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A STUDY OF THE EFFECT OF PHYSIOLOGICAL STRESSORS ON HYPOTHALAMIC REGULATION OF REPRODUCTION USING AN IN VITRO SYSTEM

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A STUDY OF THE EFFECT OF PHYSIOLOGICAL STRESSORS ON HYPOTHALAMIC REGULATION OF REPRODUCTION USING AN *IN VITRO* SYSTEM

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ABSTRACT

Stressors have a negative impact on reproductive efficiency in humans and other animals, which has an economic cost due to infertility treatments for humans and reduced income for food producers. We wished to determine the molecular pathways by which metabolic disturbances and low-level inflammation impact the hypothalamic pituitary gonadal (HPG) axis using an *in vitro* model. To this end, we used the GT1-7 cell line, an immortalized line expressing the Kiss1R receptor that responds to kisspeptin stimulation with the release of GnRH to assess the impact of stressors on the GnRH-releasing cells. Additionally, we used the KTaV-3 and KTaR-1 cell lines, immortalize lines derived from the rodent anteroventral periventricular and arcuate nuclei, respectively, to assess the impact of stressors on kisspeptin-producing cells. We modeled metabolic disease and negative energy balance by exposing cells to beta-hydroxybutyric acid (BHB) and inflammation by exposure to tumor necrosis factor alpha (TNFα [alpha]). We determined that exposure to BHB significantly increased the intrinsic production of GnRH by GT1-7 cells, but that there were no additional changes in the expression of GnRH mRNA or release of GnRH protein. Additionally, there were no significant changes in calcium signaling or in the phosphorylation of ERK1/2, suggesting that the direct effect of stressors does not impact the GnRH-producing cells of the hypothalamus. Exposure to stressors in culture did not change the expression of *kiss1* mRNA in KTaV-3 cells upon exposure to estradiol. However, KTaV-1 cells exhibited a significantly decreased expression of *kiss1* mRNA and kiss1 protein release upon exposure to both BHB and tumor necrosis factor alpha. There was also a reduction in the expression of neurokinin B (*nkb*) mRNA under these conditions, suggesting that the impact of stressors on the HPG axis may be acting through the suppression of signaling in the arcuate nucleus. Disruption of neurokinin B and kisspeptin expression in the arcuate nucleus could impact the production of tonic levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH), resulting in a deficiency in follicle production and oocyte maturation.

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INTRODUCTION

Purpose

A variety of environmental and physiological stressors can impact reproduction in humans and other mammals. Infertility in humans comes with not only an emotional cost, but a financial burden ranging from $1,182 to $38,015 per person depending on the type of treatment (Katz, Showstack et al. 2011). In food animals, reproductive inefficiency has an economic impact for both producers and consumers and is a threat to global food security. Therefore, understanding the mechanisms by which stressors impact fertility is critical to our ability to intervene and improve reproductive efficiency.

Difficulties in mammalian reproduction have been associated with stressors such as negative energy balance, metabolic disease, and inflammation. Undernutrition (Castellano, Navarro et al. 2005, Luque, Kineman et al. 2007), type II diabetes (Castellano, Navarro et al. 2006, Castellano, Navarro et al. 2006, Castellano, Navarro et al. 2009, Castellano, Roa et al. 2009), and ketosis (Huzzey et al., 2105; Albaaj et al., 2017) are all associated with reproductive difficulty. Treatment with lipopolysaccharide (LPS) to simulate inflammation suppresses the LH surge in rabbits (Akema, He et al. 2005) and rats (Iwasa et al, 2008; Castellano et al, 2010). Lipopolysaccharide-induced suppression of the LH surge is mediated by tumor necrosis factor α (TNFα) (Yoo, Nishihara et al. 1997), which has also been shown to suppress the LH surge independently of LPS.

Many of these studies have concentrated on the effect of stressors on the production of luteinizing hormone (LH) from the pituitary gland and the function of the ovary. However, the release of gonadotropins from the pituitary gland occurs in response to signals originating in the hypothalamus; these signals are further regulated by estradiol released from the ovary, forming a feedback loop. Kisspeptin and its receptor, Kiss1R, are central to the function of the hypothalamic pituitary gonadal (HPG) axis. Kisspeptin (KP) functions in the hypothalamus, binding to Kiss1R and stimulating the release of gonadotropin releasing hormone (GnRH) from the median eminence into the hypophysial portal blood, which then stimulates the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary (Han, Gottsch et al. 2005, Castellano, Navarro et al. 2006, Suzuki, Kadokawa et al. 2008). These hormones function by promoting gametogenesis and steroidogenesis. Mutations of either protein in humans or mice result in an inability to progress through puberty and reproduce effectively (de Roux, Genin et al. 2003, Seminara, Messager et al. 2003, Wahab, Quinton et al. 2011, Novaira, Sonko et al. 2014).

Kp neurons in the arcuate nucleus of the hypothalamus (ARC) nucleus express KP in a tonic manner during the estrus cycle, causing the release of follicle stimulating hormone (FSH) and allowing maturation of oocytes to occur. These nuclei respond negatively to estradiol (E2) stimulation, causing KP production to decrease as E2 secretion from the ovary increases. Alternately, KP neurons in

the anteroventral periventricular nucleus (AVPV) respond positively to E2, releasing KP in response to the E2 peak during estrus, causing the release of luteinizing hormone (LH) to generate the LH surge and thus ovulation.

In this work we wished to determine if there is an impact of stressors on the function of the neurons in the hypothalamus, either in those that produce and release kisspeptin, or those that respond to kisspeptin with the release of GnRH. Identification of the molecular pathways by which hypothalamic function is compromised by stressors would help to identify targets for intervention, potentially improving gonadal function.

Literature Review

Kisspeptin and the HPG axis

Reproduction is regulated by the hypothalamic pituitary gonadal (HPG) axis, which in turn is regulated by a variety of physiological and environmental signals. Central to this process is gonadotropin releasing hormone (GnRH), which is released from the hypothalamus into the hypophysial portal blood system, resulting in the release of the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland. These act upon the gonads, which generate gametes and produce sex hormones, forming a regulatory feedback loop. After puberty, positive feedback by estrogen increases the production of gonadotropins, causing the maturation of ovarian follicles and

ultimately resulting in an LH surge, which triggers ovulation. Afterward, sex steroids, like progesterone, feed back to negatively regulate GnRH, establishing physiological homeostasis (Figure 1).

The production and release of GnRH, and therefore FSH and LH, is responsive to circulating concentrations of estradiol. The effect of estrogen is mediated by estrogen receptor α (ERα) but not β, as mice lacking ERα are infertile, whereas mice lacking ERβ display a wild-type phenotype (Wintermantel, Campbell et al. 2006). Treatment of ovariectomized rats (Degreef, Dekoning et al. 1987) with estradiol (E2) induced surges of LH and FSH; these surges were similar to those induced by administration of GnRH, suggesting that the E2 induced surges are mediated by GnRH. Treatment of anestrous ewes (Clarke 1988) and ovariectomized ewes (Caraty, Locatelli et al. 1989) with E2 also induced release of LH and FSH, with parallel changes in GnRH. However, work done by Caraty *et al*. (Caraty, Fabre-nys et al. 1998)using E2 implants into the brains of ovariectomized ewes showed that response to E2 varied with implant location; the medial preoptic area (MPOA) responds to E2 with strong negative feedback, as did implants in the middle or caudal ventromedial nucleus (VMN) or arcuate nucleus (ARC). Implants in the estrogen receptor (ER) expressing area of the VMN resulted in a full LH surge, suggesting that different regions of the hypothalamus vary in their response to estrogen. Work done by Yeo and Herbison (Yeo and Herbison 2014) demonstrated that the targeted removal of

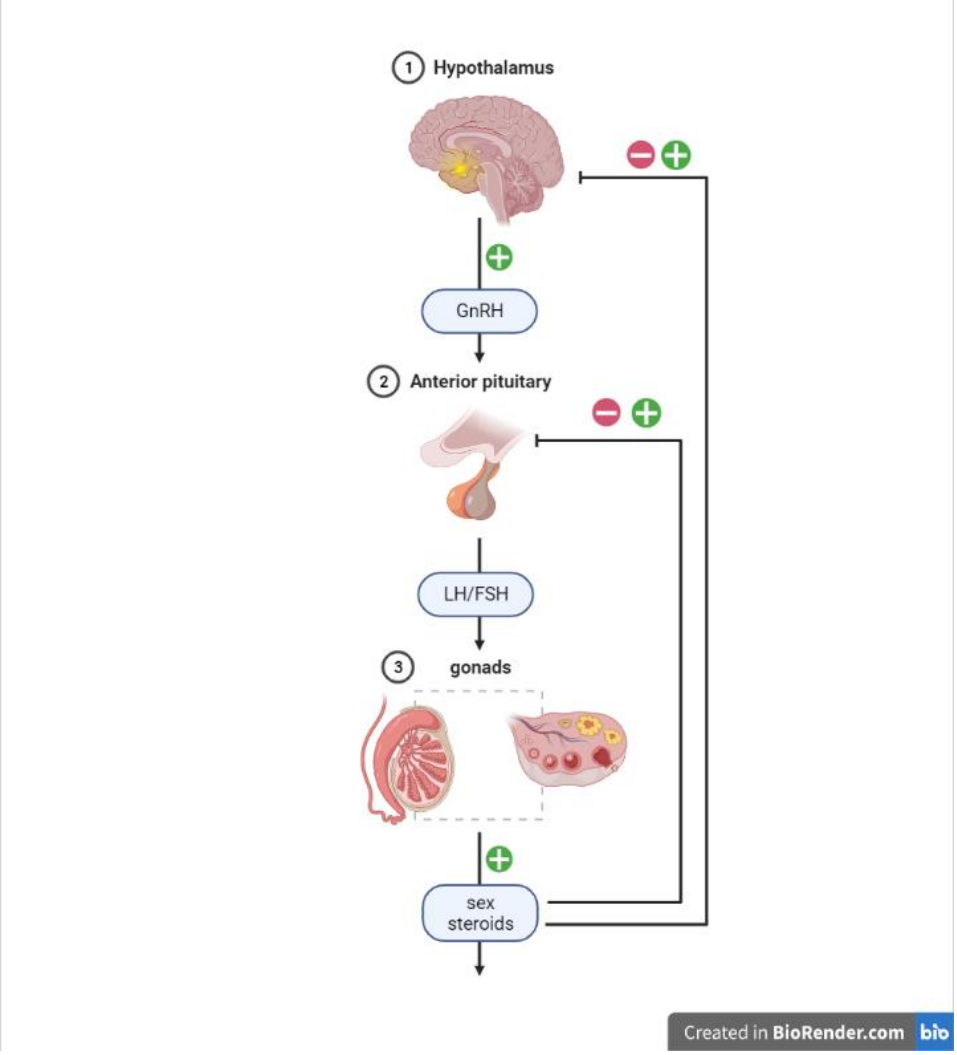


Figure 1**:** The hypothalamic-pituitary-gonadal axis. Gonadotropin releasing hormone (GnRH) is released from the hypothalamus into the hypophysial portal blood. GnRH then triggers the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland. These hormones are responsible for gametogenesis and the production of sex steroids from the gonads. Sex steroids then form a feedback loop with either positive or negative effects on various nuclei of the hypothalamus.

estrogen receptor alpha (ERα) from the arcuate nucleus (ARC) of mice resulted in ablation of chronic estrogen feedback, whereas the acute feedback was normal, highlighting the differing functions of various hypothalamic nuclei. Despite the role played by estradiol in regulating the release of GnRH, evidence illustrating the ability of GnRH neurons to respond directly to E2 stimulation is lacking. Double labeling experiments using probes against ERα and GnRH in ewes (Herbison, Robinson et al. 1993, Lehman, Ebling et al. 1993, Lehman and Karsch 1993), non-human primates (Herbison, Horvath et al. 1995, Sullivan, Witkin et al. 1995) and minks (Warembourg, Leroy et al. 1998) have established that most GnRH neurons do not express ERα, suggesting that the function of an interneuron is necessary to transmit signaling from estradiol to GnRH neurons.

The neuropeptide kisspeptin was originally discovered as an inhibitor of metastasis in melanoma cell lines (Lee, Miele et al. 1996, Ohtaki, Shintani et al. 2001) and was initially named metastin for this reason. The full length peptide of 54 amino acids is cleaved to shorter peptides of 14, 13, and 10 amino acids, all of which are equally functional as the natural ligand for the orphan receptor G protein-coupled receptor 54 (GPR54 (Kotani, Detheux et al. 2001)), also identified as hOT7T175 (Ohtaki, Shintani et al. 2001) and Axor12 (Muir, Chamberlain et al. 2001), and currently known as Kiss1R. The importance of Kiss1R to reproduction was established by its mutation in two consanguineous families with idiopathic hypogonadotropic hypogonadism (IHH), which is

characterized by the absence of spontaneous sexual development (de Roux, Genin et al. 2003, Seminara, Messager et al. 2003). Messager et al. detected the expression of Kiss1R in mouse GnRH neurons (Messager, Chatzidaki et al. 2005). Mice lacking *Kiss1R* (Funes, Hedrick et al. 2003, Seminara, Messager et al. 2003) or the kisspeptin gene (*kiss1)* (d'Anglemont de Tassigny, Fagg et al. 2007, Lapatto, Pallais et al. 2007) are phenocopies of their human counterparts, as they have a reduction in the size of the reproductive organs and are infertile. The deletion of *kiss1*, however, appears to result in a more variable phenotype than the deletion of *Kiss1R* (Lapatto, Pallais et al. 2007).

Kisspeptin is expressed in the hypothalamus of every mammalian species that has been examined, although there are structural differences between them. There is a population of kisspeptin neurons in the rostral portion of the hypothalamus, which is the preoptic area (POA) in sheep and primates. In rodents, this area consists of the anteroventricular periventricular nucleus (AVPV) and some surrounding regions, which together are referred to as the rostral periventricular region of the third ventricle (RP3V) and is more centralized and densely populated than in other mammals (Clarkson, de Tassigny et al. 2009). The second major population of kisspeptin neurons is located in the arcuate nucleus (ARC), which is the infundibular nucleus in humans. This is the most consistent population between mammalian species.

Kisspeptin neurons in the arcuate nucleus, which coexpress neurokinin B (NKB) and dynorphin (DYN), are known as KNDy neurons (Burke, Letts et al. 2006, Goodman, Lehman et al. 2007, Navarro, Gottsch et al. 2009, Wakabayashi, Nakada et al. 2010, Sakamoto, Murata et al. 2012). There is sexual dimorphism in both the expression of kisspeptin (Cheng, Coolen et al. 2010, Overgaard, Ruiz-Pino et al. 2014) and the response to the administration of senktide, an NKB receptor agonist, (Ruiz-Pino, Navarro et al. 2012), which are greater in females than males. Antagonism of the NKB receptor, Tac3R, results in the suppression of LH release. (Lehman, Hileman et al. 2013, Clarke, Li et al. 2018) NKB is released from KNDy neurons (Ramaswamy, Seminara et al. 2010, Garcia-Galiano, Schenau et al. 2012) and functions in an autocrine fashion to activate kisspeptin expression (Navarro, Gottsch et al. 2011, Sakamoto, Murata et al. 2012), ultimately resulting in the release of LH. (Ramaswamy, Seminara et al. 2010, Nestor, Briscoe et al. 2012, Sakamoto, Murata et al. 2012, Goodman, Hileman et al. 2013, Li, Millar et al. 2015). Dynorphin, on the other hand, inhibits the firing of kisspeptin neurons (de Croft, Boehm et al. 2013) and the release of LH (Goodman, Coolen et al. 2004, Wakabayashi, Nakada et al. 2010, Goodman, Hileman et al. 2013, Li, Smith et al. 2020, Liu, Li et al. 2020). The expression of *kiss1* (Li, Smith et al. 2020), the mRNA encoding NKB (*tac2)*, and mRNA encoding the NKB receptor (*tac3R)* (Navarro, Ruiz-Pino et al. 2012) are increased during progression through puberty, and it has been found that,

similarly to *KISS1R,* mutation of *TAC3R* results in normosmic idiopathic hypogonadotropic hypogonadism (nIHH) in humans (Guran, Tolhurst et al. 2009). The expression of kisspeptin in the arcuate nucleus and the anteroventral periventricular nucleus are differentially regulated by estradiol, in that the expression of *kiss1* mRNA in the ARC is suppressed by E2 while the expression of *kiss1* in the AVPV of rodents is enhanced (Smith, Cunningham et al. 2005). Knockdown of *kiss1* expression by stereotactic microinjection of antisense oligonucleotides targeting *kiss1* mRNA into the ARC or AVPV of the rat brain highlights the different functions of these two nuclei in the regulation of reproduction. This experiment demonstrated that neurons in the ARC are responsible for the tonic release of KISS1, which is necessary for the appropriate development of ovarian follicles. In the AVPV, the production of KISS1 is responsible for both the progression through puberty and the LH surge, which causes ovulation (Hu, Li et al. 2015).

Disfunction in the HPG axis

Disfunction of the HPG axis can be caused by a variety of environmental and physiological conditions, including negative energy balance, metabolic disorders, psychological or physical stress, or a combination of factors. One type of disfunction found in humans is functional hypothalamic amenorrhea (FHA), which is the absence of menstruation due to low adiposity, excessive exercise, or other stressors. This is characterized by a range of biochemical changes, including a

reduction in GnRH pulse frequency, attenuated follicular growth and maturation, and an absence of ovulation. Additionally, patients with FHA also tend to have elevated levels of corticotrophin releasing hormone (CRH), ACTH, cortisol, and endogenous opioids (Liu and Bill 2008), which can suppress the secretion of GnRH from the mediobasal hypothalamus. FHA can be seen in athletes, in which amenorrhea is likely due to low energy availability rather than other stressors (Loucks, Verdun et al. 1998), and patients suffering from anorexia nervosa, in which disordered eating leads to poor nutrition, leading to an increase in ghrelin and a decrease in leptin which is often accompanied by increased stress hormones such as cortisol (Tolle, Kadem et al. 2003, Misra, Prabhakaran et al. 2006).

The concentration of glucose in plasma is tightly regulated. Glucose is the preferred source of energy for the brain, and hypoglycemia can result in mental confusion, physical discomfort, coma, and in extreme cases, even death. The hormone insulin stimulates glucose uptake and utilization by the cells. In type I diabetes, insulin production by the pancreas is absent, while in type II diabetes, cells are resistant to the action of insulin; in both cases, the level of glucose in the plasma becomes elevated but is unavailable as an energy source. In the case of food restriction, plasma glucose levels can begin to drop as a result of insufficient food being consumed, which triggers the release of glucagon from the pancreas and suppresses the release of insulin. Glucagon stimulates glucose

production and release into the plasma from the liver, and also causes the release of free fatty acids (FFAs) from adipose tissue to be used as fuel. These FFAs typically enter the citric acid cycle to generate glucose; however, extended food restriction causes the depletion of oxaloacetate, preventing the entry of FFAs into the citric acid cycle. This favors the production of the ketone bodies acetone, acetoacetate, and β-hydroxybutyrate (BHB). Elevated levels of these ketone bodies can result in a metabolic condition called ketosis, which is common in diabetic patients and in the starvation response.

