

CONTRACEPTIVE HORMONES
-ETHINYL ESTRADIOL AND LEVONORGESTREL-
COGNITIVE EFFECT AND NORADRENERGIC ALTERATIONS

by

JEAN SIMONE

(Under the Direction of Philip V. Holmes)

ABSTRACT

The advent of fertility control by contraceptive hormones swept into the mid-twentieth century altering women's lives more than anticipated. The pharmacological mechanism of action of these potent hormones was known but not correlated to central nervous system targets with downstream consequences. Over fifty years, few studies questioned altered cognitive processing in this modified endocrinological state. Using a rat model, this dissertation uncovers different cognitive abilities that present with chronic contraceptive treatment. Our focus was the locus coeruleus, an area of the brain ascribed to attention, and its noradrenergic projection areas—hippocampus and prefrontal cortex, all sexually dimorphic. Rats, receiving three weeks of daily ethinyl estradiol 10 μ g or 30 μ g and levonorgestrel 20 μ g or 60 μ g were tested in learning and anxiety tasks relevant to the norepinephrine system. The lower doses proved to be ovulation suppressive, and the higher doses were chosen for dose-dependent observations. In the elevated-plus maze and shock-probe defensive burying, anxiolytic-like behavior was noted in the low dose groups. In the learning tests, dose-dependent effects of drug treatments were manifest,

with the low dose showing an impairment and the high dose an enhancement compared to the natural low hormone state of the rat. *In-situ* hybridization was performed for locus coeruleus tyrosine hydroxylase, the rate-limiting enzyme for the production of norepinephrine, and hippocampal brain-derived neurotrophic hormone, a neurotrophin. Both were reduced in the low dose drug-treated groups, and, when combined with behavioral results, suggested an altered noradrenergic tone. Duplicating the experiment, with the low and high dose of ethinyl estradiol only, the novel object recognition testing again showed enhanced learning in the higher dose ethinyl estradiol treated rats. Consistent with this behavior, the ethinyl estradiol 30 μ g treated rats' tissue norepinephrine level was significantly greater than both the control and the ethinyl estradiol 10 μ g group, in the prefrontal cortex—an area necessary for recent memory retrieval. These distinct dose-dependent actions of ethinyl estradiol on learning/memory and norepinephrine levels suggest a threshold to the minimal dose of ethinyl estradiol used in contraceptive hormone regimens. These findings could have significant consequences for the 10.6 million women using contraceptive hormones in the United States.

INDEX WORDS: ethinyl estradiol; levonorgestrel; contraceptive hormones; cognition; learning/memory; rats; norepinephrine; norepinephrine

transporter; norepinephrine reuptake inhibition; locus coeruleus;
prefrontal cortex; hippocampus

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DEDICATION

This dissertation is dedicated to James Terry Bell MD, my husband, who for 35 years has supported every ‘novel’ idea I came up with to explore, attempt, pursue, and succeed in. His first reaction might be “what?” However, no matter what, always he was there, backing me with perseverance when I wanted to flag, encouragement when I wanted to quit, and a huge smile when I obtained a goal. Thus, his motto of “you cannot stop living, for fear of dying” grew into my motivational mantra of ‘you cannot stop trying, for fear of failing.’ Too souls could not have been better matched.

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CHAPTER 1

1.0 Introduction

Contraceptive hormones were a seismic breakthrough in women's healthcare. Realized over the decades were unanticipated health benefits in addition to prevention of pregnancy. One often-quoted benefit is the reduction in ovarian cancer. Nevertheless, in a recent study consisting of 2,178,679 person-years of follow-up, this benefit was lost when a short duration (\leq six months of use) of first-generation contraceptive hormones were taken (Shafir et al., 2017). This example illustrates the importance of studying specifics—the dose, type, and duration—of hormone contraceptives with its relationship to an area of interest, in our case, cognition.

1.1 Contraceptive hormones and estradiol levels

A dramatic growth in family size was taking place before the introduction of contraceptives, with a rise from nearly forty percent of the women, having one-to-two children in 1910, to four or more children in 1935 (Kirmeyer & Hamilton, 2011). Contraceptive hormones were formulated to prevent ovulation and thus pregnancy, and simultaneously mimic the female menstrual cycle with a withdrawal bleed. Hormonal contraceptives stand apart from typical pharmaceutical products, for they are prescribed to more than one-hundred million healthy women worldwide (Christin-Maitre, 2012) to alter the homeostatic state rather than align it. In the 1960's, with the initiation of contraceptive hormone usage, so began the replacement of normal cyclic 17β -estradiol levels. The ovarian follicles are the source of estrogen in cycling women, which secrete

63–400 µg/24hr depending on the phase of an un-medicated cycle (Baird & Fraser, 1974). In fact, follicles produce 90% of circulating estradiol during their growth phase (Gleason, Carlsson, Johnson, Atwood, & Asthana, 2005). In contrast, with contraceptive hormone use, the growth of follicles is inhibited (Baerwald, Olatunbosun, & Pierson, 2004) and the 17β-estradiol serum level stabilizes at 30-50pg/ml throughout the next three weeks (Gaspard, Dubois, Gillain, Franchimont, & Duvivier, 1984; Mishell Jr, Thorneycroft, Nakamura, Nagata, & Stone, 1972; Vandever et al., 2008) (Figure 1.1). Further suppression of the follicular growth is obtained by the intake of greater than three weeks of contraceptive hormones (Birtch, Olatunbosun, & Pierson, 2006; Van Heusden & Fauser, 2002), resulting in even lower 17β-estradiol levels (Spona et al., 1996; Vandever et al., 2008; Willis, Kuehl, Spiekerman, & Sulak, 2006). In women 18-35 years old, serum estradiol levels drawn bi-weekly for eight weeks over the third and fourth birth control pill cycle had a median of 19.7 pg/mL (Westhoff et al., 2010). In comparison, menopausal women have an average estradiol level of less than 25 pg/mL (Burger, Hale, Robertson, & Dennerstein, 2007).

1.2 Relationship between estrogen and cognition

Estrogen therapy or higher endogenous estradiol levels in early menopausal women correlate with improved verbal memory (Galea, Frick, Hampson, Sohrabji, & Choleris, 2016; Wolf & Kirschbaum, 2002). Higher estradiol levels are commonly associated with improvement in verbal/non-spatial memory in pre-menopausal women and animal studies (Acosta, Hiroi, Camp, Talboom, & Bimonte-Nelson, 2013; Bronwyn M Graham, Ash, & Den, 2017; A.A. Walf, Koonce, Manley, & Frye, 2009; A. A. Walf, Rhodes, & Frye, 2006). Young cycling women (18- 38 years old) given exogenous

estradiol show an improvement in long-term verbal memory (Bartholomeusz et al., 2008). Craig et al. 2008, using a verbal encoding fMRI study on reproductive age women, found a highly significant positive relationship between estradiol levels and the average fMRI responses at the left inferior frontal gyrus, the activation of which is associated with successful memory. By diminishing the estradiol levels in the premenopausal women to the menopausal range using a gonadotrophin-releasing hormone agonist (GnRHa), verbal memory impairment was exemplified by a significant reduction of left frontal activity (Craig, Fletcher, Daly, Rymer, Brammer, Giampietro, Maki, et al., 2008). Normalizing the ovarian function reversed the encoding deficit—returning the pre-GnRHa left inferior frontal gyrus activity.

Alternatively, spatial learning improves in low estradiol states (Hampson, 1990; Hampson, Levy-Cooperman, & Korman, 2014; S. G. Warren & Juraska, 1997). Mental rotations scores are higher in the menses phase of the natural cycle when estradiol is low (Hausmann, Slabbekoorn, Van Goozen, Cohen-Kettenis, & Gunturkun, 2000). A recent review supports gonadal hormones' link to cognition and a biphasic effect exists in different cognitive strategies across species (Hamson, Roes, & Galea, 2016).

Neuroimaging studies of contraceptive hormone users, in contrast to naturally cycling women, differ in regards to grey matter volume, seen as increases in the prefrontal and mid-temporal areas (Belinda Pletzer et al., 2010; B. A. Pletzer & Kerschbaum, 2014), changes in executive control network connectivity (Petersen, Kilpatrick, Goharзад, & Cahill, 2014), and lateralization during verb generation (Rumberg et al., 2010).

Considering that the 17β -estradiol level is chronically suppressed, cognitive changes could become perceivable the longer one is taking contraceptive hormones.

These neuroimaging changes may represent altered brain estradiol levels. Post-mortem (accidental death) evaluation of a 19-year-old female on contraceptive hormones found decreased tissue estradiol across 17 brain regions compared to fertile women (Bixo, Backstrom, Winblad, & Andersson, 1995). Correspondingly, exogenous contraceptive hormones modulate the hypothalamic-pituitary-gonadal axis to be below the natural age threshold and cyclicity. Gonadotropic-releasing-hormone orchestrates hippocampal neurosteroid production (Brandt, N., Vierk, R., & Rune, G. M. 2013). Inhibition of the synthesis of the neurosteroid estradiol can decrease long-term potential and memory in rodent models (Grassi, Frondaroli, Dieni, Scarduzio, & Pettorossi, 2009; Tuscher et al., 2016). In a rat model of contraceptive use, central neurosteroids were significantly reduced, while slightly less so in the peripheral plasma (Follesa et al., 2002). Women on contraceptive pills, compared to cycling women, demonstrated a significant drop in serum neurosteroids in addition to estradiol (Rapkin, Morgan, Sogliano, Biggio, & Concas, 2006).

1.3 Ethinyl estradiol and levonorgestrel

This dissertation examines whether contraceptive hormones can compensate for the diminished endogenous hormones and support the relationship that exists between cognitive strategies and endogenous hormones. The commonly prescribed contraceptive analogs—ethinyl estradiol and levonorgestrel—may mimic endogenous hormones as receptor agonists (Figure 1.2). Nevertheless, ethinyl estradiol is biologically 500x more potent than 17 β -estradiol (Helgason, Damber, von Schoultz, & Stigbrand, 1982). Ethinyl estradiol has a greater affinity for the estrogen receptor than 17 β -estradiol (190:100% relative binding affinity respectively) (Blair et al., 2000). The alpha estrogen

receptor is translatable between rat to human since it is highly conserved (Katzenellenbogen, Katzenellenbogen, & Mordecai, 1979) and ethinyl estradiol binds to estrogen receptor α preferentially ($ER\alpha/ER\beta = 6.2$) (Escande et al., 2006; Heather A. Harris, Katzenellenbogen, & Katzenellenbogen, 2002).

Levonorgestrel is a second-generation progestogen with the capability of binding as an agonist to estrogen, progesterone, and to androgen receptors (Africander, Verhoog, & Hapgood, 2011; García-Becerra et al., 2006; Kloosterboer, Vonk-Noordegraaf, & Turpijn, 1988) so assessing behavior based on single receptor type is probably insufficient. The relative binding affinity of levonorgestrel is three-times more than progesterone for the progesterone receptor, while it has only fifty percent of the testosterone affinity to the androgen receptor (Philibert et al., 1999).

Both hormones can bind to nuclear/intracellular receptors and act as ligand-activated transcription factors, or to the cell membrane, G-protein coupled receptors and activate signaling cascades (Nilsen & Brinton, 2003).

Still, interaction with steroid receptors does not necessarily reflect the biological effectiveness and may vary depending on factors such as dose and route of drug administration. For example, ethinyl estradiol potency is shown to be dose-dependent for prevention of osteoporosis in menopausal women. An oral daily dose greater than 25 μ g results in a net bone gain; alternatively, a prescription below 15 μ g daily results in a net bone loss (Horsman, Jones, Francis, & Nordin, 1983). Enterohepatic circulation plays a major role in the pharmacokinetics of ethinyl estradiol/levonorgestrel, and even with a subcutaneous route bypassing the first liver passage, the dose needed for rodent

ovulation inhibition needs to be higher than expected per calculated body surface area (Kuhl, 2005).

Lastly, the dynamics of the receptors are that the biological response to steroids falls off before all receptors are occupied (W.V. Welshons et al., 2003). This phenomenon translates into, within the lower concentration range of exogenous hormone exposure, as an increase in concentration is given there is an equal increase in receptor occupation. On the other hand, as the concentration of hormone rises to higher ranges, the reciprocal receptor occupancy is not linear anymore, instead only a fractional increase in occupancy occurs (i.e., a ten-fold concentration increase results in a two-fold increase in occupancy) (Welshons et al., 2003).

Interspecies comparison of pharmacokinetics is not the only or even the most correct analysis of drug-dose effects. Rather, the comparison of the hypothalamic-pituitary axis modulation with its up/downstream adjustments in the central nervous systems may be more biologically exact and correlatable to behavioral outputs (Hart, 1990). Due to the aforementioned factors, it is imperative to study the behavioral effect synthetic analogs have on learning and memory since all hormones are not equal. As important, dose-response curves are needed to investigate the expected inverted-U response to steroids.

1.4 Learning and memory

Learning and memory most often are measured indirectly, by noting an adaptive change in animal performance or behavior. Learning and memory cannot be attributed to one functional network. Instead, learning behavior is controlled by an integration of multiple sources of information. To obtain that information, motivation, as well as attention to one's surroundings, are needed. Motivation varies with hormonal levels, as

well as with the cognitive task examined. For instance, the motivation to obtain a higher number of food pellets by more lever pressing exhibited a biphasic outcome correlated with estradiol level (Uban, Rummel, Floresco, & Galea, 2011). Low estradiol levels increased the choice for more lever pressing, hence more food; with high levels of estradiol, the opposite occurred (Uban, Rummel, Floresco, & Galea, 2011).

Appetively motivated tasks of working memory are the radial arm maze, T-maze, or operant conditioning (nose poking) whereas, aversively motivated memory tasks are the water maze and passive avoidance with foot shock (Sousa, Almeida, & Wotjak, 2006; Williams, 1998). Considering that we were evaluating the noradrenergic system, we wished to avoid an anxiogenic confound. Consequently, the motivation chosen was the natural instinct to explore novel non-food objects in a familiarized chamber (Gompf et al., 2010). As expected with steroids, hormonal biphasic outcomes can be seen in this task, such as proestrus mice perform better in novel object recognition tests and demonstrate more exploratory behavior than the low estrogen diestrus state (A.A. Walf et al., 2009).

Attentiveness can increase memory networks associated with an incident and vice-versa, a revisit to the incident or surroundings will recall these bundled details.

Attention and motivation are innately linked to changing levels of gonadal and adrenal hormones. The interplay of hormone response levels varies with the needed attentiveness of conditions. For example, in an immobilization stress paradigm, cortisol levels increased; nevertheless, estradiol treatments diminished the rise in biosynthetic enzymes for norepinephrine in the locus coeruleus—the origin of attention mechanisms (L. I. Serova, Harris, Maharjan, & Sabban, 2010). We, therefore, examined the

neurobehavioral effects of contraceptive hormones treatments in rats using a minimal stress learning model with a natural motivation to explore novelty, as well as to appropriately evaluate the neurotransmitter—norepinephrine, both at the level of the locus coeruleus and its end-targets, the prefrontal cortex, and the hippocampus.

1.5 Areas of the brain—relationship with hormones

1.51 Locus coeruleus

The locus coeruleus (LC) is located in the brainstem situated between the lateral floor of the fourth ventricle above, and the seventh cranial nerve below and has neuromodulatory links with the cerebral cortex, hippocampus, hypothalamus and thalamus, cerebellum and spinal cord (Foote, Bloom, & Aston-Jones, 1983; Loizou, 1969). The LC in female rats, which becomes a sexually dimorphic nucleus after puberty, is larger in volume, has a greater dorsoventral range, and has more neurons than males (Babstock, Malsbury, & Harley, 1997; Luque, de Blas, Segovia, & Guillamón, 1992; Pinos, Collado, Rodríguez-Zafra, et al., 2001) (Table 1). Post-pubertal ovariectomy results in a loss of this dimorphism (Pinos et al., 2001), making it essential that investigation of contraceptive hormone effects on the LC be done with intact females. The LC remains sensitive to estradiol levels through the fluctuating expression of alpha estrogen receptors and progesterone receptors (induced by estradiol) across the estrus cycle (Helena et al., 2006). The LC networks with related sexually dimorphic areas of the brain. Originating from this small nucleus is much of the noradrenergic innervation of the entire central nervous system. In study one (chapter 3), upon sectioning of the LC for *in-situ* histochemistry, we perceived a volume disparity

among the unidentified subjects, leading us to look at tyrosine hydroxylase mRNA in the LC as a window into noradrenergic activity.

1.52 Hippocampus

Multiple studies have shown a correlation between estradiol levels and hippocampal dendritic spines (Brandt, Vierk, & Rune, 2013), neuron number (C. Barha, Lieblich, & Galea, 2009) and spatial memory (C. K. Barha & Galea, 2010). Cell proliferation in the hippocampus is highest when female estradiol level is highest (Tanapat, Hastings, Reeves, & Gould, 1999) and this is accomplished via activation of both estrogen α/β receptors (Mazzucco et al., 2006). Gonadectomy in rats resulted in reduced levels of hippocampal estradiol, yet not a change in the level in the prefrontal cortex (J. Barker & Galea, 2009).

By looking at hormonally sensitive areas of the noradrenergic system, we hypothesize a biphasic result that would depend on where our doses fell on the inverted-U dose-response curve, and the cognitive strategy needed to perform the learning task (spatial vs. non-spatial). The sex steroids influence on norepinephrine production, release, and metabolism would affect its level and actions. The purpose of the study one (Chapter 3) was to define the cognitive gain or loss based on the dose of the individual drugs alone, and in combination, as well as to investigate if the drugs influenced the noradrenergic system upstream or downstream by looking at tyrosine hydroxylase and brain-derived neurotrophic factor. Due to the finding of opposing effects of the drugs doses associated with a cognitive strategy, study two (Chapter 4) included the prefrontal area for evaluations of noradrenergic tone.

1.53 Prefrontal cortex

The prefrontal cortex, one of the many noradrenergic projection areas of the LC, is involved with the focusing of recent memory. The quantity of norepinephrine released in this area can improve or diminish learning, based on the affinity of low levels binding preferentially to post-synaptic α -2A adrenergic receptors, while high levels bind to α -1 adrenergic receptors (Ramos & Arnsten, 2007). Learning is at its best under moderate noradrenergic stimulus, whereas too high or too low results in specific deficits (Berridge & Waterhouse, 2003). The importance of this coeruleo-cortical noradrenergic connection is emphasized by the prefrontal feedback circuit to the LC for ‘top-down’ control (Jodo, Chiang, & Aston-Jones, 1998). Sexual dimorphism exists in structural format of the prefrontal cortex, with shorter and less branching of dendrites in females (Kolb, B., & Gibb, R. 2015). Although estradiol treatment of post-menopausal primates increased dendritic spines in layer three of the prefrontal cortex, there was no improvement over controls in learning (Hao et al., 2007). Even so, when more diversified cognitive strategies were evaluated, a biphasic result occurred, dependent upon the estrogen level. Using a battery of cognitive tasks, menopausal women not taking hormone therapy exhibited a deficit, specifically in working memory (prefrontal cortex tasks), compared to the treated group (Keenan, Ezzat, Ginsburg, & Moore, 2001). Consistent with biphasic findings, young cycling females (mean age 26) at or near ovulation (high estrogens) showed a marked increase in perfusion in cortical areas during the testing of cognitive tasks compared to the low estrogen phase (Dietrich et al., 2001).

1.6 Assessments of noradrenergic tone

1.61 Norepinephrine activity via synthesis—tyrosine hydroxylase

Neurotransmitter activity can be evaluated indirectly by measuring precursors or biosynthetic enzyme levels. Tyrosine hydroxylase (TH) is the rate-limiting enzyme for norepinephrine synthesis and is diffusely distributed in the LC (Holets, Hökfelt, Rökaeus, Terenius, & Goldstein, 1988). In the LC, TH distribution is sexually dimorphic in that females have more TH-containing neurons than males (Luque, Guillamon, & Hwang, 1991). This is not surprising, given that the LC with its hypothalamic noradrenergic connections augments ovulation (Anselmo-Franci, Rocha-Barros, Franci, & McCann, 1999). Estrogens have been shown to increase (L. Serova, Rivkin, Nakashima, & Sabban, 2002), decrease (Maharjan, Serova, & Sabban, 2005), and have no effect on TH transcription (Tseng, Kolb, Raskind, & Miller, 1997) depending on the type of estrogen, location and route of administration. Notably, alpha and beta estrogen receptors up and downregulate the transcription of TH respectively, through the cAMP pathway (Maharjan et al., 2005). Norepinephrine concentrations can linearly inhibit TH enzyme activity, but more pertinent to our studies is the metabolite of estrogens, catechol estrogens, ability to inhibit TH with the same dose-dependent potency of norepinephrine (Lloyd & Weisz, 1978). Norepinephrine and catechol estrogens compete for the cofactor tetrahydrobiopterin needed for the production of TH (Lloyd & Weisz, 1978). All are important reasons to examine TH mRNA in the LC.

1.62 Norepinephrine activity by indirect measurements

1.62a BDNF

Estrogens may improve noradrenergic neuronal function through other receptor-independent mechanisms such as in conjunction with neurotrophins. Brain-derived neurotrophic factor (BDNF) is a determining growth factor in proliferation, differentiation, and longevity of neurons (Huang & Reichardt, 2001). These same benefits are seen with estrogen exposure and are felt to be mediated through the stimulus of BDNF (H.E. Scharfman & MacLusky, 2006). Estrogen regulates the expression of BDNF mRNA in the hippocampus and frontal cortex consistent with a putative estrogen response element on the BDNF gene (Simpkins et al., 1997; Sohrabji, Miranda, & Toran-Allerand, 1995). In vivo, after a single estrogen injection, there is a two-fold increase in BDNF mRNA expression in the cortex of ovariectomized animals (Sohrabji et al., 1995). Serum BDNF levels fluctuated over the menstrual cycle with the luteal phase the highest, except in women on contraceptive hormones where no luteal phase rise occurred (N. Pluchino et al., 2009). BDNF can be a reliable marker of norepinephrine transmitter activity (Van Hoomissen, Holmes, Zellner, Poudevigne, & Dishman, 2004). Playing a role in learning and memory, BDNF acts as a mediator of neuronal plasticity (K. S. Chen et al., 1997; M. F. Egan et al., 2003; Korte et al., 1995; Lindsay et al., 1991; Tanaka et al., 2008). For these reasons, we measured BDNF mRNA in the hippocampus in study one (Chapter 3).

Study two (Chapter 4) was completed with the same learning paradigms but with a reduced retention time to highlight the drug effect of ethinyl estradiol on cognition. With this second experiment, we explored the function of ethinyl estradiol as

norepinephrine modulators by looking at tissue levels of norepinephrine, norepinephrine transporter and catabolite normetanephrine.

1.62b Norepinephrine transporters /Norepinephrine reuptake

Norepinephrine reuptake ends the transmission of the noradrenergic signal via norepinephrine transporters (NET) (Figure 1.3). The early development of NET coincides with rapid noradrenergic synapse growth followed by pruning to adult levels, a process which doesn't reach completion until postnatal day 45 or more in rats (Murrin, Sanders, & Bylund, 2007). Inhibition of norepinephrine reuptake results in a reduced level of NET *in vitro* and an increase in noradrenergic transmission *in vivo* in the brain (Weinshenker, White, Javors, Palmiter, & Szot, 2002; Zhao et al., 2008; Zhu, Blakely, Apparsundaram, & Ordway, 1998). Ethinyl estradiol acts as a norepinephrine reuptake inhibitor (Ghraf, Michel, Hiemke, & Knuppen, 1983; Kendall, Tonge, & Leonard, 1977). Therefore we examined the tissue levels of NET as a preliminary to future immunohistochemistry and in-situ experiments.

1.62c Norepinephrine tissue levels

The noradrenergic system—the neurons, their terminals and norepinephrine levels—develops more slowly than the serotonergic system in rats, but reaches completion by its 40th postnatal day (Murrin et al., 2007). Norepinephrine levels differ in female rats from males, in that females have a higher rate of synthesis, metabolism, and turn-over (Luque et al., 1992). Punch biopsies of discrete areas of the brain across the rat estrous cycle have revealed fluctuations in norepinephrine concentrations, with a generalized trend for higher levels when estrogen is lowest (Crowley, O'Donohue, & Jacobowitz, 1978). Cortical differences in extracellular norepinephrine could allow for flexible

cognitive strategies to adjust behavior to new contingencies. The density of norepinephrine axonal varicosities, a mechanism of transmission, may be the modulator of this fine-tuning (Zoli, Jansson, Sykova, Agnati, & Fuxe, 1999). A forty percent greater varicosity density exists in the frontal cortex compared to the rest of the cortex in rats, suggesting a regional rheostat for noradrenergic tone (Agster, Mejias-Aponte, Clark, & Waterhouse, 2013). For instance, a mouse microdialysis study of norepinephrine in the medial prefrontal cortex and hippocampus after exploration of a novel cage demonstrated an increase of 97% and 84% in efflux respectively (Ihalainen, Riekkinen, & Feenstra, 1999). To research this, we measured norepinephrine by enzyme-linked immunosorbent assay.

