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Assessing the Effects of Levonorgestrel (LNG) on Neural Differentiation in the Developing Hypothalamus Using the Zebrafish (*Danio rerio*) as a Model

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A Thesis

by

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ABSTRACT

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Neurogenesis is a critical component of fetal neurodevelopment consisting of: migration, proliferation and differentiation. Health concerns from anxiety to reproductive issues have been linked to alterations of neurogenesis processes. Endocrine disrupting chemicals (EDCs) can mimic hormones vital to this process, potentially adversely affecting the susceptible fetal brain. One EDC, Levonorgestrel (LNG), is a testosterone-like progestin used in birth control methods that has a high bioavailability and long half-life. Using zebrafish embryos as a model, this lab previously discovered that an environmentally relevant LNG dose (5 ng/L) significantly increased anxiety behaviors. The hypothalamus is responsible for the development of behavior and puberty and is rich in steroidogenic receptors. Thus, I sought to determine if the behavioral changes were linked to neural changes in the hypothalamus. The present study reveals that LNG exposure before zygotic transcription until post fertilization day 5 increases neural differentiation that can be linked to behavioral changes.

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CHAPTER I

INTRODUCTION

Fetal neuronal development, and the events associated with neurogenesis in the brain, is a critical developmental step that can have a lasting impact on a species. The alteration of the normal biological processes that regulate neurogenesis have been implicated in a wide range of health concerns from behavioral pathologies (anxiety and depression) to reproductive related issues (advancing or delaying the normal timing of puberty) [1-6]. Interestingly, previous studies have suggested that the societal increase in these health pathologies can be linked to a comparable rise in anthropogenic waste, particularly endocrine disrupting chemicals (EDC). Studies support the theory that the fetal brain has an increased vulnerability to exogenous EDCs particularly those that mimic characteristics of hormone signaling needed for neurogenesis. The difficulty is identifying environmental EDCs at rational environmental and physiological levels capable of specifically targeting areas in the brain that regulate limbic and reproductive processes.

Exogenous EDCs, functioning as weak steroid agonists might be more potent during fetal development than endogenous steroids. Vertebrate studies have provided supportive evidence that steroid mimicking EDCs can stimulate hypothalamic progenitors [7-9]. Recently, the well-studied EDC bisphenol A (BPA), a weak steroid receptor agonist, was shown to advance prenatal hypothalamic development in fish [2, 10]. Furthermore, both estrogen receptor (ER) and androgen receptor (AR) are located on highly proliferative cells in the developing hypothalamus [11-14].

These cells, identified as hypothalamic radial glial cells, serve as neuronal stem cells (NCs) and progenitor cells during development [15, 16]. Interestingly, vertebrate studies show that functional ER, and AR are present in hypothalamic progenitor cells earlier than the differentiated gonads and the establishment of the HPG (hypothalamic pituitary gonadal) regulatory hormonal milieu [8, 14, 17]. If true, the prenatal hypothalamus would be particularly vulnerable to exogenous steroid-like substances. Thus, steroid-mimicking EDCs could potentially control neurogenesis earlier than developmentally planned.

Levonorgestrel (LNG) is a second-generation synthetic progestin, used in birth control and in emergency contraceptive pills such as Plan B. LNG has a high bioavailability, particularly in mediums in contact with vertebrates (including humans). It has a half-life of almost 2 years in sediment medium, 4 months in soil, and 2 months in water [18]. Efforts to reduce environmental LNG, particularly in water treatment plants are limited. LNG, structurally related to testosterone (T_4), primarily targets progesterone (PR) and AR [18-21]. LNG binding affinity for ER has been reported to be low, yet conflicting data suggest it may have some estrogenic potential, mainly via LNG metabolites [21-23]. Previous rodent studies have shown that LNG promotes progesterone and androgenic mechanisms in the hypothalamus and pituitary in rodent models [24]. Furthermore, LNG exposure in zebrafish has been shown to promote male differentiation, increase androgen-regulated genes and cause disruptions in luteinizing hormone (LH) and follicle stimulating hormone (FSH), two key reproductive hormones secreted from the brain [19, 25, 26]. For these and other reasons, it has emerged as an environmental EDC of concern.

Previous studies strongly support the possibility that LNG could alter brain development, yet the majority focus on the effects long term exposure has on gonadal differentiation and the consequences thereof. Norgestin, a sister compound of LNG, was shown, in zebrafish, to alter

transcriptional profiles potentially affecting sex development particularly in the brain and gonads [27]. LNG, via ER, was shown to disrupt *in vitro* brain development, using estrogen receptor (ER) transfected human glial cells [22]. However, to date, no *in vivo* study has identified if LNG directly targets the brain to affect neurogenesis. Recent work in the Dearth laboratory, demonstrated that an environmentally relevant dose of 5 ng/L of LNG significantly increased hyperactivity, thigmotaxis, and heart rate (all indicators of anxiety-like behavior) in zebrafish larvae at 5 days post fertilization (dpf) [18]. One measure, hyperactivity, is a known phenotypic indicator of hypothalamic disruption [2, 28-30]. Furthermore, AR and ER are implicated in the establishment of neuronal pathways that control behavior [31]. Collectively, these studies suggest that LNG might disrupt brain development. Therefore, in the present study, *my hypothesis* is that LNG targets the fetal brain, to induce hypothalamic progenitor cell differentiation to neurons (neurogenesis), thus advancing overall brain development in the zebrafish (*Danio rerio*). The following experiments were conducted.

Experiment 1: **Determine if embryonic exposure to LNG can alter the radial glial-to neuron ratio in the zebrafish brain.** Using a previously established dose, Zebrafish larvae were treated for 5 days starting at 2-3 hours post fertilization (hpf). Immunohistochemistry (IHC) was used to detect neurons versus glial cells in various regions of the brain at 5 dpf. Brains were stained with a neuronal-soma marker (alpha-HUC) and DAPI. Cells were individually counted in the preoptic area (POA), rostral hypothalamus and thalamus.

Experiment 2: **Identify if LNG increased ER expression in the hypothalamus.** ER has been implicated as a driving force in neurogenesis. It also plays a significant role in hypothalamic reproductive and limbic neuronal regulation. ER expression in brain regions in control and LNG-dosed larvae were compared via IHC fluorescent detection.

CHAPTER II

BACKGROUND

Endocrine Disrupting Chemicals (EDCs)

Environmental contaminants are widespread and range from household products, pharmaceuticals to hormones and industry by-products. EDC introduction and build up in the environment is mainly a consequence of wastewater dumping into rivers and soil. Although publications describing risks of EDCs are increasing, there has been very little regulation on EDCs in drinking water.

Collectedly, environmental EDCs (even at low concentrations) are abundant, posing a real risk to human chronic exposure [32]. During the 2000's endocrine researchers noticed a continued menarcheal age decrease originally postulated to be linked with an increase in obesity in first-world countries. Eventually, this was refuted after an epidemiologic evaluation revealed two major findings: earlier onset puberty in countries not affected by obesity and internationally adopted children developing earlier menarche compared to children in their country of origin [33]. This opened the door for more theories postulated on the impact environment (particularly EDCs) has on developmental milestones. Slight changes to the hormonal balance of the brain can cause physiological, behavioral and reproductive issues [6]. Even low parts-per-trillion concentrations of chronic EDC exposure via drinking water during neurodevelopmentally sensitive periods, like gestational and perinatal time points, have resulted in EDC transfer from

mother to developing fetus. As such, the developing brain might be particularly susceptible to EDC introduction, with effects not manifested until later in life. Supporting this, several EDCs have been linked to increasing behavioral health issues, low fertility rates, externalizing behaviors, anxiety/depression, ADHD and even precocious puberty [34, 35].

Neurogenesis: The Behavior and Puberty Link

The etiology of psychiatric disorders such as anxiety and ADHD are multifaceted and are exhibited in females at an increasingly younger age. Females that experience an earlier age of menarche (precocious puberty) also reported significantly more issues regarding anxiety, depression, bulimia, and substance abuse [36]. Altogether indicating that behavioral disorders and precocious puberty are likely linked. More recent studies have shown a correlation between aromatase concentration in specific brain regions and behaviors. In human PET scans from the medulla, amygdala and hypothalamus, high concentrations of aromatase were found to be associated with specific behaviors. This work suggested that the reproductive hormone estrogen (which regulates aromatase concentration) may influence personality [4].

Fortunately, neurochemistry is highly conserved among vertebrates, providing a wealth of knowledge to extrapolate findings [37]. Zebrafish are growing as a model organism for complex psychiatric diseases especially to identify core molecular mechanisms that are translational across vertebrate species (including humans). Hippocampal and hypothalamic functions are tightly and reciprocally connected in behavioral and hormonal processes. While extensive research linked hippocampal neurogenesis to hyper-anxiety induction in rodent models, less work has been done on the link between hypothalamic neurogenesis and anxiogenic behavior [1]. Centralized precocious and delayed puberty are often results of hypothalamic neurogenetic

dysfunction. These changes are now being correlated to behavioral changes. In several studies proliferative neurogenesis corresponds to anxiety phenotypes like increased locomotion (hyperactivity) and thigmotaxis changes due to EDC introduction during a critical window [2, 3, 28, 38, 39]. For example, early in life, after hatching (2-3 dpf), zebrafish larvae begin showing escape response swimming behavior. Behavioral changes can then be measured and used as an assessment tool for various studies [40, 41]. Work related to LNG and behavior is scarce. However, recent rodent studies have shown LNG exposure during gestation resulted in higher inactivation of ERβ with a corresponding increase in autism-like behaviors [42-44].