Elevated concentrations of beta hydroxybutyric acid (BHB) are found in the plasma of patients suffering from anorexia nervosa (Peters, Rohloff et al. 1998) because the body begins to rely on ketone bodies for energy during prolonged starvation. Elevated plasma concentrations of BHB are also found in animals such as cattle, which can enter a state of ketosis in response to the energy deficient state of early lactation. Ketosis is associated with in increased interval between calving and conception, resulting in inefficient reproduction (Gillund, Reksen et al. 2001, Huzzey, Mann et al. 2015, Albaaj, Foucras et al. 2017). Castellano *et al*. found that in rat models of undernutrition, short term food deprivation decreased the expression of *kiss1* mRNA in the hypothalamus with a small increase in the expression of *Kiss1R* mRNA (Castellano, Navarro et al. 2005). The secretion of LH in response to intracerebroventricular (i.c.v.) injection of the 10-amino acid kisspeptin peptide (KP10) in these animals is greater in

comparison to controls and plasma LH concentrations can also be restored in undernourished animals by central administration of leptin (Roa, Vigo et al. 2006). Luque *et al*. (Luque, Kineman et al. 2007) determined that not only was *kiss1* mRNA expression decreased during fasting, but *kiss1R* mRNA was as well. In sheep, feed restricted ewes exhibit an increase in the interpulse interval of LH compared to animals fed *ad libitum*, which is restored upon refeeding (Szymanski, Schneider et al. 2007) Additionally, lean animals exhibit reduced *KISS1* mRNA in the ARC compared to animals of normal weight, which is restored by leptin treatment (Backholer, Smith et al. 2010). In rats, acute fasting has been shown to decrease *kiss1* mRNA, as well as showing a downward trend in the expression of *nkb* (*tac2*) and a significant reduction of the NKB receptor (*tac3R*) mRNA in the hypothalamus (Navarro, Ruiz-Pino et al. 2012).

On the other extreme of metabolic disorders, diet induced obesity (DIO), which is often accompanied by metabolic syndrome and/or type II diabetes (T2D), also interferes with the HPG axis. These diseases tend to be characterized by high blood glucose, elevated triglycerides, and insulin resistance. In juveniles, obesity or increased BMI have the effect of advancing the onset of puberty. There is a negative correlation between age at puberty and BMI in humans, such that humans with a greater BMI complete puberty at a younger age (Kaplowitz 2007). Additionally, rats that have been fed a high fat diet from weaning also exhibit an early onset of LH pulse frequency, earlier vaginal opening, and increased levels

of *kiss1* mRNA in the hypothalamus (Li, Lin et al. 2012). In adult humans, these conditions have been associated with reduced testosterone in males (Dandona, Dhindsa et al. 2008) and a reduction of LH levels in both sexes. Type 2 diabetes has been associated with early menopause and premature ovarian failure (Anagnostis, Christou et al. 2019), although nondiabetic obese females also exhibit reduced plasma kisspeptin and LH, establishing a negative correlation between kisspeptin and body mass index (BMI) (Kolodziejski, Pruszynska-Oszmalek et al. 2018). In rat models of streptozotocin (STZ)-induced T2D, *kiss1* mRNA levels in the hypothalamus are reduced, and the resulting decrease in LH is restored by exogenous KP10. Male animals also exhibit reduced testosterone and decreased gonadotropin response to orchidectomy (ORX). KP10 administration was able to normalize post-ORX gonadotropin secretion. In females, STZ treatment resulted in persistent anestrus and decreased gonadotropin levels, a decreased response to ovariectomy (OVX), and lower levels of *kiss1* mRNA expression although responses to exogenous KP10 were preserved. Obese mice (*ob/ob*), which are deficient in leptin, exhibit obesity, hyperphagia, hyperglycemia, and impaired fertility which is accompanied by reduced *kiss1* mRNA in the hypothalamus (Ahima, Prabakaran et al. 1996). It was also determined that kisspeptin neurons express mRNA for the signaling form of the leptin receptor, *Ob-Rb*, and that treatment of these animals with exogenous leptin increased the cellular expression of *kiss1*, although not to wild type levels (Smith et al. 2006). Sanchez-Garrido determined that although both

overnutrition and high fat diet can increase body weight, only HFD leads to metabolic perturbation in a rat model. Animals fed HFD exhibited decreased expression of *kiss1* mRNA in the ARC and a decreased response to exogenous KP10. In mouse models, Zhang et al. (Zhang, Zhang et al. 2008) showed that IKKβ and NFκB, which are involved in the inflammatory process, are enriched in the hypothalamus, but only active in animals fed a high fat diet. Taken together, this body of evidence suggests that a reduction in kisspeptin is associated with both negative energy balance and obesity. Additionally, active IKKβ and NFκB were also found to be in the hypothalamus in *ob/ob* mice, suggesting a connection between metabolic disorders and inflammation, and a possible explanation why both negative energy balance and obesity could both contribute to the disfunction of the HPG.

Experiments establishing the impact of inflammation on the HPG axis have been conducted using lipopolysaccharide (LPS), which is a glycolipid located on the surface of gram-negative bacteria that stimulates the immune system in mammals and leads to an inflammatory response. Intravenous LPS injection into rats suppressed both hypothalamic *kiss1* mRNA expression (Iwasa, Matsuzaki et al. 2008, Kinsey-Jones, Li et al. 2009, Knox, Li et al. 2009, Iwasa, Matsuzaki et al. 2014, Surkin, Di Rosso et al. 2017) and LH release (Akema, He et al. 2005, Iwasa, Matsuzaki et al. 2008, Kinsey-Jones, Li et al. 2009, Castellano, Bentsen et al. 2010, Surkin, Di Rosso et al. 2017). Co-injection with the nonsteroidal anti-

inflammatory drug (NSAID) indomethacin completely blocked the suppressive effects of LPS (Iwasa, Matsuzaki et al. 2008). Injection of high doses of LPS not only decreased the expression of *kiss1* and *gnrh* mRNA, but increased the expression of mRNA for RFamide-Related Peptide-3 (*rfrp-3f),* which suppresses the HPG axis, as well as mRNA for its receptor *grp147* (Iwasa, Matsuzaki et al. 2014). Increased fever and the resultant anorexia could potentially result in negative energy balance; however, experiments done by Castellano *et al.* (Castellano, Bentsen et al. 2010) showed that LPS treatment results in a reduction in *kiss1* mRNA that is not present under metabolic stress. Furthermore, responsiveness to exogenous kisspeptin was suppressed in the presence of LPS but not metabolic stress in this system. Treatment of ovariectomized rats with intravenous (i.v.) lipopolysaccharide LPS significantly suppressed the electrical activity of neurons located in the mediobasal hypothalamus (MBH), which was accompanied by a concomitant reduction in plasma luteinizing hormone (LH). This effect was reversed by intracerebrovectrucular (i.c.v.) co-injection with an antibody against TNFα; furthermore, the suppression of both electrical activity and LH release was replicated by either i.v. or i.c.v. injection of tumor necrosis factor α (TNFα), suggesting that TNFα is responsible for the suppressive effect of LPS on LH release (Yoo, Nishihara et al. 1997). Both LPS and TNFα have similar effects on the ability of bovine ovarian theca and granulosa cells to produce and release estradiol (Williams, Sibley et al. 2008). *In vivo*, fewer animals treated with either LPS or TNFα were able to successfully ovulate. TNFα in plasma and *tnfα*

mRNA in the hypothalamus of mice increases in response to LPS administration. Blockade of the cannabinoid receptor (CB1) increased these effects but reversed the reduction of *kiss1* and *gnrh* mRNA.(Surkin, Di Rosso et al. 2017) Taken together, these data suggest that inflammation plays a role in the suppression of the HPG axis, likely through the activation of TNFα.

While both metabolic factors and inflammation can have a negative impact on the HPG axis, there is evidence that combinations of mild stressors can have the same effect. FHA is often associated with personality characteristics such as perfectionism and an intense desire for social acceptance, suggesting that psychological stress may combine with physical stressors to impede normal menstruation (Liu and Bill, 2008). In a non-human primate model, macaques that exhibited sensitivity to stress had lower LH pulse frequencies than those animals that were more resistant to stress (Herod, Pohl et al. 2011). In a separate study mimicking the lifestyle of women suffering from FHA, combinations of mild stress, diet, and exercise disrupted the menstrual cycle of monkeys whereas the individual mild stressors did not (Williams, Berga et al. 2007). In a rat model, psychological (restraint), metabolic (hyperglycemia), and inflammatory (LPS) stressors were all capable of reducing the expression of *kiss1* mRNA in the hypothalamus (Kinsey-Jones, Li et al. 2009). Each type of stress resulted in an increase in plasma corticosterone and exogenous corticosterone administration was able to reduce *kiss1* mRNA; however, *kiss1R* mRNA was increased and LH

pulsatility was unaffected. This would suggest that the response to stressors is potentially more complicated *in vivo* than simply being a response to the end product of the stress response. The potential for combinations of stressors to affect experimental outcomes makes the investigation of stress-induced suppression of mammalian reproduction complicated. Therefore, cell lines established from the nuclei of the murine hypothalamus have been developed to simplify the study of individual stressors.

Modeling the HPG axis *in vitro*

GT1-7 cells were generated from a hypothalamic tumor in transgenic mice carrying the SV40 T antigen under the control of the GnRH promoter. They express the neural markers neuron-specific enolase (NSE) and neurofilament protein (NF) but fail to express the glial markers glial fibrillary acidic protein (GFAP), myelin basic protein (MBP) and myelin proteolipid protein (PLP), establishing their neuronal origin. They respond to depolarization by the secretion of GnRH (Mellon, Windle et al. 1990). These cells also express the leptin receptor, LEPR (Zamorano, Mahesh et al. 1997) and respond to leptin exposure with the upregulation of *kiss1* mRNA (Oride, Kanasaki et al. 2017). Additionally, they express estrogen receptor α, estrogen receptor β (Roy, Angelini et al. 1999), and Kiss1R (Terasaka, Otsuka et al. 2013). The ability of GT1-7 cells to express *kiss1* mRNA appears to be dependent on supraphysiological estrogen exposure (10nM), and their response to KP10 stimulation is increased in the presence of lower concentrations of estradiol

(100pM) (Jacobi, Martin et al. 2007, Tonsfeldt, Goodall et al. 2011, Terasaka, Otsuka et al. 2013). However, there is contradictory evidence that some native GT1-7 cultures may have reduced expression of Kiss1R and the ability to respond to KP10 stimulation (Sukhbaatar, Kanasaki et al. 2013). This difference could possibly be due to the tendency of immortalized cell lines to mutate with increasing passage number. However, upon transfection with the kisspeptin receptor and a reporter plasmid, the cells did respond with stimulation of the GnRH promoter by production of luciferase. Additionally, this study found that kisspeptin increases the phosphorylation of ERK1/2, the release of cAMP, and expression of the GnRH receptor (GnRHR). GT1-7 cells can produce kisspeptin upon exposure to elevated levels of estradiol in the nanomolar range, but do not do so with lower E2 levels.

The KTaV-3 and KTaR-1 cell lines were generated from mice carrying the *Kiss-1*-GFP transgene. Explants were removed from the AVPV and ARC, respectively, and cells isolated from the explants were immortalized using the SV40-T antigen. Both lines express *kiss1*, but only KTaR1 expresses *tac2* and *pdyn* (the mRNA encoding dynorphin). Upon exposure to estradiol, KTaV-3 cells upregulate the expression of *kiss1*, while *kiss1* expression in the KTaR1 line is suppressed. This confirms that these lines are indeed isolated from separate nuclei in the mouse hypothalamus (Jacobs, Veitch et al. 2016). This three lines will allow the separate assessment of the impact of stressors on the GnRH producing neurons

from the median eminence (GT1-7) and the kisspeptin-producing cells from the AVPV (KTaV-3) and ARC (KTaR-1).

CHAPTER I
THE IMPACT OF STRESSORS ON THE PRODUCTION OF GNRH IN AN IMMORTALIZED HYPOTHALAMIC CELL LINE

Abstract

Stressors have a negative impact on reproductive efficiency in humans and other animals, which has an economic cost due to infertility treatments for humans and reduced income for food producers. We wished to determine the molecular pathways by which metabolic disturbances and low-level inflammation impact the hypothalamic pituitary gonadal (HPG) axis using an *in vitro* model. To this end, we used the GT1-7 cell line to assess the impact of stressors on the release of GnRH. We modeled metabolic disease and negative energy balance by exposing cells to beta-hydroxybutyric acid (BHB) and inflammation by exposure to tumor necrosis factor alpha (TNFα [alpha]). We determined that exposure to BHB significantly increased the intrinsic production of GnRH by GT1-7 cells, but that there were no additional changes in the expression of GnRH mRNA or release of GnRH protein. Additionally, there were no significant changes in intracellular calcium release or in the phosphorylation of ERK1/2, suggesting that the direct effect of exposure to stressors is not entirely mediated by the GnRH producing cells of the hypothalamus.

Introduction

Stressors have a negative impact on the reproductive efficiency of humans and other mammals. This comes with both an emotional and economic cost for humans struggling with infertility. Food producers are also economically

impacted, where a drop in reproductive efficiency influences food production impacts both the financial stability of the producer and global food security.

Published studies have shown that stressors such as negative energy balance and inflammation interfere with the release of luteinizing hormone (LH). In humans, functional hypothalamic amenorrhea is characterized by an absence of menstruation due to excessive exercise or disordered eating habits, both of which lead to negative energy balance (NEB). NEB in cattle can result from the energy deficient state of early lactation. Both conditions have been associated with ketosis characterized by elevated levels of beta hydroxybutyric acid (BHB). Rat models of both negative energy balance and diet induced obesity have been associated with a defect in the HPG axis. Obesity has also been associated with perturbations in the HPG axis in humans, with an elevated BMI resulting in precocious puberty in juveniles and a reduction in LH secretion in adults. Inflammation has also been shown to disrupt the HPG axis; experiments in rats, rabbits and sheep have indicated that treatment with LPS suppresses the production of LH. Further, the combination of microstressors, such as mild NEB and restraint stress, also have a negative impact on the production of LH. However, there is evidence that the suppression of LH can be rescued by treatment with exogenous GnRH, suggesting that the anterior pituitary gland functions normally. This suggests that the defect in HPG axis function possibly lies not in the ability of the pituitary gland to respond to GnRH, but in the

production of GnRH itself. In order to assess this possibility, we used the GT1-7 cell line to model the GnRH producing cells of the hypothalamus *in vitro.*

GT1-7 cells were generated from a hypothalamic tumor in transgenic mice carrying the SV40 T antigen under the control of the GnRH promoter. They express the neural markers neuron-specific enolase (NSE) and neurofilament protein (NF) but fail to express the glial markers glial fibrillary acidic protein (GFAP), myelin basic protein (MBP) and myelin proteolipid protein (PLP), establishing their neuronal origin. They respond to depolarization by the secretion of GnRH (Mellon, Windle et al. 1990). They express estrogen receptor α, estrogen receptor β (Roy, Angelini et al. 1999), and Kiss1R, and their response to KP10 stimulation is increased in the presence of estradiol (Jacobi, Martin et al. 2007, Tonsfeldt, Goodall et al. 2011, Terasaka, Otsuka et al. 2013). However, there is contradictory evidence that some native GT1-7 cultures may lack the expression of Kiss1R and the ability to respond to KP10 stimulation (Sukhbaatar, Kanasaki et al. 2013). This difference could possibly be due to the tendency of immortalized cell lines to mutate with increasing passage number. However, upon dual transfection with the kisspeptin receptor and a reporter plasmid containing the GnRH promoter, the cells did respond to kisspeptin stimulation with the expression of luciferase, establishing the function of the transfected receptor. This study established that kisspeptin increases the phosphorylation of ERK1/2, the release of cAMP, and expression of the GnRH

receptor (GnRHR), suggesting that the cells can both produce and respond to GnRH. GT1-7 cells can produce kisspeptin upon exposure to elevated levels of estradiol in the nanomolar range, but do not do so with lower E2 levels. Using the methodology established in the literature, we exposed this cell line to BHB and TNFα to simulate stress *in vitro* to elucidate the molecular mechanisms by which stressors impact the ability of GnRH-producing cells in the hypothalamus to respond to kisspeptin stimulation.

Materials and Methods

Cell lines and culture conditions

The GT1-7 cell lines were a kind gift from Pamela Mellon of the Salk Institute and Patrick Chappell of Oregon State University. Cells were grown in high glucose DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), penicillin, streptomycin, L-glutamine, (Gibco) and amphotericin B and maintained in a humidified incubator at 37ºC with 5% CO2. Upon reaching confluence, GT1-7 cells were split at a ratio of 1:3.

RNA extraction and qPCR

GT1-7 cells were plated in 60mm tissue culture dishes at a density of 5.0 x 10^4 per cm^2 and allowed to grow to 80 percent confluency in phenol red-free DMEM (Gibco) supplemented with 10% charcoal stripped FBS (Gibco). The media was then replaced with serum-free phenol red-free DMEM either alone or with 10ng/ml TNFα (Sigma Aldrich, St. Louis, MO), 6mM BHB (Acros Organics, Geel,

Belgium), or a combination of TNFα and BHB and maintained in a humidified incubator at 37ºC with 5% CO2 for twenty-four hours. Samples of media for RIA and cells for RNA extraction were collected after exposure to stressors, and this was labeled the 0h time point. The remaining cells were then stimulated with the addition of 300μM KP10 (Peptide International, Osaka, Japan) to the experimental media for 24 hours. After the stimulation period, the media was removed and stored at -80ºC for further analysis. Cells were washed once in cold PBS, then RNA was isolated using the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA). One microgram of RNA was reverse transcribed using the High-Capacity cDNA Kit (Applied Biosystems, Foster City, CA). Of this reaction, 100ng were placed into a qPCR reaction using PowerUp SYBR Green (Applied Biosystems, Foster City, CA) on an Applied Biosystems MxPro 3005P real time PCR machine using Quantitect primer sets for GnRH and GapDH (Qiagen, Hilden, Germany). The data were analyzed using the ΔΔCt method (Livak and Schmittgen 2001).

Cytosolic calcium release assay

Calcium release into the cytosol of GT1-7 cells was assayed using the Fluoforte Calcium assay kit (Enzo Life Sciences, Farmingdale, NY) according to manufacturer's instructions. Briefly, $1x10^5$ cells per well were plated in a 96-well black well plate in a 100μL volume and allowed to attach overnight in growth media. Media was removed and replaced with serum-free phenol red-free DMEM either alone or with 10ng/ml TNFα, 6mM BHB, or a combination of TNFα and

BHB and maintained in a humidified incubator at 37ºC with 5% CO2 for twenty-four hours. Media was then removed and replaced with 100µL of Fluoforte dye loading solution. Cells were incubated for one hour at room temperature, then KP-10 was added to a final concentration of 300nM. The assay was immediately placed into a Biotek Synergy HT plate reader and read at an excitation wavelength of 490mM and an emission wavelength of 525nM every one minute for thirty minutes. The data was collected using Gen5 software.

