CONSEQUENCES OF DEVELOPMENTAL AND ADULT NEUROENDOCRINE DISRUPTION TO THE HYPOTHALAMUS AND PITUITARY

BY

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DISSERTATION

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ABSTRACT

The endocrine system is a complex, interconnected web of communication that uses hormones as chemical messengers to allow distinct and geographically distant organs to coordinate physiological homeostasis. The hypothalamus and pituitary are the master regulators of this process. Healthy physiology relies on the hypothalamus and pituitary to respond appropriately to signals communicated by other endocrine organs. When external stimuli influence the development or behavior of neuroendocrine tissue, it can lead to deleterious health consequences. Often, associations between environmental exposures and negative health outcomes are reported in epidemiological data while the question of how they are causally connected remains unclear. This is the case with the two exposures on which this work expands: gestational diabetes mellitus and iodoacetic acid. Using *in vivo* and *in vitro* models, we observed the impact these exposures have on the hypothalamus and pituitary to better understand how they lead to endocrine disruption.

Gestational diabetes mellitus (GDM), a transient diabetes during pregnancy, has frequently been linked to the development of obesity in offspring, beginning in childhood and continuing into adulthood. The hypothalamus is the master regulator of energy homeostasis and despite the opportunity for an *in utero* exposure to alter the development of this region, relatively little is known about if or how it does so. The arcuate nucleus (ARC) balances feeding behavior through two competing neuron populations that either promote hunger or satiety. The ARC is bordered by a pool of

nutritionally active, stem-like progenitors called tanycytes that can influence the cellular make-up of the ARC as well as mediate the cues it sees from circulation. Additionally, the ARC lies just superior to the median eminence (ME), a region with a leaky blood brain barrier that acts as a conduit for peripheral cues about energy status. We hypothesized that GDM would influence the cellular composition and function of the ARC, ME, and/or tanycytes, potentially driving altered nutritional responsiveness in offspring. Using a genetic model of gestational diabetes, we found a reduction in the number of cells that proliferated during the onset of GDM in the ME of offspring. We also found that post-natal basal insulin signaling is elevated in the β2-tanycytes of offspring. Through a tanycytic neurosphere culture paradigm, we observed that excess insulin, a characteristic of the GDM growth environment, has a trophic effect on cells. We do not see this effect *in vivo*. This indicates that GDM's influence is likely multifactorial and ultimately not primarily driven by hyperphysiological insulin levels. Our data together reveal that GDM's influence on nutritional communication tools, rather than altered stem cell proliferation, is a key driver of hypothalamic consequence. These developmental alterations may underlie high-weight phenotype in offspring.

Turning our attention to another timepoint and exposure source, we observe the consequences of adult exposure to the water disinfection byproduct (DBP), iodoacetic acid (IAA). DBPs have been linked to a range of health consequences including increased risk of cancer, birth defects, and reproductive disruption. IAA has high formation potential, created as a byproduct of a reaction between an oxidizing disinfectant used to treat water and iodide present in the water. In previous studies, IAA

has been shown to have significant cyto- and genotoxic capabilities, as well as to induce mRNA expression of genes related to cell cycle arrest and promoting apoptosis. IAA also disrupts ovarian follicle development and estradiol synthesis *in vitro* and estradiol serum levels *in vivo*. These data suggest IAA acts as toxicant with reproductive-axis disrupting potential. Yet, previously virtually nothing was known about how IAA effects the hypothalamus and pituitary, which guide reproduction through the hypothalamic-pituitary-gonadal axis. We hypothesized that IAA exposure disrupts expression of key neuroendocrine factors and directly induce cell damage in the mouse pituitary. Using an *in vivo* exposure experiment, we found that IAA significantly elevated mRNA levels of kisspeptin (*Kiss1*) in the arcuate nucleus of the hypothalamus, while not affecting *Kiss1* in the anteroventral periventricular nucleus. It also reduced follicle-stimulating hormone (FSHβ)-positive cell number and *Fshb* mRNA expression, though not luteinizing hormone (LHβ/*Lhb*) expression. Evidence for FSHβ/*Fshb* disruption was substantiated by pituitary explant experiments which revealed the same effect and suggested that IAA acts on the pituitary directly. IAA also introduced toxicity in the pituitary, inducing DNA damage and P21/*Cdkn1a* expression *in vitro* and DNA damage and *Cdkn1a* expression *in vivo.* These data implicate IAA as a reproductive neuroendocrine disrupter with toxic capabilities in the pituitary and add weight to the call for IAA to be better studied and regulated as a potential public health concern.

With this understanding of adult exposure risks, we wanted to determine how a developmental exposure to IAA could affect the pituitary. Prior data indicating cytotoxicity, DNA damage, and cell cycle arrest-related mRNA expression in work from

other groups, combined with similar findings from our adult study, suggest IAA may be especially detrimental for developing, proliferative tissue. Prior work from our lab suggests that the pituitary is particularly vulnerable to disruption by endocrine disrupting chemicals during development. If IAA were to induce similar effects, it may permanently alter pituitary regulation of the endocrine system. As such, we wanted to survey the major hormone-building mRNA of the pituitary in this context, as well as observe its toxic effects. Until the current research, no prior data had been collected on early life exposures to IAA in the pituitary, nor, to the best of our knowledge, any tissue. Using an *in vivo* IAA exposure from conception through weaning, we tested the hypothesis that in the pituitary, developmental exposure to IAA would result in upregulation of the cell-cycle arrest gene *Cdkn1a*, suppression of the proliferative marker *Mki67*, and shifts in hormone-building mRNA. Surprisingly, we found no changes to *Ckdn1a* or to *Mki67* suggesting IAA may not be a significant threat to pituitary cellular health developmentally. We also performed qPCR for the major hormone-building mRNAs expressed by the pituitary, finding a significant increase in thyroid stimulating hormone beta subunit (*Tshb*) and a trend towards increased proopiomelanocortin (*Pomc*), though no other significant effects. Together with our findings from our adult IAA exposure study, these data suggest that IAA may be a more notable threat to adult pituitaries, though further experiments observing different pituitary functions and whole axes is warranted.

The work presented in this document explores the previously under-examined topics of hypothalamic consequences of the gestational diabetes growth environment and

neuroendocrine disruption by the water disinfection byproduct iodoacetic acid. Through these studies, we reveal the hypothalamus and pituitary to be vulnerable to environmental exposures in a way that could undermine healthy physiology.

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CHAPTER 1: Introduction

Neuroendocrine Disruption: A Necessary Lens for Understanding the Interaction Between Physiology and the Environment

In a lab setting in which we endeavor to control for as many variables as possible, it is easy to overlook the importance that the environment holds. However, in nature, bodies are constantly integrating internal and external stimuli to alter gene and protein expression and respond to those cues.

The endocrine system is a particularly interesting context in which to consider these interactions. Put very basically, the endocrine system uses hormones secreted and communicated between and within structures to allow disparate and anatomically distant organs to coordinate with each other to maintain physiological homeostasis. Hormones are messengers within the body, acting as ligands to receptors on the membranes or in the nucleus of target tissue's cells. Their binding activates signaling cascades to regulate the expression of genes and proteins in those target cells. These communications are crucial for healthy body function and for appropriate responsiveness to the outside world. Notably, endocrine axes are complex, each organ constantly responding to the others. As such, anything that could alter the function of one structure or the hormone it produces could have effects on every other component of the axis. Exogenous factors such as exposure to chemicals in the environment, altered nutritional exposure, or other environmental contexts can and do affect these

interactions, including through shifting production and binding of hormones to their receptors. Therefore, the endocrine system represents an important lens for understanding how the environment influences biology. Endocrine-disrupting external factors have the potential to irreparably alter the behavior of the organ. If the homeostatic setpoint is shifted, either by long term disruption in gene expression or cell viability, or through acting during a developmental critical period, it could permanently disrupt what the body views as “normal” and lead to suboptimal function. When these forces are exerted in neuroendocrine structures – the hypothalamus of the brain and the pituitary – the disruption can be especially influential. The hypothalamus and pituitary act as the master regulators of the endocrine system with a common “information flow” involving hormones released from the hypothalamus being communicated to the pituitary which releases its own hormones that act on other organs. It is therefore especially valuable and important to look at how the neuroendocrine system can be influenced by specific environmental factors.

In the following sections, I will expand on the question of how external stimuli can influence hormonal systems, focusing on the significance of the hypothalamus and pituitary and their roles in guiding physiology. Using two specific exposures, gestational diabetes mellitus and iodoacetic acid, and two different time points, development and adulthood, we explore the manifestations of neuroendocrine disruption in an effort to elucidate how they may lead to deleterious health outcomes.

Gestational Diabetes Mellitus: A Maternal Endocrine Context for the Developmental Origins of Health and Disease Hypothesis

Developmental Origins of Health and Disease

During pre-natal life, organisms are rapidly developing, laying down the framework that will guide how their bodies functions throughout the lifetime. The central and autonomic nervous, immune, cardiovascular, and neuroendocrine systems all have critical windows during this stage (van den Bergh, 2011). Increasingly, researchers point to associations between gestational and perinatal exposures and long-term health outcomes for the offspring in childhood and into adulthood. This connection was most prominently first put forth by D.J.P. Barker in a 1990 article synthesizing his prior work on the association between birth weight and childhood nutrition and adult cardiovascular health (Barker, 1990; Barker et al., 1989, 1990; Barker and Osmond, 1986). Since termed the Developmental Origins of Health and Disease (DOHaD) hypothesis, it has grown in popularity as a framework for understanding the lasting consequences of early life exposures. These questions are especially interesting in the context of the brain, in which the majority of the cells and intercellular connections are formed in early development and external factors acting during these windows may irreversibly alter outcomes. Within the brain the hypothalamus is especially compelling; altering its development could affect the offspring's ability to regulate physiological function, leading to permanent dysfunction.

Maternal Nutrition

As patterns in offspring outcomes are observed epidemiologically, one association that is frequently noted involves maternal nutrition. Both maternal under- and over-nutrition have been indicated as causal factors in determining offspring health later in life. Studies on the Dutch Famine draw associations between maternal malnutrition and the development of coronary artery disease, hypertension, obesity, pulmonary disease, renal disease, type 2 diabetes, depression, schizophrenia, and antisocial personality disorder in offspring (Osborne-Majnik et al., 2013). Other studies on maternal under-nutrition found similar relationships with the etiology of heart disease, obesity, and diabetes, as well as contributions to the development of osteoporosis (Gluckman et al., 2008). Children born premature, whether born of an appropriate weight or of a low weight for gestational age, are more likely to be insulin insensitive in childhood (Hofman et al., 2004) Interestingly, maternal over-nutrition carries similar risks. Maternal high fat diet has been linked to developmental heart defects and heart disease, for example (Elahi and Matata, 2017; Xue et al., 2019). Further, in a study of Australian rural women, maternal BMI, excess gestational weight gain, and hyperglycemia were all independently correlated with neonatal adiposity (Longmore et al., 2019). Maternal obesity often leads offspring born large for gestational age and with high adiposity (Ross and Desai, 2013). Macrosomic babies are at increased risk of childhood and adult obesity, coronary heart disease, hypertension, and type 2 diabetes mellitus (Elahi et al., 2009). In normal weights, as well as extreme weight, there is a continuous relationship between birth weight and future risk of developing these conditions

(Gluckman et al., 2008). Looking at these associations paints the picture that maternal nutrition can exert long-term consequences. This leads to question of how and why these consequences could occur. These outcomes converge on systems with pre-natal critical periods and in particular on endocrine and neurological health. As such, abnormal maternal growth environment could be considered a developmental endocrine disrupter with implications for neural development and long-term health consequences.

Gestational Diabetes Mellitus and the Obesity Phenotype

Gestational diabetes mellitus (GDM) is a commonly-occurring maternal context resulting in offspring over-nourishment. Briefly, this condition arises when a mother is unable to clear the glucose from her system, exposing the offspring to high glucose levels. During normal pregnancy, the mother develops insulin resistance while simultaneously undergoing beta-cell hyperplasia in the pancreas to increase insulin production (Catalano et al., 1999; Sonagra et al., 2014). This balance is thought to function to ensure the mother's body does not absorb excess glucose, depriving the baby of sufficient levels needed to grow, while working to ensure she also has enough glucose. GDM arises when the balance is disrupted and insulin resistance overwhelms pancreatic capacity (Buchanan et al., 2007). As a result, the baby is exposed to more glucose than it would in healthy conditions and, since the baby normatively has a functioning insulin system, its body increases insulin production. This high insulin/high glucose hormonal context characterizes the GDM growth environment.

GDM is relatively common, affecting an estimated 16% of pregnancies worldwide (Cho et al., 2018), with frequencies varying drastically for different populations and reaching as high as 36% for some communities (Anand et al., 2017). This is cause for concern; the consequences to offspring of GDM pregnancies are similar to those of maternal malnutrition mentioned previously, in particular, being born large for gestational age and developing obesity in childhood and later life (Boney et al., 2005; Catalano et al., 2012; Ross and Desai, 2013). The World Health Organization reports increases in obesity in the general population, as well as increases in rates of childhood obesity (WHO Obesity and overweight fact sheet, 2021; Abarca-Gómez et al., 2017). GDM may be contributing to such trends. High glucose levels *in utero* are significantly associated with childhood glucose levels and insulin resistance, independent of reason for exposure, BMI, and family history of diabetes (Scholtens et al., 2019). This suggests that maternal high glucose can induce diabetes-like symptoms, even without typically associated causes like genetic pre-disposition or high-weight. Further, obesity predisposes the individual to the development of metabolic syndrome - a cluster of abnormalities including insulin resistance, elevated triglyceride levels, hypertension, and atherosclerosis. GDM offspring tend to develop these conditions earlier than their peers as well, perhaps owing to a longer period of having obesity (Ross and Desai, 2013). GDM has also been shown to exacerbate maternal immune activation (MIA) in the developing brain (Money et al., 2018), and elevated immune response has been associated with a range of offspring outcomes, particularly in the development of neurological, mostly psychiatric, disorders through the lifetime (Estes and McAllister, 2016). This fact lends credence to

a connection between the disrupted physiological state of GDM and potential changes in the brain.

Despite the evident association and reasonable cause for concern, how GDM contributes to obesity in offspring is poorly understood. As previously mentioned, the neuroendocrine system is a valuable candidate for study for its vulnerability to perturbation and ability to guide long-term physiology and dictate health outcomes over the lifetime and, as such warrants further study.

The Hypothalamic Feeding Network: Normal Function and Potential Sources of Endocrine Disruption

The induction and cessation of feeding behavior is a complex process involving the integration of myriad signals from the body along with intercommunication within the brain. The primary seat of this integration is in the arcuate nucleus (ARC) of the hypothalamus, which contains two key neuronal populations responsible for balancing energy homeostasis. The first of these is of anorexigenic POMC neurons, which promote satiation via the release of POMC, a peptide pro-hormone, which is processed into α-melanocyte-stimulating hormone (αMSH) and activates melanocortin-4 receptors (MC4R) (Luquet et al., 2005). The other is of orexigenic NPY/AgRP neurons, which stimulate feeding through secretion of neuropeptide Y and agouti gene–related peptide (Morley et al., 1984). Agouti gene–related peptide in particular inhibits MC4R, decreasing satiation. This inhibition is reciprocated; when MC4R is activated AgRP is

inhibited (Yang et al., 2011). While activating these neuronal populations can inhibit one another, it has been shown that activation of NPY/AgRP neurons alone is sufficient to induce hunger (Cansell et al., 2012). The neurons in the ARC communicate also with other brain regions such as the ventral tegmental area (VTA), allowing reward circuitry to participate in feeding and vice versa (Wu et al., 2012). Similarly, connections to the brainstem allow for conveyance of levels of important gut hormones like cholecystokinin (CCK), ghrelin, peptide YY (PYY), and glucagon-like peptides 1/2 (GLP-1/2) (Cohen et al., 2003; Gibbs et al., 1973; Klockars et al., 2018).

The most direct mediation of feeding occurs directly in the ARC itself where its close proximity to the median eminence (ME) and third ventricle allow exposure to nutritional factors in circulation through the blood and cerebral spinal fluid, respectively. The ME sits at the base of the hypothalamus and serves as the communication point between the hypothalamus and pituitary. Due to a “leaky” blood brain barrier, the hypothalamus can receive peripheral signals from the rest of the body (Rizzoti and Lovell-Badge, 2017). Two of the most important factors for ARC response to energy needs are insulin and leptin. Secreted by the pancreas in response to an increase in glucose, insulin’s primary role is to facilitate for the uptake and processing of the glucose. It travels to the brain where it accesses the hypothalamus through the ME and acts on widely expressed insulin receptors (Bruning et al., 2000). Leptin is secreted by adipocytes, making it an important factor for communicating fat stores and energy availability. Leptin receptors are similarly widely expressed in the brain, with the highest density found in the hypothalamus (Bouret, 2010; Caron et al., 2010). Leptin and insulin resistance are

correlated with obesity and both activate POMC neurons and inhibit NPY/AgRP neurons (Lee and Blackshaw, 2014).

Importantly, insulin and leptin could influence neural development, making them relevant for understanding how a hormonally altered growth environment can lead to long-term dysregulation. Insulin receptor signaling has been shown *in vitro* to regulate dendritic spine morphogenesis (Choi et al., 2005) and neurite outgrowth (Govind et al., 2001), to be neuroprotective (Mielke et al., 2006; Aftab et al., 2017), and to act as a trophic factor (Aizenman and de Vellis, 1987). Further, insulin's neurotrophic influence has been illustrated specifically in hypothalamic stem cells (Desai et al., 2011). *In vivo*, insulin receptor density decreases drastically between the embryonic period and adulthood in some areas of the brain (Schulingkamp et al., 2000) and insulin receptor substrate-2 deficiency leads to reduced neural proliferation in the developing brain (Schubert et al., 2003). These data point to an influential role for insulin action during neural development and may indicate that a heightened level, as is likely in GDM, could influence how the hypothalamus develops. While it is possible that it would have a trophic effect in this region, the onset of GDM is late in gestation, past the height of the neurogenic window. More likely, these data are evidence that altered insulin levels could affect how the neurons themselves develop and whether they survive and form appropriate connections and are appropriately insulin-sensitive.

Similarly, leptin has been shown to play a role in early development. Leptin transport across the blood brain barrier appears to be developmentally regulated (Bouret, 2008).

Seminal studies in mice with leptin receptor mutations indicated they have a reduction in cell density in the hypothalamus in addition to other regions, along with changes in dendrite orientation and decreased dendrite density in the ARC (Bereiter and Jeanrenaud, 1979; Bereiter and Jeanrenaud, 1980; Bouret et al., 2004). When treated with leptin in the neonatal window, affected mice could recover normal connectivity patterns, but could not do so if treated in adulthood (Bouret et al., 2004). This illustrates how even after peak neurogenesis, leptin is able to influence ARC function, however plasticity is still limited to early life. Importantly, this evidence illustrates the significance of both insulin and leptin in early development and highlight how hormonal disruption can alter how the hypothalamus is able to respond to those and other hormones by altering the structure of the region itself.

Another cellular developmental source of disruption could be in effects on tanycytes. The hypothalamus is uniquely equipped to respond to the needs of the body due to these cells. Tanycytes are specialized radial glial cells, which serve as a source of post-natal stem cells that give rise to NPY and POMC neurons, in addition to glial cells (Lee et al., 2012; Rizzoti and Lovell-Badge, 2017). This neurogenic capability contrasts the lack of postnatal neurogenesis in most other brain regions. Tanycytes originate from the hypothalamic ventricular zone (HVZ) and project into the median eminence, allowing them to be particularly sensitive to changes in the circulatory environment, including being diet-responsive (Lee et al., 2012; Bolborea and Dale, 2013; Langlet, 2014; Goodman and Hajihosseini, 2015). Further, tanycytes arise late in gestation, coincident with GDM onset. A nutritionally abnormal growth environment may affect how these

cells develop, altering how the ARC could respond to hormonal signals and nutritional status.

Models of Gestational Diabetes Mellitus

One challenge in pursuing the question of how gestational diabetes could alter brain development lies in developing effective rodent models of GDM. An ideal model would display simultaneous insulin resistance and pancreatic beta-cell hyperplasia and develop glucose intolerance late in gestation. The model should also produce offspring born large for gestational age and predisposed to early postnatal and adulthood obesity. There are currently two primary categories of models most commonly used: diet-based and genetic/chemical-alteration. The former consists of feeding dams a high-fat and/or high-fructose diet for a period leading up to gestation. This model relies on work demonstrating that a high fat diet provided for a short period before mating could lead to the development of glucose intolerance whereas the same feeding paradigm would not induce intolerance in non-pregnant females (Holemans et al., 2004; Liang et al., 2010). The major downside of this model is that it does not always lead to offspring born large for gestational age, which is especially important when the focus of the study is on the etiology of the higher-weight phenotype. An example genetic model uses heterozygous leptin receptor (*Lepr*) mutant mice (*+/db*). The homozygotes for this mutation are diabetic in all contexts and are infertile, but the heterozygotes develop glucose intolerance under the stress of pregnancy, similar the human condition. This model has been shown to lead to macrosomic offspring, along with maternal glucose intolerance

and insulin resistance (Kaufmann et al., 1981; Yamashita et al., 2001). The major downside of this model is that it does not develop GDM in every lab who uses them (Plows et al., 2017; Pollock et al., 2015), making reproducibility a concern. In an example chemical model, dams are dosed with streptozotocin (STZ), which selectively kills beta-cells leading to an inability to secrete insulin to the necessary extent. This method reliably and directly accounts for pancreatic contributions to GDM etiology, providing a benefit over other methods where insulin homeostasis is disrupted incidentally. However, there is variability in effectiveness influenced by strain, age, and dose, resulting in no consensus for best protocol. Further, it has a notable weakness in that, since it affects insulin synthesis with no inherent insulin resistance, the resulting phenotype is more similar to a non-pregnant type 1 diabetic state than to a GDM state. Additionally, STZ exposure late in gestation risks acting on embryos directly (Pasek and Gannon, 2013).

These factors make studying gestational diabetes in rodent systems difficult and, at present, limited. Even so, they are the best option currently available to pursue mechanisms of GDM. Carefully choosing a model based on study requirements is necessary.

Closing a Missing Link in Understanding Gestational Diabetes Mellitus Outcomes

GDM provides an interesting context in which to consider the DOHaD hypothesis as applied to endocrine disruption. In this case, an external (to the fetus) environment –

maternal nutritional milieu – affects a hormonal state to induce changes to long term physiological health and energy homeostasis. This association needs be pursued in order to potentially address the resulting detriment to public health. In exploring this connection, the ARC of the hypothalamus, which acts as the master control for energy homeostasis is a prime candidate for developmental alteration by the abnormal hormonal growth context and as such it should be a focal topic of study.

The Influence of Iodoacetic Acid on Neuroendocrine Health with a Focus on Hypothalamic-Pituitary-Gonadal Axis Disruption

Endocrine Disrupting Chemicals in the Neuroendocrine System

As the world has become more industrialized, the presence of synthetic chemicals has necessitated a closer look at how those chemicals affect the body. Observations of their deleterious effects has led the to the growth of the field of environmental toxicology in which many of these toxicants, deemed endocrine disrupting chemicals (EDCs), have been found to act as endocrine mimics or blockers, ultimately causing physiological dysfunction by interfering with normal endocrine function. EDCs can have a range of effects in the body essentially limited only by their capacity to imitate or alter the actions of an endogenous hormonal factor. For this reason, there is important work on EDCs being carried out in virtually every sector of biology.

As previously discussed, the endocrine system, broadly, is extremely interdependent and effects in one region often have consequences in a related region. This makes the neuroendocrine system and its role in governing so many different axes an important one to study when concerned with EDCs. Not only should the hypothalamus and pituitary be studied as responders to feedback caused by direct action on an endocrine organ, but they themselves are susceptible to influence by EDCs. As an example, bisphenol-A (BPA) is among the best-studied EDCs and can be illustrative of hypothalamic-pituitary-gonadal (HPG) axis disruption, particularly due to its known estrogenic capacity (Gore et al., 2015). Early life exposure (gestation through 5 weeks) to BPA leads to increased mRNA expression of key reproduction-related genes gonadotropin-releasing hormone (*Gnrh1)* and kisspeptin (*Kiss1)* mRNA in the hypothalamus and *Fshb* mRNA in the pituitary of adult mice (Xi et al., 2011). Perinatal exposure alone leads to significant increases in anteroventral periventricular zone (AVPV) kisspeptin neuron number in male rats (Bai et al., 2011) and rostral periventricular nucleus kisspeptin-immunoreactive neurons in female mice (Naulé et al., 2014). In culture, BPA has been shown to reduce estrogen responsiveness in pituitary cells (Jeng et al., 2010). Data from our lab also support the association between BPA and pituitary function, illustrating that gestational exposure increases proliferation, likely of gonadotrophs, and either increases or decreases gonadotropin-releasing hormone receptor mRNA levels depending on dose (Brannick et al., 2012). Independent of specific reproductive effects, we also that BPA exposure can alter mRNA expression of the important developmental genes *Pit1* (Eckstrum et al., 2018) and *Icam5* (Eckstrum et al., 2016). Our findings on EDCs in the pituitary extend beyond BPA as well. We have

found that environmental botanical estrogens isoliquiritigenin and genistein, have the ability to alter cell-cycle gene and protein expression (Weis and Raetzman, 2016, 2019), displaying how exogenous hormone-like factors can influence pituitary function. These data make a strong argument for hypothalamic and pituitary vulnerability to endocrine disruption and for the HPG as a prime target.

While the current body of evidence is strong, an understanding of EDC effects in neuroendocrine tissue is noticeably lagging behind that of other tissues and in many, cases adult exposures are under-observed. Additionally, there are myriad toxicants with apparent epidemiological consequence that are yet to be examined in any tissue, at any timepoint. Pursuing questions of environmental toxicology, especially in the neuroendocrine system is a matter of public health, especially if exposure is difficult to avoid and governing bodies are ill-equipped to regulate these chemicals. Understanding their biological effects is the major tool public health advocates have to argue for such regulations.

Water Disinfection Byproducts: Friends or Foe?