1.62d Normetanephrines

The majority of the catabolism of norepinephrine occurs in the noradrenergic neuron, whereas in extraneuronal cells, conversion of norepinephrine to normetanephrines (NMN) is catalyzed by catechol-O-methyltransferase (COMT) (Eisenhofer, Kopin, & Goldstein, 2004). Catechol estrogens formed from hydroxylation of ethinyl estradiol, have a significantly greater affinity for COMT than norepinephrine; therefore, extraneuronal catabolism of norepinephrine is competitively inhibited by catechol estrogens (Breuer & Köster, 1975). In rat brain COMT tests, 2-hydroxy-ethinyl estradiol inhibited norepinephrine by 98% with the catechol estrogen to norepinephrine molar ratio of 0.3 (Breuer & Köster, 1975). All the same, inhibition of COMT does not significantly alter norepinephrine levels in rat brains (Glowinski & Axelrod, 1965), though it may prolong the action of norepinephrine (Ball & Knuppen, 1990). Norepinephrine transmission and reuptake inhibition intensify extraneuronal

metabolism of norepinephrine resulting in increased NMN (Wood, Kim, & Altar, 1987); thus our rationale for the measurement of NMN as a reflection of noradrenergic changes.

In summary, variability in female hormones can result in advantageous or disadvantageous behavioral responses. Contraceptive hormones, by follicular growth inhibition, remove fluctuations and support a low endogenous estrogen state. The purpose of the following experiments is to assess whether there is adequate compensation by contraceptive hormones to not lose cognitive agility.

1.7 Figures/Table

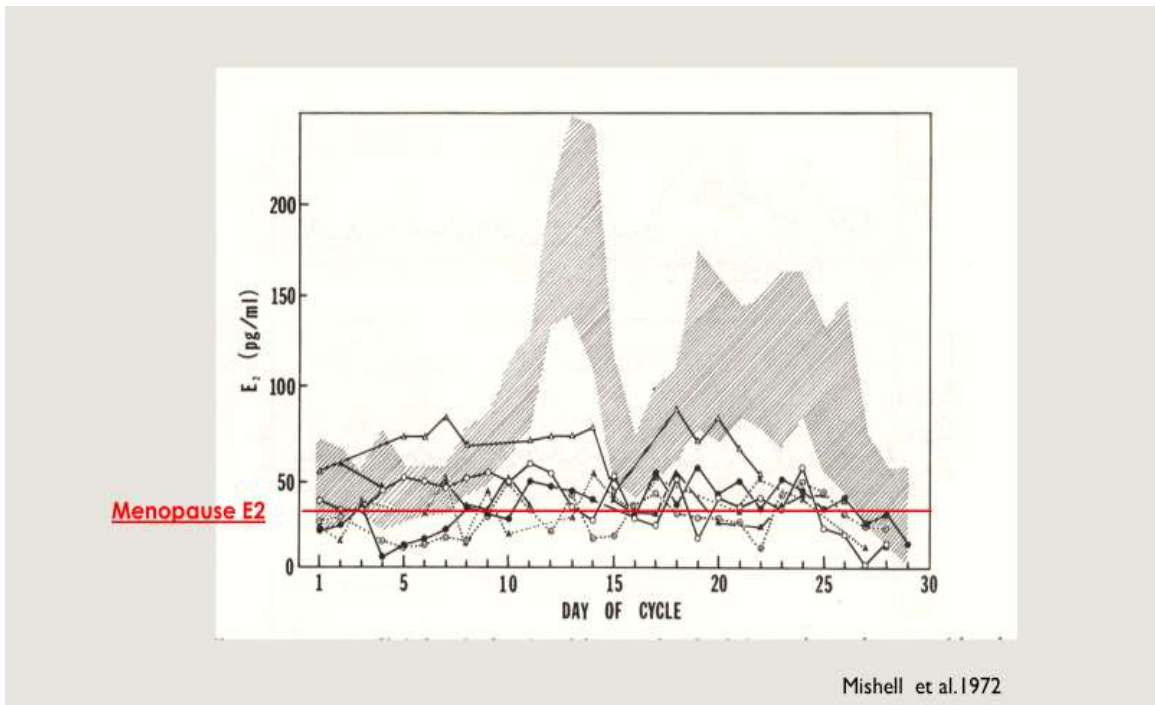


Figure 1.1 Effect of contraceptive hormones on 17β -estradiol levels

Daily serum 17β -Estradiol levels in six women taking contraceptive hormones for greater than a year. The shaded area is the expected 17β -Estradiol levels of a woman not taking contraceptive hormones (mean \pm SEM) (Adapted from Mishell et al. 1972). The red line is the expected mean high of 17β -Estradiol in menopausal women (Burger et al. 2007).

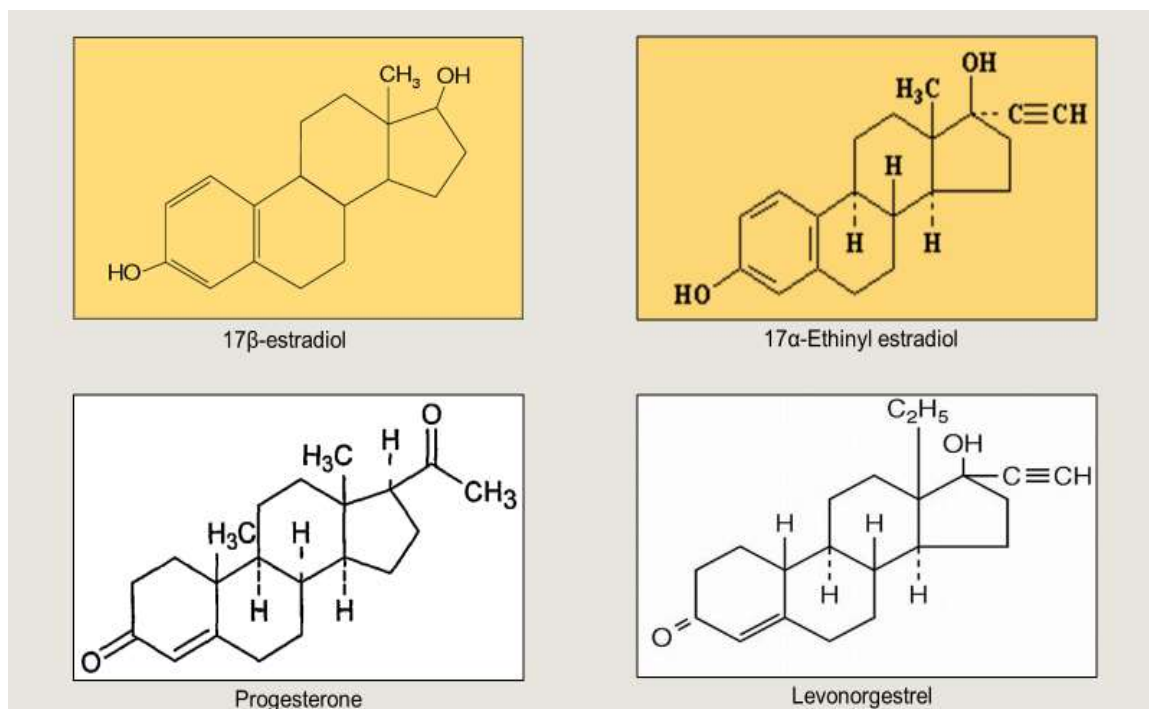


Figure 1.2 Analogs of endogenous hormones

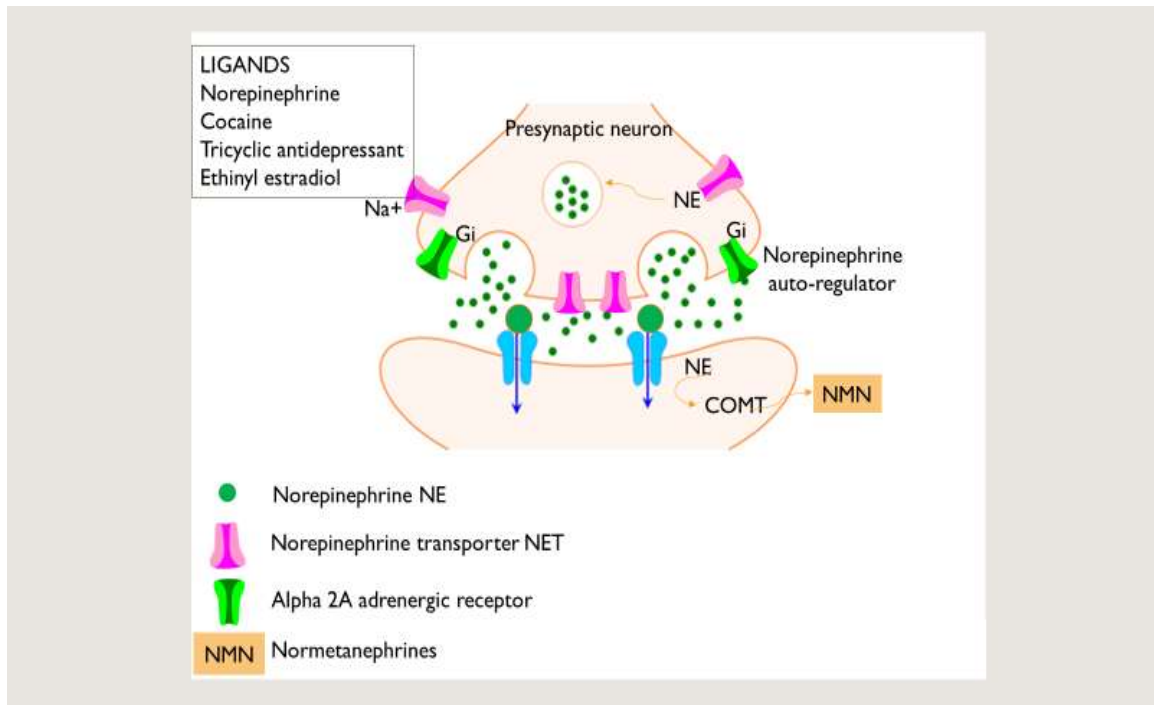


Figure 1.3 Noradrenergic synapse and the processing of norepinephrine

Norepinephrine released has several possible trajectories. It may bind as a ligand to the to an alpha or beta receptor on the postsynaptic cell and complete transmission. Norepinephrine can be taken up into the postsynaptic cell and once inside the postsynaptic cell it can be metabolized by Catechol-O-methyltransferase (COMT) as shown and normetanephrines (NMN) released. Alternatively, it may bind to a presynaptic alpha-adrenergic autoreceptor and modulate the firing of the neuron. It can bind to the norepinephrine transporters (NET) to be recycled back into the presynaptic cell. In the presynaptic cell, it can be metabolized by monoamine oxidase (MAO) and dihydroxyphenylglycol released into the bloodstream, or it can bind to vesicular monoamine transporter and be stored in vesicles. Over 70% of recaptured catecholamines are sequestered into storage vesicles rather than being metabolized because VMAT has a stronger affinity for norepinephrine than MAO (Eisenhofer, G. 2004).

Table 1: Rat Maturation Statistics

Rat	Postnatal Day
Adult age	63
Sexual Maturity	35 -50
Adult # locus coeruleus neurons	60
Adult Locus coeruleus volume	90
Adult # hippocampus neurons	0
Adult # of cortex neurons	0
Adult norepinephrine levels	42
Adult Adrenergic receptors	60
Adult Norepinephrine synapses	60
One human year \approx 14 days in rat	

Sources: Sengupta, P. (2013). Murrin, L. C., Sanders, J. D., & Bylund, D. B. (2007).

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CHAPTER 2

Literature review of contraceptive hormones' effect on cognition

2.1 Introduction

The current knowledge about female gonadal hormones' complex effects on learning and memory is extensive and diverse. The foundation of the topic's compilation of knowledge began with Beach's first ten chapters of Hormones and behavior; a survey of interrelationships between endocrine secretions and patterns of overt response (Beach, 1948). He gathered the existing data on hormones' effects on behavior, which at the time was heavy in observations of reproductive-related behavior, yet did include learning as one of the parameters. The behavioral endocrinology information at that point was enough to produce a 65-page bibliography. The next three decades added depth and breadth to the knowledge that hormones program the brain from inception and onward (reviewed in Berenbaum & Beltz, 2011; McCarthy, 2010), beginning with such prominent works by Rosenblatt, JS., Lehrman, DS., & Rheingold, HL., Grady, K., Phoenix, CH., and Young, WC. (Marler, P. 2005). Over forty years, neuroendocrinologist Samuel McCann and his co-workers defined hypothalamic control and its particular relevance to female endocrinology (Licinio, Gold, Chrousos, & Sternberg, 1997). From that strong platform, the nineties to present brought an ever-increasing volume of literature confirming the role gonadal hormones play on learning and its retention. Maki, P. (2002), Sherwin, B. (1988), and McEwen, B. (1999) examined the cognitive effects of estradiol occurring across cycle phases and spanning all ages, while animal studies added

the advantage of cellular models of estrogen's mode of action (A.A. Walf & Frye, 2006; C. Woolley, Weiland, McEwen, & Schwartzkroin, 1997; C.S. Woolley, 2007). However, findings of a large human study, WHIMS, contradicted the predictions of estrogens' cognitive advantages after menopause (Chisholm & Juraska, 2013; M. Craig, Maki, & Murphy, 2005; Resnick et al., 2009; Shumaker et al., 2003). This led to provisions being added to previously held beliefs (Chisholm & Juraska, 2013; Fischer, Gleason, & Asthana, 2014; Morrison, Brinton, Schmidt, & Gore, 2006; Sherwin, 2006). Although the benefits/detriments of estrogen's influence on cognition are still under discussion, the consequence of the premature loss of function of the ovary by oophorectomy or by medication is now known to have adverse costs on cognition (M. C. Craig et al., 2009; M. C. Craig et al., 2008; Grigorova, Sherwin, & Tulandi, 2006; McEwen, 2002; Nappi et al., 1999; Rocca et al., 2007).

Pertinent to this dissertation is the latest expansion of the information base into the area of premenopausal hormonal fluctuations modulating cognitive performance (Hampson & Morley, 2013) and how inhibition of this natural cyclic exposure to gonadal hormones may affect cognitive processing. Given the scope of accumulated general knowledge and the fifty-years use of contraceptive hormones, it is surprising to find that the research on their effects on learning and memory remain limited. The results of these limited studies are dispersed between positive and negative outcomes (A. M. Warren, Gurvich, Worsley, & Kulkarni, 2014). The aim of this systematic literature review is to present the existing evidence for the interaction between contraceptive hormones and cognitive processing, as well as the possible classical neurotransmitters that may bring about this effect.

2.2 Ethinyl estradiol

Possibly due to the assumption that all estrogens are the same, lagging behind are in-depth systematic studies regarding the cognitive impact of synthetic ethinyl estradiol (EE) (B. A. Pletzer & Kerschbaum, 2014). The studies using EE as the estrogen of choice are sparse and the results inconsistent. Frequently the result is a biphasic dose-response, consistent with receptors interaction (E. J. Calabrese & Baldwin, 2003), response to hormones (W. V. Welshons et al., 2003), and Yerkes-Dodson Law of optimum learning performance (Strom, Theodorsson, & Theodorsson, 2011; Yerkes & Dodson, 1908). For instance, 17β -estradiol has been shown to have a dose response curve in intact female rats. A moderate dose of estradiol supplementation maintained success in increasing working memory load performance whereas low estradiol supplementation did not. (Bimonte & Denenberg, 1999). Intact rats evinced improved learning in novel object recognition with the EE 30 μ g per day treatments and impaired learning with EE 10 μ g per day when compared to met-diestrus phase rats (Simone et al., 2015). In contrast, ovariectomized rats, given a high (considered so for their study) dose EE (0.3 μ g per day), but not low EE (0.125 μ g per day), or medium EE (0.18 μ g per day) had impaired spatial working memory (radial-arm and water maze) (Mennenga et al., 2015). Likewise, different treatment regimens had an impact on learning. “Cyclic,” consisting of daily subcutaneous injections, impaired reference memory at all three doses of EE, while “tonic,” an implanted capsule, did not (Mennenga et al., 2015). On the other hand, in middle age, non-human primates 10-15 years post-ovariectomies, administration of EE at the dose typically prescribed to pre-menopausal women for oral contraceptive use was able to improve learning in a working memory test (Lacreuse, Wilson, & Herndon,

2002). All of which shows that the starting range of endogenous estradiol and the quantitative dose of the supplemented analog interplay to accomplish varying results.

Studies that compare the dose of the EE component in combination pills also had mixed results. A within-subject study of women on either an EE dose of 30, or 50 μ g (with the same type of progestin) saw no difference in pre-and post-psychometric tests (Silber, Almkvist, Larsson, Stock, & Uvnas-Moberg, 1987). This study is not in accord with results seen by Beltz et al. (2015) in women taking EE 20 μ g (mean) monophasic pills contrasted with women taking EE 30 μ g tri-phasic pills (all progestins differed amongst the pill). The increase in EE dose was associated with a decrease in mental rotation performance and less expressional fluency when compared to the low EE dose pill usage (Beltz, Hampson, & Berenbaum, 2015).

Few studies look at EE's effect on cell molecular changes. Using excitotoxicity and chemotoxicity by quinolinic acid and kainic acid respectively, EE, 1, 10, or 100 μ g, was tested for neuroprotective qualities on hippocampal neurons—in ovariectomized adolescent rats (O. Picazo, Becerril-Montes, Huidobro-Perez, & Garcia-Segura, 2010). All three doses prevented neuronal loss by kainic acid, yet only the low dose 1 μ g prevented it with quinolinic acid. The authors hypothesize that the higher EE doses enhance excitatory neurotransmission and thus were synergistic with quinolinic acid actions (O. Picazo et al., 2010). In experimental autoimmune encephalomyelitis mice, oral high dose EE (50 μ g) protected against the progression of the disease by down-regulating proinflammatory cytokines (Subramanian, Matejuk, Zamora, Vandenbark, & Offner, 2003). Neonatal rat hippocampal tissue slices, pre-treated with EE before glutamate excitotoxicity resulted in increased damage in CA3 (Sato, Matsuki, Ohno, &

Nakazawa, 2002). Together, these studies suggest that EE may play a neuroprotective role from chemotoxic/inflammatory insults yet loses the benefit with excitotoxic insults. More recent studies have looked at the timing of EE exposure and emotive and cognitive learning later in life. Intact adolescent male and female rats exposed developmentally to environmental level EE (4 and 400 ng/kg/day) demonstrated more anxiety-like behavior in novel environments (Zaccaroni, Seta, Farabollini, Fusani, & Dessi-Fulgheri, 2016). Doses of EE (15µg/kg/day) by intrauterine exposure resulted in adult rats demonstrating anxiety-like and depression-like symptoms (Arabo, Lefebvre, Fermanel, & Caston, 2005; Dugard, Tremblay-Leveau, Mellier, & Caston, 2001). Consistent with EE's low affinity to alpha-fetoprotein (Hong et al., 2015), male rats exposed developmentally to 4ng/kg/day showed enhanced spatial learning in the Morris water maze (higher estrogen intrauterine exposure masculinizes neural circuits) (Corrieri, Della Seta, Canoine, & Fusani, 2007). Since EE is an endocrine-disrupting chemical, many toxicology studies exist regarding reproductive behavior and physiological changes (Zaccaroni et al., 2016), however, a review of these is not in this dissertation's domain.

2.3 Levonorgestrel

The progestin components of contraceptive hormones are often treated as if all progestins behave in the same manner. Brinton and her colleagues have proven this to be incorrect with significant variance seen between the progestins' neuro-proliferative behavior (Liu et al., 2010). Levonorgestrel (LNG), *in-vitro* significantly increased neural progenitor cells in adult rats' hippocampi and protected the neurons from glutamate-induced toxicity, whereas *in -vivo* it acted in a pro-apoptotic manner (Liu et al., 2010). As a result,

they suggested that LNG failed to constructively effect neural regeneration (Liu et al., 2010).

Some authors feel that LNG and its metabolites function as estrogen receptor alpha (ER α) agonists (García-Becerra et al., 2006), while many have shown they bind primarily to androgen receptors (Africander et al., 2011; Kloosterboer et al., 1988; Lemus et al., 1992; Liu et al., 2010; A Phillips, Demarest, Hahn, Wong, & McGuire, 1990; Audrey Phillips, Hahn, Klimek, & McGuire, 1987; Poulin, Baker, Poirier, & Labrie, 1991; Stanczyk, 2003). LNG is approximately fifty-five percent as potent as testosterone (Philibert et al., 1999). Brain emotive and cognitive changes induced by LNG may not only be related to ligand binding to hormone receptors, but also to alterations in other receptors such as GABA_A (Porcu et al., 2012), mineralocorticoid or glucocorticoid (Sitruk-Ware, 2006), or alterations in neurosteroids (Follesa et al., 2002; Sassoè-Pognetto et al., 2007).

Insofar as its androgenic qualities, findings that LNG enhanced spatial learning in intact or ovariectomized rats at a variety of ages and doses (Braden et al., 2016; Simone et al., 2015) is not surprising. Men outperform women on spatial tests (Andreano & Cahill, 2009; Hampson et al., 2016; Nowak, Diamond, Land, & Moffat, 2014), testosterone positively correlates with mental rotation (Silverman, Kastuk, Choi, & Phillips, 1999), and rats and women perform best in spatial tests in the low estrogen cycle phase (Hampson et al., 2014; S. G. Warren & Juraska, 1997). In humans, use of a more androgenic progestin in the pill resulted in a decrease in verbal fluency yet more correct answers on the mental rotation test (albeit a slower response time) than non-contraceptive users (Griksiene & Ruksenas, 2011). A similar finding was seen in a mental rotation test where users of a pill consisting of androgenic progestins besting less androgenic and

nonandrogenic progestin pill users (Wharton et al., 2008). Brain activation differs in recruitment areas for math mental rotation when contraceptive hormone users are compared to non-users (B. Pletzer, Kronbichler, Nuerk, & Kerschbaum, 2014). For numeral mental manipulation tasks, the androgenicity of the pill seems to make the contraceptive users process differently from the follicular phase and luteal phase non-users, and instead resemble male processing (B. Pletzer et al., 2014). A within-subject comparison of levonorgestrel-containing pill users demonstrated fMRI activational changes occurred in affective brain regions consistent with the subjects deterioration of mood (Gingnell et al., 2012).

2.4 Ethinyl estradiol/Progestin combinations

Research on combination contraceptive hormones (i.e. the pill) is extensive if physiological and emotive changes are the dependent variables. Since mental function correlates with emotional well-being, this review will cover some studies that look at affect and cognitive fluctuations. Whether combination contraceptive hormones (CH) initially alter circuits that control emotion resulting in cognitive modification or whether a change in cognitive perception alters the individual's emotions is difficult to distinguish. For instance, global processing (attention to whole rather than to detail) and global-local interference (inability to override the "gist" and concentrate on the "facts") are enhanced in contraceptive pill users resulting in a bias toward more abstract and less detail thinking (Belinda Pletzer, Petasis, & Cahill, 2014). In contrast, prepulse inhibition (PPI), is defined as the ability to focus on details that are pertinent for cognition and to filter out extraneous sensory information. Women on CH did not differ in PPI from women in the follicular phase when PPI is the greatest and improved delayed memory

occurred (Gogos, 2013). Consistent with this, despite being fed misinformation, the CH users were more successful in the accuracy of memory than non-users, and these authors felt CH altered the gist to ‘verbatim’ memory (Petersen, Patihis, & Nielsen, 2014). What may underlie these findings in emotive learning is the interaction between the hypothalamic-adrenal axis and the hypothalamic-gonadal axis (Kudielka & Kirschbaum, 2005; Shawn E Nielsen, Barber, Chai, Clewett, & Mather, 2015).