Neurogenesis: Differentiation

Neurogenesis consists of proliferation, differentiation, and migration. The proliferative and, to a lesser extent, migratory aspects of neurogenesis have been the most rigorously studied and well defined (thus not covered here). Differentiation is the critical step in neurogenesis when the stem/precursor cell changes to its developmentally defined cell-type, in this case a neuron. The molecular cues driving the process of neural differentiation is a lesser-known process, but critical to understanding brain development. The Wingless and Int-1 (Wnt) proteins are highly evolutionarily conserved, and integral to a large list of developmental, signal transduction and cell communication pathways signaling cascade. Canonical Wnt signaling in glial-neuronal differentiation is marked by β-catenin translocation to the nucleus and binding to Lef/Tch transcription factors [45]. Wnt signaling proteins have been identified in the presumptive hypothalamus of zebrafish as early as 18-30 hpf [46] (Figure 1), suggesting its necessity during early development. At 50 hpf the distinct hypothalamus is visible and Wnt proteins are present in mitotic progenitors and post mitotic precursors [47]. Recently, this cascade has been identified as vital to neural differentiation in multiple model organisms including zebrafish: Wnt protein

reduction leads to less differentiating neurons while activation results in the opposite, a loss of radial glial cells (RGCs) and increase of neurons [45]. It is postulated that, similar to mammalian cortex, Wnt-positive cells are progenitors committed to neural differentiation [45] but more work is needed to confirm this.

Estrogen and Aromatase B (AroB)

EDCs that function like steroids have been shown to alter brain development in vertebrates [2, 9, 10, 48]. Aromatase, an enzyme necessary for steroidogenesis, is present in the brain of all vertebrates, but highly expressed in teleost. The high capacity for aromatization is one of the reasons zebrafish are a good model for studying steroidogenic EDCs [11, 49]. In vertebrates, ER has been shown to be co-expressed with aromatase B (AroB) in hypothalamic radial glial cells and estrogen (E_2) is believed to control their differentiation and migration in a paracrine manner [49, 50]. Contrary to humans, zebrafish, have two aromatase (*cyp19a1)* genes: *cyp19a1a (cyp19a)* generating Aromatase A (AroA) mostly expressed in the gonads, and *cyp19a1b (cyp19b)* generating Aromatase B (AroB) primarily in the brain. The two *cyp19a1a/b* genes function in the biosynthesis from androgens to estrogens suggesting that the *cyp19b* gene plays a vital role for estrogens produced in the brain [51]. This gene (*cyp19b)* may influence the release of hormones from the hypothalamus, pituitary or gonads as well as the ability of estrogen to mediate androgenic effects [52].

Cyp19 genes were originally thought to be activated during sexual differentiation, but both genes have been identified in larvae before gonadal development implying that they are necessary for early development [7]. Maternal derived *cyp19a/b* mRNA is detected in unfertilized and fertilized embryos at 0 and 1.5 hpf and, like other maternal RNA, decline progressively

through 12 hpf, further supporting a role in embryogenesis [8, 17, 51, 53] An increase of transcripts during 12-24 hpf aligns with the segmentation period of embryos where the CNS begins developing [40] (Figure 1). AroB and AroA synthesis increases progressively into the early larval period (24-120 hpf) [7]; however, AroB is more abundant at all embryogenic time points [17].

Unlike *cyp19a*, the *cyp19b* gene contains an estrogen response element (ERE) promoter, which means that the presence of ER is important to AroB expression [16]. The ERE promoter suggests *cyp19b* activation could be involved in sex differentiation, a role classically characterized by *cyp19a* alone [7]. Response element factor glial-specific (GxRE) is another regulatory element that is a part of the *cyp19b* gene. Although information is scarce, it is known to be important to ER regulation, suggesting that it may reinforce estrogen induction even if estrogen is not present [49]. *Cyp19b's* nerve growth factor inducible-B protein (NBRE)/Nur77 has another ½ ERE promoter that is postulated to reinforce the intersection of vertebral neuroendocrine pathways and provides another possible mechanism by which ER regulates aromatase in the brain [17].

Estrogen receptor (ER) has two main cellular occupations; nuclear ER isoforms mediate genomic effects while G-protein ER (GpER) is a membrane receptor that mediates non-genomic effects. Embryonic transcription of the three isoforms of ER (ERα, ERβ and ERγ) and GpER is detectable between 12-24 hpf as expression of *cyp19a/b* and the segmentation period commences [12, 40] (Figure 1) Moreover, maternal transfer of ER mRNA is thought to also take place similar to mammalian ER expression [17, 50]. Implying that ER, like *cyp19a* and *cyp19b*, are necessary for embryo development.

Teleosts, like zebrafish, have high aromatase production throughout their life, making them an excellent model to study the mechanisms of neurogenesis [11, 13]. Radial glial cells (RGCs) are progenitor cells that turn into neurons, a process called neurogenesis, in the developing embryo and in adults. Dual stains for neurons and AroB expression (or GFP-*cyp19b*) has validated that AroB activity is significant to neurogenic activity and is strictly specific to RGCs (does not occur in neurons) [2, 11, 16, 23, 49, 54, 55]. AroB expressing RGCs have been identified throughout the larval and adult brain but is particularly abundant more anteriorly in: olfactory bulbs (OB), telencephalon (T), preoptic area (POA) and the hypothalamus [11, 17, 53].

The function of estrogen in the AroB/RGC neurogenesis mechanism has been studied for several years. Various mammalian models have shown a correlation between embryonic neurogenesis, high aromatase activity and high estrogen production [16]. In fact, brain areas that are high in AroB expression, like the POA and hypothalamus, align with areas that are high in GpER and ER transcription [14, 15, 49]. It is not yet understood which specific ER isoforms interact functionally with AroB/A [17] but it has been shown that ER is fully functional early in life [12]. Before 24 hpf, E_2 treatment does not affect AroB expression [11]. After 24 hpf, low doses (1 nM) of E_2, can promote AroB expression and increase functional ER in the POA and hypothalamus of embryos by activating the *cyp19b* gene via the ERE promoter [11, 23, 53, 56]. Moreover, ICI (an ER antagonist) treatment blocks *cyp19b* activation and AroB-K/O results in significant reduction of estrogen production [11, 57] further confirming estrogen dependence. Collectively, studies support the theory that AroB activation results in an estrogen-mediated autoregulatory loop in RGCs where ERE promoter activation results in *cyp19b* gene expression, AroB production, and aromatization of steroids to produce E_2 [49].

RGCs are localized near ventricles, their processes extend out and it is suspected that during proliferation, generated daughter cells migrate down RGC processes before gaining a neural phenotype in both adults and larvae [11, 23, 49, 55]. This also serves as a mechanism for neural migration especially during early development. Since AroB functions as a mediator converting testosterone to estrogen and estrogenic activity is linked to neurogenesis it has been assumed that upregulation of AroB in RGCs would result in an increase in S phase marked cells in the brain. In the diencephalon and telencephalon of adult zebrafish, dual staining of AroB and BrdU (an S phase marker) showed that most AroB-positive cells, especially in high density areas, displayed mitotic activity [49]. Coexposure to FAD, an AroB inhibitor, and a neurogenesis inducing-EDC in developing zebrafish resulted in a decrease in neural-birth [2].

However, the effects of E_2 dosing on proliferation are contradictory. Very low doses (1-10 nM) and high doses (1000 nM) inhibited proliferation [16] and AroB production after 24 hours of treatment [8]. While lower intermediate doses (10 and 100 nM) results in increased AroB production [8]. Biphasic neuroendocrine responses are common, variations are postulated to be due to dosing concentration, as well as the developmental stage and duration of exposure.

In humans, aromatase mutations that increase expression exhibit a corresponding increased estrogen activity that can result in precocious puberty/gigantomastia in girls, gynecomastia in boys and short stature in both sexes. Mutations that result in a deficiency of aromatase lead to polycystic ovaries and pubertal failure in girls, and ambiguous external genitalia and tall stature in both sexes. Moreover, placental deficiency of aromatase can produce virilization of the mother due to excess androgen and pseudohermaphroditism of the female baby [58]. These manifestations further demonstrate the vitality of aromatase to normal development

during gestation and puberty *in utero*. Estrogen facilitates proper development not only during sex differentiation but in many ways.

Androgen receptor (AR)

Androgen receptor (AR) pathways are not well defined especially prior to gonad differentiation. Only one zebrafish *ar* gene has been observed, suggesting it is a true ortholog of the human gene and, likely, has a strong pathway resemblance [14]. Paralleling ER and *cyp19b* gene expression, AR is highly expressed in the hypothalamus/POA of zebrafish (adults and larvae) and rodents. Rodent observations indicate AR has influence on endocrine and reproductive behaviors [59-62]. Recent work has identified some AR-responsive genes in the developing embryonic zebrafish, with AR expression identified as early as 1dpf in zebrafish embryos as early as 1 dpf (Figure 1) [14, 63].