The irony of many environmental toxicants is that they were often introduced as the solution to a problem, such as the need to make plastics more durable or to add fragrance to a cosmetic, however they end up having negative effects more concerning than the compounds are necessary. Water disinfection byproducts (DBPs) heighten that conundrum. Forms of water treatment have existed for thousands of years; the

connection between contaminated water and disease is easily observable. At present, most industrialized water treatment systems involve a multistep process of filtration and chemical treatment. The major downside of this is the unintended formation of water disinfection byproducts, which can occur when a disinfectant comes into contact with an organic or inorganic compound present in water. DBPs have been associated with a range of alarming consequences, though the field lacks real consensus when it comes to a nuanced and causal understanding of physiological outcomes. In epidemiological studies, DBPs correlated with an increased risk of bladder and colon cancer (Rahman et al., 2010; Villanueva et al., 2004), reproductive dysfunction (Villanueva et al., 2015), congenital anomalies (Nieuwenhuijsen et al., 2000; Narotsky et al., 2011), and first trimester *in utero* exposure, limb and bone defects (Kaufman et al., 2020). A number of *in vitro* studies lend some insight into how DBPs act on the cells, revealing they can be cyto- and genotoxic, in addition to teratogenic (Dad et al., 2013; Pals et al., 2013). These data necessitate further exploration into the consequences of DBP exposure. It is vitally important that they are further studied in order to best enable individuals, medical professionals, and regulators to make informed decisions about how to avoid or mediate any deleterious effects.

IAA as an Endocrine Disruptor

Iodoacetic acid (IAA) is formed when an oxidizing disinfectant such as chlorine comes into contact with iodide present in the water. In *in vitro* studies, it has consistently been shown to be one of the most cyto- and genotoxic DBPs (Dad et al., 2013; Plewa et al.,

2004). Building evidence suggests that it could be of particular concern for endocrine systems. *In vivo* exposure leads to disruption in multiple factors of the hypothalamic-pituitary-thyroid axis including by decreasing mRNA and protein expression of thyroid stimulating hormone receptor and sodium iodide symporter, and by decreasing T3 thyroid hormone levels (Xia et al., 2018). Some of the strongest evidence in endocrine tissues comes from reproductive toxicology. In *in vitro* studies establishing IAA toxicity, exposures were carried out in Chinese Hamster Ovary cells (Plewa et al., 2004; Dad et al., 2013). In *in vitro* murine ovarian follicles, IAA can disrupt folliculogenesis and ovarian estradiol synthesis (Jeong et al., 2016 and Gonsioroski et al., 2020). *In vivo*, IAA can cause reduced estradiol serum levels (Gonsioroski et al., 2021), and decrease ovarian weight (Xia et al., 2018). IAA has also been identified as having potential estrogenic and androgenic activity (Kim et al., 2020; Long et al., 2021). Not only does IAA carry the blunter, whole-cell implications such as killing cells or damaging DNA as seen in foundational *in vitro* studies, but it can have these more dynamic, subtler, but no less significant, effects on mRNA expression, hormone production, and tissue development in endocrine systems. The epidemiological data associating DBPs with reproductive disfunction further point to this potential (Villanueva et al., 2015). Together, these data characterize IAA as an endocrine disrupting chemical.

Until the present work, no studies focused on the brain and the pituitary, representing a significant gap in the literature. The neuroendocrine system is a vital component of endocrine axis regulation that has thus far been ignored. Alterations at any of the axis levels could result in consequences for another. Established data on the ovaries is

missing a key component: how could those changes affect the hypothalamus and pituitary and how could hypothalamic and pituitary exposures effect those tissue?

While there many contexts to explore when it comes to complications that may arise from IAA's possible neuroendocrine influences, the reproductive system, in particular, is an important initial focus. According to the National Center for Health Statistics, two of the three primary statistics typically used for reporting fertility data reached an all-time low in 2017 (Hamilton and Ventura, 2006; Hamilton et al., 2017). Many social factors such as delayed marriage and age of first pregnancy may contribute to this outcome (Mathews and Hamilton, 2000; U.S. Census Bureau, 2018), however, physiological infertility or subfertility may also be a contributor. Between 2015 and 2017,12.6% of women in the U.S, reported impaired fecundity (National Center for Health Statistics, 2017). Given this, it is worth understanding how reproductively-disruptive EDCs like IAA contribute, especially as many people are unaware that they may be exposed to chemicals that contribute to that infertility.

Neuroendocrine Control of Reproduction

To explore the mechanisms by which IAA could be affecting reproduction, it is important to consider how the hypothalamus regulates reproduction. Reproduction is broadly guided by two neuron types, those that release kisspeptin and those that release gonadotropin-releasing hormone (GnRH). Briefly, kisspeptin is released to act on GnRH neurons which release GnRH into the median eminence of the hypothalamus where it

travels to the pituitary to act on gonadotropes and stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Despite the fact that the direct action on the pituitary comes from GnRH release, kisspeptin neurons are the key sites for feedback regulation from the ovary. One of the primary mechanisms of ovarian hormone feedback is through estrogen release. GnRH neurons generally lack estrogen receptor-α (ERα), which is critical for regulatory feedback (Christian et al., 2008), however kisspeptin neurons do express ERα and are responsive to estrogen (Fergani and Navarro, 2017; Wang and Moenter, 2020). Kisspeptin neurons reside in two populations: those in the arcuate nucleus (ARC) that co-express the auto-regulatory neuropeptides Neurokinin B-Dynorphin, leading them to often be referred to as KNDy neuron (Navarro et al., 2009), and those in the anteroventral periventricular nucleus (AVPV). During non-ovulatory phases of the estrous cycle, estrogen acts on ARC kisspeptin neurons to inhibit kisspeptin (KISS1) release. Kisspeptin has a stimulatory effect on GnRH through which they maintain a low-level pulsatile release of GnRH. As ovarian follicles develop and secrete increasing levels of estrogen, there is a flipping point in which estrogen is sufficiently high and begins to instead act on AVPV kisspeptin neurons where it has a stimulatory effect, inducing a GnRH surge. This, in turn, causes a surge of LH from the pituitary, ultimately inducing ovulation. So, when considering how hypothalamic regulation could be disrupted, there are several candidates including through altering KISS1 release from the ARC, auto-regulation of KNDy neurons through neurokinin-B or dynorphin, release of KISS1 from the AVPV, or GnRH release.

Similarly, in the pituitary, there are several key factors on which to focus for their influence on the reproductive axis. Gonadotropes secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These hormones are released into circulation where they act on the ovaries to induce folliculogenesis and ovulation, respectively. Both LH and FSH share a common alpha subunit, so synthesis of their respective beta units determines relative levels of each hormone (Thompson and Kaiser, 2014). Beta-subunit synthesis is regulated by GnRH acting through the GnRH receptor, with one of the major factors in determining preference for one subunit over the other being pulse frequency. LH synthesis is favored by quick pulse frequencies (greater than one pulse per hour) and FSH synthesis is favored by slow pulse frequencies (less than 1 pulse every 2-3 hours) (Tsutsumi and Webster, 2009). However, pulse frequency is not the only factor in regulating these hormones, especially for FSH. Activins, secreted in the ovaries or pituitary, inhibins, secreted by ovarian granulosa cells, and follistatins, which are ubiquitously expressed including in ovaries and gonadotropes, have all been shown to influence FSH synthesis (Bernard et al., 2010). In this context, activins act to stimulate FSH synthesis and inhibins and follistatins to inhibit it. All taken together, if IAA were to be affecting pituitary control of reproduction, it could occur through a number of mechanisms, indirectly, through ovarian hormone feedback or hypothalamic hormone release, or directly at the level of the pituitary.

IAA as a Developmental Toxicant in the Pituitary

Prior to the presented work, virtually nothing was known about how IAA exerts an effect during developmental exposure, including in neuroendocrine tissue. As previously discussed, pioneering studies of IAA's consequences revealed it to be a cyto- and genotoxicant. Oocyte culture experiments have substantiated IAA's DNA damaging effects (Jiao et al., 2021) and studies in ovaries reveal exposure leads to an increase in pro-apoptotic and a decrease in anti-apoptotic mRNA expression (Gonsioroski et al., 2020; Gonsioroski et al., 2021). These data suggest IAA could be especially detrimental to developing tissue, as during this period the organs are growing rapidly and healthy cell division is necessary. Previous studies from our lab have illustrated pituitary vulnerability to developmental EDC exposure that can alter proliferation and mRNA expression of key developmental genes (Brannick et al., 2012; Eckstrum et al., 2016; Eckstrum et al., 2018), providing a foundation on which to research IAA as a developmental toxicant. Since IAA is carried in public water, observing how offspring of these women react to IAA exposure could inform risk in a vulnerable physiological stage of life.

The Need for a Greater Understanding of IAA Influence

With so little currently known about its influence, the room for exploration on IAA's consequences is great. Not only is this a valuable question of biology, but it's an important one for public health. Exposure to EDCs is generally unwitting with the

majority of the public left uninformed about risk. This is especially true when it comes to DBPs where many people would be unlikely to suspect their water could carry harmful chemicals that are not due to improper treatment, but rather due to the treatment itself. The most important tool scientists have to mediate the harm of these chemicals is to better understand them and inform the public about risks. Such an imperative necessitates further study of IAA.

Conclusions

In the following chapters, we will examine the question of how external factors influence hormonal contexts that can lead to deleterious health effects. This will focus on two time points and two contexts: high-glucose maternal growth environment and gestational diabetes mellitus and its effects on the arcuate nucleus of the hypothalamus (chapter 2), early adult exposure to the water disinfection byproduct iodoacetic acid (IAA) and its ability to disrupt neuroendocrine control of reproduction (chapter 3), and developmental exposure to IAA and its effects on the pituitary (chapter 4). These focuses serve as important examples for informing an understanding of how the environment interacts with hormonal systems to guide physiology and how these factors can be disrupted.

Chapter 2: Gestational Diabetes Mellitus Alters The Development Of Tanycytes And The Median Eminence In The Offspring

ABSTRACT

Gestational diabetes mellitus (GDM) poses a risk for children's health. In particular, offspring born to GDM mothers are predisposed to obesity over their lifetimes. The hypothalamus is essential for metabolic regulation and, despite the potential for *in utero* alteration, the influence of GDM on hypothalamic development has been under-explored. The hypothalamic arcuate nucleus (ARC) contains neurons that titrate feeding. It is bordered by nutritionally-active tanycytes that also act as a neurogenic pool populating the ARC. Further, the ARC sits superior to the median eminence (ME), a conduit between the circulatory system and the ARC. Pregnancy is also an immune activated state with GDM shown to exacerbate the condition. We hypothesize that GDM alters cell number and physiological function in the arcuate nucleus, tanycyte population, or median eminence and leads to increased microglial activity. Using a *Lepr*$^{+/db}$ model of GDM and hypothalamic neurosphere culture, we find reduced proliferation in the ME and increased basal insulin signaling in the β2-tanycytes of offspring as well as increased proliferation with insulin exposure in culture. These findings suggest GDM does not induce ARC or tanycyte proliferation due to excess insulin, but may impair hypothalamic sensitivity to nutritional signals in offspring.

INTRODUCTION

Gestational context plays a critical role in the development of a fetus; maternal glucose milieu and transfer is a major influence on successful fetal growth (Baeyens et al., 2016). Yet, in approximately 7% of pregnancies in the US (Pesak and Gannon, 2013) and 14% of pregnancies worldwide (Cho et al., 2018), the mother develops a condition of excess glucose – gestational diabetes mellitus (GDM). Greater maternal glucose causes the fetus' functioning pancreas to produce increased insulin to compensate (Herrera and Desoye, 2016; Castillo-Castrejon Powell, 2017). This altered nutritional environment poses a risk to the offspring. Offspring from GDM pregnancies tend to be born large for gestational age and to develop obesity in childhood, often extending into adulthood. This puts them at increased and earlier risk of developing metabolic syndrome and type 2 diabetes (Clausen et al., 2008; Clausen et al., 2009; Lowe et al., 2018; Deierlein et al., 2011; Ardıc¸ et al., 2020).

While the association is well-reported, it remains unclear what drives it. Rodent studies reveal that GDM offspring display the high-weight phenotype characteristic of the human condition, as well as higher fasting blood glucose levels in the early postnatal period and as adults, and significantly higher serum insulin and serum leptin levels, blunted leptin exposure effect, and insulin resistance as adults (Steculorum and Bouret, 2011; Schellong et al., 2019; Agarwal et al., 2019; Zhu et al., 2019). Offspring of GDM mothers also respond worse to a challenge of a high fat/high sucrose (HFHS) diet as adults, gaining more weight compared those born to lean mothers and showing

dysregulated leptin response including a higher leptin/adiponectin ratio (Talton et al., 2019), and displaying greater glucose intolerance (Agarwal et al., 2019). These data support a causal connection between GDM and dysregulated energy homeostasis in offspring, yet the mechanism is still largely unknown.

In order to interrogate this association, we focused on the development of the hypothalamus. While many there are likely many contributing factors, the hypothalamus is a prime subject of focus. It is the brain region that acts as master regulator of homeostasis in the body (Brooks, 1988). Within the hypothalamus, the arcuate nucleus (ARC) controls energy homeostasis, promoting feeding through the action of NPY/AgRP neurons and satiation through POMC/αMSH neurons (Cone, et al., 2002). The ARC surrounds the 3rd ventricle, which is lined with specialized radial glia cells with stem capabilities called tanycytes. Tanycytes play an important role in feeding behavior, acting as gatekeepers for nutritional signals as well as the source of postnatal neurogenesis in the hypothalamus, one of the few regions capable of undergoing adult neurogenesis (Lee, et al., 2012; Rizzoti and Lovell-Badge, 2017). GDM has its highest prevalence late in gestation – the third trimester for humans and around e16.5 for mice (Sonagra et al., 2014). While the canonical neurogenic period in mice lasts from approximately e12 through e17, tanycytes arise late in gestation, around day e17 in mice (Goodman and Hajihosseini, 2015). Given the timing, GDM could alter ARC neuronal populations through tanycytic neurogenesis. Further, while most of the brain is limited in exposure to factors circulating in blood due to the blood brain barrier (BBB), the ARC lies on the median eminence (ME). The ME has fenestrated capillaries through

which cells that project into the ME, including neurons and tanycytes, receive signals from the rest of the body (Goodman and Hajihosseini, 2015). Tanycytes have been shown to be diet responsive, guiding neurogenesis to regulate NPY and POMC population composition and altering their barrier, nutrient transport, and signaling functions enabling a more attuned response to nutrient levels in the body (Lee, et al., 2012; Bolborea and Dale, 2013; Langlet, 2014). These factors make the tanycytes and ME region a strong candidate for mediating the observed association between GDM and offspring outcomes.

Another consideration may be immune activation. Pregnancy is typically characterized by heighted immune activation and GDM has been shown to exacerbate it further (Ranheim et al., 2004; Morisset et al., 2011; Xu et al., 2014). The brain has dynamic microglial populations that can respond to challenges such as tissue damages or inflammation (Li and Barres, 2018) and microglia have been shown play an important role in supporting the development of the brain (Tay et al., 2017; Thion and Garel, 2017). If GDM were altering the number or activation state microglia, it may have a substantial impact on the health of the tissue potentially underlying reduced responsiveness to stimuli.

Considering these factors, we hypothesize that being born to a mother with GDM alters the development of the ARC, tanycyte population and the ME, including by altering cell number, insulin responsiveness, and microglial profile. In the present study, to test this, we utilized a mutant mouse model of GDM, *Lepr*$^{+/db}$, in which dams are heterozygous

for a leptin receptor mutation and develop GDM under the stress of pregnancy. This allows for a temporally and physiologically similar context as is observed in humans (Yamashita et al., 2001). Additionally, we utilized a hypothalamic neurosphere culture as an *in vitro* context. We used these tools to assess how GDM could influence proliferation, cell death, microglial number and activation state, and insulin signaling.

METHODS

Mice

For in vivo experiments, BKS.Cg-Dock7m +/+ Leprdb/J/ (*Lepr*$^{+/db}$) and C57BLKs/J/ (*Lepr*$^{+/+}$) mice were purchased from Jackson Laboratories and bred in house. For neurosphere culture experiments, CD-1 mice were bred from lines originally obtained from Charles River Laboratories (Charles River, CA). Mice were provided ad libitum access to Teklad 8664 rodent diet (Envigo) and water. Breeding colonies were maintained with a 12-h light/dark cycle at University of Illinois at Urbana Champaign (UIUC). All protocols were approved by the UIUC Institutional Animal Care and Use Committee.

Glucose tolerance testing

Glucose tolerance tests were performed on embryonic day 16.5 (e16.5) or e17.5. Pregnant mice were fasted for 6 hours prior to testing. Dams were then given 2g/kg

body weight glucose by intraperitoneal injection (i.p.). Blood samples were taken via tail nick and glucose levels were measured 0, 15, 30, and 60 minutes after injection using a glucose meter (Aviva Accu-Chek). Glucose concentration for *Lepr*$^{+/+}$ and *Lepr*$^{+/db}$ dams was plotted and area under the curve was calculated.

BrdU injections for cell birth-dating

Lepr$^{+/+}$ or *Lepr*$^{+/db}$ females were bred to C57BLKs/J/. Plugs were checked every morning at 0900 h. 1200 h on the day plugging was observed was considered e.5. Pregnant dams were given intraperitoneal injections of 5-Bromo-2-deoxyuridine (BrdU) (Sigma-Aldrich) at 0.1 mg/g body weight. Injections were given twice daily on e16.5, 17.5, 18.5 at 0830 h and 1730 h. Pups were collected for histological analysis on their day of birth (p0).

Tissue collection for in vivo experiments

Lepr$^{+/+}$ heads from offspring born to *Lepr*$^{+/+}$ or *Lepr*$^{+/db}$ mothers were collected on p0, skin removed and fixed overnight at 4 °C in a 3.7% formaldehyde solution (Sigma-Aldrich) diluted in phosphate-buffered saline (PBS). Tissue was dehydrated by graded washes in ethanols, followed by methyl salicylate, before embedding in paraffin wax. 6µm coronal sections were collected. Two sequential sections were mounted on each charged slide and stored for histological analysis. Two consecutive sections were mounted per slide. For immunohistochemistry, 3 slides, separated by roughly 50-60µm

spanning roughly 120-140μm of the ARC, were stained. The third ventricle was used as a marker for the median eminence, which was considered to be the region immediately inferior to the hypothalamic ventricular zone. For the purposes of counting, the ARC was constrained to a triangular region 485μm left-lateral to the third ventricle and 410μm superior to the inferior border of the third ventricle.

Hypothalamic slice culture and insulin exposure at p9

Pups born to *Lepr*$^{+/+}$ or *Lepr*$^{+/db}$ mothers were sacrificed at p9, and 2 mm coronal brain slices containing the arcuate nucleus (ARC) were generated using a neonatal brain matrix (Zivic Instruments). Brain slices were then incubated in DMEM/F12 (Gibco) for 1 hour at 37°C and exposed to vehicle or to 4μg/mL insulin for 10 minutes. Slices were fixed for 30 minutes in 4% paraformaldehyde and cryoprotected overnight in 30% sucrose/phosphate-buffered saline (PBS) prior to cryosectioning at 10μm for IHC.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Biehl and Raetzman, 2015). *Lepr*$^{+/+}$ heads from offspring born to *Lepr*$^{+/+}$ or *Lepr*$^{+/db}$ dams were collected on p0 and embedded in paraffin wax. 6 μm coronal sections were collected and mounted to slides. Sections were deparaffinized and boiled for 10 min (BrdU, Ki67, and Iba-1 experiments) or 5 min (pAKT experiment) in 10 μM citrate buffer (pH 6.0) for antigen retrieval. All slides were blocked for 1 hours in 5% normal donkey serum (Jackson

ImmunoResearch) diluted in a block containing 3% bovine serum albumen (Jackson ImmunoResearch), and 0.5% Triton-X100 in PBS. For Iba-1 experiments, 20min peroxide block step (1.5% hydrogen peroxide in PBS) preceded this block. Slides were blocked incubated in primary antibody (anti-BrdU, BD Biosciences, 1:150 dilution; anti-Ki67, BD Biosciences, 1:1000 dilution; anti-Iba-1, 1:1000 dilution, FUJIFILM Wako Pure Chemical Corporation, courtesy of Dr. Rodney Johnson; anti-pAKT 1:500 dilution, Cell Signaling) overnight at 4 °C. For all but pAKT IHCs, all slides were incubated for 1hr in a either mouse (BrdU, Ki67, 1:200) or rabbit (Iba-1, 1:300) biotin secondary. Anti-pAKT IHC slides were incubated for 1hr in rabbit-Cy3 secondary. For BrdU and Ki67 IHCs, all slides then underwent a 1hr incubation strep-Cy3 (1:200). Iba-1 IHC slides underwent streptavidin-HRP amplification using the Vectastain Elite ABC kit (Vector) and visualization by DAB staining. All slides were mounted with media containing 4′,6-diamidino-2-phenylindole (DAPI, 1:1000 dilution, Sigma-Aldrich), and visualized using a Leica DM2560 microscope. Images were taken using a Retiga 2000R color camera with Q-Capture software (Q-Imaging) and processed using Adobe Photoshop. pAKT signal intensity was quantified by ImageJ software (NIH).

TUNEL assay

TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) was used to observe cell death using an *in situ* cell death detection kit (Roche, Indianapolis IN, USA) according to the manufacturer's protocol. Briefly, slides were deparaffinized, incubated in 346nM proteinase k diluted in 10mM Tris, and incubated in TUNEL

reaction mixture at 37°C for 1hr. Slides were mounted with media containing 4′,6-diamidino-2-phenylindole (DAPI, 1:1000 dilution, Sigma-Aldrich), and visualized using a Leica DM2560 microscope. Images were taken using a Retiga 2000R color camera with Q-Capture software (Q-Imaging) and processed using Adobe Photoshop.

Quantification of cells for BrdU, Ki67, IBA-1 and TUNEL assays

The ARC, median eminence (ME) and ventricular zone were defined by standard mouse anatomical co-ordinates (Paxinos and Franklin, fourth edition). For immunohistochemistry, the number of BrdU, Ki67, Iba-1, and TUNEL positive cells were counted in 3 slides containing 2 sections each for each region. Quantified slides spanned approximately 120-140μm of the ARC. For BrdU, Ki67, and TUNEL stains the fraction of positive cells equaled the number of stained cells divided by DAPI stained nuclei. For Iba-1 stains, the total number of positive cells was reported instead of a fraction and morphology was characterized visually. Individual sections of the same slide were averaged and sexes were pooled.

Hypothalamic neurosphere culture and quantification

Hypothalamic stem cells were cultured according to a procedure adapted from Desai et al., 2011. Briefly, 2mm-diameter hypothalamic punches were isolated from 1-day-old CD-1 mice. 12 mice were pooled for each culture. The cells were washed in PBS with 4000 ng/mL added insulin and transferred to a growth media containing a base medium

of DMEM/F12 with 20ng/mL Human FGFbasic (R&D Systems), 20ng/mL Mouse EGF (R&D Systems), 100x Antibiotic-Antimycotic (Invitrogen), 2% B27 Supplement (Life Technologies), 2.92 µg/mL L-Glut (Fisher Scientific). The cells were transferred to a T25 flask and incubated in a humidified CO2 incubator for 3 days. After 3 days, the contents of the flask were transferred to a T75 flask with new growth media and expanded for 3 days. The process was repeated two more times for a total of 12 days in culture before plating. 600,000 cells from the flask were added to 9 wells of a 12 well plate for each condition. After plating in insulin-free media, 40ng/mL insulin was added to the Low Insulin condition and 4000ng/mL insulin was added to the High Insulin condition. The concentration of insulin in the High Insulin condition was chosen as it is the level normally present in the media supplement established for this culture. Three images were taken per well. Number and diameter of spheres was assessed using ImageJ software for each image (NIH). Values from the same well were averaged together. Statistics were determined by normalizing the number of cells in each size bin.

RNA Extraction and cDNA Synthesis

Three wells of neurospheres were aggregated and RNA extraction was performed using TRIzol reagent according to the manufacturer's instructions (Life Technologies). Neurospeheres were broken up by pipetting up and down in TRIzol. RNA yield and purity were determined by spectrophotometry. Using the ProtoScript M-MuLV First Strand cDNA synthesis kit (New England BioLabs, MA, USA), approximately 0.5µg of

RNA was synthesized into cDNA. A no-enzyme sample was included as a negative control for each set of synthesis reactions.

RT-qPCR for *Mki67*

RT-qPCR was performed on the cDNA samples using *Mki67* primers with sequences as follows: forward (5' to 3') – *AGTAAGTGTGCCTGCCCGAC; reverse (3' to 5')* – *ACTCATCTGCTGCTGCTTCTCC.* Data were analyzed using the double change in threshold cycle (ΔΔCT) method as previously described (Goldberg et al., 2011). Samples were normalized to *Ppia*. *Ppia* primer sequences were: forward (5' to 3') – *CAAATGCTGGACCAAACACAAACG* and reverse (3' to 5') - *GTTCATGCCTTCTTTCACCTTCC*. Student's two-tailed *t*-tests were performed to ensure there were no significant changes in *Ppia* between dosage groups, thus verifying it could suitably be used as a control gene.

Statistics

Data are represented as mean +/- SEM. Statistical significance was determined by student's *t*-test using GraphPad Prism 8.43.

RESULTS

Lepr$^{+/db}$ dams develop glucose intolerance late in gestation and produce heavier offspring than *Lepr*$^{+/+}$ dams

To confirm our model develops GDM, pregnant *Lepr*$^{+/+}$ and *Lepr*$^{+/db}$ dams were given a glucose tolerance test (GTT) on either e16.5 or e17.5. *Lepr*$^{+/db}$ dams had significantly higher blood glucose levels 30 and 60 minutes following an injection of glucose compared to wild type dams (fig. 2.1A). Their overall reduced capacity to clear blood glucose was confirmed by calculating the area under the curve of the graph of each group's glucose levels over time (fig. 2.1B). Offspring born to *Lepr*$^{+/+}$ and *Lepr*$^{+/db}$ mothers were weighed at birth and at 5 and 10 days postnatal. Offspring born to *Lepr*$^{+/db}$ mothers were significantly heavier at each time point than those born to *Lepr*$^{+/+}$ mothers (fig. 2.1C). This confirms that, in our model, offspring are born large for gestational age and maintain a higher weight in early postnatal life.