Indirectly impacting emotional learning, the use of contraceptive pills blunts stress-induced free cortisol responses (Kirschbaum, Kudielka, Gaab, Schommer, & Hellhammer, 1999). Further evidence of hypothalamic-adrenal axis alterations by CH was a decrease in the general rise of exercise-induced plasma norepinephrine (Otterstetter et al., 1999). Compared to the robust response of women non-users, CH users have less cortisol release from a physical stressor, ice water, and exhibited no post-stress enhancement of recall of the overall/details of an emotional story (Shawn E Nielsen, Ahmed, & Cahill, 2014; S.E. Nielsen, Segal, Worden, Yim, & Cahill, 2012). On the other hand, if cortisol was augmented, women not on CH exhibited a decrease in memory retrieval in cognitive tests while women on CH showed no impairment (Kuhlmann & Wolf, 2005). Perhaps, the women non-users had an adequate cortisol and the additional dose caused loss of memory capacity, while the ‘deficient in cortisol release’ CH users were raised to a ‘normal’ range and thus showed no impact. Then again, a changed response to psychosocial stress in CH users was seen by a decrease in the post-stress recall of negative word pairs accompanied by an increase in anxiety compared to non-users (Kristen L Mordecai et al., 2017). Additionally, rats and women on CH had impaired retention of the extinction of learned fear (recall), compared to the high estrogen

phase of the natural cycle (B. M. Graham & Milad, 2013) and altered neural recruitment for extinction (Merz et al., 2012).

Neuroimaging studies support some of these psycho-behavioral changes seen in CH users. A neuroimaging study, comparing women on CH for three months to naturally cycling women, found a decrease in the volume of the amygdala was positively correlated with a decreased positive affect in the CH users (Lisofsky, Riediger, Gallinat, Lindenberger, & Kühn, 2016). Women on CH versus non-users differ on neuroimaging studies in regard to grey matter volume, with increases seen in the prefrontal and mid-temporal areas, suggesting that there is a lifetime continuum effect of hormones on brain structure (Belinda Pletzer et al., 2010; B. A. Pletzer & Kerschbaum, 2014). Women typically have more overall cortical connectivity (Gong et al., 2009) and interhemispheric connectivity than men (Ingahalikar et al., 2014). Yet, despite similar estradiol levels as follicular phase naturally cycling women, CH users had less executive control network connectivity (left middle frontal gyrus, left anterior cingulate cortex) suggestive of a change in cognitive performance (Petersen, Kilpatrick, et al., 2014) since women's verbal task advantages are correlated with frontal cortices (Hamson et al., 2016). CH users exhibited altered brain area activation (right hemisphere lateralization) during an fMRI verb generation when compared to non-users (Rumberg et al., 2010).

A lengthy reproductive period—earliest menarche and latest pregnancy—were positively associated with better cognitive performance in menopausal women (Karim et al., 2016). Paradoxically, a history of use of contraceptive pills during their reproductive life significantly improved their verbal memory in menopause (Karim et al., 2016) though the dose or type of contraceptives was not assessed. Evaluation of hormonal effects on brain

structure and functionality are difficult at best, given the myriad of interactions along with the diversity of doses and drugs. The conclusion of a systematic review of CH's effect on cognition was that specificity of the CH drug and dosage needs to be incorporated in all future studies to untangle the conflicting results (A. M. Warren et al., 2014). Looking at different cognitive strategies can also lead to variable outcomes in CH's effect. In a within-subject study comparing the active phase versus the inactive phase of CH pills use, women showed an enhancement of verbal memory but not mental rotations, whereas no across-cycle difference arose for either test in non-users (K. L. Mordecai, Rubin, & Maki, 2008). Expectedly, there was no change in 17 β -estradiol, progesterone, or testosterone across the cycle for the CH users. Most plausible is that the addition of the potent EE played a part in the verbal improvement. Conversely, other authors did not see a cross cycle difference in users of CH but did in the non-users. In the high-estrogen phase (mid-cycle), there was an improvement in verbal working memory (Rosenberg & Park, 2002) and calculations in arithmetic tests (Becker, Creutzfeldt, Schwibbe, & Wuttke, 1982), yet no across cycle difference in the CH users. Further studies comparing twice cycle measurements found women on the CH pill showed no difference from non-users in verbal (paragraph recall, auditory verbal learning task, delayed matching to sample task, letter fluency, the immediate post-concussion assessment and cognitive test) nor spatial memory (mental rotation, object array, digit span) (Islam, Sparkes, Roodenrys, & Astheimer, 2008; Mihalik, Ondrak, Guskiewicz, & McMurray, 2009). The diversity of the cognitive testing (task specificity) plays a larger part on the conflicting results in across-cycle comparison as exemplified by a study using male faces vs. infant's faces for working memory in women on or off CH. Regardless if

the cycle was natural or one controlled by CH, women's efficacy of working memory for male faces was best in the high-estrogen phase of their cycle, and no such difference occurred with infant faces (Vranic & Hromatko, 2008).

Visuospatial cognition is commonly better in a low estrogen state in naturally cycling women (Hampson et al., 2014). We might, therefore, expect that CH use would improve visuospatial tasks. Evaluation of reaction time to onset of a visual/auditory stimulus (7% better) and visual short-term memory of number sequences (8.8% better) improved by CH usage while there was no change in short sustained attention (Griksiene & Ruksenas, 2009). Notable, however, was the small subject numbers (10 CH users) as well as the variety of contraceptive hormone drugs and doses involved (7 different types of combination pills). Interestingly, use of CH improved spatial attention also in a different format. A within-subject study showed that compared to the inactive pill phase of CH use, the active phase caused a shift in the typical hemispheric bias (related to the handedness) (Cicinelli et al., 2011). When asked to bisect a line into equal parts, women in the active pill phase demonstrated an improvement in the interhemispheric connection between the two visual fields (Cicinelli et al., 2011). The water-level task, like mental rotation tasks, tests spatial relations and users of CH were found to be significantly better at assessing where the water level would be with the glass tilted at different angles (McFadden, 2000). Evaluation of mid-life aged women found that the longer they had taken the CH in the past, the better they presently did on spatial tests (visuospatial battery of tests and speed flexibility-i.e. Stroop) when compared to never users (K. R. Egan & Gleason, 2012). Even post-menopausal women treated with very low doses of CH (5 μ g of EE/ 1 mg norethindrone), evinced more activation in bilateral prefrontal cortices

during a non-verbal spatial working memory task (Visual Delayed Matching to Sample task) than non-users (Y. R. Smith et al., 2006). Pletzer & Kerschbaum, (2014) felt that CH masculinized the brain and though the aforementioned studies together seem to lean toward this, one should be cautious with this generalized summary.

2.5 Neurotransmitters

The evaluation of CH neuromodulatory mechanisms could span from the actions of the drugs as ligands to the membrane/cytosolic receptors, to the actions of different neurotransmitters triggered by the drugs. Since most major neurotransmitter systems can be associated with learning and memory in some way, this review of literature out of necessity will be limited. Acetylcholine certainly is a relevant neurotransmitter given its broad cholinergic distribution to regions attributed to learning and associated with estrogen receptors (Acosta et al., 2013; M. C. Craig et al., 2009; Croxson, Kyriazis, & Baxter, 2011; Jill M Daniel & Dohanich, 2001; R. Gibbs, 2000; Hasselmo & Sarter, 2011; Packard & Teather, 1997; O Picazo, Espinosa-Raya, Jiménez-Trejo, & Suarez, 2011). Noradrenergic and dopaminergic participation in cognition (Berridge & Arnsten, 2015; Berridge, Arnsten, & Foote, 1993) correlates with the levels of gonadal estrogens in animal models (J. M. Daniel, 2006; Inagaki, Gautreaux, & Luine, 2010; Victoria N Luine, Richards, Wu, & Beck, 1998).

2.6 Cholinergic

In every aspect, cholinergic neurons are linked to cognition (as reviewed by (Hasselmo & Sarter, 2011). The diffuse distribution of cholinergic axons to neocortex and hippocampus in addition to the magnitude of release (Kehr et al., 2001) suggest it surpasses the monoamines in neurocognition (Woolf, 2006). The association of estrogen,

cognition and cholinergic input into forebrain and hippocampus has been demonstrated with spatial and learning tests (M. Craig et al., 2010; Jill M Daniel & Dohanich, 2001; R.B. Gibbs, 1996; R. Hammond, Nelson, Kline, & Gibbs, 2012; V. N. Luine, Renner, Heady, & Jones, 1986; Shughrue & Merchenthaler, 2000; Singh, Meyer, Millard, & Simpkins, 1994). EE binds to a cholinergic nicotinic receptor in rats and humans (Paradiso, Zhang, & Steinbach, 2001); it is a receptor related to memory (Hogg, Raggenbass, & Bertrand, 2003). Nevertheless, rare are studies examining CH and cholinergic pathways of learning. To my knowledge, only one study exists which showed ovariectomized rats given sc EE 0.18 μ g/day decreased cholinergic neuron counts in the basal forebrain yet exhibited no learning and memory differences in a spatial test (Mennenga et al., 2015). The effect of CH treatment, with its loss in ovarian hormones, on cholinergic neurons and their function, is very relevant. Loss of ovarian hormones for a prolonged length of time alters the ability of replacement estrogen to modulate the cholinergic learning system in the hippocampus and prefrontal cortex (Bohacek, Bearl, & Daniel, 2008).

2.7 Monoamines

Catecholamines also disperse axons to a wide range of targets. Dopamine (DA) neurons via the mesocorticolimbic pathway mediate emotive and motivational memory (Le Moal & Simon, 1991). The dorsal noradrenergic pathway to the hippocampus and the central noradrenergic pathway to the prefrontal cortex mediate attention and memory (T. Robbins & Everitt, 1995). The combination of the wide variety of norepinephrine (NE) and DA neuron targets and interconnectivity lead to influential cognitive flexibility (Berridge & Spencer, 2015; Berridge & Waterhouse, 2003; Chamberlain & Robbins,

2013; Chandler, Waterhouse, & Gao, 2014; Tully & Bolshakov, 2010). The exposure to novelty increases NE in the hippocampus and prefrontal cortex (Ihalainen et al., 1999; Jordan, Kramer, Zukas, & Petty, 1994; Kehr et al., 2001). NE is suggested to be essential for the consolidation of learned memory (Kobayashi, 2001).

Older literature has examined how estrogens (Crowley, O'Donohue, Wachslicht, & Jacobowitz, 1978) and CH effect the monoamines. Not well recognized is that synthetic estrogens such as EE are hydroxylated to catechol estrogens and through their reaction with catechol-O-methyltransferase (COMT), interfere with the biological activity of catecholamines (even more than estradiol) (Ball & Knuppen, 1990; Hiemke & Ghraf, 1982). Noteworthy as well, is EE is a competitive NE reuptake inhibitor yet is not transported into the cell (Ghraf et al., 1983). EE given to mice for three weeks markedly increased NE and DA in the forebrain (Greengrass & Tonge, 1974; Tonge & Greengrass, 1971). Equally as important, rats treated with two months of CH showed significantly decreased monoamine oxidase activity in the brain (Marchi & Cugurra, 1974).

Newer literature has examined gonadal hormones and monoamine interactions with learning extensively (Best, Rees, Barlow, & Cowen, 1992a, 1992b; Bowman, Micik, Gautreaux, Fernandez, & Luine, 2009; Etgen, Ungar, & Petitti, 1992; Inagaki et al., 2010; Jacome et al., 2010; Macbeth, Scharfman, MacLusky, Gautreaux, & Luine, 2008).

Nevertheless, consistent with other transmitters virtually no studies have looked at CH, learning, and monoamines. Regarding anti-depressant-like actions, EE was comparable to desipramine (increase NE) and fluoxetine (increase serotonin) in the forced swim test (E. Estrada-Camarena, Fernandez-Guasti, & Lopez-Rubalcava, 2003; Vega-Rivera Nelly, López-Rubalcava, & Estrada-Camarena, 2013). Noradrenergic neurobiology was the

suggested source in studies of CH users and emotive learning after sympathetic stimulating stresses (Shawn E Nielsen et al., 2015; S.E. Nielsen et al., 2012). Salivary biomarkers for norepinephrine were significantly more depressed in the CH user than the non-users (S.E. Nielsen et al., 2012).

2.8 Summary

CH despite usage by millions of women worldwide has been accepted on ‘face value’ in regard to safety and longtime effects on cognition. The thought being, if there was a significant impact on cognition, evidence would exist by now. Women who stop or switch the type of CH taken confound the issue since they are typically not selected to be evaluated individually as a group or compared amongst consistent users for cognitive adverse reactions. Differences in culture and ethnicity also blur the outcomes of studies (Vitzthum & Ringheim, 2005). Pharmacoepidemiological computational analysis of the FDA Adverse Event Reporting System found greater negative central nervous system symptoms (i.e. disturbances in consciousness) in a younger aged woman exposed to regimes that contain just LNG (S. Mizutani, Y. Noro, M. Kotera, & S. Goto, 2014). An analysis of greater than a million women from the National Prescription Register and the Psychiatric Central Research Register in Denmark is the kind of study needed to assess central nervous system side effects. They found a significantly higher risk of being on CH and anti-depressants in younger age women (as high as 2.2 RR) when compared to women never users (Skovlund, Mørch, Kessing, & Lidegaard, 2016), importantly this study captured women who had stopped using CH. The following studies in this dissertation will contribute to the much-needed literature delving out dose/drug response correlations with alterations in learning/memory and anxiety-like behavior. Animal

studies will allow us to control for the variables such as mixed drug types and doses seen in human research, as well as user bias. Animal studies benefit from maintenance of a ‘universal’ life experience, and our studies will direct the motivation to learn as a natural instinct with minimal fear.

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CHAPTER 3

Ethinyl estradiol and levonorgestrel alter cognition and anxiety in rats concurrent with a decrease in tyrosine hydroxylase expression in the locus coeruleus and brain-derived neurotrophic factor expression in the hippocampus

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3.1 Abstract

In the United States, more than ten million women use contraceptive hormones. Ethinyl estradiol and levonorgestrel have been mainstay contraceptive hormones for the last four decades. Surprisingly, there is scant information regarding their action on the central nervous system and behavior. Intact female rats received three weeks of subcutaneous ethinyl estradiol (10 or 30 $\mu\text{g}/\text{rat}/\text{day}$), levonorgestrel (20 or 60 $\mu\text{g}/\text{rat}/\text{day}$), a combination of both (10/20 $\mu\text{g}/\text{rat}/\text{day}$ and 30/60 $\mu\text{g}/\text{rat}/\text{day}$), or vehicle. Subsequently, the rats were tested in three versions of the novel object recognition test to assess learning and memory, and a battery of tests for anxiety-like behavior. Serum estradiol and ovarian weights were measured. All treatment groups exhibited low endogenous 17β -estradiol levels at the time of testing. Dose-dependent effects of drug treatment manifested in both cognitive and anxiety tests. All low dose drugs decreased anxiety-like behavior and impaired performance on novel object recognition. In contrast, the high dose ethinyl estradiol increased anxiety-like behavior and improved performance in cognitive testing. In the cell molecular analyses, low doses of all drugs induced a decrease in tyrosine hydroxylase mRNA and protein in the locus coeruleus. At the same time, low doses of ethinyl estradiol and ethinyl estradiol/levonorgestrel increased galanin protein in this structure. Consistent with the findings above, the low dose treatments of ethinyl estradiol and combination ethinyl estradiol/levonorgestrel reduced brain-derived neurotrophic factor mRNA in the hippocampus. These effects of ethinyl estradiol 10 μg alone and in

combination with levonorgestrel 20 μ g suggest a diminution of norepinephrine input into the hippocampus resulting in a decline in learning and memory.

Keywords: Learning/memory; anxiety; contraceptive hormones; tyrosine hydroxylase; brain-derived neurotrophic factor (BDNF); novel object recognition tests

3.2 Introduction

Over 46 million women have taken contraceptive hormones in the United States since their inception in 1964. Nevertheless, 30% of contraceptive pill users discontinue use, with side effects being the primary reason for dissatisfaction (Daniels & Mosher, 2013). Rarely addressed in the literature is the side effect of perceived changes in cognition. A recent review of the literature addressing cognitive impacts of contraceptive hormones found little more than 1000 studies since 1960, and of these, only 22 were judged to be methodologically robust (A. M. Warren et al., 2014). Considering the magnitude of contraceptive hormone use, the paucity of data compels further research evaluating their impacts on the brain and behavior.

Contraceptive hormones interfere with the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus (Lobo & Stanczyk, 1994), thus indirectly reducing 17β -estradiol levels (Vandever et al., 2008) via a disruption of the hypothalamic-pituitary-gonadal (HPG) axis (Gaspard et al., 1984). To illustrate, the combination of 30 μ g ethinyl estradiol (EE) and 150 μ g levonorgestrel (LNG) suppresses 17β -estradiol levels below 30 pg/ml by the third cycle (Vandever et al., 2008). Since 17β -estradiol is neuroprotective and modulates neuronal plasticity by acting through numerous cellular mechanisms within the central nervous system (Frick, 2015; McEwen, 2002), this level of suppression may be deleterious to neural function (B. M. Graham & Milad, 2013), barring a compensatory mechanism. A recent review summates the effects of gonadal steroids,

most specifically 17 β -estradiol, on cognition with the preponderance of studies showing improvements in learning and memory when adequate estradiol levels exist (V. N. Luine, 2014). A compensatory mechanism via EE may occur since EE (1, 10, or 100 μ g dose) has been shown to exhibit neuroprotective qualities by preventing kainic acid-induced neuronal loss in the hippocampus of young ovariectomized rats (O. Picazo et al., 2010).

The levels of endogenous ovarian hormones are associated with fluctuations in cognition (Rosenberg & Park, 2002) and frequently demonstrate a biphasic (low vs. high hormone level) outcome (Hampson & Morley, 2013; Hatta & Nagaya, 2009; A. A. Walf et al., 2006). For instance, spatial working memory including delayed recall, as well as tests of verbal learning and retention, tended to be more accurate during the high-estrogen phase compared to the low-estrogen phase of naturally cycling women (Hampson & Morley, 2013; S. M. Phillips & Sherwin, 1992). An fMRI study revealed attenuated activation of the neural circuitry underlying visual working memory performance in young women with pharmacologically suppressed ovarian function, compared to normally cycling women (M. Craig et al., 2008). Neuroimaging data also show that contraceptive hormones affect brain activity in emotional and cognitive circuits (Toffoletto, Lanzenberger, Gingnell, Sundström-Poromaa, & Comasco, 2014); however, across studies specific findings are not always consistent. For example, Griksiene and Ruksenas (2011) showed that verbal fluency was impaired in women on contraceptives compared to naturally cycling women. In contrast, Mordecai and colleagues (2008) did not find a difference in verbal fluency compared to cycling women. However they did show, using a within group comparison, enhanced verbal memory when women were on the

contraceptive hormones versus their hormone-free day (low 17β -estradiol state both times).

In animal behavioral studies, biphasic results are seen on cognitive tests, with synthetic hormone analogs not always demonstrating equivalence to endogenous hormones. For instance, young ovariectomized rats tested in cognitive maze tasks had an adverse outcome seen with higher doses of EE(0.3 μ g/day), yet not with lower doses (0.125 μ g/day) (Mennenga et al., 2015). Young ovariectomized rats improved in spatial learning with replacement of 17β -estradiol, whereas replacement with a synthetic selective estrogen receptor modulator showed no improvement over the control (Robert B Gibbs, Gabor, Cox, & Johnson, 2004). A reason for these dose-dependent outcomes may be attributed to the action of these drugs at receptors.

Animal experiments looking at receptor binding or gene expression show us that estrogen analogues can act as agonists or antagonists in different tissue types despite the relative binding affinity to estrogen receptors (ER) and may be dose-dependent (Ogiue-Ikeda et al., 2008). Receptors have species-dependent binding affinities. Nonetheless, rats and humans share comparable selectivity for the ER, and in both humans and rats, EE was found to have a fourfold ER alpha ($ER\alpha$) selectivity over ER beta ($ER\beta$) (Heather A Harris, Bapat, Gonder, & Frail, 2002). EE has a greater affinity for the ER than 17β -estradiol (190% to 100% relative binding affinity respectively) (Blair et al., 2000). Therefore, it is not surprising that in vitro, EE induces higher upregulation of $ER\alpha$ and $ER\beta$ gene expression compared to endogenous estrogens. Likewise, LNG stimulates ER α and β gene expression in a dose-dependent manner (Jayaraman & Pike, 2014; Rabe,

Bohlmann, Rehberger-Schneider, & Prifti, 2000). LNG has about five times the relative binding affinity of progesterone (5.41:1) for the progesterone receptor (PR) (A Phillips et al., 1990) while its metabolites diminish in PR affinity and increase in androgen receptor affinity (Lemus et al., 1992). Nevertheless, the binding affinity of hormones to their respective receptors alone does not predict neurocognitive effects (Nilsen, Deng, & Brinton, 2005; Pluchino et al., 2006). Contraceptive hormones may have actions independent of classic and membrane ER/PR, such as additional actions involving glucocorticoid, androgen, and mineralocorticoid receptors which may be cell specific (Africander et al., 2011), resulting in a non-specific pattern of hormone modulation of brain areas. These differences in receptor activation contribute to the reason that, in contrast to endogenous estrogen having substantially more positive reports of its effects on the central nervous system, contraceptive hormones have contradictory reports (Beltz et al., 2015).

The mixed results of studies examining cognitive effects of contraceptive hormones may be related to the balance between estrogen loss as a result of the suppression of ovarian function by these GnRH modulators and the dose of compensatory hormones replaced. McEwen 2002, summates that loss of estrogens via GnRH altered ovarian suppression or via surgical and natural menopause, leads to decreases in declarative memory generally reversible by replacement. The ovarian suppression induced by contraceptive hormones in young subjects is in the range of 17β -estradiol $\leq 30\text{pg/ml}$, and progesterone $<1\text{ng/ml}$ (Gaspard et al., 1984; Spona et al., 1996; Willis et al., 2006). The dose of estrogens and progestins needed to compensate is relevant since, poorer cognitive scores or dementia correspond with an increased passage of time since oophorectomy induced hormone loss

(Rocca et al., 2007). Menopausal-like levels of endogenous estradiol are associated with impaired memory as the time of deprivation increases (Brinton, 2009). Young rats in the early follicular (low) range of hormones do not perform as well on object recognition tests as behavioral estrus rats (high hormone levels) and young ovariectomized rats improved in learning with replenishment of gonadal hormones (A. A. Walf et al., 2006). In addition, reduced circulating and cerebral progesterone and allopregnanolone, caused by chronic contraceptive hormones, increased anxiety-like behavior in rats (Follesa et al., 2002; Porcu et al., 2012), while, the replacement of progesterone diminished deficits in memory seen in ovariectomized young rats (Frye et al., 2007). The present experiment expands on these previous findings by decreasing endogenous serum 17β -estradiol in intact female rats through the chronic administration of EE and LNG and assessing dose-related subsequent effects on cognition and anxiety-related behavior.

The studies also aimed to investigate the neurobiological changes associated with contraceptive hormones that may mediate alterations in mood and cognition. The locus coeruleus (LC), a steroid-sensitive nucleus of noradrenergic neurons, releases norepinephrine throughout the forebrain via the dorsal noradrenergic pathway. Norepinephrine facilitates long-term potentiation and mediates long and short term memory through multiple cellular mechanisms (Walling & Harley, 2004). Tyrosine hydroxylase, the rate-limiting enzyme for the production of norepinephrine, and the neuropeptide galanin coexist in virtually all neurons in the LC (Holets et al., 1988). Galanin inhibits neuronal activity in the LC via somatodendritic autoreceptors, thus modulating norepinephrine release and synthesis (Pieribone et al., 1995). 17β -estradiol increases galanin gene expression in the LC (Tseng et al., 1997) via its estrogen response

element on the promoter region of the gene (Howard, Peng, & Hyde, 1997). Tyrosine hydroxylase (TH) expression is similarly regulated by estrogen (Maharjan et al., 2005). In addition to their involvement in learning and memory, both neurotransmitters impact anxiety-related behaviors (Sciolino et al., 2015).

Noradrenergic modulation of the hippocampus via projections from the LC is critical for learning and memory processes. Increases in noradrenergic tone enhance the expression of brain-derived neurotrophic factor (BDNF)(Van Hoomissen et al., 2004), which may mediate cognitive functions in the hippocampus through its effects on synaptic remodeling (Mizuno, Yamada, Olariu, Nawa, & Nabeshima, 2000). Like TH and galanin, BDNF is also regulated by 17β -estradiol via an estrogen response element (Sohrabji et al., 1995). BDNF is prevalent in the hippocampus where there is a high expression of $ER\alpha/\beta$ (Helen E Scharfman & MacLusky, 2014). BDNF expression in the hippocampus fluctuates with estrus cycling in rats, resulting in the potentiation of synaptic activity at peak estradiol levels (H. E. Scharfman, Mercurio, Goodman, Wilson, & MacLusky, 2003). In women, serum BDNF levels vary over the menstrual cycle with the highest level during the luteal phase. In contrast, no luteal phase augmentation occurs in women taking contraceptive hormones (N Pluchino et al., 2009).

