ERαKO females experienced perturbances to diurnal food intake patterns by OPFR (Fig. 6A). From 2000 to 2100 h, OPFR-treated mice ate half as much HFD as their oil-treated counterparts (Fig. 6A, $P < .0001$). In turn, OPFR-treated mice consumed less HFD than LFD during the same time frame, as well as during 2100-2200 and 2300-2400 h (Fig. 6A, $P < .05$, $P < .05$, $P < .05$). Total food intake over the 96-h testing period remained unaltered by OPFR exposure, as did meal frequency, meal duration, and meal size.

PPARγKO

OPFR exposure induced subtle alterations to hourly food intake in PPARγKO male mice (Fig. 7A). During 2100-2200 h, OPFR-exposed male mice consumed more LFD than control males (Fig. 7A, P <.05). And during the same window, OPFR exposed mice consumed more LFD than HFD (Fig. 7A, P <.05), whereas control mice did not experience this diet effect. Instead, control males consumed more LFD than HFD during 2200-2300 h (Fig. 7A, P < .001). However, total food intake over 96 h was unaffected, as was meal size and frequency (Fig. 7B, C, E). While control males fed HFD spent less time in their meals compared to their LFD counterparts (Fig. 7D, $F(1,25)_{Diet}$= 18.30, P < .001), this diet effect was not seen in OPFR-treated mice, indicating an OPFR influence on meal duration. PPARγKO females also experienced a main effect of OPFR on hourly food intake patterns (Fig. 8A, $F(1,19)_{OPFR}$=6.49, P < .05). *Post*-hoc analysis revealed specific alterations during the night. During 2100-200 h and during 2300-2400 h, OPFR diminished LFD intake compared to oil-control females (Fig. 8A, P < .0001, P < .05). HFD intake was also decreased by OPFR during 1900-2000 h and 0200-0300 h (Fig. 8A, P < .05). Conversely, PPARγKO females experienced a spike in HFD consumption in OPFR-fed mice during 0400-0500 h compared to both OPFR-treated LFD mice and to control-treated HFD mice (Fig. 8, P < .001, P < .01). Unfortunately, due to an issue with excessive food chewing, the BioDAQ™ apparatus was unable to accurately measure total HFD intake over the 96-h period. Animals fed LFD did not have this issue, and OPFR was found to have no statistical effect on total ingestion (Fig. 8B). Additionally, OPFR did not alter meal duration nor size (Fig. 8D, E). Control females initiated more meals per day when fed HFD as compared to LFD (Fig.8 C, $F(1,18)_{Diet}$= .41, P < .05), but OPFR exposure eliminated this diet effect (Fig. 8C).

Glucose and Insulin Tolerance

Glucose and insulin tolerance tests (Figures 9-12) were performed to examine OPFR's impact on the body's response to sudden changes in glucose homeostasis.

ERαKO

OPFR had no direct effect on glucose tolerance, compared to oil-control counterparts. However, while diet did not significantly alter glucose tolerance in males (Fig. 9A-C), ERαKO female mice had greater area under the curve (AUC) when fed HFD than when fed LFD (Fig. 9F, $F(1,24)_{Diet} = 11.83$, $P < .05$). This means that HFD was impeding glucose uptake after being administered a bolus injection. This diet effect was not observed in OPFR-treated female mice (Fig. 9F), indicating that OPFR is reducing the effect HFD has on glucose clearance. This is further supported by the observed significant difference in HFD blood-glucose at the last time point in our tolerance test. By this point, OPFR-treated females have nearly returned to baseline glucose levels, whereas oil-control mice remain significantly elevated (Fig. 9E, $F(3,24)_{OPFR} = 4.57$, $P < .05$).

While female mice experienced diet effects in glucose tolerance, the opposite was true for insulin tolerance. Insulin tolerance in ERαKO females was unaffected by both diet and OPFR (Fig.10C, D). In control males, though, HFD resulted in greater AUC than did LFD (Fig. 10B, $F(1,27)_{Diet} = 9.20$, $P < .05$). However, this effect was not seen in OPFR-treated males. Examining Fig. 10A, we see that OPFR exposure results in elevated blood-glucose in male mice fed LFD during the last two time points ($F(3,27)_{OPFR} = 5.04$, $P < .05$, $P < .05$). This informs us that the reason why we do not observe a diet effect in OPFR treated ERαKO males is likely due to impaired insulin tolerance in OPFR-treated males fed LFD. This is supported by a statistical trending effect of OPFR on ERαKO male mice (Fig. 10A, $F(1,27)_{OPFR} = 3.89$, $P = .059$).

PPARγKO

Oil-control PPARγKO male mice exhibited HFD-induced elevation of fasting glucose (Fig.11A, $F(1,28)_{Diet}$= 17.19, *P* < .0001). Male mice exposed to OPFR, however, had equivalent LFD and HFD fasting glucose levels. This is explained by an OPFR-induced elevation of fasting glucose in male mice fed LFD (Fig. 11A, $F(1,28)_{OPFR}$=1.23, *P* < .01). Additionally, OPFR exposure also resulted in elevated blood-glucose in mice fed HFD at two time points during the glucose tolerance test (Fig. 11B, $F(3,23)_{OPFR}$= 5.14, *P* < .05, *P* < .05). This correlated to OPFR-exposed males displaying greater AUC in HFD-fed than LFD-fed mice (Fig. 11C, $F(1,23)_{Diet}$= 11.45, *P* < .01). This diet effect was not observed in oil-control males, therefore OPFR may be decreasing the ability of PPARγKO males to respond to sudden changes in glucose homeostasis. In PPARγKO female mice, HFD elevated fasting glucose, irrespective of treatment (Fig. 11D, $F(1,24)_{Diet}$= 20.41, *P* < .05, *P* < .05). There were no OPFR, nor diet effects on glucose tolerance.

Insulin tolerance AUC was unaltered by diet or OPFR in PPARγKO males (Fig. 12B), but OPFR did subtly alter the tolerance curve (Fig. 12A, $F(3,27)_{OPFR}$= 1.42). OPFR enhanced the response to insulin in male mice fed HFD at t = 15 min (Fig. 12A, *P* < .0001), bringing the curve down to match the response seen in mice fed LFD. Furthermore, while control-oil males fed HFD had a reduced response to insulin at the same time point (Fig 12A, *P* < .0001), OPFR-exposed males showed no effect of diet at any time points. This indicates that OPFR exposure is eliminating the effect of HFD to decrease insulin sensitivity in PPARγKO males. OPFR exposure resulted in reduced blood-glucose in PPARγKO females at multiple time points throughout the insulin tolerance test (Fig. 12C, $F(3,24)_{OPFR}$= 2.88, *P* < .05, *P* < .05, *P* < .05). This resulted in a significant reduction of AUC in OPFR-treated females fed LFD, compared to HFD (Fig. 12D, $F(1,23)_{Diet}$= 3.52, *P* < .05). Additionally, there was a trending decrease of LFD AUC, compared to oil-control

(Fig. 12D, $F(1,23)_{OPFR}= 3.92$, $P = 0.10$). Overall, this suggests that OPFR increases insulin sensitivity in PPARγKO females fed LFD.

3.5 Discussion

The current focus on sensitive developmental exposure windows has resulted in a lack of understanding for how OPFR impacts energy homeostasis throughout adult life. In particular, there are key knowledge gaps in the mechanisms of endocrine disruption. Both ERα and PPARγ are proposed OPFR targets, and this study further implicates these nuclear receptors as targets for OPFR toxicity. This discussion will heavily reference our previous study using the same concentration and methodology of OPFR exposure in wildtype mice (Chapter 2, Vail et al., 2020, *in press*). A table summarizing direct effects of OPFR in this study is provided (Table 1).

ERα knockout mice

Consistent with previous findings (Heine et al. 2000; Bian et al. 2019), complete knockout of ERα resulted in an overall increased weight gain of both male and female mice, respective to wildtype (WT) mice (refer to Chapter 2, Figures 1 and 2). ERαKO males responded to HFD with significantly increased adiposity and bodyweight gain compared to mice fed LFD, but no effect of OPFR-treatment was observed (Fig.1A, D). However, in our previous study, WT males exposed to OPFRs exhibited an increased fat mass and bodyweight gain when fed HFD, compared to their control-treated counterparts (Chapter 2, Fig 1A, C, Vail et al., 2020 *in press*). The absence of these observed effects in ERαKO males suggests that ERα plays a vital role in facilitating OPFR effects on fat deposition and bodyweight gain. ERα is an important regulator of energy homeostasis,

and the observed lack of OPFR action may be due to its inability to target and disrupt ERα regulation of adiposity and bodyweight. Further, OPFR-treated ERαKO male mice experienced a decreased feeding efficiency when fed LFD, compared to control mice (Fig. 1C). This effect was not seen in WT mice, indicating that OPFRs are acting through a pathway not directly influenced by ERα, but perhaps associated. A toxic effect in a knockout model appearing where there was none in WT mice suggests that the action of knocking out the target gene produced alterations in inherent signaling pathways, compensating for the loss of the gene. In the case of ERα, compensatory pathways such as ERβ (also known to interact with OPFRs), or membrane estrogen receptors such as G protein-coupled estrogen receptor (GPER) or G_q--coupled membrane estrogen receptor (Gq-mER) may be upregulated and perhaps more sensitive to OPFR action, all of which are capable of regulating energy homeostasis (Mauvais-Jarvis et al. 2013; Shi et al. 2013a; Prossnitz and Hathaway 2015; Roepke et al. 2010). It is also possible that, in the absence of ERα, OPFRs may be acting preferentially upon PPARγ. ERβ-null mice have been shown to exhibit upregulated PPARγ signaling (Foryst-Ludwig et al. 2008), but ERαKO models are not known to have the same effect. However, Yasrebi et al. (2017) reported an interaction between ERαKO genotype and diet, wherein expression of PPARγ was unaltered in HFD-fed ERαKO females, when control WT mice experienced a tripling in expression (Yasrebi et al. 2017). An altered response to HFD may change how OPFRs interacts with PPARγ signaling in ERαKO mice.

While male ERαKO mice and all WT mice demonstrated increased bodyweight gain from being fed HFD (Fig. 1A; Chapter 2 Figures 1A and 2A, Vail et al., 2020 *in press*), control ERαKO females appeared to experience a greater bodyweight gain when fed LFD, resulting in no statistical difference between LFD and HFD control mice (Fig. 2A). This would be considered a genotype effect. It is interesting, however, that OPFR exposure

restored the diet-effect in female ERαKO mice. This could be explained as an estrogenic effect of OPFRs on alternative estrogen receptors (ERs) to restore WT patterns. This hypothesis is based in the idea that without the "catabolic" actions of ERα, body physiology shifts towards a more "anabolic" pattern. However, alternative ERs may be recruited to compensate for the loss of ERα, though at lesser efficacy. Potentially, OPFRs may be causing increased activity of these alternative pathways, aiding their ability to compensate for the lack of ERα. This hypothesis is further supported by our findings that OPFRs decreases caloric intake on HFD, as well as reduces fat mass in female ERαKO mice fed LFD (Fig. 2B, D). These OPFR effects were not seen in WT mice, and again, could therefore be a result of alternative ER activation by OPFR.

The BioDAQ™ apparatus used for detailed analysis of feeding behavior returned little effect of OPFR on ingestive patterns in male ERαKO mice. The only discernable impact appears to be a decrease in how often mice were initiating HFD meals. This is observable by a significant increase in meal frequency in ERαKO males fed HFD, compared to LFD in control-treated mice, whereas OPFR-treated mice showed equivalent frequencies between diets (Fig. 5C). Our previous experimentations revealed a decreased production of ghrelin in WT males exposed to OPFRs (Table 1, Chapter 2 Fig. 9C, Vail et al., 2020 *in press*). ERα signaling in the gut is shown to induce ghrelin production, a powerful orexigenic hormone (Sakata et al. 2006). Additionally, estrogen replacement therapy in post-menopausal patients increases circulating ghrelin (Kellokoski et al. 2005). Therefore, the global loss of ERα is likely affecting ghrelin production, and may therefore be interacting with how OPFR exposure modulates ghrelin to produce our observed effects on reduced feeding initiation. Additionally, WT males also experienced greater HFD-induced insulin levels (Chapter 2, Fig. 9A, Vail et al., 2020 *in press*). Because insulin signaling reduces food intake (Woods et al. 2006) and is estrogenically regulated (Gupte

et al. 2015; Shen et al. 2014), it is possible that insulin signaling in the absence of ERα may be more vulnerable to OPFR dysregulation, resulting in decreased meal frequency in knockout mice.

Female ERαKO mice also displayed few direct effects of OPFRs in ingestive behavior (Fig. 6). In the previous WT study, OPFR exposure modestly impacted the temporal intake of food in female mice, decreasing HFD intake during the night (Chapter 2, Fig. 6E, Vail et al., 2020 *in press*). These effects were replicated in ERαKO mice, suggesting that the mechanism of OPFR disruption of diurnal feeding patterns is not through ERα. However, while WT females experienced a significant decrease in meal frequency and total intake of HFD compared to control (Chapter 2, Fig. 6A, B, Vail et al., 2020 *in press*), significant effects were not observed within ERαKO mice (Fig. 5B, C). This indicates a potential role for ERα as an OPFR target in these parameters.

Lastly, ERαKO mice were tested for both glucose and insulin tolerance. ERαKO males displayed no significant effect of OPFRs on glucose tolerance, as was also true for the WT experiments (Fig. 9A, B, C; Chapter 2 Fig 7A, B, C, Vail et al., 2020 *in press*). However, insulin tolerance appears altered by OPFR in ERαKO male mice fed LFD. The LFD tolerance curve is significantly elevated in the latter half of the test, and nearly matches HFD data (Fig. 10A). This is further represented in a significant difference in area under the curve (AUC) between diets in control-treated males, where no marked effect was observed within OPFR-treated animals (Fig. 10B). This effect was not seen in WT mice and should be attributed to OPFR action on targets alternative to ERα. Female ERαKO mice displayed similar tolerance to glucose injection to the WT mice in the previous study (Fig. 9D, E, F; Chapter 2 Fig. 7D, E, F, Vail et al., 2020 *in press*). Some subtle differences are present, but none notable enough to conclude OPFR disruption of glucose tolerance. No observable effects of OPFR were found in the insulin tolerance test.

Overall, there appears to be no sex-specificity for OPFR action through ERα or proposed alternative ER pathways. Male and female ERαKO mice each experienced their own share of OPFR perturbations, with predominant effects on bodyweight gain and body composition, and modest effects on ingestive behaviors. From these data we conclude that ERα appears to be involved in mediating some, but not all, of the ingestive dysregulation elicited by OFPR exposure.

Neuronal PPARγ knockout mice

Neuronal knockout of PPARγ has been shown to limit the result of increased weight gain when fed HFD (Lu et al. 2011), and our findings report similar trends of an approximate 10% less weight gain in HFD-fed PPARγKO mice than WT counterparts (Figures 3A, 4A; Chapter 2 Figures 1A, 2A,, Vail et al., 2020 *in press*). PPARγKO males did not see any direct effects of OPFR exposure on weight gain nor adiposity (Fig. 3A, D). However, in WT mice, OPFR-treated males experienced greater weight gain and fat mass than control when fed HFD (Chapter 2, Fig. 1A, C, Vail et al., 2020 *in press*). Therefore, we can conclude that OPFR-induced adiposity and weight gain in males can be attributed, in part, to interaction with PPARγ. PPARγ is well-known for its endogenous regulation of adipose tissue and lipid metabolism (Janani and Ranjitha Kumari 2015; Wang 2010). Therefore, it follows that OPFR disruption of PPARγ manifests as dysregulated fat accumulation. Importantly, ERα was also implicated in OPFR dysregulation of weight gain and fat mass. This highlights the capacity of OPFRs to have converging effects on endpoints regulated by multiple receptor-mediated pathways.

Whereas WT females displayed no effects of OPFRs on bodyweight nor body composition, HFD-fed PPARγKO females exposed to OPFR exhibited decreased fat mass compared to their control-treated counterparts (Fig. 4D). This represents an interaction of

genotype and OPFR, whereas the loss of neuronal PPARγ sensitizes mice to OPFR action on fat deposition. Further, control-treated PPARγKO females experienced roughly 6-fold greater feeding efficiency than control-treated WT mice (Fig. 4C, Chapter 2 Fig. 2B, Vail et al., 2020 *in press*), indicating that mice lacking neuronal PPARγ more readily translate energy intake into bodyweight gain. However, OPFR treatment reduced LFD feeding efficiency to that seen in WT females. One possible explanation for these findings is that the loss of neuronal PPARγ targets may be shifting central actions of OPFR onto estrogenic pathways, and, if OPFR is acting agonistically, this may result in the observed decrease in adiposity and feeding efficiency in PPARγKO mice. Another hypothesis could be that without neuronal PPARγ, OPFR is acting to a higher degree on peripheral PPARγ. This hypothesis is weakened, though, by the knowledge that brain knockout of PPARγ does not result in altered peripheral PPARγ expression (Fernandez et al. 2017; Lu et al. 2011). With so many unknown variables, it becomes impossible to conclude with any certainty the exact mechanisms underlying these findings.

Feeding behavior in both WT and PPARγKO male mice appears to be unaffected by OPFR exposure (Fig. 8). However, there is a difference between WT and PPARγKO female mice. WT females fed HFD initiated fewer meals and consequently ate less when exposed to OPFRs (Chapter 2, Fig 6A, B, Vail et al., 2020 *in press*). Meal frequency in PPARγKO mice was equivalent between control- and OPFR-treated mice (Fig. 8C), indicating that the effect of OPFR to reduce feeding initiation is in part, through neuronal PPARγ interaction. OPFR-treatment in PPARγKO mice also resulted in disrupted hourly feeding patterns (Fig. 8A). WT females were largely unaffected by OPFR treatment, excepting minor time-specific differences (Chapter 2, Fig. 6E, Vail et al., 2020 *in press*). Again, this presents as a novel effect of OPFRs in the absence of neuronal PPARγ.

In WT mice, HFD conferred typical impaired glucose tolerance, but was unaltered by OPFR exposure (Chapter 2, Fig. 7, Vail et al., 2020, *in press*). However, in control-treated PPARγKO mice, glucose tolerance was unaffected by diet (Fig. 11). This presents as a genotype effect, indicating that neuronal PPARγ is important in conferring impaired glucose sensitivity due to diet-induced obesity. This is effect was also observed by Lu et al. (2012), but is contested by Fernandez et al (2017), who showed identical tolerance curves between WT and brain-PPARγKO mice fed HFD. Regardless, pertaining to OPFR exposure, our current study found that OPFR treatment restored HFD-induced impairment of glucose tolerance in male PPARγKO mice (Fig. 11B, C). This implies that OPFRs may be acting on targets alternative to brain PPARγ to mimic the role PPARγ plays in regulating glucose tolerance. Glucose tolerance in female mice were unaffected by OPFR exposure.

Neuronal knockout of PPARγ also impacts insulin tolerance. As seen in our previous WT experimentation, HFD increases AUC in tolerance tests, a marker of insulin insensitivity (Chapter 2, Fig 8 B, D, Vail et al., 2020 *in press*). However, without central PPARγ, control-treated mice display no significant difference in insulin tolerance AUC (Fig. 12 B, D). This is concluded to be a genotype effect and signifies an important role for neuronal PPARγ in the development of HFD-induced insulin intolerance. Interestingly, male PPARγKO mice were further protected against HFD-insulin intolerance when exposed to OPFR, displaying identical insulin tolerance curves and AUC whether fed LFD or HFD. Furthermore, female mice experienced a marked reduction in insulin tolerance when fed LFD, conferring an impressive response to insulin injection (Fig. 12C, D). Overall, OPFR exposure appears to sensitize male and female mice to insulin on HFD and LFD, respectively. While the mechanistic aspect of these results remains undeterminable without further investigations, again, it is possible that the loss of neuronal PPARγ as an OPFR target may increase OPFR action on other pathways that govern

glucose homeostasis, resulting in our observed findings. POMC neurons within the arcuate are essential regulators of hepatic glucose production and are thusly one potential site for OPFR disruption (Caron et al. 2018; Shi et al. 2013b). Importantly, these neurons also express PPARγ, and selective knockout of POMC-PPARγ has been demonstrated to improve glucose metabolism and reduce body weight, fat mass, and food intake when fed HFD (Long et al. 2014). Glucose homeostasis is also impacted within our brain-specific PPARγKO mice, and it appears that the loss of neuronal PPARγ further exposes arcuate control of energy homeostasis to OPFR disruption.

Generally, our experimentations with brain-specific PPARγKO animals produced more novel effects of OPFRs, than our hypothesized absence of effects compared to WT animals. What we can conclude from this is that while some actions of OPFRs can be attributed to neuronal PPARγ (feeding initiation, adiposity, weight gain), most effects appear to be through pathways alternative to central PPARγ.

3.6 Conclusion

Our findings collectively demonstrate differential effects of OPFRs on energy homeostasis and feeding behavior within either ERα or brain-specific PPARγ knockout models. Most effects were sex-dependent, though male and female mice were equally sensitive to OPFR disruption within these knockout models. Within this study, both ERα and neuronal PPARγ are implicated as conduits for OPFR toxicity. Interestingly, both knockout models displayed novel effects of OPFRs on energy homeostasis that is not present within WT mice. These results remain difficult to explain with any certainty; however, it is clear that the loss of either total ERα or central PPARγ sensitizes the animals to OPFR disruption. ERα and PPARγ are both nuclear receptors that, upon activation,

bind to respective response elements to initiate transcription of target genes (Bjornstrom and Sjoberg 2005; Janani and Ranjitha Kumari 2015). Importantly, both ERα and PPARγ demonstrate the reciprocal ability to bind to and activate response elements associated the other receptor (Keller et al. 1995; Wang and Kilgore 2002). The apparent crosstalk between ERα and PPARγ may be contributing to our observations in knockout mice. Both models only lack the receptor, and their associated response elements remain intact. It is therefore possible that activation of intact PPARγ in ERαKO mice may result in transactivation of estrogen response elements, and vice versa within PPARγKO mice. These interactions may play a role in the appearance of novel OPFR effects in our knockout mice. In addition, the loss of ERα or PPARγ may increase OPFR action on the remaining receptors governing energy homeostasis, resulting in novel disruptions.

Overall, this study represents novel insight to the intersection of OPFR endocrine disruption of homeostatic endpoints regulated by ERα and PPARγ. Dysregulation of energy homeostasis can result in metabolic disorders such as obesity, diabetes, and metabolic syndrome. Therefore, it is important to understand the toxicological mechanisms through which OPFR exposure may be pre-dispositioning human populations to such conditions. To aid this study's current understanding of OPFR interaction with ERα and neuronal PPARγ, further experimentations will be conducted, including hypothalamic gene expression, serum peptide hormone levels, and examination of metabolism and energy expenditure (we were unable to use the CLAMS for the KO studies due to a pinworm infestation in the KO colonies). Future studies should then build upon these results with more mechanistic endpoints, such as electrophysiological examination of NPY, POMC, and kisspeptin neurons within the same knockout animals and selective knockout of ERα and PPARγ in specific hypothalamic neuronal populations.

Continued research into the mechanisms of OPFR endocrine disruption will provide a better understanding of its long-term potential for adverse effects on human health.

3.7 Acknowledgements

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Tables

Endpoint	Males		Females	
	LFD	HFD	LFD	HFD
Bodyweight Gain	n.s.	↑↑	n.s.	n.s.
Feeding Efficiency	n.s.	n.s.	n.s.	n.s.
Fat Mass	n.s.	↑↑	n.s.	n.s.
Lean Mass	n.s.	↓↓	n.s.	n.s.
96 h Food Intake	n.s.	n.s.	n.s.	↓↓
Hourly Food Intake	n.s.	↓↑	n.s.	↓↑
Meal Frequency	n.s.	n.s.	n.s.	↓↓
Meal Duration	n.s.	n.s.	n.s.	n.s.
Meal Size	n.s.	n.s.	n.s.	n.s.
Fasting Glucose	n.s.	n.s.	n.s.	n.s.
Glucose Tolerance	n.s.	n.s.	n.s.	n.s.
Insulin Tolerance	n.s.	n.s.	n.s.	n.s.

Table 1. Summary of WT data comparing control- and OPFR-treated groups.
Up arrows denote an OPFR-induced increase and down arrows denote an OPFR-induced decrease. One arrow indicates a modest effect and two arrows indicate a strong effect. One up and one down arrow indicates a mixed effect dependent on time of day. N.S. = not significant.

Figures

Figure 1. Physiological parameters in ERαKO males orally dosed with an OPFR mixture (1 mg/kg bw) for ~7 weeks. **(A)** % Body Weight Gain over 4 weeks; **(B)** Energy Intake; **(C)** Feeding Efficiency; **(D)** Body composition % Fat Mass; **(E)** Body composition % Lean Mass. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparisons test. Uppercase letters denote diet effects within EDC group. Lowercase letters denote OPFR effects within diet (a=P<.05; b=P<.01; c=P<.001; d=P<.0001). Data (n=8) are presented as mean ± SEM.

Figure 2. Physiological parameters in ERαKO females orally dosed with an OPFR mixture (1 mg/kg bw) for ~7 weeks. **(A)** % Body Weight Gain over 4 weeks; **(B)** Energy Intake; **(C)** Feeding Efficiency; **(D)** Body composition % Fat Mass; **(E)** Body composition % Lean Mass. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparisons test. Uppercase letters denote diet effects within EDC group. Lowercase letters denote OPFR effects within diet (a=P<.05; b=P<.01; c=P<.001; d=P<.0001). Data (n=8) are presented as mean ± SEM.