The GDM growth environment led to a reduction in newly born and/or slowly proliferating cells in the median eminence of offspring during the onset of GDM.

To observe the effects of GDM on the energy homeostasis region of the hypothalamus of the offspring, we examined cell genesis. By injecting dams with BrdU after the onset of GDM and immunostaining for BrdU using IHC on the day of birth (fig. 2.2A), we were able to observe the number of cells that were either slowly proliferating or became

postmitotic in the period shortly following injection. We observed BrdU immunostaining in the ME as the ME is an important conduit through which signals regarding circulating nutrients in blood are communicated to the ARC. There was an apparent reduction in the number of positive cells in the ME of offspring born to *Lepr*$^{+/db}$ mothers compared to those born to *Lepr*$^{+/+}$ mothers (fig. 2.2B, C). In observing the BrdU-positive cells in the ARC, a region containing energy-homeostasis neurons, we observed no significant difference between *Lepr*$^{+/db}$ and *Lepr*$^{+/+}$ offspring (fig. 2.2B, C). In order to observe consequences to tanycyte proliferation, we quantified BrdU-positive cells along the hypothalamic ventricular zone (HVZ). Since tanycytes are believed to have different roles based on their subtype, with β2 being a primary source of neurogenesis, and the subtypes can be defined geographically, we split our analysis into a α1, α2, and β1-containing region and a β2-containing region. Further distinction between α1, α2, and β1 regions was not feasible due to lack of morphological indicators. There seemed to be no obvious differences between *Lepr*$^{+/db}$ offspring and *Lepr*$^{+/+}$ offspring in either grouping (fig. 2.2D, E). For all regions, we quantified positive cells to verify the apparent results of our immunohistochemistry. The ME of offspring of *Lepr*$^{+/db}$ mothers contained significantly fewer positive cells compared to offspring of *Lepr*$^{+/+}$ mothers (fig. 2.2F). There was no significant difference in the number of positive cells in the ARC (fig. 2.2G). There was similarly no significant difference in quantification of the tanycyte populations (fig. 2.2H, I).

GDM did not lead to changes in postnatal cell proliferation nor changes in postnatal apoptosis.

To determine if the proliferative effects seen in the ME persisted through birth and if there were any effects on proliferation in the tanycytes, we performed an IHC for Ki67, a proliferative marker, in tissue collected from post-natal day 1 mice. We saw no apparent differences in the ME between the offspring born to *Lepr*$^{+/db}$ mothers and those born to *Lepr*$^{+/+}$ mothers (fig. 2.3A, B). We quantified this, confirming there were no differences between groups (fig. 2.3G). We also observed positive cells in the HVZ, where the tanycytes reside. We found no apparent difference in number between positive cells in the offspring of *Lepr*$^{+/db}$ *and Lepr*$^{+/+}$(fig. 2.3C, D) mothers, quantified in (fig. 2.3H). As both leptin and insulin have previously been shown to have protective or population-maintaining effects in neural tissue (Bereiter and Jeanrenaud, 1979; Bereiter and Jeanrenaud, 1980; Mielke et al., 2006; Aftab et al., 2017), and we could anticipate that high leptin and insulin levels may be present in the GDM context, we wanted to observe apoptosis in the ME where we had seen apparent proliferative effects in our BrdU experiment. We used terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining to detect apoptotic cells in the offspring of *Lepr*$^{+/db}$ *and Lepr*$^{+/+}$ mothers (fig. 2.3E, F). When quantified, we found no difference in TUNEL staining (fig. 2.3I).

The GDM growth environment increased baseline insulin signaling while leaving the ability to induce insulin signaling following insulin administration intact.

As previously discussed, tanycytes are important metabolic sensors and there is evidence to support their role in insulin sensitivity. The high-glucose GDM environment could train this physiological role to react in a dysregulated manner after birth. Using hypothalamic slices cultured from p9 offspring of *Lepr*$^{+/+}$ and *Lepr*$^{+/db}$ mothers, we looked at basal levels of phospho-AKT, a component of the insulin signaling pathway. The *Lepr*$^{+/db}$ offspring hypothalamic slices expressed a significantly higher basal level of pAKT than in slices from *Lepr*$^{+/+}$ offspring (fig. 2.4A, B, C). In both groups, staining was confined to the β2 tanycytes and ME. Following an insulin bath, both *Lepr*$^{+/+}$ and *Lepr*$^{+/db}$ offspring expressed insulin induced pAKT at similar levels (fig. 2.2D, E and F). Insulin signaling was still largely confined to the tanycytes and ME. Insulin signaling was still largely confined to the tanycytes and ME.

GDM did not increase microglia presence nor change activation state

Since GDM is associated with immune activation in mothers, we wanted to determine if there was a difference in microglia number and activation state in the median eminence of the experimental and control groups. Microglia morphology generally relates to its activity state, with longer, thinner projections aiding in the tissue-sensing resting state of quiescent microglia. Activated microglia are categorized by larger cell bodies and denser, shorter projections (Fernández-Arjona et al., 2017). Iba-1

immunohistochemistry, which shows microglial morphology, appeared unchanged between experimental and control conditions (fig. 2.5A, B). We quantified positive staining by total number of microglia and activation state. For this analysis, we grouped the two morphologies considered most active, termed ‘active’ and ‘stout processes’ into a general ‘activated’ category. We found no difference between offspring of *Lepr*$^{+/db}$ mothers and *Lepr*$^{+/+}$ mothers in total number of microglia, nor activated microglia (fig. 2.5C).

Hypothalamic progenitor cells exposed to insulin proliferate more and create more neurospheres than those exposed to lower levels.

Prior data on hypothalamic stem cells suggest insulin could act as a trophic factor (Desai and Ross, 2011). In order to observe these effects, we utilized a neurosphere culture to grow hypothalamic stem cells. These cultures are thought to primarily consist of tanycytes and are characterized by the formation non-adherent clusters of proliferative cells. Neurospheres grown from hypothalamic punches were exposed to 40 ng/mL or 4000 ng/mL insulin (fig. 2.6A, B). Counting the neurospheres and binning them by diameter showed a greater number of total spheres and of larger spheres in the higher insulin condition compared to the lower insulin condition (fig. 2.6C). mRNA expression of the proliferative marker *Mki67* was greater in the higher insulin condition than the lower (fig. 2. 6D).

DISCUSSION

In the present work, we show changes in the hypothalamus resulting from being born to a mother with GDM. While many prior studies suggest a connection between the GDM context and long-term influence on feeding behavior, little is understood regarding how it influences the hypothalamus. The arcuate nucleus of the hypothalamus functions as the master regulator of energy homeostasis, guiding feeding behavior. Due to the significance of critical periods in the developing brain, factors that alter neural development often have irreversible consequences that guide how the organism functions. While many factors contribute to metabolic homeostasis, the hypothalamus is a particularly important subject of focus because of these critical periods and because of its role in integrating many signals from a number of organs. For this study, we elected to focus on major factors in temporally confining the consequences of developmental alteration – cell proliferation and survival – as well as insulin signaling in early postnatal life.

Neurogenesis is generally limited to a very constrained period, referred to as the neurogenic window. However, limited neurogenesis occurs in the hypothalamus through tanycytes, which act as a post-natal stem cell population, arising on the third ventricle and migrating to populate the ARC (Kokoeva et al., 2005; Shimogori et al., 2010; Haan et al., 2013). Tanycytes are thought to arise around embryonic day 17 (Goodman and Hajihosseini, 2015). This time point, late in gestation and at the tail end of the canonical neurogenic window, coincides with the onset of GDM (Sonagra et al., 2014). Cell

proliferation is typically guided by specific active factors promoting an increase or decrease in proliferation. Once those cells are differentiated, they influence the function of the region. Thus, observing how GDM could affect proliferation in the ARC is an important factor in understand its potential influence on function. Utilizing BrdU injections, we were able to observe the number and location of cells that proliferated or were progeny of cells that proliferate during the onset of gestational diabetes. We found no changes in the number of positively stained cells in the HVZ nor ARC. These data suggest GDM exerts its influence differently than dietary treatments such as postnatal or gestational exposure to high fat diet which do affect proliferation and might have acted through a similar process (Chang et al., 2008; Lee et al., 2012). These results may occur because neither canonical neurogenesis nor tanycyte neurogenesis are at their peak during maternal GDM (Goodman and Hajihosseini, 2015) - thus any shifts would have to be fairly robust to be reliably observed. Further, the migration of the newly-produced cells from the HVZ to the ARC may dilute any effect on either region. Overall, our findings suggest that the GDM growth environment does not affect the proliferative population of the ARC or neurogenic tanycyte pool. We followed up on this observation by focusing on factors that may undermine successful communication to this region.

The ME is a vital conduit of nutritional signals from the periphery to the hypothalamus (Goodman and Hajihosseini, 2015; Rizzoti and Lovell-Badge, 2017). We find that GDM reduced the number of BrdU-positive cells in this region of offspring. ME composition is fairly heterogeneous, containing projections from the rest of the hypothalamus and from

tanycytes, as well as neuronal, glial, and endothelial cell bodies (Rodríguez et al., 2010; Langlet, 2014; Rizzoti and Lovell-Badge, 2017). It is relatively sparsely populated suggesting even minimal loss may have functional significance. Which cells are affected is unclear, though preliminary data suggest they are not HuC/D-positive neurons (data not shown). Regardless of the affected cell type(s), the observed influence of GDM on ME cell number is notable for the critical role the ME plays in communicating signals from circulation to the parenchyma. We then wanted to better clarify whether this effect resulted from the direct influence of GDM or whether the effect could be persistent, lasting through birth. Immunostaining for Ki67, a marker of proliferation at the time of tissue harvest, we found no significant changes in the ME, suggesting that the active onset of GDM is necessary for its proliferative influence.

With these findings, we wanted to determine whether cell death was also a factor. Prior studies have suggested insulin and leptin, both of which could be elevated in the context of a GDM offspring, have neuroprotective effects (Bereiter and Jeanrenaud, 1979; Bereiter and Jeanrenaud, 1980; Mielke et al., 2006; Aftab et al., 2017) and the changes we see in BrdU-positive cells could be remediated by a compensatory reduction in cell death. Alternatively, GDM might have a toxic influence on the tissue, inducing cell death. Using TUNEL to stain apoptotic cells, we found no difference between control and GDM-exposed groups in any assessed region. Notably, we observed very little apoptosis in either group, leading to high variability between individuals and making detecting any differences more unlikely. TUNEL indicates apoptosis at the point of tissue harvest; it is possible that GDM induced apoptosis

during the prenatal period and the affected cells were cleared before the tissue was harvested. As such, we cannot make claims about GDM onset. However, it is apparent that day-of-birth cell death is not a major consequence of GDM exposure.

To further explore how GDM may alter offspring physiology, we focused on one key factor in glucose homeostasis: insulin signaling. GDM can lead to excess insulin production from the fetal pancreas (Herrera and Desoye, 2016; Castillo-Castrejon and Powell, 2017) and in later life, insulin signaling in the hypothalamus helps to signal satiety. In p9 hypothalamic slice cultures, we found that basal levels of phospho-AKT (pAKT), a downstream factor in the insulin signaling cascade, were elevated in the β2-tanycyte-containing region in GDM offspring. pAKT expression was almost entirely confined to ME projections, which is consistent with prior data suggesting this region and the β2-tanycytes, specifically, are critical for successful nutritional communication (Balland et al., 2014; Elizondo-Vega et al., 2015; Goodman and Hajihosseini, 2015) and may be influential in insulin signaling in particular (Yoo et al., 2020). Prior research in orexin knockout mice indicates an induction in basal pAKT in the hypothalamus may be associated with high fat diet-induced obesity (Tsuneki et al., 2008). This induction could have similar implications with GDM. Following an insulin challenge, both groups increased pAKT expression indicating that the β2-tanycytes and the ME broadly are not impaired in responding appropriately to exogenous insulin at this age. With prior evidence that this region is a vital conveyor of nutritional information and data tying increased hypothalamic basal pAKT to obesity, our findings suggest dysregulated ME/β2-tanycyte baseline insulin signaling could be a contributing factor in the obesity phenotype observed in GDM offspring.

Another candidate for GDM to exert an influence is through immune activation. Pregnancy is characterized by a heightened immune state and GDM has been shown to exacerbate the effect (Ranheim et al., 2004; Morisset et al., 2011; Xu et al., 2014). Though the brain was once considered immune privileged, it has become apparent that dynamic microglial populations can alter their activation state in response to immune and inflammatory challenges, including maternal immune activation (MIA; Li and Barres, 2018). MIA is linked to neural developmental consequences in offspring (Estes and McAllister, 2017; Money et al., 2018) and microglia have been shown to play an important role in supporting the development of the brain (Tay et al., 2017; Thion and Garel, 2017). If GDM were to alter microglial number or activation state they may act hyperactively or eventually become unresponsive to appropriate stimuli, ultimately harming the tissue. Staining for microglial marker Iba-1, we found no difference in number or activation state, as determined by the degree of ramification, in the ME microglial populations between groups. Notably, population heterogeneity in morphology and marker profiles suggest developmentally, microglial morphology may be a less reliable marker of activation and function than in adulthood (Thion and Garel, 2017; Li et al., 2019) and we did not assess for other macrophages. However, these data would point towards minimal influence of immune activation on the health of the hypothalamus resultant from being born to a mother with gestational diabetes.

In contextualizing our *in vivo* results, we followed-up on prior research indicating insulin could have a trophic effect in neural stem cells (Desai and Ross, 2011). Despite likely

elevated fetal insulin, we saw no change in proliferation in the tanycyte population. We utilized a culture paradigm that selects for post-natal hypothalamic stem cells, generally thought to be tanycytes, exposing them to either 40 ng/mL and 4000 ng/mL, the latter being the concentration typically used in neurosphere cultures. Higher insulin increased the number and size of the neurospheres formed, indicating increased proliferation. This was supported by mRNA expression of *Mki67*, which was significantly higher in the high-insulin condition. These results are predictably out of line with our previously discussed data. *In vivo*, insulin levels may not be sufficiently hyper-physiological to drive results or proliferation may be constrained by other cellular factors that are lost in a purified culture with *in vivo* special relationships disrupted. From this, we can tell that GDM's influence is likely multifactorial and potentially targets tanycytic nutrient sensing, rather than stem cell, functions.

Together, our data support that maternal GDM can target how the hypothalamus of the offspring is able to receive and respond to cues about nutritional availability in the body. The GDM growth environment leads to a reduction in the number of cells in the ME as well as to increased pAKT in the ME and β2-tanycyte projections in the absence of insulin. Either of these factors could affect how the hypothalamus is able to sense and respond to the nutritional needs of the body. These data contribute to an understanding of why being born to a mother with GDM predisposes offspring to be overweight later in life.

ACKNOWLEDGEMENTS

The data shown in Fig. 2.1 and Fig. 2.4 were generated by Karen Weis and used with permission.

FIGURES

Fig. 2.1: *Lepr*$^{+/db}$ dams develop glucose intolerance late in gestation and produce heavier offspring than *Lepr*$^{+/+}$ dams.

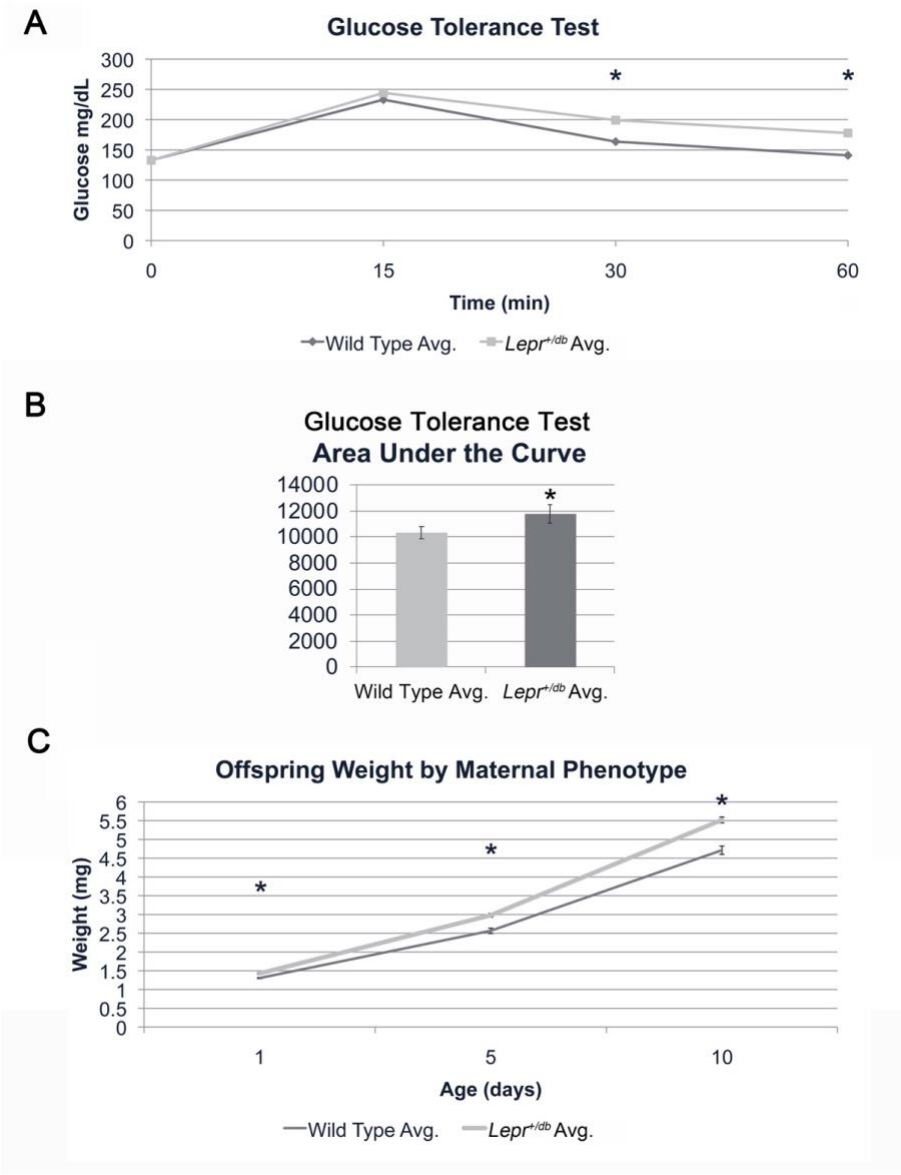


Fig. 2.1: Lepr$^{+/db}$ dams develop glucose intolerance late in gestation and produce heavier offspring than Lepr$^{+/+}$ dams. Pregnant dams with either a *Lepr*$^{+/+}$ (wild type) or *Lepr*$^{+/db}$ genotype were given a glucose tolerance test (GTT) on embryonic day 16.5 or 17.5 which indicated higher blood glucose concentrations at 30 and 60min after glucose administration in *Lepr*$^{+/db}$ (A). Quantifying by area under the curve of the GTT chart confirmed *Lepr*$^{+/db}$ dams were glucose intolerant compared to *Lepr*$^{+/+}$ dams (B). Pups born to *Lepr*$^{+/db}$ dams where significantly heavier than those born to *Lepr*$^{+/+}$ dams at post-natal day 1, 5, and 10 (C). N=5-7; *, p≤.05.

Fig. 2.2: The GDM growth environment led to a reduction in newly born and/or slowly proliferating cells in the median eminence of offspring during the onset of GDM.

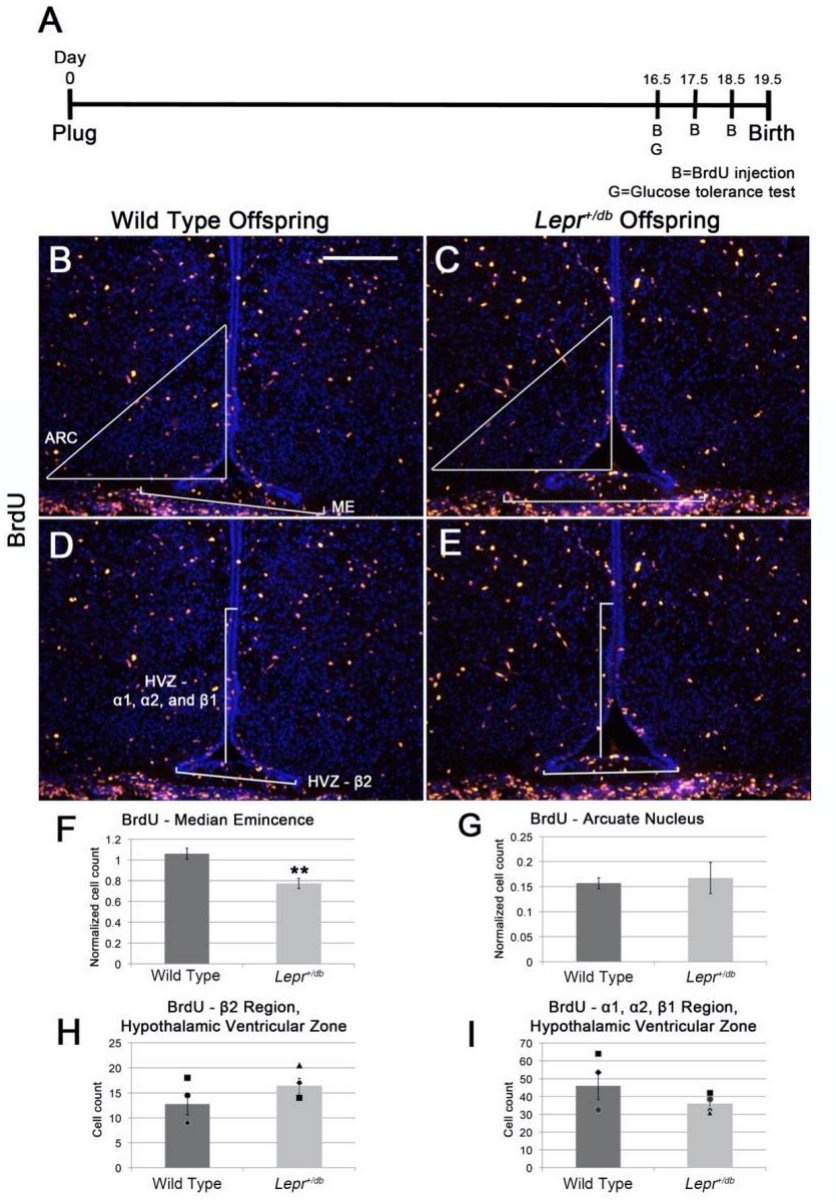


Fig. 2.2: The GDM growth environment led to a reduction in newly born and/or slowly proliferating cells in the median eminence of offspring during the onset of GDM. Pregnant dams were injected with 5-Bromo-2-deoxyuridine (BrdU) twice daily at 0830 H and 1730 H on embryonic days 16.5, 17.5, and 18.5 (A). Immunohistochemistry for BrdU indicated a greater number of positive cells in the median eminence (ME) of offspring born to wild type dams compared to *Lepr*$^{+/db}$ dams with no distinct difference in the arcuate nucleus (ARC) (B and C). It appeared there was no marked difference in positive cells in the hypothalamic ventricular zone (HVZ), the region in which tanycytes reside, between the two groups (D and E). Quantifying the number of BrdU positive cells in the median eminence confirmed a greater number in the ME of wild type offspring compared to *Lepr*$^{+/db}$ offspring (F). Quantification also confirmed no significant difference in BrdU positive the ARC between the two groups (G). Counting cells residing on the inferior border of the HVZ, which contains the β2-subtype of tanycytes, indicated no significant difference between offspring born to wild type and GDM mothers (H). There was similarly no significant difference in the number of labeled cells in the region of the HVZ containing the α and β1 tanycytes between the two groups (I). Number of BrdU positive cells normalized to total number of cells (F and G). Scale bar = 200μm (B-E). N=4; **, p≤.01.

Fig. 2.3: The GDM growth environment did not lead to changes in postnatal cell proliferation nor changes in postnatal apoptosis.