Previous studies demonstrate $10\mu\text{g}/\text{rat}/\text{day}$ EE and $20\mu\text{g}/\text{rat}/\text{day}$ LNG are the lowest individual doses found to inhibit ovulation in intact rodents (P. Andrews et al., 2002; Bennink, Skouby, Bouchard, & Holinka, 2008; Kumar, Koide, Tsong, & Sundaram, 2000; Muhn, Krattenmacher, Beier, Elger, & Schillinger, 1995; Schindler et al., 2003). Contraceptive steroid doses for ovulation inhibition treatment are higher than expected when compared to body surface area equivalent dose because of the more extensive

enterohepatic circulation and more rapid elimination of drugs in rat (Emudianughe, Caldwell, Sinclair, & Smith, 1983). Based on the hypothesis that both low and high doses of EE and LNG would reduce endogenous 17β -estradiol in intact female rats, we predicted that their effects on cognitive functions and anxiety-related behavior would be dose-dependent (opposing effects at low and high dosages)(V. Calabrese et al., 2012). As dose-dependent biphasic functions of estrogen receptors have been previously reported (E. J. Calabrese & Baldwin, 2003; C. Frye et al., 2012). Sprague-Dawley female rats received EE and LNG by daily injections for three weeks and subsequently tested for anxiety-like behavior in study 1 and learning and memory in study 2. *In situ* hybridization for mRNA for TH and galanin in the LC and BDNF in the hippocampus were examined in both studies. In study 1, Elisa testing was performed for galanin protein. In study 2, Elisa testing was completed for BDNF and TH protein.

3.3 Materials and Methods

3.31 Subjects and drug treatments

Approval from the University of Georgia Animal Care and Use Committee was obtained prior to the experiment. Female virgin Sprague-Dawley rats (n=136) 175 – 200 grams, 60-90 days (Harlan Inc. Indianapolis, IN) were used. This age was specifically selected because the continuous increase in LC neurons stops in females after postnatal day 60 (Pinos, Collado, Rodriguez-Zafra, et al., 2001). The rats were acclimated to the vivarium for one week prior to experimentation and housed 2-5/cage with a 12-hour reversed light/dark cycle. The rats had access to chow and water ad-libitum. In order to avoid exogenous estrogenic activity other than that of the specified investigational regimen, animals were fed a minimal phytoestrogen diet (Harlan Inc. 2016 Teklad Global). The

rats were not tested for cyclicity by vaginal smears during the experiments to avoid stimulus-induced activation of the LC neurons and a change in noradrenergic tone (Poletini et al., 2012).

Rats were randomly assigned to one of the following drug conditions: vehicle, EE 10 µg, EE 30 µg, LNG 20 µg, LNG 60 µg, EE + LNG 10/20 µg, or EE + LNG 30/60 µg. Drugs were suspended in 5% ethanol in organic sesame oil and given daily by subcutaneous injections (0.2ml) for 21 days during the first few hours of the dark cycle. The same individuals handled rats for injections as testing, and experimenters were naïve to drug conditions.

Behavioral tests occurred between 0900h and 1700h, within 24 hours of the last injection.

Upon completion of testing, rats were rapidly decapitated by the guillotine while under deep carbon dioxide sedation. The brains were rapidly harvested and hemi-dissected.

One-half was flash frozen in dry ice, then stored in -80°C freezer. Dissected from the other half, were the medial preoptic area, the total hippocampus and the rostral pons containing the LC. These samples were flash frozen and stored at -80°C. Collected trunk blood remained on ice until centrifugation at 2500 rpm for 30 minutes at 4°C (Beckman Model T J-6). Aliquots of serum were stored at -20°C till assayed. The gross appearance of the uterus was recorded. The ovaries were removed and frozen. For study 1, the oviducts were dissected from the ovaries, sandwiched between two slides and evaluated under a dissecting microscope for oocytes. Since no oocytes were seen, this was not repeated in study 2. For study 1, immediate post-mortem vaginal smears were taken to estimate cycle day (Marcondes, Bianchi, & Tanno, 2002). Since there was a 95%

agreement between the serum estradiol level and the vaginal smear obtained at the same time, this was not repeated for Study 2.

3.32 Behavior tests

In Study 1, rats were tested for anxiety-like behavior on the elevated plus maze (EPM) and shock probe defensive burying (SPDB) tests. The order for the tests was EPM followed by SPDB after an hour rest with food and water. The start date for the 21-day drug administration had been staggered so that four to six animals were tested per day. The animals were sacrificed one hour after SPDB testing ended. A total of 136 rats were used for these experiments (see Table 1). Total number of rats for study 1 was 68, (vehicle control n=20, EE10 μ g n=8, EE30 μ g n=8, LNG 20 μ g n=8, LNG 60 μ g n=8, EE/LNG 10/20 μ g n=8, EE/LNG 30/60 μ g n=8). In Study 2, rats were tested for cognitive function in object recognition trials—novel object recognition (NOR), novel object placement recognition (NOP), and novel object context recognition (NOC). The total number of rats for study 2 was 68, and they were randomly assigned into the groups corresponding to the aforementioned anxiety study. The order of the cognitive tests were NOR, followed by NOP and lastly NOC since test order had been previously shown to be insignificant (Hui-Yin, Ahmad, Azmi, & Makmor-Bakry, 2014). Since only six rats were tested per day, each rat had an hour rest before being tested in the next object recognition test. Spontaneous locomotion was examined in an open field test one week preceding object recognition trials. Investigation of object preference bias for stimulus objects was assessed for each rat and was performed on day 10, allowing for drug(s) steady-state. For all trials, lighting was 25-30 lux on the apparatus' floor with background white noise of 70 decibels unless otherwise stated. Video recording of behavior occurred by a direct

overhead webcam (Microsoft or Logitech) and a single trained investigator naïve to condition scored all videos. For inter-rater reliability, an additional naïve investigator remained in the test room behind an opaque partition and scored behavior in real time for all experiments. Rats were habituated to the testing areas for 30 minutes prior to testing. Each rat was tested once per behavioral test. The order of rats was randomized to prevent order effects. Between every trial, all equipment and boxes were cleaned with Vimoba (Quip Laboratory) and allowed to air dry.

3.32a Behavioral tests of anxiety: elevated plus-maze (EPM) and shock-probe defensive burying (SPDB)

EPM: The maze utilized was a wooden structure painted black matte. The apparatus included two open arms (45 x 9 cm) and two closed arms (45 x 9 x 38 cm) extending from a central platform (9 x 9 cm). It was elevated 50 centimeters (cm) above the floor. Following the placement of the rats on the center platform facing an open arm opposite the experimenter, the number of entries and time spent in each arm were recorded. An arm entry was defined as the presence of all four feet in the arm.

Shock-probe defensive burying (SPDB): Each rat was placed in a covered clear polycarbonate cage (20 x 40 x 20 cm) that contained a shock prod (extending 6 cm into the cage; 2 cm above 5 cm of wood shavings). The rats were delivered a single mild shock of 2 mA DC (E13-08, Coulbourn Instruments, Allentown, PA) upon initial contact with the prod. During the 15-minute test, parameters evaluated were latency to prod, latency to return to the prod, exploratory prod contact time, and time spent freezing and burying.

3.32b Behavioral tests of learning and memory: object recognition

The test chambers (60 x 40 x 33 cm, Sterilite) containing Sani-chip bedding had opaque walls with a clear vented acrylic lid. The objects used for the recognition tests varied in size, shape and material (metal, glass, plastic or ceramic). The maximum size was 15.5 cm high and 7 cm wide. All objects were glued to the bottom of a small jar that could be fixed in place by screwing the jar to a lid in a static position, thereby preventing displacement of the object. For the familiarization trial, the particular object/location of the object was randomized, and it was never repeated for a given rat. Exploration time was scored when the rat whisked/sniffed within 2 cm from the edge or touched the object. Neither climbing on/over nor rearing on the object was counted.

The novel object recognition tests were a modified version of Ennaceur and Delacour, (1988.) The novel object context and object placement tests were formatted similarly to Dix and Aggleton, (1999). The format of all tests included the following phases: 1) A familiarization phase (T1) – in which the rat was allowed to explore two identical objects for five minutes and exploration time was scored for each object. 2) A retention phase - immediately following familiarization in which the rat was returned to its home cage with food and water for 45 minutes. 3) A test phase (T2) –in which the rat is returned to the test chamber. During this phase, a novel object or the location of a T1 object had been changed while a duplicate T1 object is in the same location as the familiarization phase. Exploration time was scored for individual objects for 3 minutes.

For the novel object recognition (NOR) test, the objects' position remained the same for both familiarization (T1) and testing phase (T2). Placement of the objects was counterbalanced across treatment groups. For the novel place recognition (NOP) test, the novelty involved moving one of the duplicate T1 objects to another quadrant of the test

chamber for T2. The novel context recognition (NOC) test employed two T1 phases, each conducted in separate rooms: the room used for NOR and NOP, which was illuminated with 30 lux and had plain white walls, and an additional room illuminated with 15 lux with a variety of vibrant colored black-light posters on the walls. For the first T1 phase, the rat was placed in a box with two identical objects for five minutes in either of the rooms. For the second T1 phase, the rat was immediately brought to the other room and familiarized an additional five minutes with two identical objects different from the previous room. The retention phase was five minutes for this test. For T2, the rat was placed in one of the two rooms within a test chamber containing an object from each room. The novel object was the object not previously seen in that specific room (out of context). The room choice was counterbalanced among the rats.

3.33 Cell Molecular studies

In study 1, Elisa for galanin protein and *in situ* hybridization for TH, BDNF, and galanin mRNA were performed. In study 2, Elisa for TH and BDNF protein were performed as well as *in situ* hybridization for TH, BDNF, and galanin mRNA.

3.33a ELISA

Protein extraction buffer used contained 100 mM Tris/HCl, (pH 7- 7.2), 1 M NaCl, 2% Triton X-100, 2% bovine serum albumin, 4 mM EDTA.Na², 0.1% sodium azide with 250ul of Cocktail Protease Inhibitors (1:200 ratio; Millipore). Tissue was homogenized (PowerGen 125, Fischer Scientific) in 500ul of cold Protein Extraction Buffer (1:8 Wt./vol.). The homogenates were centrifuged for 45 minutes at approximately 1500g at 4°C. The supernatant was collected for testing.

ELISA kits for estradiol (Biovendor or Calbiotech), tyrosine hydroxylase (My BioSource), galanin (Peninsula), and BDNF (ChemiKine, Millipore) were performed according to manufacturer's recommendations. Absorbance was read immediately on a Vmax Kinetic microplate reader (Molecular Devices, Menlo Park, CA.). SoftPro Max 2.41 generated the curve fitting algorithm.

3.33b *In situ* Hybridization

In situ hybridization was performed as previously described (Sciolino et al., 2015).

Oligonucleotide probes (Oligos Etc.) for galanin

(5'-GAAGGTAGCCAGCGCTGTTCAGGGTCCAGCCTCTCTTCTCCTTT-3'),

tyrosine hydroxylase

(5'-CGTGGGCCAGGGTGTGCAGCTCATCCTGGACCCCCTCCAAGGAGCGCT-3') and BDNF

(5'-AGTTCCAGTGCCTTTTGTCTATGCCCCTGCAGCCTTCCTTGGTGTAACCC-

3') were radiolabeled at the 3' end with ³⁵S-dATP (1 mCi; Perkin Elmer, Boston, MA)

using tailing buffer, CoCl₂, and terminal deoxynucleotransferase (Roche, Indianapolis,

IN). Unincorporated radionucleotide was removed using column separation (Micro Bio-

Spin P30 in Tris, Bio-Rad, Hercules, CA) and bound radionucleotide was stabilized using

1M dithiothreitol. The hybridization buffer containing the radiolabeled probe included

25% formamide, 72mMNaCl, 3.2mM Tris-HCl, 0.0032mM EDTA, 0.001% sodium

pyrophosphate, 0.004% sodium dodecyl sulfate, 0.002 mg/mL heparin sulfate, and 2%

dextran sulfate. Each section was then covered with 35µl of the probe mixture and

coverslipped with parafilm, then incubated for 24 hours at 37°C. Following

hybridization, slides underwent a series of washes in 1% SSC and 2% SSC-formamide

(50:50) at 40°C and room temperature. Slides were air-dried, and opposed to ³⁵S-sensitive film (Kodak BioMax MR, Rochester, NY). Densitometry was performed using NIH Image (Bethesda, MD, <http://rsb.info.nih.gov/nih-image/>) by tracing regions of interest. Mean grayscale values were obtained from 2-4 sections per subject.

Densitometry was performed by an investigator blind to subject condition. Average optical density overlying the white matter of the hippocampus and the center of the pons, where no labeled mRNA was detected, was used to estimate the background.

3.4 Statistical analyses

All scores were converted to percent of metestrus-diestrus vehicle control to account for variability across multiple experiments and data were analyzed by one-way ANOVAs followed by two-tailed planned *t*-tests comparing metestrus-diestrus vehicle control to each drug. Effect size for ANOVAs were described by partial eta squared (R^2). Cohen *d* described effect size for unpaired *t*-tests with the general guidelines that effect size is considered small (if Cohen *d* = 0.2), medium (*d*=0.5; clinically significant) and large (*d*=0.8)(Cohen, 1977). When the comparison (*t*-test) was significant, the percentage of nonoverlap between the two distributions (U_1) or the percent of the treatment group above/below the mean of the control group (U_3)(Cohen, 1977), was used for further description of the effect size significance. The pre-selected critical value for statistical significance was $p \leq 0.05$. Data are presented as means \pm SEM. For both studies, the exclusion criteria for removal of an outlier was a false discovery rate (FDR) of 1% in behavioral data and 2% FDR in cell molecular data since the variance is smaller (GraphPad Prism 6.05, 2014). Therefore, no more than 1% (2%) of the identified outliers would be false, such as they might occur in just the tail of the distribution. In other words,

at least 99% (98%) identified outliers would belong to another distribution. All other exclusions are described in the result section of the individual test. The total number of rats was decreased to 126 since ten rats were excluded due to high serum estradiol levels and vaginal smears that were proestrus.

Time spent on the novel object (location) against time spent on the previously seen object (location) during the first two minutes of the test was assessed in the vehicle groups by paired t-tests. If subjects spent significantly more time exploring the new object (location), they were considered to have remembered the familiar T1 object (learning). To further assess learning and memory we used a Preference Index (PI) of greater than 50% as evidence of learning. PI was defined as the ratio of novel object exploration time to total object exploration time (previously seen object plus novel object).

3.5 Results

3.51 Ovarian Function

The majority of the subjects in the vehicle groups' were determined to be in the metestrus, or early diestrus (met-di) based on serum 17β -estradiol ($\mu \leq 10.0$ pg/ml)(Nequin, Alvarez, & Schwartz, 1979; A. A. Walf et al., 2006). This majority is the expected distribution by chance (60-65 hours/96 hours of a cycle is met-di (Long & Evans, 1922)) and by synchronous cycling effect of group housing (McClintock, 1981). Precluded from the control group were the rats with an estradiol level in the proestrus range and post-mortem vaginal smears that were read as proestrus, which is the expected dispersal across a four-day cycle (ten out of the forty vehicle-treated rats). Thus, all control subjects in these experiments represent the natural low phase of endogenous 17β -

estradiol, and all data are presented as a percentage of the met-di control. In the evaluation of serum 17β -estradiol levels, seven rats were removed as outliers and these were dispersed across the drug groups whereas five, all in the low dose drug groups, were not able to be analyzed by Elisa due to hemolysis. The 17β -estradiol level (Figure 3.1A) in the lower dose groups align with the 17β -estradiol range of the met-di percent of control ($F_{(6, 105)} = 8.218, p < 0.0001, R^2 = 0.3195$; EE: $t_{(42)} = 1.150, p = 0.2567$ Cohen $d = 0.38$; LNG: $t_{(40)} = 1.787, p = 0.08$; Cohen $d = 0.63$; EE/LNG: $t_{(40)} = 0.2792, p = 0.78$, Cohen $d = 0.10$). Lowering the estradiol level to the early follicular range is typical of contraceptive hormone actions. Note: in study 1, the mean estradiol levels of all the low dose drug groups', but not the met-di vehicle group, were statistically less when compared to the *proestrus* percent of control ($F_{(7, 58)} = 6.053, p < 0.0001, R^2 = 0.4221$; EE: $t_{(16)} = 3.136, p < 0.01$, Cohen $d = -1.58$; LNG: $t_{(16)} = 3.425, p < 0.01$, Cohen $d = -1.72$; EE/LNG: $t_{(16)} = 3.394, p < 0.01$, Cohen $d = -1.71$; met-di vehicle: $t_{(18)} = 1.714, p = 0.1037$). The higher dose groups all exhibited statistically lower 17β -estradiol compared to met-di percent of control ($F_{(6, 105)} = 8.218, p < 0.0001, R^2 = 0.3195$; EE: $t_{(43)} = 3.568, p < 0.001$, Cohen $d = -1.15, U_1 = 60\%$; LNG: $t_{(44)} = 2.103, p < 0.05$, Cohen $d = -0.67, U_1 = 41\%$; EE/LNG: $t_{(41)} = 3.859, p < 0.001$, Cohen $d = -1.31, U_1 = 65\%$).

In study 1, the drug-treated rats post-mortem vaginal smears were exclusively met-di except for the EE groups where three were read as estrus and one as proestrus. These readings were inconsistent with the low estradiol levels in these rats and could be due to a local estrogenic effect of the drug. Andrews et al., 2002, found these similar vaginal smear findings at a dose as low as $\approx 2\mu\text{g}/\text{rat}/\text{day}$ of EE and assessed these changes to be a drug effect. In study 1, the oviducts had been examined in all rats, and no oocytes were

seen. In both studies, ovaries were weighed (Figure 3.1B) and found to be statistically less in the high dose LNG ($F_{(6,119)} = 3.382$, $p < 0.01$, $R^2 = 0.1457$; $t_{(44)} = 3.043$, $p < 0.01$, Cohen $d = -0.96$, $U_1 = 53\%$). The mean ovary weights were met-di vehicle = 0.032 grams (g), EE 10 μ g = 0.033g, EE 30 μ g = 0.040g, LNG 20 μ g = 0.031g, LNG 60 μ g = 0.025g, EE/LNG 10/20 μ g = 0.032g, EE/LNG 30/60 μ g = 0.032g. In total, our results together with previous papers supporting that these drug regimens were anti-ovulatory doses, suggests the rats to be in a low endogenous estradiol state associated with suppression of the HPG axis. Drug-induced uterine ballooning (EE 10 μ g $n=5$, EE 30 μ g, $n=2$) and ovarian enlargement (only in the EE 30 μ g group, $n=16$) were noted in the EE groups. These uterine findings are consistent with changes seen with even a single low dose of estrogen in rats (Hart, 1990) yet exhibit variability across studies (Andrews et al., 2002).

3.52 Behavioral tests of anxiety

In the EPM, no drug-treated rats were excluded. However, one of the ten control rats was excluded as an outlier. In the SPDB, no control rats were found to be an outlier.

However, several drug-treated rats' behaviors fell in the 1% outlier range.

3.52a EPM

Low dose EE, LNG, and the low dose combination EE/LNG decreased anxiety-like behaviors in the EPM, with significantly more entries ($F_{(6,50)} = 8.599$, $p < 0.0001$, $R^2 = 0.5078$; EE: $t_{(15)} = 3.061$, $p < 0.01$, Cohen $d = 1.58$, $U_1 = 71\%$; LNG: $t_{(15)} = 3.921$, $p = 0.001$, Cohen $d = 1.11$, $U_1 = 59\%$; EE/LNG: $t_{(15)} = 3.284$, $p = 0.005$, Cohen $d = 1.70$, $U_1 = 75\%$) and time spent ($F_{(6,50)} = 8.231$, $p < 0.0001$, $R^2 = 0.4969$; EE: $t_{(15)} = 3.814$, $p = 0.001$, Cohen $d = 3.78$, $U_1 = 96\%$; LNG: $t_{(15)} = 3.374$, $p < 0.01$, Cohen $d = 1.75$, $U_1 = 75\%$; EE/LNG: $t_{(15)}$

=3.970, $p = 0.001$, Cohen $d=2.05$, $U_1=81\%$) in the open arms (figure 3.2 A & B) compared to the vehicle.

3.52b SPDB

The low EE groups exhibited anxiolytic behavior, spending significantly less time burying ($F_{(6, 42)} = 2.469$, $p < 0.05$, $R^2 = 0.2607$; EE: $t_{(14)} = 2.454$, $p < 0.05$, Cohen $d = -1.35$, $U_1 = 66\%$; EE/LNG: $t_{(14)} = 2.444$, $p < 0.05$, Cohen $d = -1.35$, $U_1 = 66\%$) (Figure 3.2C).

Instead, the low EE groups spent significantly more time in contact with the prod ($F_{(6, 49)} = 3.142$, $p = 0.01$, $R^2 = 0.2778$; EE: $t_{(16)} = 2.741$, $p = 0.01$, Cohen $d = 1.38$, $U_1 = 67\%$; EE/LNG: $t_{(16)} = 2.446$, $p < 0.05$, Cohen $d = 1.23$, $U_1 = 62\%$) (Figure 3.2D). Conversely, the higher EE only group exhibited more anxious behavior with significantly more freezing ($F_{(6, 45)} = 4.628$, $p = 0.001$, $R^2 = 0.3816$; EE: $t_{(14)} = 2.276$, $p < 0.05$, Cohen $d = 1.26$, $U_1 = 63\%$) (Figure 3.2E).

3.53 Behavioral tests for object bias and locomotion

No significant differences in object bias or exploration during the familiarization phases were observed. In the open field test, there were no exclusions and there was no significant difference amongst the groups in time spent in the peripheral or central boxes (perimeter time: $F_{(6, 61)} = 1.171$, $p = 0.3337$, $R^2 = 0.1033$; central time: $F_{(6, 61)} = 0.8951$, $p = 0.5044$, $R^2 = 0.0809$). The EE groups' total number of boxes entered was significantly less than the vehicle ($F_{(6, 61)} = 3.888$, $p < 0.01$, $R^2 = 0.2766$; EE low: $t_{(26)} = 2.550$, $p < 0.05$, Cohen $d = -1.11$, $PS = 78\%$, $U_1 = 59\%$; EE high: $t_{(26)} = 2.602$, $p = 0.01$, Cohen $d = -1.13$, $PS = 78\%$, $U_1 = 59\%$; EE/LNG low: $t_{(26)} = 2.735$, $p = 0.01$, Cohen $d = -1.19$, $PS = 79\%$, $U_1 = 61\%$). Their gait was observed to be slow, possibly as a result of uterotrophic effects. The reduction of locomotor activity in the open field with EE groups was not a

confounding factor as we did not observe a decrease in locomotion in the OR tests (NOR: $F_{(6, 57)} = 1.568$, $p = 0.1732$, $R^2 = 0.1417$; NOP: $F_{(6, 58)} = 1.881$, $p = 0.0995$, $R^2 = 0.1629$; NOC: $F_{(6, 58)} = 1.326$, $p = 0.2603$, $R^2 = 0.1206$) compared to vehicle.

3.54 Behavioral tests of learning and memory

In NOR test, no control rats were excluded but four were excluded in the drug-treated groups due to rat climbing out of the test box, not exploring both objects, or testing error. In NOP test, two rats were excluded in the control group for lack of exploration of both objects and one in the drug-treated groups was excluded for climbing out of the test box. In NOC test, one rat was excluded in the control due to testing error while one was an outlier, and one was excluded in the drug-treated groups due to testing error.

3.54a Novel object recognition test

The vehicle group spent significantly more time exploring the novel than the familiar object as indicated by the vehicle groups' PI being greater than 50% ($\mu = 62.47 \pm 2.287$ SEM, $t_{(19)} = 4.576$, $p < 0.001$ one-tailed) (Figure 3.3C). The low dose groups showed a decrease in PI compared to the vehicle group ($F_{(6, 57)} = 4.103$, $p = 0.001$, $R^2 = 0.3016$; EE: $t_{(25)} = 2.430$, $p < 0.05$, Cohen $d = -1.11$, $U_1 = 59\%$; LNG: $t_{(25)} = 2.189$, $p < 0.05$, Cohen $d = 1.00$, $U_1 = 55\%$; EE/LNG: $t_{(24)} = 2.087$, $p < 0.05$, Cohen $d = -0.85$, $U_1 = 49\%$) (Figure 3.3A). The high dose EE group showed a significant increase in PI ($F_{(6, 57)} = 4.103$, $p = 0.001$, $R^2 = 0.3016$; $t_{(26)} = 2.655$, $p = 0.01$, Cohen $d = 1.15$, $U_1 = 60\%$) compared to the vehicle group (Figure 3.3A).