Figure 3. Physiological parameters in PPARγKO males orally dosed with an OPFR mixture (1 mg/kg bw) for ~7 weeks. **(A)** % Body Weight Gain over 4 weeks; **(B)** Energy Intake; **(C)** Feeding Efficiency; **(D)** Body composition % Fat Mass; **(E)** Body composition % Lean Mass. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparisons test. Uppercase letters denote diet effects within EDC group. Lowercase letters denote OPFR effects within diet (a=P<.05; b=P<.01; c=P<.001; d=P<.0001). Data (n=8) are presented as mean ± SEM.

Figure 4. Physiological parameters in PPARγKO females orally dosed with an OPFR mixture (1 mg/kg bw) for ~7 weeks. **(A)** % Body Weight Gain over 4 weeks; **(B)** Energy Intake; **(C)** Feeding Efficiency; **(D)** Body composition % Fat Mass; **(E)** Body composition % Lean Mass. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparisons test. Uppercase letters denote diet effects within EDC group. Lowercase letters denote OPFR effects within diet (a=P<.05; b=P<.01; c=P<.001; d=P<.0001). Data (n=8) are presented as mean ± SEM.

Figure 5. Analysis of feeding behaviors in neuron-specific ERαKO males orally dosed with an OPFR mixture (1 mg/kg bw) for ~5 weeks. **(A)** hourly food intake; **(B)** 96 h total food injested **(C)** meals/day; **(D)** meal duration; **(E)** meal size. Data were analyzed by a two-way ANOVA (B-E) and a repeated-measures three-way ANOVA (A) with post-hoc Newman-Keuls test. Uppercase letters denote diet effects within EDC group; and lowercase letters denote OPFR effects within diet (a=P<.05; b=P<.01; c=P<.001; d=P<.0001). Data (n=5-8 for all groups) are presented as mean ± SEM.

Figure 6. Analysis of feeding behaviors in neuron-specific ERαKO females orally dosed with an OPFR mixture (1 mg/kg bw) for ~5 weeks. **(A)** hourly food intake; **(B)** 96 h total food injested **(C)** meals/day; **(D)** meal duration; **(E)** meal size. Data were analyzed by a two-way ANOVA (B-E) and a repeated-measures three-way ANOVA (A) with post-hoc Newman-Keuls test. Uppercase letters denote diet effects within EDC group; and lowercase letters denote OPFR effects within diet (a=P<.05; b=P<.01; c=P<.001; d=P<.0001). Data (n=5-8 for all groups) are presented as mean ± SEM.

Figure 7. Analysis of feeding behaviors in neuron-specific PPARγKO males orally dosed with an OPFR mixture (1 mg/kg bw) for ~5 weeks. **(A)** hourly food intake; **(B)** 96 h total food injested **(C)** meals/day; **(D)** meal duration; **(E)** meal size. Data were analyzed by a two-way ANOVA (B-E) and a repeated-measures three-way ANOVA (A) with post-hoc Newman-Keuls test. Uppercase letters denote diet effects within EDC group; and lowercase letters denote OPFR effects within diet (a=P<.05; b=P<.01; c=P<.001; d=P<.0001). Data (n=5-8 for all groups) are presented as mean ± SEM.

Figure 8. Analysis of feeding behaviors in neuron-specific PPARγKO females orally dosed with an OPFR mixture (1 mg/kg bw) for ~5 weeks. **(A)** hourly food intake; **(B)** 96 h total food injested **(C)** meals/day; **(D)** meal duration; **(E)** meal size. Data were analyzed by a two-way ANOVA (B-E) and a repeated-measures three-way ANOVA (A) with post-hoc Newman-Keuls test. Uppercase letters denote diet effects within EDC group; and lowercase letters denote OPFR effects within diet (a=P<.05; b=P<.01; c=P<.001; d=P<.0001). Data (n=5-8 for all groups) are presented as mean ± SEM.

Figure 9. Glucose tolerance tests in ERαKO mice orally dosed with an OPFR mixture (1 mg/kg bw) for ~6 weeks. **(A)** Male fasting glucose; **(B)** Male GTT; **(C)** Area under the curve (AUC) of Male GTT; **(D)** Female fasting glucose; **(E)** Female GTT; **(F)** Area under the curve (AUC) of Female GTT. Data were analyzed by a two-way ANOVA (A, C, D, F) or a repeated-measures, three-way ANOVA (B, E) with post-hoc Newman-Keuls multiple comparisons test. Uppercase letters denote diet effects within EDC group and lowercase letters denote EDC effect within diet (a=P<.05; b=P<.01; c=P<.001; d=P<.0001). Data (n=8 for all groups) are presented as mean ± SEM.

Figure 10. Insulin tolerance tests in ERαKO mice orally dosed with an OPFR mixture (1 mg/kg bw) for ~6 weeks. **(A)** Male ITT; **(B)** Area under the curve (AUC) of Male ITT; **(C)** Female GTT; **(D)** Area under the curve (AUC) of Female ITT. Data were analyzed by a two-way ANOVA (B,D) or a repeated-measures, three-way ANOVA (A, C) with post-hoc Newman-Keuls multiple comparisons test. Uppercase letters denote diet effects within EDC group and lowercase letters denote EDC effect within diet (a=P<.05; b=P<.01; c=P<.001; d=P<.0001). Data (n=8 for all groups) are presented as mean ± SEM.

Figure 11. Glucose tolerance tests in PPARγKO mice orally dosed with an OPFR mixture (1 mg/kg bw) for ~6 weeks. **(A)** Male fasting glucose; **(B)** Male GTT; **(C)** Area under the curve (AUC) of Male GTT; **(D)** Female fasting glucose; **(E)** Female GTT; **(F)** Area under the curve (AUC) of Female GTT. Data were analyzed by a two-way ANOVA (A, C, D, F) or a repeated-measures, three-way ANOVA (B, E) with post-hoc Newman-Keuls multiple comparisons test. Uppercase letters denote diet effects within EDC group and lowercase letters denote EDC effect within diet (a=P<.05; b=P<.01; c=P<.001; d=P<.0001). Data (n=8 for all groups) are presented as mean ± SEM.

Figure 12. Insulin tolerance tests in PPARγKO mice orally dosed with an OPFR mixture (1 mg/kg bw) for ~6 weeks. **(A)** Male ITT; **(B)** Area under the curve (AUC) of Male ITT; **(C)** Female GTT; **(D)** Area under the curve (AUC) of Female ITT. Data were analyzed by a two-way ANOVA (B,D) or a repeated-measures, three-way ANOVA (A, C) with post-hoc Newman-Keuls multiple comparisons test. Uppercase letters denote diet effects within EDC group and lowercase letters denote EDC effect within diet (a=P<.05; b=P<.01; c=P<.001; d=P<.0001). Data (n=8 for all groups) are presented as mean ± SEM.

Figure 1

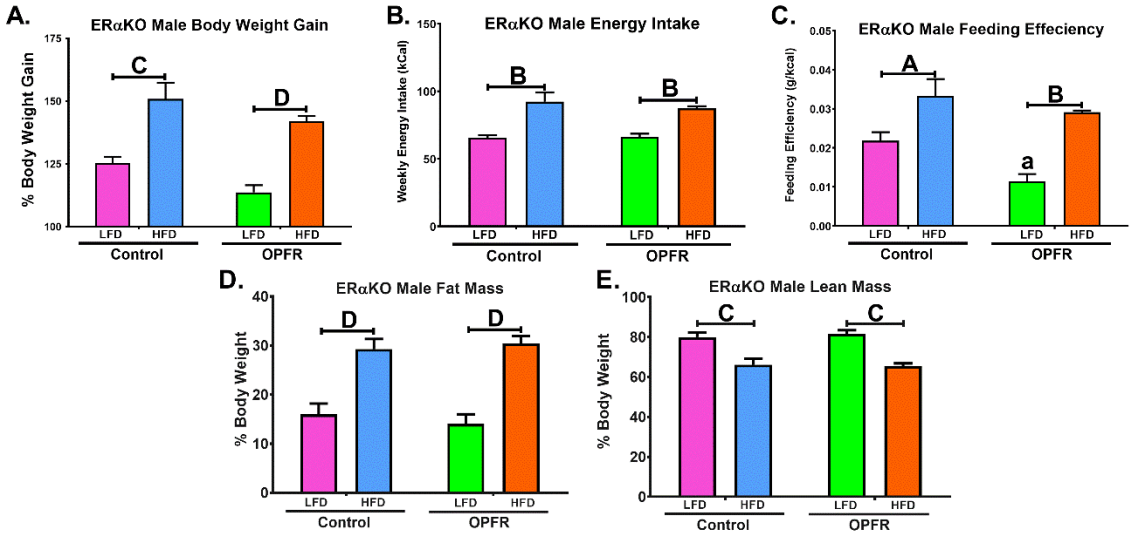

A.
ERαKO Male Body Weight Gain
% Body Weight Gain
175
150
125
100
C
D
LFD
HFD
LFD
HFD
Control
OPFR
B.
ERαKO Male Energy Intake
Weekly Energy Intake (kCal)
150
100
50
0
B
B
LFD
HFD
LFD
HFD
Control
OPFR
C.
ERαKO Male Feeding Effeciency
Feeding Efficiency (g/kcal)
0.05
0.04
0.03
0.02
0.01
0.00
A
B
a
LFD
HFD
LFD
HFD
Control
OPFR
D.
ERαKO Male Fat Mass
% Body Weight
40
30
20
10
0
D
D
LFD
HFD
LFD
HFD
Control
OPFR
E.
ERαKO Male Lean Mass
% Body Weight
100
80
60
40
20
0
C
C
LFD
HFD
LFD
HFD
Control
OPFR

Figure 2

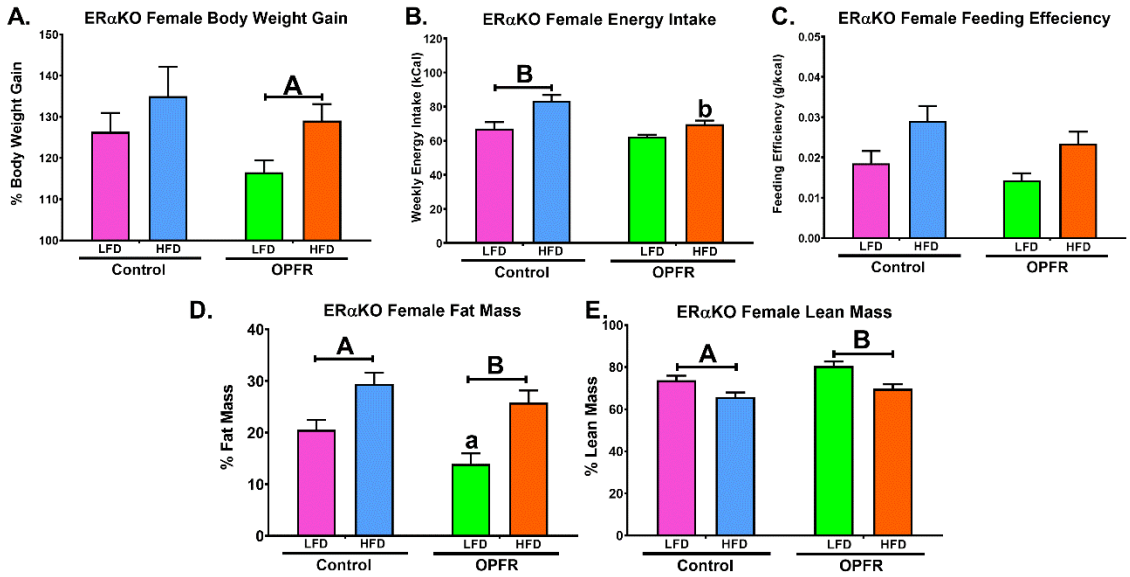

A.
ERαKO Female Body Weight Gain
% Body Weight Gain
150
140
130
120
110
100
A
LFD
HFD
LFD
HFD
Control
OPFR
B.
ERαKO Female Energy Intake
Weekly Energy Intake (kCal)
120
100
80
60
40
20
0
B
b
LFD
HFD
LFD
HFD
Control
OPFR
C.
ERαKO Female Feeding Effeciency
Feeding Efficiency (g/kcal)
0.05
0.04
0.03
0.02
0.01
0.00
LFD
HFD
LFD
HFD
Control
OPFR
D.
ERαKO Female Fat Mass
% Fat Mass
40
30
20
10
0
A
B
a
LFD
HFD
LFD
HFD
Control
OPFR
E.
ERαKO Female Lean Mass
% Lean Mass
100
80
60
40
20
0
A
B
LFD
HFD
LFD
HFD
Control
OPFR

Figure 3

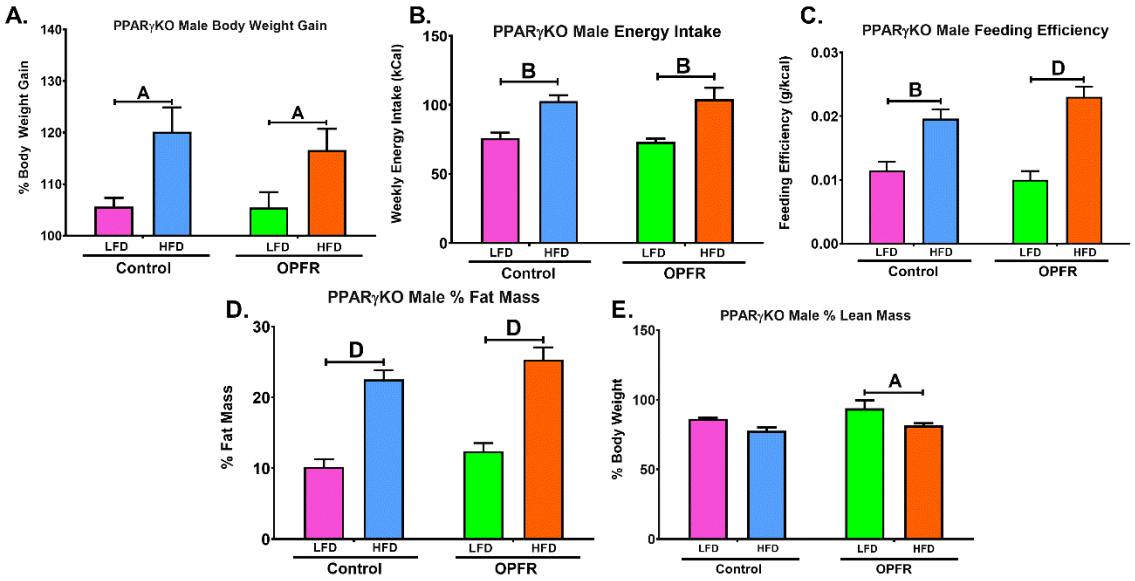

A.
PPARγKO Male Body Weight Gain
% Body Weight Gain
140
130
120
110
100
A
A
LFD
HFD
LFD
HFD
Control
OPFR
B.
PPARγKO Male Energy Intake
Weekly Energy Intake (kCal)
150
100
50
0
B
B
LFD
HFD
LFD
HFD
Control
OPFR
C.
PPARγKO Male Feeding Efficiency
Feeding Efficiency (g/kcal)
0.03
0.02
0.01
0.00
B
D
LFD
HFD
LFD
HFD
Control
OPFR
D.
PPARγKO Male % Fat Mass
% Fat Mass
30
20
10
0
D
D
LFD
HFD
LFD
HFD
Control
OPFR
E.
PPARγKO Male % Lean Mass
% Body Weight
150
100
50
0
A
LFD
HFD
LFD
HFD
Control
OPFR

Figure 4

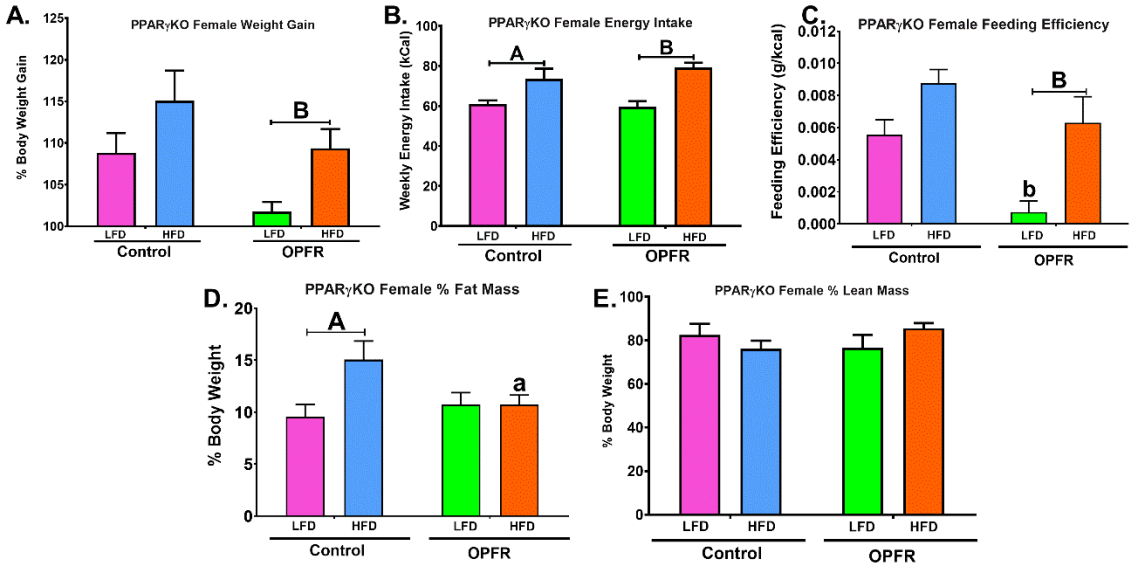

A.
PPARγKO Female Weight Gain
% Body Weight Gain
125
120
115
110
105
100
B
LFD
HFD
LFD
HFD
Control
OPFR
B.
PPARγKO Female Energy Intake
Weekly Energy Intake (kCal)
100
80
60
40
20
0
A
B
LFD
HFD
LFD
HFD
Control
OPFR
C.
PPARγKO Female Feeding Efficiency
Feeding Efficiency (g/kcal)
0.012
0.010
0.008
0.006
0.004
0.002
0.000
B
b
LFD
HFD
LFD
HFD
Control
OPFR
D.
PPARγKO Female % Fat Mass
% Body Weight
20
15
10
5
0
A
a
LFD
HFD
LFD
HFD
Control
OPFR
E.
PPARγKO Female % Lean Mass
% Body Weight
100
80
60
40
20
0
LFD
HFD
LFD
HFD
Control
OPFR

Figure 5

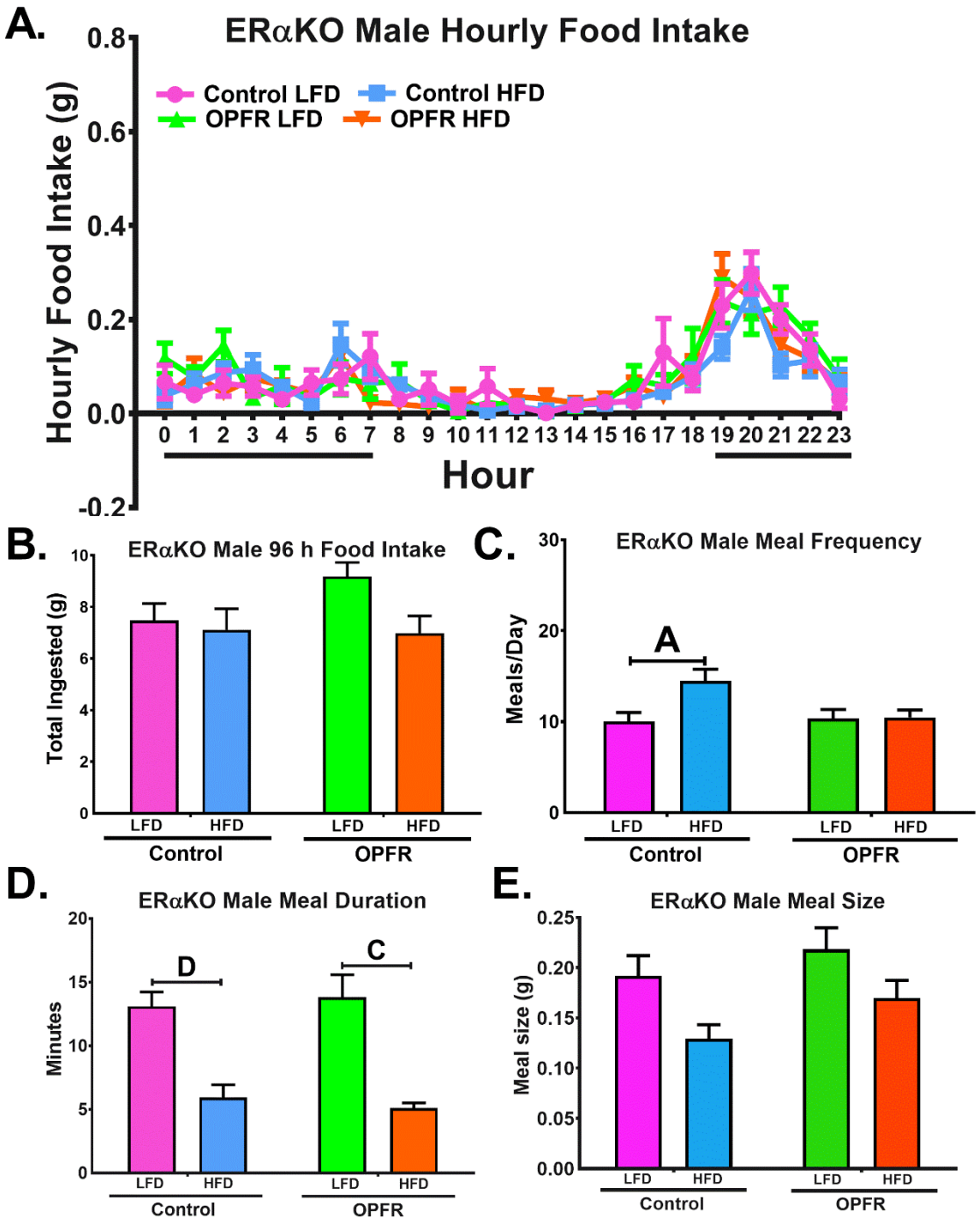

A.
ERαKO Male Hourly Food Intake
Control LFD
Control HFD
OPFR LFD
OPFR HFD
Hourly Food Intake (g)
0.8
0.6
0.4
0.2
0.0
-0.2
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Hour
B.
ERαKO Male 96 h Food Intake
Total Ingested (g)
10
8
6
4
2
0
LFD
HFD
LFD
HFD
Control
OPFR
C.
ERαKO Male Meal Frequency
Meals/Day
30
20
10
0
A
LFD
HFD
LFD
HFD
Control
OPFR
D.
ERαKO Male Meal Duration
Minutes
20
15
10
5
0
D
C
LFD
HFD
LFD
HFD
Control
OPFR
E.
ERαKO Male Meal Size
Meal size (g)
0.25
0.20
0.15
0.10
0.05
0.00
LFD
HFD
LFD
HFD
Control
OPFR

Figure 6

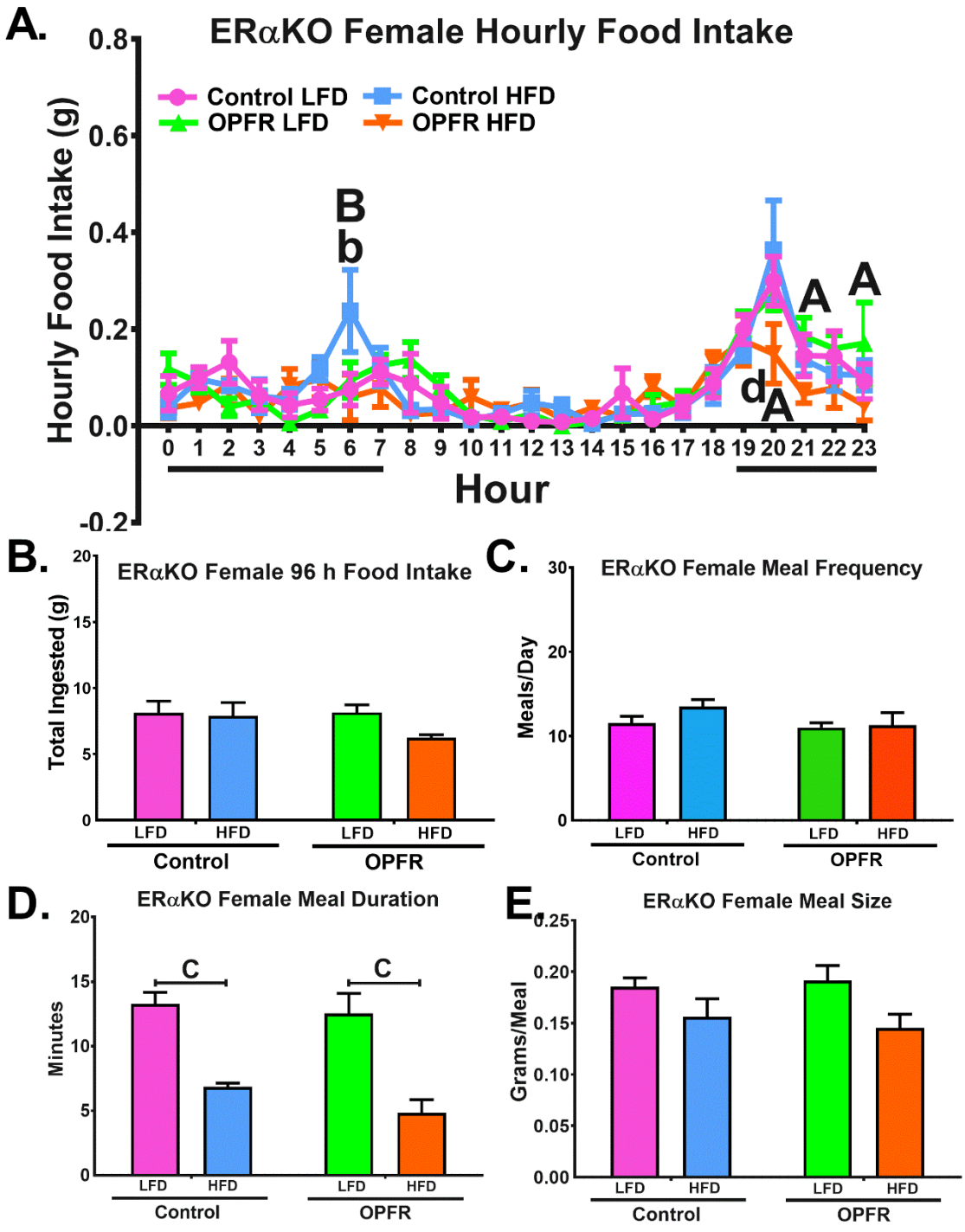

A.
ERαKO Female Hourly Food Intake
Control LFD
Control HFD
OPFR LFD
OPFR HFD
Hourly Food Intake (g)
0.8
0.6
0.4
0.2
0.0
-0.2
B
b
A
A
d
A
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Hour
B.
ERαKO Female 96 h Food Intake
Total Ingested (g)
20
15
10
5
0
LFD
HFD
LFD
HFD
Control
OPFR
C.
ERαKO Female Meal Frequency
Meals/Day
30
20
10
0
LFD
HFD
LFD
HFD
Control
OPFR
D.
ERαKO Female Meal Duration
Minutes
20
15
10
5
0
C
C
LFD
HFD
LFD
HFD
Control
OPFR
E.
ERαKO Female Meal Size
Grams/Meal
0.25
0.20
0.15
0.10
0.05
0.00
LFD
HFD
LFD
HFD
Control
OPFR