Western blotting

GT1-7 cells were plated in 100mm tissue culture dishes at a density of 5.0 x 10^4 per cm^2 and allowed to grow to 80 percent confluency in phenol-red free DMEM supplemented with 10% charcoal stripped FBS. The media was then replaced with serum-free phenol-red free DMEM either alone or with 10ng/ml TNFα, 6mM BHB, or a combination and maintained in a humidified incubator at 37ºC with 5% CO2 for 24 hours. GT1-7 cells were then stimulated with the addition of 300µM kisspeptin-10 to the experimental media for 15 minutes before protein collection. The media was removed, and cells were washed once in cold PBS, then suspended in 1mL PBS and centrifuged at 300G for 10 minutes. The supernatant was removed, and the pellet was suspended in 300µL cold RIPA buffer (Thermo Scientific, Rockford, IL) containing proteinase inhibitors (Thermo Scientific). The suspended cells were sonicated on ice for 20 seconds, then allowed to rest on ice for 30 minutes before centrifugation at 12,000 x G for 10 minutes. The supernatant was removed to a fresh tube and protein was quantified using a

Pierce BCA protein assay (Thermo Scientific, Rockford, IL). Twenty-five micrograms of protein were loaded onto a 10% polyacrylamide gel and electrophoresed at 100V for 90 minutes. The gel was then transferred to nitrocellulose (Thermo Scientific, Rockford, IL) on ice at 100V for 90 minutes. The blot was stained with Ponceau's reagent (Sigma Aldrich, St. Louis, MO) to confirm protein transfer, then washed in nanopure water to remove the stain and blocked for one hour at room temperature with gentle agitation in 1% casein blocking buffer (Alfa Aesar, Ward Hill, MA). Blots containing protein from GT1-7 cells were then probed with antibodies against ERK1/2 (4695S) and phospho-ERK1/2 (4370S) (Cell Signaling Technology, Danvers, MA) at a dilution of 1:1000 in 1% casein blocking buffer overnight at 4ºC with gentle agitation. After incubation with primary antibody, the blots were washed three times for 10 minutes each with tris-buffered saline containing Triton-X100 (TBST) then probed with an HRP conjugated anti-rabbit secondary antibody (Thermo Scientific) at a dilution of 1:10000 for one hour at room temperature with gentle agitation. Blots were once again washed three times for 10 minutes each in TBST, then exposed to ECL Prime Western blotting detection reagent (Amersham, Little Chalfont, Buckinghamshire, UK) for 5 minutes. Data was collected using a (reader info) and analyzed using ImageJ software.

Radioimmunoassay (RIA)

RIA was performed on media collected from control and kisspeptin stimulated GT1-7 cells according to the method described in Tonsfeldt et al. Duplicate

100µL samples of media collected from GT1-7 cells in 6cm dishes were incubated with EL-14 primary antibody against GnRH for 48 hours. The samples were then incubated with ^{125}I-labeled GnRH for an additional 48 hours. Three milliliters of absolute ethanol were added to all samples and standards, which were then centrifuged at 600 x G for 15 minutes at 4ºC. The resulting pellets were analyzed on a gamma counter (Packard, Palo Alto, CA) by counting for one minute per sample.

Statistics

Results were analyzed by two-way analysis of variance (ANOVA) using JMP software and by Mann Whitney analysis using SPSS software. A p-value less than 0.05 was considered significant.

Results

The expression of *gnrh* in response to kisspeptin in high glucose media

We wished to determine if stressors impacted the ability of GT1-7 cells to respond to kisspeptin (KP10) stimulation by upregulation of *gnrh1* mRNA. Cells were exposed to 6mM BHB, 12mM BHB, 10ng/mL TNFα, or combinations thereof for twenty-four hours prior to stimulation with 300nM KP10. There was a small but significant increase of 17% (p=0.02) when comparing control cells at time zero (after twenty-four hours exposure to stressors but prior to addition of KP10) to control cells after KP10 exposure, a small increase of 27% between

control cells and those exposed to 6mM BHB that did not reach significance (p=0.06), and an increase of 75% (p=0.05) between control + KP10 cells and cells exposed to 6mM BHB + KP10. Addition of KP10 to the 6mM BHB group also increased *gnrh* mRNA by 63% (p=0.01). There was an increase in the GnRH production of cells exposed to TNF compared to control cells of 37% (p=0.04), but no significant increase in *gnrh* mRNA in TNF cells when exposed to KP10 (p=0.97). There were no statistically significant changes in *gnrh* mRNA between any other experimental groups (Figure 2).

We then wished to determine if there was a change in the expression of *kiss1R* mRNA under these same conditions. There was a significant increase of 23% in *kiss1R* mRNA upon exposure to 6mM BHB (p=0.04), but not 12mM BHB (p=0.44). Exposure to TNFα increased the expression of *kiss1R* mRNA by 58% (p=0.03). There were no significant increases between other groups. Upon addition of KP10, the 6mM BHB group showed an additional increase of 74% (p≤0.01), but this increase did not occur in any other groups. (Figure 3.)

Given the published data that show an increased response to KP10 stimulation in GT1-7 cells exposed to estradiol, we decided to repeat the previous experiment in media containing 100pM E2 (Tonsfeldt, Goodall et al. 2011). There was an increase of 30% in *gnrh* mRNA in control cells exposed to KP10 that did not reach significance (0.06). The expression of *gnrh* mRNA more than doubled in cells exposed to 6mM BHB (181%, p = 0.05), but upon exposure to KP10, the expression of *gnrh* dropped by 43%, although this change did not reach

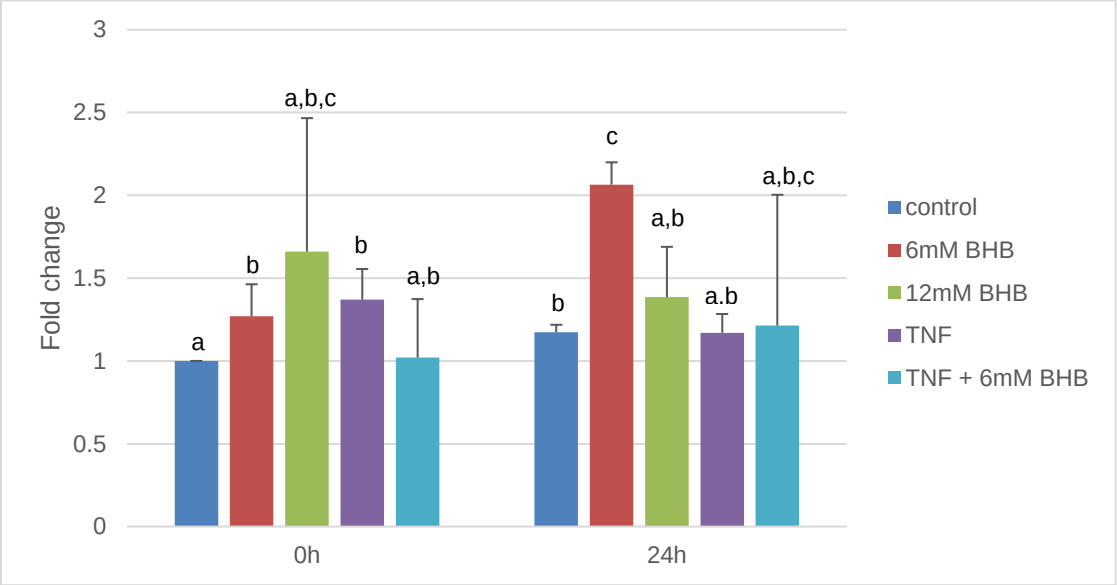


Figure 2: Change in *gnrh* mRNA in GT1-7 cells upon stimulation with kisspeptin under the influence of stressors. Cells were incubated with 6mM or 12mM BHB, 10ng per milliliter TNFα, or combinations thereof for twenty-four hours prior to stimulation with 300μM KP10. The change in *gnrh* mRNA expression was assessed with qPCR (n=3). Experimental groups with different superscript letters are considered statistically different ($p<0.05$)

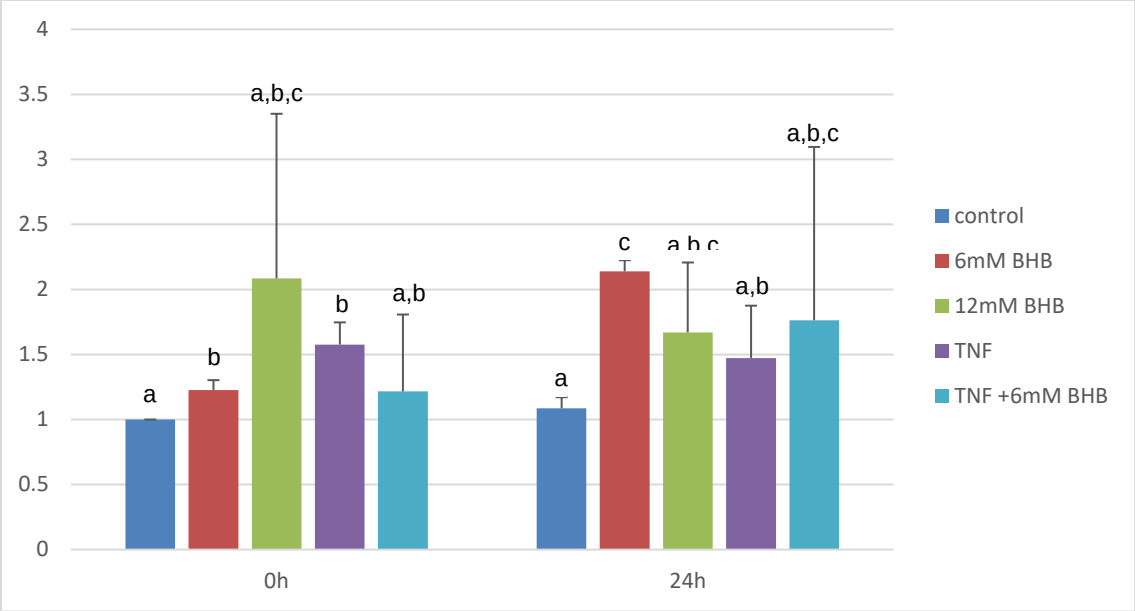


Figure 3: Change in *kiss1R* mRNA in GT1-7 cells upon stimulation with kisspeptin under the influence of stressors. Cells were incubated with 6mM or 12mM BHB, 10ng per milliliter TNFα, or combinations thereof for twenty-four hours prior to stimulation with 300μM KP10. The change in *gnrh* mRNA expression was assessed with qPCR (n=3). Experimental groups with different superscript letters are considered significantly different ($p<0.05$).

significance (p=0.06). There were no other significant changes between any other experimental groups, either between control cells vs. stressor groups, or between control cells exposed to KP10 vs. stressor groups exposed to KP10 (Figure 4).

Upon examining the expression of *kiss1R* mRNA in these samples, there is an increase in control cells upon exposure to KP10 of 24% which does not reach significance (p=0.24). There is a very large increase of 962% in cells exposed to 6mM BHB which does not reach significance (p=0.25). However, as was seen in the expression of *gnrh* mRNA, the expression of *kiss1R* mRNA is decreased by 81% in cells exposed to 6mM BHB upon stimulation with KP10. There was very little change between other experimental groups, regardless of KP10 exposure (Figure 5). Stimulation of GnRH releasing cells by kisspeptin results in both the release of intracellular calcium, which triggers the release of stored GnRH protein, and the phosphorylation of ERK1/2, which is part of the kiss1R signaling pathway. Because the assessment of *gnrh* mRNA by qPCR was variable and contained excessive standard error, we decided to assess these components of the kiss1R signaling pathway. First, we used protein extracted from GT1-7 cells exposed to 6mM BHB, 10ng/mL TNFα, or BHB and TNFα together prior to kisspeptin stimulation to assess the phosphorylation of ERK1/2 after 30 minutes of stimulation by KP10. There was not a significant difference in the phosphorylation of control samples versus the samples from cells exposed to

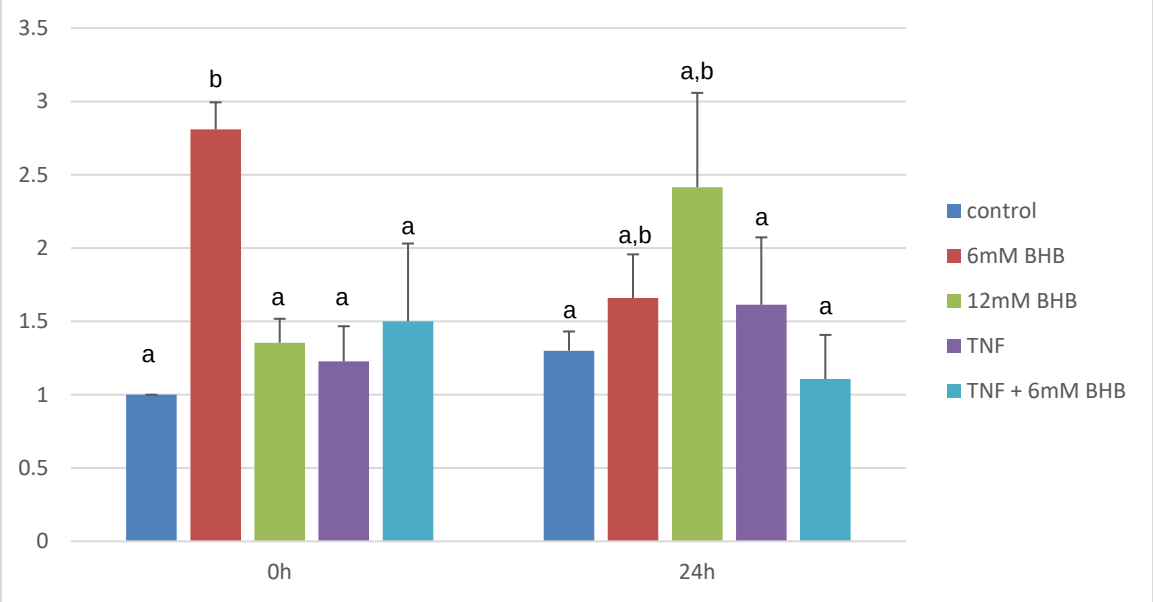


Figure 4: Change in *gnrh* mRNA in GT1-7 cells exposed to estradiol upon stimulation with kisspeptin under the influence of stressors. Cells were incubated with 6mM or 12mM BHB, 10ng per milliliter TNFα, or combinations thereof in media containing 100pM E2 for twenty-four hours prior to stimulation with 300μM KP10. The change in *gnrh* mRNA expression was assessed with qPCR (n=3). Experimental groups with differing superscript letters are considered significantly different ($p<0.05$).

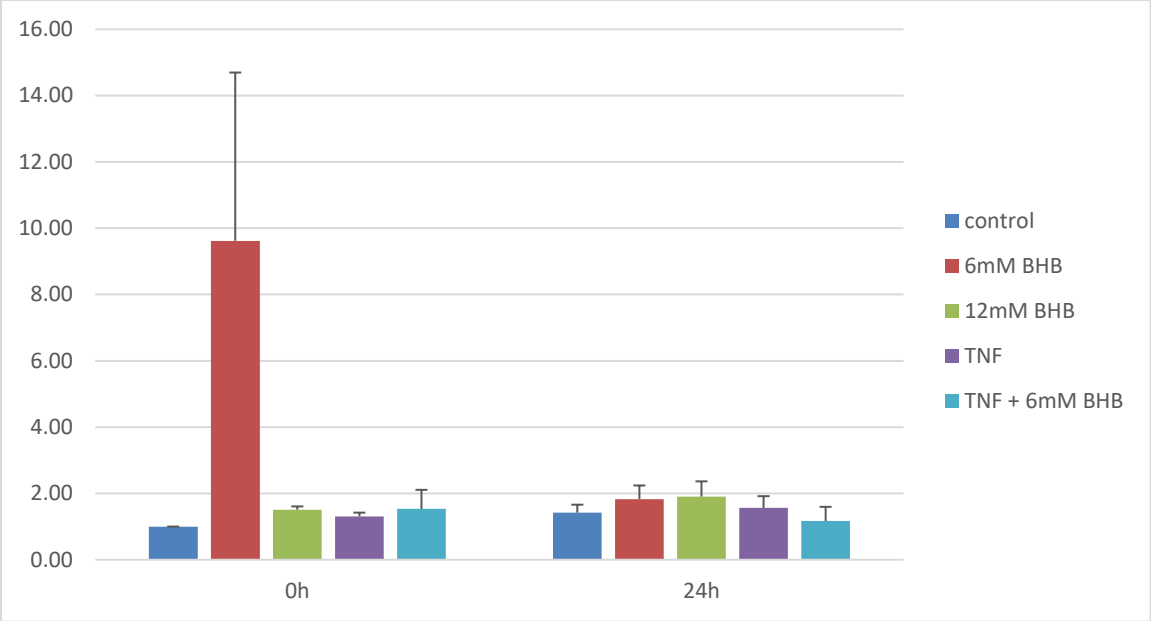
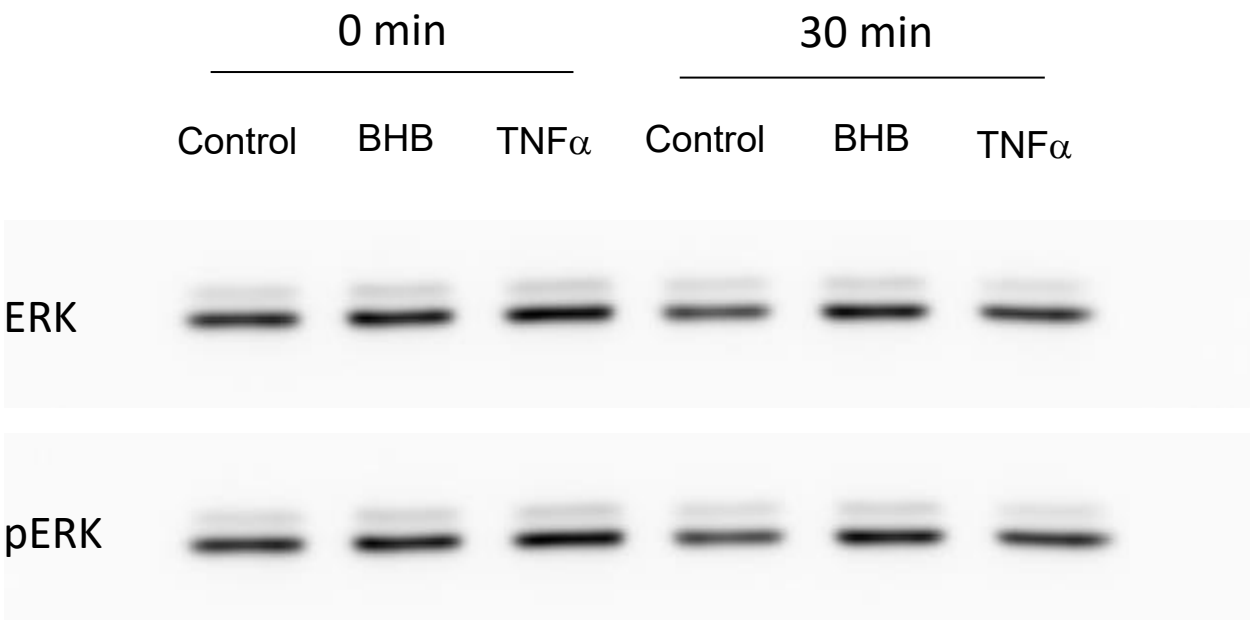


Figure 5: Change in *kiss1R* mRNA in GT1-7 cells exposed to estradiol upon stimulation with kisspeptin under the influence of stressors. Cells were incubated with 6mM or 12mM BHB, 10ng per milliliter TNFα, or combinations thereof for twenty-four hours in media containing 100pM E2 prior to stimulation with 300μM KP10. The change in *gnrh* mRNA expression was assessed with qPCR (n=3). Experimental groups with differing superscript letters are considered significantly different ($p<0.05$).

stressors for twenty-four hours at the zero time point prior to kisspeptin stimulation. Additionally, although there seemed to be a reduction in the phosphorylation status of ERK1/2 between samples taken pre- and post-KP10 stimulation, this failed to reach significance due to standard error (Figure 6a and 6b).