Testosterone (T) and a non-aromatizable androgen, dihydrotestosterone (DHT), have been utilized to discover the mechanisms of androgen-mediated AroB. Androgen mediated *cyp19b* activation was assumed to be induced by estrogen (ER) after AroB conversion rather than AR [13,]. At high concentrations, T and DHT (100-1000 nM) increased *cyp19b* expression in a similar magnitude to E_2 and in similar brain areas [13]. Recent work showed that the 3-beta-hydroxysteroid dehydrogenase (3β-hsd) gene is expressed early in life (24-28 hpf) in vivo, possibly converting DHT to a β-diol via 3β-hydroxysteroid dehydrogenase leading to indirect activation of ER (Figure 1) [14, 64]. The exerted *cyp19b* activation was blocked with ICI treatment suggesting ER was the activated receptor mediating expression [23]. However, at lower doses (10 nM) DHT was less potent and T was not effective at inducing *cyp19b* expression [13], again showing that higher doses may exert a biphasic response with androgen treatment. 11-

ketotestosterone (11-KT) is another non-aromatizable androgen that has recently been recognized as necessary for male zebrafish sexual development [65]. Interestingly, 11-KT does not activate *cyp19b* gene transcription, instead binding specifically to AR (rather than ER directly) suggesting that AR-mediated *cyp19b* is more likely still through ER activation [13].

Progesterone Receptor (PR)

Very little is known regarding progesterone receptors (PR) in neurogenesis. Similar to ERs, PR expression is found in RGCs in the telencephalon and anterior hypothalamus, suggesting progesterone-like EDCs may act on development [12, 56]. Aro-B positive RGCs contain ER and PR. Estrogenic stimulation of Aro-B RGCs could be like the mammalian brain and lead to increased synthesis of E_2, which could trigger up-regulation of PR in the RGCs [15]. Progesterone exposure has been shown to case precocious puberty in males but not influenced sex differentiation [25]. It is likely that of the three (progesterone, estrogen, androgen), progesterone plays a lesser role in steroid driven development.

Levonorgestrel (LNG)

The EDC levonorgestrel (LNG) is classified as a progestin, a synthetic form of progesterone, that is still being understood. Used in combination with synthetic estrogens, such as ethinylestradiol (EE2), LNG functions as a major synthetic hormone aiding in emergency contraception or birth control pills. Unlike estrogens, the effective removal of progestins in water has not been extensively studied [32]. Water intended for human consumption has been found to be significantly contaminated by pharmaceuticals (including EDCs) at a higher amount. Amounts can vary by country with some samples of LNG at concentrations of 1-10 ng/L [66] and some reports reaching 50 ng/L [48]. More worrisome perhaps, is that a guide with specifications on

risk assessment is not available [66]. Current chemical and biological water treatment methods are not able to significantly remove LNG to trace levels contributing to its environmental persistence. In fact, the intermediate products may have higher toxicity than the parent molecule [67].

Progestins, like LNG, have been shown to target neuroendocrine-sensitive brain areas such as the POA-hypothalamus and pituitary. Unlike progesterone, progestins do not specifically interact with one receptor; rather, they can activate ERs, ARs, PRs and glucocorticoid receptors (GRs) [68]. GRs are developed early in life and are thought to play a role in neurogenesis both in embryos and adults. Recently, these shifts in neurogenesis have also been linked to various behavioral changes including those involved in stress responses [69]. Sex steroids play a significant role in brain/sexual maturation in embryogenesis and sex determination, EDCs can act on these pathways and disrupt normal development. For example, at low concentrations transcriptional effects were present after introduction of LNG, showing its potential to affect brain and sex development during embryogenesis [32].

LNG is structurally like testosterone and most studies agree that it does not exhibit high affinity for ERs in adult zebrafish or mammals, but mostly exhibits androgenic effects. During the last larval stage, 21-25 dpf the gonads are bipotential but can be identified as presumptive ovary/testis by 25 dpf [26, 70]. At 40 dpf the gonads are determined and are mature by 90 dpf [70]. LNG at low environmentally relevant doses (5-10 ng/L) significantly decreased AroA transcription, resulting in a shift in the androgen:estrogen ratio and induction of a 100% male population. Flutamide co-exposure with LNG decreases the male ratio suggesting an AR-mediated pathway [19, 71]. The same LNG doses exhibited indications of precocious puberty in the

tissues of male larvae before normal sexual development by induction of male-fate genes, suppression female germ cell function, and *cyp19a* down regulation [19, 25].

Research identifying the effects of LNG and other testosterone-like progestins on *cyp19b* gene expression is contradicting. *In vitro* studies showed LNG to be ineffective at *cyp19b* induction unless co-dosed with a known estrogenic-progestin ethinyl estradiol (EE2) [22]. *In vivo* studies have shown LNG can induce a concentration-dependent activation of *cyp19b*, although at high concentrations. Compared to other progestins, LNG exhibited a lower estrogenic binding ability/response and does not bind directly to ERs suggesting estrogenic effects are likely due to metabolization into estrogenic metabolites [22, 23]. LNG has displayed both estrogenic and non-estrogenic properties; the effects of estrogenic metabolites could account for LNG's contrasting proposed mechanisms as well as the high-dose levels that are tested.

Zebrafish as a Model Organism

The Zebrafish (*Danio Rerio*) serves as a model experimental organism, in part, to its low maintenance cost and high fecundity, with females producing 100-500 embryos per clutch [72]. Fertilized embryos are transparent, allowing for simple morphological observation and characterization under the microscope. In contrast to mammalian model organisms, zebrafish embryo development is *ex utero*, allowing visualization of early developmental occurrences. Sexual maturity in zebrafish is reached at six months but embryonic development is much faster, with most organs grown by 5 days post fertilization (dpf) and endocrine system development beginning as early as 2 dpf. Notably, zebrafish embryonic development is comparable to human fetal development during the second trimester [2]. Although there are some limitations, the

endocrine system in zebrafish is significantly well conserved in comparison to mammalian systems thus, this model has become a staple for developmental neuroendocrine research [73].

There are four developmental stages of zebrafish: embryo (until ~72 hpf), larvae (~3-30 dpf), juvenile (1-3 months), and sexually mature adult fish. The CNS develops rapidly in zebrafish with several rhombomeres developed, including the diencephalon, by 18 hpf and the hypothalamus rudiment forming by 24 hpf [39]. This rapid neural development allows for easy assessment of neurodevelopment and high throughput of chemical screening. Thus, zebrafish are a strong model to explore neurodevelopmental disorders [37, 74].

Zebrafish maintain the pattern of brain structure conservation and allow for deciphering of early hypothalamic development due to rapid and external embryogenesis. Although teleosts lack a portal blood vessel system to deliver hormones from hypothalamus to pituitary, the direct neural populations that innervate the pituitary are confirmed to be analogous to humans [75]. There are three regions of the hypothalamus: the first region begins with the preoptic area (POA) and is followed by the rostral hypothalamus (Hr) proper. The POA-Hr controls developmental homeostasis, reproduction and, importantly, puberty/hormonal cycles [33, 45]. The onset of puberty is controlled by GnRH secretion from the POA-Hr to the pituitary, where signals for LH and FSH inhibition/excitation occur and are responsible for puberty [6, 33].

CHAPTER III

MATERIALS AND METHODS

Zebrafish Husbandry

All protocols and procedures were approved by the Institutional Animal Care and Use Committee at the University (IACUC) of Texas Rio Grande Valley. A cross strain of adult AB and TL zebrafish males and females were kindly donated by Bruce Riley from Texas A&M University, College Station, Texas. Adults were fed 3 times a day on weekdays: twice daily with Wardley Advanced Nutrition Tropical Fish flake food at 10:30 a.m. and 4:30 p.m., and once with hatched artemia at 2:00 p.m. Fish were fed flake food once on weekends at 12:00 p.m and were allowed to feed freely for 5 minutes to prevent overfeeding [76]. Lighting was connected to timers to ensure that zebrafish were maintained on a 14 hour light: 10 hour dark circadian clock. Water quality parameters were maintained according to [39] with a conductivity of ~500 ppm to ensure optimal breeding. These parameters including conductivity, temperature and pH of the system were checked weekly using the API Freshwater Master kit. The system was maintained at optimal levels using water heaters (25-29°C) and 10% water changes were conducted as needed. A 200 µm filter pad collecting feces and large debris was changed weekly or as needed. The 50 µm canister was changed weekly and the activated carbon canister was changed every two weeks [77].

Breeding

Embryo media (E3) (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, and methylene blue) was prepared fresh from stock aliquots weekly and filtered before being used during breeding experiments [78]. Breeding occurred twice a week on Tuesdays and Thursdays. Adult zebrafish were placed in breeding cages after evening feed on Mondays and Wednesdays at a 1:1 ratio. Fish were separated by a divider and breeding cages remained on system to prevent temperature fluctuations. At the onset of light on breeding days, dividers were lifted and fish were allowed to breed for 30 minutes or until embryos were produced.

Justification of Dose

Dose curves for estrogen and LNG were previously conducted using a hyperactivity analysis where an x-ray viewing box, fluorescent light source, and recording camera was set up with a black curtain surrounding it. It was determined that the environmentally relevant dose of 5 ng/L LNG was the lowest dose that induced a hyperactive behavioral phenotype [18]. A dose curve of environmentally relevant estrogen concentrations determined 100 nM of 17β-Estradiol (E_2) was to be used as a positive control. The vehicle control was the dose of EtOH (0.01%) based on the highest dose of either E_2 or LNG.

Experimental Design

After adults were bred, embryos were collected with a sifter, rinsed with E3, and plated in 96-well plates. E3 that was deposited during plating was removed using a 200 µL pipette without disturbing the embryo and replaced with the control or treatment dose between 0-3 hours of fertilization. Embryos used for experiments were plated in 96 well plates and dosed with either

control (E3), negative control (tricaine), positive control (100 nM E_2), or 5 ng/L of LNG. The outer columns and rows of the 96 well plate were not plated with embryos but were plated with embryo media to help prevent evaporation of wells containing embryos. Embryos were housed in an incubator at 28°C until 5 dpf, when they were removed and checked under a microscope for life, abnormalities and hatching. Any abnormalities were noted, hyperactivity-recording commenced, and larvae were collected.