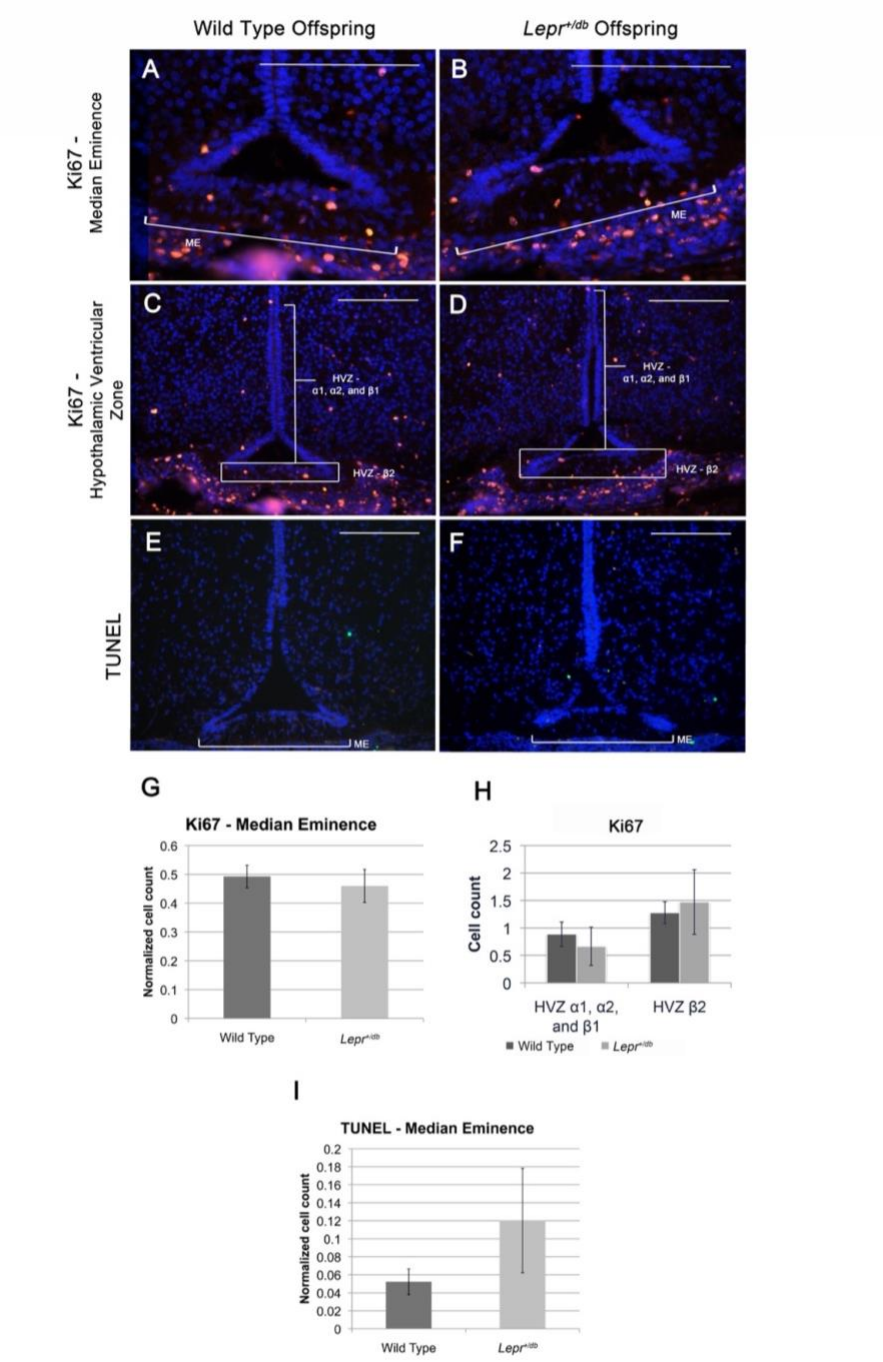


Fig. 2.3: The GDM growth environment did not lead to changes in postnatal cell proliferation nor changes in postnatal apoptosis.
Immunohistochemistry for the proliferation marker Ki67 indicated no apparent differences in proliferation in the ME between the two conditions (A, B). There was also no difference in cells in either the dorsal, α1-, α2-, and β1-, nor the β2-tanycyte-containing regions (C, D). There was further no apparent difference in median eminence terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, which detects apoptosis (E, F). These findings were substantiated by quantification (G, H, and I). Scale bar = 200μm (A-F). N=3.

Fig. 2.4: Observed *in vitro*, the GDM growth environment altered baseline insulin signaling while not affecting induction ability.

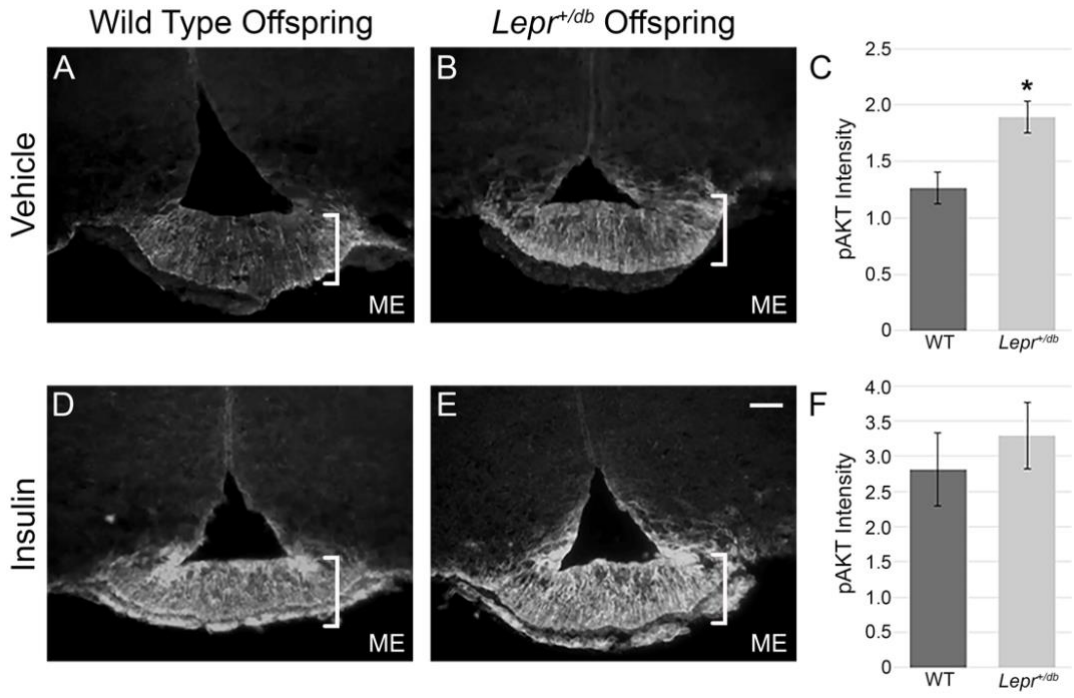


Fig. 2.4: Observed *in vitro*, the GDM growth environment altered baseline insulin signaling while not affecting induction ability.
Immunohistochemistry for pAKT, a readout of insulin signaling, indicates greater baseline insulin signaling in the median eminence (ME, brackets) and β2-tanycyte containing region of offspring born to *Lepr*$^{+/db}$ mothers compared to those born to wild type mothers at postnatal day 9 (A, B), quantified in C. Bathing the slices in insulin induced pAKT levels similarly in both groups (D, E), quantified in F. N=3-4; * indicates P<0.05. Scale bar = 50 µm

Fig. 2.5: The GDM growth environment did not increase microglia presence or activation state.

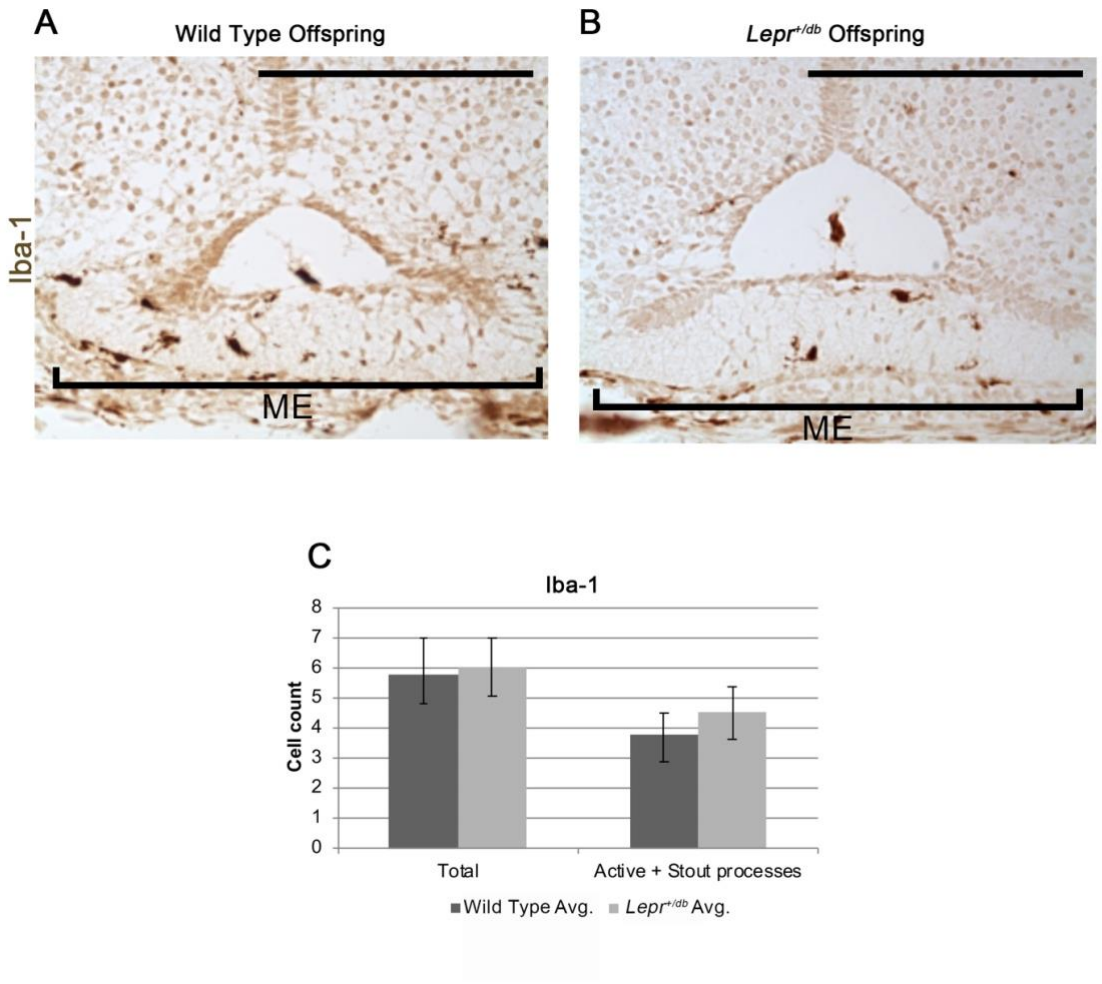


Fig. 2.5: The GDM growth environment did not increase microglia presence or activation state. Immunohistochemistry for Iba-1, a microglial marker, was carried out to assess number and morphology, an indicator of activation state, of microglia in the median eminence (A, B). Quantifying the number of total Iba-1 positive cells and the combined number of cells in the two most active morphological bins suggested no significant difference between the two groups (C). Scale bar = 200µm (A, B). N=4.

Fig. 2.6: Hypothalamic progenitor cells exposed to high insulin levels proliferate more and create more neurospheres than those exposed to lower levels.

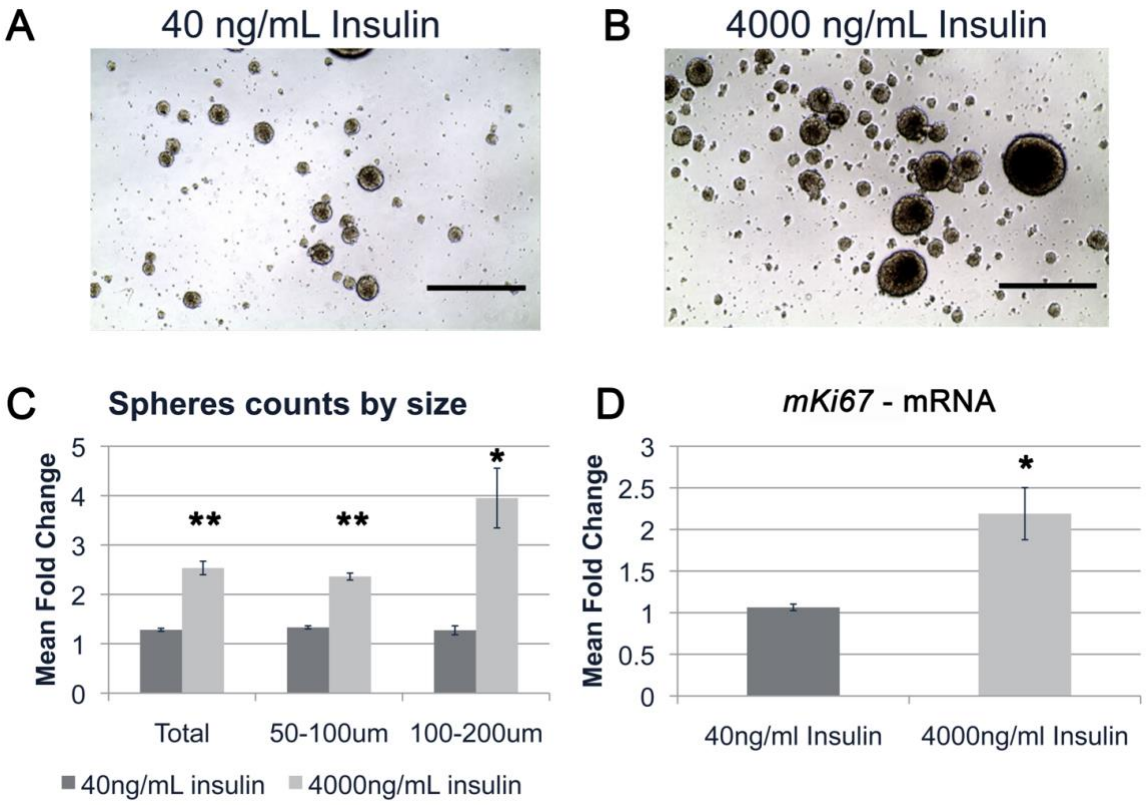


Fig. 2.6: Hypothalamic progenitor cells exposed to high insulin levels proliferate more and create more neurospheres than those exposed to lower levels. Neurospheres were grown from tissue harvested from postnatal day 1 mice and exposed to either 40ng/mL or 4000ng/mL insulin (A, B). Neurospheres were quantified and binned by diameter and an indicator of both how successfully new neurospheres were created and how proliferative each neurosphere was. This revealed that exposure to higher insulin levels lead to more, as well as larger, spheres (C). qPCR for *Mki67*, a marker of proliferation, similarly indicated significantly higher proliferation in the higher insulin condition over the lower insulin condition (D). Scale bar = 400μm (A, B). N=4; *, p≤.05; **, p≤.01.

Chapter 3: Iodoacetic Acid, a Water Disinfection Byproduct, Disrupts Hypothalamic and Pituitary Reproductive Regulatory Factors and Induces Toxicity in the Female Pituitary[1]

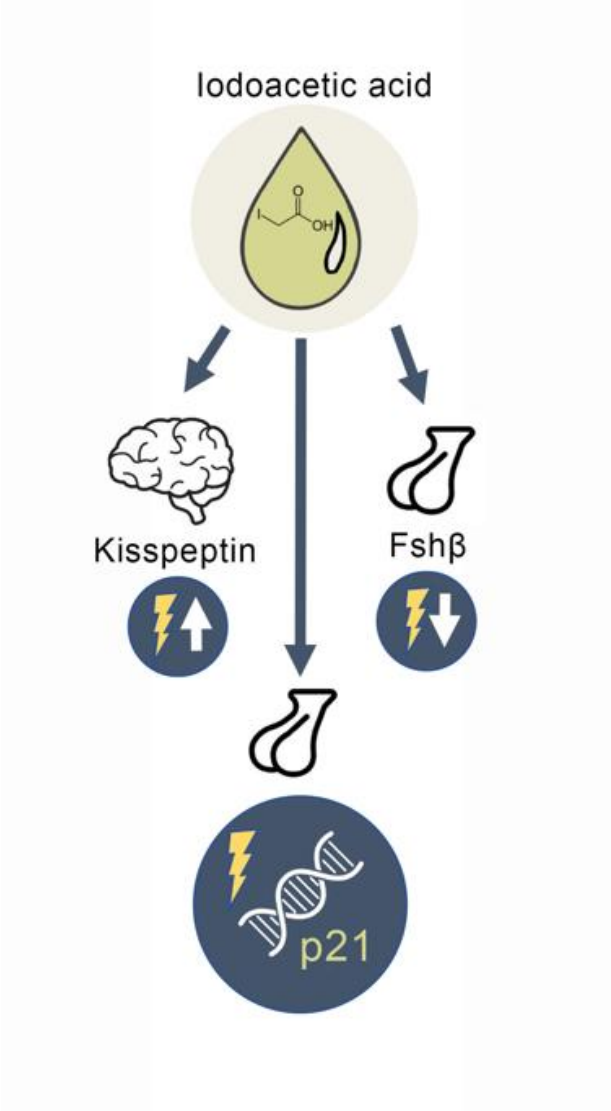
ABSTRACT

Iodoacetic acid (IAA) is a water disinfection byproduct (DBP) formed by reactions between oxidizing disinfectants and iodide. *In vitro* studies have indicated that IAA is one of the most cyto- and genotoxic DBPs. In humans, DBPs have been epidemiologically associated with reproductive dysfunction. In mouse ovarian culture, IAA exposure significantly inhibits antral follicle growth and reduces estradiol production. Despite this evidence, little is known about the effects of IAA on the other components of the reproductive axis: the hypothalamus and pituitary. We tested the hypothesis that IAA disrupts expression of key neuroendocrine factors and directly induces cell damage in the mouse pituitary. We exposed adult female mice to IAA in drinking water *in vivo* and found 0.5 and 10 mg/L IAA concentrations lead to significantly increased mRNA levels of kisspeptin (*Kiss1*) in the arcuate nucleus, while not affecting *Kiss1* in the anteroventral periventricular nucleus. Both 10mg/L IAA exposure *in vivo* and 20μM IAA *in vitro* reduced follicle-stimulating hormone (FSHβ)-positive cell number and *Fshb* mRNA expression. IAA did not alter luteinizing hormone (LHβ) expression *in vivo*, though exposure to 20μM IAA decreased expression of *Lhb* and glycoprotein

[1] This work was published in a lightly modified form in: Gonzalez, R.V.L., Weis, K.E., Gonsioroski, A.V., Flaws, J.A., Raetzman, L.T., Iodoacetic Acid, a Water Disinfection Byproduct, Disrupts Hypothalamic, and Pituitary Reproductive Regulatory Factors and Induces Toxicity in the Female Pituitary, Toxicological Sciences, 2021;, kfab106, https://doi.org/10.1093/toxsci/kfab106

hormones, alpha subunit (*Cga)* mRNA *in vitro.* IAA also had toxic effects in the pituitary, inducing DNA damage and P21/*Cdkn1a* expression *in vitro* (20μM IAA) and DNA damage and *Cdkn1a* expression *in vivo* (500mg/L). These data, implicate IAA as a hypothalamic-pituitary-gonadal axis toxicant and suggest the pituitary is directly affected by IAA exposure.

GRAPHICAL ABSTRACT



INTRODUCTION

Water disinfection byproducts (DBPs) exemplify a common driver of toxicological research: compounds initially intended for a productive end that have deleterious unintended consequences. Chemicals used to treat water to prevent potentially deadly water-borne illness can react with matter already present in the water, forming DBPs. *In vitro* studies using Chinese Hamster Ovary (CHO) and human epithelial cells have driven discoveries that a range of DBPs have significant genotoxic, cytotoxic, and teratogenic capabilities (Plewa et al., 2004; Plewa et al., 2010; Dad et al., 2013; Pals et al., 2013). Despite evidence of potential risk and knowledge of human exposure, DBPs are largely understudied, especially in whole tissue and *in vivo* contexts.

One such DBP, iodoacetic acid (IAA), is of particular concern. IAA is formed as a byproduct of a reaction between an oxidizing disinfectant, like chlorine, and iodide. As chlorine is widely utilized and iodide occurs naturally in water, there is a high risk of IAA formation in public water supplies (Richardson, 2005; Dong et al., 2019). In the previous *in vitro* studies, IAA was found to be one of the most cyto- and genotoxic DBPs tested. In cultured fibroblast cell lines, IAA introduced significant DNA damage and when the IAA-treated cells were inoculated into mice *in vivo*, the mice developed tumors, indicating it has significant tumorigenic potential (Wei et al., 2013). IAA exposure can also induce DNA damage in cultured oocytes (Jiao et al., 2021). *In vitro* work focused on ovarian follicle cultures shows that IAA can disrupt folliculogenesis and estradiol synthesis (Jeong et al., 2016; Gonsioroski et al., 2020). While existing *in vivo* studies

are very limited, prior research demonstrates early adult exposure to IAA in rodents can lead to decreased ovarian weight and disrupted hypothalamic-pituitary-thyroid (HPT) axis function (Xia et al., 2018). Together, there is strong rationale to think that IAA could have deleterious effects on tissue health and in particular on endocrine tissues. However, this question requires further exploration.

The neuroendocrine system is a vital mediator of interconnected axes in which consequences at any level could mean result in hormonal alterations at another. Yet at present, the hypothalamus and pituitary are unstudied in the context of IAA exposure. Further, they could be prime targets of IAA action. Prior studies from our lab have illustrated that the pituitary is directly sensitive to environmental toxicants that can interfere with cell viability and induce cell cycle arrest through P21/C*dkn1a* induction (Weis and Raetzman, 2016; Weis and Raetzman, 2019). This may be especially relevant considering IAA's previously established cyto- and genotoxic mechanisms. Additionally, the hypothalamus and pituitary have been shown to be vulnerable to disruption by endocrine disrupting chemicals (EDCs) especially during development. Further, this EDC action can be specifically consequential for HPG axis function (Gore et al., 2015). IAA has been shown to act as an EDC with potential estrogenic and androgenic activity (Kim et al., 2020; Long et al., 2021). Not only would it be feasible for IAA to affect the hypothalamus and pituitary, but such effects could be deleterious for physiology.

We took a two-pronged approach to address the impact of IAA in the hypothalamus and pituitary: pursuing effects on hormone-related gene and protein expression in both regions and toxic effects in the pituitary. Based on the strength of previously discussed data on the ovaries and ovarian cells, we focused our hormone-regulation experiments on contributions to the hypothalamic-pituitary-gonadal (HPG) axis. In the hypothalamus, kisspeptin-expressing neurons in the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) act to stimulate the release of gonadotropin-releasing hormone (GnRH) from neurons in the medial preoptic area (mPOA). ARC kisspeptin neurons maintain a pulsatile release of GnRH and are negatively regulated by ovarian estradiol feedback during the majority of the estrous cycle. Conversely, AVPV kisspeptin neurons are stimulated by estrogen and induce the GnRH surge that ultimately leads to ovulation. GnRH acts on pituitary gonadotropes, which also receive feedback from the ovaries, to induce the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). It is LH and FSH that are ultimately released into circulation to guide ovarian physiology. As this chain of effectors illustrates, there are numerous points at which IAA exposure could interfere with hypothalamic and pituitary contributions to the HPG axis.

Interrogating these effects will be an important step in understanding and addressing the consequences of IAA exposure. Thus, we tested the hypothesis that IAA acts as an HPG-disrupting toxicant by interfering with the expression of key factors in the hypothalamus and pituitary and leading to cytotoxic and/or genotoxic damage in the pituitary.

METHODS AND MATERIALS

Animal Care

For *in vivo* dosing experiments, cycling female CD-1 (postnatal day 26) were purchased from Charles River Laboratories (Charles River, CA) and housed at the University of Illinois at Urbana-Champaign, College of Veterinary Medicine Animal Facility. For explant culture experiments, CD-1 mice were bred from lines originally obtained from Charles River Laboratories (Charles River, CA) and housed at the University of Illinois at Urbana-Champaign, Burrill Hall Animal Facility. Both sets of animals were kept under 12h light-dark cycles and provided ad libitum access to water and Teklad 8664 rodent diet (Envigo). All procedures were approved by the University of Illinois, Urbana-Champaign, Institutional Animal Care and Use Committee.

In Vivo Dosing

Female CD-1 mice were aged to 40 days. At P40, mice were divided into groups and given varying doses of IAA (Sigma-Aldrich) dissolved in drinking water. This exposure mechanism was chosen to best replicate the human context in which the primary source of exposure is drinking water. For initial *in vivo* dosing, group were as follows: No IAA added (Control), 0.5mg/L, 10mg/L, 100mg/L, or 500mg/L IAA, corresponding to approximate ingestion levels of 3.48, 69, 490, and 1700µg of IAA per mouse per day (Gonsioroski et al., 2021). Although human exposure levels have not been well

established, prior studies have indicated IAA levels in tap water can be as a high as 2.18µg/L (Wei et al., 2013), with total haloacetic acid, the class to which IAA belongs, levels as high as 136µg/L (Srivastav et al., 2020). Overall human exposure to IAA is likely much higher, as food and dermal contact from swimming pools and bath water further introduce DBPs into an individual's environment (Chowdhury et al., 2014; Allen et al., 2021). Mice were given ad libitum access to this drinking water for 35-40 days. The dosing experiments were repeated with two groups: no IAA added (Control) and 500mg/L IAA and again with two groups: no IAA added (Control) and 10mg/L IAA.

Tissue Collection

For *in vivo* experiments, whole brains and pituitary glands were harvested from 75–80-day old IAA-dosed and control female mice in diestrus. Brains were flash frozen on dry ice shortly following decapitation and stored at -80° C. Regions of interest were dissected from frozen brains. Sectioning caudal to rostral, regions were identified using The Mouse Brain in Stereotaxic Coordinates: Fourth Edition (Paxinos and Franklin, fourth edition). Starting approximately 150µm caudal of the arcuate nucleus (ARC), 2 overlapping punches were collected, incorporating the ARC, using a 1.25mm diameter biopsy punch. Punches were approximately 2mm in depth spanning approximately Bregma -2.91mm to approximately Bregma -.91mm. Brains were then sectioned rostrally until morphological markers indicated a region approximately 100µm caudal to the beginning of the anteroventral periventricular nucleus (AVPV). A single 1.25mm diameter, approximately 1mm-deep, biopsy punch incorporating the AVPV and the

medial preoptic nucleus (mPOA) was collected. This punch spanned approximately Bregma -0.11mm to approximately Bregma 0.89mm. Sections were collected immediately preceding and following punches to confirm location. For qPCR analysis, pituitary glands were collected into RNA Later (Thermo Fisher) and stored at -20° C. For *in vivo* IHC experiments, pituitary tissue was fixed for 60 min. in 3.7% formaldehyde/phosphate buffered saline (PBS), then cryoprotected in in 30% sucrose/ PBS before embedding in Optimal Cutting Temperature (Tissue-Tek) and storage at -80° C until sectioning.

Pituitary Explant Cultures

Whole pituitaries were harvested from female postnatal day 40 or 41 CD-1 mice for *in vitro* explant culture as described in Weis and Raetzman, 2016. For each exposure group, one to four pituitaries were placed on Millicell CM 6-well plate culture inserts (Millipore) and cultured in DMEM/F12 medium containing 10% charcoal stripped Fetal Bovine Serum (FBS, Sigma), and 10,000 IU Penicillin/10,000µg/ml Streptomycin (Fisher Scientific). Explants were exposed to varying levels of IAA in culture media based on randomly assigned treatment group beginning from initial plating and ending at harvest 48 hours later. For initial qPCR experiments, IAA treatments were: 0.2 µM IAA, 2µM IAA, 20µM IAA, all diluted in DMSO as a vehicle. Control cultures received only 0.1% DMSO. These dosages were chosen to generally correspond with ovary cultures performed in Jeong et al., 2016 and Gonsioroski et al., 2020. Specific values were chosen in order to create a concentration gradient. After establishing a concentration

curve in initial experiments, 20μM IAA was selected for subsequent cultures. For all IHC experiments, comparison groups were 0.1% DMSO (control) and 20μM IAA. For qPCR analysis, following harvest, explants were submerged in TRIzol and stored at -80° C. These cultures were repeated four times such that presented data represent individuals from four independent cultures. For IHC analysis, explants were washed in PBS, then fixed in 3.7% formaldehyde/phosphate buffered saline (PBS) for 30 min. before cryoprotecting in 30% sucrose/PBS. They were then embedded in Optimal Cutting Temperature (Tissue-Tek) and stored at -80° C until sectioning. These cultures were repeated four times such that presented data represent individuals from three to four independent cultures.