3.54b Novel place recognition test

In the vehicle group the PI was not significantly greater than 50% ($\mu = 51.36$, $t_{(17)} = 0.4512$, $p = 0.3288$ one-tailed) (Figure 3.3C) and there was no discrimination seen

between the old and new location of the objects, as shown by the non-significant *t*-test ($t_{(34)}=0.05219$, $p=0.9587$, Cohen $d=0.02$, $U_1=1\%$). Additionally, there was no significant difference between groups observed for PI ($F_{(6, 58)} = 0.5843$, $p=0.7414$, $R^2=0.0570$).

3.54c Novel context recognition test

The vehicle group spent significantly more time exploring the novel than the familiar object as indicated by the vehicle groups' PI being greater than 50% ($\mu=55.91\pm 3.259$ SEM, $t_{(17)}=1.814$, $p<0.05$ one-tailed, Cohen $d=0.62$, $U_1=39\%$) (Figure 3.3C). All groups except for the EE 10 μ g group ($\mu=46.68$) had a PI greater than 50%. The low dose LNG group showed a statistically higher PI ($F_{(6, 58)} = 1.687$, $p=0.1405$, $R^2=0.1486$; $t_{(23)}=2.087$, $p<0.05$, Cohen $d=0.97$, $U_1=54\%$) than the met-di vehicle group (Figure 3.3B).

3.55 Cell Molecular studies

3.55a TH *in situ* hybridization/ELISA

Excluded from the TH mRNA studies, were two control data (leaving 28 control) and five drug-treated data, due to the mRNA labeling being equivalent to the background, while one drug-treated data was an outlier. TH mRNA in the LC was significantly decreased in all the low dose groups ($F_{(6, 111)} = 10.82$, $p<0.0001$, $R^2=0.3690$; EE: $t_{(42)}=5.178$, $p<0.0001$, Cohen $d=-1.39$, $U_1=67\%$; LNG: $t_{(40)}=2.761$, $p<0.01$, Cohen $d=-0.93$, $U_1=52\%$; EE/LNG: $t_{(41)}=2.832$, $p<0.01$, Cohen $d=-0.93$, $U_1=52\%$) (Figure 3.4A). In contrast, high dose EE groups showed a significant increase in TH mRNA ($F_{(6, 111)} = 10.82$, $p<0.0001$, $R^2=0.3690$; EE: $t_{(40)}=2.102$, $p<0.05$ Cohen $d=0.71$, $U_1=43\%$; EE/LNG: $t_{(41)}=2.465$, $p<0.05$, Cohen $d=0.80$, $U_1=47\%$) (Figure 3.4A). In study 2, TH protein was evaluated in the LC and one control data was lost due to inadequate volume

and two drug-treated data were outliers. Consistent with the mRNA, the low EE dose groups showed a decrease in TH protein in the LC ($F_{(6, 58)} = 2.195$, $p=0.05$, $R^2=0.1851$; EE: $t_{(24)} = 2.385$, $p<0.05$, Cohen $d=-1.10$, $U_1=59\%$; EE/LNG: $t_{(25)} = 2.912$, $p<0.01$, Cohen $d=-1.26$, $U_1=63\%$) (Figure 3.4B).

3.55b Galanin *in situ* hybridization/ELISA

Excluded from the mRNA studies were two control data (leaving 28 control) and six drug-treated data due to mRNA labeling being equivalent to the background, while two drug-treated data were outliers. In the LC, the high dose EE group as well as both high and low dose EE/LNG groups showed statistically increased galanin mRNA ($F_{(6, 109)} = 8.110$, $p<0.0001$, $R^2= 0.3087$; EE: $t_{(41)} = 3.413$, $p<0.01$, Cohen $d=1.12$, $U_1=59\%$; EE/LNG 10/20 μ g: $t_{(41)} = 2.910$, $p<0.01$, Cohen $d= 0.95$, $U_1=53\%$; EE/LNG 30/60 μ g: $t_{(41)} = 2.928$, $p<0.01$, Cohen $d=0.96$, $U_1=53\%$) (Figure 3.5A), whereas the LNG 60 μ g group exhibited significantly less ($F_{(6, 109)} = 8.110$, $p< 0.0001$ $R^2= 0.3087$; $t_{(42)} = 2.639$, $p=0.01$, Cohen $d=-0.85$, $U_1=49\%$) when compared to the control. In study 1, Elisa for galanin protein was performed, and it had one outlier exclusion. The findings for the galanin protein were EE 10 μ g and EE/LNG 10/20 μ g groups had a statistical increase in protein ($F_{(6, 50)} = 6.915$, $p < 0.0001$, $R^2=0.4535$; EE: $t_{(16)} = 2.194$, $p<0.05$ Cohen $d=1.10$, $U_1=59\%$; EE/LNG: $t_{(16)} = 2.935$, $p<0.01$, Cohen $d= 1.48$, $U_1=70\%$) (Figure 3.5B).

3.55c BDNF *in situ* hybridization/ELISA

For the subdivisions of the hippocampus mRNA studies, the exclusions were secondary to the radio-labeling being equivalent to the background. Compared to the met-di control, BDNF mRNA was significantly decreased in the EE 10 μ g and the EE/LNG 10/20 μ g groups in the **total hippocampus** ($F_{(6, 116)} = 3.087$, $p<0.01$, $R^2=0.1377$; EE: $t_{(42)} = 2.777$,

$p < 0.01$, Cohen $d = -0.90$, $U_1 = 52\%$; EE/LNG: $t_{(43)} = 2.838$, $p < 0.01$, Cohen $d = -0.90$, $U_1 = 52\%$) as well as each Ammon's horn divisions—**dentate** ($F_{(6, 116)} = 2.068$, $p = 0.06$, $R^2 = 0.0966$; EE: $t_{(42)} = 1.972$, $p = 0.05$, Cohen $d = -0.64$, $U_1 = 40\%$; EE/LNG: $t_{(43)} = 2.118$, $p < 0.05$, Cohen $d = -0.55$, $U_1 = 35\%$), **CA3** ($F_{(6, 116)} = 1.720$, $p = 0.12$, $R^2 = 0.0817$; EE: $t_{(42)} = 2.043$, $p < 0.05$, Cohen $d = 0.66$, $U_1 = 42\%$; EE/LNG: $t_{(43)} = 2.030$, $p < 0.05$, Cohen $d = -0.65$, $PS = 67\%$, $U_1 = 40\%$), **CA2** ($F_{(6, 116)} = 3.466$, $p < 0.01$, $R^2 = 0.1520$; EE: $t_{(42)} = 3.059$, $p < 0.01$, Cohen $d = -1.02$, $U_1 = 55\%$; EE/LNG: $t_{(44)} = 2.713$, $p < 0.01$, Cohen $d = -0.86$, $U_1 = 51\%$), **CA1** ($F_{(6, 117)} = 2.940$, $p = 0.01$, $R^2 = 0.1310$; EE: $t_{(43)} = 3.192$, $p < 0.01$, Cohen $d = -1.03$, $U_1 = 56\%$; EE/LNG: $t_{(44)} = 2.904$, $p < 0.01$, Cohen $d = -0.92$, $U_1 = 52\%$) (Figure 3.6 A, B, C, D, & E). The BDNF protein (excluded was one drug-treated rat due to dropped specimen) was significantly decreased in the total hippocampus in EE/LNG 30/60 μ g group ($F_{(6, 60)} = 2.040$, $p = 0.07$, $R^2 = 0.1694$; EE/LNG: $t_{(26)} = 2.508$, $p < 0.05$, Cohen $d = -1.09$, $U_1 = 58\%$) (Figure 3.6F).

3.6 Discussion

A favored method of birth control among young women is the contraceptive pill. Of the studies examining psychological effects of contraceptive hormone usage in reproductive-age women, the majority of the outcomes are generalized into mood changes (Kurshan & Epperson, 2006) rather than differentiated into emotional and cognitive domains. This study attempted to identify behavioral outcomes of contraceptive hormone treatments by focusing on dose-dependent effects of standard contraceptive drugs and their combinations in behavioral assays of anxiety and cognition.

As in reproductive-age women given contraceptive hormones, EE and LNG administration to intact female rats involves a complex interplay between the subtraction

of endogenous 17β -estradiol and the addition of potent exogenous drugs. As expected, endogenous 17β -estradiol was lowered to the metestrus-diestrus range by the low doses, further depressed by high dose regimens of EE and LNG and were consistent with previous findings that hormone contraceptives decrease estradiol levels to approximately 70% of proestrus percent of control levels (B. M. Graham & Milad, 2013).

Approximating our findings, the Graham and Milad (2013) study had daily vaginal smears comparable in the metestrus phase of the cycle. In our study, no evidence of ovulation was found with a chronic regimen of anti-ovulatory drug doses of EE and LNG (P. Andrews et al., 2002; Bennink et al., 2008; Kumar et al., 2000; Muhn et al., 1995).

The absence of oocytes in the control rats was consistent with the lack of estrus terminal vaginal smears, the phase when ovulation occurs commonly (Nequin et al., 1979).

Similar doses of EE ($10\mu\text{g}/\text{rat}/\text{day}$) subcutaneously for 28 days has been shown to induce a higher incidence of apoptotic corpora lutea in the ovary, indicative of inhibition of the HPG axis (P. Andrews et al., 2002). Despite all rats in our study being in a low endogenous 17β -estradiol state, there was a biphasic behavioral and cell molecular response. As previously stated, it is common for the estrogen receptor to exhibit biphasic dose-response interactions (E. J. Calabrese & Baldwin, 2003). The dose-dependent effects observed in these experiments may be attributed to variance in gonadal steroid receptor activation in rats treated with low dose contraceptive hormones compared to rats treated with higher doses. This dose-response may represent a possible re-establishment of homeostasis by the low dose of EE compensating for the loss of endogenous 17β -estradiol, whereas the high dose effects may reflect receptor activation beyond that occurring in the met-di vehicle-treated rats.

In both tests for anxiety-like behavior (EPM and SPDB), low doses of the drugs (EE 10 μ g, LNG 20 μ g, or EE/LNG 10/20 μ g) were anxiolytic with significant large effect sizes whereas, high doses were ineffective. Graham and Milad, 2013, found similar findings when they performed a series of cross-species experiments with women and rats on contraceptive hormones in fear conditioning and learned extinction paradigms. The women were undergoing treatment with combined monophasic oral contraceptives while the rats received treatments of LNG (100 or 500 μ g/kg/day) [66]. The authors found that in rats, 100 μ g/kg/day LNG (\approx 20 μ g/rat/day) significantly impaired learning in an extinction recall task compared to the met-estrus vehicle controls. Furthermore, their study points out the difference in results obtained between treatments with 17 β -estradiol and EE in associative memory formation. Women taking contraceptives had impaired learning either in the consolidation of the fear or diminished retention of the extinction memory when compared to the high estrogen phase, naturally cycling controls yet, this deficit was remedied when serum estradiol levels were restored. Other biphasic effects include the antidepressant-like action of EE in ovariectomized rats at single low doses (2.5 and 5.0 μ g/rat) but not at a single high dose (10 μ g/rat) in the forced swim test (E. Estrada-Camarena et al., 2003). Chronic treatment with a higher dose (EE 30 μ g/day and LNG 125 μ g/day) in rats, showed increase anxiety in the EPM (Follesa et al., 2002), further supporting dose-response effects of contraceptive hormones on anxiety. In our SPDB test, similar anxiolytic behavior was seen. Planned comparisons with the vehicle group showed a significant decrease in burying with a concomitant increase in prod contact in the low dose EE and EE/LNG groups. While the higher dose of EE (30 μ g/day)

resulted in a significant rise (88 % of the treatment group are above the mean of the control group-Cohen U_3) in the anxiogenic-like behavior of freezing.

This study is the first showing a decrease in TH mRNA and protein was associated with anxiolytic behavior in rats given low but not high-dose contraceptive drugs. In the EPM, anxiogenic behavior was associated with rising TH mRNA and protein. The relationship between TH expression in the LC and performance in the anxiety tests is consistent with the well-established role of noradrenergic activity in anxiety-related behavior. Estrogen-induced changes in TH mRNA are not surprising given the overlap of the estrogen response element with the cAMP response element, a vital regulator of basal expression of TH (Kvetnansky, Sabban, & Palkovits, 2009). However, long term 17β -estradiol injections of $25\mu\text{g}/\text{kg}/\text{day}$ ($\cong 5\mu\text{g}/\text{rat}/\text{day}$) in ovariectomized rats does not affect the expression of TH mRNA in the LC. Short-term treatment with higher doses of $80\mu\text{g}/\text{kg}/\text{day}$ ($\cong 16\mu\text{g}/\text{rat}/\text{day}$) raises TH mRNA expression by 300% (Sabban, Maharjan, Nostramo, & Serova, 2010). In contrast, our long-term daily injection of $10\mu\text{g}/\text{rat}/\text{day}$ of EE, a more potent analog of 17β -estradiol, resulted in a significant decrease in TH mRNA. Altogether, this suggests that an alternative mechanism of action other than dose-dependent estrogen promoter activation may be in play. The behavior seen in the anxiety as well as learning paradigms in conjunction with the TH changes are reflective of noradrenergic modulation. The influence of EE on TH expression may involve its interactions with the norepinephrine transporter. 17β -estradiol is not an efficient norepinephrine re-uptake inhibitor as it requires higher doses than EE –the most effective re-uptake inhibitor of the estrogens (Ghrif et al., 1983). Therefore, EE can function as an anti-depressant by its competition for the re-uptake transporters for norepinephrine

(Ghraf et al., 1983; Vega-Rivera Nelly et al., 2013). Norepinephrine re-uptake inhibitors such as tricyclic anti-depressants (TCAs) decrease expression of TH mRNA in the LC (Nestler, McMahon, Sabban, Tallman, & Duman, 1990), consistent with the TH mRNA findings of this study. This mechanism of contraceptive hormones can be explored further by looking at the dose-response seen in the object recognition tests.

In the novel object recognition test, high dose EE significantly enhanced learning.

Improved performance in object recognition tests associated with a high estrogen state (A. A. Walf et al., 2006) is possibly secondary to upregulation of synaptogenesis via estrogen alpha receptor (ER α) in hippocampal neurons (Rune et al., 2002). EE acts as a potent ER α agonist with EE having a four-fold selectivity over ER β (Heather A Harris et al., 2002) and 500 times the potency of 17 β -estradiol (Helgason et al., 1982). The high dose of EE (30 μ g) may be functioning as a potent ligand whereas the low dose (10 μ g) is not, due to a dose-response effect. In female rodents, all phases of estrous have recognized the novel object with a one-hour retention period (Sutcliffe, Marshall, & Neill, 2007). Despite a comparable retention period, the lower dose EE and LNG groups are unable to learn, shown by failing to reach a significantly greater than 50% PI, as well as all having a significant decrease in PI from the vehicle group (of each treatment group—86%-EE, 84%-LNG, 79%-EE/LNG is below the mean of the control-Cohen U₃).

Given that stimulation of the LC and the resultant increase in norepinephrine transmission enhances memory (Tully & Bolshakov, 2010), a mechanism involving a decrease in activation of the LC may be in effect with the low-dose drugs.

The LC has two phases of firing: a low discharge rate—tonic associated with vegetative, homeostasis type behavior such as eating or grooming, and a high discharge rate—phasic

associated with processing salient environmental cues or selective focusing to attend to the environment (Berridge & Waterhouse, 2003). The tonic state of the LC would explain the lack of preference for the novel (salient focus) or decay in memory of a familiar object. Norepinephrine re-uptake inhibitors, such as tricyclic antidepressants and contraceptive hormones, feedback to somatodendritic α_2 -adrenoceptors and thus reduce the firing rate of the LC (Grandoso, Pineda, & Ugedo, 2004; Vega-Rivera Nelly et al., 2013). It is by this means, that extrasynaptic norepinephrine binding to the α_2 -adrenergic receptors can exert local regulation of norepinephrine release (Berridge & Waterhouse, 2003). In the low dose groups, the decrease in TH mRNA and protein with concurrent behavior suggestive of a reduction in norepinephrine output would be consistent with tonic firing of the LC secondary to the activation of the inhibitory α_2 -adrenoreceptors. Frith et al., 1985, found that healthy volunteers given an IV dose of an α_2 -adrenergic agonist, were impaired only in acquisition of novel associations, not in other measures of short and long term memory, and this type of learning is more vulnerable to disruption of adrenergic transmission. In order to confirm this mechanism of contraceptive hormones—alteration of noradrenergic tone via LC modulation—future experiments will examine the reversal of impaired learning in the low dose drug groups with a selective α_2 -receptor antagonist, idazoxan.

Considering the essential role played by LC activation, the link between norepinephrine and emotional memory may be modulated by a decrease in TH (seen in this study), as well as an increase in galanin release. The low dose EE groups, but not the high, showed a significant rise in galanin protein in the LC. Galanin neuropeptide release may inhibit norepinephrine transmission as suggested by the behavior in the low dose groups in our

models. Interestingly however, possibly promoted by the potent estrogen EE, the galanin mRNA expression was increased in the high dose EE groups (EE, and EE/LNG) yet decreased in the high dose LNG alone. The discordance between the galanin mRNA and the protein suggests that regulation of galanin is multifactorial and involves transcriptional control as well as post-transcriptional mechanisms. Therefore, the decrease in anxiety and learning suggestive of a change in noradrenergic tone may be due to the summation of galanin release and the drugs acting via somatodendritic autoreceptors.

Novel context recognition is a sensitive test of visuospatial memory. Only the LNG 20 μ g group displayed a greater degree of learning when compared to the vehicle. The mechanism of action could be via PR since LNG has a relatively high progesterone receptor affinity compared to progesterone, however, metabolites of LNG decrease till they have no affinity (Lemus et al., 1992). Walf et al. (2006) showed that learning improves with higher progesterone levels in object recognition tests. To avoid a confounding variable, the vehicle rats in the proestrus were not included as controls since that is the high progesterone phase of the cycle (Nequin et al., 1979), whereas the met-di vehicle rat and drug-treated rats should be in equivalent low progesterone states. Chronic contraceptive hormones in rats (EE 30 μ g and LNG 125 μ g individually) significantly reduce progesterone (Porcu et al., 2012). At the same time, LNG is unable to be metabolized to allopregnanolone—the progesterone metabolite (Stanczyk, 2003) that in the hippocampus is a mechanism suggested for the increased learning seen in high progesterone states (A. A. Walf et al., 2006). LNG is reduced to a similar chemical configuration, 5 β -LNG then further to 3 α 5 α -LNG and 3 β 5 α -LNG which act as less

potent anxiolytics than allopregnanolone (O. Picazo, Fernandez-Guasti, Lemus, & Garcia, 1998). Previous studies, as does this study, point to a bimodal tendency of allopregnanolone and the similar LNG metabolites (extensively reviewed by Andréen, et al. 2009). According to the EPM/SPDB behavioral results, our low dose drugs-EE 10 μ g, LNG 20 μ g may fall in the portion of the inverted U-shaped curve where anxiolytic-like effects are seen, whereas, a decrease in allopregnanolone secondary to EE 30 μ g or LNG 125 μ g alone (Porcu et al., 2012) or in combination (Follesa et al., 2002) in rats increases anxiogenic behavior in the EPM. Alternatively, a decrease in this neurosteroid due to low dose contraceptive use in women did not result in an increase in reported anxiety (Rapkin et al., 2006). Likewise, the effects of allopregnanolone on memory have been shown to be bimodal. Rat studies demonstrate that increases in allopregnanolone in behavioral estrus has memory benefits (A. A. Walf et al., 2006), yet the significant decrease in allopregnanolone seen in rats given chronic oral treatment of EE/LNG (30/125 μ g/day) did not detract from spatial learning in the Morris water maze when compared to intact rats (Santoru, et al., 2014). We cannot directly address this however, for allopregnanolone levels were not evaluated in this study.

A more likely explanation is that LNG is working through androgen receptors for, it has ample affinity (44 times the relative binding affinity to androgen receptors than progesterone)(Kloosterboer et al., 1988; A Phillips et al., 1990), its metabolite 5 α -LNG increases in relative binding affinity to androgen receptors (1.2 times that of LNG) (Lemus et al., 1992) and LNG is 70% as potent as testosterone (Audrey Phillips et al., 1987). Though LNG is capable of working at many steroid receptors, its primary action is exerted through the androgen receptors as an agonist (Africander et al., 2011; Poulin et

al., 1991). In human studies, there is enhanced visuospatial aptitude in users of contraceptive hormones containing 19-nortestosterone derivatives (i.e. LNG) (Wharton et al., 2008). Women on oral contraceptives perform better on spatial tests than women in the progesterone phase of the cycle (McCormick & Teillon, 2001).

The EE 10 μ g and EE/LNG 10/20 μ g groups demonstrated a notable decrease in BDNF mRNA in all the subfields of the hippocampus (Figure 6 B, C, D, E); these same groups exhibited decreased learning in the NOR test (Figure 3A). The decline in hippocampal BDNF may be indicative of reduced norepinephrine input. Hippocampal BDNF mRNA was positively associated with TH mRNA in the LC, as would be expected from our previous findings of noradrenergic impact on adaptations in BDNF gene expression (Van Hooymissen et al., 2004). Hippocampal cell cultures show that induction of BDNF expression varies with the level of norepinephrine exposure; a higher concentration of norepinephrine is required for maximal expression of BDNF (M. Chen, Nguyen, Pike, & Russo-Neustadt, 2007). Norepinephrine functioning via β -adrenergic receptors activates BDNF facilitated pro-survival avenues and is thus neuroprotective (Counts & Mufson, 2010). Furthermore, norepinephrine release from the LC is vital for acquisition, consolidation, and retrieval of a memory, and this is accomplished via β -adrenergic receptors (extensively reviewed by Sara, 2009). Exemplified in our study, increases in BDNF mRNA were associated with decreases in the anxiolytic-like behavior (open arm exploration and prod contact). In other words, an increase in BDNF mRNA corresponds with anxiety-like behavior—a resultant excitatory effect of higher levels of BDNF (Helen E Scharfman & MacLusky, 2014). BDNF levels are highest at peak estradiol levels. Therefore, another mechanism attributing to the decrease in BDNF in the EE 10 μ g

groups may be that this specified dose of EE can not compensate for the decreased serum 17β -estradiol levels. 17β -estradiol deprivation has been shown to reduce BDNF mRNA expression (Singh, Meyer, & Simpkins, 1995).

Hormonal manipulation of the BDNF mRNA does not totally explain the changes seen in this study, since higher BDNF expression is seen in intact rats when progesterone is low or when exogenous estradiol is given with progesterone in ovariectomized rats (R.B. Gibbs, 1998). Additionally, in rat cerebrocortical cultures, LNG increases BDNF mRNA (Jayaraman & Pike, 2014). Therefore, it suggests the decrease in mRNA seen in the low dose contraceptive drug groups is related to the change in noradrenergic tone. On the same note, it is difficult to explain the significant decrease in BDNF protein seen in the total hippocampus with the combined contraceptive hormones (when progesterone should be low and potent exogenous estrogen and progestin are given). BDNF protein is elevated in high estradiol phases of the rat cycle and the range for total hippocampal BDNF protein in an intact 3 month old rat ≈ 27 pg/mg which could be reproduced with replacement of 17β -estradiol in a young ovariectomized rat (Kiss et al., 2012). In our study, the met-di control had a mean BDNF protein of 30.9 pg/mg ± 1.4 SEM yet, a decrease from this occurred when combination synthetic estrogen and progestins were given. Gibbs (1999), saw a decrease in BDNF protein specific to the hippocampus when they gave ovariectomized rats estradiol and progesterone as compared to a vehicle ovariectomized control. Plasma BDNF protein, reflective of central BDNF is decreased to follicular levels in women on combined contraceptive hormones with a circadian low at four in the afternoon (N Pluchino et al., 2009), the time at which our rats were sacrificed.