Intracellular calcium release was assessed both with and without the addition of estradiol to culture media. The release of calcium into the cytosol was negligible in all experimental groups in the absence of estradiol (data not shown). However, intracellular calcium was detected in all groups in the presence of estradiol (Figure 7.) Fluorescence was increased over time in all groups over the course of ten minutes. At every time point measured, groups containing either 6mM or 12mM BHB had an increased amount of calcium release compared to control. In all groups containing TNFα, regardless of the presence of BHB, calcium release was reduced compared to control. However, none of the changes at any time point reached statistical significance. We then assessed the release of GNRH protein into the media of GT1-7 cells upon stimulation of kisspeptin by radioimmunoassay. The amount released from each sample was very close to the detection limit of the assay, resulting in excessive standard error. There was no significant difference between the gnrh secreted in any experimental

a.



b.

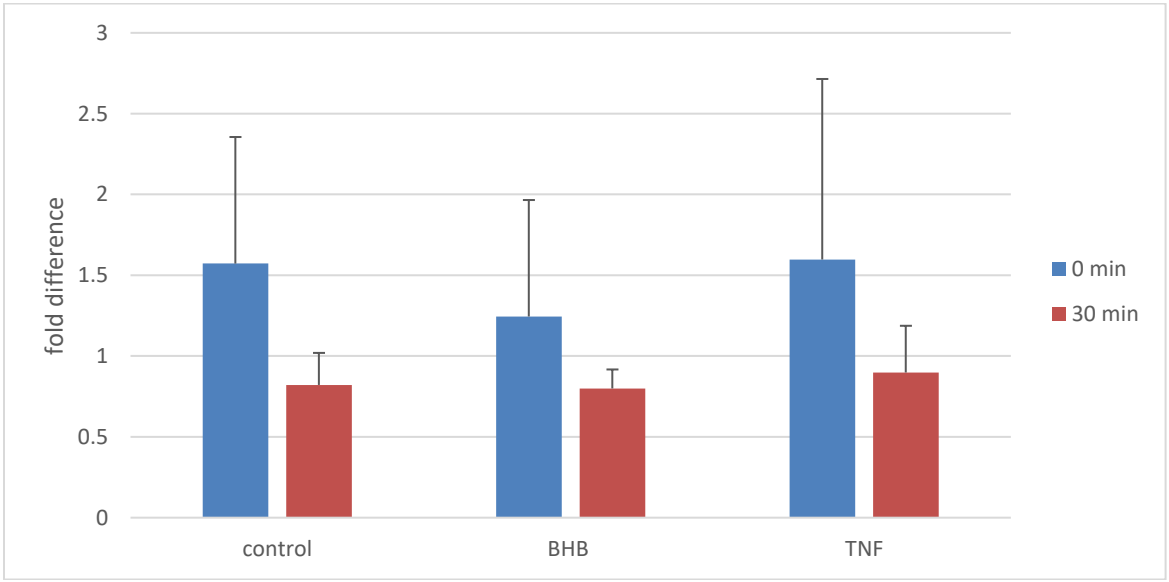


Figure 6: Assessment of ERK1/2 phosphorylation upon kisspeptin stimulation in GT1-7 cells exposed to stressors. Cells were exposed to stressors in media containing 100pM E2 for twenty-four hours, then stimulated with KP10 for 30 minutes. a) Representative western blot and b) quantitation of three replicates (n=3) by Image J.

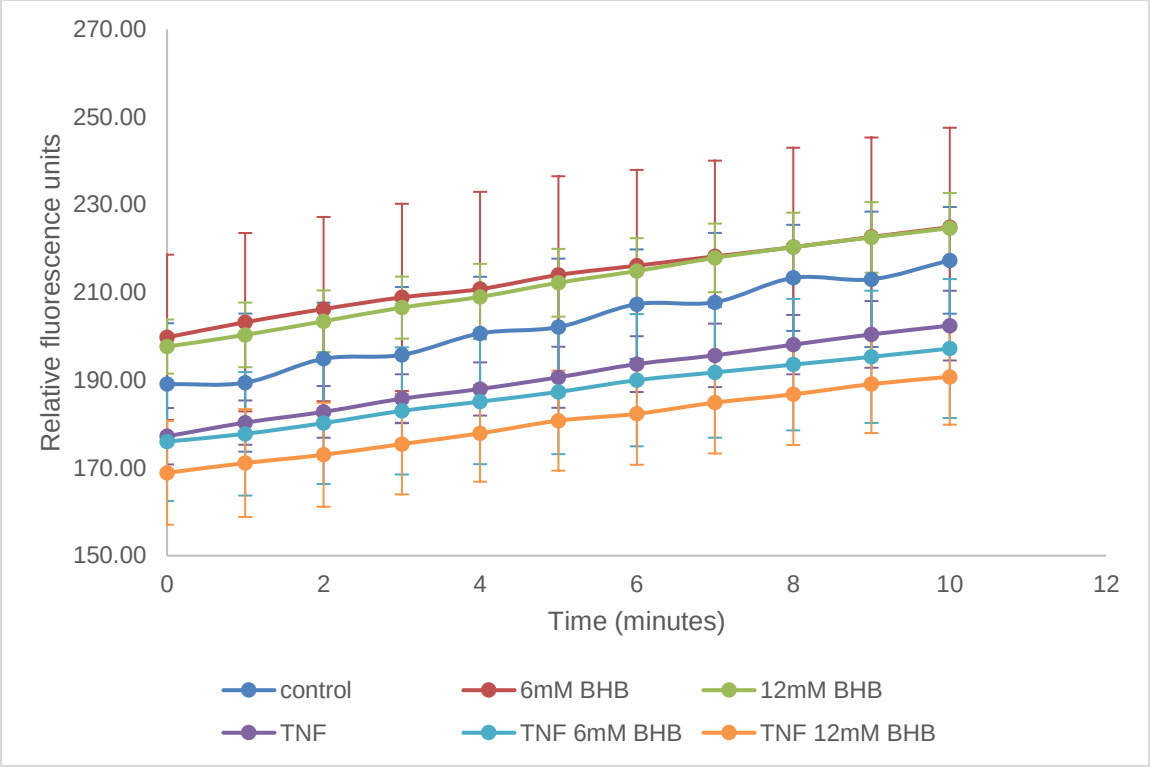


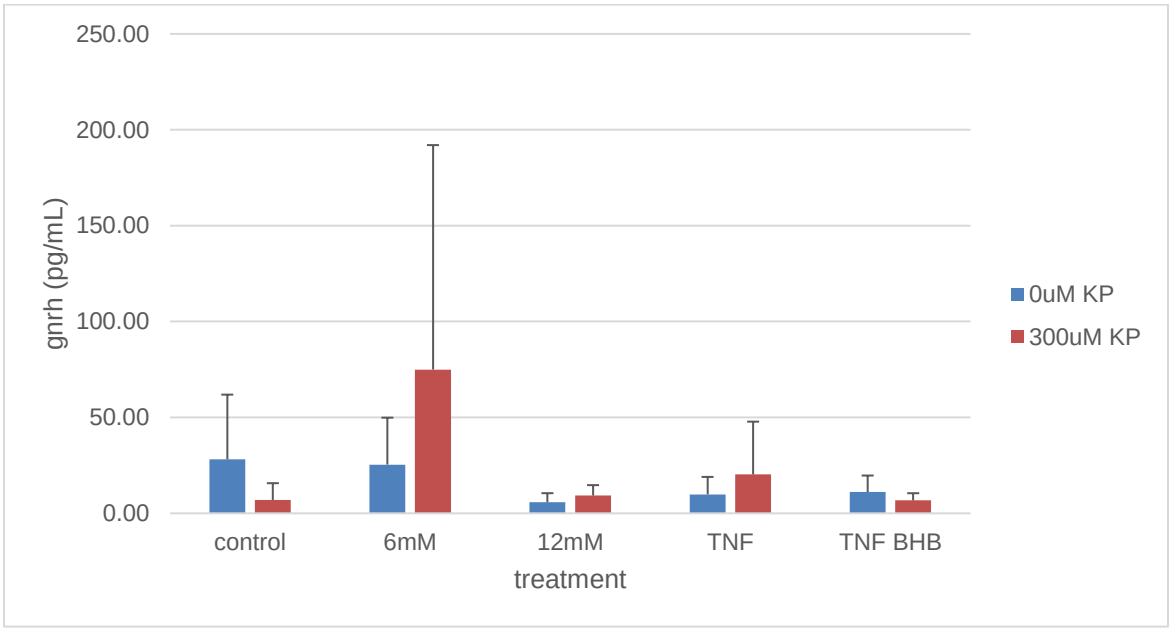
Figure 7: The release of intracellular calcium in response to kisspeptin stimulation in GT1-7 cells exposed to stressors. Cells were exposed to stressors in media containing 100pM E2 for 24 hours then stimulated by the addition of 300nM kisspeptin. Fluorescence was read immediately every minute for ten minutes (n=3).

conditions using this assay. KP10 did not appear to stimulate the release of gnrh protein into the media in the absence (Figure 8a) or presence (Figure 8b) of estradiol. There was an excessive amount of standard error in the measurement of individual samples and in the assessment of replicates.

The expression of *gnrh* mRNA in response to kisspeptin stimulation in low glucose media

There is published evidence suggesting that GT1-7 cells are impacted by the amount of glucose present in the media (Laeger, Poehland et al. 2012), so we decided to determine if low glucose growth media containing 100pM estradiol would allow us to more accurately detect any differences imposed by the presence of BHB or TNFα. We first assessed the relative amount of *gnrh* mRNA produced by GT1-cells in the presence of low glucose media or low glucose media containing either 6mM BHB or 10ng/mL TNFα. We compared samples exposed to stressors and control samples at the 0h time point to determine if exposure to stressors changed the baseline production of *gnrh* mRNAThe amount of *gnrh* mRNA was reduced in cells exposed to 6mM BHB vs. control at the 0 time point by 31% ($p = 0.45$) and increased in cells exposed to TNFα vs. control by 35% ($p = 0.16$) There was an increase in the amount of *gnrh* mRNA produced at four hours compared to zero hours by all groups that did not reach statistical significance (control 61%, $p = 0.38$; BHB 84%, $p = 0.16$; TNF 90%, $p = 0.25$), then a reduction at each time afterward. There was no statistical difference

a.



b.

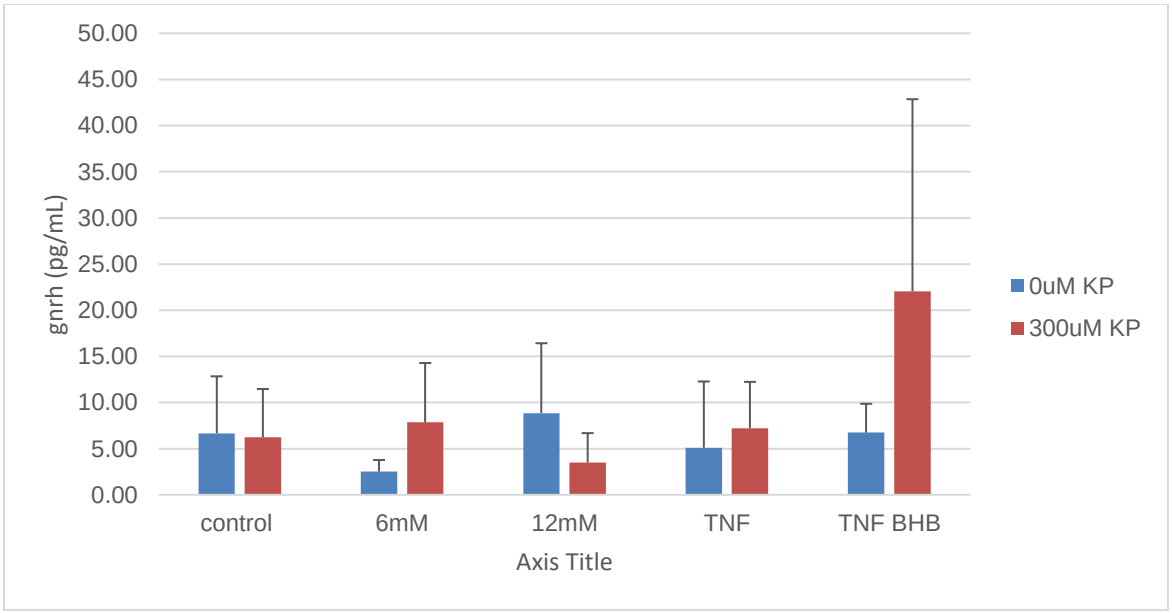


Figure 8: The release of gnrh from GT1-7 cells exposed to stressors in response to KP10 stimulation. Media without E2 (a) or with E2 (b) was collected and assayed by RIA.

between experimental groups at four hours or any time point thereafter (Figure 9.) We then used protein extracted from GT1-7 cells exposed to 6mM BHB, 10ng/mL TNFα in low glucose media to assess the phosphorylation of ERK1/2. The baseline level of phosphorylation in low glucose was relatively lower than that of high glucose media (data not shown), but there was not an increase in phosphorylation between cells at 0 minutes vs. 30 minutes, nor was there a change in the phosphorylation status between any experimental groups (Figure 10.) Although it has been shown that GT1-7 cells express kiss1R and respond to KP10 stimulation with upregulation of *gnrh* mRNA and release of gnrh protein, at least one lab has shown that kiss1R can be absent, likely due to the changes that can occur in immortalized cell lines (Sukhbaatar, Kanasaki et al. 2013). Because of this, we decided to check for the expression of kiss1R in our GT1-7 culture. We compared protein isolated from the GT1-7 line with protein from HEK293T cells, which express kiss1R, and determined that in our hands, kiss1R is undetectable in the GT1-7 cells by Western blot using this particular antibody, suggesting that Kiss1R expression may be below the limit of detection in our line, at least in the absence of estradiol. This could explain the subdued response to kisspeptin in the control cells, either at the level of mRNA or protein, in our cultures (Figure 11).

Discussion

GT1-7 cells have been used as a model for the hypothalamus in many different applications due to their neural phenotype and ability to release GnRH in

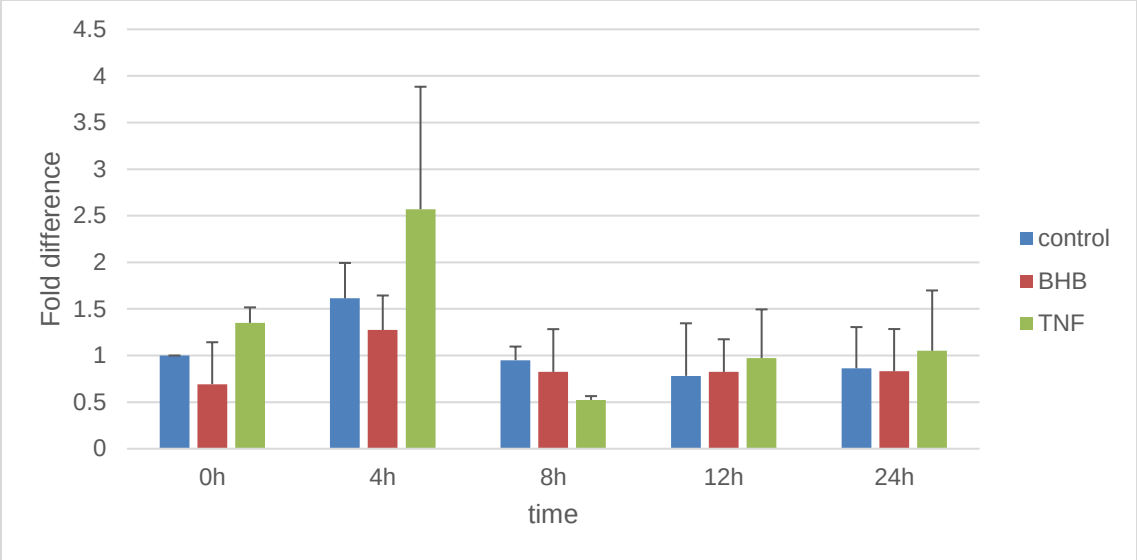
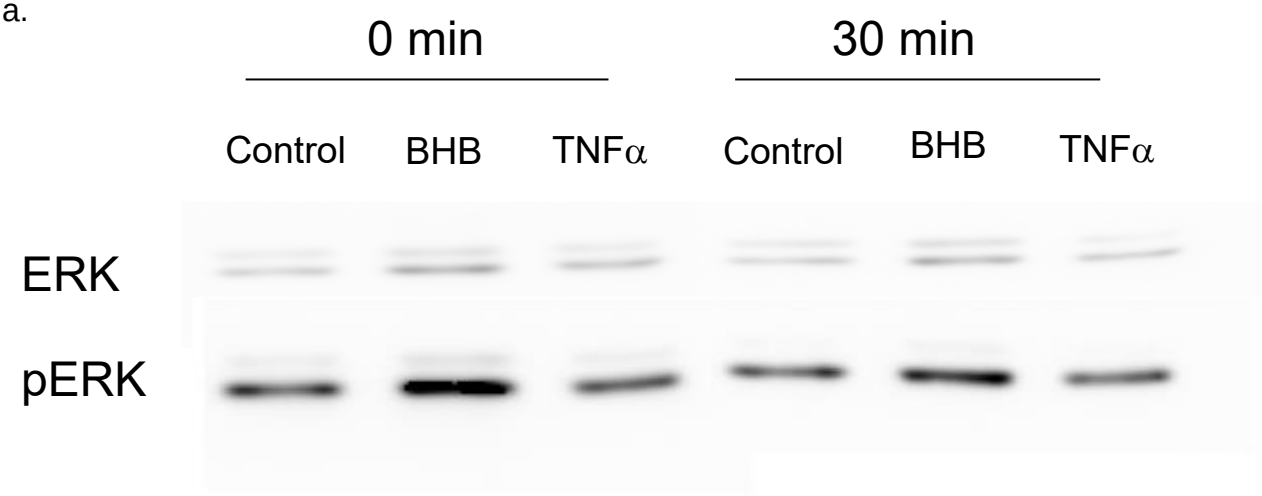


Figure 9: The expression of *gnrh* mRNA in GT1-7 cells exposed to stressors in low glucose media upon stimulation with KP10. Cells were incubated with 6mM or 12mM BHB, 10ng per milliliter TNFα, or combinations thereof in low glucose media containing 100pM E2 for twenty-four hours prior to stimulation with 300μM KP10. The change in *gnrh* mRNA expression was assessed with qPCR (n=3). Experimental groups with differing superscript letters are considered significantly different ($p<0.05$).

a.



b.