Figure 7

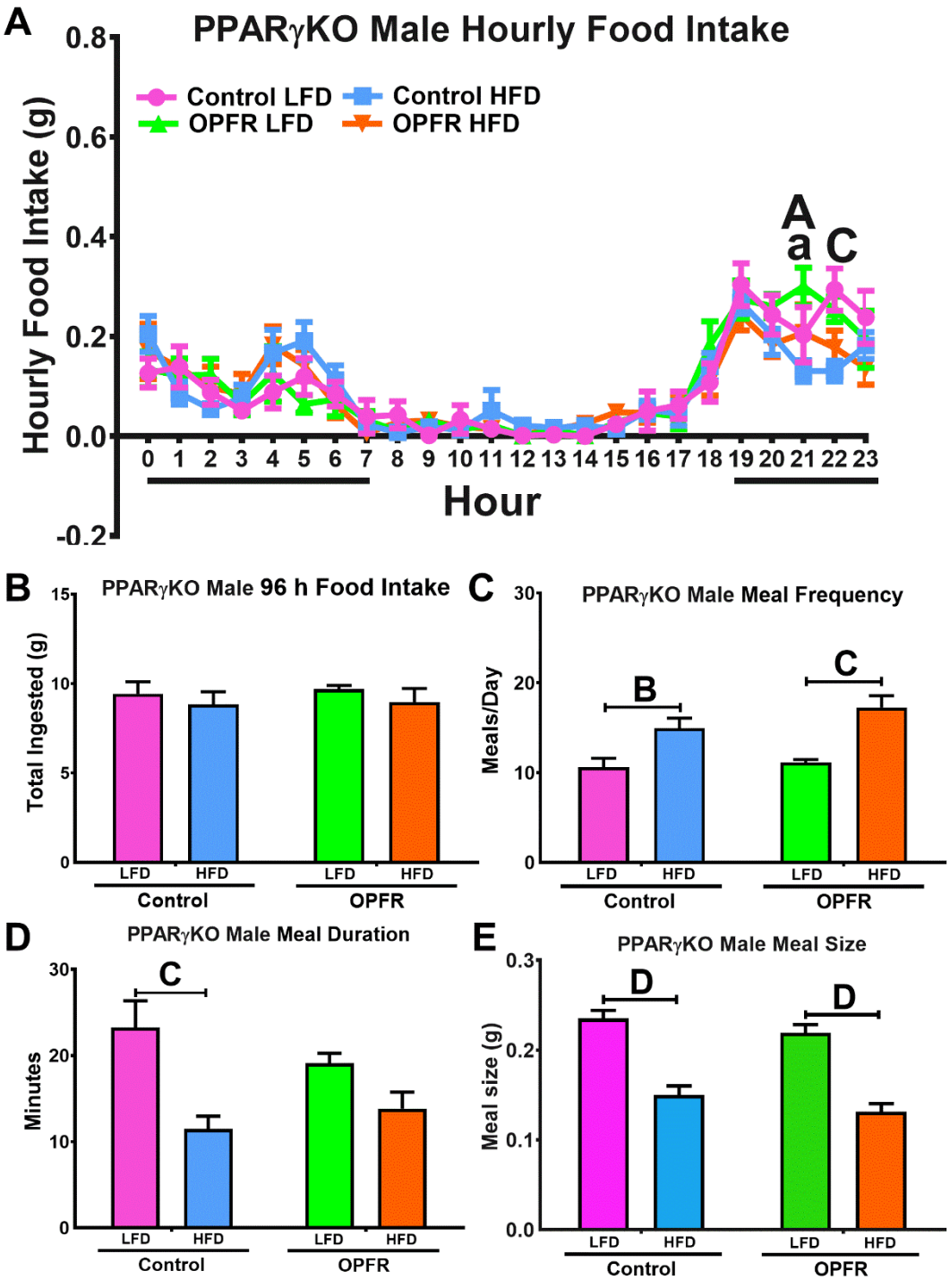

A
PPARγKO Male Hourly Food Intake
Control LFD
Control HFD
OPFR LFD
OPFR HFD
Hourly Food Intake (g)
0.8
0.6
0.4
0.2
0.0
-0.2
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Hour
A
a C
B
PPARγKO Male 96 h Food Intake
Total Ingested (g)
15
10
5
0
LFD HFD LFD HFD
Control OPFR
C
PPARγKO Male Meal Frequency
Meals/Day
30
20
10
0
B
C
LFD HFD LFD HFD
Control OPFR
D
PPARγKO Male Meal Duration
Minutes
30
20
10
0
C
LFD HFD LFD HFD
Control OPFR
E
PPARγKO Male Meal Size
Meal size (g)
0.3
0.2
0.1
0.0
D
D
LFD HFD LFD HFD
Control OPFR

Figure 8

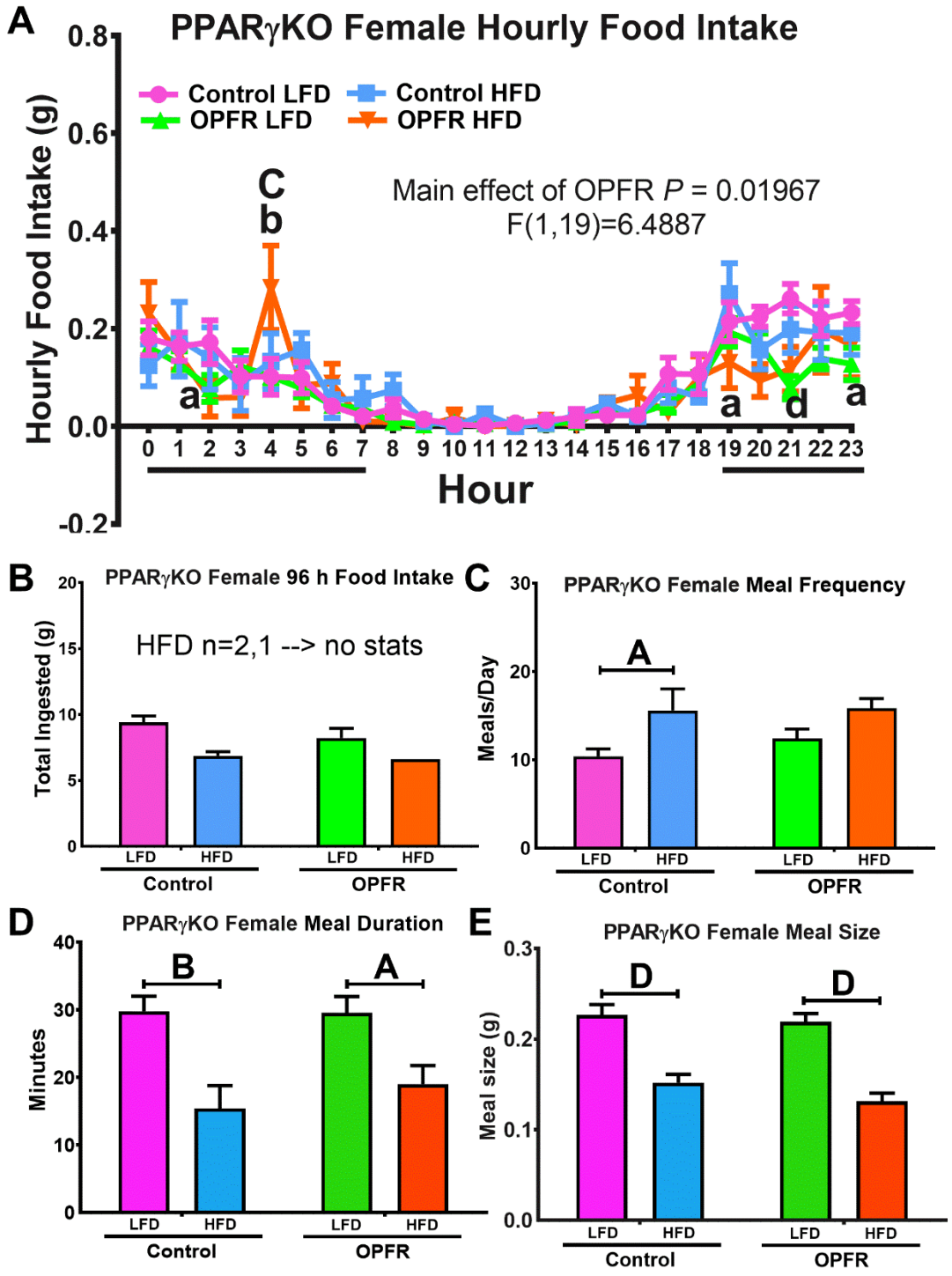

A
PPARγKO Female Hourly Food Intake
Control LFD
Control HFD
OPFR LFD
OPFR HFD
Main effect of OPFR P = 0.01967
F(1,19)=6.4887
Hourly Food Intake (g)
Hour
B
PPARγKO Female 96 h Food Intake
HFD n=2,1 --> no stats
Total Ingested (g)
C
PPARγKO Female Meal Frequency
Meals/Day
D
PPARγKO Female Meal Duration
Minutes
E
PPARγKO Female Meal Size
Meal size (g)
LFD
HFD
Control
OPFR

Figure 9

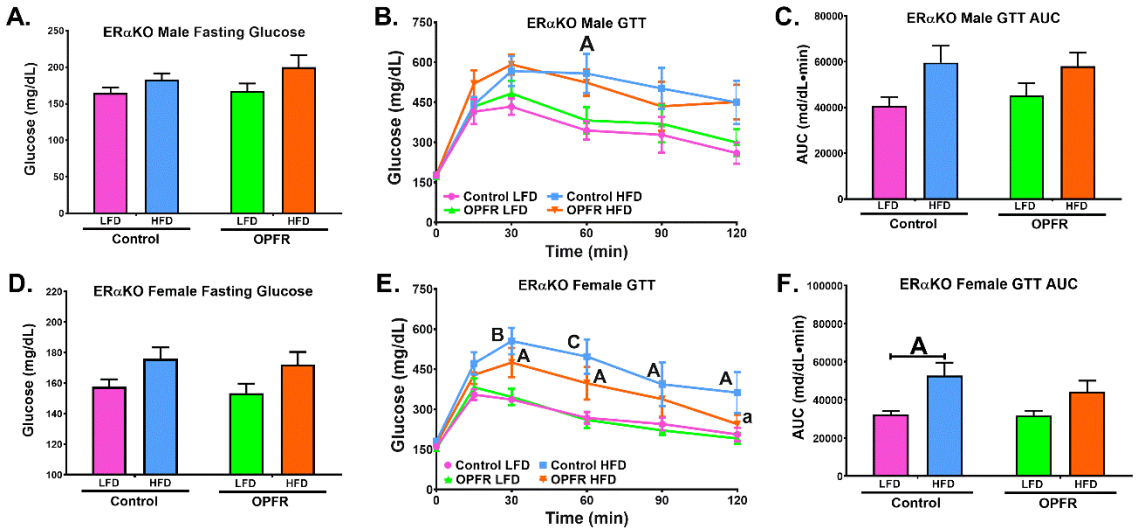

A.
ERαKO Male Fasting Glucose
Glucose (mg/dL)
LFD
HFD
Control
OPFR
B.
ERαKO Male GTT
Glucose (mg/dL)
Time (min)
Control LFD
Control HFD
OPFR LFD
OPFR HFD
C.
ERαKO Male GTT AUC
AUC (md/dL•min)
D.
ERαKO Female Fasting Glucose
E.
ERαKO Female GTT
F.
ERαKO Female GTT AUC

Figure 10

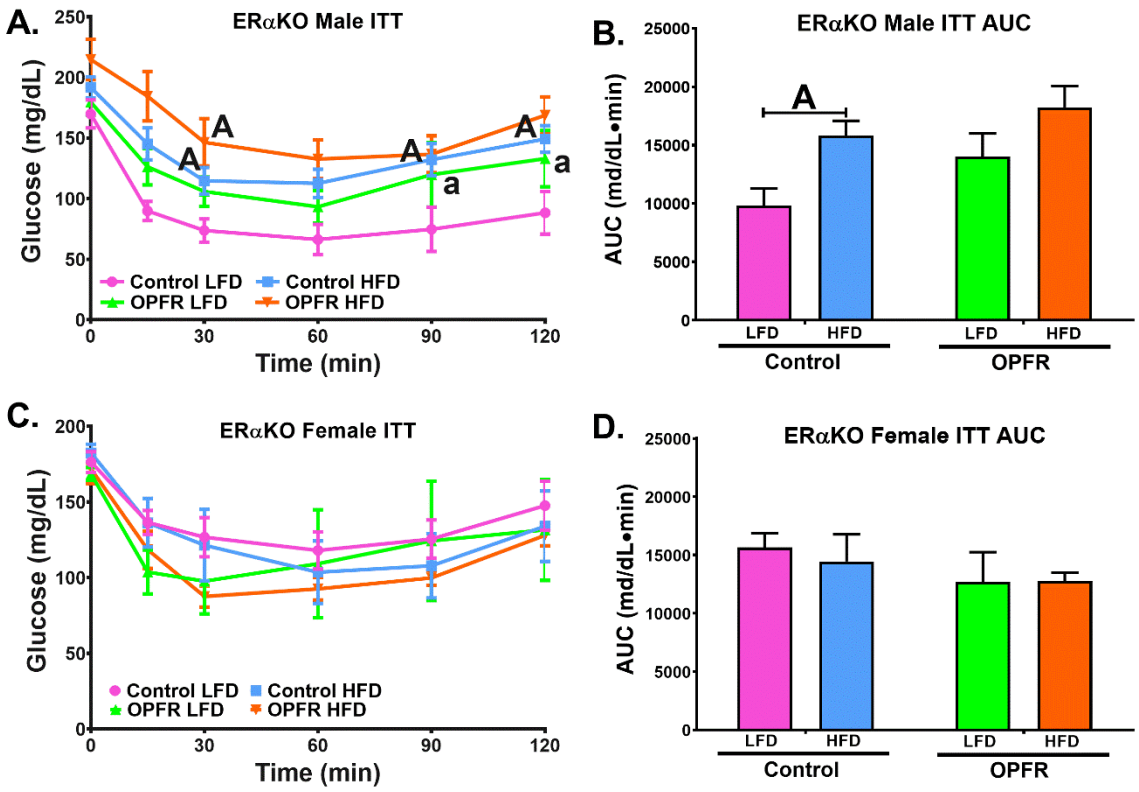

A.
ERαKO Male ITT
Glucose (mg/dL)
Time (min)
Control LFD
Control HFD
OPFR LFD
OPFR HFD
B.
ERαKO Male ITT AUC
AUC (md/dL•min)
LFD
HFD
Control
OPFR
C.
ERαKO Female ITT
D.
ERαKO Female ITT AUC

Figure 11

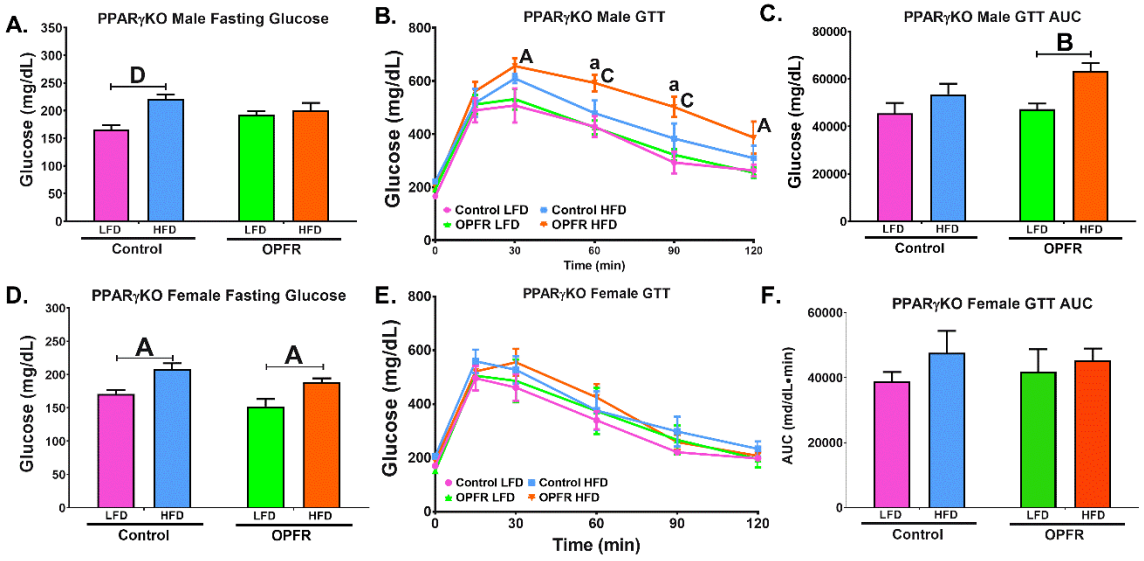
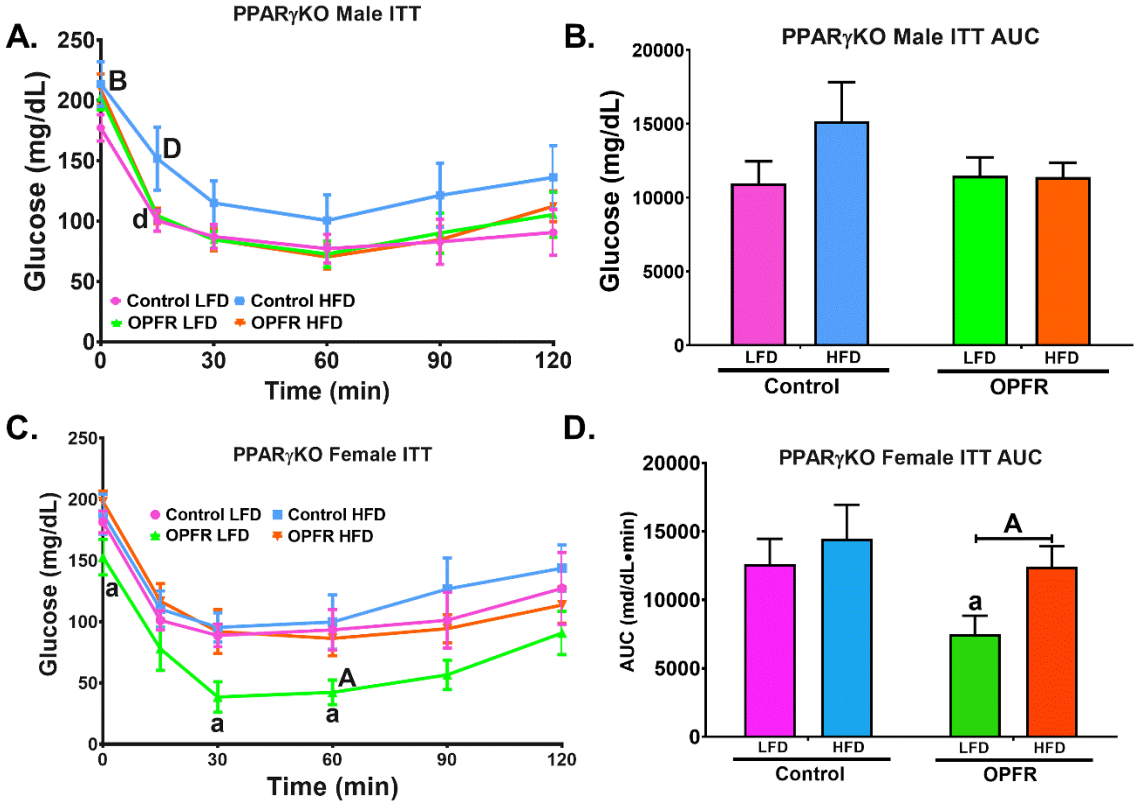

A.
PPARγKO Male Fasting Glucose
Glucose (mg/dL)
D
LFD
HFD
Control
OPFR
B.
PPARγKO Male GTT
Glucose (mg/dL)
A
a
C
Control LFD
Control HFD
OPFR LFD
OPFR HFD
Time (min)
C.
PPARγKO Male GTT AUC
Glucose (mg/dL)
B
D.
PPARγKO Female Fasting Glucose
Glucose (mg/dL)
A
E.
PPARγKO Female GTT
Glucose (mg/dL)
Time (min)
F.
PPARγKO Female GTT AUC
AUC (md/dL•min)

Figure 12


A.
PPARγKO Male ITT
Glucose (mg/dL)
Time (min)
Control LFD
Control HFD
OPFR LFD
OPFR HFD
B
D
d
B.
PPARγKO Male ITT AUC
Glucose (mg/dL)
LFD
HFD
Control
OPFR
C.
PPARγKO Female ITT
Glucose (mg/dL)
Time (min)
a
A
D.
PPARγKO Female ITT AUC
AUC (md/dL•min)

CHAPTER 4: ORGANOPHOSPHATE FLAME RETARDANTS EXCITE ARCUATE MELANOCORTIN CIRCUITRY GOVERNING ENERGY HOMEOSTASIS IN ADULT MICE AND INCREASE NEURONAL SENSITIVITY TO GHRELIN IN FEMALES

4. Organophosphate flame retardants excite arcuate melanocortin circuitry governing energy homeostasis in adult mice and increase euronal sensitvity to ghrelin in females

4.1 Abstract

Organophosphate flame retardants (OPFRs) are an expanding class of chemicals that have become near ubiquitous in the modern environment. While these flame retardants provide valuable protection against flammability of household items, they are increasingly implicated as an endocrine disrupting chemical (EDC), specifically through interactions with steroid estrogen receptors (ERs) and nuclear receptor peroxisome proliferator-activated receptor (PPAR) γ. We have previously reported that exposure to a mixture of OPFRs causes sex-dependent disruptions of energy homeostasis through alterations in ingestive behavior and activity in adult mice. Because feeding behavior and energy expenditure are largely coordinated by the hypothalamus, we hypothesized that OPFR disruption of energy homeostasis may occur through EDC action on melanocortin circuitry within the arcuate nucleus. To this end, we exposed male and female transgenic mice expressing green fluorescent protein in either NPY/AgRP or POMC neurons to a common mixture of OPFRs {triphenyl phosphate, tricresyl phosphate, and tris(1,3-dichloro-2-propyl)phosphate; each 1 mg/kg bw/day} for 4 weeks. We then electrophysiologically examined neuronal properties using whole-cell patch clamp technique. OPFR exposure depolarized the resting membrane of NPY neurons and dampened a hyperpolarizing K^+ current known as the M-current within the same neurons from female mice. These neurons were further demonstrated to have increased sensitivity to ghrelin excitation, which more potently reduced the M-current in OPFR-exposed females. POMC neurons from female

mice exhibited elevated baseline excitability and are indicated in receiving greater excitatory synaptic input when exposed to OPFRs. Together, these data support a sex-selective effect of OPFR to increase neuronal output from the melanocortin circuitry governing feeding behavior and energy expenditure and give reason for further examination of OPFR impact on human health.

4.2 Introduction

Flame retardants (FRs) are a now-ubiquitous class of compounds used in common household and workplace products such as furniture, toys, electronics, foodstuffs, plastics and resins (Li et al. 2019; Peng et al. 2020; Yang et al. 2019; Young et al. 2018). However, these compounds are not chemically bound to their products and will escape into household dust, allowing for inadvertent human exposure through inhalation and ingestion (Wei et al. 2015). Previously, polybrominated diphenyl ethers (PBDE) dominated the FR market. However, due to health concerns, PBDEs have been phased out of use in the United States since 2004, and consequentially, the concentrations have diminished in human populations (Zota et al. 2013). In response, the usage of alternative FRs increased, notably the class of organophosphate FRs (OPFRs). Global production of OPFRs rose from 186,000 tons in 2001, to 680,000 tons by 2015, resulting in broad-scale exposure to these lesser-known FRs (Yang et al. 2019; Worldwide flame retardants market to reach 2.8 million tonnes in 2018 2015; van der Veen and de Boer 2012). Indeed, sampling from civilian populations has revealed biologically relevant levels of OPFRs in human serum (680-709 ng/m), urine (1-10 ng/ml), and breast milk (1-10 ng/ml) (Butt et al. 2014; Hoffman et al. 2017; Ma et al. 2017; Ma et al. 2019; Meeker et al. 2013). With the rise in exposure comes increased concern for the long-term toxicological impact of OPFRs, and emerging research over the past decade has revealed neurotoxic, reprotoxic, immunotoxic,

obesogenic and endocrine disruptive actions of OPFRs (Dishaw et al. 2011; Patisaul et al. 2013; Kylie et al. 2018; Belcher et al. 2014; Pillai et al. 2014; Liu et al. 2013b; Liu et al. 2012; Hu et al. 2019; Steves et al. 2018) .