3.7 Conclusion

The fifty-year availability of contraceptive hormones has allowed for their usage by nearly 200 million women worldwide (Chadwick, Burkman, Tornesi, & Mahadevan, 2012). The systemic effects of these drugs have been studied extensively while central nervous system effects are less understood. Multiple studies have reviewed the benefits of ovarian steroids on cognitive performance. Contraceptive hormones suppress ovarian hormones, although they may potentially compensate for this loss if the exogenous hormones dose is sufficient. A recent pharmacoepidemiological analysis of LNG-induced adverse reactions found that negative central nervous system symptoms were associated with younger age (Sayaka Mizutani, Yousuke Noro, Masaaki Kotera, & Susumu Goto, 2014), when ovarian function is at its prime. This paper explored contraceptive hormones' effects on young reproductive-age intact rats by examining dual behavior paradigms—learning/memory and anxiety, as well as delving into the mechanisms by which the change in behavior might occur. Our findings show contraceptive hormone treatment in rat exhibit dose-dependent responses in TH, galanin, and BDNF gene and/or protein expression, as well as in learning and memory, suggesting an ability to modulate the noradrenergic tone of the brain. This study demonstrates that, depending on the dose given, synthetic contraceptive hormones imperfectly mimic endogenous hormones. In conclusion, the current trend toward lowering the dose of EE in contraceptive formulations and decreasing endogenous 17β -estradiol levels by fewer hormone-free days, may not be without cognitive consequences.

3.8 Figures

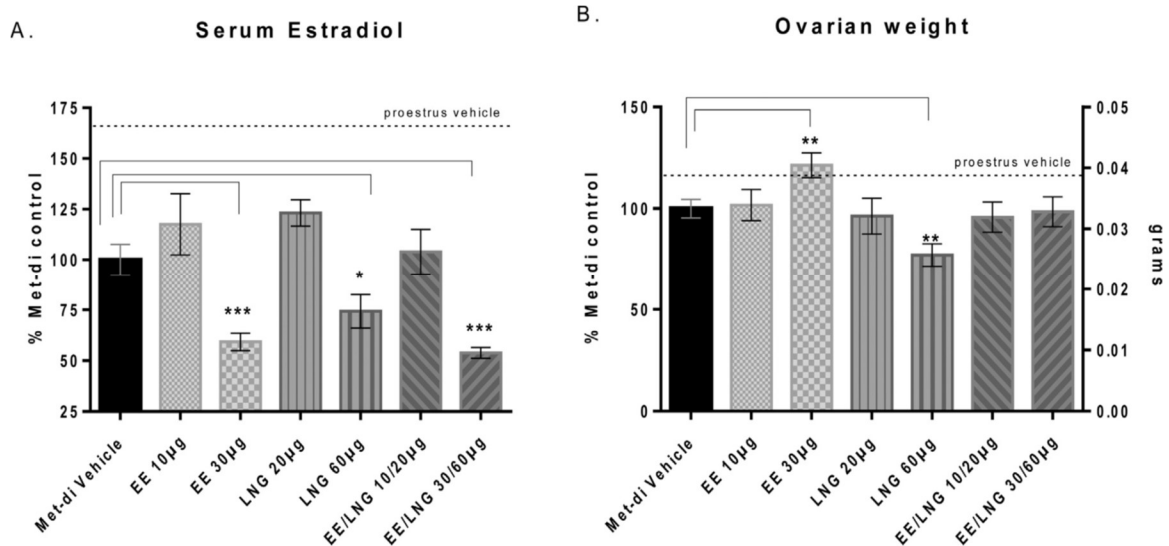


Figure 3.1 Serum estradiol levels and ovarian weights. (Study 1 and 2)

In the high dose drug groups (n : EE = 15, LNG = 16, EE/LNG = 13) estradiol (E2) levels were suppressed significantly lower than that of the metestrus-diestrus (met-di) control (n = 30). Note: the mean for vehicle rats considered to be proestrus by E2 level and vaginal smears is shown by a dotted line ($\mu = 167.4 \pm 13.78$ SEM % met-di control, $n = 10$); they were excluded from the control (A). The high dose ethinyl estradiol ($n = 16$) increased the ovarian weight while the high dose levonorgestrel ($n = 16$) induced a significant decrease in ovarian weight (B). Data is shown as % of met-di control \pm SEM (*= $p \leq 0.05$, **= $p \leq 0.01$, ***= $p \leq 0.001$ compared to met-di vehicle).

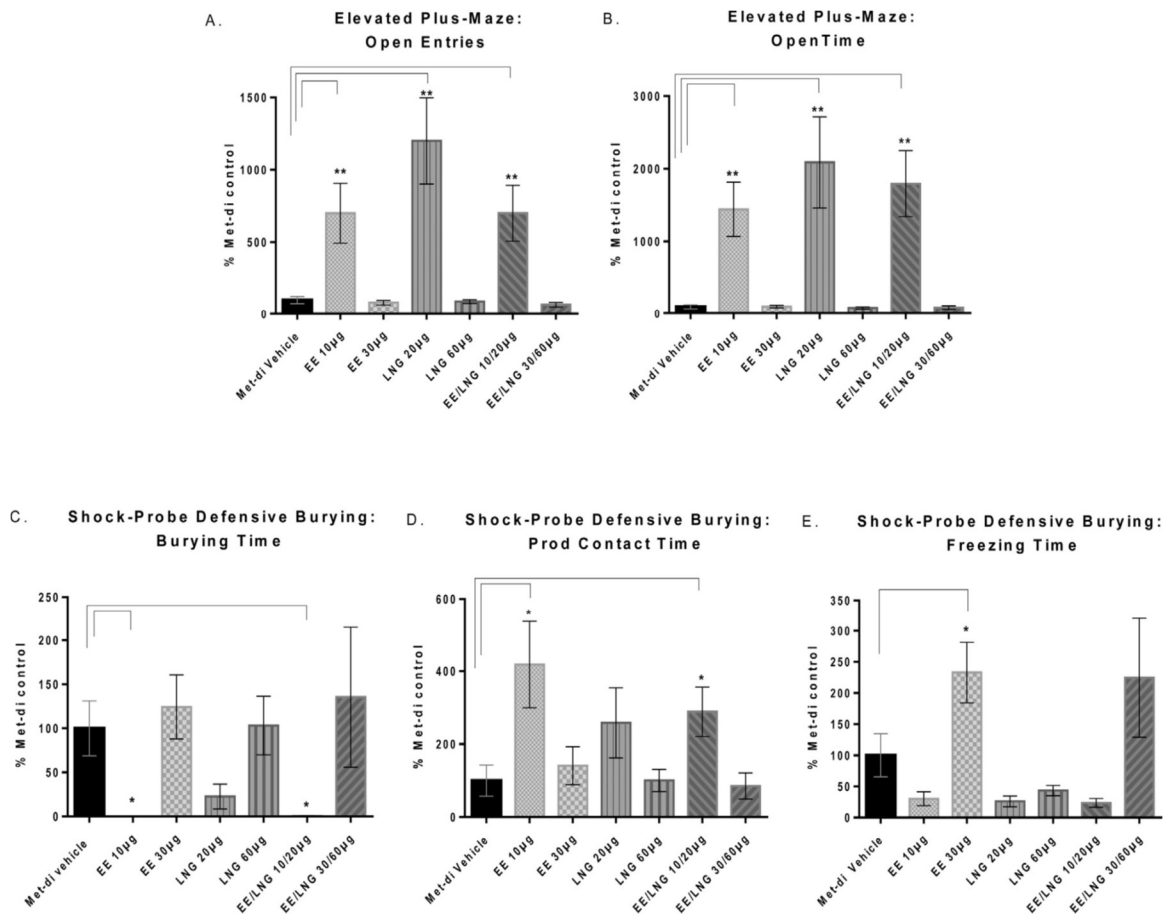


Figure 3.2 Behavioral tests of anxiety: elevated plus-maze and shock-probe defensive burying. (Study 1)

In both behavioral tests of anxiety an anxiolytic effect was exhibited in ethinyl estradiol 10 µg and ethinyl estradiol/levonorgestrel 10/20 µg groups as seen by a significant increase in open arm entries (met-di control $n = 9$, drugs $n = 8$) (A), more time spent in the open arms (B), less time spent burying the prod in response to the shock (met-di control $n = 10$, drugs $n = 6$) (C) and a substantial increase in the amount of time spent in contact with the prod (drugs $n = 8$) (D). The levonorgestrel 20 µg dose similarly was anxiolytic in the elevated plus-maze ($n = 8$) (A and B) but not significantly so in the shock-probe defensive burying paradigm ($n = 7$) (C–E). Alternatively, the ethinyl

estradiol 30 μg ($n = 6$) induced an anxiogenic response to the noxious stimulus as demonstrated by a significant increase in the amount of freezing time (E). Data is shown as % of met-di control \pm SEM (*= $p \leq 0.05$, **= $p \leq 0.01$, compared to met-di vehicle).

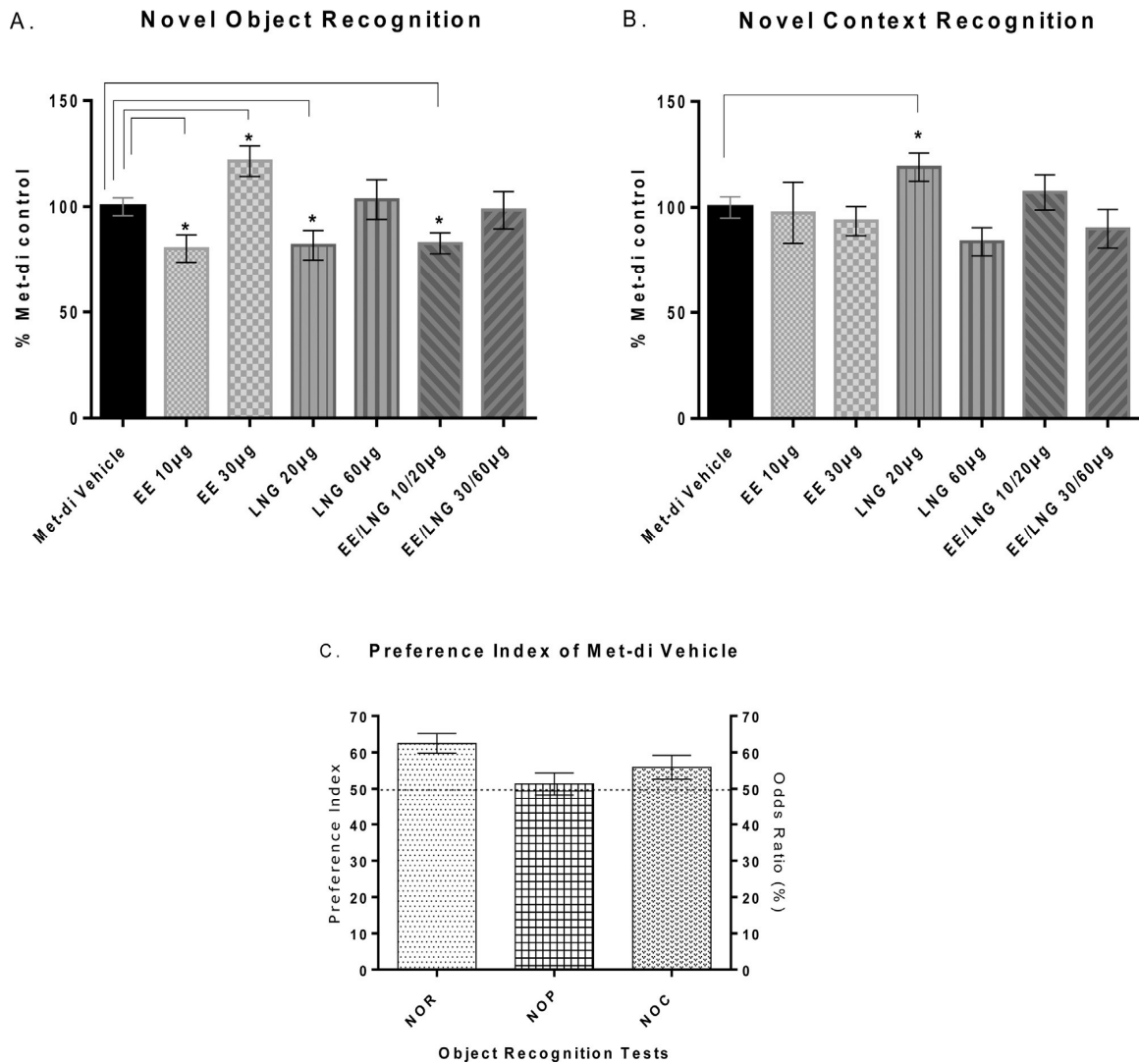


Figure 3.3 Behavioral tests of learning and memory: novel object recognition and novel context recognition. (Study 2)

Ethinyl estradiol 10 μ g, levonorgestrel 20 μ g, and ethinyl estradiol/levonorgestrel 10/20 μ g groups showed impaired learning and memory as evidenced by a decrease in preference and exploration of a novel vs. a familiar object as compared to vehicle (met-di control $n = 20$, EE = 7, LNG = 7, EE/LNG = 6) (A). Conversely, high dose ethinyl estradiol 30 μ g ($n = 8$) showed a greater preference index (exploration time of novel

object/total exploration time of both objects) compared to vehicle (A), suggesting a higher acuity in learning and memory. On the novel context recognition assessment, the levonorgestrel 20 μ g group ($n = 7$) improved in visuospatial memory compared to the met-di control ($n = 18$) as indicated by a higher preference index for the novel object (out of context with the room) (B). The odds ratio of performing in this object recognition task are typically 50% with greater than 50% preference index signifying learning and memory. The met-di control rats were able to evince learning in the novel object recognition test (NOR) ($n = 20$) and novel context recognition (NOC) ($n = 18$) but not in the novel place recognition (NOP) ($n = 18$) (C). Data is shown as % of met-di control \pm SEM (*= $p \leq 0.05$, compared to met-di vehicle).

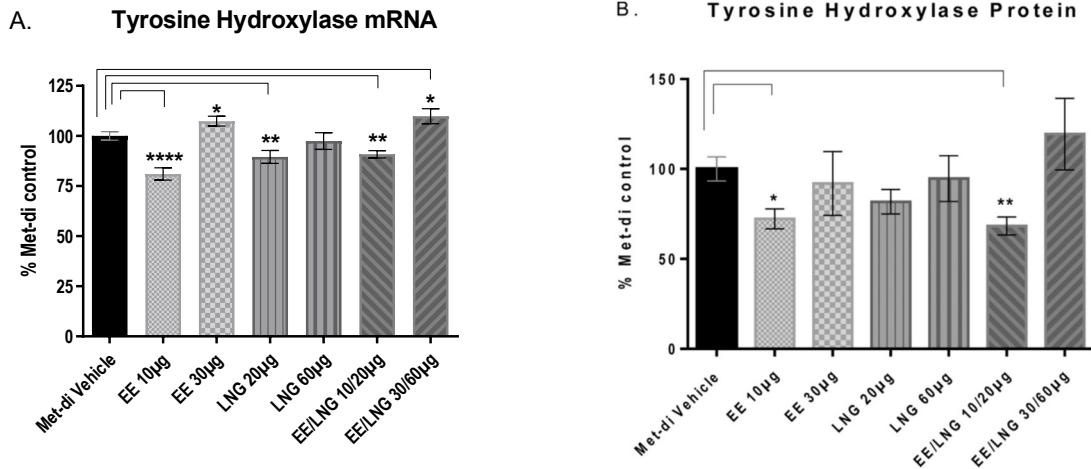


Figure 3.4 Cell molecular studies of tyrosine hydroxylase.

The ethinyl estradiol 10 µg, levonorgestrel 20 µg, and ethinyl estradiol/levonorgestrel 10/20 µg groups when compared to the met-di vehicle showed a decrease in tyrosine hydroxylase mRNA expression in the locus coeruleus, while the ethinyl estradiol/levonorgestrel 30/60 µg group showed an increase (study 1 and 2 met-di $n = 28$, EE = 16, LNG = 14, EE/LNG10/20 = 15, EE/LNG 30/60 = 15) (A). Similarly, in study 2 in which learning and memory paradigms were tested, the tyrosine hydroxylase protein in the ethinyl estradiol 10 µg ($n = 7$), and ethinyl estradiol/levonorgestrel 10/20 µg ($n = 8$) groups showed reduced protein levels in the locus coeruleus as compared to the met-di vehicle ($n = 19$) (B). Data is shown as % of met-di control \pm SEM (*= $p \leq 0.05$, **= $p \leq 0.01$, ****= $p \leq 0.0001$).

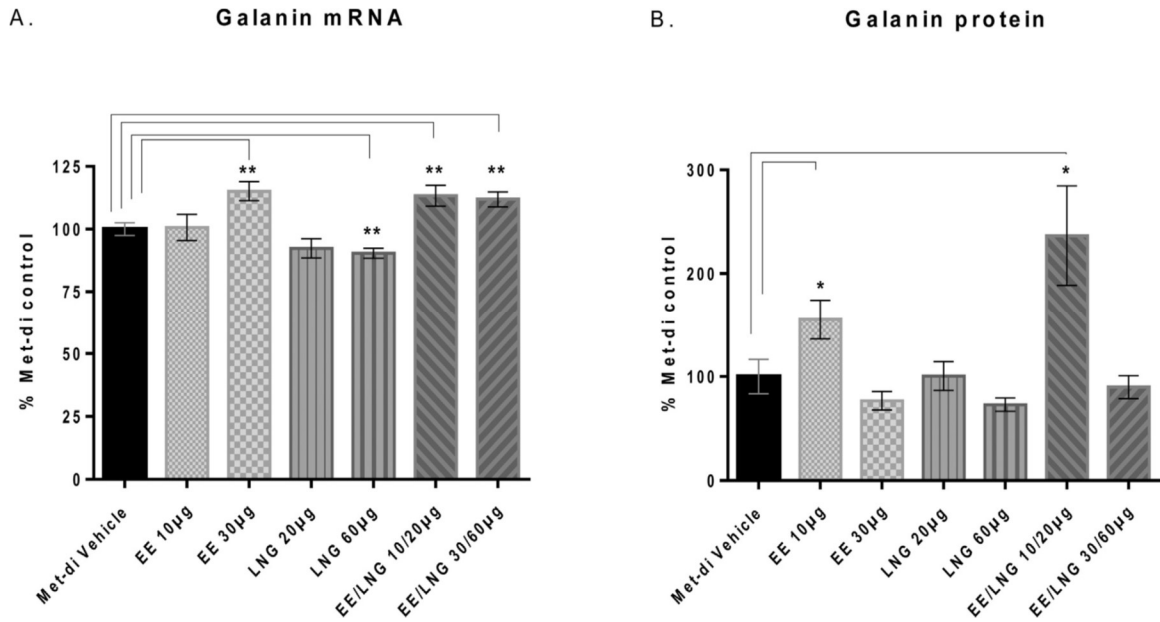


Figure 3.5 Cell molecular studies of galanin.

Galanin is present in 80% of neurons in the locus coeruleus and is modulated by estradiol. The high dose ethinyl estradiol groups (EE 30 µg, $n = 15$ and EE/LNG 30/60 µg, $n = 15$) as well as the low dose ethinyl estradiol/levonorgestrel 10/20 µg group ($n = 15$) showed a significant increase in galanin mRNA expression, while levonorgestrel 60 µg ($n = 16$) alone showed a decrease from the met-di vehicle group ($n = 28$) (A). The levels of galanin protein measured were significantly higher in the low dose groups- ethinyl estradiol 10 µg ($n = 8$) and ethinyl estradiol/levonorgestrel 10/20 µg ($n = 8$) groups when compared to met-di vehicle ($n = 10$) (B). Data is shown as % of met-di control \pm SEM (*= $p \leq 0.05$, **= $p \leq 0.01$).

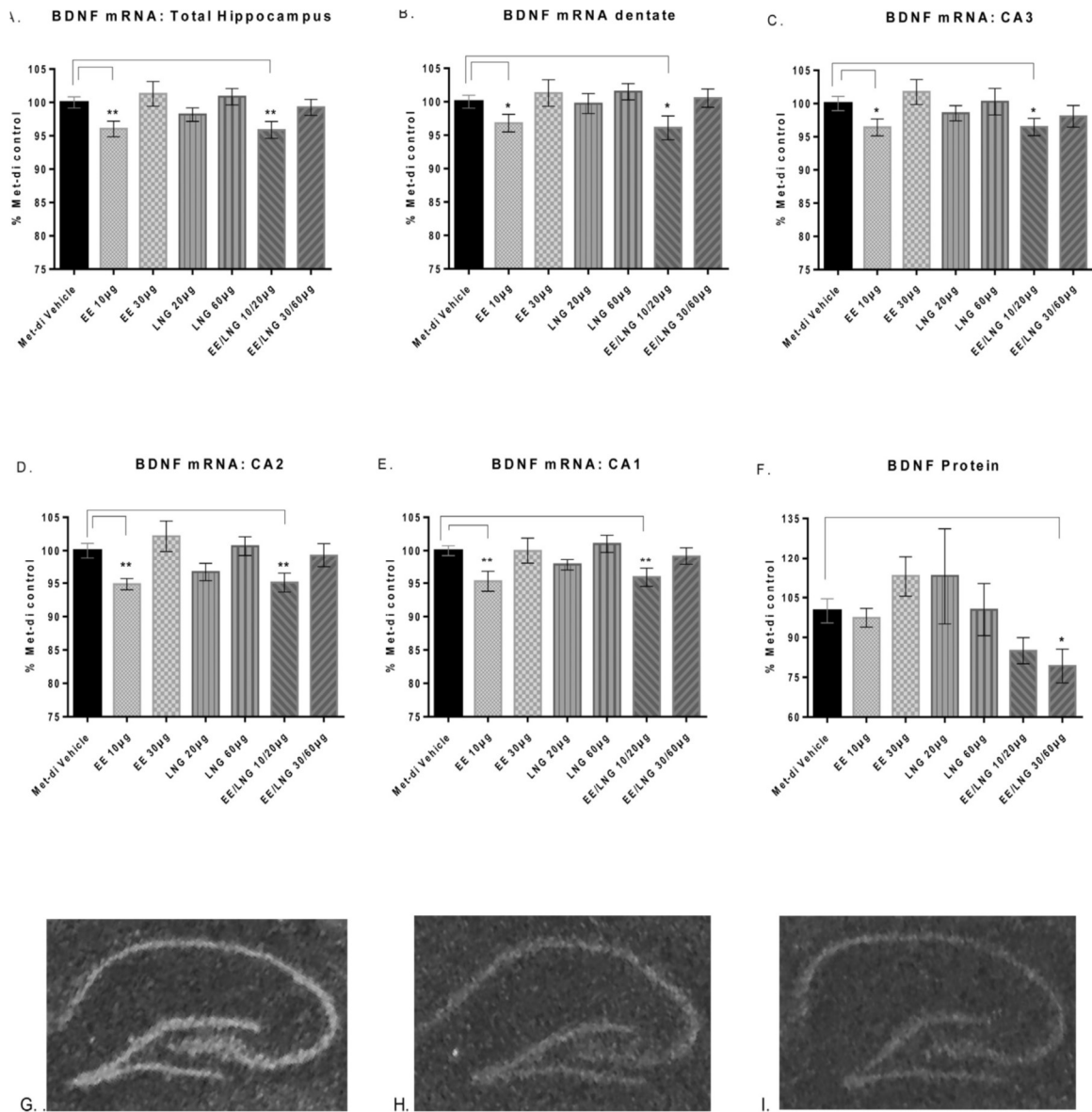


Figure 3.6 Cell molecular studies of brain-derived neurotrophic factor (BDNF).

BDNF mRNA was markedly reduced compared to the met-di vehicle ($n = 29-30$) in the ethinyl estradiol 10 μg ($n = 15$) and ethinyl estradiol/levonorgestrel 10/20 μg groups ($n = 15-16$), both in the total hippocampus as well as each part of Ammon's horn—dentate, CA1, CA2, and CA3 (A–E). BDNF protein was significantly reduced in the ethinyl estradiol/levonorgestrel 30/60 μg group ($n = 8$) compared to met-di vehicle ($n = 20$) (F).

BDNF mRNA expression was detected in each of the major cell layers of the hippocampus. Note the increase in hybridization signal detected in the hippocampus of the met-di vehicle treated rat relative to the low dose ethinyl estradiol groups. In situ hybridization BDNF mRNA expression in the vehicle (G), ethinyl estradiol 10 μg (H), and ethinyl estradiol/levonorgestrel 10 μg /20 μg (I). Data is shown as % of met-di control \pm SEM (*= $p \leq 0.05$, **= $p \leq 0.01$).