Immunohistochemistry

All pituitary tissue was sectioned to 12μm using a cryostat (Leica). For each individual, 3 slides evenly spaced through the tissue with two sections on each slide were selected. Immunostaining was performed using antibodies for FSHβ (diluted 1:100, National Hormone and Peptide Program, AF Parlow), LHβ (diluted 1:100, National Hormone and Peptide Program, AF Parlow), P21 (diluted 1:500; BD Pharmingen), and γH2AX (1:500; Cell Signaling). Slides were air dried for 5 min. and fixed in 3.7% formaldehyde/PBS for an additional 10 min. For P21 and γH2AX immunostaining, antigen retrieval was performed by immersing slides in 0.01M sodium citrate pH 6.0 at approximately 95°C for 5 min (*in vitro* samples) or for 10 min. followed by a 15min. cooling period (*in vivo* samples). For P21 staining using 3-3′-diaminobenzidine staining (DAB), slides were

submerged in 1.5% hydrogen peroxide in PBS for 20 min (*in vitro* slides) or 30min (*in vivo* slides). For *in vivo* P21 staining experiments, slides underwent a 1-hour block period specific to mouse-on-mouse antibodies in which they were incubated in 2% donkey anti-mouse IgG (Jackson ImmunoResearch). Before primary antibody treatment, all slides were blocked for 1 hour using 5% normal donkey serum (Jackson ImmunoResearch) diluted in a block containing 3% bovine serum albumen (Jackson ImmunoResearch), and 0.5% Triton-X100 in PBS. Primary antibodies were applied to slides overnight at 4 °C. For all stains, a negative control slide was included that substituted incubation in block for primary antibody. Sections were then incubated in biotin conjugated anti-rabbit secondary antibodies (Jackson ImmunoResearch) for 1 hour at room temperature, followed by incubation with streptavidin cy3, for 1 hour at room temperature. Slides were mounted using antifade mounting medium (0.1 M Tris pH 8.5, 20% glycerol, 8% polyvinyl alcohol, 2.5% 1,4-diazabicyclo[2.2.2]octane) containing the nuclear stain 4′,6-Diamidino-2-Phenylindole, dihydrochloride (DAPI), and visualized with a fluorescent microscope (Leica). Sections were then incubated in biotin-conjugated anti-mouse (P21) or anti-rabbit (all others) secondary antibodies (Jackson ImmunoResearch) for 1 hours at room temperature. For P21, following secondary incubation, slides underwent streptavidin-HRP amplification using the Vectastain Elite ABC kit (Vector) and visualization by DAB staining. These slides were then dehydrated and coverslips were affixed using Permount. These slides were visualized using a brightfield microscope (Leica).

Quantification of Immunohistochemistry Results

FSHβ, LHβ, and *in vivo* P21 experiments were quantified as follows. For each individual, 6-8 images were be taken of each section on a given slide. In a subset of images, DAPI-positive cells were quantified to determine heterogeneity of total number of cells per image. After determining number of cells per image where largely homogeneous and total number did not significantly differ between individuals, only immunopositive cells were counted and included in analysis. Number of immunopositive cells across images of same slide were averaged together. The three slides were summed together to get a representative quantification for each individual. For γH2AX experiments, quantification was performed by counting the total number of immunopositive cells in a given section, averaging the sections of the same slide together, and taking a sum of counts per individual.

RNA Extraction and cDNA synthesis

For hypothalamic punches and pituitary glands, RNA extraction was performed using TRIzol reagent according to the manufacturer's instructions (Life Technologies). Hypothalamic punches were broken up by pipetting up and down in TRIzol. Pituitary glands were homogenized in TRIzol to disrupt the tissue. RNA yield and purity were determined by spectrophotometry. Using the ProtoScript M-MuLV First Strand cDNA synthesis kit (New England BioLabs, MA, USA), approximately 0.5μg of RNA was synthesized into cDNA. When limited by the amount of RNA yield, 6μl of RNA

suspended in H_2O, the maximum allowed by the kit, was used. A no-enzyme sample was included as a negative control for each set of synthesis reactions.

RT-qPCR

RT-qPCR was performed on the cDNA samples using gene-specific primers. Results were analyzed using the double change in threshold cycle (ΔΔCT) method as previously described (Goldberg et al., 2011). *Gnrh1* and *Kiss1* in AVPV/POA mRNA samples were normalized to control gene *Ppia*. ARC and all pituitary mRNA samples were normalized to *Gapdh*. Student's two-tailed *t*-tests were performed to ensure there were no significant changes in *Ppia* nor *Gapdh* between dosage groups, thus verifying it could suitably be used as a control gene. Primer sequences for each gene are shown in Table 3.1.

Statistical analyses

All statistics were performed using Graph Pad Prism 8.2.1. Variance was assessed using an F-test of equality of variance for two-group analyses and Bartlett's test for those with three or more groups. With three or more groups, significance was determined using a one-way ANOVA followed by Dunnett's post-hoc analysis when variance was equal and using a Welsh's ANOVA followed by a Dunnett's T3 post-hoc analysis when variance was unequal. Post-hoc analyses compared experimental groups to controls. For analyses with a significant difference in variance between two

groups, statistics were performed on log-transformed data. When only one experimental condition was used, a Student's t-test was used to compare the experimental group with controls.

RESULTS

IAA increases mRNA expression of the hypothalamic hormone kisspeptin in the ARC *in vivo* while leaving other key hormones unchanged

In the hypothalamus, two regions, the ARC and the AVPV, express kisspeptin which act on GnRH neurons to control pulsatile release of GnRH or the GnRH surge, respectively. We assessed kisspeptin (*Kiss1)* mRNA in both these regions *in vivo* and found that IAA did not alter expression at any dosage in the AVPV (fig. 3.1A), but at 0.5 and 10mg/L IAA significantly increased expression in the ARC (fig. 3.1B). Finding this change in mRNA in the ARC, we wanted to determine if mRNA of the known autoregulatory peptides released by ARC kisspeptin neurons, neurokinin B (*Tac2)* and dynorphin (*Pdyn)*, were changed, potentially contributing to the resulting shift in *Kiss1* (Navarro et al., 2009). We found no changes in *Tac2* mRNA nor *Pdyn* mRNA (fig. 3.1C, D). The hypothalamus responds in a homeostatic manner to feedback from estrogen released from the ovaries, which inhibits kisspeptin neurons in the ARC and stimulates them in the AVPV. To assess one indicator of potential contributions of altered sensitivity to estrogen feedback, we assessed mRNA of estrogen receptor-α (*Esr1*) in the ARC, finding no change between any dosage and controls (fig. 3.1E). As kisspeptin neurons

act on GnRH neurons to stimulate the release of GnRH which ultimately acts on pituitary gonadotropes to release LH and FSH, we also looked at GnRH (*Gnrh1*) mRNA, which indicated no difference between any condition and controls (fig. 3.1F).

***In vivo*, IAA significantly reduced *Fshb* mRNA and FSHβ protein expression, while preserving expression of other key reproductive hormone-related genes and proteins**

We wanted to examine IAA's effects on the pituitary, as it is the other major component of neuroendocrine regulation of reproduction. FSH and LH are made up of a shared alpha-subunit, glycoprotein hormones, alpha subunit (CGA), and differing beta-subunits which determine the final hormone produced. Levels of FSH beta-subunit mRNA **(***Fshb*)**,** were significantly reduced between the control and 10mg/L IAA conditions (fig. 3.2A). LH beta-subunit mRNA (*Lhb*)*,* expression did not significantly differ between any IAA level and controls (fig. 3.2B). CGA (*Cga*) *e*xpression was not altered by IAA *in vivo* (fig. 3.2C). Observing a significant reduction in mRNA with 10mg/L IAA exposure, we wanted to observe protein expression of FSHβ at that dosage using IHC (fig. 3.2D, E). We found a significant reduction in number of FSHβ-positive cells in individuals exposed to IAA compared to controls (fig. 3.2F). We also assessed protein expression of LHβ, comparing control and 10mg/L IAA conditions (fig. 3.2G, H). The numbers of immunopositive cells were not significantly different when quantified (fig. 3.2I).

In vitro, IAA significantly reduces _Fshb_, _Lhb_, and _Cga_, but not _Gnrhr_ mRNA, as well as FSHβ, but not LHβ protein expression.

To observe the effects of IAA directly on the pituitary in the absence of signals from the hypothalamus and the rest of the reproductive axis, we carried out whole pituitary cultures, exposing explants to varying levels of IAA. Assessing mRNA expression of *Fshb* on a range of IAA concentrations revealed a significant difference between controls and those exposed to 20μM IAA (fig. 3.3A). We also assessed *Lhb*, comparing control and 20μM IAA conditions, finding a significant reduction in expression with IAA exposure compared to control (fig. 3.3B). We found a similar reduction in *Cga* mRNA levels, but no difference in *Gnrhr* mRNA expression with IAA exposure compared to control (fig. 3.3C). Immunohistochemistry for FSHβ suggested an apparent decrease in the number of positively-stained cells between explants exposed to DMSO alone and those exposed to 20μM IAA (fig. 3.3D, E). This visual difference was confirmed as a significant reduction by quantifying immunopositive cells (fig. 3.3F). We also performed immunohistochemistry for LHβ (fig. 3.3G, H). There was no significant difference between groups when quantifying number of positive cells (fig. 3.3I).

IAA exposure induced DNA damage both _in vivo_ and _in vitro_.

One potential mechanism for IAA to contribute deleteriously to the health of the tissue is through DNA damage (Jiao et al., 2021). We carried out immunohistochemistry for the marker of DNA damage γH2AX in mice exposed to 500mg/L compared to controls (fig.

3.4A, B). Quantifying the number of immunopositive cells in the entire pituitary showed a significant increase in γH2AX in the group exposed to 500mg/L IAA (fig. 3.4C). Assessing γH2AX-positivity in our pituitary explant cultures revealed a marked increase in DNA damage in those exposed to 20μM IAA compared to control (fig. 3.4D, E).

IAA increased P21 protein expression *in vitro* and P21 (*Cdkn1a*) mRNA both *in vivo* and *in vitro*. IAA also increased expression of *p53 in vitro*, but not *in vivo*.

Prior data from our lab have illustrated that P21 induction may be a particularly relevant component of the pituitary response to toxicants (Weis and Raetzman, 2016; Weis and Raetzman, 2019) as well as being induced following DNA damage (Abbas and Dutta, 2009). We assessed P21 mRNA (*Cdkn1a*) in our *in vivo* samples, finding a significant increase in expression levels with exposure to 500mg/L IAA compared to control (fig. 3.5A). qPCR for *Cdkn1a* expression *in vitro* showed increased expression in all exposure groups compared to controls (fig. 3.5B). Finding an increase in mRNA at 500mg/L IAA *in vivo*, we assessed protein expression comparing this group to controls (fig. 3.5C, D). Quantifying the number of cells immunopositive for P21 showed no significant difference between groups (fig. 3.5E). *In vitro*, this assay revealed a strikingly apparent increase in P21-positive cells in the IAA-treated condition compared to controls (fig. 3.5F, G). DNA damage is associated with p53-mediated induction of P21. Having observed both increases in both DNA damage and P21/*Cdkn1a,* we evaluated mRNA expression of *p53. In vivo,* we found no significant changes between

experimental and control conditions (fig. 3.5H). *In vitro*, mRNA expression of p53 was significantly increased with IAA exposure (fig. 3.5I).

IAA had no effect on proliferation as assessed by *Mki67* mRNA levels, or apoptosis assessed via *Bax* and *Bcl2* levels.

As P21 is a cell cycle arrester, we wanted to determine the influence of its activation by IAA on proliferation and cell death. mRNA of the proliferative marker Ki67 (*Mki67*) was unchanged by any IAA exposure level *in vivo* (fig. 3.6A) or in our explant culture (fig. 3.6B). P21 is known to have a dynamic influence on apoptosis, at times promoting it and at others inhibiting it (Kreis et al., 2019). To observe whether IAA altered cell death, we assessed expression levels of the pro-apoptotic marker *Bax* (fig. 3.6C) and the anti-apoptotic marker *Bcl2* (fig. 3.6D), neither of which were altered by IAA exposure to 20μM IAA *in vitro*. Often, the ratio of *Bax* to *Bcl2* mRNA is reported as an indicator of apoptosis in the tissue. This ratio was not altered by IAA in our *in vitro* context (fig. 3.6E).

DISCUSSION

Epidemiological studies have linked increased DBP exposure to negative impacts on sperm morphology and concentration in males, and menstrual cycle length and follicular phase length in women (Villanueva et al., 2015). In cell lines and mouse ovarian cells, IAA has been shown to have toxic effects. However, little is known about

neuroendocrine contributions to negative reproduction-related outcomes with IAA exposure. Thus, we studied how IAA affects the hypothalamus and pituitary to better understand its potential to disrupt the HPG axis.

In hypothalamic tissue harvested from mice exposed to a range of IAA dosages in their drinking water, we first focused on *Kiss1* in the ARC and the AVPV. IAA reduced expression of *Kiss1* mRNA in the ARC (0.5mg/L, 10mg/L IAA), the seat of the GnRH pulse generator. *Kiss1* mRNA reduction is not likely due to local changes in autoregulatory peptides since *Tac2* and *Pdyn* were not affected by IAA exposure (Navarro et al., 2009; Moore et al., 2018). Additionally, ovarian estrogen, acting through estrogen receptor-α (*Esr1*), is one of the primary negative regulators of *Kiss1* ARC expression. However, we found no change in *Esr1* mRNA with IAA and analysis of sera collected in a parallel study indicated estradiol was unchanged at the dosages at which we observed *Kiss1* reduction (Gonsioroski et al., 2021). These data suggest that *Kiss1* repression by IAA occurs either directly on *Kiss1* or through an unidentified mechanism. Despite the change in *Kiss1*, we found no change in *Gnrh1* mRNA in response to IAA, though we did not explore protein level changes or shifts in pulsatility. Collectively, our data reveal IAA disrupts hypothalamic contributions to the HPG via reducing ARC *Kiss1* mRNA expression.

In the pituitary, the two central factors in reproductive control are FSH and LH, which influence ovarian steroid hormone production, and guide follicle maturation and ovulation, respectively. Thus, they are prime targets for HPG axis interference. Both *in*

vivo (10mg/L IAA) and *in vitro* (20μM IAA***),*** *Fshb* mRNA expression and the number of FSHβ-positive cells were significantly reduced with IAA exposure. Seeing changes in both contexts suggest IAA targets the pituitary directly. We cannot be certain how these findings translate to circulating FSH levels. The most accurate assessment would require frequent serum collections representing the entire estrous cycle - during which peak FSH expression occurs in the early morning of estrus (Ongaro et al., 2021) - rather than the single diestrus time point collected from these mice (Gonsioroski, et al., 2021). However, a reduction in both mRNA and protein suggests the possibility that the ovaries could see reduced FSH levels. With long term exposure, such a reduction could lead to impaired ovarian follicle development and reduced ovarian estradiol synthesis (Bernard et al., 2010). This could potentially compound the direct ovarian impact of IAA revealed by prior *in vitro* studies (Jeong et al., 2016; Gonsioroski et al., 2020). We do not know the ultimate consequence of this observation for fertility in general; however, there was a borderline-significant change in estrous cyclicity with the same IAA exposure in which we observe FSHβ/*Fshb* reduction, potentially indicating a contribution of pituitary effects to HPG dysregulation (Gonsioroski et al., 2021).

The mechanism by which IAA leads to these changes in FSHβ/*Fshb* is currently unknown. Paradoxically, if *Kiss1* mRNA reduction were the causal mechanism for the reduction *in vivo*, it should lead to an increase, not a decrease in expression. Similarly, the change does not appear to be due to ovarian estradiol feedback; levels were unaffected in the dosages at which we observed FSHβ/*Fshb* reductions (Gonsioroski et al., 2021). Pursuing this in future studies will provide valuable insight.

Interestingly, the number of LHβ-positive cells and *Lhb* mRNA *in vivo* were unchanged by IAA. One of the primary mechanisms by which gonadotropins are regulated is through GnRH pulse frequency; LH synthesis is favored by quick pulses and FSH synthesis is favored by slow ones (Tsutsumi and Webster, 2009; Thompson and Kaiser, 2014; Coss, 2018). Observing a shift in FSHβ, but not LHβ expression suggests a regulatory mechanism specific to FSH and that ovulatory and other LH-driven reproductive functions may be spared. Several FSH-regulating factors that could underly this outcome have been characterized. Ovarian and pituitary activins promote FSH synthesis, whereas ovarian inhibins and ubiquitously expressed follistatins inhibit it (Bernard et al., 2010). Analysis of sera harvested from the same mice used in this study showed no difference in inhibin B with IAA exposure (Gonsioroski et al., 2021), though inhibin A was not assessed. Future experiments will be vital to clarifying the mechanisms by which IAA influences FSHβ/*Fshb*.

In addition to disrupting hormone production, IAA has been shown to have direct toxic influences on ovarian tissue and in various cell types. Therefore, we examined the potential for cyto- and genotoxic effects in the pituitary both systemically *in vivo* and directly *in vitro*. IAA exposure substantially increased DNA damage *in vitro* and at 500mg/L IAA *in vivo*, as evidenced by an increase in cells immunostaining for γH2AX. This finding is in line with other studies, most notably genotoxicity observed in ovarian cells (Plewa et al., 2010, Jiao et al., 2021). Aggregated DNA damage has been linked to premature cellular aging, which can include telomere attrition, changes in gene

expression, and impaired DNA damage repair (López-Otín et al., 2013). Further, evidence suggests that DNA damage is a key contributor to pituitary tumorigenesis (Chesnokova et al., 2008; Ben-Shlomo et al., 2020; Chesnokova et al., 2020). These prior data would suggest that IAA could contribute to a tumorigenic environment or potentially to dysfunction resulting from the consequences of premature aging in the pituitary, especially with persistent exposure.

DNA damage induces the cell-cycle arrester P21 (*Cdkn1a*), which is important in the context of pituitary response to cellular damage. Studies suggest it aids in constraining pituitary tumor growth and may contribute to the observation that pituitary tumors, unlike most types of tumors, are frequently benign (Chesnokova et al., 2008; Chesnokova and Melmed, 2009). In two studies from our lab on pituitary phytoestrogen exposure, *Cdkn1a* was increased (Weis and Raetzman, 2016; Weis and Raetzman, 2019), suggesting it is part of the pituitary response to select toxicant exposure. Expression of *Cdkn1a* was significantly increased following IAA exposure both *in vivo* and *in vitro.* These data mirror the effect that IAA had on *Cdkn1a* expression in ovarian follicle cultures (Gonsioroski et al., 2020), though not in *in vivo* ovaries collected from the same animals in which we see induction in the pituitary (Gonsioroski et al., 2021). Assessing P21-immunopositivity *in vitro* revealed a marked increase in P21-positive cells*. In vivo* there was no significant difference in the number of P21-positive cells. It may be the case that in the systemic context, IAA affects P21 at too subtle a degree to be observable in our assay. However, with longer-term exposure, as could be the case with everyday drinking water, P21 protein could be induced, especially since we do see

consequences at the mRNA level. DNA damage induces P21 through p53, P21's primary transcriptional regulator (Jung et al., 2010; Huarte, 2016; Karimian et al., 2016). Interestingly, we observed an increase in *p53* mRNA expression *in vitro* where DNA damage and P21 were obviously induced, but not *in vivo* where P21 changes were not apparent. This suggests that IAA introduces DNA damage which then invokes P21. Inhibiting p53 in culture revealed that doing so eliminated the IAA-induced increase in P21 expression, confirming that P21/*Cdkn1a* induction by IAA is likely due to its role in increasing DNA damage (Supplemental Figure 1).

P21 pathway's primary action is to arrest the cell-cycle, decreasing proliferation. It can also stimulate DNA repair and apoptosis (Abbas and Dutta, 2009; Herranz and Gil, 2018). We assessed both proliferative (*Mki67*) and apoptosis-related mRNAs (*Bax* and *Bcl2*). Surprisingly, *Mki67* was not altered *in vivo* or *in vitro,* where P21 induction was obvious at both the mRNA and protein levels. P21/*Cdkn1a* induction by IAA may be insufficient to lead to cell-cycle arrest in enough cells to detectably alter mRNA. In adulthood, cells in the rodent pituitary show limited proliferation (Laporte et al., 2021) and this may contribute to subtlety of effects. *In vitro* analysis showed that apoptosis was also not affected by IAA exposure. These data would suggest that IAA-induced DNA damage and P21 activation would not ultimately affect the number of cells in the tissue, but may still affect their function through P21's involvement in gene transcription (Abbas and Dutta, 2009). Interestingly, it also shows that unlike in previous cell culture and ovarian experiments, IAA may not be cytotoxic in the pituitary (Plewa et al., 2004; Plewa et al., 2010, Dad et al., 2013; Gonsioroski et al., 2020; Gonsioroski et al., 2021).

Notably, body, uterine, ovarian, and liver weights were unchanged in these mice, suggesting any cytotoxicity in these tissues is also insufficient to change overall organ weight (Gonsioroski et al., 2021).

It appears that the mechanisms by which IAA induced DNA damage and P21/*Cdkn1a*, and those by which it reduced FSHβ/*Fshb* are independent, as they occurred at different exposure levels. It is common when studying toxicants to see lower dose effects that are not observed at higher dosages and vice versa (Vandenberg, 2014). One proposed mechanism for this involves the toxicant interfering with hormone receptor activity until a certain exposure concentration at which the body recognizes it and clears it from the system relieving the burden, or shuts down the receptor, reversing the effect. At higher levels, the toxicant might induce more broad toxic effects such as genotoxicity, as is reflected in our DNA damage results, through a mechanism distinct from how it acts at lower doses.

Prior to this study, virtually nothing was known about how IAA influences neuroendocrine systems. Our data reveal that IAA decreases expression of key mRNA related to reproduction including kisspeptin in the arcuate nucleus of the hypothalamus and *Fshb* in the pituitary, as well as reduces the number of FSHβ-positive cells, a key component of the hormone necessary for guiding ovarian follicle maturation. We have also shown that IAA can introduce DNA damage and induce P21/*Cdkn1a* expression in the pituitary. These data provide an important foundation for further study of IAA as a reproductive environmental toxicant.

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Conflicts of Interest (COI)

The authors have no conflicts of interest to declare.

FIGURES AND TABLE

Fig. 3.1: IAA increases mRNA expression of key hypothalamic hormone kisspeptin in the ARC while leaving other key hormones unchanged.

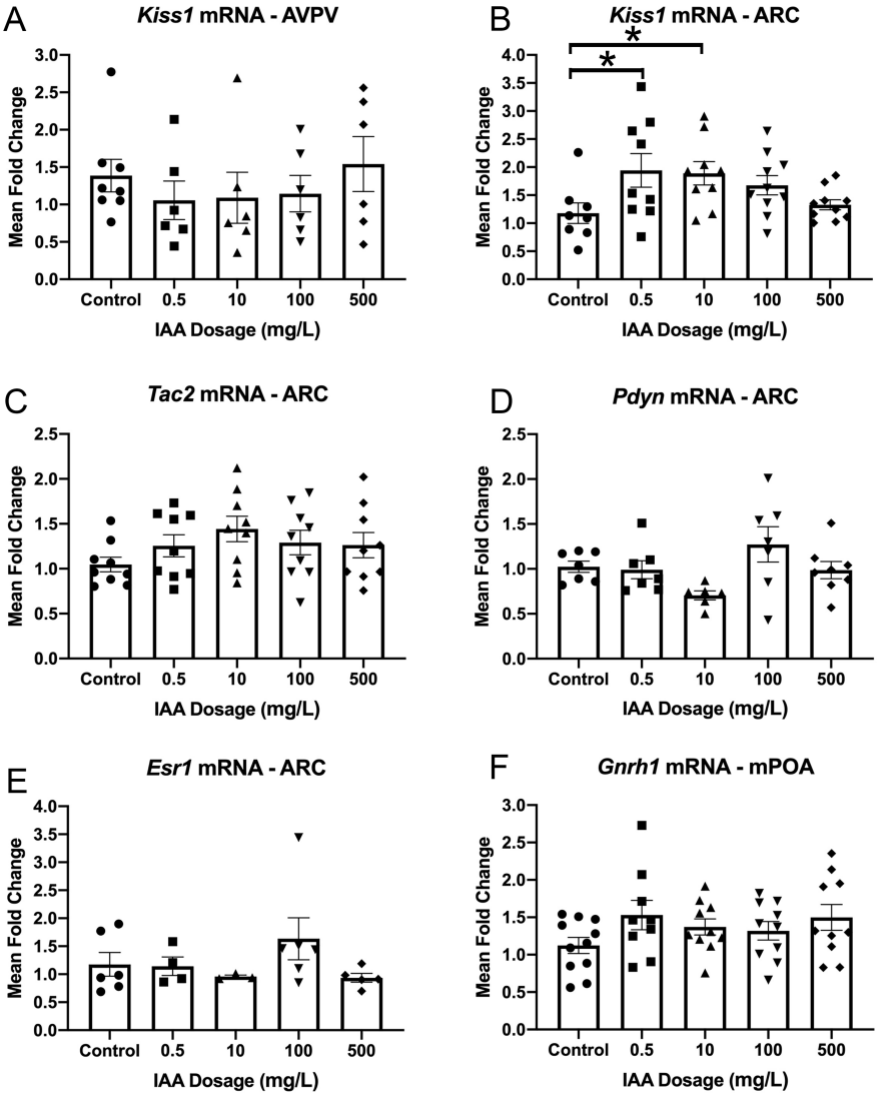


Fig. 3.1: IAA increases mRNA expression of key hypothalamic hormone kisspeptin in the ARC while leaving other key hormones unchanged. Female CD-1 mice were exposed to varying levels of IAA in their drinking water for 45 days. mRNA expression of kisspeptin (*Kiss1)* was assessed in the arcuate nucleus (ARC) and found to be significantly increased at 0.5 and 10mg/L dosages compared to controls (A, N=9-10). *Kiss1* was also assessed in the anteroventral periventricular zone (AVPV) and found unchanged (B, N=6-8). mRNA expression of the neuropeptides neurokinin B (*Tac2)* and dynorphin (*Pdyn*) were similarly unchanged between controls and any IAA condition (C, N=9; D, N=6-8). Estrogen receptor-a (*Esr1*) expression in the ARC was unchanged (E, N=3-6). Expression of gonadotropin-releasing hormone (*Gnrh1)* was also unchanged (F; N=10-11). One-way ANOVA for ARC *Kiss1*, p=0.02, *, p≤0.05 by Dunnett's post-hoc analysis.