The disruptive capacity of OPFRs is likely due to mechanistic actions on nuclear receptors, with estrogen receptors (ERs) and peroxisome proliferator-activated receptor (PPAR) γ being primary targets for endocrine dysregulation. Indeed, commonly used OPFR tris(1,3-dichloro-2-propyl)phosphate (TDCPP) is an antagonist for human ERs, and leads to the *in vitro* upregulation of ERα–regulated genes (Liu et al. 2013a). Furthermore, triphenyl phosphate (TPP) is another OPFR known to activate human ERα, ERβ, PPARγ, as well as pregnane X receptor, and demonstrates antagonistic action on human androgen receptor and glucocorticoid receptor (Kojima et al. 2013; Pillai et al. 2014; Tung et al. 2017). Likewise, tricresyl phosphate (TCP) is capable of the same nuclear interactions as TPP, except that it is not known to activate ERβ nor PPARγ (Kojima et al. 2013). However, in another study, TDCPP, TPP, and TCP all acted as antagonists to ERα, preventing estrogen (E2) binding, indicating a potential mixed capacity of disruption for these compounds (Liu et al. 2012). OPFRs' mixed and broad spectrum of endocrine targets is likely causative for its diverse endpoints, but contextually, disruption of any of these receptor-mediated pathways can lead to metabolic dysregulation and disruptions of energy homeostasis. As such, the rise in human exposure to OPFR becomes increasingly concerning, especially when considering the relatively concomitant epidemic of obesity in developed nations, with its associated conditions such as diabetes and metabolic syndrome.

The maintenance of energy homeostasis is a complex process. At its hub is the hypothalamus and its associated neuroendocrine signaling pathways. Within the hypothalamus are multiple nuclei containing discrete neuronal subgroups that collectively

function to synthesize and respond to energy status signals from the periphery (Williams et al. 2001). The hypothalamus then integrates these signals with reward input from the limbic forebrain for execution of feeding behavior by the hindbrain (Berthoud 2002; Grill and Hayes 2012). The arcuate nucleus (ARC) of the hypothalamus is of particular importance in sensing and responding to energy state due to its location adjacent to a leaky segment of the blood brain barrier. This allows for ARC neurons to directly sense and respond to peripheral signals such as glucose, insulin, leptin, and ghrelin (Schwartz et al. 2000; Saper et al. 2002), and, in turn, exposes the neurons as a potential site of toxicity for endocrine disruptors.

ARC regulation of energy homeostasis is controlled via reciprocal interactions between two neuronal subpopulations: orexigenic neuropeptide Y (NPY) neurons and anorexigenic proopiomelanocortin (POMC) neurons. Their combined inputs to the paraventricular nucleus (PVN) and the lateral hypothalamus (LH) influence food intake (Arora and Anubhuti 2006; Nahon 2006). Selective optogenetic activation of NPY neurons elicits intense feeding within minutes, while the converse is true for POMC activation (Aponte et al. 2011). As to be expected, the feeding behavior invoked by NPY activation is intensified by the “hunger hormone”, ghrelin, and inhibited by insulin and leptin, which in turn, are activational signals for POMC neurons (Baldini and Phelan 2019). The NPY-POMC circuit also self-regulates. NPY neurons co-express agouti-related peptide (AgRP), which prevents α–melanocyte stimulating hormone (α-MSH; a cleavage product of proopiomelanocortin) from binding onto melanocortin 4 (MC4) receptors in target, PVN and LH neurons (Cone 2005; Williams et al. 2001; Adan et al. 2006). Additionally, NPY neurons synapse with POMC neurons, providing inhibitory tone onto their counterparts, suppressing the anorexigenic activity when energy resources are low (Williams et al. 2001; Cone 2005). Another ARC neuronal subpopulation that interacts with this circuit are KNDy neurons, which co-express the signaling peptides kisspeptin, neurokinin B, and dynorphin.

These neurons influence the balance of NPY/POMC actions by direct glutamate excitation of POMC neurons, and indirect GABA inhibition of NPY neurons (Fu and van den Pol 2010; Nestor et al. 2016). While only POMC and KNDy neurons are known to express ERα, all three neurons are modulated by the ovarian hormone 17-β-estradiol (E2) (Qiu et al. 2018; Stincic et al. 2018). In addition, the nuclear receptor PPARγ plays a role in ARC-driven energy homeostasis. PPARγ is expressed in both POMC and NPY neurons and is implicated in driving NPY-induced feeding behavior, as well as POMC inhibition (Sarruf et al. 2009; Stump et al. 2016; Garretson et al. 2015).

In a previous study, we dosed adult mice with a sub-chronic exposure to OPFRs, and observed sex-dependent alterations in body-weight gain, energy intake, and ARC gene expression (Krumm et al. 2018). OPFR upregulated ARC expression of ERα and altered expression of *Npy* and *Pomc*, while inducing 4- to 8-fold increases in expression of the potassium channel genes *Kcnq2*, *-3*, and *-5*, as well as insulin, leptin, and ghrelin receptors. KCNQ channels (KCNQ2, -3, and -5) form heteromultimers to produce the M-current, which is a constitutive, subthreshold, voltage-gated, outwardly-rectifying K^+ current. KCNQ5 is predominantly expressed in the brain and co-assembles with KCNQ3, indicating potential selectivity in comprising neuronal M-currents (Robbins 2001; Delmas and Brown 2005). The M-current in NPY neurons is sensitive to energy state and is diminished in fasted male and female mice, increasing NPY neuronal activity leading to feeding behavior (Roepke et al. 2011). Additionally, the M-current in NPY neurons is sensitive to estrogenic modulation through direct augmentation of KCNQ5 expression and M-current activity in female mice, accomplishing an inhibition of NPY neuronal excitability (Roepke et al. 2011). Conversely, ghrelin suppresses the M-current in NPY neurons and increases neuronal excitability (Yasrebi et al. 2016).

While the study of OPFR toxicity is progressing quickly, most studies focus on the developmentally sensitive windows of exposure. However, there is little data providing evidence of OPFR capacity to disrupt adult homeostasis. Considering the increasingly ubiquitous commercial usage of OPFRs, there is need to evaluate the toxicological impact of these compounds across the lifespan. Furthermore, the ability of OPFRs to interact with ERs and PPARγ is of particular concern for this ARC circuit, as OPFR dysregulation is likely to have cascading effects on energy homeostasis that may increase the risk for obesity and its associated disorders, such as metabolic syndrome and type 2 diabetes mellitus. To address this need and to further extend our previous study, we selectively targeted NPY and POMC neurons, hypothesizing that adult exposure to a mixture of three common OPFRs (TPP, TCP, and TDCPP) sex-dependently disrupts arcuate melanocortin circuitry governing energy homeostasis. To test this hypothesis, we utilized neuronal harvesting for cell-type specific gene expression and electrophysiology whole-cell patch-clamp techniques to examine neuronal excitability and hormone sensitivity.

4.3 Materials & Methods

Animal Care and Experimental Design

All animal experiments followed National Health Institute guidelines and have been approved by The Rutgers University Institutional Animal Care and Use Committee. GFP-NPY and EGFP-POMC mice {originally provided by Dr. Bradford Lowell, Harvard University (Yasrebi et al. 2016), and Dr. Malcom Low, University of Michigan Medica School, respectively} were selectively bred in-house and maintained in controlled temperature and photoperiod (12 h: 12 h) conditions and provided free access to water and standard, low-phytoestrogen lab chow (Lab Diets, 5V75). Adult mice (>6 weeks) were housed in littermate pairs and weighed weekly. Mice were dosed orally daily for 4-5 weeks

with selected OFPRs (1 mg/kg bw/day) dissolved in sesame oil and added to a rehydrated peanut butter vehicle (test groups) or with an equivalent amount of plain sesame oil as control. Final dosing was completed ~30 minutes before euthanasia, which was performed at 0930-1100 h. Mice used for cell harvesting were injected with ketamine (50 μL of 100 mg/ml, i.p.) prior to decapitation. In compliance with the approved protocol, mice used for electrophysiology did not receive ketamine injection to eliminate the neurophysiological affects. To control for fluctuating gonadal steroid levels, female mice were euthanized in diestrus as determined by vaginal cytology. The vagina was flushed with approximately 100-200 μL water, and the flush was evaluated microscopically for determination of cycle stage based on cell types present (Cora et al. 2015; Byers et al. 2012).

Test Materials

A singular mixture of three selected OPFRs was used to approximate human exposures to mixed OPFR commercial use. OPFRs utilized were tricresyl phosphate (TCP, CAS no. 1330-78-5; purity 99%; purchased from AccuStandard, New Haven, CT), and triphenyl phosphate (TPP, CAS no. 115-86-6; purity 99%) and tris (1,3-dichloro-2-propyl)phosphate (TDCPP, CAS no. 13674-87-8; purity 95.6%) (both purchased from Sigma-Aldrich, St. Louis, MO). 100 mg of each OPFR was dissolved in the same 1 ml acetone (Sigma) to make a for long term storage. 100 μl of this stock solution were then added to 10 ml of sesame oil (Sigma-Aldrich) to create a 1 mg/ml mixture of OPFR-oil. Control-oil was created by adding 100 μl acetone. Each mixture was then left stirring for 48-72 h to completely evaporate the acetone. Based on body weight, the resulting mixtures were then added to a vehicle dehydrated peanut butter (~50 mg). This generated a rehydrated peanut butter product that contained either 1 mg/kg bw OPFR, or equivalent

amount of OPFR-free vehicle. Mice were supplied peanut butter rehydrated with either OPFR-oil or control-oil on a daily at 090-1100 h, to be consumed orally. Tetrodotoxin (TTX), a selective and reversible sodium channel blocker, and ghrelin were purchased from Tocris (Bristol, UK) and were dissolved in water. XE-991 (Tocris), a selective KCNQ channel blocker, was dissolved in dimethyl sulfoxide (Sigma). Mouse leptin was purchased from the National Hormone and Peptide Program (Torrance, CA, US) and dissolved in phosphate-buffered solution.

Cell Harvesting

GFP-NPY and EGFP-POMC mice that selectively express green fluorescent protein in NPY or POMC neurons were used to harvest neurons for cell-specific gene expression analysis. Afterwards, the brain was quickly extracted from the skull and submerged into 4°C oxygenated (95% O2, 5% CO2) high-sucrose artificial cerebrospinal fluid (aCSF), containing (in mM): 208 sucrose, 2 KCl, 1.25 NaH_2PO_4, 10 glucose, 26 $NaHCO_3$, 10 HEPES, 2 $MgSO_4$, 1 $MgCl_2$ (pH 7.3, 300 mOsm). After a 3-min incubation in ice-cold aCSF, four coronal slices (250 μm) were taken from the basal hypothalamus and then transferred to an auxiliary chamber where they were kept at room temperature (25°C) in aCSF without sucrose, containing (in mM): 124 NaCl, 5 KCl, 2.6 NaH_2PO_4, 10 glucose, 26 $NaHCO_3$, 10 HEPES, 2 $MgSO_4$, 2 $CaCl_2$ (pH 7.3, 300 mOsm). Slices were given ~1 h recovery time until cell dispersal. To disperse neurons, the arcuate (ARC) nucleus was microdissected and exposed to 1 mg/ml protease in 10 ml aCSF (37°C) for 16 min to break down extracellular connective tissue. The tissue was washed free of protease with four washes of low-calcium (1mmol $CaCl_2$) aCSF, before washing twice more in regular aCSF. Three Pasteur pipettes were flame polished with decreasing tip diameter and used to carefully triturate the tissue by successive flushing through the Pasteur pipettes. The suspended individual neurons were dispersed onto a glass-bottomed petri dish and

allowed to settle for 12 min before being continually perfused with fresh aCSF at a rate of ~2 ml/min. GFP-NPY or EGFP-POMC neurons were visualized using a Leica DM-IL inverted fluorescent microscope (Leica Microsystems, Wetzlar, Germany). Individual neurons were then harvested into the tip of a pipette by applying low negative pressure using the Xenoworks manipulator system (Sutter Instruments, Novato, CA, USA). The pipette contents were then expelled into a siliconized microcentrifuge tube containing 1 µl 5X Superscript III Buffer (Life Technologies), 15 U RNasin (Promega), 0.5 µl 100 mM DTT, and DEPC-treated water in 8 µl volume. Per animal, a total of 60 GFP-NPY or 60 EGFP-POMC neurons were collected in 12 pools of 5 neurons. All cell harvesting procedures were carried out in an RNAse-free environment.

RNA harvested from the pools of neurons were reverse transcribed to produce a cDNA library. This process was accomplished by first adding 400 ng anchored oligo(d)T (Invitrogen), 0.625 mM dNTPs (Takara Biotechnology), 100 ng random hexamers (Promega) to the sample pools, and then denaturing the RNA at 65°C for 5 min, before cooling on ice for 5 min to allow the oligos to anneal. Next, 50 U of Superscript III Reverse Transcriptase (RT) (Invitrogen), 3 µl 5x Superscript Buffer (Invitrogen), 10 mM DTT (Invtrogen), , 15 U RNasin (Progema), and in DEPC-water (GeneMate) was added to create a total volume of 25 µl for the process of reverse transcription. The reverse transcription protocol used is as follows: 5 min at 25°C, 60 min at 50°C, 15 min at 70°C. As control, two samples containing 25 ng of hypothalamic RNA were reverse transcribed. One hypothalamic RNA sample received RT and acted as the positive control, while the other hypothalamic RNA sample did not receive RT, and acted as our negative control. Furthermore, during the harvesting process, one sample was collected containing a singular neuron. This sample also did not receive RT and acted as an additional negative control. The products of reverse transcription result in single-stranded, cDNA amplimers,

which were subsequently used for gene expression analysis (*Npy*, *Agrp*, *Ghsr*, *Lepr*, *Kcnq-2*, *Kcnq-3*, and *Kcnq-5* in NPY-GFP cDNA, and *Pomc*, *Cart*, *Trpc5*, *Lepr*, *Kcnq-2*, and *Kcnq-3* in EGFP-POMC cDNA) using real-time quantitative polymerase chain reaction (qPCR).

Real-time Quantitative PCR

Primers for these experiments were designed to span exon-exon junctions using Clone Manager 5 to reduce DNA contamination and were synthesized by Life Technologies. Refer to Table 1 for the complete list of primer sequences used in these experiments. For pool samples, 4 μl of cDNA template was amplified using either SsoAdvanced SYBR Green (BioRad, Hercules, CA) or PowerSYBR Green master mix (Life Technologies) with CFX-Connect Real-time PCR instrument (BioRad). Standard curves for each primer pair were prepared using serial dilutions of basal hypothalamic cDNA in triplicate to determine efficiency [$E = 10^{(-1/m)}-1$, m = slope] of each primer pair. All efficiencies, expressed as % efficiency, were approximately equal (one doubling per cycle, 90-110%, Table 1).

Amplification of all genes followed this protocol: 95°C initial denaturing for 10 min (Power SYBR) or 3 min (SsoAdvanced) preceding 45 cycles of amplifications alternating between 94°C for 10 s (denaturing) and 60°C for 45 s (annealing). The process was finished with a melting point analysis of 60 cycles of 95°C for 10 s, then a 65°C to 95°C ramp (in 0.5°C steps) for 5 s. *Gapdh* (Glyceraldehyde-3-phosphate-dehydrogenase) and *Actb* (β-actin) were used as reference genes for SsoAdvanced or PowerSYBR qPCR, respectively. In addition to the no RT controls, one water well blank was also analyzed in each run. For each 96-well plate, all 12 animals (n = 6 OPFR-oil group, n = 6 control-oil group) were analyzed for two target genes and a reference gene. Individual pools were

run in duplicate, and each animal was run in triplicate (3 pools/animal) per plate. Relative gene expression was calculated by comparing the average target gene expression of the three pools to that of the average reference gene expression from the same three pools. Relative gene expression was calculated using the δδCt method (Schmittgen and Livak 2008) by normalizing to control samples and comparing between treatment groups within each cell type.

Whole-cell Patch Clamp Recording

GFP-NPY and EGFP-POMC mice that selectively express fluorescent protein in NPY or POMC neurons were used to specifically record electrophysiological properties from both neuronal subpopulations in the arcuate nucleus of the hypothalamus. The preparation of hypothalamic brain slices for electrophysiology was identical to our cell harvesting protocol as outlined in 2.3, but without the need for an RNAse-free environment. Slices were maintained in a room temperature, oxygenated (25°C, 95% O_2, 5% CO_2) aCSF auxiliary chamber for at least 1 h recovery time, before use in electrophysical recording. A single slice was transferred to a shallow, glass-bottomed recording chamber which was mounted on an Olympus BX51W1 upright microscope with enhanced video output utilizing infrared-differential interference contrast (IR-DIC). GFP-NPY neurons were identified with an Exfo X-Cite 120 Series fluorescence light source (Mississauga, Ontario, Canada) using blue excitation light and IR-DIC through a 40X water-immersion lens. The recording chamber was continually perfused with warm (35°C), oxygenated aCSF at 1.5 mil/min.

Whole-cell voltage and current clamp recordings were performed using pipettes made of borosilicate glass and pulled using a Narishige PC-10 two-stage, micropipette gravity puller (Amityville, NY, USA). Pipettes were filled with normal internal solution consisting of (in mM): 10 NaCl, 128 K-gluconate, 1 $MgCl_2$, 10 HEPES, 1 ATP, 1.1 EGTA; 0.25 GTP (pH

7.3; 300 mOsm) with 3.5-6.5 MΩ resistance. A Hum Bug 50/60 Hz Noise Eliminator (Quest Scientific North Vancouver, Canada), and Axopatch 200A amplifier, Digidata 1322A Data Acquisition System, and pCLAMP software [version 9.2 (Molecular Devices, San Jose, CA, USA) were used for data acquisition and analysis. Pipette positioning was accomplished with aid of an MPC-200 micropipette manipulator system (Sutter Instruments, Navato, CA, USA). Input resistance, series resistance, and membrane capacitance were monitored throughout experiments and only cells with stable series resistance (<30 MΩ, <20% change), and an input resistance > 500 MΩ were used for analysis. The access resistance was 80% compensated, and liquid junction potential (10 mV) was corrected. I-V plots were generated from a holding potential of -60 mV, stepping to -50 to -140 mV in 10 mV increments with 1 s intervals.

The input resistance was determined from the I-V plot as the ratio of the voltage (-60 to -80 mV)/current (pA). The M-current was measured in voltage clamp using a deactivation protocol which records the currents elicited during 250 ms voltage steps from -25 to -75 mV after a 250 ms prepulse at -20 mV stepped from a holding potential of -60 mV. This protocol includes membrane voltage at which the maximal M-current is generally induced (Roepke et al. 2011). Current deactivation was measured as the difference in initial (<10 ms) and sustained current (>240 ms) of the current trace. The M-current was calculated as the difference between control (TTX only, 1 μM, 5 min) and XE-991 (40μM, +TTX 1μM, 10 min) conditions.

The pCLAMP software threshold function was used to detect action potentials and spontaneous excitatory post-synaptic potentials and currents (sEPSPs and sEPSCs, respectively). These data were measured during the last 3-5 min of each treatment section to allow capture of the fullest treatment effect. As an example: measurements were taken during ~ mins 4-7 during the 7 min recording of 1 nM ghrelin. The threshold minimum for

a sEPSP was a 2 mV depolarization, and the threshold minimum for a sEPSC was 25 pA. Capturing these small changes in potential and current requires a very steady baseline, giving reason for the flexible window (last 3 min of exposure) of when representative measurements were taken. General excitability in female POMC neurons was measured as resting membrane potential (RMP), action potential frequency, and frequency and amplitude of sEPSPs and sEPSCs. Under current clamp, RMP, action potentials and sEPSPs were first measured for ~7 min before switching to voltage clamp to record sEPSCs for another ~7 min.

Ghrelin sensitivity in female NPY neurons was measured in both voltage clamp (M-current) and current clamp (membrane potential depolarization and firing rates) in the absence of TTX. The firing rate and membrane potential were continuously measured under control (7 min) and ghrelin (1 nM, 7 min, and 100 nM, 7 min) conditions with one ~1 min interruption to record the M-current midway through the recording. Leptin sensitivity in male POMC neurons was measured only in current clamp to record membrane potential and firing rates in the absence of TTX. The firing rates and membrane potential were continuously measured under control (7 min) and leptin (100 nM, 7 min).

Data Analysis

All data were analyzed using GraphPad Prism software (GraphPad Software, LA Jolla, CA) and are shown with mean ± SEM. Effects were considered significant at $\alpha \leq 0.05$. I-V plots were analyzed by a repeated-measures, two-way ANOVA with Holm Sidak's multiple comparison test. All other data were analyzed with unpaired, Student's t-test testing treatment effect vs control.

4.4 Results

Gene expression

GFP-NPY and EGFP-POMC mice fed control-oil and OPFR-oil mixture (1 mg/kg bw/day TCP, TPP, and TDCPP) for 4-5 weeks were analyzed for neuron-specific gene expression using the cell harvesting protocol. In males, OPFR decreased *Npy* expression to nearly half that of control, and increased *Kcnq-5* two-fold in NPY neurons (Fig. 1A, $P < .001$, $P < .05$). OPFR also elicited a trending increase in expression of leptin receptors *Lepr* in POMC neurons (Fig. 1C, $p = .0845$). In females, OPFR exposure doubled the expression of ghrelin receptors *Ghsr* in NPY neurons (Fig. 1B, $P <.05$), with a trending inhibition of *Cart* in POMC neurons (Fig. 1D, $P = .0616$). These data indicate OPFRs exert a sex-dependent effect on arcuate neuronal expression of neuropeptides and hormone receptors and suggest that OPFR exposure perturbs the activity of M-current in NPY neurons and hormone sensitivity in NPY and POMC neurons. Therefore, we based the following electrophysiological studies upon on these results.

M-Current Activity

Male and female GFP-NPY mice exposed to OPFR-oil or control-oil were euthanized and hypothalamic brain slices were obtained for electrophysiological characterization of the M-current.

Male NPY-GFP mice

Despite the upregulated *Kcnq-5* expression (Fig. 1A), we saw no effect of OPFRs on the M-current in NPY neurons from male mice (Fig. 2). However, OPFR treatment elevated RMP by 10.17 ± 3.11 mV in male NPY neurons (Fig. 2D, $P <.01$), suggesting a more excitable baseline state. When comparing RMP before and after bath-exposure to

XE-991, we saw no difference in the depolarization elicited by XE-991 (Fig. 2E), indicating that the depolarization induced by KCNQ channel blockage was not altered by OPFR. Overall, OPFR exposure was shown to have no effect on NPY M-current in adult male mice, but may alter neuronal excitability.

Female NPY-GFP Mice

Despite no significant alterations in *Kcnq* channel expression in Fig. 1, female mice exposed to OPFRs exhibited a diminished M-current compared to control females (Fig. 3A & B). There was an overall effect of OPFR and an interaction of OPFR and voltage ($F(1,23)=5.09_{OPFR}$, $P < .05$; $F(9,207)_{OPFR*voltage}$, $P < .001$), with reductions of XE-991-sensitive current at voltage steps -45 through -35 mV ($P < .05$), with a maximal peak reduction of 51.06 ± 21.74 pA by OPFR (Fig. 3C, $P < .05$) at -40 mV ($P < .05$). Similar to their male counterparts, NPY neurons from female mice exhibited a depolarized baseline RMP (Fig. 3D, 8.41 ± 3.37 mV $P <.05$), suggesting a more excitable neuron by OPFR exposure, potentially through the observed reduction in the M-current. However, OPFR exposure did not result in a (Fig. 3E). OPFRs did not alter input resistance in NPY neurons from female mice (Fig. 3F).

NPY sensitivity to Ghrelin in Female Mice

The elevated *Ghsr* expression seen in NPY neurons from female mice (Fig. 1B) informed our hypothesis that OPFR may be inducing increased production of ghrelin receptors in females, sensitizing NPY neurons to ghrelin signaling. For these experiments, we perfused hypothalamic brain slices from female GFP-NPY mice to a low dose (1 nM) and a high dose (100 nM) of ghrelin. Consistent with our qPCR findings in Fig. 1B, we observed a greater depolarization (+6.90 ± 2.82 mV) by 1 nM ghrelin in OPFR-treated

females and a trending increase by 100 nM ghrelin (Fig. 4B, $P < .05$, $P = .0850$). There were no differences in the frequency of action potentials evoked by ghrelin (Fig. 4D).