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CHAPTER 4

Dose-dependent effects of ethinyl estradiol on norepinephrine levels and object
recognition memory in female rats

To be submitted to *Hormones and Behavior*

4.1 Abstract

Subtle changes in norepinephrine signaling promote flexibility in cognition-driven behaviors. Norepinephrine underlies working memory, which can be measured by performance in object recognition tests (ORT). Ethinyl estradiol (EE), potentially due to its action as a norepinephrine reuptake inhibitor, was previously shown by us to have a dose-dependent effect in ORT. Sprague-Dawley intact female rats received EE (10 or 30 $\mu\text{g}/\text{rat}/\text{day}$) or vehicle subcutaneously for 21 days. Open field and rotarod testing showed equivalent exploration across the regimens and ruled out motoric side effects of the drug, respectively. Rats were tested in novel object and novel context object recognition to assess learning and memory. Serum 17β -estradiol, tissue norepinephrine, normetanephrines, and norepinephrine transporter (NET) were assessed by ELISA. All groups were in a low 17β -estradiol state at the time of testing. Previously, this lab proved ovulation inhibition with similar doses. In both ORT, a dose-dependent response was seen with the EE30 μg group, learning better than the 10 μg group in the novel object, but significantly worse than control in the novel context test. When compared to control, EE30 μg -treated rats' norepinephrine tissue levels were elevated in the prefrontal cortex—an area necessary for recent memory retrieval. Levels were decreased in the hippocampus where spatial memory is predominantly dependent. This effect of EE may be a NET mechanism—a change occurred in NET levels in the locus coeruleus. Our findings of divergent dose-dependent actions of EE on norepinephrine and learning suggests a need for a minimal dose limit of EE used in contraceptive hormone regimens.

4.2 Introduction

The subtleties of cognitive processing allow for multitudes of behavioral responses adaptable to external circumstances. One neurotransmitter credited with aiding in cognition-driven behavior is norepinephrine (Chandler et al., 2014; T. W. Robbins & Arnsten, 2009). Findings within the last decade show that locus coeruleus-norepinephrine terminal fields differ in their varicosity density, thus allowing precise adjustments in transmitter release (Agster et al., 2013; Chandler, Lamperski, & Waterhouse, 2013). Additionally, with the locus coeruleus (LC) modulating the release of norepinephrine (NE) by phasic and tonic firing, specific behavioral actions multiply in complexity and intricacy. For instance, increasing or decreasing NE transmission may escalate exploratory behavior or allow habituation to novel stimuli respectively (Arnsten, 2000; Bouret & Sara, 2005). Further plasticity is obtainable by location, type, affinity, and density of adrenergic receptor across brain regions. For example, the cortical density of α_2 adrenergic receptors (α_2 AR) is almost four times its hippocampal density (Subhash, Nagaraja, Sharada, & Vinod, 2003). As ascribed by Arnsten (2000), the location of the NE stimulus can result in contrasting behavioral outcomes despite stimulating the same type of receptor. Noradrenergic stimulation of α_2 AR in the hippocampus impairs mnemonic performance in the water maze (Sirvio, Riekkinen, Vajanto, Koivisto, & Riekkinen, 1991), while stimulation of post-synaptic α_2 AR receptors in the prefrontal cortex improve working memory (Kawaura, Karasawa, Chaki, & Hikichi, 2014). NE transmission exhibits a regional dose-response which adds to the total diversity obtainable across the brain. Since α_2 AR have a greater affinity for NE, lower doses of

NE trigger α 2AR receptors preferentially over alpha-1 or beta adrenergic receptors (Fig 4.1 A&B)(Arnsten, 2000). Furthermore, reduced quantities of NE may increase the number of postsynaptic α 2AR in the cerebral cortex (C. M. Lee, Javitch, & Snyder, 1983) allowing for improved memory via these favored receptors. In summation, varying levels of NE may transduce into opposing dose-dependent behaviors in memory function tests. Estrogens also can alter NE's effect on working memory. Suggestive of an increase in NE via NE re-uptake inhibition, estradiol treatment of ovariectomized female rats regulates α 2AR receptors across the cortex differentially (Karkanas, Li, & Etgen, 1997). In the prefrontal cortex, estradiol decreased mRNA and density of α 2AR, but there was no change in the hypothalamus (Karkanas et al., 1997). EE, like estradiol, has been shown by direct and indirect methods to be a norepinephrine reuptake inhibitor (NRI). EE inhibits NE uptake on the presynaptic membrane in a competitive manner without being transported itself (Ghraf et al., 1983; Kendall et al., 1977). As predicted by its structure, EE's potency as an inhibitor is in the moderate range based on its K_i value (Koe, 1976). Acting like an NRI, low doses of EE (25ug/kg) in ovariectomized mice caused an increase in normetanephrines in the forebrain (cortex, hippocampus) (Greengrass & Tonge, 1974). In intact female rats, biweekly doses of mestranol (50 μ g/100g wt. which according to Goldzieher & Brody, (1990), 50 μ g mestranol is bioequivalent to 35 μ g of EE) reduced α 2AR in the frontal cortex and the LC (Shackelford Jr, McConnaughey, & Iams, 1988). The same occurs in the cortex, LC and the hippocampus with chronic high dose treatment with NRIs (C. B. Smith, Garcia-Sevilla, & Hollingsworth, 1981; Subhash et al., 2003). EE, together with its metabolites, increases the biological activity of catecholamines in many ways (Marchi & Cugurra, 1974), most significantly by being a

reuptake inhibitor (Ball & Knuppen, 1990). Specifically, metabolites of EE have a higher affinity for catechol-O-methyltransferase than NE, thus may inhibit the metabolism of NE in brain tissues (Breuer & Köster, 1975). In vitro, the molar ratio of EE metabolite to norepinephrine was 1:3 to result in 98% inhibition of NE metabolism (Breuer & Köster, 1975).

EE has been the exclusive estrogen in contraceptives for 40 plus years. The use of contraceptive hormones in the reproductive-age range substitutes natural hormones, such as 17 β -estradiol, with synthetic hormones. The inhibition of ovulation diminishes the 17 β -estradiol to an early follicular level (22-39 pg/ml) (Gaspard et al., 1984; Spona et al., 1996; Willis et al., 2006), and EE is the potent add-back therapy. EE is 500 times biologically equipotent as 17 β -estradiol (Helgason et al., 1982). The study of EE's effect on learning has opposing outcomes due to differing doses, tests performed and animal models used (as reviewed by Beltz, Hampson, & Berenbaum, 2015; Gogos, Wu, Williams, & Byrne, 2014; Warren, Gurvich, Worsley, & Kulkarni, 2014). For instance, Simone et al. 2015, found dose-dependent effects for EE in intact female rats such that the low dose resulted in a decrease in learning in the novel object recognition test while the high dose demonstrated an improvement in learning. The opposite results were obtained in tests of memory with the Morris water maze in ovariectomized rodents on a tenfold lower dose of EE (Mennenga et al., 2015). For human studies, Griksiene and Ruksenas (2011) showed that verbal fluency was impaired in women on contraceptives compared to naturally cycling women. In contrast, Mordecai et al. (2008) did not find a difference in verbal fluency in women on contraceptive hormones compared to natural

cycling women. The NRI capabilities of EE and the versatility of norepinephrine transmission may account for these behavioral differences.

Estrogen's impact on hippocampal learning has long been explored and is widely accepted as beneficial (as reviewed in Daniel, 2006; Frick, 2009; Luine, 2014). Whereas, estrogen's action on the prefrontal contribution to memory has only recently been acknowledged (Galea et al., 2016). Novel object recognition (NOR) tests are considered a model for working memory (Ennaceur & Delacour, 1988). At the least, NOR tests involve hippocampal and prefrontal cortical processing via noradrenergic input (Broadbent, Gaskin, Squire, & Clark, 2010; Clark & Martin, 2005; R. S. Hammond, Tull, & Stackman, 2004; Nirogi et al., 2012). NOR tests use the rat's innate sense of exploration without distraction, fear cues or food deprivation, all of which may incur more subcortical input (Dornelles et al., 2007). The hippocampus has been linked to recent memory reclamation and the prefrontal cortex to remote and recent memory retrieval (Frankland & Bontempi, 2005; Murchison et al., 2004; Nelson, Cooper, Thur, Marsden, & Cassaday, 2011). Specifically, interactions between the prefrontal cortex and hippocampus are important in detecting novel stimuli, and the noradrenergic tone delineates the overall responsiveness to external events (Nieuwenhuis, Aston-Jones, & Cohen, 2005). Attention frames a memory. Accordingly, in NOR tests with short retention phases, recruitment of the hippocampus alone or in synergy with the prefrontal cortex would depend on noradrenergic input.

To explore the noradrenergic effect of EE, we treated Sprague-Dawley intact female rats with two doses of EE (10 or 30 $\mu\text{g}/\text{rat}/\text{day}$) for three weeks. The rats were then tested in novel object/context recognition models of learning and memory. Levels of NE,

normetanephrines (NMN), and norepinephrine transporter (NET) were measured by enzyme-linked immunosorbent assay (ELISA) in the prefrontal cortex, LC, and hippocampus.

4.3 Materials and Methods

4.3.1 Subjects and drug regimens

Intact, female, nonparous Sprague-Dawley rats (Harlan) 60 to 90 days old, weighing 175-200 grams (Harlan Inc., Indianapolis, IN) were selected since rats reach their adult reproductive capacity by 55 to 90 days (Moore, In: Rollin, & Kesel, 1996; Sengupta, 2013). Additionally, the continuous increase in the number of LC neurons stops in females after postnatal day 60 (Pinos, Collado, Rodriguez-Zafra, et al., 2001). NE levels reach adult levels in the rat brain by post-natal day 42 (Shaywitz, Anderson, & Cohen, 1985), temporally with the complete noradrenergic system—transmitters, transporters and receptors (Murrin et al., 2007). Rats housed three to four per cage, were acclimatized for a week. Throughout the experiment, they were kept under standard laboratory conditions, with a reverse 12 h light/dark cycle (lights on from 1900 - 0700 hour) and handled daily. Approval of University of Georgia Animal Care and Use Committee was obtained before commencement of the experiment. The rats had access to chow and water *ad-libitum*. Due to the nature of the hormonal investigation, animals were fed a minimal phytoestrogen diet (Harlan Inc., 2016 Teklad Global) to avoid exogenous estrogenic intake. Additionally, to prevent stimulus-induced activation of the LC neurons and a change in noradrenergic tone, the rats were not tested for cyclicity by vaginal smears during the experiments (Poletini et al., 2012). Rather, serum estradiol levels were tested

at the time of sacrifice to show evidence of cycle phase or inhibition of ovulation as reported previously in our lab (Simone et al., 2015).

Rats (24) were randomly assigned a daily 0.2ml subcutaneous drug regimen of either 10 or 30 μg of EE (suspension of 5%EtOH and sesame oil) or vehicle alone (vehicle $n = 9$, EE_{10 μg} $n = 7$, EE_{30 μg} $n = 8$). EE 10 μg has previously been shown to prevent ovulation, and decrease endogenous estradiol levels in rats, thereby mimicking oral contraception in women without being supraphysiological (Kumar et al., 2000; Muhn et al., 1995; Spona, Schneider, Bieglmayer, Schroeder, & Pirker, 1979). The higher dose was chosen for a dose-response evaluation. The dose level was selected to account for the rapid metabolism of steroids by the rat liver (Coelingh Bennink, Heegaard, Visser, Holinka, & Christiansen, 2008). Throughout the study, the investigators were blinded to the drug regimen.

4.32 Behavioral Tests

Behavioral testing occurred between 0900 h and 1700 h. Since test order is shown to be non-significant (Hui-Yin et al., 2014), the order of the cognitive tests were novel object recognition (NOR), followed by novel object context recognition (NOC). Secondary to the staggered start date of injections, only 6-7 rats were tested per day; allowing each rat an hour rest before being tested in the next object recognition test. Unless otherwise stated, for all behavioral testing, lighting was 25–30 lux on the apparatus' floor with background white noise of 70 dB. Behavior was scored in real-time by a hidden investigator naïve to condition and was video recorded by a direct overhead webcam (Microsoft or Logitech). For inter-rater reliability, another trained naïve investigator scored the recorded videos. Before testing, all rats were habituated to the test area for 30-

minutes. Each rat was tested once per behavioral test. Data from some rats on some measures could not be collected or was considered invalid due to being statistical outliers. To prevent order effects, the order of rats was randomized. Between every trial, all equipment and testing chambers were cleaned with Vimoba (Quip Laboratory) and air dried.

4.32a Behavioral tests of locomotion and motility

Open Field Test: The testing chamber (60 × 40 × 33 cm, Sterilite) used for the open field exploration was the same box utilized for the object recognition tests. Day 25, the rats were placed in the chamber to explore for 10-minutes. Time spent in the perimeter or center squares (31 × 31 cm) and total distance traveled (locomotion) were analyzed. A second 10-minute habituation occurred within three days of the novel object testing.

Rotarod: The rotarod test, was used to rule out any drug-induced motor ataxia. Rats were placed on an accelerating rod starting at 4 rpm, with an arbitrary endpoint of 60 seconds at 40 rpm. The rats were scored on latency to fall, as well as the coordinated movement of all four limbs. The rats received three consecutive trials for acclimation, rested for 15-minutes then tested.

4.32b Behavioral tests of learning and memory

Novel Object/Context Recognition test: In object recognition tasks, the novelty is based on a change of one of the objects between trials (Ennaceur & Delacour, 1988). Testing was the same as our earlier experiment (Simone et al., 2015). The stimulus objects varied in size, shape, and material (metal, glass, plastic or ceramic). The largest height and width was 15.5 x 7 cm, and object bias had been ruled out before object choice. Objects were glued to the bottom of a small jar that could be screwed to a jar-lid fixed to the

floor, in one of the four quadrants of the chamber. The floor was covered in Sani-chips. The object and its placement was randomized and counterbalanced across treatment groups and never repeated for a given animal. Locomotion was defined by the rat's ambulatory time (secs) while exploration time was scored when the rat whisked/sniffed within 2 cm of the object center. Excluded was time spent climbing/sitting on objects. In the context recognition test, the novelty is based on the room surroundings (Dix & Aggleton, 1999). The test chamber—with its clear, vented, acrylic lid and translucent sides—allowed for visualization of the white walls in room one (white) and the black posters on all white walls in room two (poster). To further distinguish the two rooms, the white room was illuminated to 30 lux on the chamber floor, while the black poster room was lit to a 15 lux.

The format of recognition tests included three phases: first, familiarization phase (T1), 5-minutes; second, retention phase, 30-minutes for NOR; and third, test phase (T2), 3-minutes. In T1, the rat explored two identical objects. Return of the rat to its home cage with food/water access was the retention phase. In T2, the rat was placed in a chamber with one duplicate T1 object and the replacement of the other with a novel object; both in the same locations. In the context object recognition task, there are dual 5-minute familiarization phases back-to-back. One T1 for each room (white and poster) with paired objects differing between rooms. The retention phase is only 5-minutes due to the working memory demand. For T2, the rat was placed in a chamber randomized to one of the two rooms. The chamber contained one T1 object from each room in the same positions, resulting in one object out of context; consequently, it is novel to the rat.

The first two of the three-minute T2 phase were used as our final score since this has been shown to be the most sensitive period of exploration (Dix & Aggleton, 1999; Mumby, Gaskin, Glenn, Schramek, & Lehmann, 2002). Preference Index (PI), is defined as the ratio of novel object exploration time to total object exploration time (same object plus novel object). A PI of greater than 50% is evidence of learning defined as more time spent on novel object exploration.

Upon completion of the trials, the rats were euthanized. After deep anesthesia with CO₂, blood was obtained by a transcardial puncture. The blood was centrifuged in a serum separator tube at 2500 rpm for 30 min at 4°C (Beckman Model T J-6). The serum was aliquoted and stored at -20°C until analysis. Transcardial perfusion was performed with phosphate-buffered saline (PBS) and heparin, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. The brain was rapidly extracted, and hemi-dissected. One-half was processed in PFA at 4°C for 24 hours; immersed in 30% sucrose solution for 24 hours, then stored in -80°C freezer. Dissected from the remaining hemibrain, were the prefrontal cortex, hippocampus, and the dorsal rostral pons, containing the LC (fig. 4.2). All were stored at -80°C until further testing. The uterus was examined for ‘ballooning’ as a measure of hormone effect. The ovaries were removed and frozen until weighed.

4.33 Cell Molecular studies: ELISA Studies

Terminal serum estradiol was tested with ELISA (Calbiotech) which had a sensitivity of <3 pg/mL and intra-assay and interassay CVs of 3.1% and 9.9%. Dissected brain areas were weighed and analyzed per manufacturer’s recommendations with the following kits: Norepinephrine Research (Rocky Mountain Diagnostics, Inc. BA E-5200) Sensitivity 1.3 pg/ml, intra-assay CVs of 11.7; Normetanephrine Plasma ELISA Fast Track (Rocky

Mountain Diagnostics, Inc. BA E-8200, sensitivity of 23 pg/mL, intra-assay and interassay CVs of 9.5% and 10.6%; and Norepinephrine transporter (US Biological #152892), sensitivity of 0.112ng/ml, intra-assay and interassay CVs of <10.0% and <12.0%.

For ELISAs, the homogenization buffer was RIPA solution (25 mM Tris-HCL, 150 mM NaCl, 2 mM EDTA, 1% Triton-X-100, 1% Sodium deoxycholate, .1% SDS, pH 7.4) with 250 µl of Cocktail Protease Inhibitors (1:100 ratio; Millipore) (1:10 Wt./vol.) or PBS (1:2 Wt. /vol.), depending on the region. The extraction buffer for NE and NMN's was 0.01N HCl in the presence of 1mM EDTA and 4mM sodium metabisulfite (a 1:4 or 1:5 Wt. /vol.). Tissue was homogenized (PowerGen 125, Fischer Scientific) in the cold protein extraction buffers and centrifuged for 30-minutes 2500 rpm at 4°C (Beckman Model T J-6); then, the supernatant was decanted off and aliquoted for ELISA testing. As the NMN kit had plasma-based standards, the supernatants were diluted with plasma-based equalizing reagent (courtesy of Rocky Mountain Diagnostics), 1:2 to 1:4 depending on the area being evaluated. Absorbance was read on a microplate reader (Vmax Kinetic, Molecular Devices, Menlo Park, CA.) at 450nm and SoftPro Max 2.41 generated the curve-fitting algorithm.

4.4 Statistical Analysis

Only rats found to be in the in metestrus-early diestrus (met-di) based on serum 17β-estradiol level (Goodman, 1978; Nequin et al., 1979; A. A. Walf et al., 2006) were included as the control group. All scores were converted to percent of control and analyzed by ANOVA's, as well as, two-tailed planned *t*-tests. The effect size was described by partial eta squared (R^2) and Cohen *d* with the general guidelines that effect

size is considered small (if Cohen $d = 0.2$), medium ($d = 0.5$; clinically significant) and large ($d = 0.8$) (Cohen, 1977). Statistical exclusions were defined as outliers by GraphPad Prism 7.01, 2016 as a false discovery rate (FDR) of 1% in behavioral data and 2% in ELISA data since the variance is smaller. For statistical significance, the pre-selected critical value was $p \leq 0.05$, while $p \leq 0.10$ was considered a marginal effect.

4.5 Results

4.51 Ovarian Function

The vehicle mean 17β -estradiol was less than 10 pg/ml and the drug-treated rats had low 17β -estradiol levels ($\mu = 3.748$ and 4.339 for the $10\mu\text{g}$ and $30\mu\text{g}$ EE respectively) as expected for ovulation inhibition. There was no group mean difference ($F_{(2, 20)} = 1.100$, $p = 0.35$, $R^2 = 0.10$) (Fig. 4.3A). The ovarian mean weights were equivalent (**vehicle** = 0.040 g, **EE $10\mu\text{g}$** = 0.044 g, **EE $30\mu\text{g}$** = 0.043 g) resulting in no statistical difference between the groups ($F_{(2, 21)} = 0.400$, $p = 0.68$, $R^2 = 0.04$) (Fig. 4.3B). Ballooning of the uterus was seen exclusively, as well as in most of the drug-treated rats (**EE $10\mu\text{g}$** : 4 out of 7, **EE $30\mu\text{g}$** : 7 out of 8). Together, these findings are consistent with our previous study, which given the same drug and doses, evinced no oocytes within the oviducts (Simone et al., 2015).

4.52 Behavioral tests

Open field test: There was no significant group difference in the mean perimeter-time ($F_{(2, 19)} = 1.584$, $p = 0.23$, $R^2 = 0.14$; **EE $10\mu\text{g}$** : $t_{(12)} = 0.9047$, $p = 0.38$, Cohen $d = 0.48$; **EE $30\mu\text{g}$** : $t_{(13)} = 2.05$, $p = 0.06$, Cohen $d = 1.06$). Correspondingly, although EE $30\mu\text{g}$ drug-treated rats spent marginally more center-time when compared to vehicle rats ($F_{(2, 19)} = 1.603$, $p = 0.22$, $R^2 = 0.14$; **EE $10\mu\text{g}$** : $t_{(12)} = 0.9154$, $p = 0.38$, Cohen $d = 0.48$; **EE $30\mu\text{g}$** : $t_{(13)} = 2.05$, $p = 0.06$, Cohen $d = 1.06$).

(13) = 2.066, $p = 0.06$, Cohen $d = 1.07$) there was no mean difference center time nor perimeter-time between the two EE groups (Center-time: $t_{(13)} = 0.74$, $p = 0.47$, Cohen $d = 0.38$; Perimeter-time: $t_{(13)} = 0.742$, $p = 0.47$, Cohen $d = 0.38$). Locomotion—as scored by the number of squares entered (8cm^2)— was definitively equivalent between the groups ($F_{(2, 19)} = 0.045$, $p = 0.95$, $R^2 = 0.004$, **EE 10 μg** : $t_{(12)} = 0.2409$, $p = 0.81$, Cohen $d = 0.13$; **EE 30 μg** : $t_{(13)} = 0.2605$, $p = 0.80$, Cohen $d = 0.13$; **EE 30 μg vs EE 10 μg** : $t_{(13)} = 0.02505$, $p = 0.98$, Cohen $d = 0.01$).

Novel Object Recognition: During T1, locomotion (seconds), evaluated to rule out a lack of motivation, was not significantly different among the groups ($F_{(2, 18)} = 0.514$, $p = 0.61$, $R^2 = 0.05$; **EE 10 μg** : $t_{(12)} = 0.4964$, $p = 0.63$, Cohen $d = 0.27$; **EE 30 μg** : $t_{(13)} = 0.588$, $p = 0.57$, Cohen $d = 0.29$; **EE 30 μg vs EE 10 μg** : $t_{(11)} = 0.9504$, $p = 0.36$, Cohen $d = 0.54$) (Fig. 4.4A). Furthermore, during T1 all groups explored the two objects equally ($F_{(5, 36)} = 0.3957$, $p = 0.85$, $R^2 = 0.05$; **vehicle**: $\mu_{\text{obj1}} = 41.42 \pm 2.326$ SEM, $\mu_{\text{obj2}} = 40.2 \pm 5.569$, $t_{(14)} = 0.2023$, $p = 0.84$, Cohen $d = 0.10$; **EE 10 μg** : $\mu_{\text{obj1}} = 34.28 \pm 6.066$, $\mu_{\text{obj2}} = 42.88 \pm 6.251$, $t_{(10)} = 0.9874$, $p = 0.35$, Cohen $d = 0.57$; **EE 30 μg** : $\mu_{\text{obj1}} = 42.68 \pm 4.867$, $\mu_{\text{obj2}} = 44.82 \pm 7.428$, $t_{(12)} = 0.2407$, $p = 0.81$, Cohen $d = 0.12$). In T1, rats require 10 seconds of object interaction for reliable discrimination of objects in T2 (Akkerman et al., 2012); all included rats succeeded in this (Fig. 4.4B).

In T2, the vehicle group exceeded the 50% PI for discrimination between the same and novel object, exhibiting learning ($\mu = 66.83 \pm 3.158$ SEM, $t_{(7)} = 5.329$, $p < 0.01$, Cohen $d \approx 2.66$) (Fig. 4.5A). The two drug groups also met the greater than 50% criterion (**EE 10 μg** : $\mu = 60.73 \pm 1.831$ SEM, $t_{(5)} = 5.861$, $p < 0.01$, Cohen $d \approx 3.38$; **EE 30 μg** : $\mu = 72.47 \pm 3.652$, $t_{(6)} = 6.153$, $p < 0.01$, Cohen $d \approx 3.29$) (Fig 4.5A). In our preceding study, we saw

a decrease in learning in the NOR test by the low dose EE group. In this study, there was a negative marginal trend (one-tailed t comparison $p = 0.08$) in learning and memory in the EE 10 μ g group compared to vehicle ($F_{(2, 18)} = 3.29$, $p = 0.06$, $R^2 = 0.27$; **EE 10 μ g**: $t_{(12)} = 1.524$, $p = 0.15$, Cohen $d = -0.86$). Statistical power was weakened with the loss of one rat in each group. The former NOR study, exhibited a statistical increase in learning by EE 30 μ g group; however, in this study our findings were understated ($F_{(2, 18)} = 3.29$, $p = 0.06$, $R^2 = 0.27$; **EE 30 μ g**: $t_{(13)} = 1.175$, $p = 0.26$, Cohen $d = 0.60$). Yet, like the previous study we saw a significant dose-response between the two EE doses. The higher 30 μ g treated rats learned significantly better than the lower 10 μ g treated rats ($F_{(2, 18)} = 3.29$, $p = 0.06$, $R^2 = 0.27$; **EE 30 μ g vs EE 10 μ g**: $t_{(11)} = 2.723$, $p < 0.05$, Cohen $d = 1.56$) (Fig 4.5B).