Immunohistochemistry (IHC)

Zebrafish larvae were sacrificed at 5 dpf by ice-submersion for ~10 minutes. Larvae were immediately collected and placed in fixative for 18 hours in 4% PFA. Before tissue processing, tissues were placed in labeled tissue cassettes and rinsed for 5 minutes, 4 times with 1X PBS using a benchtop orbital shaker. After paraffin infiltration was complete, a paraffin embedding machine was used to make transversely oriented larval blocks and sectioned at 3-4 μm.

For neurogenesis experiments, five fish were sectioned and 5-8 slides were stained with a primary neural marker (orange, HuC 1:200), Alexa Fluor 488 (1:1000) and DAPI (1:1000) for each treatment (n=5). Anatomical position of each slide was determined under the microscope to select slides for staining. Anatomical identification was set posterior to the anterior commissure (foremost area of POA) and the foremost area of the pretectum (last area of the rostral hypothalamus). Identification of estrogen receptor experiments followed a similar design; however, 3 fish (n=3) were chosen for this study to provide preliminary data. Antibodies for this section of the experiments are as follows, estrogen receptor primary antibody (red, ERα 1:50), Alexa Fluor 647 (1:1000), and DAPI (1:000). Antibodies were validated by adding secondary antibodies alone to ensure specification. All images were obtained via confocal microscope.

Neuronal Cell Counting

After staining, 5-8 serial sectioned brains were counted at the rostral hypothalamus and thalamus. ImageJ was installed with the plugin "Cell Counter" and used to perform all counts. In each section, cells were considered negative if only their nuclei were visible (DAPI only) and positive if there was an orange (HuC+DAPI) or red (ER+DAPI) outline. Cells were recorded and added to obtain the total cell count. Then, net positive, negative and total were collected from the counts and used at the value for data analysis. Positive percentages were calculated as such: Positive cells/ total cells = percent positive cells.

Statistical Analysis

All experiments were tested in JMP, a statistical analysis software. Percentages were inputted in JMP and presented as decimal values for ease of manipulation. One-way ANOVA was used to determine the significance between the means of Control, E_2 and LNG treatment groups for the HuC experiments and between means of Control and LNG treatment groups for the E_2 staining experiment. If significance was determined, a Tukey's post-hoc test was used to test all pairwise differences. In tables and graphs, letters were used to signify which treatments were significantly different and p-values were provided either in black (p-value between 0.01 and 0.05) or red (p-value of less than 0.01).

CHAPTER IV

RESULTS

Previously, our lab used quantitative structure-activity relationship (QSAR) to identify LNG as a viable EDC, based on structure, predicted activity and environmental bioavailability. Findings indicated LNG had a long half-life, was likely non-biodegradable and would not be removed via wastewater treatment [18]. E_2 was selected as a positive control; the 100 nM dose had a significant increase of swimming velocity ($p < 0.01$). We showed LNG exposure led to a significant increase of swimming velocity ($p < 0.001$) and heart rate ($p < 0.05$) compared to control groups [18]. Thus, it was concluded that LNG may have the potential to affect neurogenesis (Figure 2).

Experiment 1: Determine if embryonic exposure to LNG can alter the radial glial-to neuron ratio in the zebrafish brain.

Exp. 1A. Creating a histological roadmap of hypothalamic structures in the larval zebrafish (Dearth Bioatlas)

To determine if LNG had an effect on neurons, a histological map of the hypothalamus and associated brain structures was needed. The rostral hypothalamus (rh) is located in the diencephalon and known to consist of two overlapping areas, the POA and the rostral hypothalamus proper (Hr) [79]. After an extensive literature review, it became clear that there were

few references for identifying specific brain areas to study in larval zebrafish, particularly transverse histological stains at 5 dpf. Figure 3 shows representative images of zebrafish brains at 4 and histological images 5dpf to previously established bioatlas: Penn-State's 4 dpf Bioatlas [80] and the Atlas of Early Zebrafish Brain Development (Atlas of Early ZF.) [81]. Penn-State's Bioatlas provided clear brain structure-landmarks but lacked serial sectioning and specific brain labeling. While Atlas of Early ZF. provided clear brain labeling specific to larval day but lacked ease of morphological identification (RNA-staining was used instead of H&E). The Dearth Lab Bioatlas provides a combination of the two: serial sections, defined structures (H&E), and specific labeling (Figure 3, right column). Creating the Dearth Bioatlas allowed me to conduct my experiments in a precise and controlled manner and served as a reference for our lab's future work.

The POA-Hr (Figure 3) that encompasses the rh, is an area critical to reproduction, onset of puberty, and is composed of a high network of dopaminergic neurons early in life [82]. Increased hyperactivity was already identified in LNG dosed fish in our previous work [18]. Altogether, both ours and others' work led me to hypothesize this area to be important to behavioral-neuroendocrine change sensitive to EDCs. Thus, I focused my study on identifying potential neural changes in this area. The dorsal thalamus (DT) is not involved with neuroendocrine functions but is found in the same sections as the POA-Hr, (Figure 3) allowing for direct comparison between two brain areas. Additionally, a previous study worked to identify neural-sensitive areas to EDC exposure and found changes in the rh and no changes to the thalamus, tectum and hindbrain [2]. As such, we decided to restrict cell counts to three areas: the POA-Hr (encompassing the rh) and the dorsal thalamus to serve as a comparison control of neurogenesis in the Hr. The Dearth Lab Bioatlas was directly compared to IF stained (HuC and DAPI) control

treated 5 dpf larvae to identify brain structures under immunofluorescence (Figure 4). In (Figure 4 - Column 1) shows the area just posterior to the anterior commissure (AC) where the POA, eminentia thalami (EmT), and migrated area of the EmT (M3) first emerges. At this point, there is an introduction of lateral forebrain bundles (lfbs) (solid, eosin-stained columns mirrored on the left and right of the brain) but the presence of the pallium (P) remains. The POA continues (Figure 4 - Column 2), the lfbs and EmT are still present and more brain structures are easily identifiable such as the epiphysis (E), habenula (Ha), tectal ventricle (TVe), and subcommissural organ (SCO). When the post optic commissure (poc) (Figure 4 - Column 3) becomes visible, there is a small window where there is still a POA presence until the Hr emerges. In column 3, there is a clear bundle of cells under the poc, this is the emergence of the Hr and represents POA completion and the presence of the Hr and thalamus. At this point you may be able to visualize the habenular commissure (HaC) and for a short period, the ventral thalamus (VT) will be just under the DT before the DT encompasses the thalamic area.

After the poc, the rostral hypothalamus proper (Hr) is identifiable by its hook-like shape at the very bottom of the brain to the two mirrored protrusions under the posterior tuberculum (PT) (Figure 4 - Column 4). Above the PT on both sides there are divots where the migrated pretectal area (M1) cluster of cells are on the white matter. At this portion of the brain the DT lies between the PT and the posterior commissure (pc) (below and above it respectively) (Figure 4 - Column 4). The Hr hook-like shape extends and becomes bulbous (Figure 4 - Column 5); this is an indicator marking the end of the Hr, further sectioning would emerge the intermediate hypothalamus. The brain is noticeably different at this point as there is the introduction of several structures that indicate the rostral-most area of the midbrain such as: the tectum opticum (TeO) and two white matter finger-like projections that point towards the midline of the brain, the

pretectum (Pr) and fasciculus retroflexus (fr). The DT is still present and is located between the two projections and the PT. (Figure 4 - Column 4 and 5).

EXP 1B. Identifying the number of neurons in the hypothalamus and thalamus.

Before assessing neurogenesis, I sought to determine if there was variation in the total number of cells between treatment groups in the thalamus and the rh. Means for the total cell count (Table 1) for each treatment group (n=5) in the hypothalamus and thalamus were tested against each other for significance. The analysis determined no significant change between treatment or brain region (Figure 5). Ensuring any potential changes that could be identified between treatment groups were not due to differences in pool size or total brain cell proliferation. Additionally, there was no identification of counting data skewing that could indicate bias.

Next, I sought to prove my hypothesis that LNG-dosed larvae would exhibit a change in the rostral hypothalamus. The means of percent positive neurons (Table 1) were tested for significance between the three treatment groups in the hypothalamus and the thalamus (n=5). As expected, there was no change in positive neural cells in the thalamus between treatments (Figure 6, Figure 7). This confirmed previous work (Kinch et al. 2015) that found no neurogenesis in the thalamus and proved that the thalamus could serve as a negative control in studies focused on hypothalamic neurogenesis. In the rostral hypothalamus, there was a strong significant increase in the percentage of neurons (p-value < 0.0025) in LNG-treated larvae compared to control larvae (Figure 6, Figure 7). This change represented for the first time that LNG does in fact, induce neurogenesis after chronic, low dose exposure confirming our hypothesis. Surprisingly, even though E_2 dosing induced hyperactivity, there was no change in

the number of neurons present; in fact, it was closer in relation to the control group (Figure 6, Figure 7).

These results are mostly consistent with this lab's previous hyperactivity results. Indicating that the rostral hypothalamus is susceptible to EDC disturbance and these disturbances can alter behavior. Additionally, behavioral phenotypes like hyperactivity can serve as a marker for neuro-endocrine changes in development but further molecular work should be utilized.