We also measured spontaneous excitatory post-synaptic potentials (sEPSPs) before and after ghrelin exposure. sEPSPs are small depolarizations that are sub-threshold to evoking an action potential, and the amplitude of sEPSP, a marker of a post-synaptic effect, indicates the sEPSP strength. In regard to ghrelin, an increase in sEPSP amplitude would indicate a direct effect of ghrelin signaling leading to an increase in excitability and depolarization. However, we saw no alterations in ghrelin induced amplitude (data not presented). We did observe a decrease in ghrelin-induced sEPSP frequency, an indicator of a pre-synaptic effect. OPFR caused an 80% reduction of ghrelin-induced sEPSP frequency, in response to both 1 nM and 100 nM ghrelin (Fig. 4F, $P < .05$, $P < .01$). These data indicate that OPFRs are impinging on ghrelin signaling through neurons upstream of the patched NPY neurons.

Using the M-current protocol described earlier, we also observed that 100 nM ghrelin reduced peak current by -65.29 ± 24.81 pA and -94.34 ± 25.89 pA, in both control- and OPFR-treated mice, respectively (Fig. 5D, $P < .01$; Fig. 5F, $P < .001$). However, since we could not use XE-991 in these experiments, the suppressed K^+ current may not constitute all of KCNQ channel activity. Of note, 1 nM ghrelin reduced the M-current maximal peak by -66.31 ± 26.25 pA in OPFR-treated mice, whereas no significant reduction was seen in control-treated mice (Fig. 5E, $P < .05$, vs. Fig. 5C). Furthermore, ghrelin (100 nM) perfusion suppressed the M-current more in OPFR-treated mice than in control-treated mice (Fig. 5F vs. Fig. 5D). This demonstrates OPFR potential in sensitizing NPY neurons to ghrelin in female mice.

POMC excitability in Male and Female Mice

We characterized the effect of OPFR exposure on general POMC excitability using current and voltage clamp. RMP, input resistance, action potential frequency, and the frequency and amplitude of both sEPSPs and sEPSCs (spontaneous excitatory post-synaptic currents) were recorded. OPFR exposure did not alter POMC excitability or intrinsic properties in male mice (data not shown). In females, OPFR did not affect RMP (Fig. 6B), nor input resistance (data not presented), but did induce a striking, +2.88 ± 1.109 Hz increase in action potential frequency (Fig. 6C, P <.05). OPFR augmented the number of action potentials being fired by POMC neurons, as well as increased the total population of active POMC neurons, compared to control (Fig. 6D). Furthermore, POMC neurons also exhibited a higher frequency in both sEPSPs (+ .362 ± .172 Hz, Fig. 6G, P <.05) and sEPSCs (+.500 ± .141 Hz, Fig. 6I, P < .01), indicating that OPFR is altering arcuate synaptic relationships to increase excitatory signaling onto POMC neurons. In addition, OPFR augmented the amplitude of sEPSPs by 2.34 ± .665 mV and had a trending effect on sEPSCs (Fig. 6H, P < .01; Fig. 6J, P = .0806). An increase in amplitude correlates to a direct effect of OPFR on POMC neurons making them more responsive to glutamate release. Overall, these data indicate that OPFRs are making POMC neurons more excitable.

POMC neuron sensitivity to Leptin in Male Mice

Lastly, we explored the potential for OPFR to increase sensitivity to leptin in POMC neurons in male mice, a decision informed by our findings in Fig. 1. We recorded RMP, input resistance, action potential frequency, and frequency and amplitude of sEPSPs in POMC neurons. Counter to our above findings in female mice, POMC neurons in male mice did not display any of the baseline excitability effects caused by OPFR (data not presented). There was no alteration of baseline RMP or resistance input (data not

presented), and no significant effect of OPFR on the depolarization effect of 100 nM leptin on POMC neurons (Fig. 7A & B). There may be a positive trend toward increased action potential (Fig. 7C &D) and sEPSP frequency (Fig. 7E & F), but unfortunately, with the onset of COVID-19 lockdown, we were unable to obtain sufficient power to concretely determine these results.

4.5 Discussion

Our current understanding of OPFRs' influence on homeostasis is almost exclusively informed by developmental exposure data. Perinatal exposures have revealed an array of effects including neurotoxicity and disruptions of thyroid endocrine signaling, hepatic metabolism, and energy homeostasis (Wang et al. 2019a; Wang et al. 2019b; Wang et al. 2015; Baldwin et al. 2017; Du et al. 2016). Such effects, as shown in a study by Green et al. (2018) predisposed rats to type-2 diabetes (Green et al. 2017). Despite the ever-growing body of literature surrounding endocrine disrupting chemicals (EDCs), there are yet to be satisfactory explorations of how EDC exposure modifies endocrine pathways throughout adulthood and particular knowledge gaps exist concerning OPFRs. Our findings help address these gaps, providing critical insight into the mechanisms of OPFR EDC-action.

Collectively, our research demonstrates that even sub-chronic OPFR exposure in adult mice is sufficient to cause significant disruptions in the neuronal circuitry governing energy homeostasis. Previously, we observed sex-dependent, transcriptomic alterations of arcuate (ARC) genes involved in energy homeostasis, in particular, receptors for the circulating hormones leptin, ghrelin, and 17-β-estradiol (E2) (Krumm et al. 2018). In addition to being capable of crossing the blood-brain-barrier, OPFRs accumulate within the brain (Marco, S. and Buckley, B., Department of Toxicology; Rutgers University, 2019.

Collaborative, unpublished data), allowing chronic interactions with ARC estrogen and PPARγ receptors governing energy homeostasis. Together, these findings informed our hypothesis that OPFRs' influence on energy homeostasis is initiated centrally, through disruption of hypothalamic signaling.

One potential mechanism for the negative outcomes associated with adult OPFR exposures is modulation of E2 signaling in the arcuate (Krumm et al. 2018). E2 is a powerful anorexigenic hormone, and its endogenous receptor, ER, is widely expressed in the hypothalamus and concentrated in the ARC nucleus (Shughrue et al. 1997). ERα activation is known to inhibit NPY-stimulated feeding, and excite anorexigenic POMC neurons via rapid, membrane-bound receptors, in addition to classical nuclear receptor-mediated transcriptional activation (Stincic et al. 2018; Thornton et al. 1994; Roepke et al. 2008; Smith et al. 2013). OPFRs are known to interact with estrogen receptors (ERs) (Pillai et al. 2014; Kojima et al. 2013; Tung et al. 2017; Liu et al. 2013a), and, in our previous adult OPFR study, we found that mice lacking ERα exhibited a reduction in gene expression induced by OPFR. For these reasons, we hypothesize that the dysregulation of estrogenic regulation of energy homeostasis is a primary target for OPFR action. One mechanism by which E2 influences energy homeostasis is through activation of what is known as the M-current in NPY neurons. As mentioned earlier, the M-current is a constitutive, subthreshold, voltage-gated, outwardly-rectifying K^+ current that acts to hyperpolarize neurons and decrease excitability. In NPY neurons, the M-current is sensitive to energy state and under control of estrogenic signaling. The M-current in NPY neurons from ovariectomized female mice treated with estradiol benzoate was elevated compared to control females, which acts to reduce NPY excitability (Roepke et al. 2011).

In our previous study (Krumm et al. 2018), we found increases in expression of M-current channel subunits *Kcnq2*, *-3*, and *-5*. In the current study, we also observed an

increase in *Kcnq* expression in NPY neurons by OPFR exposure (Fig. 1). Therefore, we first explored OPFRs' effects on the M-current in NPY neurons in both male and female mice. Because E2 does not regulate the M-current in female POMC neuron (Roepke et al. 2012) we did not record POMC M-current in this study. Despite the increase in *Kcnq5*, male mice did not exhibit any OPFR effects on the M-current (Fig. 2). While OPFR did not alter NPY M-current, we did observe a baseline reduction (10 ± 3.11 mV) in the resting membrane potential (RMP), indicating that OPFR may be increasing NPY excitability through mechanisms alternative to the M-current in male mice.

However, in females, OPFR exposure did suppress the M-current, highlighting the sex-specific nature of OPFR disruption. Sub-chronic OPFR exposure in females sharply decreased the M-current, with maximal reductions across voltage steps -45 to -35 mV. Additionally, OPFR decreased NPY RMP (8 ± 3.37 mV), together indicating that OPFR is acting to increase NPY excitability through reductions in inhibitory M-current in female mice. Because E2 endogenously augments the M-current in female mice, we hypothesize that our observed reduction in M-current is a result of OPFR interference of E2 control over the M-current. The sex-dependent nature of this dysregulation is interesting, and may be due to differential regulation of the M-current by E2 between the sexes. However, since the only existing literature of estrogenic regulation of NPY M-current exists within females, this remains to be determined.

Another hormone that influences the M-current in NPY mice is ghrelin (Yasrebi et al. 2016). Ghrelin is an orexigenic brain-gut peptide hormone that is secreted by the stomach into systemic circulation, where it is then able to cross into the ARC via the leaky portion of the blood-brain-barrier. Ghrelin powerfully stimulates food intake by binding to its endogenous receptor, growth hormone secretagogue receptor (GHSR). GHSR is expressed throughout the brain, and within NPY neurons and acts to directly depolarize NPY neurons (Cowley et al. 2003; Hashiguchi et al. 2017) as well as inducing

transcriptional activation of NPY genes to induce feeding and weight gain (Chen et al. 2004; Kamegai et al. 2000, 2001). One of the mechanisms through which ghrelin depolarizes NPY neurons is the M-current. We have previously reported that in male mice ghrelin inhibits NPY M-current resulting in neuronal depolarization (Yasrebi et al. 2016). Furthermore, the actions of ghrelin appear to be regulated by estrogen. In intact females, the orexigenic effects of ghrelin are reduced during proestrus, when circulating E2 is high, indicating that the anorexigenic actions of E2 may extend to dampening ghrelin responsiveness (Sakurazawa et al. 2013). Additionally, exogenous administration of 1 μg estradiol in ovariectomized female mice is reported to increase ARC *Ghsr* expression (Frazao et al. 2014), presumably as a negative feedback response to E2 inhibition of ghrelin signaling.

Because of these studies, we hypothesized that OPFR is disrupting the neuronal response to ghrelin in female NPY neurons. In our study, ghrelin depolarized NPY neurons, and did so with a greater magnitude within OPFR-treated females in respect to control females (Fig. 4B). Furthermore, the stimulatory effects of ghrelin may be contributed, in part, to an inhibition of NPY M-current. We observed that OPFR exposure causes a basal reduction in the M-current in NPY females. In measuring the neuronal response to ghrelin, we found that the ghrelin-induced reduction in NPY M-current activity was greater in OPFR-treated females compared to control females (Fig. 5). These findings indicate a potential sex-dependent, ghrelin-mediated pathway for OPFR dysregulation of the M-current within NPY neurons.

Interestingly, despite OPFR augmenting ghrelin depolarization of NPY neurons, we observed a decrease in the frequency of spontaneous excitatory post-synaptic potentials (sEPSPs) in response to ghrelin. sEPSPs are small depolarization spikes sub-threshold to producing an action potential, that are produced by the recorded neuron in response to synaptic input. In OPFR-treated female mice, NPY neurons displayed a lower frequency

of sEPSPs than did neurons from control females, in response to both 1 and 100 nM ghrelin (Fig. 4F). A lowered sEPSP frequency means fewer neuronal responses to synaptic stimuli and is therefore a marker of reduced synaptic input onto the recorded neuron. At first, it appears somewhat counter-intuitive that OPFR is diminishing synaptic input onto NPY neurons, yet still resulting in greater depolarization. We hypothesize that this is a result of sub-chronic OPFR augmentation of compensatory ghrelin signaling originating in KNDy neurons (ARC neurons that express neuropeptides kisspeptin, neurokinin B, and dynorphin) (Conde and Roepke 2019).

ARC KNDy neurons are most well-known for their role in reproduction as the luteinizing hormone pulse-generator (Plant 2019; Wang and Moenter 2020). However, reproduction has high resource demands, and the relationship between reproduction and energy homeostasis is linked, in part, through KNDy action. KNDy neurons both sense, and help regulate energy homeostasis, and murine knock-out models of the kisspeptin receptor, *Kiss1r*, result in increased bodyweight-gain and adiposity, and decreased energy expenditure in females (Tolson et al. 2016; Tolson et al. 2014). Furthermore, KNDy neurons are under estrogenic regulation and their selective ablation blocks the effects of E2 restoration on post-ovariectomy weight-gain in female rats (Mittelman-Smith et al. 2012). KNDy neurons communicate with both NPY and with POMC neurons (Backholer et al. 2010), directly exciting POMC neurons and indirectly inhibiting NPY neurons (Nestor et al. 2016; Qiu et al. 2016; Fu and van den Pol 2010). Overall, the literature suggests a role of KNDy neurons in reinforcing the anorexigenic activity of E2.

KNDy neurons are also sensitive to ghrelin, which causes excitation in part, through dampening of KNDy M-current (Conde and Roepke 2019). Ghrelin excitation of these neurons is also E2-regulated (Frazao et al. 2014; Yang et al. 2016; Qiu et al. 2018), and thusly vulnerable to EDC action by OPFRs. As OPFR treatment decreased the frequency of sEPSPs in response to ghrelin – an inhibitory, indirect effect on a ghrelin-associated

pathway – we hypothesize that this effect is occurring through KNDy neurons, which indirectly inhibit NPY neurons (Fu and van den Pol 2010). Additionally, as our previous study revealed that OPFR increases *Kiss1* (Krumm et al. 2018), future studies will characterize the effects of OPFR on KNDy neuronal activity and ghrelin sensitivity.

In addition to OPFR effects on NPY, we also observed a striking elevation in POMC neuronal activity in female mice (Fig. 6). While OPFR did not alter resting membrane potential, it did induce a 4-fold increase in action potential frequency, as well as enhancing amplitude and frequency of sEPSPs, and spontaneous excitatory post-synaptic currents (sEPSCs) frequency. These data point at both pre- and post-synaptic mechanisms through which OPFR is augmenting POMC excitation, which is indicative of an estrogenic effect (Stincic et al. 2018). This too, is in alignment with our theory that OPFR is influencing KNDy output, for KNDy neurons excite POMC neurons through vesicular release of glutamate (Nestor et al. 2016; Fu and van den Pol 2010). We hypothesize that OPFR may be augmenting the endogenous E2 signaling pathways that govern KNDy excitability – perhaps through sensitizing KNDy neurons to activation by ghrelin – to provide additional excitatory synaptic input onto POMC neurons.

Our findings reveal excitatory effects of OPFR on both NPY and on POMC neurons, which makes for an interesting collective excitation of ARC pathways. One possible explanation for these results is that the increased NPY excitation experienced in OPFR-treated female mice is resulting in compensatory action by KNDy neurons to augment POMC activity. Recall that the actions of ghrelin on KNDy neurons are in opposition to the net effect ghrelin has on stimulating food intake and reducing energy expenditure. Because E2 augments ghrelin excitation of KNDy neurons (Frazao et al. 2014; Yang et al. 2016; Qiu et al. 2018), this appears to be a mechanism through which E2 asserts inhibitory feedback on orexigenic signaling. Therefore, it is possible that the mechanism

of OPFR dysregulation of energy homeostasis lies within estrogenic interactions that influence ghrelin signaling pathways.

Furthermore, ghrelin is known to decrease energy expenditure (Lv et al. 2018), and in a similar, adult OPFR study, we found that OPFR decreased energy expenditure and decreased nighttime activity of female mice (Chapter 2, Vail et al., 2020, *in publication*). Additionally, OPFR also demonstrated an interesting dysregulation of water intake within both males and females. Because fluid homeostasis and intake timing is influenced by both estrogen (Santollo et al. 2013; Krause et al. 2003; Kisley et al. 1999; Jonklaas and Buggy 1984) and ghrelin (Mietlicki et al. 2009; Hashimoto et al. 2010; Plyler and Daniels 2017), it is possible that OPFR disruption of diurnal drinking patterns could also be attributed to effects on these pathways. While our current study reports OPFR effects primarily in females, the same previous study observed increased body-weight gain and fat mass within male mice exposed to OPFR, with no body mass effect on females (Chapter 2, Vail et al., 2020, *in publication*). Instead, female mice exhibited a decreased intake of high-fat diet (Chapter 2, Vail et al., 2020, *in publication*). These apparent disparate results are indicative of potential mixed actions of OPFR, and suggest that dysregulation of ghrelin signaling is likely not the only aspect of OPFR endocrine disruption.

Another possible mechanism of OPFR actions is through PPARγ. PPARγ is a nuclear receptor involved in lipid and glucose homeostasis (Janani and Ranjitha Kumari 2015) and is known to interact with OPFRs (Kojima et al. 2013; Pillai et al. 2014). Indeed, in our previous adult OPFR study, female mice exhibited subtle effects on respiratory exchange ratio, a marker of altered lipid metabolism rate (Chapter 2, Vail et al., 2020 *in publication*). One way that PPARγ can sense and respond to lipid state is through agonistic binding activity of endogenous lipid ligands (Grygiel-Górniak 2014). Certain lipids can bind to and

activate PPARγ, and if OPFRs are doing the same, they could be mimicking the effects of a heightened lipid-load. Furthermore, both NPY and POMC neurons express PPARγ (Sarruf et al. 2009), indicating neuronal routes for potential PPARγ disruption. Importantly, agonistic binding of PPARγ-specific ligand rosiglitazone (ROSI) increases expression of *Npy* and *AgRP*, and was sufficient to induce feeding and hoarding behaviors in male mice and hamsters (Garretson et al. 2015). Additionally, brain-specific knockout of PPARγ conferred resistance to diet-induced obesity (Lu et al. 2011). Using a Cre-recombinase system to selectively knock out PPARγ within POMC neurons, Stump et al. (2016) found that without POMC-PPARγ, intraperitoneal administration of ROSI was unable to elicit its orexigenic effects. This indicates POMC-PPARγ as an important mechanism through which PPARγ modulates central regulation of feeding behavior. Additionally, our findings in Chapter 3 using whole-brain knockout of PPARγ also indicates a role for this receptor in OPFR-induced dysregulation of energy homeostasis. These studies outline an overall orexigenic effect of neuronal PPARγ, and highlight a secondary measure through which OPFRs could be modulating ARC control over energy homeostasis.

Lastly, we measured OPFR effects on leptin sensitivity within POMC neurons from male mice. While these exploratory studies did not reveal conclusive effects, repeat studies are needed for sufficient power to confidently declare that OPFR has no effects on leptin signaling within male mice.

Collectively, these data indicate that OPFR exposure preferentially affects females more so than males, possibly as a consequence of actions involving estrogen connections. NPY M-current and ghrelin sensitivity were only disrupted within females, and the excitatory effect of OPFR on POMC neurons was also only observed within females. Because estrogen signaling pathways are highly sexually dimorphic, our observed sex-dependent effects support our theory of estrogenic disruption by OPFR. In

addition, our underlying hypothesis that OPFR is interacting with estrogenic regulation of KNDy actions is also supported by our observed sex-specific effect. While ARC *Kiss1* expression is similar between sexes (Kauffman et al. 2007), knockout of its receptor *Kiss1r* increases bodyweight-gain and adiposity and decreases energy expenditure only within females (Tolson et al. 2016; Tolson et al. 2014). We propose that one possible explanation for these effects is through dysregulated KNDy action onto NPY and POMC neurons, potentially through a ghrelin-mediated pathway.

4.6 Conclusion

In summary, our findings highlight a female-specific effect of increased NPY and increased POMC neuronal activity, with increased sensitivity to ghrelin signaling. These results detail an interesting net increase in ARC output. Because NPY and POMC have oppositional endpoints, this makes it difficult to declare a clear effect on energy homeostasis. It may come down to a difference in tone, and over time, OPFR exposure may culminate in a significant shift in energy homeostasis. Indeed, using the same OPFR mixture, we have previously reported increased weight gain, and decreased energy expenditure within adult male and female mice, respectively (Chapter 2, Vail et al., 2020, *in publication*). Collectively, past and current findings raise concerns for the ability of OPFR exposure to induce cascading effects that, over time, may manifest into serious conditions such as metabolic syndrome, diabetes, and diet-induced obesity. Considering the relatively short, 4-week exposure of our current study, a chronic, 6-month follow-up study would be very informative for understanding the long-term impact of homeostatic dysregulation by OPFRs.

OPFRs remain the predominant flame retardant in usage within the United States. It took over two decades of research before OPFRs' predecessors, polybrominated diphenyl

ethers, were removed from U.S. markets due to health concerns. It stands to reason that continued investigation will therefore be a necessity to enable future regulatory limits providing appropriate human safety protection. Our research has further highlighted the potential for OPFRs to affect metabolic control through ARC KNDy neurons, and future studies would greatly benefit from investigating these neurons more directly, specifically though the use of patch-clamp electrophysiological techniques. Lastly, while this study focused on OPFR disruption of ARC circuitry in regards to feeding, there are many other aspects of homeostasis and reproduction that these neurons also govern, which may be important for understanding the full scope of OPFRs' impact on human health.

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Tables

Table 1. List of primers for quantitative real-time PCR

Gene Name	Product Length	Primer Eff. (%)	Primer sequence	Base Pair #	Accession #
Actβ	63	100.7	F:GCCCTGAGGCTCTTTTCCA R:TAGTTTCATGGATGCCACAGGA	849-867 890-911	NM_007393.3
Agrp	146	105	F:CTCCACTGAAGGGCATCAGAA R:ATCTAGCACCTCCGCCAAA	287-307 414-432	NM_007427.2
Cart	169	95	F:GCTCAAGAGTAAACGCATTCC R:GTCCCTTCACAAGCACTTCAA	227-297 425-445	NM_013732
Gapdh	98	93.1	F:TGACGTGCCGCCTGGAGAAA R:AGTGTAGCCCAAGATGCCCTTCAG	778-797 852-875	NM_008084.2
Ghsr	122	103	F:CAGGGACCAGAACCACAAAC R:AGCCAGGCTCGAAAGACT	1003-1022 1107-1124	NM_177330
Kcnq-2	171	105	F:GGTGCTGATTGCCTCCATTG R:TCCTTGCTGTGAGCGTAGAC	644-663 795-814	NM_133322
Kcnq-3	94	105	F:GCTGCTGGAAACCTTTGC R:ACGCCAGCCTTTGTATCG	474-491 550-567	NM_152923.1
Kcnq-5	99	101	F:GGGCACAATCACACTGACAAC R:GAAATGCCAAGGAGTGCGAAG	915-935 993-1013	NM_023872.2
Lepr	149	104.8	F:AGAATGACGCAGGGCTGTAT R:TCCTTGTGCCCAGGAACAAT	3056-3075 3185-3204	NM_146146.2
Npy	182	100	F:ACTGACCCTCGCTCTATCTC R:TCTCAGGGCTGGATCTCTTG	106-125 268-287	NM_023456
Pomc	200	103	F:GGAAGATGCCGAGATTCTGC R:TCCGTTGCCAGGAAACAC	145-164 327-344	NM_008895
Trpc5	195	103.3	F:TGGTAGTGCTGCTGAATATG R:TGAACCAGTTGCCAAGATAG	2241-2260 2461-2435	NM_009428

Table 2. Cell parameters for each experiment

	Control				OPFR			
	RMP (mV)	Rin (gΩ)	RMP (mV)	Rin (gΩ)	RMP (mV)	Rin (gΩ)	RMP (mV)	Rin (gΩ)
	Baseline		XE-991		Baseline		XE-991	
NPY ♂	-69.8 ± 2.2	1.1 ± 0.0	-59.7 ± 3.1[a]	0.9 ± 0.1[a]	-59.6 ± 2.2[B]	1.2 ± 0.2	-51.2 ± 3.0[a]	1.2 ± 0.2
NPY ♀	-67.7 ± 2.7	0.8 ± 0.1	-63.4 ± 3.4	0.8 ± 0.2	-59.3 ± 2.0[A]	0.9 ± 0.1	-53.1 ± 3.0	0.6 ± 0.0
	Baseline		100 nM Ghrelin		Baseline		100 nM Ghrelin	
NPY ♀	-65.3 ± 2.0	0.3 ± 0.0	-62.2 ± 2.0	0.3 ± 0.0	-67.3 ± 1.9	0.3 ± 0.0	-59.0 ± 2.3[a]	0.3 ± 0.1
	Baseline		100 nM Leptin		Baseline		100 nM Leptin	
POMC ♂	-63.5 ± 1.7	0.3 ± 0.0	-60.5 ± 2.4	0.3 ± 0.0	-64.3 ± 2.0	0.2 ± 0.0	-59.1 ± 1.7	0.3 ± 0.0
	Baseline				Baseline			
POMC ♀	-64.5 ± 1.5	0.3 ± 0.0	N/A	N/A	-64.1 ± 2.8	0.3 ± 0.0	N/A	N/A

Table 2. Resting membrane potential (RMP) and input resistance (Rin) values for all recordings before and after exposure to XE-991, 100 nM ghrelin, and 100 nM leptin, respectively. Uppercase letters in superscript denote comparisons between control-oil and OPFR-oil baseline values. Lowercase letters denote comparisons of before and after exposure to perfused drugs XE-991, ghrelin, or leptin. Data were analyzed by unpaired, parametric Student's t-test (A=P<.05; B=P<.01; C=P<.001; D=P<.0001), and are presented as mean ± SEM.

Figures

Figure 1. Relative arcuate gene expression in NPY neurons **(A,B)**, and POMC neurons **(C,D)** collected from male and female mice exposed to OPFR (1 mg/kg) for 4 wks. Data were analyzed by unpaired, parametric Student's t-test (A=P<.05; B=P<.01; C=P<.001; D=P<.0001). Data (n=6 animals, 3 pools per animal) are presented as mean ± SEM.