Fig. 3.2: *In vivo*, IAA significantly reduced FSHβ protein and *Fshb* mRNA expression, while preserving expression of other key reproductive hormone-related genes and proteins

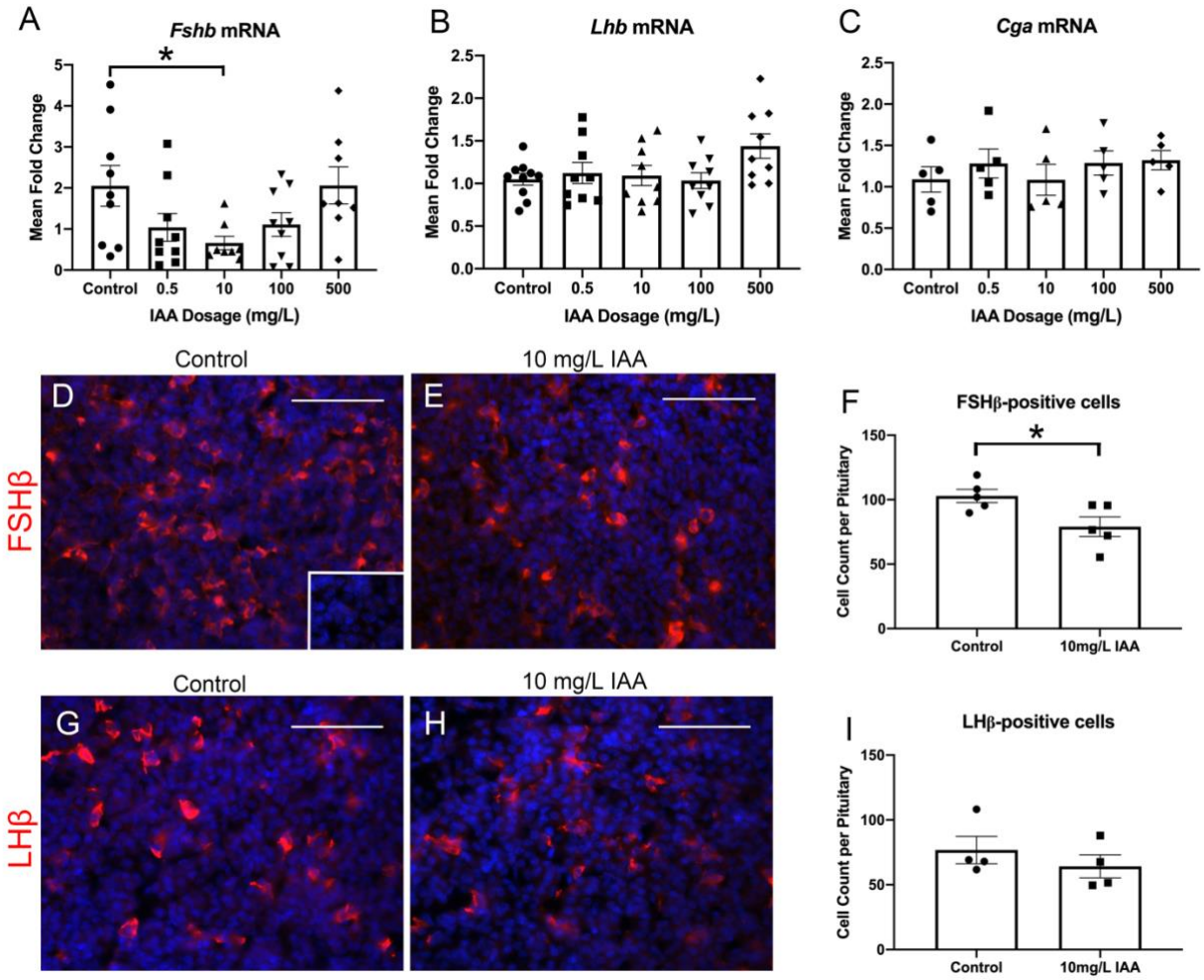


Fig. 3.2: *In vivo*, IAA significantly reduced FSHβ protein and *Fshb* mRNA expression, while preserving expression of other key reproductive hormone-related genes and proteins. qPCR analysis was performed on pituitary tissue harvested from mice exposed to a range of IAA levels in their drinking water. *Fshb* mRNA levels were significantly decreased in the 10mg/L IAA condition compared to controls (A, N=9-10). mRNA analysis revealed no impact of IAA exposure on *Lhb* (B, N=10) or *Cga* (C, N=5). Immunohistochemistry (IHC) for FSHβ was carried out on pituitary tissue harvested from mice exposed to either 0mg/mL or 10mg/L IAA in their drinking water (D and E, N=5). The number of positively stained cells were quantified revealing a significant reduction in the IAA exposed group (F, N=5). IHC for LHβ was performed to compare control and 10mg/L IAA groups (G and H, N=4). Quantifying positively stain cells indicated no significant difference between groups (I, N=4). Negative control for IHC stains (inset in panel D). One-way ANOVA for *Fshb* mRNA, p=0.04,*, p≤0.05 by Dunnett's post-hoc analysis. T-test analysis for FSHβ counts,*, p≤0.05. Scale bars = 75μM.

Fig. 3.3: *In vitro*, IAA significantly reduces *Fshb*, *Lhb*, and *Cga*, but not *Gnrhr* mRNA, as well as FSHβ, but not LHβ protein expression.

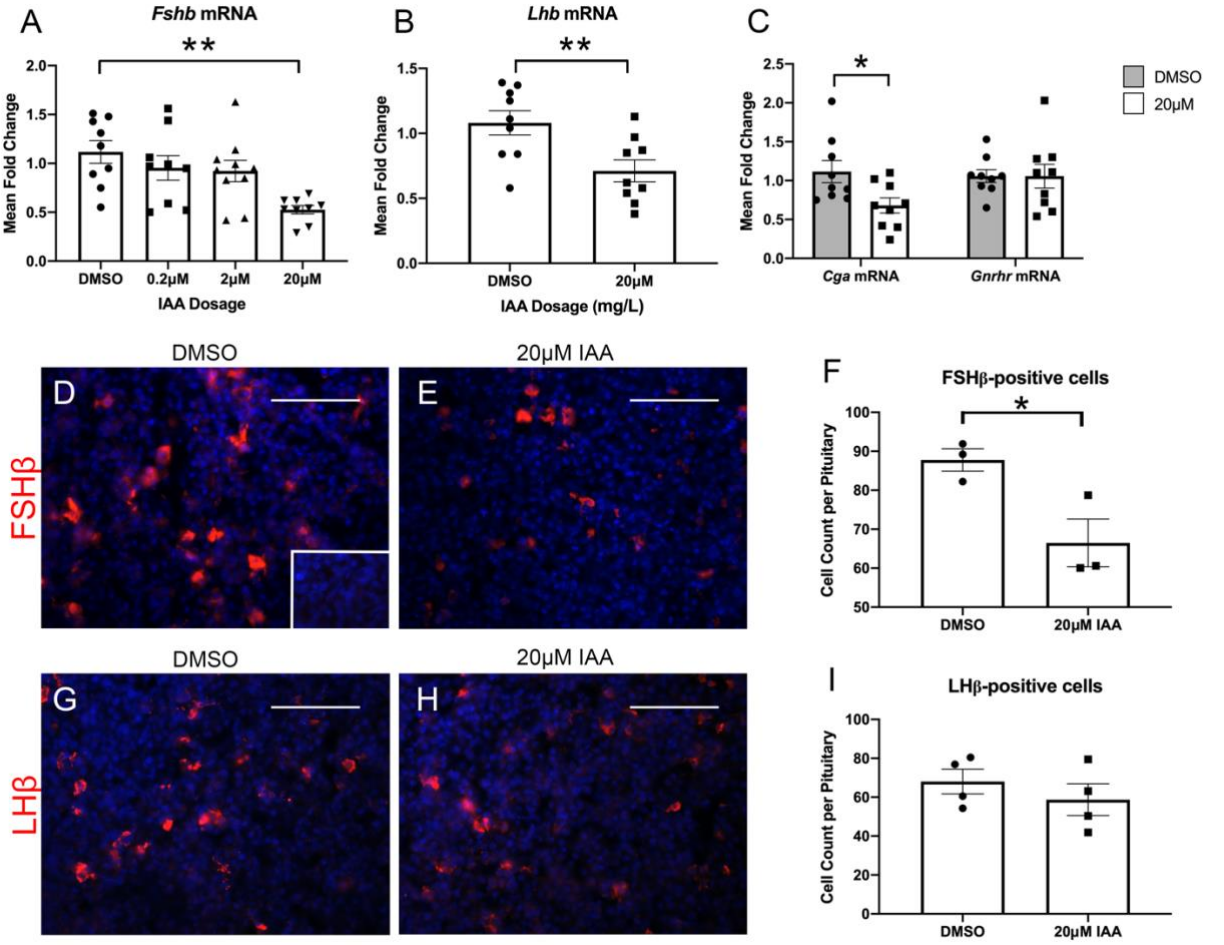


Fig. 3.3: In vitro, IAA significantly reduces Fshb, Lhb, and Cga, but not Gnrhr mRNA, as well as FSHβ, but not LHβ protein expression. qPCR analysis for *Fshb* was performed on explant cultures exposed to a range of IAA concentrations. *Fshb* mRNA levels were significantly decreased in the 20μM IAA condition compared to controls (A; N=9-10). mRNA expression was assessed for *Lhb*, *Cga*, and *Gnrhr* in an explant culture comparing DMSO-only treated explants with those treated with 20μM IAA. *Lhb* was significantly reduced in the IAA-treated condition (B; N=9). *Cga* was reduced with IAA treatment compared to controls as well, while *Gnrhr* was unchanged between conditions (C; N=9). An IHC for FSHβ on mice and exposed to either DMSO vehicle alone or 20μM IAA in culture for 48 hours revealed an apparent reduction in FSH-positive cells in the IAA-exposed condition compared to controls (D and E, N=3). This was verified by quantification (F, N=3). IHC for LHβ suggested no apparent change between DMSO and 20μM groups (G and H, N=4). Quantification showed there was no significant difference in number of protein-positive cells (I, N=4). Negative control for IHC stains (inset in panel D). Welsh's ANOVA for *Fshb* p=0.0003, **, p≤0.01 by Dunnett's T3 post-hoc analysis. T-test analyses for *Lhb*, *Cga* and FSHβ, *, p≤0.05; **, p≤0.01. Scale bars = 75μM.

Fig. 3.4: IAA increased DNA damage *in vivo* and in IAA-exposed explants

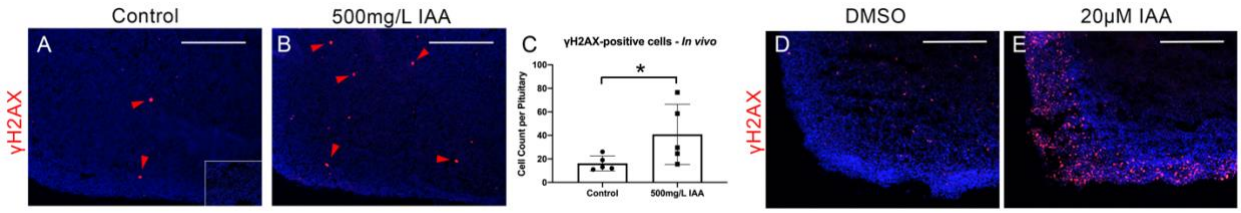


Fig. 3.4: IAA increased DNA damage *in vivo* and in IAA-exposed explants. IHC for the DNA damage marker γH2AX was performed on pituitary tissue collected from adult female mice exposed to 0mg/l IAA or to 500mg/L IAA *in vivo* (A and B, N=5). Quantifying the number of γH2AX-positive cells in the whole pituitary showed a significant increase in the IAA exposed group (C, N=5). Pituitaries exposed to either DMSO alone or 20µM IAA in whole explant cultures were also immunostained for γH2AX which revealed a visually marked increase in the number of positive cells (D and E; N=3). Negative control is shown as an inset in panel A**.** T-test analysis ,*, p≤0.05. Scale bars = 250µM.

Fig. 3.5: IAA increased P21 (*Cdkn1a*) mRNA *in vivo* and *in vitro*, and P21 positive cells *in vitro*. IAA also increased expression of *p53 in vitro*.

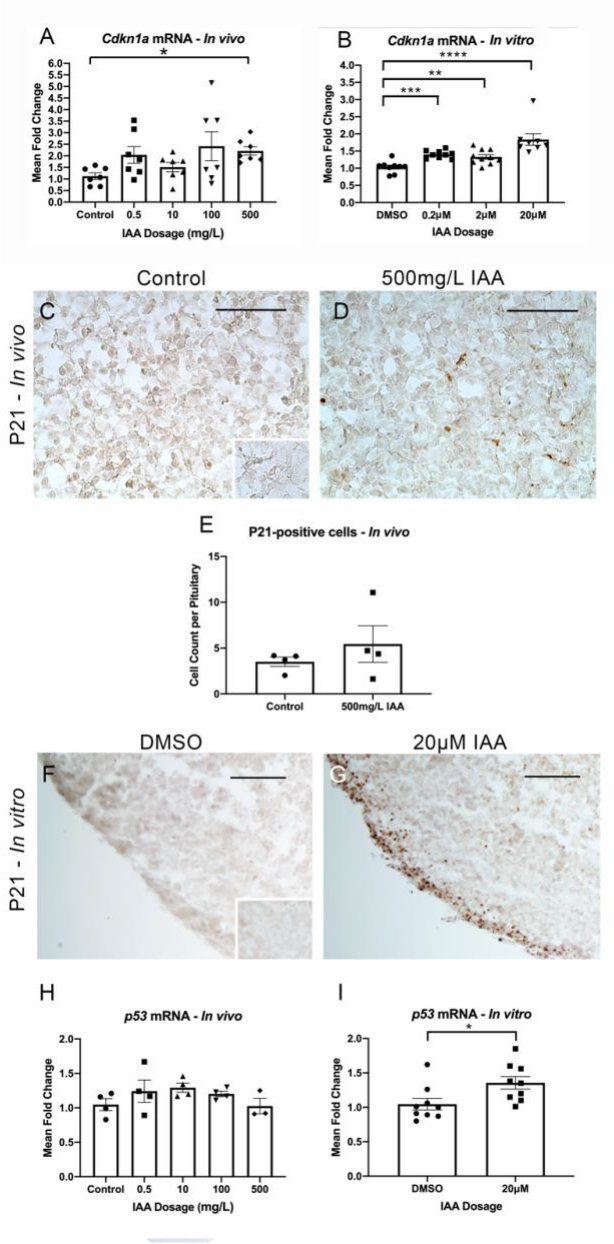


Fig. 3.5: IAA increased P21 (*Cdkn1a*) mRNA *in vivo* and *in vitro*, and P21 positive cells *in vitro*. IAA also increased expression of *p53 in vitro*. mRNA for P21 (*Cdkn1a*) was significantly higher in mice exposed to 500mg/L IAA *in vivo* compared to controls (A, N=7). There was a significant increase in *Cdkn1a* expression compared to controls at all doses of IAA *in vitro* (B, N=8-10). IHC for P21 was performed on control and 500mg/L IAA-exposed pituitary samples harvested *in vivo* (C and D, N=4). The number of positive cells was quantified, finding no difference between groups (E, N=4). P21 was also assessed in *in vitro* samples, which indicated a visually marked increase in the number of positive cells in the IAA condition (F and G, N=3). mRNA expression of p53 was assessed *in vivo* and showed no change between groups (H, N=3-4). *In vitro, p53* mRNA expression was significantly increased with IAA exposure (I, N=9). Negative control for IHC stains (inset in panels C and F***).*** Welsh's ANOVA for *Cdkn1a in vivo*, p=0.005, *, p≤0.05 by Dunnett's T3 post-hoc analysis. One-way ANOVA for *Cdkn1a in vitro*, p<0.0001, *, p≤0.05; **, p≤0.01; ***, p≤0.001; ****, p≤0.0001 by Dunnett's post-hoc analysis. T-test for *p53*, *, p≤0.05. Scale bars = 75μM (A, B), Scale bars = 250μM (D, E).

Fig. 3.6: IAA had no effect on proliferation as assessed by *Mki67* mRNA levels, nor did affect apoptosis assessed via *Bax* and *Bcl2* levels *in vitro*.

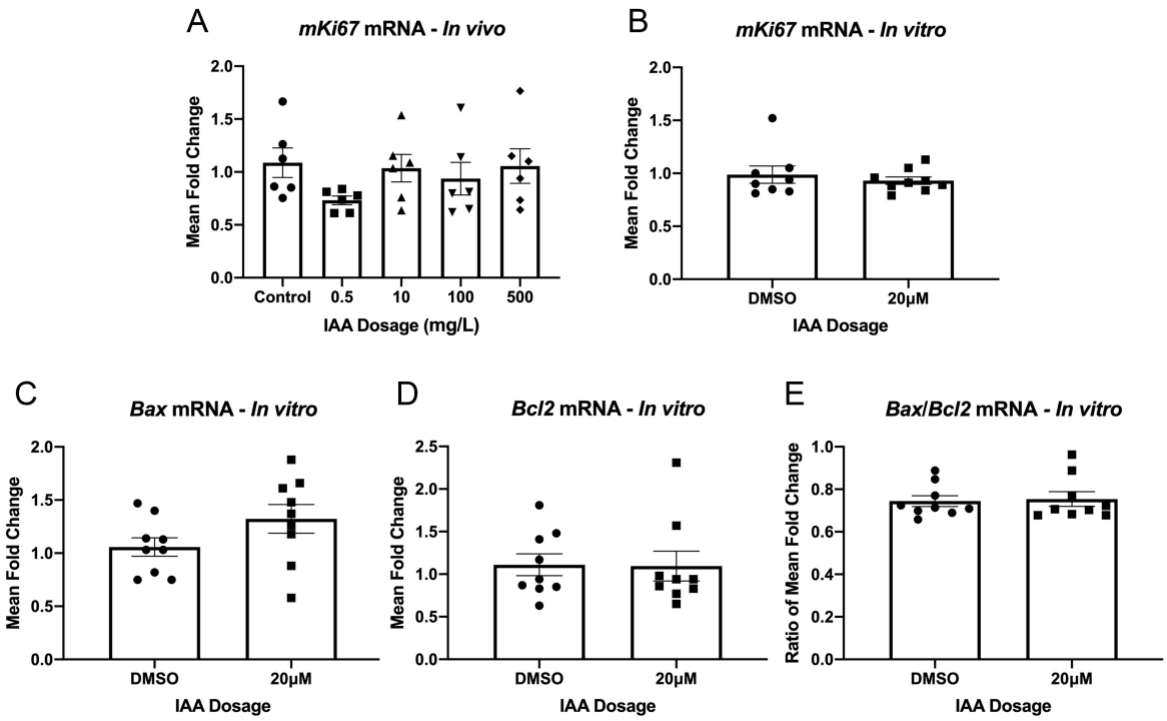


Fig. 3.6: IAA had no effect on proliferation as assessed by Mki67 mRNA levels, nor did affect apoptosis assessed via *Bax* and *Bcl2* levels *in vitro*. qPCR analysis for the proliferative marker Ki67 (*Mki67*) was unchanged in any IAA exposure level *in vivo* (A; N=6). *Mki67* expression was similarly unaffected by IAA exposure in explant culture (B; N=8-9). mRNA levels for the pro-apoptotic *Bax* (C; N=9) and the anti-apoptotic *Bcl2* (D; N= 9) were unchanged *in vitro*. The ratio of *Bax* to *Bcl2* was also unchanged by IAA exposure *in vitro* (E; N=9).

Table 3.1: qPCR primer sequences

Gene Name	*Forward 5' to 3'*	*Reverse 5' to 3'*
Bax	*TGAAGACAGGGGCCTTTTTG*	*AATTCGCCGGAGACACTGG*
Bcl2	*ATGCCTTTGTGGAACTATATGGC*	*GGTATGCACCCAGAGTGATGC*
Cdkn1a	*TTGGAGTCAGGCGCAGATCCACA*	*CGCCATGAGCGCATCGCAATC*
Cga	*GTATGGGCTGTTGCTTCTCC*	*GTGGCCTTAGTAAATGCTTTGG*
Esr1	*AATTCTGACAATCGACGCCAG*	*GTGCTTCAACATTCTCCCTCCTC*
Fshb	*TGGTGTGCGGGCTACTGCTAC*	*ACAGCCAGGCAATCTTACGGTCTC*
Gapdh	*GGTGAGGCCGGTGCTGAGTATG*	*GACCCTTTTGGCTCCACCCTTC*
Gapdh	*GGTGAGGCCGGTGCTGAGTATG*	*GACCCTTTTGGCTCCACCCTTC*
Gnrh1	*AGCCAGCACTGGTCCTATGG*	*CAGTACATTCGAAGTGCTGGGG*
Gnrhr	*ATGATGGTGGTGATTAGCC*	*ATTGCGAGAAGACTGTGG*
Kiss1	*TGCTGCTTCTCCTGT*	*ACCGCGATTCCTTTTCC*
Lhb	*CCCAGTCTGCATCACCTTCAC*	*GAGGCACAGGAGGCAAAGC*
Mki67	*AGTAAGTGTGCCTGCCCGAC*	*ACTCATCTGCTGCTGCTTCTCC*
p53	*CCAGCCACTCCATGGCCC*	*TGCACAGGGCACGTCTTCGC*
Pdyn	*GAGGTTGCTTTGGAAGAAGGC*	*TTTCCTCTGGGACGCTGGTAA*
Ppia	*CAAATGCTGGACCAAACACAAACG*	*GTTCATGCCTTCTTTCACCTTCC*
Tac2	*TTCCACAGAAACGTGACATGC*	*GGGGGTGTTCTCTTCAACCAC*

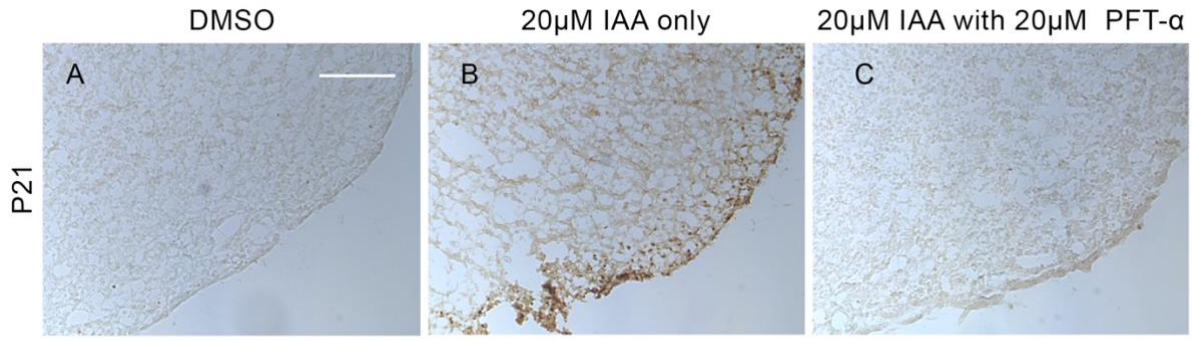
SUPPLEMENTAL METHODS:

p53-Inhibitor Experiment

Pituitaries were harvested from p40 or p41 female CD1 mice. For each exposure group, one to four pituitaries were placed on Millicell CM 6-well plate culture inserts (Millipore) and cultured in DMEM/F12 medium containing 10% charcoal stripped Fetal Bovine Serum (FBS, Sigma), and 10,000 IU Penicillin/10,000µg/ml Streptomycin (Fisher Scientific). Explants were divided into three groups: DMSO, 20µM IAA only (IAA), or

20μM IAA with 20μM of the p53-inhibitor pifithrin-α (PFT-α). IAA and PFT-α were dissolved in DMSO while the DMSO group received only 0.1% DMSO. There was an 8-hour pre-treatment period in which DMSO and IAA groups saw only media with .1% DMSO and the PFT-α group was exposed to 20μM PFT-α. After pre-treatment, explants were incubated in their respective group's media content for 48hrs. After this time, pituitaries were preserved for P21 IHC analysis as described in main methods. Culture was repeated a second time such that data shown in supplemental figure 1 represent three individuals from two independent cultures for each group.