Exp 1C. Identifying the difference between the number of neurons in preoptic area (POA) POA versus the rostral hypothalamus proper

After determining neurogenesis was localized to the rostral hypothalamus I sought to determine if there was a neural population difference between the two areas comprising my rh counts: the POA and Hr. In the POA (Figure 8-C), there was a significant increase in positive neurons in the LNG treated tissue compared to the control (p-value < 0.0126). Additionally, in the Hr (Fig. 8-D) there was a strong significant difference between control and LNG treated tissue (p-value < 0.0057). However, in the Hr there was also a strong significant difference between Estrogen and LNG treated tissue (p-value < 0.0031) further indicating E_2 dosing did not result in neurogenesis but rather, mimicked control neural numbers (Figure 8-D).

Experiment 2: Identify if LNG increased ER expression in the hypothalamus.

Determining the pathways of neurogenesis has been difficult, most work suggests that the ER pathway is a likely source. We wanted to explore if LNG dosing would increase estrogen receptor (ER) presence. We observed no significant difference in the HuC-strained neural cells between control and estrogen-treated larvae at 5 dpf. Therefore, we only investigated the LNG

treated group compared to controls (n=3). Interestingly, there was no difference in the total number of LNG cells that stained positive for ER in the thalamus or the rostral hypothalamus (Figure. 9).

CHAPTER V

DISCUSSION

In recent years EDC exposure via chronic drinking water has increased, coinciding with a shift in behavioral disorders and precocious puberty. EDC doses exceeding typical exposure amounts can result in obvious morphological defects suggesting general toxicity rather than endocrine-disrupting effects. This lab's previous work identified no difference in morphology or hatching in LNG-dosed embryos compared to controls [18]. This aligns with human LNG-epidemiological studies and zebrafish LNG-focused work that show there has been no connection to major physical and cognitive defects after fetal exposure to LNG [19, 83]. To our knowledge, this study demonstrates for the first time that environmentally relevant chronic LNG exposure can affect neurogenesis that is synonymous with a phenotypic behavioral shift. My study investigated the impact of LNG exposure on the size of the progenitor pool in larval fish in the rostral hypothalamus by measuring the number of differentiated neurons.

Experimental designs vary between studies and are likely another reason one chemical does display consistent findings. Many studies tend to overlook the importance of early embryo exposure, a period where many important developmental switches are turning on concurrently (Figure1). To mimic fetal EDC exposure we dosed immediately after fertilization (0-3 hpf) (before zygotic transcription or neural cell fate determination (Figure 1), and utilized chronic exposure as opposed to short interval dosing. Moreover, rather than inadvertently observing toxic

effects, we opted for a lowest observed effect concentration (LOEC) dose. Our LNG dose was observed recently as a LOEC dose in other studies [19, 25] and aligned with the concentrations observed in nature [20].

Previous work in this lab found both LNG (5 ng/L) and E_2 significantly increased hyperactivity [18], thus suggesting an effect on neurogenesis. To test this, I used HuC staining to analyze neurogenesis occurrence. In the present study, we did not mark neurogenesis by the presence of AroB (RGCs) as so many studies have done, but rather neural presence specifically indicating neural differentiation. Estrogen affects the *cyp19b* autoregulatory loop, a major part of the neurogenesis mechanism, so the absence of E_2's effect on the number of neurons in the rostral hypothalamus was puzzling. However, *cyp19b* encodes for AroB which is largely synonymous with RGCs and not neurons [55]. Thus, previous studies using AroB as an indicator of neuronal number might be misleading. Early embryogenic studies (0-2 dpf) have shown that 100 nM E_2 stimulates AroB production in the POA/Hr [8, 11, 84]. Indicating an EDC sensitivity and predicting neural changes. RGCs are known precursors to neurons and are necessary for neurogenesis, but the exact mechanism of neurogenesis is largely unknown, especially in early development. However, there have been contrasting reports of the E_2/AroB effect on proliferation. In zebrafish, E_2 and estrogenic EDCs surprisingly resulted in a decrease in proliferation and migration at very low doses (1 nM) [15] and higher doses (1000 nM) [16].

It is likely in this study that E_2 dosed larvae exhibited no change in brain for two possible reasons. One, the estrogenic presence increased AroB presence in RGCs and inhibited proliferation or migration as seen previously. Two, the change resulting from AroB increase influences hyperactivity, increases the potential to differentiate but does not commit to differentiation because another unknown activator is not present. More work is needed to

determine this difference; it would be interesting to consider LNG + E_2 co-exposure or LNG + ICI in order to determine if neurogenesis is not affected or stifled. Although I cannot rule out the possibility, it is unlikely that the lack of change is simply due to a biphasic response as both higher doses and lower doses also showed increased AroB presence and inhibited proliferation. Xenoestrogens, like BPA and EE2, showed similar trends [2, 9, 12, 15], suggesting instead that LNG is not mechanistically like them.

Molecular mechanisms of LNG are not well elucidated. Some studies have shown LNG can induce xenoestrogenic effects such as an increase in *cyp19b* AroB expression after exposure [10, 22, 23]. Although it is possible for LNG to act as an estrogen, it is more likely these findings are a result of a biphasic response. The tested doses for LNG in these studies are 6 to 20,000x higher than our very low dose (5 ng/L) with no induction of *cyp19b* until ~10-100 nM (2,000 to 20,000 x higher than our dose) [23]. Moreover, structural analysis has postulated that LNG is not estrogenic but works through progestagenic or androgenic means [20], aligning with this lab's previous QSAR findings [18]. PR is present early in zebrafish larvae but the role it plays in neurogenesis is unknown. In adults there is a higher concentration of PR in the POA-Hr, if this translates to larvae it is possible LNG could specifically affect this area [56]. Another possibility is that LNG activates more than one receptor but it or its metabolites are preferential to one. For instance, one study found androgen dosing resulted in an estrogenic response (vitellogenin increase) but an all-male population (androgenic response) later in life. It was speculated that there was aromatization of some androgen molecules but not all so both pathways were affected [21].

Progestins derived from testosterone, like LNG, are more mechanistically androgenic [68]. Effects of low-dose LNG exposure are scarce and tend to focus on sexual differentiation rather

than neuroendocrine development. Exposure to 5 and 5.5 ng/L increased androgenic genes significantly, decreased oogenic dependent genes, resulted in 100% male sex ratio, and displayed an increase in AR binding affinity [19, 25]. Recently, studies have discovered that environmentally relevant doses of LNG can also result in manifestations of precocious puberty. Along with 100 % male differentiation, 5.5 ng/L exposed fish exhibited a shift in LH and FSH expression (high at 44 dpf and low at 55 dpf) compared to control and tissue samples revealed indications of testicular development before normal sexual maturation [25, 26]. However, exposure was from 20-80 dpf not accounting for early developmental shifts that could occur. Another study that exposed larvae with 5 ng/L from 2 hpf (before zygotic transcription) to 144 dpf resulted again in 100% male bias [19]. In my study LNG dosing displayed neuronal increase compared to control in the rostral hypothalamus, an area that directly innervates the pituitary and controls development and puberty [75]. The shift in neural differentiation indicates for the first time that LNG can induce indications of precocious hypothalamic development (neural shift) as early as 5 dpf.

The Wnt pathway has been identified as necessary to neural differentiation during embryogenesis, specifically, differentiation to dopaminergic neurons in the early hypothalamus [75]. Dopaminergic neurons are present by 18 hpf and the hypothalamus is high in dopaminergic neurons by 3 dpf [82]. An increase of which is linked to increased aggression, anxiety and ADHD [75] while decreased dopamine neurons is correlated with hypoactivity behaviors in zebrafish [85]. Although most work is focused on cancer pathways, the Wnt pathway has exhibited interaction abilities with steroid receptors [86]. Potentially, LNG influences Wnt/AR and the increased neural differentiation is specific to a dopaminergic neuron increase aligning with behavioral changes (hyperactivity). Moreover, after EDC exposure, the Wnt pathway has been linked to male-favored sex differentiation and a precocious pubertal shift [87].

In summary, the *ex utero* and rapid development of zebrafish offer an excellent model to examine potential developmental changes that can be induced after EDC exposure. We suggest early-life behaviors can be assessed in larvae and serve as a screening method for determining potential neuroendocrine changes elicited after exposure. The hyperactive behavioral change induced after 100 nM E_2 brings us to assume that a neuroendocrine shift is occurring, but this change is not indicative of proliferation or differentiation confirmed in this study and aligning with previous work [15, 16, 88]. Additionally, using AroB as a marker for neurogenesis could be misleading as AroB expression increase is not necessarily synonymous with HuC or BrdU changes [88]. HuC (neural stain) may serve as a better primary molecular marker especially when analyzing neural differentiation. This study shows that LNG can induce seemingly minute changes to development that can alter brain processes in the developing embryo resulting in behavioral changes, neurogenesis, and eventually precocious puberty [19, 25]. Recent evidence has observed low-dose LNG exposure is able to affect DNA methylation of developmental, epigenetic regulatory and carcinogenic pathways across three generations in a teleost, *M. beryllina* [89] adding to the potential long-term risks associated with LNG exposure. To our knowledge, ours represents the lowest dose of LNG shown to illicit neurogenesis during early development.