Fig. 2. Electrophysiological parameters and M-current of NPY neurons in male mice orally dosed with an OPFR mixture (1 mg/kg) for 4 weeks. **(A)** M-current protocol and representative traces. From a holding potential of -60mV, a voltage jump to -20 mV (250 ms) was followed by steps from -25 to -75 mV in 5 mV increments (250 ms). Current was recorded before and after 10 min incubation with 40 mM XE-991, in the presence of TTX (1 mM). This protocol calculates current relaxation, the difference between instantaneous and steady state (arrows), as a measurement of channel activity. **(B)** The XE-991-sensitive current was calculated by subtracting the post-XE-991 current relaxation, from the baseline current relaxation, and is representative of the M-current. **(C)** XE-991-induced change in resistance input; **(D)** Baseline resting membrane potential (RMP); **(E)** Recording-paired presentation of pre- and post-XE-991 RMP. Data were analyzed by a 2-way ANOVA with post-hoc Holm Sidak's mutliple comparisons test (B), and by an unpaired, parametric Student's t-test (C-E) (A=P<.05; B=P<.01; C=P<.001; D=P<.0001). Data (n=15;11 neurons) are presented as mean ± SEM.

Fig. 3. Electrophysiological parameters and M-current of NPY neurons in female mice orally dosed with an OPFR mixture (1 mg/kg) for 4 weeks. **(A)** Representative M-current traces; **(B)** The XE-991-sensitive current was calculated by subtracting the post-XE-991 current relaxation, from the baseline current relaxation, and is representative of the M-

current. **(C)** Baseline resting membrane potential (RMP); **(D)** Max peak of XE-991-sensitive current; **(E)** Baseline-XE-991 RMP paired relationships for all recordings in Fig. 2; **(F)** XE-991-induced change in resistance input. Data were analyzed by a 2-way ANOVA with post-hoc Holm Sidak's mutliple comparisons test (B), and by an unpaired, parametric Student's t-test (C-F) (A=P<.05; B=P<.01; C=P<.001; D=P<.0001). Data (n=13;12 neurons) are presented as mean ± SEM.

Fig. 4. Ghrelin dose-response from NPY neurons in female mice orally dosed with an OPFR mixture (1 mg/kg) for 4 weeks. **(A)** Representative traces of ghrelin responses in female NPY neurons; **(B)** Ghrelin-induced depolarization; **(C)** Ghrelin-induced action potential firing (AP); **(D)** Recording-paired presentation of (C); **(E)** Representative traces of spontaneous excitatory post-synaptic potentials (sEPSP); **(F)** Ghrelin-induced sEPSPs; (G) Recording-paired presentation of **(G)**. Data were analyzed by an unpaired, parametric Student's t-test (A=P<.05; B=P<.01; C=P<.001; D=P<.0001). Data (n=10;11 neurons) are presented as mean ± SEM.

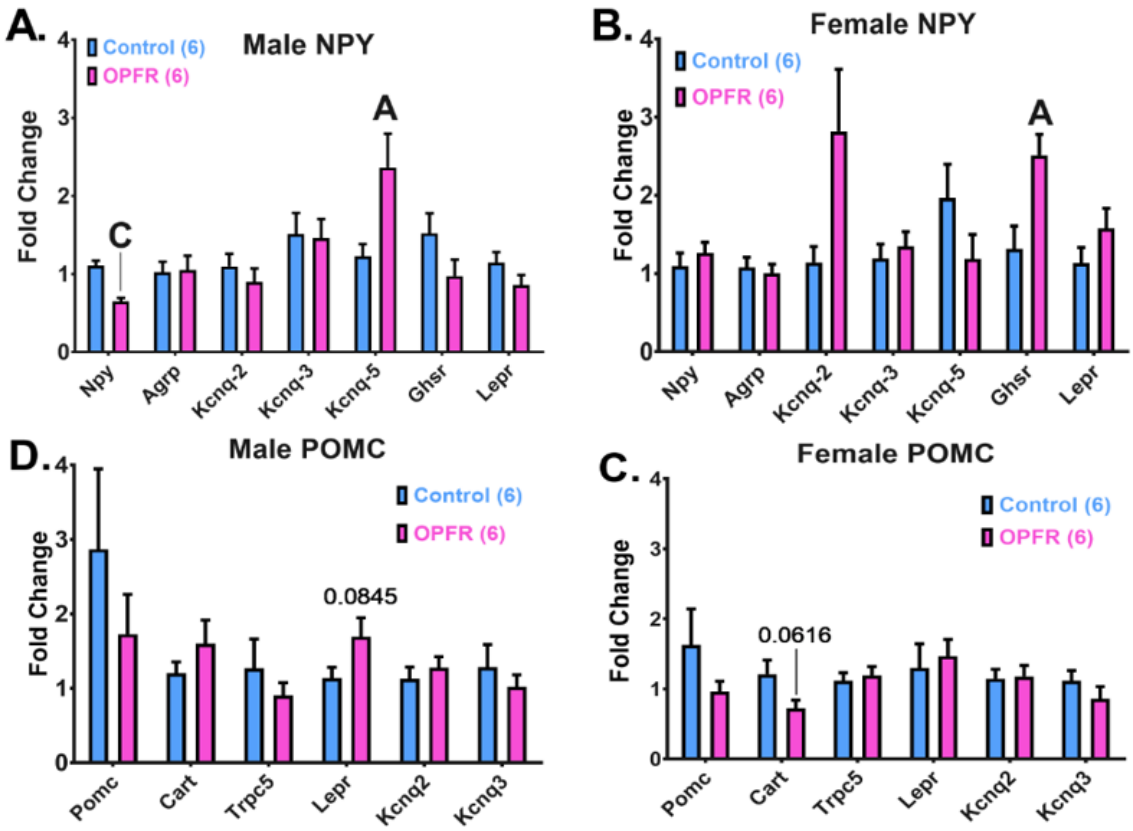
Fig. 5. Ghrelin dose-response from NPY neurons in female mice orally dosed with an OPFR mixture (1 mg/kg) for 4 weeks. **(A)** Representative traces of the M-current protocol before and after 100 nM Ghrelin; **(B)** Baseline current in Oil and OPFR; **(C)** Oil currents: baseline, and in response to 1 or 100 nM Ghrelin; **(D)** OPFR currents: baseline, and in response to 1 or 100 nM Ghrelin. Data were analyzed by a two-way ANOVA with post-hoc Holm Sidak's multiple comparisons. Data (n=11;11 neurons) are presented as mean ± SEM.

Fig. 6. Basal electrophysiological recordings of POMC neurons in female mice orally dosed with an OPFR mixture (1 mg/kg) for 4 weeks. **(A)** Representative traces of baseline

action potential (AP) firing; **(B)** Resting membrane potential; **(C)** AP frequency; **(D)** Ratio of actively firing cells; **(E)** Representative traces of spontaneous post-synaptic potentials (sEPSP); **(F)** sEPSP amplitude; **(G)** sEPSP amplitude; **(H)** Representative traces of spontaneous post-synaptic currents (sEPSC); **(I)** sEPSC frequency; **(J)** sEPSP amplitude. Data were analyzed by an unpaired, parametric Student's t-test. Data (n=19;18 total neurons) are presented as mean ± SEM.

Fig. 7. 100 nM Leptin repsonse from NPY neurons in male mice orally dosed with an OPFR mixture (1 mg/kg) for 4 weeks. **(A)** Leptin-induced depolarization; **(B)** Recording-paired presentation of (A); **(C)** Leptin-induced action potentials; **(D)** Recording-paired presentation of (C); **(E)** Leptin-induced sEPSP; **(F)** Recording-paired presentation of (E). Data were analyzed by an unpaired, parametric Student's t-test (A=P<.05; B=P<.01; C=P<.001; D=P<.0001). Data (n=9;7 total neurons) are presented as mean ± SEM.

Figure 1


A.
Male NPY
Control (6)
OPFR (6)
Fold Change
C
A
Npy
Agrp
Kcnq-2
Kcnq-3
Kcnq-5
Ghsr
Lepr
B.
Female NPY
Control (6)
OPFR (6)
Fold Change
A
Npy
Agrp
Kcnq-2
Kcnq-3
Kcnq-5
Ghsr
Lepr
D.
Male POMC
Control (6)
OPFR (6)
Fold Change
0.0845
Pomc
Cart
Trpc5
Lepr
Kcnq2
Kcnq3
C.
Female POMC
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Cart
Trpc5
Lepr
Kcnq2
Kcnq3

Figure 2

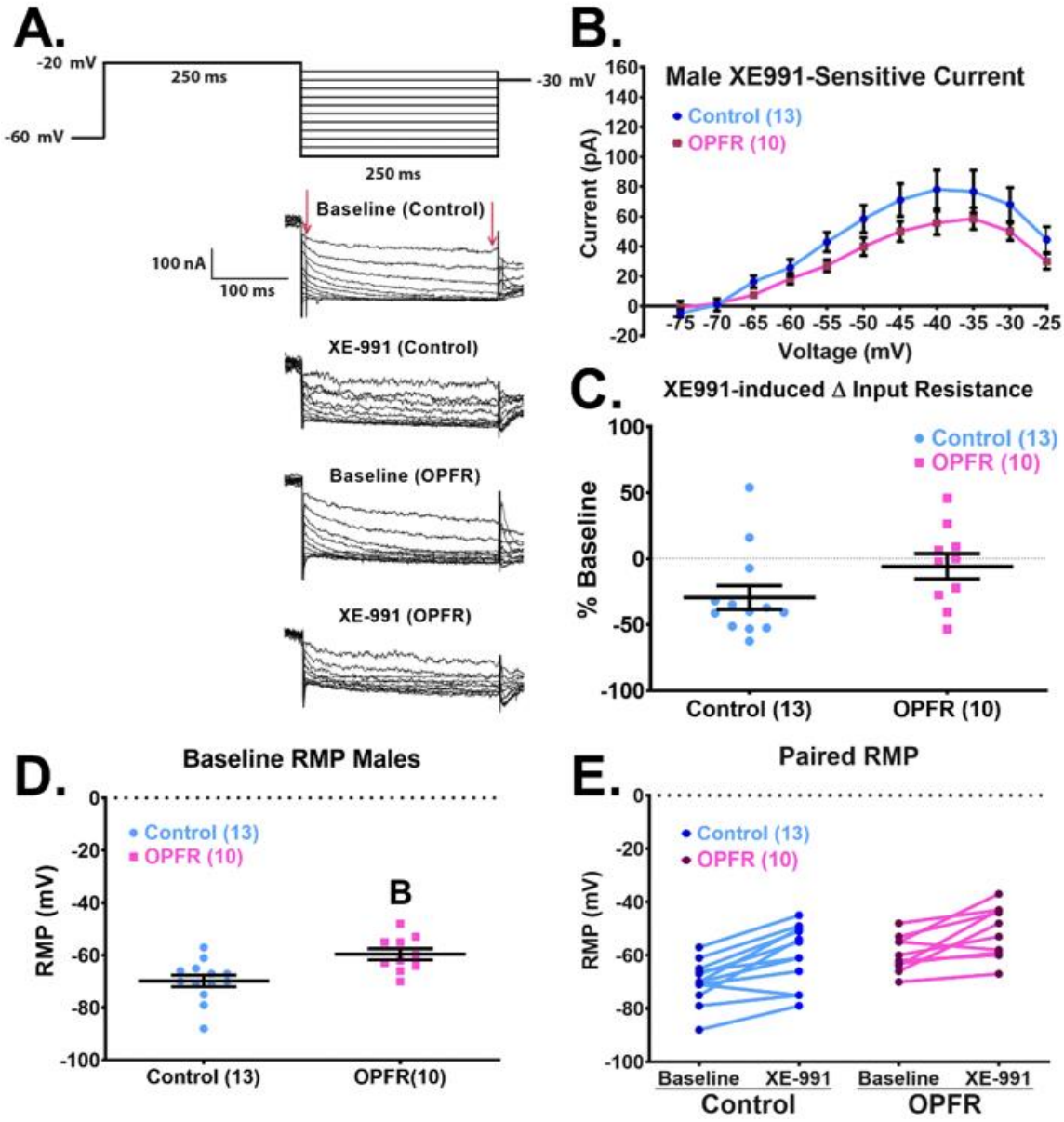

A.
-20 mV
250 ms
-30 mV
-60 mV
250 ms
Baseline (Control)
100 nA
100 ms
XE-991 (Control)
Baseline (OPFR)
XE-991 (OPFR)
B.
Male XE991-Sensitive Current
Control (13)
OPFR (10)
Current (pA)
Voltage (mV)
C.
XE991-induced Δ Input Resistance
Control (13)
OPFR (10)
% Baseline
Control (13)
OPFR (10)
D.
Baseline RMP Males
Control (13)
OPFR (10)
RMP (mV)
B
Control (13)
OPFR(10)
E.
Paired RMP
Control (13)
OPFR (10)
RMP (mV)
Baseline
XE-991
Baseline
XE-991
Control
OPFR

Figure 3

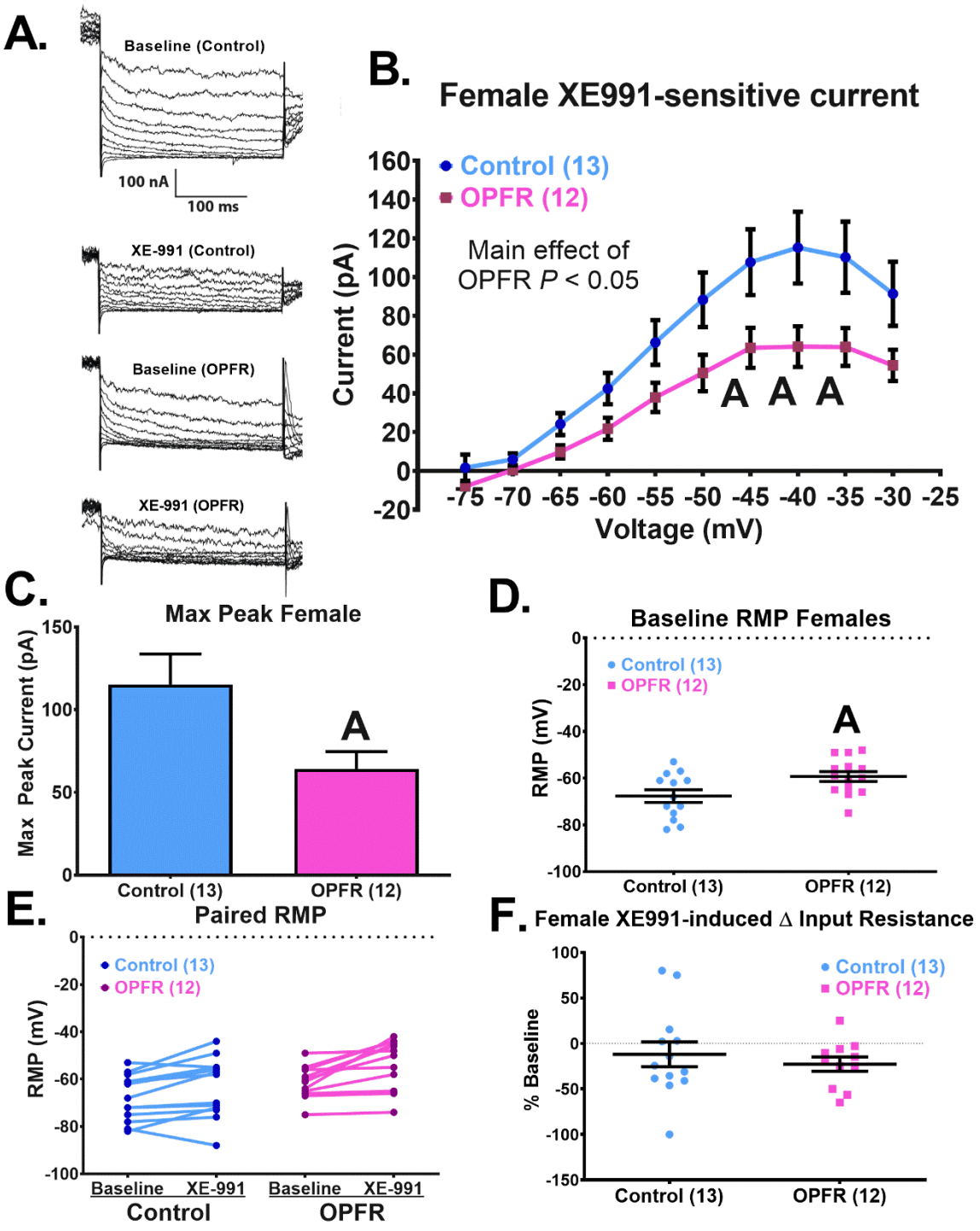

A.
Baseline (Control)
100 nA
100 ms
XE-991 (Control)
Baseline (OPFR)
XE-991 (OPFR)
B.
Female XE991-sensitive current
Control (13)
OPFR (12)
Main effect of
OPFR P < 0.05
Current (pA)
Voltage (mV)
A A A
C.
Max Peak Female
Max Peak Current (pA)
A
Control (13)
OPFR (12)
D.
Baseline RMP Females
Control (13)
OPFR (12)
RMP (mV)
A
Control (13)
OPFR (12)
E.
Paired RMP
Control (13)
OPFR (12)
RMP (mV)
Baseline XE-991 Baseline XE-991
Control
OPFR
F. Female XE991-induced Δ Input Resistance
Control (13)
OPFR (12)
% Baseline
Control (13)
OPFR (12)

Figure 4

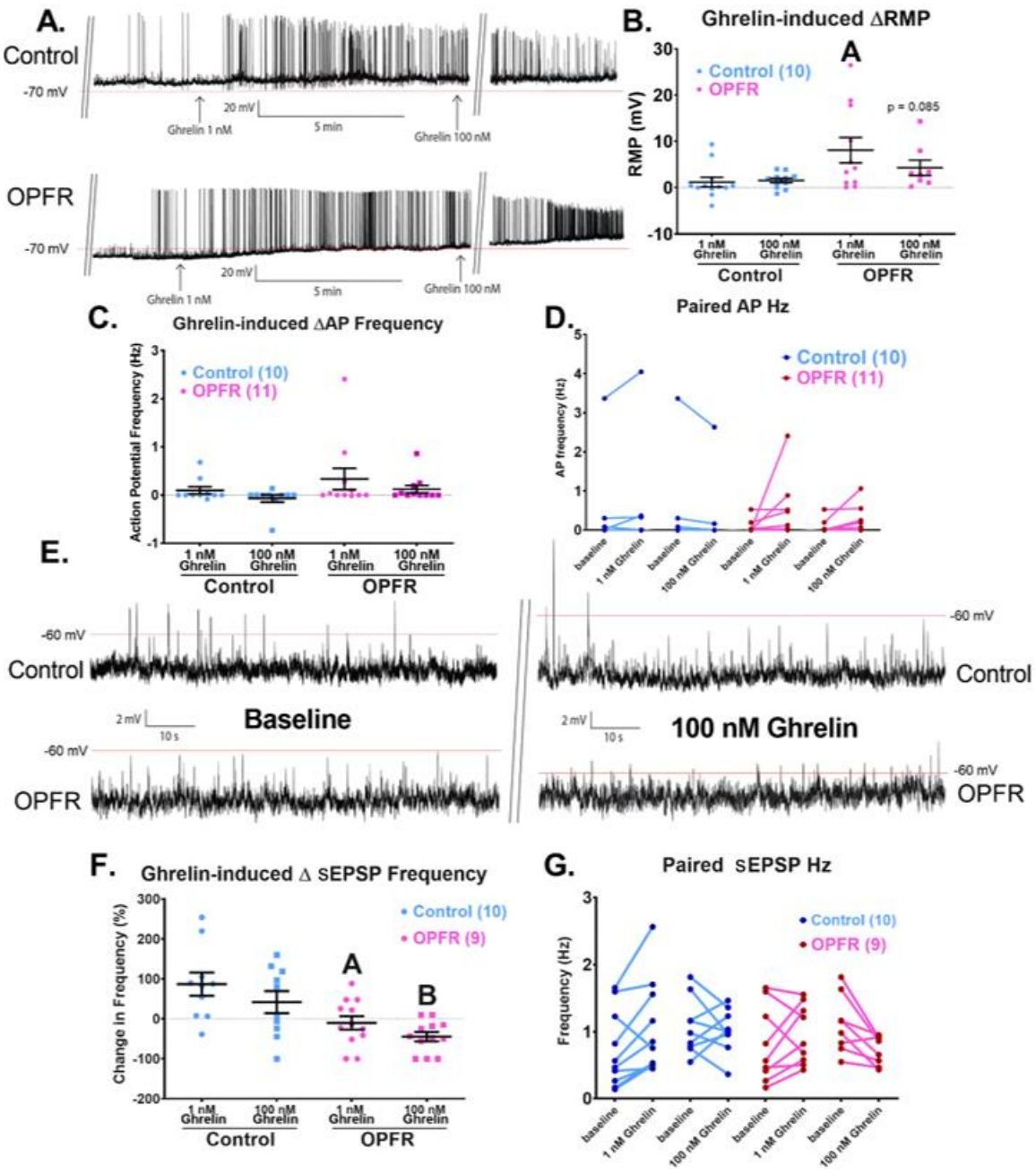

A.
Control
-70 mV
20 mV
5 min
Ghrelin 1 nM
Ghrelin 100 nM
OPFR
-70 mV
B.
Ghrelin-induced ΔRMP
Control (10)
OPFR
p = 0.085
RMP (mV)
1 nM Ghrelin
100 nM Ghrelin
Control
OPFR
C.
Ghrelin-induced ΔAP Frequency
Control (10)
OPFR (11)
Action Potential Frequency (Hz)
D.
Paired AP Hz
Control (10)
OPFR (11)
AP frequency (Hz)
baseline
1 nM Ghrelin
100 nM Ghrelin
E.
-60 mV
Control
2 mV
10 s
Baseline
OPFR
100 nM Ghrelin
F.
Ghrelin-induced Δ sEPSP Frequency
Control (10)
OPFR (9)
Change in Frequency (%)
G.
Paired sEPSP Hz
Frequency (Hz)

Figure 5

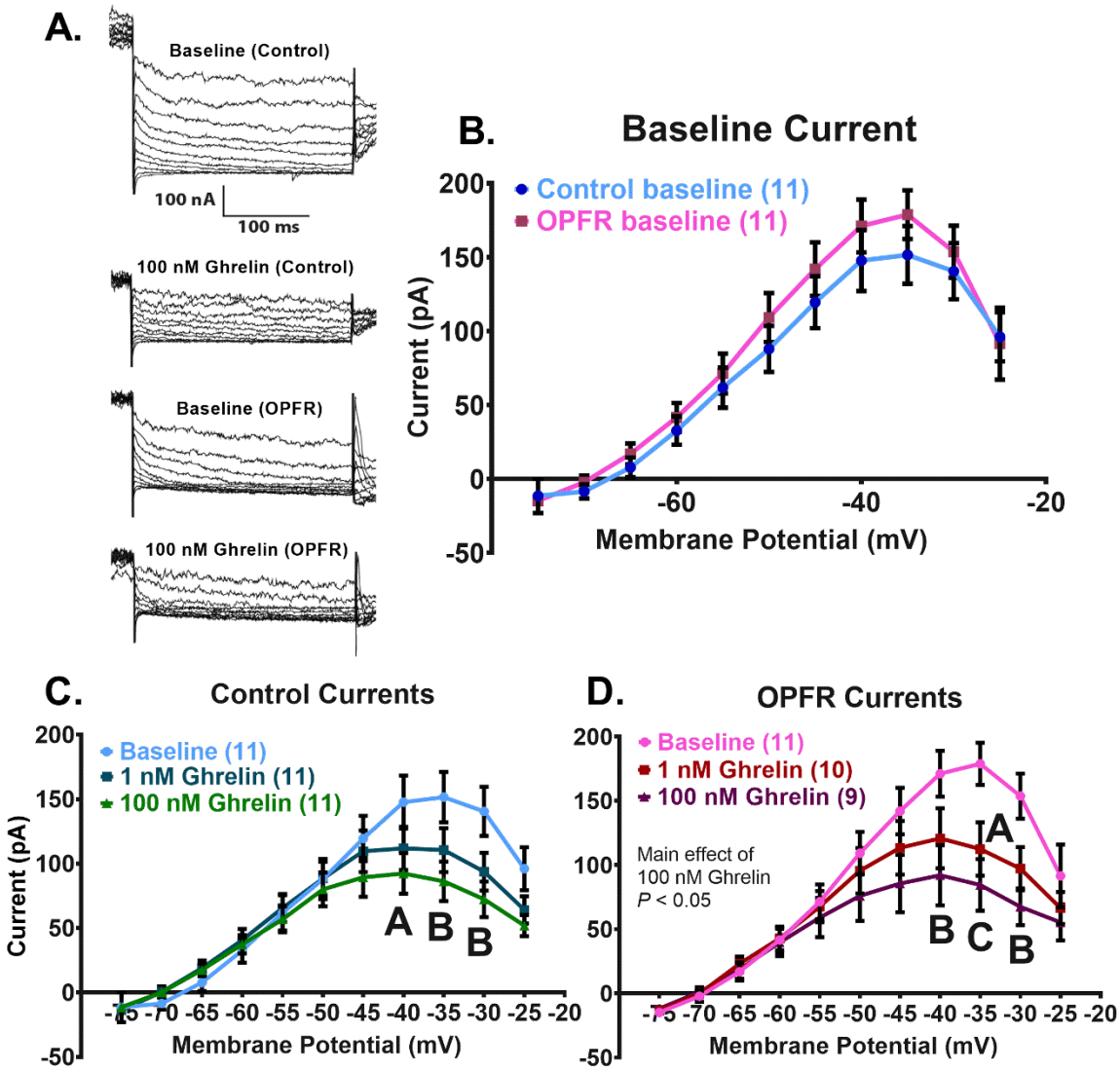

A.
Baseline (Control)
100 nA
100 ms
100 nM Ghrelin (Control)
Baseline (OPFR)
100 nM Ghrelin (OPFR)
B.
Baseline Current
Control baseline (11)
OPFR baseline (11)
Current (pA)
Membrane Potential (mV)
C.
Control Currents
Baseline (11)
1 nM Ghrelin (11)
100 nM Ghrelin (11)
A B B
Current (pA)
Membrane Potential (mV)
D.
OPFR Currents
Baseline (11)
1 nM Ghrelin (10)
100 nM Ghrelin (9)
Main effect of
100 nM Ghrelin
P < 0.05
A
B C B
Membrane Potential (mV)