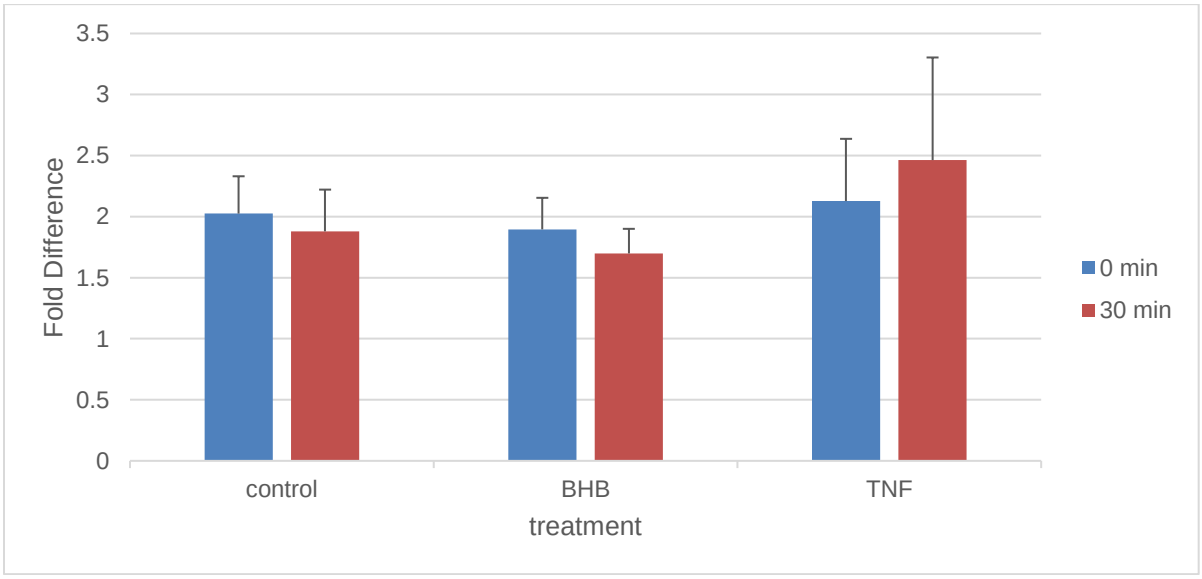


Figure 10: Assessment of ERK1/2 phosphorylation upon kisspeptin stimulation in GT1-7 cells exposed to stressors in low glucose media. Cells were exposed to stressors in low glucose media containing 100pM E2 for twenty-four hours, then stimulated with KP10 for 30 minutes. The phosphorylation status of ERK1/2 was assessed by Western blotting. a) Representative Western blot, b) quantitation of signal in three replicates by Image J.

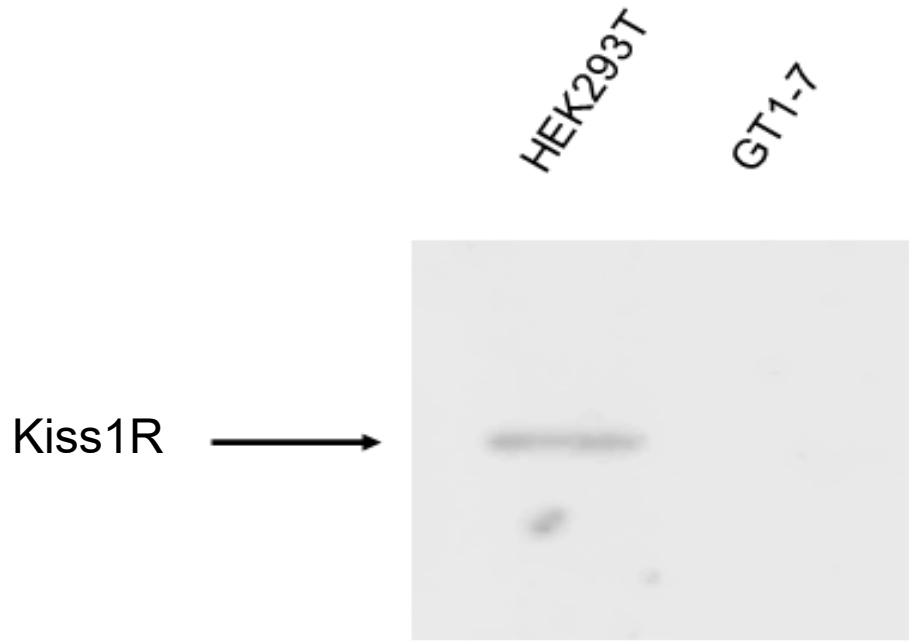


Figure 11: The expression of kiss1R in HEK293T and GT1-7 cells. Protein was extracted from HEK293T and GT1-7 cells in normal growth media and quantitated by Western blot.

response to depolarization (Mellon, Windle et al. 1990). They respond to kisspeptin stimulation by an upregulation of gnrh mRNA and a release of GnRH protein. Their response to KP10 stimulation is increased in the presence of estradiol (Jacobi, Martin et al. 2007, Tonsfeldt, Goodall et al. 2011, Terasaka, Otsuka et al. 2013). However, there is contradictory evidence that some native GT1-7 cultures may lack the expression of Kiss1R and the ability to respond to KP10 stimulation (Sukhbaatar, Kanasaki et al. 2013). This difference could possibly be due to the tendency of immortalized cell lines to mutate with increasing passage number. However, upon dual transfection with the kisspeptin receptor and a luciferase reporter conjugated to the GnRH promoter, the cells did respond with stimulation of the GnRH promoter and luciferase expression. Additionally, this study found that kisspeptin increases the phosphorylation of ERK1/2, the release of cAMP, and expression of the GnRH receptor (GnRHR).

In our hands, there was a small but statistically significant increase in the expression of *gnrh* mRNA in response to kisspeptin in the absence of estradiol. However, in the presence of 100pM estradiol, while this small increase remained, it did not reach statistical significance. This minimal response to kisspeptin stimulation in the control might be partially due to a reduced expression of the kisspeptin receptor protein. However, an upregulation in the expression of *gnrh* mRNA might also be masked by the intrinsic ability of GT1-7 cells to produce and release GnRH in a pulsatile manner. GT1-7 cells have been shown to release GnRH in discrete, rhythmic pulses (Krsmanovic, Stojilkovic et al. 1993, Weiner

and Delaescalera 1993). The spontaneous propagated action potentials that trigger this release are calcium dependent and can be suppressed by removal of calcium from the media. Additionally, these cells also express the GnRH receptor, which allows the GnRH released into the media to act in an autocrine fashion. While this amplifies and helps to synchronize the pulsatile release of GnRH, it is not required (Krsmanovic, Stojilkovic et al. 1993, Charles and Hales 1995). While the release of GnRH from GT1-7 cells occurs in normal growth media in a perfusion culture system, the ability to release GnRH in a pulsatile fashion in static culture is dependent upon cell synchronization by either serum shock or serum starvation (Wetsel, Valenca et al. 1992, Chappell, White et al. 2003). The initial release of GnRH after cell synchronization is dependent upon intracellular calcium stores (Krsmanovic, Stojilkovic et al. 1993), while the propagation of action potentials requires extracellular calcium to be imported through L-type calcium channels (Weiner and Delaescalera 1993, Charles and Hales 1995, Chen, Javors et al. 2004). Therefore, the exposure of our GT1-7 cells to stressors in a serum free medium that contains calcium likely triggered the intrinsic ability of the cells to produce *gnrh* mRNA and release GnRH protein into the media, even low or potentially absent levels of GnRH protein.

Upon exposure to BHB, the intrinsic production of *gnrh* mRNA in GT1-7 cells was increased slightly but significantly in basal media and to a much greater extent in media containing 100pM E2. Surprisingly, while there was a significant increase

in *gnrh* mRNA upon exposure to kisspeptin in basal media, this increase did not occur with exposure to E2. These changes were dependent on the glucose concentration of the media, as they did not occur in experiments conducted in low glucose media. Cheng et al. determined that the exposure of GT1-7 cells to adiponectin reduces GnRH signaling via activation of AMPK(Cheng, Wen et al. 2011). It has been determined that exposure of GT1-7 cells to 6mM BHB can suppress the activation of AMPK in both high and low glucose media, with a singular exception at 4 hours in high glucose media. BHB elicits a faster increase in intracellular calcium in cells in high glucose media, whereas in low glucose media only a marginal influx of calcium is detected (Laeger, Poehland et al. 2012). Given this data, it is possible that BHB suppresses the activity of AMPK in high glucose media and increases the calcium influx, enhancing the intrinsic production of *gnrh* mRNA in GT1-7 cells. Additionally, it has been shown *in vivo* that in a rat model of undernutrition, while the expression of *kiss1* is decreased in the hypothamus, the expression of *kiss1R* is increased (Castellano, Navarro et al. 2005). While the levels of plasma BHB were not assessed in the rat *in vivo* model, in other models of undernutrition plasma BHB is increased (Szymanski, Schneider et al. 2007, Hosseini, Behrendt et al. 2012, Huzzey, Mann et al. 2015, Albaaj, Foucras et al. 2017)If this is the case, then one would expect that exposure to BHB would increase the expression of *kiss1R* in a hypothalamic cell line such as GT1-7. In our model, we see an increase in *kiss1R* mRNA expression in GT1-7 cells grown in high glucose media, with an even greater

increase upon stimulation with kisspeptin, which could possibly explain the elevation in *gnrh* mRNA expression in BHB exposed cells upon stimulation with kisspeptin in high glucose media. Further experiments to determine the increase in *kiss1R* mRNA is accompanied by an increase in Kiss1R protein upon exposure to BHB may help explain the response of GT1-7 cells to this stressor. GT1-7 cells are able to produce *kiss1* mRNA in response to high levels of estradiol, so an assessment of the expression of *kiss1* mRNA in response to BHB may be warranted to determine if the combination of lower estradiol levels and subclinical concentrations of BHB could have the same effect.

We did not see a change in the expression of *gnrh* mRNA in cells incubated with TNFα regardless of exposure to estradiol, KP10, or glucose. This is unexpected because Sarchielli *et al*. (Sarchielli, Comeglio et al. 2017) determined that exposure to TNFα blocks the depolarization of primary GnRH producing neurons in response to KP10 stimulation. Given the ability of TNFα to inhibit the response to KP stimulation in culture in combination with *in vivo* studies suggesting that TNFα inhibits the release of gonadotropins, we expected that the production of gnrh mRNA would be reduced in our system. It is possible that the excessive production of GnRH in GT1-7 cells may have masked any effect that exposure to TNFα may have had on the cells' ability to respond to kisspeptin stimulation. On the other hand, the expression of *kiss1R* mRNA is increased in GT1-7 cells exposed to TNFα in high glucose media. This would suggest that if exposure to

TNFα would decrease the cells' ability to respond to KP10, it might not be due to a suppression of Kiss1R expression. However, the possibility remains that *kiss1R* mRNA may not be translated into Kiss1R protein, which would inhibit the cells' ability to appropriately respond to KP10 stimulation.

We did not see significant changes in the release of intracellular calcium from the endoplasmic reticulum in any of the experimental conditions studied. In the case of calcium release, we were unable to detect calcium changes during the log phase of calcium release due to limitations in our detection equipment. Assessment in real time with perifusion culture may be a more sensitive method to determine if stressors impact this facet of Kiss1R signaling. However, the intrinsic ability of these cells to produce GnRH has been shown to be dependent on calcium signaling, and therefore our assay may be ineffective in detecting any changes due to high background. This same difficulty may explain the lack of changes in the phosphorylation status of ERK1/2 in this study, as the pathway may be active prior to the addition of the KP ligand to the culture system.

In our hands, we were unable to detect Kiss1R in unstimulated GT1-7 cells in normal growth media. While the expression of the receptor is below the level of detection under this condition, the slight increase in *gnrh* mRNA in response to KP10 suggests that the receptor may be present at some level. The possibility remains that the addition of estradiol to the cells would increase the expression

of Kiss1R, as Tonsfeldt et al. have determined that Kiss1R expression can oscillate in a circadian manner upon exposure to estradiol (Tonsfeldt, Goodall et al. 2011). It is worth assessing whether the expression of Kiss1R protein can change in response to BHB, which may explain the change in *gnrh* mRNA expression in response to BHB exposure.

CHAPTER II
THE IMPACT OF STRESSORS ON THE PRODUCTION OF KISSPEPTIN IN AN *IN VITRO* MODEL OF THE ARCUATE NUCLEUS

Abstract

Stressors have a negative impact on reproductive efficiency in humans and other animals, which has an economic cost due to infertility treatments for humans and reduced income for food producers. We wished to determine the molecular pathways by which metabolic disturbances and low-level inflammation impact the hypothalamic pituitary gonadal (HPG) axis using an *in vitro* model. To this end, we used the KTaV-3 and KTaR-1 cell lines to assess the impact of stressors on the release of kisspeptin from the rodent anteroventral periventricular and arcuate nuclei, respectively. We modeled metabolic disease and negative energy balance by exposing cells to beta-hydroxybutyric acid (BHB) and inflammation by exposure to tumor necrosis factor alpha (TNFα). Exposure to stressors in culture did not change the expression of *kiss1* mRNA in KTaV-3 cells upon exposure to estradiol. However, KTaV-1 cells exhibited a significantly decreased expression of *kiss1* mRNA and kiss1 protein release upon exposure to both BHB and tumor necrosis factor alpha. There was also a reduction in the expression of neurokinin B (*nkb*) mRNA under these conditions, suggesting that the impact of stressors on the HPG axis may be acting through the suppression of signaling in the arcuate nucleus. Disruption of neurokinin B and kisspeptin expression in the arcuate nucleus could impact the production of tonic levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH), resulting in a deficiency in follicle production and oocyte maturation.

Introduction

Kisspeptin (KP) and Kiss1R are central to the function of the HPG axis. Kisspeptin functions in the hypothalamus, binding to Kiss1R and stimulating the release of GnRH into the hypophysial portal blood, which then stimulates the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary (Han, Gottsch et al. 2005, Castellano, Navarro et al. 2006, Suzuki, Kadokawa et al. 2008). These hormones function by promoting gametogenesis and steroidogenesis. Mutations of either protein (KP or Kiss1R) in humans or mice result in an inability to progress through puberty and reproduce effectively (de Roux, Genin et al. 2003, Seminara, Messager et al. 2003, Wahab, Quinton et al. 2011). Kisspeptin neurons in the arcuate (ARC) nucleus express the protein in a tonic manner during the estrous cycle, causing the release of FSH and allowing maturation of oocytes to occur. These nuclei respond negatively to estradiol (E2) stimulation, causing KP production to decrease as E2 secretion from the ovary increases. Alternately, KP neurons in the anteroventricular periventricular (AVPV) nucleus respond positively to E2, releasing KP in response to the E2 peak during estrous, causing the release of LH to generate the LH surge and thus ovulation (Smith, Cunningham et al. 2005, Smith, Li et al. 2009).

Metabolic disease such as undernutrition (Castellano, Navarro et al. 2005, Luque, Kineman et al. 2007), type II diabetes (Castellano, Navarro et al. 2006,

Castellano, Navarro et al. 2009, Castellano, Roa et al. 2009), and ketosis (Huzzey, Mann et al. 2015, Albaaj, Foucras et al. 2017) have been associated with disruption of the hypothalamic pituitary gonadal (HPG) axis. Inflammation has a similar suppressive effect on the HPG axis, as shown in animal models in which treatment with lipopolysaccharide (LPS; endotoxin) suppresses the LH surge in rabbits (Akema, He et al. 2005) and rats (Iwasa, Matsuzaki et al. 2008, Castellano, Bentsen et al. 2010). The effect of LPS on the LH surge is mediated by TNFα (Yoo, Nishihara et al. 1997) , which has also been shown to suppress the LH surge independently of LPS.

Many of the studies investigating the impact of stressors on reproduction have concentrated on the effect of stressors on the production of LH from the anterior pituitary gland and the function of the ovary. However, the anterior pituitary releases LH in response to signals originating in the hypothalamus; these signals are further regulated by E2 released from the ovary, forming a feedback loop. Identification of the molecular pathways by which hypothalamic function is compromised in these nuclei by stressors would help to identify targets for intervention, potentially improving gonadal function. Deficits in the production of LH due to negative energy balance have been shown to be rescued by treatment with exogenous GnRH, suggesting that the pituitary gland is able to respond to GnRH normally. In previous *in vitro* experiments in our lab using the GnRH-producing cell line GT1-7 have suggested that stressors do not have a significant

impact in suppressing the production on GnRH from hypothalamic cells. This might indicate that stressors have in impact on the production of kiss1, which triggers the production of GnRH in the kiss1R expressing cells of the hypothalamus. In this work, we wished to determine if there is an impact of stressors on the function of the Kp neurons located in the AVPV and ARC nuclei of the hypothalamus and their ability to respond to E2 signaling.

As an *in* vitro model, we used two immortalized mouse cell lines (KTaV-3 and KTaR-1) (Jacobs, Veitch et al. 2016) as a model to investigate the impact of stressors on the AVPV and ARC. The KTaV-3 line was isolated from the AVPV of the mouse and has been shown to positively express Kp in response to estradiol. In the KTaR-1 line, which was isolated from the ARC, exposure to estradiol negatively regulates the expression of Kp. Using these lines, we will mimic stressors in culture using tumor necrosis factor alpha (TNFα) to simulate inflammation, beta-hydroxybutyrate (BHB) to simulate negative energy balance (a type of metabolic stress),

Materials and Methods

Cell lines and culture conditions

KTaV-3 and KTaR-1 cells were a gift from Patrick Chappell of Oregon State University. Cells were grown in high glucose DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), penicillin, streptomycin, L-glutamine, (Gibco) and amphotericin B and maintained in a humidified incubator at 37ºC with 5%

CO2. Upon reaching confluence, the cells were split 1:10 for maintenance in culture. Cells at a passage of less than 20 were used in experiments.