SUPPLEMENTAL FIGURE 3.1:



Supplemental fig. 3.1: Inhibiting p53 negates effects of IAA exposure in inducing P21 expression. Immunohistochemistry for P21 was carried out on explant culture tissue exposed to DMSO, 20μM IAA alone, or 20μM IAA with 20μM pifithrin-α (PFT-α) for 48hrs in culture. The control group exposed to only DMSO showed virtually no positive staining (A). 20μM IAA-exposed tissue had noticeable staining on the periphery of the tissue (B). Adding 20μM PFT-α along with 20μM IAA negated any effect of IAA on P21 expression such that the tissue looked similar to that of the DMSO alone group (C). N=3, Scale bar = 100μm

Chapter 4: Developmental Exposure To Water Disinfection Byproduct Iodoacetic Acid Induces Pituitary mRNA Expression Of The Thyroid Stimulating Hormone Beta Subunit

ABSTRACT

Water disinfection by products (DBPs) result from unintended reactions between treatment disinfectants and substances present in the water. Iodoacetic acid (IAA) may be of particular concern. In cell culture studies, IAA was identified as having significant cyto- and genotoxic capabilities. Whole organ culture and systemic-exposure studies revealed it can alter gene expression of hormone-related genes in endocrine tissues, including reducing *Fshb* levels in the pituitary. Increases in expression of the cell cycle arrester P21/*Cdkn1a* suggested IAA may be especially consequential for proliferative tissues. Until the present study, studies have not examined the effects of IAA on the developing pituitary. This is noteworthy since the pituitary is vulnerable to proliferation-related disruption during development and, if its gene expression were to be altered, it could result in consequences for a number of endocrine axes. We tested the hypothesis that in the pituitary, developmental exposure to IAA would result in upregulation of *Cdkn1a*, suppression of the proliferative marker *Mki67*, and shifts in hormone-building mRNA. We found that in developmentally exposed mice thyroid stimulating hormone beta (*Tshb*) levels were significantly increased with 500mg/L IAA exposure. There was no effect, however, on TSHβ-positive cell number. *Pomc* mRNA levels were trending towards an increase with 500mg/L IAA in developmentally exposed mice. *Fshb*, *Lhb*,

Prl/PRL, and *Gh* levels were unchanged. Surprisingly, *Cdkn1a* and *Mki67* levels were also unaffected. These data suggest IAA may be a bigger threat to adult pituitaries than to developing pituitaries, though further research is warranted.

INTRODUCTION

Water disinfection byproducts (DBPs) are a relatively recently emphasized potential environmental toxicant. Disinfectants used to sanitize water and make it safe for human consumption can react with substances present in the water, forming a resultant byproduct. Foundational research in cell culture found that DBPs can have significant cytotoxic, genotoxic, and teratogenic effects (Plewa et al., 2004; Plewa et al., 2010; Dad et al., 2013; Pals et al., 2013). In such studies, one DBP, iodoacetic acid (IAA), was frequently identified as being among the most deleterious. IAA forms from the reaction between an oxidizing disinfectant, such as the widely-utilized chlorine, and iodide present in the water. As iodide is characteristic of salt water, coastal communities are at particular risk of exposure (Richardson, 2005; Dong et al., 2019). At present, there is no standard known level of exposure, however limited data suggest IAA levels in tap water can be as a high as 2.18μg/L (Wei et al., 2013). Total haloacetic acid level, the class to which IAA belongs, are as high as 136μg/L (Srivastav et al., 2020). Further, dermal contact through swimming and bathing, as well as additional oral exposure from food consumption are known exposure routes (Chowdhury et al., 2014; Allen et al., 2021). This exposure is concerning as research increasingly indicates IAA can be toxic.

Building off the previously mentioned data focused on cell cultures, studies from our lab and others have suggested that IAA poses organ-level and systemic risks. Interestingly, these data point to IAA as particularly relevant for endocrine tissues. *In vitro*, exposure to IAA leads to reduced folliculogenesis and estradiol secretion in the ovaries (Jeong et al., 2016; Gonsioroski et al., 2020). Reduced estradiol levels were also identified in serum *in vivo* (Gonsioroski et al., 2021). IAA exposure also impaired oocyte development in culture (Jiao et al., 2021), lead to thyroid dysregulation *in vivo* (Xia et al., 2018) and, in data from our lab, caused neuroendocrine changes in hormone-related gene and protein expression (Gonzalez et al., 2021). In addition to endocrine disruption, much of the existing research highlights IAA's role as a genotoxicant, as seen in cultured oocytes (Jiao et al., 2021) and both the *in vitro* and *in vivo* pituitary (Gonzalez et al., 2021). Additionally, IAA has been linked to changes in cell cycle-regulatory and apoptosis-related genes in ovarian follicle culture and *in vivo* ovaries (Gonsioroski et al., 2020; Gonsioroski et al., 2021). Changes to cell cycle-arrester P21/*Cdkn1a* were observed in the pituitary in prior data from our lab (Gonzalez et al., 2021). Together, these data build towards a picture of IAA as an endocrine system disruptor with strong potential to interfere with tissue health.

Notably, these prior studies had all been in adults *in vivo* or in cultured tissues and organs harvested from adult animals. This leaves open a major question: how does IAA affect the body during development? We wanted to address this question in the context of the pituitary, a structure that acts as the master regulatory gland for the endocrine system and, as such, is extremely influential in guiding physiology. The pituitary is

particularly vulnerable to perturbation during development. During this time, environmental toxicant exposures could influence cell proliferation and cell fate determination (Brannick et al., 2012; Eckstrum et al., 2018). As IAA has previously been shown to induce P21/*Cdkn1a,* it may be especially influential during development by discouraging cell proliferation. Additionally, if IAA targets hormone production-related functions, it may permanently alter the body's homeostatic set point during critical windows in which endocrine axes are being established. Both a reduction in cells and a reduction in the type or behavior of those cells could lead to long term dysregulation in a number of physiological functions.

Cells in the pituitary fall into five primary hormone-producing cell types, each of which are believed to secrete only one hormone category. Briefly, corticotropes secrete proopiomelanocortin (POMC) and guide the stress axis. Gonadotropes, secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and regulate the hypothalamic gonadal axis. Lactotropes secrete prolactin (PRL), the key hormone that allows for lactation. Somatotropes secrete growth hormone (GH) and are influential in bone and muscle development. The final category, thyrotropes secrete thyroid stimulating hormone (TSH) and make up the pituitary component of the pituitary-thyroid-axis. Prior data from our lab has shown that IAA targets FSHβ/*Fshb,* the beta-subunit that determines production of FSH. However, prior to this study, nothing was known about how developmental exposure to IAA could affect expression of FSHβ or any other pituitary hormone.

To address the question of how a developmental exposure to IAA effects the pituitary, we utilized an *in vivo* exposure in which dams consumed varying levels of IAA in their drinking water from conception through pup weaning. Pup pituitaries were harvested for analysis. We hypothesized that exposure to IAA during this period would lead to alterations in gene expression of key pituitary hormones, as well as an increase in *Cdkn1a* mRNA expression and reduce cell proliferation.

METHODS AND MATERIALS

Animal Care and Dosing

Cycling female CD-1 mice were obtained from Charles River Laboratories (Charles River, CA) and housed at the University of Illinois at Urbana-Champaign, College of Veterinary Medicine Animal Facility. The animals were kept under 12hr light-dark cycles and allowed ad libitum access to water and Teklad 8664 rodent diet (Envigo). Mice were divided in groups and provided varying levels of IAA in their drinking water. The groups were as follows: No IAA added (Control), 10mg/L, 100mg/L, or 500mg/L IAA. This corresponded to approximate ingestion levels of 3.48, 69, 490, and 1700µg of IAA per mouse per day (Gonsioroski et al., 2021). After 35 days, mice were mated to an unexposed male. During pregnancy, mice were singly-housed. For the duration of gestation through postnatal day 21 (PND21, typical day of weaning), cage water continued to be dosed with the corresponding level of IAA.

Tissue Collection

Pituitary glands were harvested from PND21 IAA-dosed and control female offspring of exposed dams. Dams were harvested on the first day in diestrus after their pups reached PND21. Tissue was either flash frozen on dry ice shortly following decapitation and stored at -80° C (for qPCR experiments) or preserved in 3.7% formaldehyde/phosphate buffered saline (PBS) for 60 min., then cryoprotected in in 30% sucrose/ PBS before embedding in Optimal Cutting Temperature (Tissue-Tek) and storage at -80° C until sectioning (for IHC experiments).

Adult *in vivo* and *in vitro* exposures

For analysis of adult exposed tissues, procedures were carried out as described in Gonzalez et al., 2021. Briefly, for in vivo studies, female CD1 mice were aged to 40 days and exposed to 0 (control), 0.5, 10, 100, or 500mg/L IAA in their drinking water for 35-40 days and harvested in diestrus. Pituitaries were stored in RNA Later (Thermo Fisher) and kept at -20° C until extraction of RNA and cDNA synthesis. For *in vitro* studies, briefly, whole pituitaries were harvested from female postnatal day 40 or 41 CD-1 mice and cultured in DMEM/F12 medium containing 10% charcoal stripped fetal bovine serum (FBS, Sigma), and 10,000 IU Penicillin/10,000µg/ml Streptomycin (Fisher Scientific). Explants were exposed to varying levels of IAA diluted in culture media based and incubated for 48 hours. IAA treatments were: 0.2 µM IAA, 2µM IAA, or 20µM IAA, all diluted in DMSO as a vehicle. Control cultures received only 0.1% DMSO.

Following the culture period, pituitaries were flash frozen and stored at -80° C until processed for RNAL extraction and cDNA synthesis.

Immunohistochemistry

All pituitary tissue was sectioned to 12μm using a cryostat (Leica). For each individual, 3 slides evenly spaced through the tissue with two sections on each slide were selected. Immunostaining was performed using antibodies for TSHβ (diluted 1:100, National Hormone and Peptide Program, AF Parlow) and PRL (diluted 1:100, National Hormone and Peptide Program, AF Parlow). Slides were air dried for 5 min. and fixed in 3.7% formaldehyde/PBS for a 10 min post-fixation period. All slides were blocked for 1 hour using 5% normal donkey serum (Jackson ImmunoResearch) diluted in a block containing 3% bovine serum albumen (Jackson ImmunoResearch), and 0.5% Triton-X100 in PBS before primary antibody treatment. Slides were incubated in primary antibodies overnight at 4°C. For all stains, a negative control slide was included that substituted incubation in block for primary antibody. Sections were then incubated in biotin conjugated anti-rabbit secondary antibodies (Jackson ImmunoResearch) for 1 hour at room temperature, followed by incubation with streptavidin cy3, for 1 hour at room temperature. Slides were mounted using antifade mounting medium (0.1 M Tris pH 8.5, 20% glycerol, 8% polyvinyl alcohol, 2.5% 1,4-diazabicyclo[2.2.2]octane) containing the nuclear stain 4′,6-Diamidino-2-Phenylindole, dihydrochloride (DAPI), and visualized with a fluorescent microscope (Leica).

Quantification of Immunohistochemistry Results

TSHβ and PRL experiments were quantified as follows. For each individual, 4-7 images were taken of each section on a given slide. A Cell Profiler (Broad Institute) pipeline was utilized to quantify the number of DAPI-stained and the number of protein-positive cells for each image. Images were not included in the analysis if the DAPI-staining revealed compromised tissue integrity or any other factor that would contribute to inaccurate quantification. The number of protein-positive cells was divided by the number of DAPI-stained cells to get a normalized cell count of the image. After, all images for each slide were averaged together. Normalized averages for each slide were added together to get an overall normalized average count for each individual.

RNA Extraction and cDNA synthesis

For pituitary glands, RNA extraction was performed using TRIzol reagent according to the manufacturer's instructions (Life Technologies). Pituitary glands were homogenized in TRIzol to disrupt the tissue. RNA yield and purity were determined by spectrophotometry. Using the ProtoScript M-MuLV First Strand cDNA synthesis kit (New England BioLabs, MA, USA), approximately 0.5µg of RNA was synthesized into cDNA. When limited by the amount of RNA yield, 6µl of RNA suspended in H_2O, the maximum allowed by the kit, was used. A no-enzyme sample was included as a negative control for each set of synthesis reactions.

RT-qPCR

RT-qPCR was performed on the cDNA samples using gene-specific primers. Results were analyzed using the double change in threshold cycle (ΔΔCT) method as previously described (Goldberg et al., 2011). Student's two-tailed *t*-tests were performed to ensure there were no significant changes in *Gapdh* between dosage groups, thus verifying it could suitably be used as a control gene. Primer sequences for each gene are shown in Table 4.1.

Statistical analyses

All statistics were performed using Graph Pad Prism 8.2.1. For qPCR analyses, statistics were performed on log-transformed ΔCT values. Variance was assessed using a Bartlett's test. Significance was determined using a one-way ANOVA followed by Dunnett's post-hoc analysis when variance was equal and using a Welsh's ANOVA followed by a Dunnett's T3 post-hoc analysis when variance was unequal. Post-hoc analyses compared experimental groups to controls. For cell count analyses, t-tests compared normalized cell counts from control and 500mg/L IAA groups.

RESULTS

IAA induced *Tshb* expression in developmentally exposed and adult mice while in vitro *Tshb* and thyroid hormone receptor expression was unchanged *in vivo*.

Prior data suggests the hypothalamic-pituitary-thyroid axis may be vulnerable to perturbation by IAA (Xia et al., 2018). We observed levels of the mRNA that encodes the unique beta-subunit that determines TSH expression, *Tshb*. *Tshb* was significantly induced with 500mg/L IAA exposure in developmentally exposed PND21 female mice (fig. 4.1A). IHC for TSHβ showed the normalized number of positive cells was unchanged between control and 500mg/L IAA conditions in these animals (fig. 4.1B-D). Adult exposure to 500mg/L IAA also led to an increase in *Tshb* mRNA expression (fig. 4.1E). However, *Tshb* was not altered in adult pituitary cultures exposed to varying levels of IAA (fig. 4.1F). Observing *Tshb* induction *in vivo*, but not *in vitro*, we looked for indications of contributions from systemic alterations within the thyroid hormone axis. Developmental exposure to IAA did not affect expression of thyroid hormone alpha (*Thra*) nor thyroid hormone beta (*Thrb*) receptors in PND21 animals (fig. 4.1G, H). *Aldh1a1* mRNA has been shown to increase in response to an increase in thyroid hormone (Gil-Ibáñez et al., 2014; Stoney et al., 2015). *Aldh1a1* was unchanged in our PND21 samples (fig. 4.1I).

Of the other four key pituitary hormones, only *Pomc* was borderline induced with IAA exposure, *Fshb*, *Lhb*, and *Prl* were unchanged with IAA exposure.

mRNA for *Fshb*, the beta-subunit unique to FSH, was reduced with adult exposure to IAA (Gonzalez et al., 2021). However, developmental exposure had no observable effect on *Fshb* mRNA expression (fig. 4.1A). Luteinizing hormone beta subunit, *Lhb,* expression was similarly unchanged (fig. 4.1B). An ANOVA comparing proopiomelanocortin (*Pomc*) levels in IAA-exposed PND21 individuals and controls showed a significant overall change between groups. A Dunnett's post-hoc test showed a trend towards a significant increase in expression between the control and 500mg/L dosage of IAA groups (fig. 4.2C). Prolactin (*Prl*) mRNA levels were not significantly altered (fig. 4.2D). IHC for PRL showed no change in the protein-positive cell count between groups (fig. 4.2E-G). Growth hormone (*Gh*) mRNA was unchanged by IAA exposure (fig. 4.2H).

mRNA levels of the cell-cycle arrester *Cdkn1a* and the proliferative marker *Mki67* were unchanged by developmental exposure to IAA.

Adult exposure to IAA significantly increased *Cdkn1a* expression *in vivo* (Gonzalez et al., 2021). In developmentally exposed animals, though, *Cdkn1a* mRNA expression is unchanged. Development is a pro-proliferative period that could be especially vulnerable to perturbation by changes in cell cycle arrest (fig. 4.3A). Expression of

Mki67 mRNA, a general cell cycle marker, was unchanged following IAA exposure (fig. 4.3B).

DISCUSSION

Prior studies on the consequences of exposure to iodoacetic acid suggested it could have deleterious effects on endocrine organs. Further, one of its best characterized mechanisms of action - inducing expression of cell cycle arrest genes - could be especially detrimental for developing tissue. We thus studied the consequences of developmental exposure to the water disinfection byproduct iodoacetic acid, focusing on the key mRNA related to hormones produced by each of the five pituitary hormone-secreting cell types and on induction of the cell cycle arrest gene *Cdkn1a*.

Our study uncovered an increase in *Tshb* mRNA in mice developmentally exposed to IAA. The increase in *Tshb* in response to IAA exposure also occurs in adult female pituitaries as well. We have reason to believe IAA's effects occur primarily at the level of the thyroid. We assessed *Tshb* levels in cultured pituitaries, collected as part of Gonzalez et al., 2021. In these cultured pituitaries, in the absence of systemic context, expression was not elevated. While there is currently no available data on the relative aggregation of IAA in the thyroid compared to the pituitary, the thyroid's reliance on iodide could make it a particularly vulnerable target of IAA, a byproduct of an iodide-involved reaction. One published study suggests IAA could dysregulate the hypothalamic-pituitary-thyroid (HPT) axis in the adult. Xia et al., 2018 showed that IAA

could target the thyroid to reduce thyroid stimulating hormone receptor (TSHR/*Tshr)* and sodium-iodide symporter (NIS/*Nis*) expression in adult rats. This would lead to hypothyroidism. However, this study did not make any observations of changes in pituitary gene or protein expression. Interestingly, Xia et al., 2018 found TSH serum reductions following IAA exposure, but only in males. TSH levels are often one of the most sensitive indicators of thyroid-level changes to hormone production, such that in humans they are the primary test used to assess functional thyroid hormone (TH) levels (Sheehan, 2016). Although we have not analyzed serum TSH levels in our study, we might expect it to be increased if *Tshb* mRNA is increased. This would be more consistent with an impairment of thyroid function. Low TH would lead to increased TSH because of the loss of negative feedback inhibition.

Besides its effect on *Tshb* mRNA expression, circulating TH also affects expression of several genes in the pituitary, including the thyroid hormone receptor subunits. Focusing on our PND21 samples, we find no difference in mRNA expression of thyroid hormone receptor alpha (*Thra*)*,* which is expressed widely throughout the pituitary, or thyroid hormone receptor beta (*Thrb*), which is expressed primarily in thyrotropes. This may mean that thyroid hormone levels themselves are not reduced. Alternatively, the change may be too subtle to meaningfully alter receptor levels or possibly, by the time the individual reaches PND21 having been exposed to IAA for their whole lives, their receptors may have adapted to a normal level. In a similar vein, we observed no change in TSHβ protein-positive cell number, indicating that potentially hormone levels are changed in the absence of cell number or that hormone protein levels may be more

resistant to alteration compared to mRNA levels. Additionally, mRNA for *Aldh1a1*, a well characterized TH response gene (Gil-Ibáñez et al., 2014; Stoney et al., 2015) did not change in this context. It is important to note, however, that *Aldh1a1* may only be sensitive to TH increases, unable to fall below a baseline expression with reduced TH. Nevertheless, the change in *Tshb* mRNA we observe is an example of endocrine disruption and an example of HPT axis dysfunction. To further confirm our findings, we plan to look at *Trh* mRNA expression in the hypothalamus of developmentally IAA exposed female mice. We would expect that *Trh* mRNA may be elevated like *Tshb* because it is also under negative feedback control from TH.

The HPT is just one of a number of axes guided by the pituitary. We wanted to carry out a survey of the effects of developmental IAA exposure in each of the other axes as well. In our prior study of adult IAA exposure, we found that both mRNA and protein of FSHβ was reduced with IAA exposure (Gonzalez et al., 2021). Here, we did not see the same result. There was also a very high degree of variability in mRNA levels, with some individuals within the control group displaying over 20 times the expression compared to others. There was similarly no change in *Lhb* mRNA. These results may be influenced by the mice's early stage of puberty in which some mice have vaginal opening, where others do not (Gonsioroski, personal communication). This means some animals may have drastically different levels of reproductive hormones compared to others. As such, this time period is a difficult one for observing changes to pituitary reproductive hormones. The pituitary component of the hypothalamic-pituitary-adrenal axis is characterized by expression of proopiomelanocortin (*Pomc*) by corticotropes. Here, we

find that exposure to IAA has a borderline significant effect inducing expression, suggesting IAA may play a role in dysregulating the stress axis, however the physiological consequences would require further study. We also observed prolactin (*Prl*) mRNA was variable in expression, possibly to due to hormonal differences between mice because *Prl* mRNA is influenced by estradiol levels (Maurer, 1982; Zárate and Seilicovich, 2010). mRNA of growth hormone (*Gh*) was unchanged by IAA exposure. Overall, our results suggest that the consequences of developmental exposure to IAA on pituitary hormone mRNA is subtle or absent.

Finally, we were interested in whether there were changes in *Cdkn1a* and *Mki67* with an early life exposure to IAA. In adult-exposed mice, we observed an increase in *Cdkn1a* mRNA, likely induced by DNA damage, but no related decrease in the proliferation marker Ki67 (*Mki67*) (Gonzalez et al. 2021). This is despite one of the primary consequences of P21/*Cdkn1a* pathway induction being cell cycle arrest (Abbas and Dutta, 2009; Herranz and Gil, 2018). We thought it may be the case because the pituitary is not very proliferative in the adult. However, during the early weeks of postnatal life, the pituitary almost doubles in size (Laporte et al., 2021). PND21 would fall at the end of this period with markedly less proliferation than in the first postnatal week, but likely higher levels than in adult exposure, making any potential effects of IAA exposure more consequential. However, developmental exposure as observed at PND21 had no effect on *Cdkn1a* nor *Mki67* levels, suggesting that pups exposed through maternal consumption are spared IAA's genotoxic effects. However,

assessment of earlier timepoints would be required to ensure that no effects went unobserved due to harvesting tissue at weaning.

Notably, *Tshb* and borderline *Pomc* induction both occur in the group exposed to 500mg/L IAA, the highest tested level. Between this fact and an absence of change in other hormones, it may be that the mother's body is fairly successful at filtering the IAA she consumes and exposure isn't heavily transferred to pups. This may also contribute to variability observed in the data as each dam may have slightly different capacities to process IAA. Broadly, we find that at most concentrations of IAA, maternally-exposed pups seem to have largely healthy pituitaries. This is an optimistic finding in regard to the risk of IAA exposure. Early development is a vulnerable time with high potential for long term consequences. An absence of exposure consequences could be met with relief. Still, the full consequence of IAA exposure is not known. By looking at individuals at weaning, we may not be capturing the most vulnerable period. Further, IAA remains far from fully observed even in varied systems in adulthood. Therefore, it is vital we continue to probe the question of IAA exposure risks in as many contexts as possible.

FIGURES AND TABLE

Fig. 4.1: IAA reduced *Tshb* expression in *in vivo* exposed mice while *in vitro Tshb* and thyroid receptor expression was unchanged.

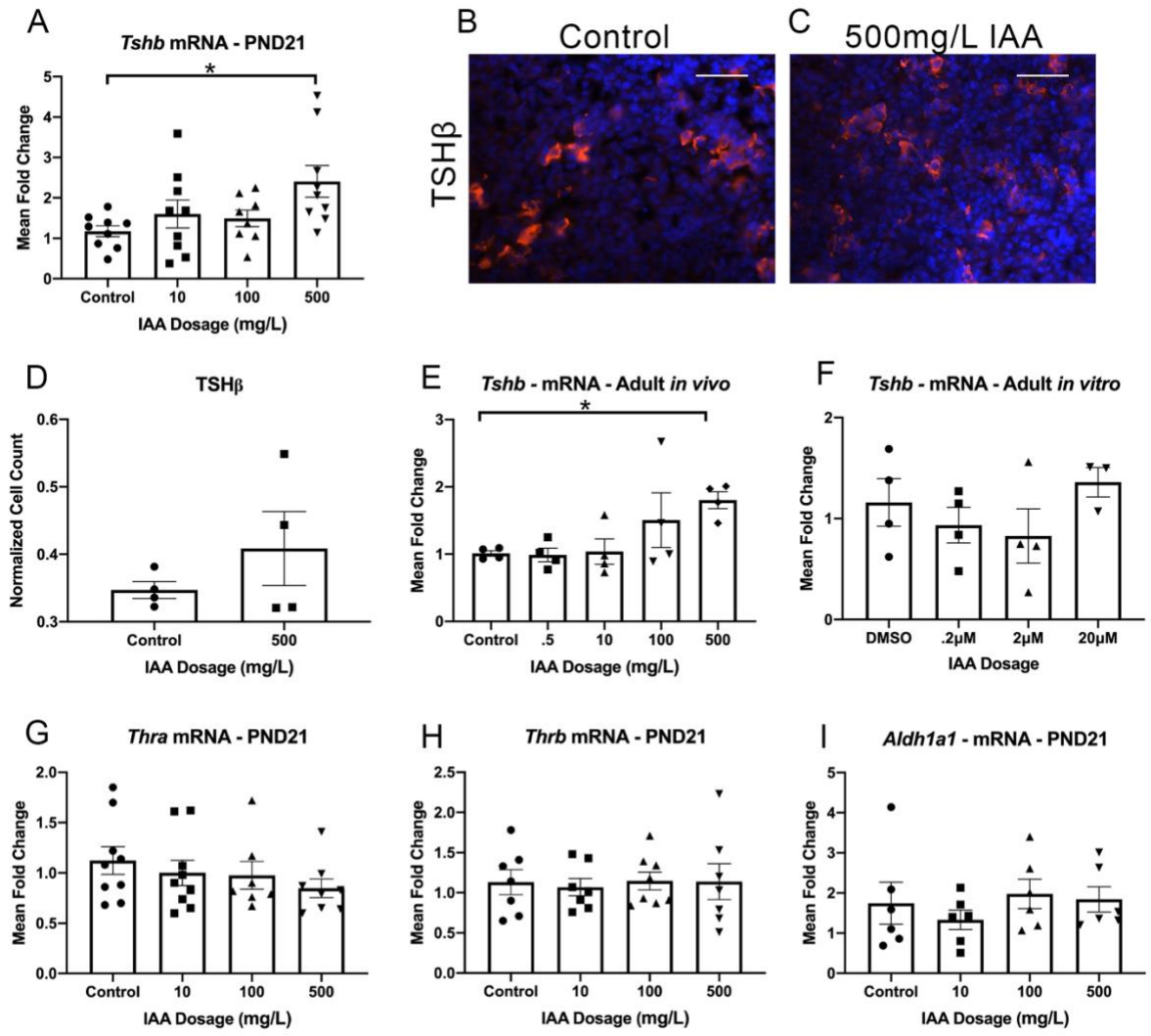


Fig. 4.1: IAA reduced *Tshb* expression in *in vivo* exposed mice while *in vitro Tshb* and thyroid receptor expression was unchanged. qPCR for *Tshb* was performed on whole pituitary samples harvested from postnatal day 21 pups exposed to a range of IAA dosages through their mother from conception until day of weaning. 500mg/L IAA significantly increased mRNA expression levels (A; N=9). IHC for TSHβ was performed comparing control and 500mg/L IAA groups (B, C; N=4). Quantifying normalized number of positively-stained revealed no difference between groups (D; N=4). qPCR for *Tshb* in pituitaries exposed to a range of IAA dosages in adulthood showed a significant increase in expression in those exposed to 500mg/L IAA (E; N=4). *Tshb* mRNA levels were assessed in cultured pituitary tissue finding no difference between any exposure level and controls (F; N=4). Assessing mRNA levels of thyroid hormone receptor alpha (*Thra*) in individuals exposed to IAA developmentally showed no change in any exposure group (G; N=7-9). Thyroid hormone receptor beta (*Thrb*) was similarly unchanged in any group (H; 7-8). mRNA for *Aldh1a1*, a TH responsive gene, was not changed by developmental IAA exposure in any group compared to controls (I; N=6). Welsh's ANOVA for PND21 *Tshb*, p=.0368, Dunnett's T3 post hoc analysis, p=.0450; Welsh's ANOVA for Adult *in vivo Tshb*, p=.0118, Dunnett's T3 post hoc analysis, p=.0111; Scale bar = 100 μM (B,C).