Figure 6

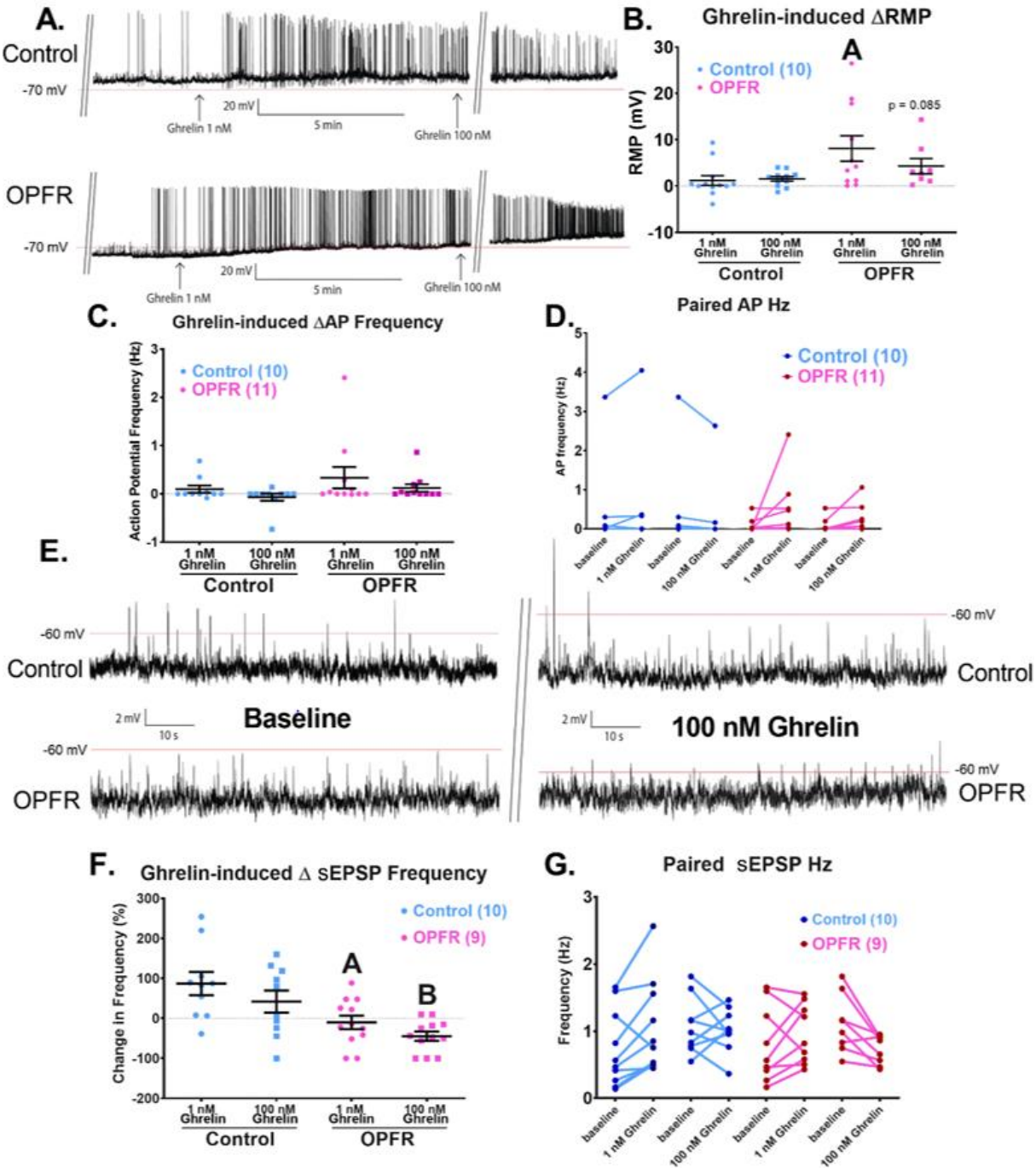
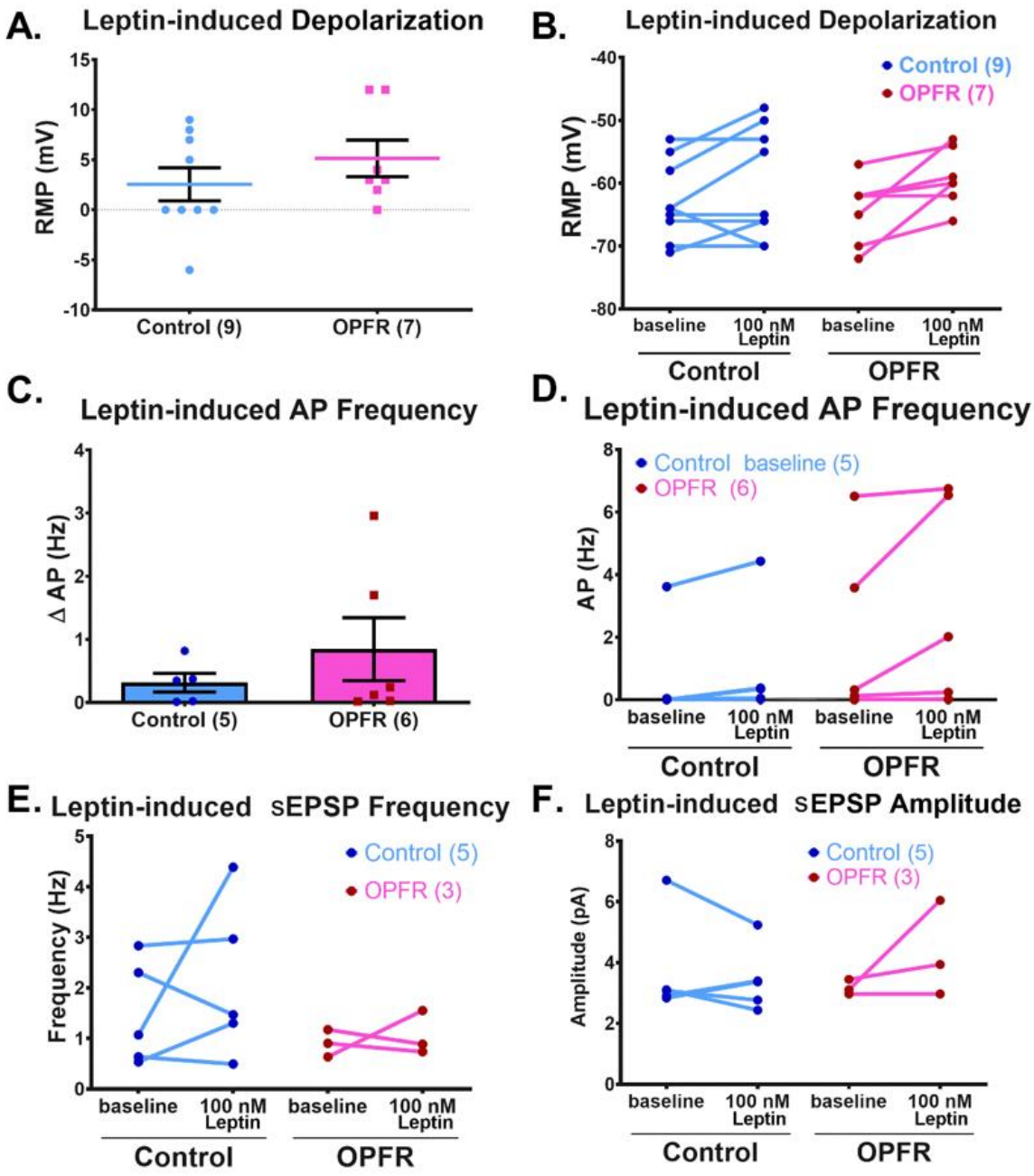

A.
Control
-70 mV
20 mV
5 min
Ghrelin 1 nM
Ghrelin 100 nM
OPFR
-70 mV
B.
Ghrelin-induced ΔRMP
Control (10)
OPFR
p = 0.085
RMP (mV)
1 nM Ghrelin
100 nM Ghrelin
Control
OPFR
C.
Ghrelin-induced ΔAP Frequency
Control (10)
OPFR (11)
Action Potential Frequency (Hz)
D.
Paired AP Hz
AP frequency (Hz)
baseline
1 nM Ghrelin
100 nM Ghrelin
E.
-60 mV
Control
2 mV
10 s
Baseline
100 nM Ghrelin
OPFR
F.
Ghrelin-induced Δ sEPSP Frequency
Change in Frequency (%)
G.
Paired sEPSP Hz
Frequency (Hz)

Figure 7


A.
Leptin-induced Depolarization
RMP (mV)
15
10
5
0
-5
-10
Control (9)
OPFR (7)
B.
Leptin-induced Depolarization
Control (9)
OPFR (7)
RMP (mV)
-40
-50
-60
-70
-80
baseline
100 nM Leptin
baseline
100 nM Leptin
Control
OPFR
C.
Leptin-induced AP Frequency
Δ AP (Hz)
4
3
2
1
0
Control (5)
OPFR (6)
D.
Leptin-induced AP Frequency
Control baseline (5)
OPFR (6)
AP (Hz)
8
6
4
2
0
baseline
100 nM Leptin
baseline
100 nM Leptin
Control
OPFR
E.
Leptin-induced sEPSP Frequency
Control (5)
OPFR (3)
Frequency (Hz)
5
4
3
2
1
0
baseline
100 nM Leptin
baseline
100 nM Leptin
Control
OPFR
F.
Leptin-induced sEPSP Amplitude
Control (5)
OPFR (3)
Amplitude (pA)
8
6
4
2
0
baseline
100 nM Leptin
baseline
100 nM Leptin
Control
OPFR

CHAPTER 5: SUMMARY

5. Summary

The expanding human exposure to endocrine disrupting chemicals such as OPFRs demonstrates the need for critical examinations of their toxicological impact. In the current studies, we characterize the physiological and neuroendocrine impact of OPFRs on energy homeostasis in adult mice. We show that adult mice display significant, sex-dependent effects. We show that WT males gain more weight and have higher adiposity when fed HFD, highlighting the potential of OPFRs to be obesogenic. Female mice did not display an OPFR-effect on bodyweight, and instead, were shown to have reduced energy expenditure and altered diet and fluid consumption patterns. These collective observations lead us to conclude that adult exposure to OPFRs disrupts homeostatic functions of fat storage and usage, feeding behavior, activity levels, and fluid regulation. Two major nuclear receptors are implicated in these dysregulations: PPARγ and ERα. PPARγ is an essential regulator of lipid homeostasis, and altered adiposity suggests disruption of PPARγ pathways. While PPARγ signaling does converge with homeostatic functions of feeding and energy expenditure, regulation of these pathways is primarily associated with estrogen and ERα. Because our tested OPFRs TPP, TCP, and TDCPP are shown to interact with both PPARγ and ERα, we hypothesize that OPFR disruption of energy homeostasis is occurring through these pathways. To further test this hypothesis, we utilized brain-specific, and global knockout mouse models for PPARγ, and ERα, respectively.

Using the knockout models, we demonstrated that both PPARγ and ERα are necessary for OPFR impact on weight gain and adiposity within adult males. Further, we reported novel effects of OPFR exposure in knockout genotypes, not present within WT animals. Among these are decreased fat mass in both ERαKO and PPARγKO females

and reduced caloric intake, and reduced feeding efficiency in ERαKO and PPARγKO females, respectively. These data indicate catabolic actions of OPFRs on female mice lacking key receptors regulating energy homeostasis. While interesting, it remains difficult to conclude the true mechanistic origins of such observations; however, the results are in alignment with estrogenic endpoints of OPFR exposure, perhaps acting on alternative estrogenic signaling pathways such as ERβ or membrane-bound ERs. Additionally, we find that the loss of PPARγ signaling within the brain confers a genotype effect of eliminating the effect of HFD on glucose and insulin tolerance. This effect is also reported by Lu et al. (2011). PPARγ is expressed in ARC NPY and POMC neurons, and its selective knockout within POMC neurons is reported to improve glucose tolerance (Long et al. 2014). Interestingly, we report that within males, OPFR exposure replicates the effect of HFD on glucose tolerance seen in WT mice, whereas within females, OPFR exposure strikingly reduces insulin tolerance when fed LFD. These findings suggest that OPFRs help restore a sensitivity to diet, eliminating the protective effect that neuronal knockout of PPARγ conferred to glucose homeostasis.

Future research will want to explore these findings in more detail, perhaps through the use of the Cre-lox system to selectively knockout ERα and PPARγ within specific ARC neuronal subpopulations, such as POMC, NPY, and KNDy neurons. Adding a level of specificity will eliminate many variables that confound our current study and help towards identifying the neuronal role in OPFR dysregulation of energy homeostasis.

Because both ERα and PPARγ are expressed in and regulate melanocortin circuitry governing central regulation of energy homeostasis, we also examine herein the effects of adult OPFR exposure on NPY and POMC neuronal excitability. Previous findings implicate the potential for OPFRs to alter hypothalamic activity of a hyperpolarizing K^+ channel known as the M-current (Krumm et al. 2018). The current dissertation supports

these findings, showing that adult exposure to OPFRs diminishes the M-current within NPY neurons from female mice. Additionally, we are the first to report that ghrelin reduces the M-current in NPY neurons, and that OPFR exposure augments the effect of ghrelin to excite NPY neurons within female mice. Interestingly, while we observe a greater direct effect of ghrelin to excite these neurons, we also show that excitatory neuronal input to NPY neurons decreases following ghrelin perfusion in OPFR exposed females. The dichotomy of this observation is perhaps explained through OPFR excitation of another ARC neuronal subtype, KNDy neurons.

KNDy neurons are estrogenically regulated and communicate with NPY and POMC neurons to influence hypothalamic control of energy homeostasis (Backholer et al. 2010; Mittelman-Smith et al. 2012). KNDy neurons are excited by ghrelin, a process that is modulated by E2 (Frazao et al. 2014; Yang et al. 2016; Qiu et al. 2018; Conde and Ropeke 2019). Subsequently, excited KNDy neurons directly stimulate POMC neurons, and indirectly inhibit NPY neurons (Nestor et al. 2016; Qiu et al. 2016; Fu and van den Pol 2010). In this dissertation, we hypothesize that OPFRs are influencing estrogenic regulation of KNDy neurons to alter their response to ghrelin. This could explain how OPFR exposures augments typical excitation of NPY neurons by ghrelin, in addition to dampening excitatory input from other neurons. If OPFRs are exciting KNDy neurons, their increased signaling could decrease excitatory input to NPY neurons. Supporting this theory is our additional findings that POMC neurons from female mice are markedly excited by OPFR exposure. If OPFRs are estrogenically exciting KNDy neurons, our observations may be a product of their resulting excitatory input onto POMC neurons. Regardless of the hypothetical origin, this dissertation outlines an overall increase in ARC neuronal output in female mice, potentially brought upon through estrogenic interactions of OPFRs within KNDy neurons.

While this dissertation highlights striking alterations in melanocortin circuitry activity within female mice, we do not report the same actions of OPFRs within male mice. OPFRs do not appear to affect the M-current in NPY neurons from males, however, we do show that these neurons exhibit a baseline depolarized state, indicating OPFR-increased neuronal excitability through a mechanism alternate to the M-current. We observe no impact of OPFRs on baseline POMC activity, and neither do we report any significant effects of OPFR on leptin sensitivity within the same neurons. Admittedly, due to COVID-19 closures our leptin studies suffer from low experimental power and there may yet be OPFR effects within male POMC neurons. However, what remains evident is that OPFRs demonstrate a clear, sex-dependent ability to alter melanocortin circuitry regulating energy homeostasis.

The neurological disruption brought upon by adult OPFR exposure can be linked with our physiological observations of perturbed metabolic endpoints. Aside from the obvious impact OPFR disruption of NPY and POMC neurons will have on feeding behavior, ARC dysregulation is also associated with peripheral roles in modulating energy expenditure, metabolism, adiposity, and glucose homeostasis. POMC neurons are essential regulators of hepatic glucose production, brought upon by leptin excitation (Caron et al. 2018; Smith et al. 2018). OPFR disruption of typical POMC activity may therefore be impacting glucose homeostasis and altering circulating peripheral signals of energy status such as leptin, insulin, and ghrelin, as seen in Chapter 2. In the same study, we report increased adiposity in male mice. Adipose tissue is an endocrine organ that plays an important role in relaying peripheral signals of energy status to the brain for hypothalamic regulation of energy homeostasis. This connection is bi-directional, and modulated by sympathetic outflow originating from the hypothalamus. As reviewed by Zhang et al (2014), hypothalamic NPY reduces sympathetic nervous system outflow, which promotes adipogenesis and fat accumulation while simultaneously inhibiting

brown adipose tissue deposition associated with the expenditure of energy through thermogenesis. Our observed weight gain and adiposity within OPFR-exposed male mice could therefore be attributed to neuronal origins we describe in Chapter 4.

In conclusion, our studies represent important scientific evidence that adult exposure to OPFRs is capable of impairing homeostatic regulation of energy balance. These studies are the first to demonstrate physiological effects within adult mammalian models and are the first to pair these findings with electrophysiological examination of the neuronal mechanisms behind OPFR disruption. The collective content of this dissertation will provide a necessary foundation for future examination of the lifetime impact of OPFRs on energy homeostasis. Dysregulated energy homeostasis is the first step in acquiring serious health conditions such as metabolic syndrome, obesity, and diabetes. Thus, this dissertation indicates OPFRs as a critical human health concern and outlines the importance of continued research to understand the full scope of their toxicological potential.

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CHAPTER 6: APPENDICIES

6. Appendicies

Appendicies are an additional review of membrane-initiated estrogen signlaning within the central nervous system

6.1 Membrane-initated estrogen signaling via Gq-coupled GPCR in the central nervous system

Abstract

The last few decades have revealed increasing complexity and depth to our knowledge of receptor-mediated estrogen signaling. Nuclear estrogen receptors (ERs) ERα and ERβ remain the fundamental dogma, but developing research targeting membrane-bound ERs urges for a more expanded view on ER signaling. ERα and ERβ are also involved in membrane-delineated signaling alongside membrane-specific G protein-coupled estrogen receptor 1 (GPER1), ER-X, and the Gq-coupled membrane ER (Gq-mER). Membrane ERs are responsible for eliciting rapid responses to estrogen signaling, and their importance has been increasingly indicated in central nervous system (CNS) regulation of such functions as reproduction, energy homeostasis, and stress. While the Gq-mER signaling pathway is well characterized, the receptor structure and gene remains uncharacterized, although it is not similar to the nuclear ERα/β. This review will describe the current knowledge of this putative membrane ER and its selective ligand, STX, from its initial characterization in hypothalamic melanocortin circuitry to recent research exploring its role in the CNS outside of the hypothalamus.

Introduction

Estrogen receptors (ER) ERα and ERβ were initially discovered through their regulation of gene expression via action of their main endogenous estrogen, 17β-estradiol (E2) (Green et al. 1986; Mosselman et al. 1996). Over the past 30 years, ER have been thought to signal primarily through long-term transcriptional regulation of the brain, mediating neuronal circuitry formation during developmental stages, or through targeted activation of gene expression in the adult brain later in life (Arnold 2009). These transcriptional events, often called the "classical" ER signaling pathway, are initiated by the formation of ERα and ERβ steroid binding–dependent hetero- or homodimers. These dimers translocate to the nucleus to bring about transcriptional activation through their interaction with the DNA binding site known as the estrogen response element (ERE) or with other DNA-bound transcription factors such as activator protein 1 (AP-1) and specificity protein-1 (SP-1) (O'Malley and Tsai 1992; Marino et al. 2006; Safe and Kim 2008; Jacobson et al. 2003; Roepke et al. 2011).

However, it has become clear that there also exists a rapid, membrane-initiated, E2-mediated actions independent of the classic nuclear signaling pathway. Even before the discovery of ERα and ERβ, these rapid actions of E2 were observed in the uterus and the hypothalamus, both sites in which it is important for there to be rapid responses to hormonal signaling (Kelly et al. 1977; Kelly et al. 1984; Szego and Davis 1967). Only within the last 15 years have these fast-acting E2 actions been mechanistically investigated and shown to have physiological significance (Kelly and Levin 2001; Mermelstein and Micevych 2008; Qiu et al. 2006b; Vasudevan et al. 2005; Ronnekleiv and Kelly 2005; Lebesgue et al. 2010). Membrane-mediated effects of E2 signaling triggers various intracellular cascade pathways, including protein kinase C (PKC), protein kinase A (PKA), phosphatidylinositol-3 kinase (PI3K), and mitogen-activated protein kinase (MAPK) leading to protein phosphorylation, gene transcription, and regulation of ion

channel and neuronal excitability (Lee et al. 2008; Lagrange et al. 1997; McNatty et al. 1979; Roepke et al. 2009; Zhou et al. 1996).

The focus of this review will be on a specific, putative membrane ER that is a Gq protein-coupled receptor called the Gq-mER, which has been extensively characterized in hypothalamic proopiomelanocortin (POMC) neurons (Qiu et al. 2006b; Qiu et al. 2003; Qiu et al. 2008; Conde et al. 2016). In guinea pig and mouse POMC neurons, E2 attenuates the baclofen response (GABA-B receptor activation) within minutes. This rapid action of E2 is mimicked by E2 conjugated to bovine serum albumin (E2-BSA), indicating a membrane-initiated mechanism of disinhibition, and is blocked by the ER antagonist ICI 182,780, indicating that the signaling is mediated by an ER. Baclofen, as a GABA-B receptor agonist, has an inhibitory effect on neuronal excitability by activating a G-protein inward-rectifying potassium (GIRK) channel; thus, E2's inhibition of the baclofen response reduces the inhibitory GABAergic tone. Furthermore, STX, an agonist for the Gq-mER, can activate the same Gq protein-phospholipase C (PLC)-PKC-PKA pathway activated by E2 (See Figure 1). Together, these results demonstrate that STX as a specific agonist of the Gq-mER capable of eliciting electrophysiological changes in POMC excitability that mimic E2.

Since the characterization of STX in hypothalamic POMC neurons, focus has shifted to other hypothalamic and extrahypothalamic neurons to determine if the E2- and STX-sensitive Gq-mER controls neurological functions elsewhere in the brain and to identify the physiological consequences of the Gq-mER activation. Over the past decade, research has shown direct effects of Gq-mER activation on energy homeostasis (Roepke et al. 2009; Conde et al. 2016; Qiu et al. 2006b; Roepke et al. 2010; Roepke et al. 2008), thermoregulation (Roepke et al. 2010), reproductive behavior (Christensen and Micevych 2013; Micevych and Dewing 2011), regulation of gonadotropin releasing hormone (GnRH) neurons (Kenealy et al. 2011; Kenealy and Terasawa 2011; Zhang et al. 2010), and

corticotropin releasing hormone (CRH) neurons (Hu et al. 2016) as well as other neurological functions in the central nervous system (CNS).

Gq-mER signaling in the hypothalamic melanocortin circuitry

E2 is generated by the ovaries and other peripheral tissues (McNatty et al. 1979) and travels to the hypothalamus to interact with neuronal populations to control homeostatic functions. The primary role of E2 is to control reproduction by controlling output of the gonadotropin releasing hormone (GnRH) neurons in the preoptic area (POA) of the hypothalamus. However, E2 also has a number of secondary functions, most notably in energy homeostasis and temperature regulation {previously reviewed in (Radovick et al. 2012; Mauvais-Jarvis et al. 2013)}. E2 is anorectic, and estrogen deficiency is strongly correlated with decreased energy expenditure and increased weight gain (Asarian and Geary 2002; Clegg et al. 2007; Butera and Czaja 1984). In fact, decreased fertility and altered menstrual cycle patterns are linked with obesity (Johnson et al. 1994; Li et al. 1999), indicating that sex hormones, specifically E2, play a role in energy homeostasis. Decreased levels of E2 are implicated in causing the accumulation of fat in postmenopausal women (Davis et al. 2012). Furthermore, mutations resulting in dysfunctional ERs show distinct patterns of obesity, hyperinsulinemia, and type 2 diabetes in humans (Smith et al. 1994). Increased feeding behaviors and weight gain are also observed in overiectomized rodents, which are reversible with supplementation of E2 to pre-ovariectomy levels (Asarian and Geary 2002; Hong et al. 2009). Likewise in male rodents, a decrease in E2 signaling observed in ERα knockout (KO) rodents results in similar patterns of fat accumulation (Heine et al. 2000; Ribas et al. 2010). However, conditional ERα KO mice that express an ERα lacking a functional DNA-binding (ERE targeting) domain exhibit a phenotype similar to wild-type females (Park et al. 2011). This suggests that substantial nongenomic E2 signaling is important in energy homeostasis.