RNA extraction and qPCR

KTaV-3 and KTaR-1 cells were plated in 60mm tissue culture dishes at a density of 5.0 x 10^4 per cm^2 and allowed to grow to 80 percent confluency in phenol red-free DMEM (Gibco) supplemented with 10% charcoal stripped FBS (Gibco). The media was then replaced with serum-free phenol red-free DMEM either alone or with 10ng/ml TNFα (Sigma Aldrich, St. Louis, MO), 6mM BHB (Acros Organics, Geel, Belgium), or a combination of TNFα and BHB and maintained in a humidified incubator at 37ºC with 5% CO2 for twenty-four hours. The media was then switched to the same conditions with the same conditions with the addition of 50pM estradiol for KTaV-3 cells and 5pM estradiol for KTaR-1 cells (Sigma Aldrich, St. Louis, MO). Cells were collected prior to estradiol treatment at 0 hours, then after estradiol at 4, 8, 12 and 24 hours. At each time point, the media was removed and stored at -80ºC for further analysis. Cells were washed once in cold PBS, then RNA was isolated using the IBI Scientific Total RNA Mini Kit (Dubuque, IA). One microgram of RNA was reverse transcribed using the High-Capacity cDNA kit (Applied Biosystems, Foster City, CA). Of this reaction, 100ng were placed into a qPCR reaction using PerfeCta SYBR Green (Quantabio, Beverly, MA) on an Applied Biosystems MxPro 3005P real time PCR machine using primers against Kiss1 (Forward GCTCTGGTGAAGTACGAACTCTGA, Reverse GACACAGAGGAGAAGCAGCA, Tac2 (Forward

ATCCAACGCTCTTGAGATCA, Reverse CATCTTACCTTACTGAGCCTCC), and PPIA (Forward AAGTTCCAAAGACAGCAGAAAAC, Reverse CTCAAATTTCTCTCCGTAGATGG) (Tonsfeldt et al.). The data were analyzed using the ΔΔCt method.

ELISA

Media samples from KTaV-3 and KTaR-1 cells were dried in a vacuum centrifuge for 6 hours, then resuspended in 250μL PBS. The samples were then centrifuged for 20 minutes to remove any particulate matter and were analyzed using the LSBio ELISA kit according to manufacturer's directions. Briefly, 100μL of sample were loaded in duplicate onto the 96-well plate and incubated at 37ºC for two hours. One hundred microliters were of Detection Reagent A were added to each well, mixed gently, and incubated at 37ºC for one hour. Wells were then aspirated, washed, and incubated with 100μL of Detection Reagent B at 37ºC for one hour.

Statistics

Data from RNA experiments were analyzed by a Mann Whitney test in SPSS> Data from ELISA were analyzed by two-way analysis of variance (ANOVA) using SPSS software. A p-value of less than or equal to 0.05 was considered significant.

Results

The impact of stressors on an in vitro model of the arcuate nucleus

We sought to determine if physiological stressors had an impact on the function of the arcuate nucleus of the hypothalamus, which is responsible for the tonic expression of kisspeptin *in vivo*. To this end, we used the KTaR-1 cell line (Jacobs et al.), which expresses neurokinin B and dynorphin, and downregulates kisspeptin upon exposure to estradiol. Cultures were exposed to serum free media containing 6mM BHB, 10ng/mL TNFα*,* or a combination thereof for 24 hours prior to exposure to 5pM E2. Cells were collected at 0, 4, 8, 12, and 24 hours, and the expression of *kiss1* mRNA was assessed by qPCR. There was a significant reduction in *kiss1* mRNA expression in cells exposed to BHB versus control (54%, p=0.05), TNF versus control (37%, p=0.01), and BHB/TNF versus control (33%, p=0.002). Upon exposure to estradiol, *kiss1* mRNA expression in control cells was reduced by 49% (p=0.02), but there was no decrease in cells exposed to BHB (p=0.97) and a decrease of 32% in cells exposed to TNFα that did not reach significance (p=0.35). There was also a decrease between the 0h and 4h time points in cells exposed to a combination of BHB and TNFα of 55% that also failed to reach significance (p=0.49). There was no significant difference between control cells and stressed cells at any other time point. There was an increase in all experimental conditions at 24 hours versus 0 hours (control p=0.52; BHB p=0.05; TNFα p=0.19) that only reached significance in the case of

BHB, suggesting that the cells were released from E2-induced suppression of *kiss1* mRNA at this time (Figure 12).

Because the difference in *kiss1* mRNA expression in the presence of stressors was detected at the 0h timepoint, we wished to determine if the expression of neurokinin B (*nkb, tac2*) or dynorphin (*pdyn)* m*RNA* was impacted at this time point upon exposure to BHB or TNFα. Neurokinin B acts in an autocrine fashion on KNDy neurons to stimulate the expression of *kiss1* mRNA and the production of *kiss1.* Dynorphin also acts in an autocrine fashion but suppresses the expression of *kiss1.* (Ramaswamy, Seminara et al. 2010, Wakabayashi, Nakada et al. 2010, Garcia-Galiano, Schenau et al. 2012, Goodman, Hileman et al. 2013). In our hands, *pdyn* mRNA was undetectable by qPCR in under any experimental condition. The expression of *nkb* mRNA was significantly lower in cells exposed to TNFα (60%, $p \leq 0.001$); *nkb* was lower to a similar extent in cells exposed to BHB by 61% ($p=0.08$), but this number failed to reach significance (Figure 13.)

Finally, we wished to quantitate the KISS1 protein released from KTaR1 cells in response to E2 exposure. Because of the low levels present, media was

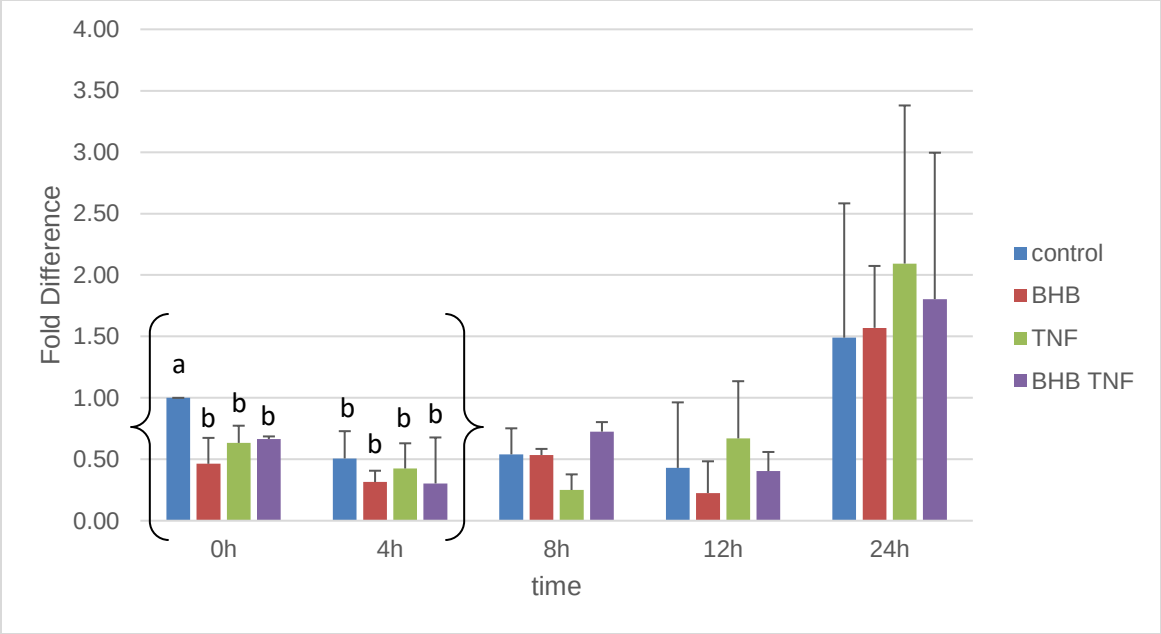


Figure 12: The expression of *kiss1* mRNA in KTaR-1 cells exposed to stressors prior to stimulation by estradiol. KTaR-1 cells were exposed to 6mM BHB, 10ng/mL TNFα, or a combination for twenty-four hours prior to stimulation by 5pM E2. The expression of *kiss1* mRNA was assessed by qPCR (n=3). Experimental samples with differing superscript letters are considered significant ($p<0.05$).

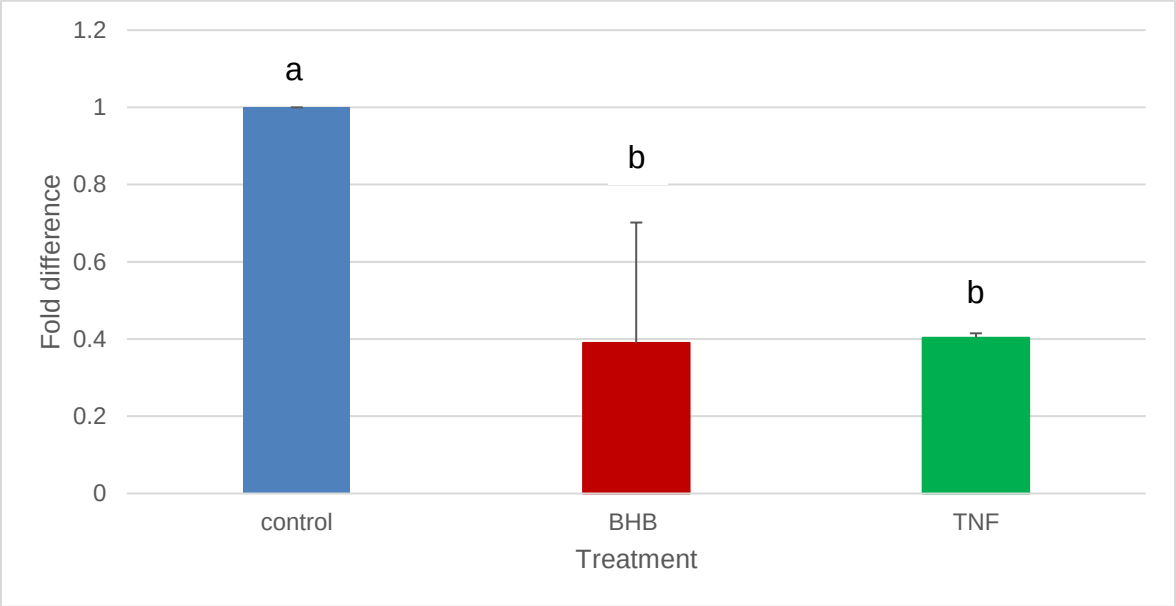


Figure 13: The expression of *nkb* mRNA in KTaR-1 cells exposed to stressors for 24 hours. KTaR-1 cells were exposed to 6mM BHB, 10ng/mL TNFα, or a combination for twenty-four hours. The expression of *kiss1* mRNA was assessed by qPCR (n=3). Samples with differing superscript letters are considered significantly different ($p<0.05$)

collected and concentrated in a vacuum centrifuge prior to protein measurement by ELISA. At the 0h time point, there was no significant difference between protein present in control media versus media containing BHB. The protein present in media containing TNFα was reduced by 28.1% in comparison to control media, but this value did not reach significance. Upon exposure to 5pM E2, protein release by KTaR1 cells in control media was significantly reduced by 92.9 percent ($p=0.006$), as was the protein released into the media containing BHB ($p=0.036$) and TNFα ($p=0.007$). There was no significant difference between the protein content of control media and media containing either BHB or TNFα at the 4h time point (Figure 14).

The impact of stressors on an *in vitro* model of the anteroventricular periventricular nucleus

Having determined that stressors do impact the ability of an *in vitro* model of the arcuate nucleus to produce *kiss1* mRNA, we then wished to determine if they could also impact the function of the AVPV, which is responsible for the kiss1 surge. This nucleus responds positively to estradiol *in vivo*. We used the KTaV-3 cell line (Jacobs, Veitch et al. 2016), which upregulates kisspeptin upon exposure to estradiol. Cultures were exposed to serum free media containing 6mM BHB, 10ng/mL TNFα, or a combination thereof for 24 hours prior to exposure to 25pM E2. Cells were collected at 0, 4, 8, 12, and 24 hours, and the expression of *kiss1* mRNA was assessed by qPCR (Table 1). While each replicate did respond

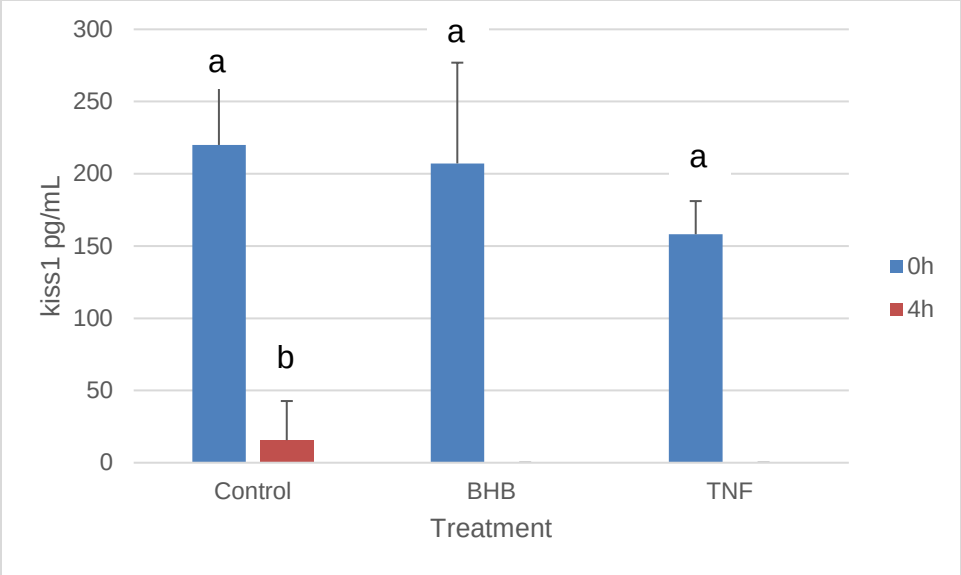


Figure 14: The release of kisspeptin from KTaR-1 cells after exposure to estradiol. KTaR-1 cells were exposed to 6mM BHB, 10ng/mL TNFα, or a combination for twenty-four hours prior to stimulation by 5pM E2. The release of kisspeptin into the media was assessed by ELISA (n=3). Experimental groups with differing superscript letters indicate significant difference ($p<0.05$).

Table 1: The expression of *kiss1* mRNA in KTaV-3 cells after exposure to estradiol. KTaV-3 cells were exposed to 6mM BHB, 10ng/mL TNFα, or a combination for twenty-four hours prior to stimulation by 25pM E2. The expression of *kiss1* mRNA was assessed by qPCR.

time	media	kiss1 mRNA relative expression	p value vs. control 0h	p value by time
0h	control	1.00 +/- 0.00		
	BHB	0.82 +/- .09	0.22	0.22
	TNF	1.19 +/- 1.12	0.85	0.85
4h	control	0.93 +/- 0.72	0.91	
	BHB	0.58 +/- 0.22	0.22	0.61
	TNF	0.82 +/- 0.80	0.80	0.90
8h	control	2.53 +/- 2.84	0.59	
	BHB	2.54 +/- 2.52	0.55	1.00
	TNF	1.91 +/- 2.14	0.65	0.83
12h	control	4.18 +/- 3.31	0.40	
	BHB	2.56 +/- 2.48	0.54	0.64
	TNF	2.85 +/- 2.56	0.49	.
24h	control	2.53 +/- 0.16	0.05	
	BHB	1.55 +/- 0.88	0.54	0.35
	TNF	1.68 +/- 0.56	0.34	0.26

positively to the addition of E2 beginning at the 8h time point and continuing through 24 hours, the fold increase varied by replicate which resulted in a large standard error and the inability to reach significance (Figure 15). We then decided to assess the ratio of stressor to control at each time point in individual replicates (Table 2). Compared to controls, cells exposed to BHB showed a reduction in *kiss1* expression of approximately 25% (p=0.51) at 4 hours, an increase of 20%45% (p=0.57) at 8 hours, a decrease of 45% (p=0.16) at 12 hours, and a decrease of 38% (p=0.40) at 24 hours, none of which reached statistical significance. Cells exposed to TNFα showed a reduction of approximately 22% (p=0.44) at 4 hours, 24% (p=0.02) at 8 hours, 36% (p=0.13) at 12 hours, and 34% (p=0.32) at 24 hours. Of these, only the reduction in *kiss1* expression at 8 hours reached significance (Figure 16). We then determined whether exposure to stressors changed the release of kiss1 protein from KTaV-3 cells into the media after 24 hours of exposure to 25pM E2. Contrary to our expectations, kiss1 protein was slightly reduced upon exposure to E2 under all conditions (control 22.5%, p = 0.17; BHB 27.74%, p=0.23; TNF 14.77%, p=0.64), although this reduction did not reach statistical significance (Figure 17).

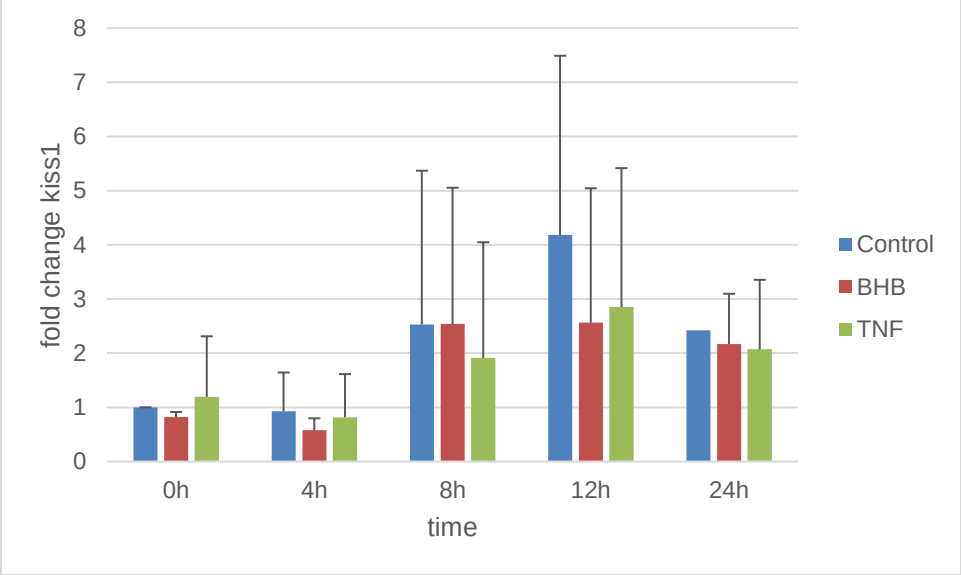


Figure 15: The expression of *kiss1* mRNA in KTaV-3 cells. KTaV-3 cells were exposed to 6mM BHB, 10ng/mL TNFα, or a combination for twenty-four hours prior to stimulation by 5pM E2. The expression of *kiss1* mRNA was assessed by qPCR (n=3).