Fig. 4.2: Of the other four key pituitary hormones, only *Pomc* mRNA was borderline induced with IAA exposure, *Fshb*, *Lhb*, and *Prl* were unchanged.

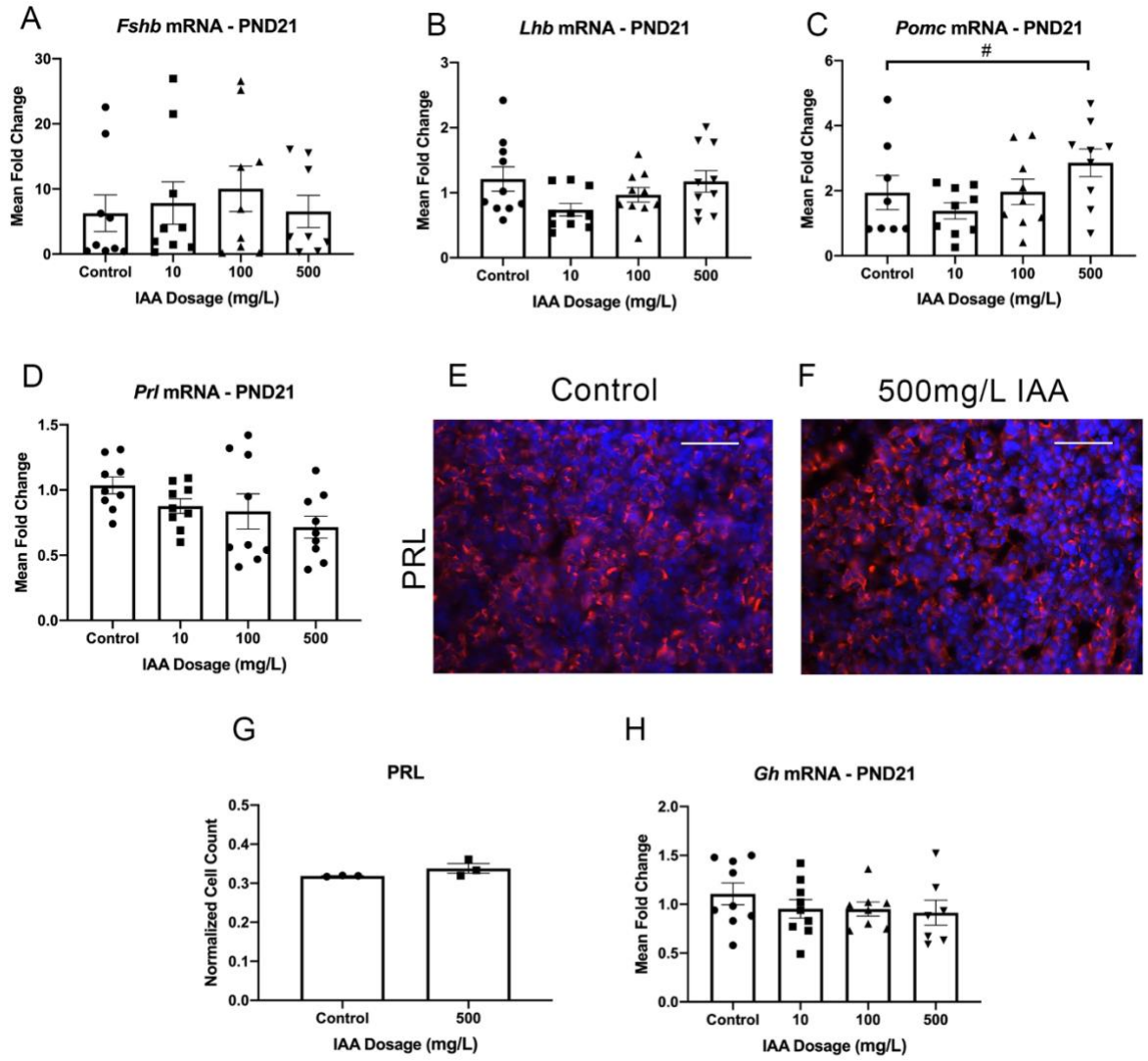


Fig. 4.2: Of the other four key pituitary hormones, only *Pomc* mRNA was borderline induced with IAA exposure, *Fshb*, *Lhb*, and *Prl* were unchanged. qPCR analysis of *Fshb* mRNA showed no change between controls and any dosage of IAA in developmental exposure (A; N=9). mRNA levels of *Lhb* were similarly unchanged by IAA exposure (B; N=9). There was a borderline increase in *Pomc* expression in 500mg/L IAA-exposed individuals compared to controls, as assessed by qPCR (C; N=8-9). *Prl* levels were unchanged by IAA (D; N=9). IHC was carried out for PRL and showed no difference in the number of Lactotropes between IAA-exposed individuals and controls (E-G, N=3). qPCR for *Gh* showed IAA had no effect on mRNA expression levels (H, N=9). #, one-way ANOVA for *Pomc*, p=.0402, Dunnett's post hoc analysis, p=.0564; Scale bar = 100 µM (E,F).

Fig. 4.3: mRNA levels of the cell-cycle arrester *Cdkn1a* and the proliferative marker Mki67 were unchanged by developmental exposure to IAA.

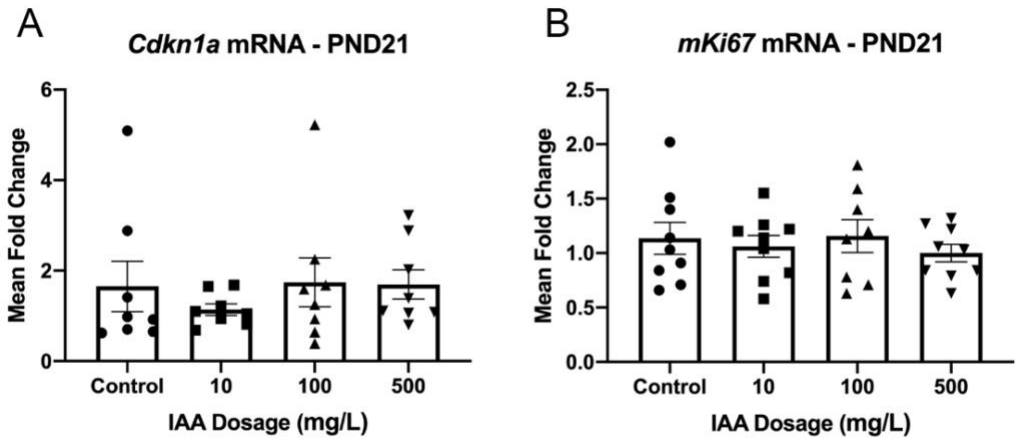


Fig. 4.3: mRNA levels of the cell-cycle arrester *Cdkn1a* and the proliferative marker *Mki67* were unchanged by developmental exposure to IAA. In order to observe effects on cell cycle arrest and proliferation in PND21 mice exposed developmentally to IAA, mRNA levels of *Cdkn1a* and *Mki67* were assessed. There was no effect of exposure on *Cdkn1a* levels (A; N=8), nor *Mki67* (B; N=8-9).

Table 4.1: qPCR primer sequences

Gene Name	*Forward 5' to 3'*	*Reverse 5' to 3'*
Aldh1a1	*GCTGGCTACAATGGAGGCACTC*	*CATGAGCATTGGAAAATTCCAGGG*
Cdkn1a	*TTGGAGTCAGGCGCAGATCCACA*	*CGCCATGAGCGCATCGCAATC*
Fshb	*TGGTGTGCGGGCTACTGCTAC*	*ACAGCCAGGCAATCTTACGGTCTC*
Gapdh	*GGTGAGGCCGGTGCTGAGTATG*	*GACCCTTTTGGCTCCACCCTTC*
Gh	*AACTGAAGGACCTGGAAGAG*	*GGCGTCAAACTTGTCATAGG*
Lhb	*CCCAGTCTGCATCACCTTCAC*	*GAGGCACAGGAGGCAAAGC*
Mki67	*AGTAAGTGTGCCTGCCCGAC*	*ACTCATCTGCTGCTGCTTCTCC*
Pomc	*TGTACCCCAACGTTGCTGAG*	*AGGACCTGCTCCAAGCCTAA*
Prl	*TCAGCCCAGAAAGCAGGGACA*	*GGCAGTCACCAGCGGAACAGA*
Trha	*CTACGTCAACCACCGCAAACAC*	*CTGCACTTCTCTCTCCTTCATCAG*
Trhb	*TGGTGCACTGAAGAATGAGC*	*AGTGGTACCCTGTGGCTTTG*
Tshb	*GCCGTCCTCCTCTCCGTGCTT*	*AGTTGGTTCTGACAGCCTCGTG*

CHAPTER 5: Conclusions and Future Directions

Often, the body is regarded as a context independent from the environment, especially in biological research. This neglects a vital layer of understanding how bodies function, being that they are constantly responding to external stimuli. As the neuroendocrine system regulates physiology, it is one of the most important frames through which to view this question. Additionally, due to critical windows, neuroendocrine developmental context can play a significant role in determining homeostatic setpoints that govern long-term patterns in physiology. In the presented work, we illustrated two exposures that disrupt endocrine function (gestational diabetes, chapter 2 and iodoacetic acid, chapters 3 and 4) and two time points (development and adulthood). In doing so, we aimed to bridge the gap between data suggesting an exposure contributes to a physiological outcome and understanding how that contribution is exerted.

Gestational diabetes mellitus alters the development of tanycytes and the median eminence in the offspring

The gestational diabetes mellitus (GDM) growth environment has been frequently associated with adverse metabolic outcomes, predisposing offspring to higher weight and to the quality of life-limiting conditions to which it is associated, like metabolic syndrome (Clausen et al., 2008; Clausen et al., 2009; Lowe et al., 2018; Deierlein et al., 2011; Ardıc¸et al., 2020). The presence of this phenotype beginning in childhood points towards GDM having a deleterious influence on the development of energy

homeostasis-regulatory hypothalamic functions. However, the hypothalamus is relatively understudied in this context. In chapter 2, we identified the consequences of being born to a mother with GDM, focused on the arcuate nucleus (ARC) of the hypothalamus, its unique postnatal stem cell population that also has nutrient sensing responsibilities – the tanycytes – and the median eminence (ME), the region through which signals from the body are conveyed to the hypothalamus. In the work presented, we illustrated for the first time that GDM targets key cellular and signaling tools contributing to the hypothalamus' ability to respond to nutritional signals from the periphery.

Our study identified reduced ME proliferation in the context of GDM offspring. Using a mouse model of gestational diabetes, we employed BrdU labeling to identify proliferating cells during the onset of GDM. We found fewer BrdU-positive cells in the ME of pups born to mothers with GDM. This is noteworthy as the hypothalamus integrates nutritional signals from the body, conveyed through the ME, to guide energy homeostasis (Goodman and Hajihosseini, 2015). The cell population in this region is fairly heterogeneous, comprised of neurons, glia, and endothelial cells (Rodríguez et al., 2010; Langlet, 2014; Rizzoti and Lovell-Badge, 2017). While it is currently unclear which cell types are lost, the relatively low cell density points to each cell having a greater significance than those in more densely and homogenously populated regions. Future studies would benefit from following up on this observation to identify the affected cell type and asses its function. This finding illustrated for the first time that GDM targets

median eminence, which may inhibit successful nutritional communication and underly the high weight phenotype seen in offspring.

Through this study, we also demonstrated for the first time that baseline insulin signaling is elevated in the superior border of the median eminence in offspring of GDM pregnancies. This region contains the β2-tanycyte population, however in the present experiment, we cannot say for certain that the β2-tanycytes were the only cells displaying this behavior. A future study should include co-staining for a known β2-tanycyte marker, clarifying this question. Tanycytes are known to be responsive to nutritional signals (Balland et al., 2014; Elizondo-Vega et al., 2015; Goodman and Hajihosseini, 2015) and may be influential in insulin signaling in particular (Yoo et al., 2020). An elevated basal level of signaling, in which no insulin is present to induce it, could indicate a decoupling of stimuli and the cell's nutrient responsiveness. Prior research in orexin knockout mice illustrated that an induction in basal pAKT in the hypothalamus could be associated with high fat diet-induced obesity (Tsuneki et al., 2008). In the GDM context, it could similarly be indicative of dysregulated feeding.

While these data represent a valuable step in understanding how GDM influences offspring physiology, there is, of course more work to be done. One significant limiting factor in pursuing this question further is the lack of a reliable, fully descriptive GDM model, as described in the introduction to this paper (Chapter 1, 2.5: Models of Gestational Diabetes Mellitus). In our study, we were able to replicate the key factors of temporally-appropriate maternal insulin resistance and heavy offspring, however not

every group has had the same success. Developing such a model would significantly progress the field. Our data suggest future studies would benefit from focusing on hypothalamic nutritional sensing, including the development of the median eminence. This region is relatively poorly studied and it may be a key factor in how GDM exerts an effect. The ME is able to act as a communication point due to leaky a leaky blood brain barrier (Rizzoti and Lovell-Badge, 2017). Future studies could, for example, explore whether its barrier functions are altered by GDM. Additionally, we focused on insulin signaling, but leptin signaling is also an important component of dysregulated feeding behavior and that may prove an interesting subject of study. Additionally, to the best of our knowledge, no studies have utilized a GDM model to observe physiological outcomes and hypothalamic function at multiple timepoints. The latest timepoint observed in our study was postnatal day 9; it would be valuable to know, empirically, whether the insulin signaling effects we observe in early postnatal life persist into adulthood or whether any other trends emerge. The question of how the GDM growth environment alters hypothalamic development and function is far from fully resolved and there are many specific questions still to be answered. However, our research valuably contributes to bridging the gap specifically by highlighting the hypothalamus' nutritional communication resources, an often-overlooked lens.

Iodoacetic acid, a water disinfection byproduct, disrupts hypothalamic and pituitary reproductive regulatory factors and induces toxicity in the female pituitary

Water disinfection is a vital public health service, however there is reason to believe it poses a risk to human health. Water disinfection byproducts can form as a consequence of unintended reactions between disinfectants and substances present in water. These compounds, called water disinfection byproducts, or DBPs, have been linked to a range of epidemiological consequences, notably for reproduction (Villanueva et al., 2015). They have also been found in cell culture to have significant cyto- and genotoxic capabilities (Plewa et al., 2004; Plewa et al., 2010; Dad et al., 2013; Pals et al., 2013). Iodoacetic acid (IAA) has been identified as one of the most toxic DBPs in these cultures. Further, IAA has been shown in whole organ cultures and *in vivo* contexts to have deleterious effects on reproductive endocrine tissue in particular. Despite their roles as master regulators of the HPG axis, prior to the work described in this document, no previous studies had focused on IAA's consequences for the hypothalamus and pituitary. Chapters 3 and 4 represent the first to do so, illustrating adult and developmental exposures, respectively.

In chapter 3, we observed IAA's capacity to induce mRNA expression of a key hypothalamic reproduction-regulatory gene for the first time. The hypothalamus regulates reproductive endocrine function primarily through two neuronal types, the kisspeptin (*Kiss1*) expressing neurons which reside in the ARC and the anteroventral periventricular nucleus (AVPV), and gonadotropin-releasing hormone (GnRH) neurons

which reside in the medial preoptic nucleus. Estrogenic feedback from the ovaries acts on kisspeptin neurons to promote the pulsatile release of GnRH (ARC) during nonovulatory periods or the GnRH surge (AVPV). We find elevated *Kiss1* expression *in vivo* in the ARC population, indicating at the hypothalamic level, IAA interferes with appropriate regulation of pulsatile GnRH during non-ovulatory phases of the estrous cycle. It is still unknown, however, how IAA induces these effects. We observed no changes in autoregulatory mRNA from ARC *Kiss1* neurons. We also saw no significant change in mRNA for estrogen receptor alpha, the major receptor responsible for conveying estradiol levels from the ovaries, and there were no observed changes in serum estradiol at the dosage we observe hypothalamic results reported in a parallel study on the same animals (Gonsioroski et al., 2021). This suggests that ovarian estradiol feedback may not be the primary driver of *Kiss1* results. Future studies should attempt to elucidate the relationship. Hypothalamic slice cultures could provide a valuable tool, honing in on whether the effects are systemic or at the level of the pituitary and allowing for more targeted manipulations. Additionally, future studies should focus on KISS1 protein. This was not feasible in our study due to limitations of the tissue preservation methods utilized, but is nonetheless valuable information for understanding how the hypothalamus reacts to IAA.

Interestingly, when looking at the level of the pituitary, it is apparent the two neuroendocrine organs are influenced through independent mechanisms. If elevated ARC *Kiss1* levels were inducing pituitary-level effects *in vivo*, they would be expected to increase *Lhb* and *Fshb* expression. Instead, we saw a decrease in the beta-subunit that

determines follicle-stimulating hormone (FSH; *Fshb*) mRNA and protein (FSHβ). This decrease was not accompanied by a luteinizing hormone (LH; *Lhb)* shift despite the two mRNA being expressed in the same cell type, gonadotropes, and sharing a regulatory mechanism, GnRH pulse frequency (Tsutsumi and Webster, 2009; Thompson and Kaiser, 2014; Coss, 2018). Using a pituitary explant system, we observed similar consequences for *Fshb*/FSHβ levels. Suggests that at least part of the mechanism by which IAA suppresses *Fshb*/FSHβ is direct at the level of the pituitary. There are a number of known mechanisms that differentially regulate FSH and LH. Namely, inhibitory inhibins and follistatins, and stimulatory activins. Serum levels of inhibin B were assessed in a parallel study on the same animals which found no changes (Gonsioroski et al., 2021). Inhibins are not produced locally in the pituitary and preliminary qPCR assays for activins and follistatins in the pituitary failed to give any indication of alteration. mRNA for activin receptors and for *Nr4a3*, an orphan receptor and GnRH-dependent negative-regulator of FSH secretion (Terashima et al., 2020), similarly showed no evidence of potential shifts (data not shown). It may be the case that IAA altered expression of these genes enough to affect *Fshb*/FSHβ, but levels returned to normal by the time of tissue harvest. Future experiments will be vital to clarifying our observations, including through assessing serum levels of inhibin A, activins, and follistatins as they may be altered, and protein expression of the latter two in the pituitary, which could be less adaptable to influence than mRNA. Beyond these factors, to the best of our knowledge, no other mechanisms of FSH regulation absent of LH regulation are known. In the explant exposure context, we also see a reduction in *Lhb*, though not in LHβ, and in *Cga,* the alpha-subunit common to *Fshb* and *Lhb*. This

indicates a potential direct mechanism for IAA to decrease other hormone-building mRNAs for which the *in vivo* context is able compensate. Future studies should focus on how IAA targets these mRNA directly and what factors contribute to the differences in expression observed *in vivo* compared to *in vitro.*

One of the best characterized consequences of IAA exposure is DNA damage (Plewa et al., 2010; Jiao et al., 2021). Using a γH2AX immunohistochemistry assay, we found that IAA exposure significantly introduced DNA damage in pituitary tissue. While this was previously observed in ovarian cultured cells, this was the first example of such an effect *in vivo* and in the pituitary. DNA damage is known to activate the P21/*Cdkn1a* pathway in a p53-dependent manner (Abbas and Dutta, 2009; Herranz and Gil, 2018). P21 has been shown to be an influential constraint for tumors in the pituitary, contributing to their unique cancer-resistance (Chesnokova et al., 2008; Chesnokova and Melmed, 2009). Additionally, *Cdkn1a* was upregulated in ovarian follicle cultures exposed to IAA (Gonsioroski et al., 2020). In our study, we found upregulation of Cdkn1a in both *in vivo* and *in vitro* contexts, as well as a marked induction of P21 *in vitro*. We found this protein induction does not occur when treated with a p53-inhibitor, suggesting that IAA introduces DNA damage, which then activates P21/*Cdkn1a*. To the best of our knowledge, this is the first such example in any tissue. One of the primary functions of the P21 pathway is cell cycle arrest and, interestingly, we saw no changes in mRNA for proliferative marker *Mki67*. The pituitary undergoes a precipitous drop in proliferation in the first few postnatal weeks (Laporte et al., 2021), so this observation may be due to a limited number of cells proliferating in the first place. The P21 pathway

also engages in a number of other functions including DNA damage repair and transcriptional regulation (Karimian et al., 2016). Future studies should explore whether the observed P21 increase affects these or other outcomes.

The data presented in Chapter 4 represent a part of the only *in vivo* developmental IAA exposure study carried out on any tissue, to the best of our knowledge. We aimed to do a survey of how this exposure influenced mRNA expression of the six major hormones secreted by the five primary hormone types in the pituitary. They are as follows, gonadotropes which secrete FSH and LH, thyrotropes which secrete thyroid stimulating hormone (TSH), corticotropes that secrete proopiomelanocortin (POMC), lactotropes which secrete prolactin (PRL), and somatotropes which secrete growth hormone (GH). Of these, *Pomc* mRNA was trending towards an increase, while the novel significant finding was in mRNA for the beta-subunit that dictates TSH production, *Tshb*. We found that providing IAA in dam drinking water from conception through postnatal day 21, the day of weaning, lead to an upregulation of *Tshb* in pups. We also found this result when assessing mRNA levels in adult-exposed tissue harvested for the study in Chapter 3, while finding no change in the pituitary explant experiments from that study. This suggests *Tshb* effects are not direct at the level of pituitary and may instead be thyroidal. In support of this idea, previous research on the hypothalamic-pituitary-thyroid axis showed IAA exposure could disrupt key factors within the axis, including T3 levels, which were decreased by exposure (Xia et al., 2018). Future studies should focus on the thyroid more directly and completely, in conjunction with a pituitary study to clarify any relationship present.

While our findings on developmental exposures are important, there are caveats that warrant further study of this question. We observed both mRNA and protein of *Tshb*/TSHβ and *Prl*/PRL, however, we did not observe protein levels of other hormones which may have shifted. Importantly, we did not observe hormone release, a potential major factor in altered pituitary function. Subsequent studies should follow-up on this potential. Further, future studies should observe effects of developmental exposure in adulthood as it is possible for long-term consequences to become apparent over time.

CONCLUSION

The studies presented in chapters 2, 3, and 4, expand our understanding of the consequences of neuroendocrine disruption. We demonstrated that the hypothalamus and pituitary are vulnerable to perturbations by environmental stimuli in development as well as in adulthood. We illustrated how important it is to look beyond the bounds of traditional mechanisms of endocrine disruption – hormone agonism and antagonism – to the myriad other ways that exposures can alter how these tissues are able to carry out their jobs. Through these data, we showed that the support systems the hypothalamus has to enable communication of peripheral cues can be altered by the *in utero* environment and that this could be an overlooked venue through which early life exposures cause long-term consequences. We also showed that an environmental toxicant with a best-known mechanism of action being through cyto- and genotoxic effects can nevertheless target hormone production and endocrine axes. Both GDM and

IAA illustrate how increased research can illuminate the unknown cause through which an environmental stimulus can lead to a negative physiological effect. Finally, it is our hope that this work also adds to the body of knowledge on which we can build towards improved human health.

Table 5.1: Summary of IAA exposure results

Gene/protein	Adult exposure, *in vivo*	Adult exposure, *in vitro*	Developmental exposure, *in vivo*
Fshb	decrease	decrease	n.c.
FSHβ	decrease	decrease	
Lhb	n.c.	decrease	n.c.
LHβ	n.c.	n.c.	
Cga	n.c.	decrease	
Gnrhr		n.c.	
γH2AX	increase	increase (qual.)	
Cdkn1a	increase	increase	n.c.
P21	n.c.	increase (qual.)	
Mki67	n.c.	n.c.	n.c.
Bax		n.c.	
Bcl2		n.c.	
Tshb	increase	n.c.	increase
TSHβ			n.c.
Trha			n.c.
Trhb			n.c.
Aldh1a1			n.c.
Pomc			increase (trend)
Prl			n.c.
PRL			n.c.
Gh			n.c.

Table 5.1: Summary of IAA data results. For listed genes, “increase” and “decrease” refer to changes in mRNA expression between IAA-exposed conditions compared to controls. For listed proteins, “increase” and “decrease” refer to changes in number of protein-positive cells in IAA-exposed conditions compared to controls. N.C. indicates no observed change. Gray boxes represent unassessed genes/proteins in that exposure context. All positive results are significant unless labeled with “(trend)” to indicate there was a trend towards a significant change or “(qual.)” to indicate that the results were not quantified and the change was visually marked.

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CHAPTER 1: Introduction

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CHAPTER 2: Gestational Diabetes Mellitus Alters The Development Of Tanycytes And The Median Eminence In The Offspring

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CHAPTER 3: Iodoacetic Acid, A Water Disinfection Byproduct, Disrupts Hypothalamic And Pituitary Reproductive Regulatory Factors And Induces Toxicity In The Female Pituitary

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Chapter 4: Developmental Exposure To Water Disinfection Byproduct Iodoacetic Acid Induces Pituitary mRNA Expression Of The Thyroid Stimulating Hormone Beta Subunit

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Chapter 5: Conclusions and Future Directions

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