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APPENDIX

APPENDIX

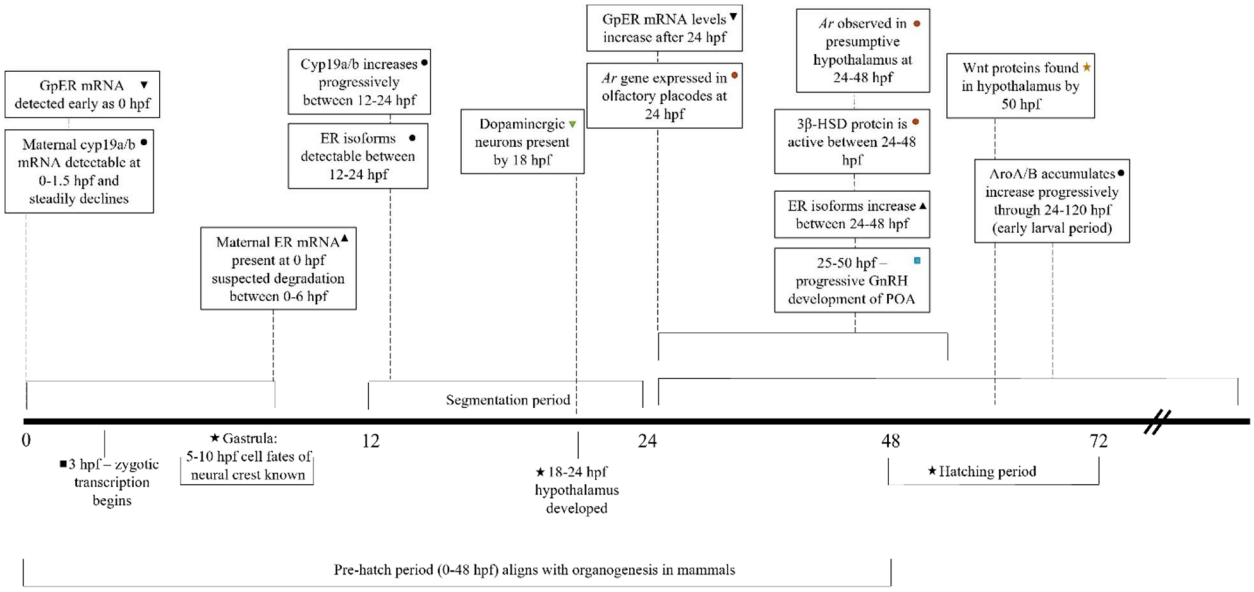

Figure 1. Overview of vital time points in the developing zebrafish

●Callard et al. 2001 ▲Kishida et al. 2001 ■Diotel et al. 2010 ★Kimmel et al. 1995
▼Schweitzer and Driever 2009 ●Gorelick et al. 2008 ■Zhao et al. 2013 ★Wang et al. 2009
▼Shi et al. 2013

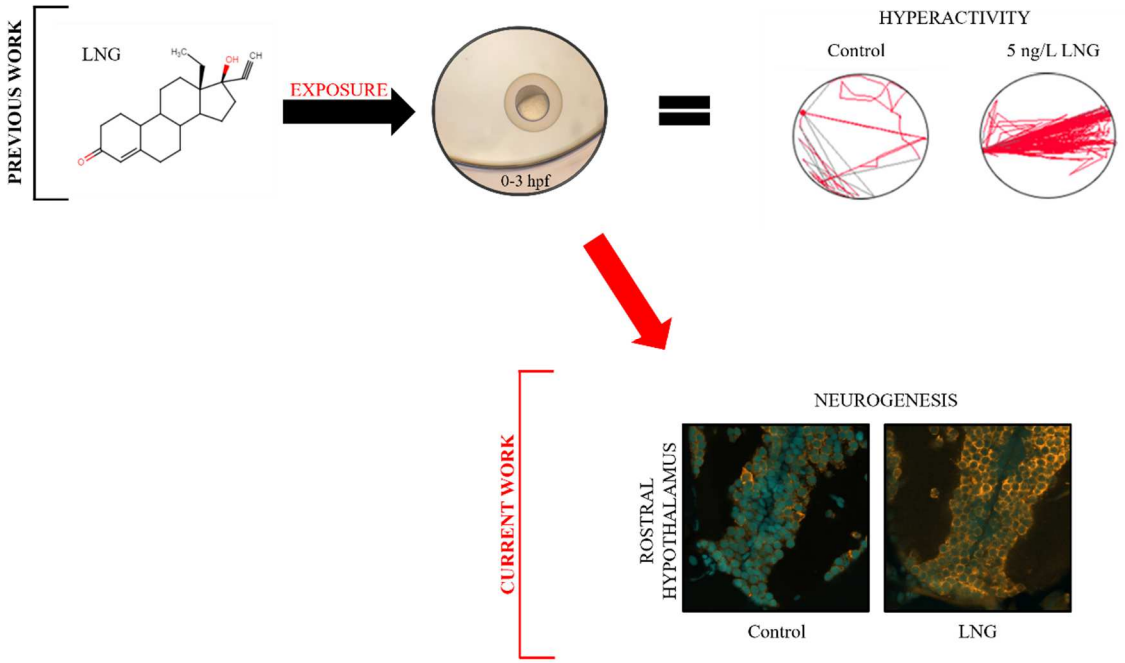

Figure 2. Experimental overview

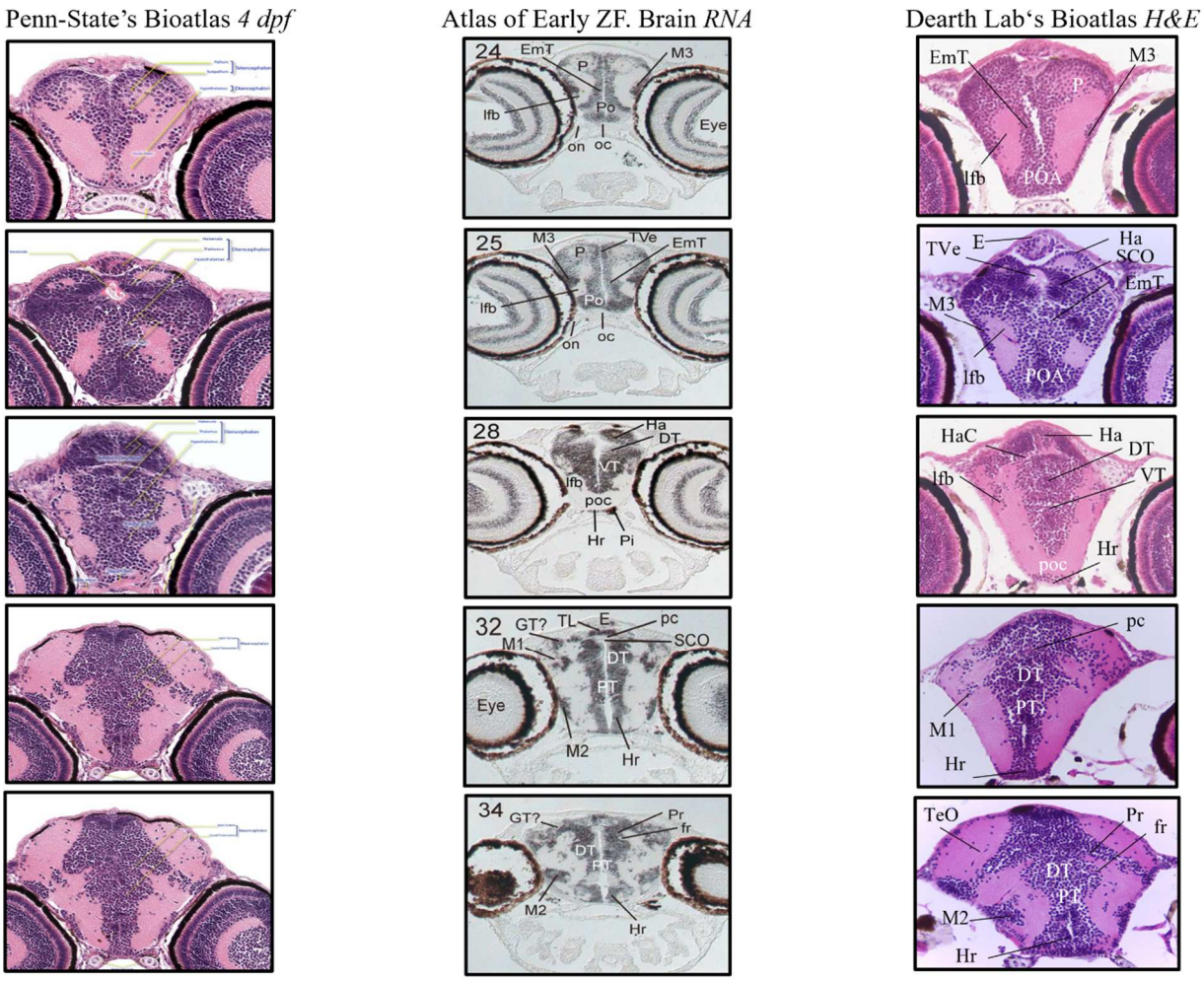

Figure 3. Establish an atlas for the rostral hypothalamic (rh) area in 5 dpf zebrafish larvae (transverse orientation).

A dpf Bioatlas from Penn-State (left column) was used as a guide for major brain structures but lacked specific labeling and serial sections. The second reference (middle column) was the "Atlas of Early Zebrafish Brain Development" in 5 dpf larvae which provided specific labeling of the brain but lacked the easy identification of structures that H&E provides. The Dearth Lab Bioatlas H&E (right column) provides easy brain structure identification, serial section, and thorough labeling.