Table 2: Relative expression of *kiss1* mRNA in KTaV-3 cells. The relative expression of *kiss1* mRNA in each stressor condition at each time point was assessed relative to the *kiss1* mRNA expression in the control. Assessment was done for each replicate and the results averaged. A p-value less than 0.05 was considered significant.

time	media	kiss1 mRNA relative expression	p-value
0h	control	1.00 +/- 0.00	
	BHB	0.82 +/- 0.09	0.22
	TNF	1.19 +/- 1.12	0.85
4h	control	1.0 +/- 0.00	
	BHB	0.76 +/- 0.35	0.51
	TNF	0.78 +/- 0.26	0.44
8h	control	1.0 +/- 0.0	
	BHB	1.20 +/- 0.36	0.56
	TNF	0.76 +/- 0.01	0.02
12h	control	1.0 +/- 0.0	
	BHB	0.55 +/- 0.16	0.16
	TNF	0.64 +/- 0.11	0.13
24h	control	1.0 +/- 0.0	
	BHB	0.62 +/- 0.38	0.4
	TNF	0.67 +/- 0.26	0.32

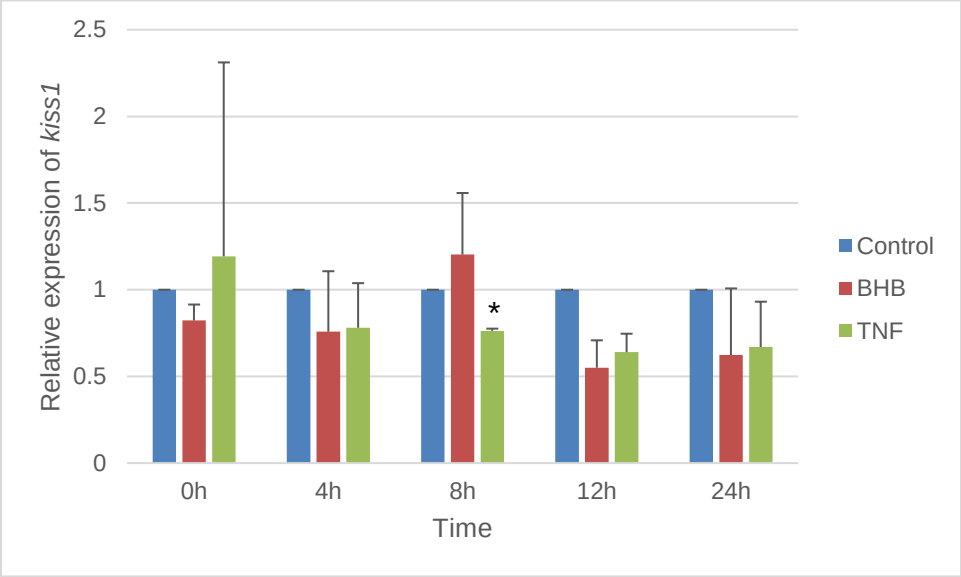


Figure 16: The ratio of stressor to control *kiss1* mRNA expression in KTaV-3 cells. The expression of *kiss1* mRNA in each experimental condition was divided by the expression in the control sample at the same time point for each replicate, and the resulting ratios from each replicate were averaged.

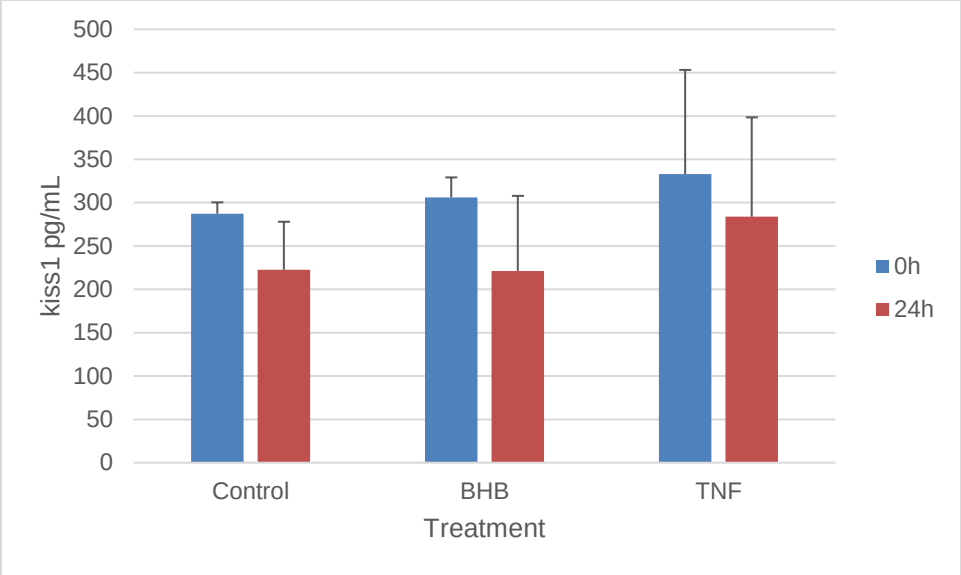


Figure 17: The release of kisspeptin from KTaV-3 cells after exposure to estradiol. KTaV-3 cells were exposed to 6mM BHB, 10ng/mL TNFα, or a combination for twenty-four hours prior to stimulation by 25pM E2. The release of kisspeptin into the media was assessed by ELISA.

Discussion

In this work, we have shown that exposure to stressors impacts the ability of cells derived from the arcuate nucleus to appropriately produce both *kiss1* mRNA and *nkb* mRNA. Exposure to 6mM BHB or 10ng/mL TNFα for twenty-four hours reduces the expression of both *kiss1* and *nkb* mRNA prior to the addition of 5pM estradiol to the media. Addition of estradiol reduces the expression of *kiss1* mRNA in control cells by 49 percent, but no further significant reduction is seen in cells exposed to BHB or TNFα. Given that nkb acts in an autocrine fashion to upregulate *kiss1*, the suppression of *nkb* mRNA upon exposure to stressors suggests that this may be the mechanism by which *kiss1* mRNA is suppressed. The reduction of *kiss1* mRNA in this model is not mirrored by a reduction in kiss1 protein released into the media at the 0h time point in our model, although the release of kiss1 into the media was appropriately suppressed by exposure to estradiol in all experimental conditions. The presence of kiss1 protein in the media at the 0h time point may be due to the performance of this experiment in static culture. The kiss1 protein release could change over the course of twenty-four hours, but it is possible that the protein released shortly after the addition of stressors could still be present when the 0h time point samples were collected. This could be assessed by analysis of the media at several time points over the course of twenty-four hours with the addition of fresh media at each time point to determine if the release of kiss1 protein is suppressed over time upon exposure

to stressors. Alternately, the experiment could be performed in perfusion culture, which would more closely replicate the movement of bodily fluids *in vivo*.

We did not see significant suppression of *kiss1* mRNA in the KTaV-3 (AVPV) cell line, aside from a small but significant suppression in the relative expression of *kiss1* at eight hours by TNFα. The expression of *kiss1* mRNA increased in all experimental replicates at eight hours, although the level of increase was variable. The kiss1 protein released into the media of these cells was assessed at twenty-four hours and no significant increase in response to estradiol exposure was detected. It is possible that the release should be assessed over an extended period of time, analyzing the protein release at several earlier and later time points. Additionally, the performance of these experiments in perfusion rather than static culture may give a better picture of the impact of stressors on the function of these cells as it more closely replicates the *in vivo* setting. The difference between the pattern of expression of *kiss1* mRNA in KTaV-3 cells seen by Jacobs et al. and that seen in our hands may also be related to the manner of cell synchronization, in that Jacobs et al. used serum shock with 50% FBS for two hours, while cells in our system were synchronized in serum free media for twenty-four hours. The possibility exists that a change in cell synchronization might alter the response times to estradiol exposure. The lack of change in kiss1 protein expression between the 0h and 24h time points may be due to a negative feedback mechanism. Assessment of earlier time points may

show a changed pattern of protein expression in KTaV-3 cells similar to the alteration in the *kiss1* mRNA expression in our system.

While the AVPV is responsible for the kisspeptin surge that ultimately results in LH-induced ovulation, the tonic release of kisspeptin from the ARC is responsible for the selection and maturation of ovarian follicles. As follicles mature, they release increasing amounts of estradiol. If the expression of kisspeptin in the arcuate nucleus is suppressed, ovarian follicles might not mature appropriately, which would ultimately result in an absence of ovulation. In most of the systems studied with either metabolic or inflammatory stress, altered expression of *kiss1* mRNA is detected in the ARC. KTaR-1 cells behave similarly in response to exposure to BHB or TNFα with a suppression of *kiss1* mRNA expression, supporting the idea that the production of kiss1 by the ARC is critical for the HPG axis to function normally and that a defect in *kiss1* expression in the ARC may be common to different types of stressors.

CONCLUSION

Physiological stressors such as negative energy balance, diet induced obesity, and low-level inflammation have a negative effect on the function of the hypothalamic pituitary gonadal axis. These stressors have an impact on the reproductive efficiency of both humans and other animals. *In vivo* studies have shown that metabolic disorders and inflammation are both associated with reduced levels of plasma luteinizing hormone. Since this defect can be rescued with exogenous gonadotropin releasing hormone (GnRH), it is likely that the hypothalamic pituitary gonadal (HPG) axis is impacted by stressors at the level of the hypothalamus. In this work, we used three cell lines as an *in vitro* system to assess if stressors impact the ability of hypothalamic nuclei to adequately function.

GT-7 cells, which can produce GnRH in response to kisspeptin stimulation, were used to assess the impact of stressors on GnRH function. We determined that exposure to BHB significantly increased the intrinsic production of GnRH by GT1-7 cells, but that there were no additional changes in the expression of GnRH mRNA or release of GnRH protein. Additionally, there were no significant changes in calcium signaling or in the phosphorylation of ERK1/2. However, this cell line has the ability to spontaneously produce and release GnRH in a pulsatile manner upon cell synchronization in static culture. Additionally, we were unable to detect the presence of kiss1r protein in our cultures, potentially due to the

accumulation of mutations resulting in the loss of expression in our hands. While the ability of the cells to spontaneously produce GnRH could make the detection of GnRH production due to kisspeptin stimulation difficult, it might be possible to repeat these experiments with a line that has been transfected with a construct used to overexpress kiss1r which has been shown to be successful by Sukhbaatar et al. Additionally, since cell synchronization is necessary for the initiation of periodic GnRH expression, exposure to stressors could be done in complete growth media to suppress *gnrh* mRNA expression until the cells are stimulated by kisspeptin.

We also wished to determine the impact of stressors on the kisspeptin-producing cells of the arcuate nucleus and anteroventricular periventricular nucleus. As an *in vitro* model, we used two immortalized mouse cell lines (KTaV-3 and KTaR-1) (Jacobs, Veitch et al. 2016) as a model to investigate the impact of stressors on the anteroventricular periventricular nucleus (AVPV) and arcuate nucleus (ARC). The KTaV-3 line was isolated from the AVPV of the mouse and has been shown to positively express kisspeptin in response to estradiol. In the KTaR-1 line, which was isolated from the ARC, exposure to estradiol negatively regulates the expression of kisspeptin. We determined that exposure to 6mM BHB or 10ng/mL TNFα for twenty-four hours reduces the expression of both *kiss1* and *nkb* mRNA prior to the addition of 5pM estradiol to the media in KTaR-1 cells, but that there was not a significant impact on KTaR-3 cells aside from a slight suppression of

kiss1 mRNA expression by TNFα at a single time point. *In vivo*, however, it is not possible to entirely separate the function of these two hypothalamic nuclei. Disruption of tonic kisspeptin production in the ARC would have an effect on the maturation of ovarian follicles, which produce increasing amounts of estradiol as they mature. If the follicles were not able to mature appropriately and the level of estradiol necessary to generate a surge of kisspeptin from the AVPV is not produced, then ovulation will not occur.

Both KTaR-1 and KTaV-3 cell lines express the core clock genes *bmal1* and *per2*. KTaV-3 cells exhibit a rhythmic expression of these genes after serum synchronization regardless of estradiol exposure, while the rhythmic expression of *kiss1* is initiated by exposure to E2. KTaR-1 cells exhibit the same pattern as KTaV-3 cells, but this pattern shifts in response to E2 exposure (Jacobs, Veitch et al. 2016) There is evidence that inflammation and ketosis have an impact on the expression of clock genes in various tissue types, including brain. The possibility exists that the expression of these genes in the KTaR-1 cell line might be altered by exposure to BHB or TNFα. A future experiment might assess the levels of *bmal1*and *per2* over a twenty-four hour time course to determine if the expression pattern changes in response to stressors and if the expression of core clock genes is related to the expression of *nkb* mRNA.

Although *in vitro* experiments are very useful to tease apart molecular pathways, they cannot provide the entire picture of what happens *in vivo.* Therefore, an *in vivo* experiment using a mouse model would be a valuable addition to the current *in vitro* data. Mice cycle very quickly, and their cycles can be determined by vaginal cytology. It has already been established that the LH surge in mice occurs during the night hours since mice are nocturnal, and mating occurs at night. Wild type mice can be food restricted as a model of negative energy balance, fed a high fat diet as a model of diet induced obesity, or treated with LPS as a model of inflammation. In these models, the estrus cycle would be tracked by vaginal cytology to determine it changes in response to physiological stress. Additionally, blood samples would be taken to assess the level of LH in the plasma in correlation with the estrus cycle. Finally, mice would be sacrificed at different times of day and the expression of *kiss1* and *nkb* mRNAs assessed by *in situ* hybridization to determine if stress changes the expression pattern of these genes, and if the change is similar between the stressor types. Understanding the molecular basis of stress-induced perturbation of the HPG axis could provide insight into the interventions that might correct this phenomenon.

REFERENCES

Ahima, R. S., et al. (1996). "Role of leptin in the neuroendocrine response to fasting." Nature **382**(6588): 250-252.

Akema, T., et al. (2005). "Lipopolysaccharide increases gamma-aminobutyric acid synthesis in medial preoptic neurones in association with inhibition of steroid-induced luteinising hormone surge in female rats." Journal of Neuroendocrinology **17**(10): 672-678.

Albaaj, A., et al. (2017). "High somatic cell counts and changes in milk fat and protein contents around insemination are negatively associated with conception in dairy cows." Theriogenology **88**: 18-27.

Anagnostis, P., et al. (2019). "Early menopause and premature ovarian insufficiency are associated with increased risk of type 2 diabetes: a systematic review and meta-analysis." European Journal of Endocrinology **180**(1): 41-50.

Backholer, K., et al. (2010). "Kisspeptin Cells in the Ewe Brain Respond to Leptin and Communicate with Neuropeptide Y and Proopiomelanocortin Cells." Endocrinology **151**(5): 2233-2243.

Burke, M. C., et al. (2006). "Coexpression of dynorphin and neurokinin B immunoreactlivity in the rat hypothalamus: Morphologic evidence of interrelated function within the arcuate nucleus." Journal of Comparative Neurology **498**(5): 712-726.

Caraty, A., et al. (1998). "Evidence that the mediobasal hypothalamus is the primary site of action of estradiol in inducing the preovulatory gonadotropin releasing hormone surge in the ewe." Endocrinology **139**(4): 1752-1760.

Caraty, A., et al. (1989). "BIPHASIC RESPONSE IN THE SECRETION OF GONADOTROPIN-RELEASING HORMONE IN OVARIECTOMIZED EWES INJECTED WITH ESTRADIOL." Journal of Endocrinology **123**(3): 375-382.

Castellano, J. M., et al. (2010). "Kisspeptins: Bridging energy homeostasis and reproduction." Brain Research **1364**: 129-138.

Castellano, J. M., et al. (2010). "Acute inflammation reduces kisspeptin immunoreactivity at the arcuate nucleus and decreases responsiveness to kisspeptin independently of its anorectic effects." American Journal of Physiology-Endocrinology and Metabolism **299**(1): E54-E61.

Castellano, J. M., et al. (2006). "Ontogeny and mechanisms of action for the stimulatory effect of kisspeptin on gonadotropin-releasing hormone system of the rat." Molecular and Cellular Endocrinology **257-8**: 75-83.

Castellano, J. M., et al. (2005). "Changes in hypothalamic KiSS-1 system and restoration of pubertal activation of the reproductive axis by kisspeptin in undernutrition." Endocrinology **146**(9): 3917-3925.

Castellano, J. M., et al. (2006). "Expression of hypothalamic KiSS-1 system and rescue of defective gonadotropic responses by kisspeptin in streptozotocin-induced diabetic male rats." Diabetes **55**(9): 2602-2610.

Castellano, J. M., et al. (2009). "Alterations in Hypothalamic KiSS-1 System in Experimental Diabetes: Early Changes and Functional Consequences." Endocrinology **150**(2): 784-794.

Castellano, J. M., et al. (2009). "KiSS-1/kisspeptins and the metabolic control of reproduction: Physiologic roles and putative physiopathological implications." Peptides **30**(1): 139-145.

Chappell, P. E., et al. (2003). "Circadian gene expression regulates pulsatile gonadotropin-releasing hormone (GnRH) secretory patterns in the hypothalamic GnRH-secreting GT1-7 cell line." Journal of Neuroscience **23**(35): 11202-11213.

Charles, A. C. and T. G. Hales (1995). "MECHANISMS OF SPONTANEOUS CALCIUM OSCILLATIONS AND ACTION-POTENTIALS IN IMMORTALIZED HYPOTHALAMIC (GT1-7) NEURONS." Journal of Neurophysiology **73**(1): 56-64.

Chen, E. C., et al. (2004). "Dependence of 3 ',5 '-cyclic adenosine monophosphate-stimulated gonadotropin-releasing hormone release on intracellular calcium levels and L-type calcium channels in superfused GT1-7 neurons." Journal of the Society for Gynecologic Investigation **11**(6): 393-398.

Cheng, G. L., et al. (2010). "The Kisspeptin/Neurokinin B/Dynorphin (KNDy) Cell Population of the Arcuate Nucleus: Sex Differences and Effects of Prenatal Testosterone in Sheep." Endocrinology **151**(1): 301-311.

Cheng, X. B., et al. (2011). "GnRH secretion is inhibited by adiponectin through activation of AMP-activated protein kinase and extracellular signal-regulated kinase." Endocrine **39**(1): 6-12.

Clarke, I. J. (1988). "GONADOTROPIN-RELEASING HORMONE-SECRETION (GNRH) IN ANESTROUS EWES AND THE INDUCTION OF GNRH SURGES BY ESTROGEN." Journal of Endocrinology **117**(3): 355-360.

Clarke, I. J., et al. (2018). "Continuous Kisspeptin Restores Luteinizing Hormone Pulsatility Following Cessation by a Neurokinin B Antagonist in Female Sheep." Endocrinology **159**(2): 639-646.

Clarkson, J., et al. (2009). "Distribution of Kisspeptin Neurones in the Adult Female Mouse Brain." Journal of Neuroendocrinology **21**(8): 673-682.

d'Anglemont de Tassigny, X., et al. (2007). "Hypogonadotropic hypogonadism in mice lacking a functional Kiss1 gene." Proceedings of the National Academy of Sciences of the United States of America **104**(25): 10714-10719.

Dandona, P., et al. (2008). "Hypogonadotrophic hypogonadism in type 2 diabetes." Aging Male **11**(3): 107-117.

de Croft, S., et al. (2013). "Neurokinin B Activates Arcuate Kisspeptin Neurons Through Multiple Tachykinin Receptors in the Male Mouse." Endocrinology **154**(8): 2750-2760.

de Roux, N., et al. (2003). "Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54." Proceedings of the National Academy of Sciences of the United States of America **100**(19): 10972-10976.

Degreef, W. J., et al. (1987). "LEVELS OF LH-RELEASING HORMONE IN HYPOPHYSEAL STALK PLASMA DURING AN ESTROGEN-STIMULATED SURGE OF LH IN OVARIECTOMIZED RATS." Journal of Endocrinology **112**(3): 351-359.