POMC and neuropeptide Y (NPY) neurons are necessary in mediating the balance between anorectic and orexigenic signaling in energy homeostasis, respectively (Aponte et al. 2011; Luquet et al. 2005). E2 control of energy homeostasis is, in part, a CNS-mediated process (Butera and Czaja 1984; Ahdieh and Wade 1982). Specifically, E2 has important interactions with arcuate POMC and NPY neurons (Pelletier et al. 2007; Roepke 2009). POMC mRNA decreases in postmenopausal women (Abel and Rance 1999), and in studies with ovariectomized rodents, *Pomc* mRNA is increased following E2 treatment, leading to an increase in the expression of the POMC-derived peptide, β-endorphin (Roepke et al. 2008; Thornton et al. 1994). Furthermore, the increase in NPY mRNA and peptide expression in rodents post-ovariectomy is reversed after E2 dosing (Shimizu et al. 1996). These findings indicate that the arcuate POMC and NPY neurons are important targets in the anorectic signaling pathways of E2.

While genomic E2 pathways certainly play a significant part in mediating its anorectic effect, there is mounting evidence supporting the existence and role of the Gq-mER in mediating the response to E2. Genomic pathways typically require a time frame of hours to days for effects to be observed in mammals; however, E2 dosing directly into the third ventricle results in an attenuation of food intake in just 4-6 h in fasted, ovariectomized rodents (Gao et al. 2007; Qiu et al. 2007). This suggests the presence of a fast-acting ER in E2 signaling. The Gq-mER was characterized using both guinea pig and mouse models using whole-cell patch-clamp techniques to record hypothalamic neuronal activity in brain slices (Qiu et al. 2006b; Qiu et al. 2003). These studies found a significant attenuation of the activity of a GABA-B receptor agonist, baclofen, within minutes after E2 perfusion. When administered alone, baclofen-induced activation of the GABA-B receptor initiates a K^+ efflux through the GIRK channel in hypothalamic neurons. In POMC neurons, GABA-B signaling acts to inhibit neuronal excitability, hence the attenuation of the baclofen response following E2 perfusion acts to disinhibit GABA-B tone and increase the

excitability of POMC neurons, potentially augmenting in the anorectic effects of E2. E2-BSA perfusion recapitulates E2's attenuation of the baclofen response (Qiu et al. 2003). In addition, an ER-specific antagonist ICI 182,780 blocks the effects of E2 at subnanomolar affinity (Qiu et al. 2003), suggesting that the receptor is not ERα36, which has been shown to activate ERa36 in some breast cancer cells (Zhang et al. 2012). Furthermore, the estrogenic attenuation of the baclofen response is mimicked by stimulating G protein-coupled downstream signaling (activation of adenylyl cyclase) and by direct activation of PKA. Inhibition of PKC and PKA blocks the Gq-mER pathway (Lagrange et al. 1997; Qiu et al. 2003). Lastly, PI3K has also been indicated in this pathway as inhibitors wortmannin and LY294002 significantly reduced the baclofen response in POMC neurons (see Figure 1) (Malyala et al. 2008).

To selectively target this pathway and its role in neurophysiology, Kelly and colleagues (Qiu et al. 2003) developed a selective Gq-mER agonist called STX. STX is a diphenylacrylamide that is structurally similar to 4-hydroxytamoxifen and does not bind to either ERα or ERβ. STX mimics E2 attenuation of the baclofen response (at ~20x greater potency; STX: EC_{50} = 2.6nM, E2: EC_{50} = 46nM) in wild-type (WT) and ERα or ERβ KO mice and was blocked by ICI 164,384, indicating that STX acts through an ER that is not ERα or ERβ. Lastly, PKA and PKC inhibitors all blocked the attenuation of the baclofen response by STX (Qiu et al. 2006b). Together, these results characterize STX as a specific agonist of a novel Gq-mER capable of mimicking E2 in eliciting electrophysiological changes in POMC excitability.

Orexigenic NPY neurons also demonstrate a rapid response to E2, in part through the actions of a complementary Gq-mER pathway (Smith et al. 2013). NPY neurons will respond to baclofen in the same way that POMC neurons do; however, the effect of E2 on the baclofen response in NPY neurons is variable. Interestingly, some NPY neurons

show an attenuated baclofen response similar to POMC neurons, while other NPY neurons exhibit an opposing, hyperpolarizing effect. When explored further, it was revealed that NPY neurons express a rapid ERα-mediated signaling pathway in addition to the Gq-mER signaling pathway that impinges on GIRK-GABA-B interactions (Smith et al. 2013). Perfusing NPY neurons with propyl pyrazole triol (PPT), a potent ERα agonist, resulted in an attenuation of the baclofen response in ovariectomized mouse brain slices. In contrast to PPT, STX elicited an augmentation of the baclofen response in NPY neurons that is eliminated with co-administration of ICI 164,384 (see Figure 1) (Smith et al. 2013). These results suggest that rapid E2 signaling in NPY neurons has two modes of action through either the membrane-bound ERα or Gq-mER and that the individual NPY neuronal response to E2 signaling, either a suppression or augmentation of GABA-B tone, may be dependent on the relative expression of ERα or Gq-mER, respectively. One possible explanation for this differential response is that Gq-mER is responsible for the control of energy homeostasis, while ERα is involved in a different E2-mediated process such as reproduction or thermoregulation (Smith et al. 2013).

Gq-mER is involved in the hypothalamic control of reproduction

Arcuate POMC neurons are well known for regulating energy homeostasis, but a subpopulation of the cells is also involved in mediating aspects of female sexual behavior. These POMC neurons project to the median preoptic nucleus (MPN) and release the opioid peptide β-endorphin. β-endorphin activates μ-opioid receptors expressed in MPN neurons signaling for inhibition of sexual receptivity (lordosis) (Sanathara et al. 2011; Sinchak et al. 2015). E2 is known to regulate β-endorphin expression and release (Thornton et al. 1994; Sinchak et al. 2015; Sinchak and Micevych 2001; Dewing et al.

2007), and recent research proposes that this effect is also mediated by membrane-initiated estrogen signaling.

The opioid receptor-like (ORL) 1 receptor is a Gi-coupled receptor that is highly expressed in the hypothalamus and in arcuate POMC neurons. Orphanin FQ, also known as nociceptin (OFQ/N), the endogenous ligand for the ORL1 receptor, regulates cell excitability by increasing postsynaptic potassium currents, inhibiting postsynaptic calcium currents, and by diminishing presynaptic neurotransmitter release (Connor et al. 1996; Emmerson and Miller 1999; Wagner et al. 1998; Altier et al. 2006; Werthwein et al. 1999). OFQ/N signaling via ORL-1 receptors inhibits POMC neurons, and Conde *et al.* (2016) hypothesized that E2 regulates POMC excitability by attenuating inhibitory ORL-1 signaling that induces a large outward potassium currents to hyperpolarize and reduce neuronal excitability in POMC neurons. This inhibitory effect is attenuated with pre-treatment with E2, as well as E2-BSA, demonstrating E2's inhibitory effect on ORL-1 signaling. This effect was blocked by ICI 182,780 and mimicked by both PPT and STX. This indicates that the signaling is ER-mediated, initiated at the membrane, and involves ERα and Gq-mER, alone or simultaneously. Furthermore, inhibiting PI3K eliminated the attenuation of ORL-1 induced currents, and inhibitors of PLC, PKC, and PKA blocked the estrogenic effect. In contrast, activation of PLC, PKC, or PKA all recapitulated E2's attenuation of ORL-1 signaling (Conde et al. 2016). These data indicate the role of membrane-bound ERα as well as Gq-mER in attenuating the inhibitory effects of OFQ/N signaling in POMC neurons, which project to the MPN and release β-endorphin to inhibit lordosis.

Gonadotropin-releasing hormone (GnRH) neurons, localized to the preoptic area in the rodent, coordinates and integrates hypothalamic signals to directly regulate reproduction. GnRH neurons secrete GnRH into the capillaries of the hypophyseal portal

system, which transport the neurohormone to the anterior pituitary to stimulate release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the gonadotropic cells. LH and FSH travel to the gonads to control gametogenesis and steroid production (Constantin 2011). E2 and progesterone signal back to the hypothalamus as negative feedback throughout the estrous cycle, thus compromising the hypothalamic-pituitary-gonadal (HPG) axis. During the proestrous stage, elevated E2 produced by the dominant follicles stimulates a positive feedback loop to induce the GnRH and LH surges and initiate ovulation. Steroidal regulation of the GnRH pulse generator is essential for fine-tuning reproductive cyclicity and function (Constantin 2011; Watanabe et al. 2014).

The pulsatile action of GnRH neurons is an innate characteristic, as immortalized GnRH (GT1-7 cells) in culture also show timed bursts of secretion and activity (Funabashi et al. 2001). This is further emphasized in studies wherein ovariectomized females, released from steroidal feedback, exhibit a consistent, basal pulsatile GnRH release approximately every 30-40 min in rodents and up to 1 hour in sheep and monkey models (Krsmanovic et al. 1992; Martinez de la Escalera et al. 1992; Wetsel et al. 1992; Terasawa et al. 1999; Duittoz and Batailler 2000). The exact mechanism that controls this innate pulsatility is not yet known, but calcium signaling seems to be key. Oscillations of intracellular calcium concentrations ($[Ca^{2+}]_i$) correlate with GnRH secretion and other neuronal activity; however, differences are reported depending on the model (Constantin et al. 2010). Overall, the idea is that depolarization of GnRH neurons activates voltage-sensitive, T-type Ca^{2+} channel influx, and the rise in $[Ca^{2+}]_i$ stimulates bursts of GnRH release (Watanabe et al. 2014; Constantin 2011; Constantin et al. 2010; Constantin et al. 2009; Kelly and Wagner 2002). This current is produced by 3 channel subunits encoded by the *Cacna1g* (Cav3.1), *Cacna1h* (Cav3.2), and *Cacna1i* (Cav3.3) genes. All three genes are expressed in GnRH neurons with an expression profile of Cav3.3 > Cav3.2 > Cav3.1 based on single-cell RT-PCR in ovariectomized female mice (Zhang et al. 2009).

Reproduction is often considered a long process, not in need of the fast-acting responses to hormonal control of such behaviors like feeding. However, recent research indicates that despite this nature, the rapid signaling of Gq-mER plays a role. Early studies showed that GnRH neurons do not contain nuclear ERα (Shivers et al. 1983), leading to a hypothesis that estrogenic control over GnRH activity is mediated through presynaptic neurons that do express nuclear ERα and produce kisspeptin and other regulators of GnRH neurons (Kenealy and Terasawa 2011). However, the recent discovery that ERβ is expressed in GnRH neurons (Hrabovszky et al. 2007) and the exploration of membrane-initiated E2 signaling are overturning the old dogma that GnRH neurons do not directly sense circulating E2.

Over the past decade, GnRH neuronal excitability has been demonstrated using calcium imaging and other techniques to show that E2 has both excitatory and inhibitory effects via either direct or indirect (presynaptic) mechanisms (Temple and Wray 2005; Temple et al. 2004; Wintermantel et al. 2006; Abe et al. 2008; Chu et al. 2009; Lagrange et al. 1995). Some of these effects are rapid, membrane-initiated E2 responses potentially through the Gq-mER. E2-BSA was able to mimic an increase in GnRH neuronal activity and synchronization and was reversed by co-perfusion with ICI 182,780 or the pertussis toxin, which blocks G-protein coupled receptors (GPCR), indicating that this effect was produced by a membrane-bound estrogenic GPCR (Temple et al. 2004). Using similar approaches, other studies were able to show varying responses to E2, and the variability in effect indicates that GnRH neurons may have specific subpopulations, depending on their differential expression of ER types (Chu et al. 2009; Lagrange et al. 1995; Temple et al. 2004; Temple and Wray 2005). Expression profiles within the neurons may determine through which receptor signaling pathway (ERβ, GPER, Gq-mER, etc.) E2 regulates GnRH neuronal excitability and synchronization.

The mechanism by which the excitability and synchronization of GnRH neurons is regulated is believed to be through calcium signaling. Influx of Ca^{2+} into the cell via T-type Ca^{2+} ion channels is mediated by E2. E2 regulates expression of T-type channel subtypes (Cav3.1, Cav3.2, and Cav3.3) within the arcuate nucleus and increases peak T-type Ca^{2+} current density in arcuate neurons (Qiu et al. 2006a; Bosch et al. 2009). Regulation of Cav3.1 expression is dependent on ERα, and regulation of Cav3.2 is dependent on ERα and ERβ (Bosch et al. 2009). Furthermore, E2 regulates channel activity and subunit expression in GnRH neurons using patch clamp electrophysiology coupled with single-cell type quantitative real-time PCR from EGFP-tagged GnRH transgenic mice. Interestingly, STX mimicked E2's regulation of subunit expression (an increase in Cav3.3), indicating that the Gq-mER also plays a role in the activity of T-type channels in the hypothalamus in addition to ERα and ERβ (see Figure 1) (Zhang et al. 2009). STX also modulates GnRH neurons from primate models. Treatment with 10 nM STX increased $[Ca^{2+}]_i$ oscillation frequency and synchronized the frequency of these oscillations. STX also increased the percentage of stimulated cells and augmented GnRH release, although at lower magnitude than E2. Additionally, ICI 182,780 and the PLC inhibitor U73122 blocked the STX-induced $[Ca^{2+}]_i$ oscillation, further indicating the role of Gq-mER (Kenealy et al. 2011).

GnRH is a vital hormone in regulating the reproduction cycle and sexual receptivity. Because STX and the Gq-mER pathway were shown to be involved in regulating GnRH neurons, the next step is to determine whether the receptor has a physiological effect on reproduction. E2 is an essential hormone in regulating reproduction, most often acting as negative feedback in the HPG axis but also having rapid, local effects on sexual behaviors. In males, sexual motivation has been shown to be rapidly regulated by E2, in a time period less than most protein production (Shang et al. 2000; Cross and Roselli 1999). This indirectly suggests that E2 is acting via a nongenomic pathway to regulate male sexual

motivation. However, STX was shown to have no effect in both rats and quails (Seredynski and Balthazart 2015; Christensen and Micevych 2013). GPER and ERα are also not involved. This effect on male sexual motivation appears to be mediated primarily through ERβ and its interaction with the metabotropic glutamate receptor 1a (mGluR1a) (Seredynski and Balthazart 2015). In female rats, however, STX stimulated sexual receptivity and induced activation and internalization of μ-opioid receptors in the medial preoptic nucleus, an action necessary for producing lordosis. If an antagonist to mGLuR1a was pre-administered, internalization of μ-opioid receptors were not seen (Christensen and Micevych 2013; Micevych and Dewing 2011). This suggests that the putative Gq-mER interacts with mGluR1a to rapidly activate cellular signaling to augment lordosis behavior.

Gq-mER activity in corticotropin-releasing hormone and extrahypothalamic neurons

In a recent study from our laboratory (Hu et al. 2016), activation of the Gq-mER in paraventricular corticotropin-releasing hormone (CRH) neurons increased neuronal excitability in female mice. CRH neurons play a key role in the hypothalamus-pituitary-adrenal (HPA) axis, which mediates hormonal adrenal action, and E2 directly modulate these neurons (Vamvakopoulos and Chrousos 1993; Roy et al. 1999). Through use of whole-cell electrophysiology, Hu *et al.* (2016) found a rapid attenuation of the M-current (a voltage-dependent, inwardly rectifying K^+ current) in CRH neurons after perfusion of exogenous E2. The M-current is a constitutively active hyperpolarizing current that suppresses neuronal excitability, so inhibition of the M-current by E2 leads to an excitation of CRH neurons. Co-administration of E2 with inhibitors of the Gq-PLC-PKC-PKA signaling pathway blocked E2's attenuation of the M-current. Furthermore, a selective ER

inhibitor also blocked inhibition, and STX mimicked the actions of E2 (see Figure 1). This study additionally examined the *in vivo* effects of Gq-mER signaling by injecting ovariectomized mice with E2 or STX, which elicited an increase in c-fos mRNA expression in CRH neurons and a corresponding rise in plasma corticosterone. This evidence indicates that Gq-mER signaling is involved in the regulation of CRH neurons and the HPA axis and may participate in E2's observed effect on HPA-associated mood disorders (Young and Korszun 2002; da Silva et al. 2014).

Sex differences in pain perception and analgesic drug efficacy are present in both animal models and humans and in the prevalence of pain-related diseases such as fibromyalgia, migraines, and arthritis (Unruh 1996; Fillingim et al. 2009; Greenspan et al. 2007; Yunus 2002; van Vollenhoven 2009; Peterlin et al. 2011). The mechanistic nature of these differences is not well known, but estrogens may be an important factor. E2 attenuate GPCR-mediated antinociception (Nag and Mokha 2004; Claiborne et al. 2006). Recent evidence indicates that fast-acting, membrane-bound receptors such as the Gq-mER may contribute to this effect (Nag and Mokha 2014). Nag *et al.* (2014) found that STX rapidly attenuated antinociception induced by the α2-adrenoceptor agonist, clonidine, as measured by the tail flick test in ovariectomized female rats in a dose-dependent manner. STX and other drugs were intrathecally administered 5 min prior to clonidine, and the resulting decrease in latency observed with STX treatment indicates an inhibition of antinociception or an increase in pain perception. The ER antagonist ICI 182,780 blocked this effect and E2-BSA mimicked the effects of STX. Furthermore, STX increased spinal cord levels of phosphorylated extracellular signal regulated kinase (ERK), and *in vivo* inhibition of ERK phosphorylation with U0126 blocked the attenuation effect of STX on antinociception. PKA and PKC phosphorylation were altered by STX, indicating

that ERK is the primary mediator of the STX-initiated, Gq-mER signaling cascade that inhibits the antinociceptive actions of clonidine.

Accumulating evidence has shown that estrogens have important beneficial effects in the aging body that protect against cardiac incidents, ischemic injury recovery, and neurodegeneration (Green and Simpkins 2000; Brann et al. 2007; DonCarlos et al. 2009). Consequently, this lead to the idea of using synthetic estrogens as a hormone replacement therapy for postmenopausal women, who experience a decline in estrogen production. However, there is considerable controversy about the use of hormone replacement therapy as prolonged exposure to estrogens increases risk of breast cancer and thrombosis (Wu 2005; Wren 2009). Lebesgue *et al.* (2010) hypothesized that non-classical estrogenic ligands, such as STX and the GPER selective agonist G1, might exhibit these neuroprotective effects of E2, while reducing or eliminating the deleterious side-effects of synthetic estrogens that target nuclear ER. In that study, middle-aged rats were subjected to 10 min of global ischemia followed immediately by reperfusion eight weeks after ovariectomy. Rats were immediately injected with either E2, G1, or STX directly into the lateral ventricle. Hippocampal neuronal survivability was assessed one week later. All estrogenic ligands were neuroprotective (55-60% survivability vs. 15% in the controls). A single systemic injection of E2 was also shown to be protective (~50%). This work suggests that activation of estrogen-responsive GPCR (using STX or G1) might be a useful replacement for standard hormone therapy and reduce the susceptibility to stroke in postmenopausal women.

In a recent paper by Gray *et al.* (2016), the neuroprotective effects of STX were examined in the context of amyloid β (Aβ) toxicity in neuroblastoma cell lines as well as primary hippocampal neurons from wild-type and Alzheimer model Tg2576 mice (Gray et al. 2016). STX reduced cell death, mitochondrial dysfunction, dendritic simplification, and

synaptic loss from levels seen in control Aβ-exposed cells. In primary neurons, STX also increased ATP as well as mitochondrial gene expression in both genotypes. This paper indicated that STX can be neuroprotective against Aβ toxicity and may also prove to be useful outside this Alzheimer model, as protective effects were reported in the absence of Aβ. Supporting these data are reports of Gq-mER-initiated PKA, ERK, and PI3K signal manipulations increasing mitochondrial activity and protecting against the impaired bioenergetic states caused by Aβ toxicity (see Figure 1) (Sarkar et al. 2015; Zhao et al. 2014)

Lastly, cortical neurons in primary culture from rat pups sorted by sex, 5-min E2 pretreatment protected against glutamate toxicity 24 h later in neurons from females, but from males (Bryant and Dorsa 2010). ERα and ERβ were expressed in these cultures and the ERα-selective agonist PPT replicated these effects while ERα antagonist methyl piperidino pyrazole (MPP) blocked them. The ERβ selective agonist diarylpropiolnitrile (DPN) exhibited a small protective effect in both female- and male-derived neurons. Membrane-delineated receptor mechanisms were also tested via STX and G1 administration. STX was neuroprotective against glutamate toxicity in both female- and male-derived cortical neurons, while G1 had no significant effect. Interestingly, E2-BSA was also shown to not have an effect. These results indicate that the sexually dimorphic neuroprotective effect by estrogenic compounds is primarily mediated by ERα, and not by other ER receptors (ERβ or GPER), while the Gq-mER is neuroprotective against glutamate toxicity in both female- and male-derived cortical neurons.

Conclusions

Research continues to accumulate supporting the significance of membrane-initiated estrogen signaling, and it is becoming increasingly evident that these receptors play more of a role than previously assumed. Also, their involvement in neurophysiology is complex and interlaced with "classical" ERα/β nuclear-initiated and membrane-initiated signaling. As the compound becomes more readily available, STX is proving to be an essential tool for analyzing how the Gq-mER modulates the well-characterized melanocortin pathway, as well as extra-hypothalamic neurons. While the effects of STX and Gq-mER activation are easily examined, the structure and gene sequence of the putative Gq-mER are still unknown. Therefore, receptor identification is of paramount importance as it will provide another tool with which to explore the expanding topic of rapid estrogen signaling.

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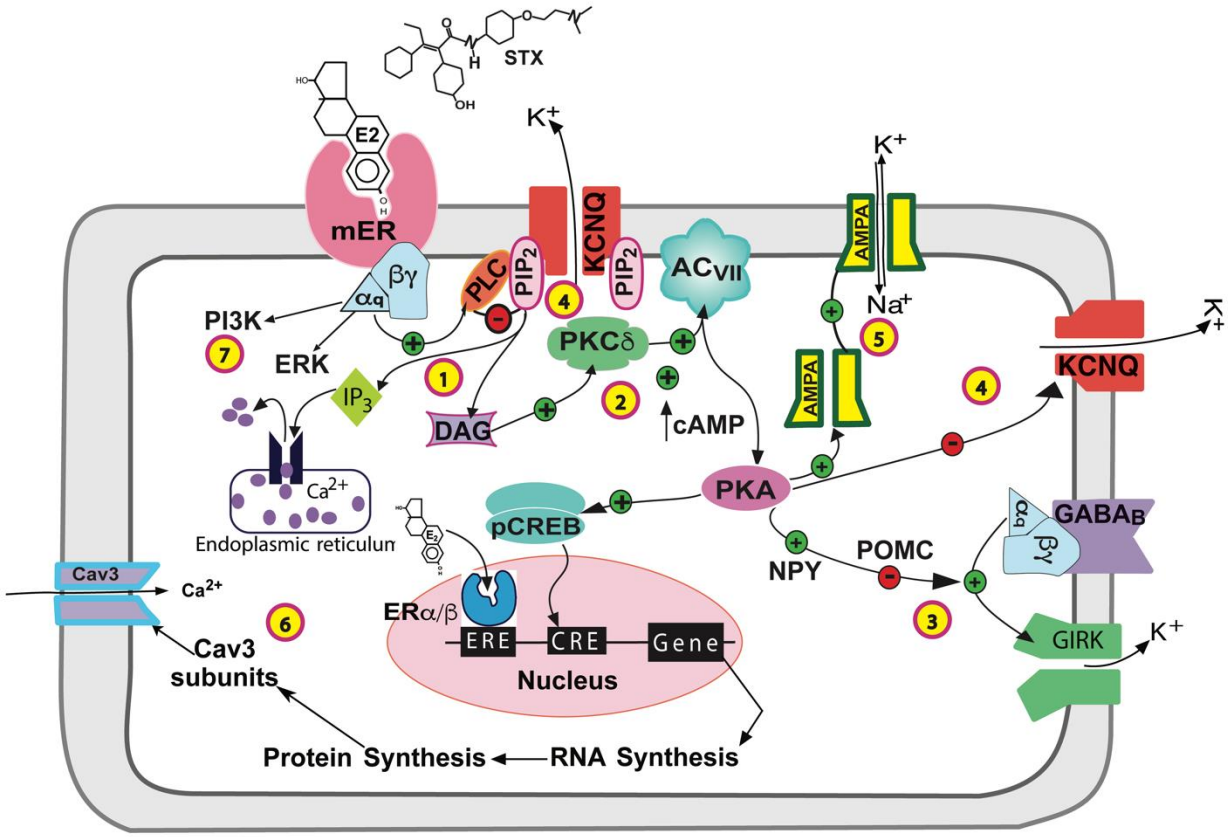
Figures

Figure 1



A model cell illustrating the Gq-mER signaling pathway in hypothalamic neurons. (1) E2 (or STX) activates a membrane-associated ER (mER) that is Gq-coupled. The G□q protein activates PLC to catalyze the hydrolysis of membrane-bound PIP_2 into IP_3 and DAG. (2) DAG activates PKC□ to augment adenylyl cyclase (AC_{VII}) activity and generate cAMP, which in turn activates PKA. (3) In POMC neurons, activation of PKA attenuates the GABA-B receptor-mediated activation of GIRK channels, while in NPY neurons, PKA activation potentiates GIRK channel activity. (4) In CRH neurons, PKA phosphorylates KCNQ subunits to inhibit the M-current, which may also be inhibited by the hydrolysis of PIP_2 in the membrane into free IP_3 and DAG. (5) PKA activation may also lead to the phosphorylation of AMPAR subunits augmenting membrane trafficking and recruitment to rapidly increase the amplitude of AMPAR currents in CRH neurons. (6) STX also increases the expression of Cav3 (T-type Ca^{2+}) channels in GnRH neurons, presumably through the PKA-mediated phosphorylation of pCREB, leading to gene regulation through the cAMP response element (CRE). (7) Activation of the Gq-mER has also been associated with PI3K and ERK signaling pathways in hypothalamic and hippocampal neurons.