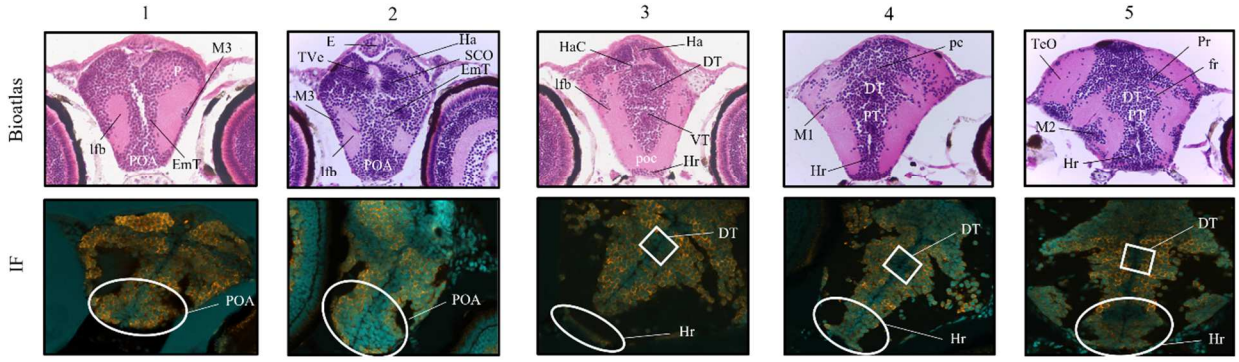

Figure 4. Direct comparison of the bioatlas to immunofluorescence (IF) to define areas of interest.

The creation of our bioatlas was a necessary reference when counting neurons in the POA, rostral hypothalamus proper (Hr) and thalamus in larval brains. In the IF row, rh areas have been circled in white while the dorsal thalamus was boxed in white. Columns 1 and 2 represent the area posterior to the AC where the POA is located and where the thalamus has not yet emerged. Column 3 exhibits the beginning of Hr presence just posterior to the poc and the area where the thalamus is introduced. Columns 4 and 5 display clear Hr and thalamic presence. The structures in the column 5 are the furthest Hr area that can be used in our counts before the intermediate hypothalamus is emerges.

Bioatlas Abbreviations - AC - anterior commissure, DT - dorsal thalamus, E - epiphysis, EmT - eminentia thalami, fr - fasiculus retroflexus, Ha - habenula, HaC - habenular commissure, Hr - rostral hypothalamus proper, lfb - lateral forebrain bundle, M1 - migrated pretectal area, M2 - migrated posterior tubercular area, M3 - migrated area of the EmT, P- pallium, pc - posterior commissure, POA - preoptic area, poc - postoptic commissure, Pr - pretectum, PT - posterior tuberculum, SCO - subcommissural organ, TeO - tectum opticum

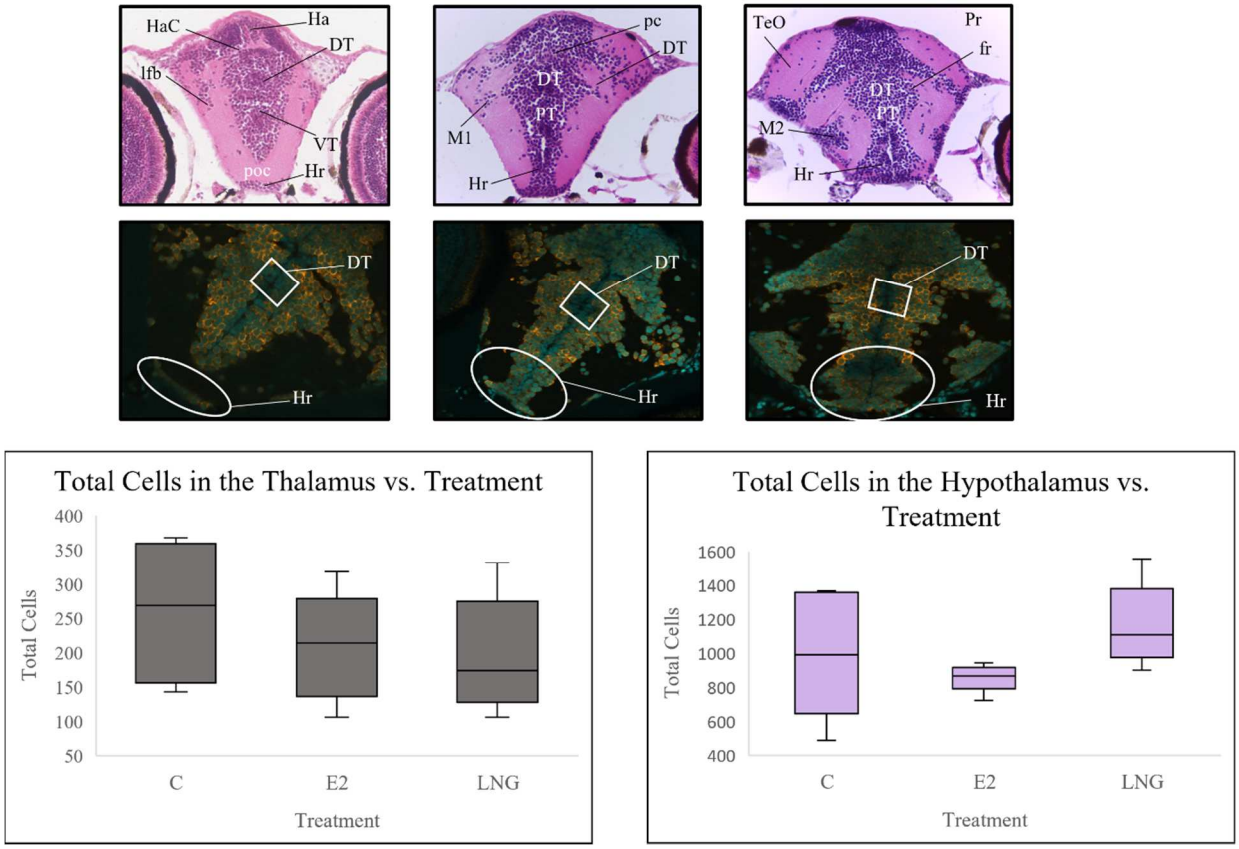

Figure 5. There is no change in the total cell number between treatments in the thalamus and rostral hypothalamus.

The total number of cells in the thalamus and hypothalamus of 5 dpf zebrafish larvae were counted after treatment with either Control, 100 nm Estrogen, or 5 ng/L LNG. Analysis confirmed there was no statistical difference in total cell number between treatment or brain area. (One-way ANOVA);n=5.

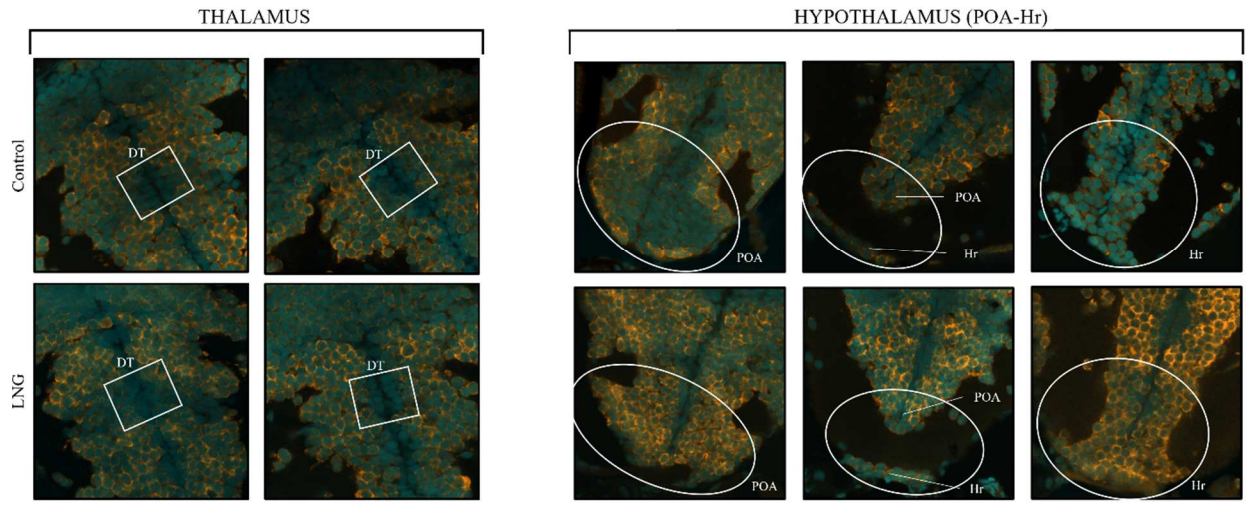

Figure 6. Representative images of thalamic and hypothalamic area in control and LNG treated tissue.

IF stain representing no significant change between HuC-positive cells between Control and LNG treated 5 dpf larval brains in the thalamus. Hypothalamic representative images showing the increase in HuC (neural) positive cells in LNG treated tissue compared to Control treated tissue from the POA to Hr.