Funes, S., et al. (2003). "The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system." Biochemical and Biophysical Research Communications **312**(4): 1357-1363.

Garcia-Galiano, D., et al. (2012). "Kisspeptin Signaling Is Indispensable for Neurokinin B, but not Glutamate, Stimulation of Gonadotropin Secretion in Mice." Endocrinology **153**(1): 316-328.

Gillund, P., et al. (2001). "Body condition related to ketosis and reproductive performance in Norwegian dairy cows." Journal of Dairy Science **84**(6): 1390-1396.

Goodman, R. L., et al. (2004). "Evidence that dynorphin plays a major role in mediating progesterone negative feedback on gonadotropin-releasing hormone neurons in sheep." Endocrinology **145**(6): 2959-2967.

Goodman, R. L., et al. (2013). "Kisspeptin, Neurokinin B, and Dynorphin Act in the Arcuate Nucleus to Control Activity of the GnRH Pulse Generator in Ewes." Endocrinology **154**(11): 4259-4269.

Goodman, R. L., et al. (2007). "Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin a and neurokinin B." Endocrinology **148**(12): 5752-5760.

Guran, T., et al. (2009). "Hypogonadotropic Hypogonadism due to a Novel Missense Mutation in the First Extracellular Loop of the Neurokinin B Receptor." Journal of Clinical Endocrinology & Metabolism **94**(10): 3633-3639.

Han, S. K., et al. (2005). "Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty." Journal of Neuroscience **25**(49): 11349-11356.

Herbison, A. E., et al. (1995). "DISTRIBUTION OF ESTROGEN RECEPTOR-IMMUNOREACTIVE CELLS IN MONKEY HYPOTHALAMUS - RELATIONSHIP TO NEURONS CONTAINING LUTEINIZING-HORMONE-RELEASING HORMONE AND TYROSINE-HYDROXYLASE." Neuroendocrinology **61**(1): 1-10.

Herbison, A. E., et al. (1993). "DISTRIBUTION OF ESTROGEN RECEPTOR-IMMUNOREACTIVE CELLS IN THE PREOPTIC AREA OF THE EWE - COLOCALIZATION WITH GLUTAMIC-ACID DECARBOXYLASE BUT NOT LUTEINIZING-HORMONE-RELEASING HORMONE." Neuroendocrinology **57**(4): 751-759.

Herod, S. M., et al. (2011). "Treatment with a CRH-R1 antagonist prevents stress-induced suppression of the central neural drive to the reproductive axis in female macaques." American Journal of Physiology-Endocrinology and Metabolism **300**(1): E19-E27.

Hosseini, A., et al. (2012). "Differential effects of propionate or beta-hydroxybutyrate on genes related to energy balance and insulin sensitivity in bovine white adipose tissue explants from a subcutaneous and a visceral depot." Journal of Animal Physiology and Animal Nutrition **96**(4): 570-580.

Hu, M. H., et al. (2015). "Relative Importance of the Arcuate and Anteroventral Periventricular Kisspeptin Neurons in Control of Puberty and Reproductive Function in Female Rats." Endocrinology **156**(7): 2619-2631.

Huzzey, J. M., et al. (2015). "Associations of peripartum markers of stress and inflammation with milk yield and reproductive performance in Holstein dairy cows." Preventive Veterinary Medicine **120**(3-4): 291-297.

Iwasa, T., et al. (2008). "Decreased expression of kisspeptin mediates acute immune/inflammatory stress-induced suppression of gonadotropin secretion in female rat." Journal of Endocrinological Investigation **31**(7): 656-659.

Iwasa, T., et al. (2014). "Hypothalamic Kiss1 and RFRP gene expressions are changed by a high dose of lipopolysaccharide in female rats." Hormones and Behavior **66**(2): 309-316.

Jacobi, J. S., et al. (2007). "17-beta-estradiol directly regulates the expression of adrenergic receptors and kisspeptin/GPR54 system in GT1-7 GnRH neurons." Neuroendocrinology **86**(4): 260-269.

Jacobs, D. C., et al. (2016). "Evaluation of Immortalized AVPV- and Arcuate-Specific Neuronal Kisspeptin Cell Lines to Elucidate Potential Mechanisms of Estrogen Responsiveness and Temporal Gene Expression in Females." Endocrinology **157**(9): 3410-3419.

Kaplowitz, P. (2007). BMI and the Onset of Puberty. When Puberty Is Precocious: Scientific and Clinical Aspects. O. H. Pescovitz and E. C. Walvoord**:** 137-150.

Katz, P., et al. (2011). "Costs of infertility treatment: results from an 18-month prospective cohort study." Fertility and Sterility **95**(3): 915-921.

Kinsey-Jones, J. S., et al. (2009). "Down-Regulation of Hypothalamic Kisspeptin and its Receptor, Kiss1r, mRNA Expression is Associated with Stress-Induced Suppression of Luteinising Hormone Secretion in the Female Rat." Journal of Neuroendocrinology **21**(1): 20-29.

Knox, A. M. I., et al. (2009). "Neonatal Lipopolysaccharide Exposure Delays Puberty and Alters Hypothalamic Kiss1 and Kiss1r mRNA Expression in the Female Rat." Journal of Neuroendocrinology **21**(8): 683-689.

Kolodziejski, P. A., et al. (2018). "Serum Levels of Spexin and Kisspeptin Negatively Correlate With Obesity and Insulin Resistance in Women." Physiological Research **67**(1): 45-56.

Kotani, M., et al. (2001). "The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54." Journal of Biological Chemistry **276**(37): 34631-34636.

Krsmanovic, L. Z., et al. (1993). "EXPRESSION OF GONADOTROPIN-RELEASING-HORMONE RECEPTORS AND AUTOCRINE REGULATION OF NEUROPEPTIDE RELEASE IN IMMORTALIZED HYPOTHALAMIC NEURONS." Proceedings of the National Academy of Sciences of the United States of America **90**(9): 3908-3912.

Laeger, T., et al. (2012). "The ketone body beta-hydroxybutyric acid influences agouti-related peptide expression via AMP-activated protein kinase in hypothalamic GT1-7 cells." Journal of Endocrinology **213**(2): 193-203.

Lapatto, R., et al. (2007). "Kiss1(-/-) mice exhibit more variable hypogonadism than Gpr54(-/-) mice." Endocrinology **148**(10): 4927-4936.

Lee, J. H., et al. (1996). "KiSS-1, a novel human malignant melanoma metastasis-suppressor gene." Journal of the National Cancer Institute **88**(23): 1731-1737.

Lehman, M. N., et al. (1993). "DISTRIBUTION OF ESTROGEN RECEPTOR-IMMUNOREACTIVE CELLS IN THE SHEEP BRAIN." Endocrinology **133**(2): 876-886.

Lehman, M. N., et al. (2013). Neuroanatomy of the Kisspeptin Signaling System in Mammals: Comparative and Developmental Aspects. Kisspeptin Signaling in Reproductive Biology. A. S. Kauffman and J. T. Smith. **784:** 27-62.

Lehman, M. N. and F. J. Karsch (1993). "DO GONADOTROPIN-RELEASING-HORMONE, TYROSINE HYDROXYLASE-IMMUNOREACTIVE, AND BETA-ENDORPHIN-IMMUNOREACTIVE NEURONS CONTAIN ESTROGEN-RECEPTORS - A DOUBLE-LABEL IMMUNOCYTOCHEMICAL STUDY IN THE SUFFOLK EWE." Endocrinology **133**(2): 887-895.

Li, Q., et al. (2015). "Evidence that Neurokinin B Controls Basal Gonadotropin-Releasing Hormone Secretion but Is Not Critical for Estrogen-Positive Feedback in Sheep." Neuroendocrinology **101**(2): 161-174.

Li, Q., et al. (2020). "Expression of genes for Kisspeptin (KISS1), Neurokinin B (TAC3), Prodynorphin (PDYN), and gonadotropin inhibitory hormone (RFRP) across natural puberty in ewes." Physiological Reports **8**(5).

Li, X. F., et al. (2012). "High-Fat Diet Increases LH Pulse Frequency and Kisspeptin-Neurokinin B Expression in Puberty-Advanced Female Rats." Endocrinology **153**(9): 4422-4431.

Liu, J. H. and A. H. Bill (2008). Stress-associated or functional hypothalamic amenorrhea in the adolescent. Menstrual Cycle and Adolescent Health. C. M. Gordon, C. Welt, R. W. Rebar et al. **1135:** 179-184.

Liu, Y. L., et al. (2020). "Dynorphin and GABA(A) Receptor Signaling Contribute to Progesterone's Inhibition of the LH Surge in Female Mice." Endocrinology **161**(5).

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method." Methods **25**(4): 402-408.

Loucks, A. B., et al. (1998). "Low energy availability, not stress of exercise, alters LH pulsatility in exercising women." Journal of Applied Physiology **84**(1): 37-46.

Luque, R. M., et al. (2007). "Regulation of hypothalamic expression of KiSS-1 and GPR54 genes by metabolic factors: Analyses using mouse models and a cell line." Endocrinology **148**(10): 4601-4611.

Mellon, P. L., et al. (1990). "IMMORTALIZATION OF HYPOTHALAMIC GNRH NEURONS BY GENETICALLY TARGETED TUMORIGENESIS." Neuron **5**(1): 1-10.

Messager, S., et al. (2005). "Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54." Proceedings of the National Academy of Sciences of the United States of America **102**(5): 1761-1766.

Misra, M., et al. (2006). "Role of cortisol in menstrual recovery in adolescent girls with anorexia nervosa." Pediatric Research **59**(4): 598-603.

Muir, A. I., et al. (2001). "AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1." Journal of Biological Chemistry **276**(31): 28969-28975.

Navarro, V. M., et al. (2009). "Regulation of Gonadotropin-Releasing Hormone Secretion by Kisspeptin/Dynorphin/Neurokinin B Neurons in the Arcuate Nucleus of the Mouse." Journal of Neuroscience **29**(38): 11859-11866.

Navarro, V. M., et al. (2011). "Regulation of NKB Pathways and Their Roles in the Control of Kiss1 Neurons in the Arcuate Nucleus of the Male Mouse." Endocrinology **152**(11): 4265-4275.

Navarro, V. M., et al. (2012). "Role of Neurokinin B in the Control of Female Puberty and Its Modulation by Metabolic Status." Journal of Neuroscience **32**(7): 2388-2397.

Nestor, C. C., et al. (2012). "Evidence of a Role for Kisspeptin and Neurokinin B in Puberty of Female Sheep." Endocrinology **153**(6): 2756-2765.

Novaira, H. J., et al. (2014). "Disrupted Kisspeptin Signaling in GnRH Neurons Leads to Hypogonadotrophic Hypogonadism." Molecular Endocrinology **28**(2): 225-238.

Ohtaki, T., et al. (2001). "Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor." Nature **411**(6837): 613-617.

Oride, A., et al. (2017). "GLP-1 increases Kiss-1 mRNA expression in kisspeptin-expressing neuronal cells." Biology of Reproduction **97**(2): 240-248.

Overgaard, A., et al. (2014). "Disparate Changes in Kisspeptin and Neurokinin B Expression in the Arcuate Nucleus After Sex Steroid Manipulation Reveal Differential Regulation of the Two KNDy Peptides in Rats." Endocrinology **155**(10): 3945-3955.

Peters, A., et al. (1998). "Fetal hemoglobin in starvation ketosis of young women." Blood **91**(2): 691-694.

Ramaswamy, S., et al. (2010). "Neurokinin B Stimulates GnRH Release in the Male Monkey (Macaca mulatta) and Is Colocalized with Kisspeptin in the Arcuate Nucleus." Endocrinology **151**(9): 4494-4503.

Roa, J., et al. (2006). "Hypothalamic expression of KiSS-1 system and gonadotropin-releasing effects of kisspeptin in different reproductive states of the female rat." Endocrinology **147**(6): 2864-2878.

Roy, D., et al. (1999). "Estrogen directly represses gonadotropin-releasing hormone (GnRH) gene expression in estrogen receptor-alpha (ER alpha)- and ER beta-expressing GT1-7 GnRH neurons." Endocrinology **140**(11): 5045-5053.

Ruiz-Pino, F., et al. (2012). "Neurokinin B and the Control of the Gonadotropic Axis in the Rat: Developmental Changes, Sexual Dimorphism, and Regulation by Gonadal Steroids." Endocrinology **153**(10): 4818-4829.

Sakamoto, K., et al. (2012). "Central Administration of Neurokinin B Activates Kisspeptin/NKB Neurons in the Arcuate Nucleus and Stimulates Luteinizing

Hormone Secretion in Ewes During the Non-Breeding Season." Journal of Reproduction and Development **58**(6): 700-706.

Sarchielli, E., et al. (2017). "Tumor Necrosis Factor-alpha Impairs Kisspeptin Signaling in Human Gonadotropin-Releasing Hormone Primary Neurons." Journal of Clinical Endocrinology & Metabolism **102**(1): 46-56.

Seminara, S. B., et al. (2003). "The GPR54 gene as a regulator of puberty." New England Journal of Medicine **349**(17): 1614-U1618.

Smith, J. T., et al. (2005). "Regulation of Kiss1 gene expression in the brain of the female mouse." Endocrinology **146**(9): 3686-3692.

Smith, J. T., et al. (2009). "Kisspeptin Neurons in the Ovine Arcuate Nucleus and Preoptic Area Are Involved in the Preovulatory Luteinizing Hormone Surge." Endocrinology **150**(12): 5530-5538.

Sukhbaatar, U., et al. (2013). "Kisspeptin induces expression of gonadotropin-releasing hormone receptor in GnRH-producing GT1-7 cells overexpressing G protein-coupled receptor 54." General and Comparative Endocrinology **194**: 94-101.

Sullivan, K. A., et al. (1995). "GONADOTROPIN-RELEASING-HORMONE NEURONS IN THE RHESUS MACAQUE ARE NOT IMMUNOREACTIVE FOR THE ESTROGEN-RECEPTOR." Brain Research **685**(1-2): 198-200.

Surkin, P. N., et al. (2017). "Participation of hypothalamic CB1 receptors in reproductive axis disruption during immune challenge." Journal of Neuroendocrinology **29**(8).

Suzuki, S., et al. (2008). "Direct kisspeptin-10 stimulation on luteinizing hormone secretion from bovine and porcine anterior pituitary cells." Animal Reproduction Science **103**(3-4): 360-365.

Szymanski, L. A., et al. (2007). "Changes in insulin, glucose and ketone bodies, but not leptin or body fat content precede restoration of luteinising hormone secretion in ewes." Journal of Neuroendocrinology **19**(6): 449-460.

Terasaka, T., et al. (2013). "Mutual interaction of kisspeptin, estrogen and bone morphogenetic protein-4 activity in GnRH regulation by GT1-7 cells." Molecular and Cellular Endocrinology **381**(1-2): 8-15.

Tolle, V., et al. (2003). "Balance in ghrelin and leptin plasma levels in anorexia nervosa patients and constitutionally thin women." Journal of Clinical Endocrinology & Metabolism **88**(1): 109-116.

Tonsfeldt, K. J., et al. (2011). "Oestrogen Induces Rhythmic Expression of the Kisspeptin-1 Receptor GPR54 in Hypothalamic Gonadotrophin-Releasing Hormone-Secreting GT1-7 Cells." Journal of Neuroendocrinology **23**(9): 823-830.

Wahab, F., et al. (2011). "The kisspeptin signaling pathway and its role in human isolated GnRH deficiency." Molecular and Cellular Endocrinology **346**(1-2): 29-36.

Wakabayashi, Y., et al. (2010). "Neurokinin B and Dynorphin A in Kisspeptin Neurons of the Arcuate Nucleus Participate in Generation of Periodic Oscillation of Neural Activity Driving Pulsatile Gonadotropin-Releasing Hormone Secretion in the Goat." Journal of Neuroscience **30**(8): 3124-3132.

Warembourg, M., et al. (1998). "Estrogen receptor and progesterone receptor-immunoreactive cells are not co-localized with gonadotropin-releasing hormone in the brain of the female mink (Mustela vison)." Cell and Tissue Research **291**(1): 33-41.

Weiner, R. I. and G. M. Delaescalera (1993). "PULSATILE RELEASE OF GONADOTROPIN-RELEASING-HORMONE (GNRH) IS AN INTRINSIC PROPERTY OF GT1 GNRH NEURONAL CELL-LINES." Human Reproduction **8**: 13-17.

Wetsel, W. C., et al. (1992). "INTRINSIC PULSATILE SECRETORY ACTIVITY OF IMMORTALIZED LUTEINIZING-HORMONE-RELEASING HORMONE-SECRETING NEURONS." Proceedings of the National Academy of Sciences of the United States of America **89**(9): 4149-4153.

Williams, E. J., et al. (2008). "The Effect of Escherichia coli Lipopolysaccharide and Tumour Necrosis Factor Alpha on Ovarian Function." American Journal of Reproductive Immunology **60**(5): 462-473.

Williams, N. I., et al. (2007). "Synergism between psychosocial and metabolic stressors: impact on reproductive function in cynomolgus monkeys." American Journal of Physiology-Endocrinology and Metabolism **293**(1): E270-E276.

Wintermantel, T. M., et al. (2006). "Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility." Neuron **52**(2): 271-280.

Yeo, S.-H. and A. E. Herbison (2014). "Estrogen-Negative Feedback and Estrous Cyclicity Are Critically Dependent Upon Estrogen Receptor-alpha Expression in the Arcuate Nucleus of Adult Female Mice." Endocrinology **155**(8): 2986-2995.

Yoo, M. J., et al. (1997). "Tumor necrosis factor-alpha mediates endotoxin induced suppression of gonadotropin-releasing hormone pulse generator activity in the rat." Endocrine Journal **44**(1): 141-148.

Zamorano, P. L., et al. (1997). "Expression and localization of the leptin receptor in endocrine and neuroendocrine tissues of the rat." Neuroendocrinology **65**(3): 223-228.

Zhang, X. Q., et al. (2008). "Hypothalamic IKK beta/NF-kappa B and ER stress link overnutrition to energy imbalance and obesity." Cell **135**(1): 61-73.

VITA

Lisa L. Amelse was born in Chicago, IL and moved to Loudon, TN at the age of two. She was the first of three children born to Ricarda E. and Donald T. Amelse. After graduating Loudon High School, she attended The University of the South in Sewanee, TN, graduating in 1993 with a Bachelor of Science in Biology. She then attended The University of Texas Graduate School of Biomedical Science in Houston, TX, graduating with a Master of Science in Biomedical Science in 1997. In the intervening years before returning to graduate school at the University of Tennessee in Knoxville, she worked at M.D. Anderson Cancer Center in Houston, TX, Vanderbilt University in Nashville, TN, and the University of Tennessee in Knoxville. She married Bryan K. Binkley and had two children, Hannah Elise and William Theodore. She currently lives with her family in Loudon, TN with two dogs, two cats, two parakeets, and a couple of rats. In her spare time, she is the Scoutmaster of Scouts BSA Troop 355, the first female BSA troop in Loudon County, and serves as a mentor for tnAchieves, helping high school seniors meet the requirements to secure the Tennessee Promise last dollar scholarship.