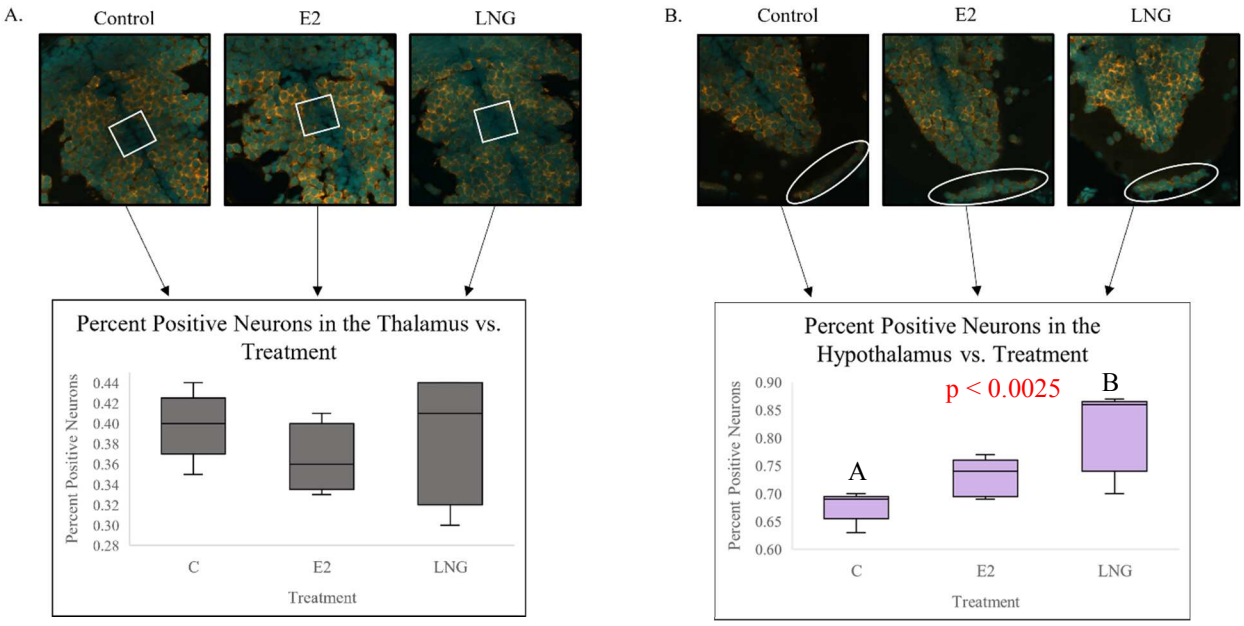

Figure 7. The percentage of positive neurons is specific to the rostral hypothalamus.

The number of positive and negative cells in the thalamus and hypothalamus were counted and percentages were calculated for each larvae. (A) No significant difference in positive cells between treatment in the thalamus was found. (B) In the hypothalamus there was a strong significant increase of positive cells between the Control (A) and LNG treatment group (B). (One-way ANOVA, Tukey's HSD); n=5.

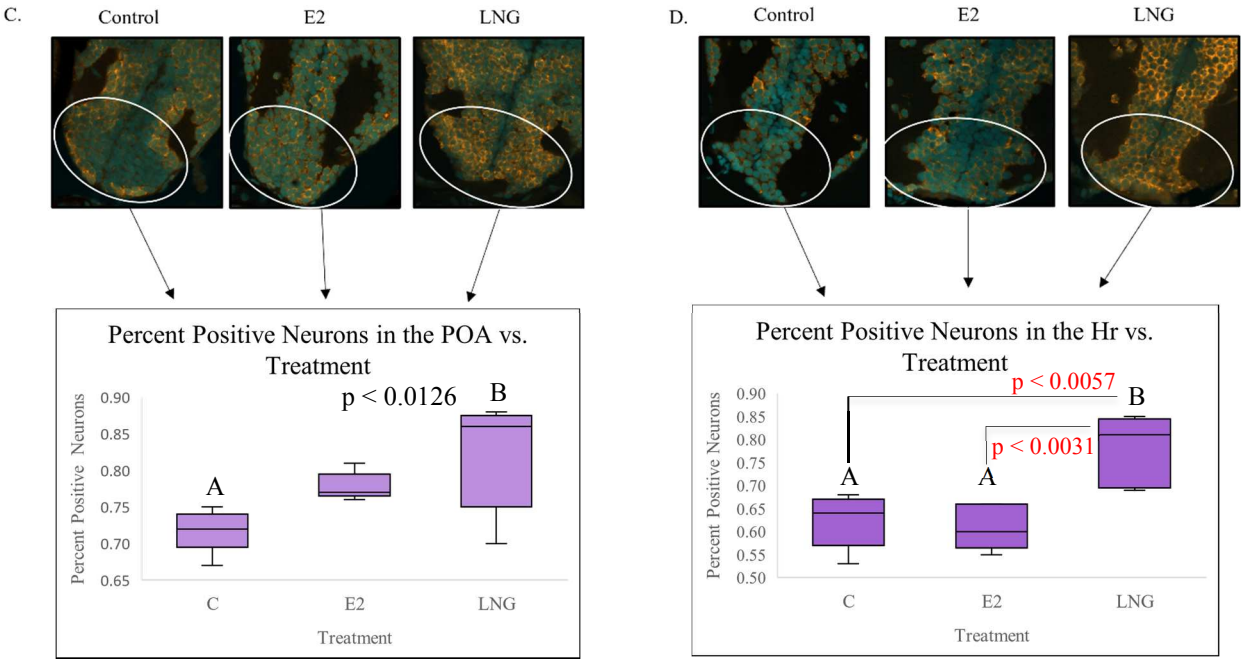

Figure 8. There is a significant increase in positive neurons in both the POA and rostral hypothalamus proper (Hr).

In the POA (C), there was a significant increase in LNG (B) treated brain compared to Control (A). Similarly, in the Hr (D), strong significance was found between LNG treated brain (B) and both Control and Estrogen treated brain (A). (One-way ANOVA, Tukey's HSD); n=5.

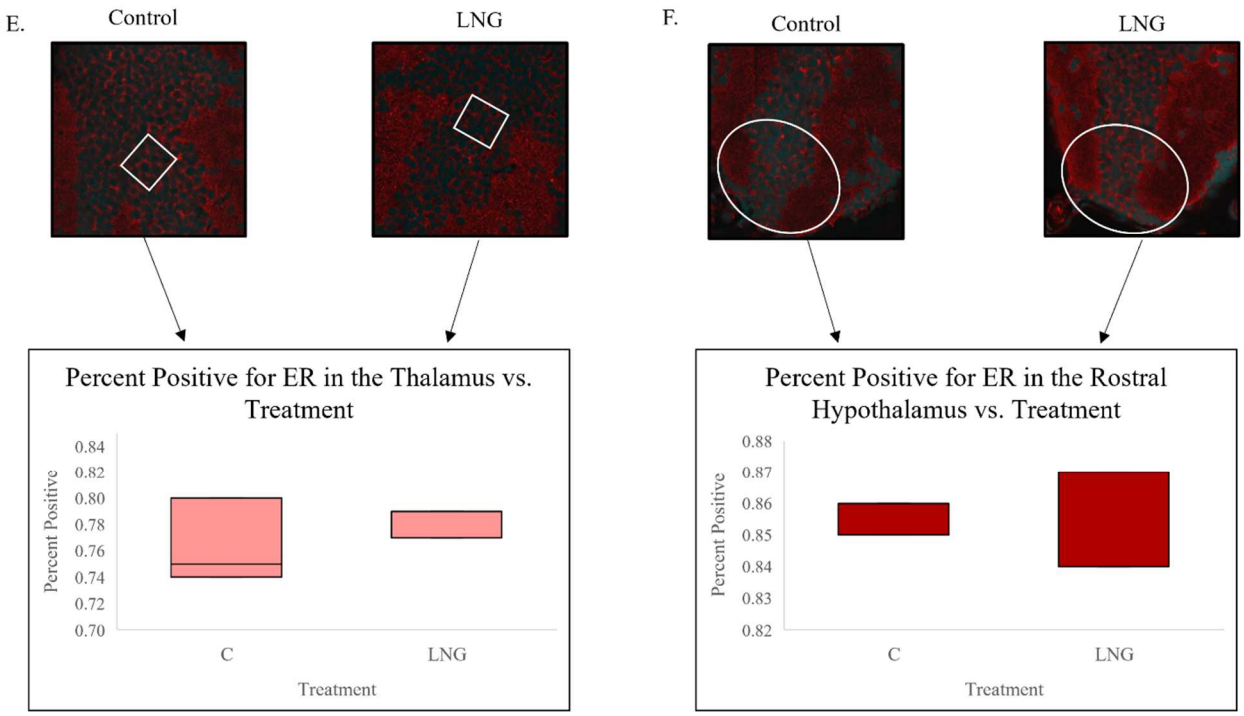

Figure 9. There is no significance in the presence of estrogen receptor in the thalamus and rostral hypothalamus.

There was no significant difference in ER presence between Control and LNG-treated larvae in either the thalamus (J) or the rostral hypothalamus (K). (One-way ANOVA); n=3.

Table 1. Overview of neural data using means.

	Mean Total Cell Count		Mean Percent Positive Neurons		Mean Percent Positive Within Hypothalamic Areas	
Treatment	Hypothalamus	Thalamus	Hypothalamus	Thalamus	POA	Rostral Hypothalamus
C	1000	260	67.8%	39.8%	71.8%	62.4%
E2	856	209	73%	36.6%	77.8%	61%
LNG	1163	196	81.2% *	38.6%	82.2% *	77.8% *

BIOGRAPHICAL SKETCH

Gabriella Hinojosa received her Bachelor of Science in Biology with a Minor in Psychology at the University of Texas Rio Grande Valley in the Summer of 2019. She then completed her Master of Science in Biochemistry and Molecular Biology at the University of Texas Rio Grande Valley in May of 2022. Her current email address is gabriella.hinojosa01@utrgv.edu.