Novel object context: The NOC has two 5-minute T1 phases to be evaluated. The greater than ten secs exploration of each object in the white room was adequate for T2 discrimination (Fig. 4.6A). Equivalent object 1 &2 exploration occurred ($F_{(5, 36)} = 1.619$, $p = 0.1801$, $R^2 = 0.1835$; **vehicle**: μ obj1 = 36.2 ± 4.245 SEM, μ obj2 = 33.46 ± 4.744 , $t_{(12)} = 0.4307$, $p = 0.67$, Cohen $d = 0.23$; **EE 10 μ g**: μ obj1 = 33.86 ± 4.123 , μ obj2 = 24.45 ± 2.543 , $t_{(12)} = 1.942$, $p = 0.08$, Cohen $d = 1.04$; **EE 30 μ g**: μ obj1 = 43.47 ± 8.076 , μ obj2 = 37.87 ± 4.043 , $t_{(12)} = 0.6197$, $p = 0.55$, Cohen $d = 0.37$) (Fig. 4.6A). Likewise in the poster room, object exploration time was adequate for T2 discrimination (Fig. 4.6B) and acceptable object 1&2 exploration occurred ($F_{(5, 36)} = 1.534$, $p = 0.2037$, $R^2 = 0.1757$, **vehicle**: μ obj1 = 40.95 ± 7.698 SEM, μ obj2 = 28.13 ± 5.386 , $t_{(12)} = 1.364$, $p = 0.1977$, Cohen $d = 0.73$; **EE 10 μ g**: μ obj1 = 49.83 ± 10.05 , μ obj2 = 33.45 ± 5.828 , $t_{(12)}$

= 1.41, $p = 0.1839$, Cohen $d = 0.75$; **EE 30 μ g**: μ obj1 = 50.57 ± 6.618 , μ obj2 = 48.98 ± 9.215 , $t_{(12)} = 0.1397$, $p = 0.8912$, Cohen $d = 0.07$) (Fig. 4.6B).

To simplify, T2 locomotion is described rather than the two-room T1 phase. There clearly was no discrepancy in locomotion between the groups ($F_{(2, 18)} = 0.0016$, $p = 0.9984$, $R^2 = 0.0001$; **EE 10 μ g**: $t_{(12)} = 0.006$, $p = 0.9953$, Cohen $d = 0.01$; **EE 30 μ g**: $t_{(12)} = -0.072$, $p = 0.9434$, Cohen $d = 0.10$; **EE 30 μ g vs EE 10 μ g**: $t_{(12)} = 0.040$, $p = 0.9683$, Cohen $d = 0.06$).

In NOC T2, the vehicle group exploration of the novel context object did exceed the 50% PI criterion for learning ($\mu = 55.18 \pm 3.367$ SEM, $t_{(6)} = 1.54$, $p = 0.1745$, Cohen $d \approx 0.82$) (Fig. 4.7A). In the drug-treated groups, only the EE 10 μ g group exceeded the 50% PI ($\mu = 58.15 \pm 6.30$ SEM, $t_{(6)} = 1.294$, $p = 0.2433$, Cohen $d \approx 0.69$), while the EE 30 μ g group did not evince learning, seen by a PI < 50% ($\mu = 44.17 \pm 3.55$ SEM, $t_{(6)} = 1.642$, $p = 0.1517$, Cohen $d \approx -0.87$) (Fig 4.7A). There was no difference between the vehicle and EE 10 μ g group in the ability to prefer the novel object over the same object ($F_{(2, 18)} = 2.559$, $p = 0.1052$, $R^2 = 0.2214$; **EE 10 μ g**: $t_{(12)} = 0.4151$, $p = 0.6854$, Cohen $d = 0.22$) (Fig 4.7B). On the other hand, the EE 30 μ g group demonstrated a significant decrease in their ability to prefer the novel over the same context object compared to the vehicle group ($F_{(2, 18)} = 2.559$, $p = 0.1052$, $R^2 = 0.2214$; **EE 30 μ g**: $t_{(12)} = 2.251$, $p < 0.05$, Cohen $d = -1.20$) (Fig 4.7B). There was a trend for the lower EE group to learn better in the context learning than the higher group ($F_{(2, 18)} = 2.559$, $p = 0.1052$, $R^2 = 0.2214$; **EE 10 μ g vs EE 30 μ g**: $t_{(12)} = 1.933$, $p = 0.08$, Cohen $d = 1.03$) (Fig. 4.7B).

Rotarod: The rotarod test confirmed there was no drug-induced alterations in locomotion. Latency to fall times (secs) showed no group differences ($F_{(2, 21)} = 0.676$, $p = 0.5194$, R^2

= 0.0605; **EE 10 μ g**: $t_{(14)} = 1.21$, $p = 0.2465$, Cohen $d = 0.58$; **EE 30 μ g**: $t_{(15)} = 0.8807$, $p = 0.3924$, Cohen $d = 0.42$; **EE 30 μ g vs EE 10 μ g**: $t_{(13)} = 0.2837$, $p = 0.7811$, Cohen $d = 0.15$). Asynchronous walking was not evident in any group.

4.53 Cell Molecular studies: ELISAs

Norepinephrine: The quantity of rats' brain tissue was reduced by one per group. In the LC, no difference in NE was observed between the vehicle-treated rats and the drug-treated rats ($F_{(2, 18)} = 1.156$, $p = 0.3369$, $R^2 = 0.1139$; **EE 10 μ g**: $t_{(12)} = 0.8178$, $p = 0.4294$, Cohen $d = 0.45$; **EE 30 μ g**: $t_{(13)} = 1.448$, $p = 0.1713$, Cohen $d = 0.76$) (Fig. 4.8). Similarly, no dose-dependent difference between the two drug groups occurred (**EE 30 μ g vs EE 10 μ g**: $t_{(11)} = 0.6392$, $p = 0.5358$, Cohen $d = 0.36$).

In the hippocampus, there was a significant difference between the means in the hippocampal area; the EE 30 μ g drug-treated rats showed a significant decrease in the NE level compared to vehicle ($F_{(2, 15)} = 3.887$, $p < 0.05$, $R^2 = 0.3413$; **EE 10 μ g**: $t_{(10)} = 1.839$, $p = 0.09$, Cohen $d = -1.06$; **EE 30 μ g**: $t_{(10)} = 2.196$, $p = 0.05$, Cohen $d = -1.27$) (Fig. 4.9). There was not a significant difference between the high and low dose EE because of the trend toward a decrease NE level in the low dose EE ($t_{(10)} = 0.969$, $p = 0.3554$, Cohen $d = 0.56$).

In the prefrontal cortex, there was a significant difference among the group means presented in this brain area ($F_{(2, 16)} = 8.203$, $p < 0.01$, $R^2 = 0.5063$). NE rose with the EE treatments and was significantly greater than vehicle in the EE 30 μ g group (**EE 10 μ g**: $t_{(11)} = 1.921$, $p = 0.08$, Cohen $d = 1.04$; **EE 30 μ g**: $t_{(11)} = 3.523$, $p < 0.01$, Cohen $d = 1.88$) (Fig. 4.10). Additionally, there was a dose-response difference noted between the two

levels of drugs ($t_{(10)}=2.299$, $p < 0.05$, Cohen $d = 1.33$) such that a step up to 30 μ g resulted in a significant increase in NE.

Normetanephrines: The LC NMN results did follow the trend of the LC NE results ($F_{(2,11)} = 3.17$, $p = 0.08$, $R^2 = 0.3656$; **EE 10 μ g:** $t_{(8)}=0.313$, $p = 0.76$, Cohen $d = 0.20$; **EE 30 μ g:** $t_{(7)}=2.219$, $p=0.06$, Cohen $d = 1.50$) (Fig. 4.8).

Evaluation of the hippocampal tissue for NMN also mimicked the hippocampal NE results, with its group differences and significant decrease in NMN in the high dose EE ($F_{(2,19)} = 6.987$, $p < 0.01$, $R^2 = 0.4238$; **EE 10 μ g:** $t_{(13)}=1.599$, $p = 0.13$, Cohen $d = 0.83$; **EE 30 μ g:** $t_{(13)}=3.929$, $p < 0.01$, Cohen $d = 2.10$) (Fig. 4.9). This also follows in the dose-dependent decrease in NMN seen between the drug treatments (**EE 30 μ g vs EE 10 μ g:** $t_{(12)}=2.178$, $p = 0.05$, Cohen $d = 1.16$) (Fig. 4.9).

In the prefrontal cortex, the EE 30 μ g group was insufficient in power after the loss of four outliers, while there were two outliers in the vehicle group. The NMN levels for EE 10 μ g mimic the rise in NE levels seen in the prefrontal cortex but does not reach significance (**EE 10 μ g:** $t_{(10)}=1.527$, $p = 0.1578$, Cohen $d = 0.88$) (Fig. 4.10).

Norepinephrine transporter: For the LC area, a between-group difference was seen in this brain area, with the low dose EE showing a significant rise in NET protein ($F_{(2,19)} = 4.166$, $p < 0.05$, $R^2 = 0.30$; **EE 10 μ g:** $t_{(12)}=2.216$, $p < 0.05$, Cohen $d = 1.17$; **EE 30 μ g:** $t_{(14)}=0.007$, $p = 0.99$, Cohen $d = 0.00$) (Fig. 4.8). This rise in NET in the low dose group contrasted significantly from the no change in NET seen in the EE 30 μ g dose and proved to be statistically different between the drug doses (**EE 30 μ g vs EE 10 μ g:** $t_{(12)} = 2.582$, $p < 0.05$, Cohen $d = 1.32$) (Fig.4.8).

In the hippocampus, a decrease in the magnitude of NET protein (ng/mg) compared to the LC is consistent with the LC being predominately noradrenergic in function (vehicle: μ (HC) = 0.13 ± 0.02 , μ (LC) = 0.86 ± 0.16 SEM; $t_{(15)} = 4.731$, $p < 0.001$, Cohen $d = -2.22$). However, within the hippocampus, there was no group mean differences in NET protein ($F_{(2, 21)} = 0.3019$, $p = 0.74$, $R^2 = 0.0279$; **EE 10 μ g**: $t_{(14)} = 0.5653$, $p = 0.58$, Cohen $d = 0.28$; **EE 30 μ g**: $t_{(15)} = 0.2594$, $p = 0.79$, Cohen $d = -0.12$) nor between the drug group doses (**EE 30 μ g vs EE 10 μ g**: $t_{(13)} = 0.7153$, $p = 0.49$, Cohen $d = 0.38$) (Fig 4.9).

The remaining prefrontal supernatant was insufficient to perform the NET ELISA.

4.6 Discussion

One-quarter of women using contraception choose a hormonal method which equates to 10.6 million women in the U.S. (Jones, Mosher, & Daniels, 2012). Of those contraceptive hormone users, approximately 3.5 million women used a formulation with less than 30 μ g EE, while 42,000 women used one with no EE (Hall & Trussell, 2012). This work has clinical significance, with its examination of dose-dependent cognitive effects of the principal estrogen in contraceptive therapy.

Endogenous 17 β -estradiol levels are expected to decrease to early follicular levels as a result of contraceptive hormone use, an effect that occurs with the inhibition of ovulation (Vandever et al., 2008). EE 10 μ g has been proven to inhibit ovulation in intact rats (P. Andrews et al., 2002; Coelingh, et al. 2008; Simone et al., 2015). In agreement with our previous study, both low and high drug treatments brought about low 17 β -estradiol levels (Fig 4.3A). Therefore, we used met-di rats, shown to be in a low estradiol state, as controls (Goodman, 1978).

The estrogen state of the subject is particularly relevant since variable outcomes of cognitive testing are seen depending on the estrogen phase of the subject. Specifically, improved working memory frequently is observed during the high-estrogen phase of a menstrual cycle (Hampson & Morley, 2013; Rosenberg & Park, 2002). Similar findings exist in rodent studies. For example, rats in the high estrogen state of estrus demonstrate a greater non-spatial working memory of novel in the object recognition test (A. A. Walf et al., 2006). Improved cognitive function has been seen in humans (Brinton, 2009; Rocca et al., 2007) as well as rats (Jacome et al., 2010; Takuma et al., 2007; A. A. Walf et al., 2006) when 17β -estradiol is supplemented in a low endogenous estradiol state. Not all studies show comparable results, though, and depend on which memory tests or type of estrogens utilized. Contraceptive hormone users, compared to naturally cycling women, had outcomes ranging from impaired verbal fluency (Griksiene & Ruksenas, 2009), to no difference seen (K. L. Mordecai et al., 2008). At different estrogen levels, visuospatial memory tests commonly show the opposite results from verbal memory.

Correspondingly, women on contraceptive hormones with low endogenous estrogen perform better on spatial tests (Beltz et al., 2015; McCormick & Teillon, 2001; Wharton et al., 2008). Our findings in NOC followed these findings with the higher EE group unable to reach a greater than 50% preference for the novel object.

NOR tests use the rats' innate proclivity to seek out novelty in its environment as an assessment of short-term memory. The shorter the retention phase, the greater the evidence of learning. Novel object tests can effectively test for delay-dependent impairment of declarative memory (Baker & Kim, 2002; Clark, Zola, & Squire, 2000; Nemanic, Alvarado, & Bachevalier, 2004). With a 30-minute retention interval, the

preference for the novel object appears to be the same as with a 2-minute retention interval (Hale & Good, 2005). A short retention phase can result in twice the amount of time exploring the novel to the familiar object (Clark & Martin, 2005). However, preference for the novel object declines as the retention interval increases in rodents. (Hale & Good, 2005). Previously with a 45-minute retention phase, we saw a decrease in learning in the EE 10 μ g-treated rats compared to 30 μ g treatment in the object recognition tests. This current study with its 30-minute retention phase was designed to diminish the delay-dependent impact on working memory, allowing the drug functionality to be more conspicuous. We found that the PI of the vehicle or drug groups was non-significantly changed with the 45-minute vs 30-minute retention intervals ($\mu_{(\text{vehicle } 45 \text{ min})} = 62.47 \pm 2.725$, $\mu_{(\text{vehicle } 30 \text{ min})} = 66.83 \pm 3.158$, $t_{(26)} = 0.9139$, $p = 0.37$; $\mu_{(\text{EE } 10\mu\text{g } 45\text{min})} = 53.69 \pm 4.323$, $\mu_{(\text{EE } 10\mu\text{g } 30\text{min})} = 60.73 \pm 1.831$, $t_{(11)} = 1.41$, $p = 0.19$; $\mu_{(\text{EE } 30\mu\text{g } 45 \text{ min})} = 70.47 \pm 4.169$, $\mu_{(\text{EE } 30\mu\text{g } 30 \text{ min})} = 72.47 \pm 3.652$, $t_{(13)} = 0.3552$, $p = 0.73$). We had anticipated the 30-minute retention phase in this study would negate the decrease in learning seen in the EE 10 μ g group. Instead, there was a persistent decrement (albeit, marginal in this study) in the PI in the EE 10 μ g group compared to its respective control. With the EE 10 μ g treatment, 82 % rats were below the mean PI of the vehicle group (Cohen's U_3), regardless of the more simplistic delay interval. Gonadal-intact females in any cycle phase significantly discriminate between novel and familiar at retention intervals as much as six times greater (three hours) or as little as six times less (5 minutes) than our 30-minute inter-trial delay (Sutcliffe et al., 2007). Galea et al. 2001, found that estrogen supplementation had no effect in ovariectomized rodents in a delay-dependent memory

task. Consequently, the subtle decrease in PI in this study—in similar 17β -estradiol-state rats—appears to be independent of working memory demand.

Furthermore, EE causes no motoric effects as demonstrated by the open field and rotarod findings confirming the cognitive effect is drug-dependent. In our earlier NOR experiment the PI was statistically different for the two EE groups, (**EE 30 μ g vs EE 10 μ g**: $t_{(13)} = 4.22$ $p = 0.001$, Cohen $d = 2.20$) as was found to be true in this study (**EE 30 μ g vs EE 10 μ g**: $t_{(11)} = 2.723$, $p < 0.05$, Cohen $d = 1.55$). The EE 30 μ g groups selected the novel object over the familiar object a greater amount of the time than the EE 10 μ g, which is 93% of the EE 30 μ g group will be above the EE 10 μ g mean (Cohen U_3). This is consistent with other authors' findings of rodents' dose-dependent effects of estrogen in the NOR test. Moderate range/dose of estrogen facilitates memory, and lower does not (inverted U) (Inagaki et al., 2010; Paris & Frye, 2008; van Goethem et al., 2012; A.A. Walf et al., 2009). In other models of learning, supplementation to a physiological estrogen state proved to be beneficial (Singh et al., 1994). Non-replenishment of estrogen in an ovariectomized deprivation rodent model rendered the rat incapable of discriminating between familiar and novel objects in a NOR task with a 4-hour retention phase delay (when before ovariectomy they could) (Wallace, Luine, Arellanos, & Frankfurt, 2006). Our results support earlier findings that in a chronically low endogenous hormone state, a threshold for the beneficial effect of EE on short-term memory processing may exist.

Like endogenous estradiol, this dose-response of EE may change per strategies used, spatial vs. non-spatial. Contextual learning—distinguishing novel vs. familiar objects—by the room surrounds, may be considered a variant of visuospatial memory. We found a

significant difference between the EE 30 μ g and the vehicle group, with the EE 30 μ g group not reaching the 50% PI; EE 30 μ g did not distinguish the novel out-of-context object over the in-context object. In contrast, the lower dose EE-treated rats exhibited learning in the contextual paradigm, both in the ability to exceed the 50% PI, as well as a marginally significant increase ($p = 0.08$, Cohen $d = 1.03$) in novel preference compared to the EE 30 μ g group. These results are consistent with the finding that women in low estrogen states perform better in spatial aptitude than when in the high-estrogen phase of the menstrual cycle (Hampson, 1990). Beltz et al. 2015, specifically looked at the effect of EE on spatial abilities by comparing normal cycling women to women taking contraceptive hormones. Results showed that women on a birth control pill with a median range of 20 μ g (monophasic) of EE performed better in spatial tests (mental rotations) than one with a median range of 35 μ g (tri-phasic). Improvement in working-reference memory tasks are inversely related to estrogen levels in rodents and humans (C. A. Frye & Sturgis, 1995; Galea et al., 2001; Hampson et al., 2014; Maki, Rich, & Rosenbaum, 2002). It has been shown to heighten if the task increased in complexity (Hampson et al., 2014; K. Phillips & Silverman, 1997). Higher luteal phase estrogen was associated with poorer scores on mental rotation tests compared to contraceptive users and menses phase cycling women in accord with our findings (McCormick & Teillon, 2001). Though this estrogen level differential on spatial memory is not conclusive across all studies (Gordon & Lee, 1993; Kozaki & Yasukouchi, 2009).

In spatial-novel object tasks, the influence of circulating estrogen levels on performance of the rat is inconclusive. Sutcliffe et.al. 2007, found female rats tested in novel place recognition—where the novelty is the change in location of the familiar objects—

performance was enhanced in the low estrogen phase of the rats' cycle compared to the high. Conversely, Paris and Frye (2008), found in the same memory model, that intact rats in high estrogen states (behavioral proestrus) outperformed rats in a low estrogen state (diestrus). Attributing the outcomes in these studies only to estrogen levels can be shortsighted, for many other variables come into play, including 'cross-talk' between all gonadal steroids/receptors (Fox, 1975; Rabinowitz, Cohen, Finn, & Stackman, 2014; Strom et al., 2011). Our earlier research in intact rats demonstrated that the androgenic progestin—levonorgestrel—improves NOC performance in the low dose-treated rats (20µg/rat/day); yet not in the high (60µg /rat/day) (Simone et al., 2015). These experiments support a dose-response curve to endogenous/exogenous steroids influence on spatial-contextual recognition tests. Nevertheless, the oppositional results seen in NOR and NOC may represent differences in prefrontal and hippocampal recruitment. Areas involved in memory processing of object recognition tests—object, location, and context—include the prefrontal cortex, the hippocampus and adjacent cortical areas (G. R. Barker, Bird, Alexander, & Warburton, 2007). Specific brain regions may play more important roles for different types of memory. For example, hippocampal functioning appears to be more relevant for spatial and thus contextual learning (Goulart et al., 2010; I. Lee, Hunsaker, & Kesner, 2005; Matus-Amat, Higgins, Barrientos, & Rudy, 2004; Rudy, Barrientos, & O'Reilly, 2002), with deficits impairing memory (Mumby et al., 2002). Although, there is some controversy whether inactivation of the hippocampus compromises NOR (Ainge et al., 2006; Broadbent et al., 2010; Clark et al., 2000), a consensus exists that there is a loss in NOC learning (Dere, Huston, & De Souza Silva, 2007; Mumby et al., 2002; Norman & Eacott, 2005). In fact, Gilbert & Kesner (2002)

demonstrated that the specific pairing of the object with its location (what and where) requires the hippocampus, whereas the identity of the object alone does not. Moreover, Piterkin P. et al. (2008) found that when the context of the room was changed, rats with hippocampal lesions were unable to reach a significantly greater than 50% PI. This preference for the in-context object is consistent with our findings in the EE 30 μ g-treated rats. Our results in NOC suggest a change in hippocampal processing associated with the dose of EE. The hippocampus is highly sensitive to the regional estrogenic tone (J.M. Daniel, Fader, Spencer, & Dohanich, 1997; Hamson et al., 2016; Mazzucco et al., 2006; Prange-Kiel et al., 2008; C. S. Woolley, 1998); this may include the potent estrogen—EE—though it is much less studied (Beltz et al., 2015; Lacreuse et al., 2002). Dose differential in spatial and object learning may also be linked to a change in transmitter release or binding to receptors at select areas of the brain.

The coeruleo-cortical noradrenergic system has been shown to be necessary for cognitive flexibility in novel and context learning (Aston-Jones & Cohen, 2005; Tait et al., 2007). EE has been shown to have direct and indirect consequences on the NE levels in select areas of the brain (Ghraf et al., 1983; Greengrass & Tonge, 1974; Kendall et al., 1977; Tonge & Greengrass, 1971). Marchi & Cugurra (1974) demonstrated a decrease in monoamine oxidase enzyme activity in the brain after a month of contraceptive hormones treatments, which has typically been correlated with the EE component. The second enzyme that metabolizes NE—catechol-O-methyltransferase (COMT) is also decreased by estrogen therapy (McDermott, Liu, Ade, & Schrader, 2015). Furthermore, NE levels can be altered by the action of EE as an NRI. In a comprehensive review of NETs, Eisenhofer 2001, points out that “catecholamine transporters function as important

modulators of neuronal transmission, and differences or changes in the efficiency of uptake can exert profound effects on the intensity, duration, and spread of transmitter action.” Lastly, the NE biosynthetic enzyme, tyrosine hydroxylase, may be altered by estrogens (Sabban et al., 2010; L. Serova et al., 2002), which is not surprising given the existence of an estrogen response element in the promotor region of the tyrosine hydroxylase gene (Kvetnansky et al., 2009). Our earlier data showed an EE dose-response on tyrosine hydroxylase mRNA in the LC, with EE 10 μ g treatment decreasing mRNA thus possibly modulating NE levels into different regions (Simone et al., 2015). In this study, we examined NE, normetanephrine, and NET protein levels at three brain regions.

NE can be released in the LC in response to physiological stimuli and drug treatments, as well as in response to the rate of firing of the LC noradrenergic neurons (Singewald & Philippu, 1998). Given this, we saw no significant group differences in LC-norepinephrine levels. This lack of dissimilarities may be a consequence of local parameters, such as a higher density of somatodendritic autoreceptor α 2AR in the LC than in other areas of the brain (Boyajian, Loughlin, & Leslie, 1987). An initial increase in NE in synaptic or axon collaterals may feed back to autoreceptors, decrease the firing rate, and normalize the NE level in the LC. Microdialysis studies of low versus high norepinephrine reuptake inhibitors have opposing dose responses in cortical areas as seen in our study. A dose of 0.8mg/kg of desipramine completely inhibited the firing of the LC (Alba-Delgado et al., 2012) and decreased cortical NE, which resolved with α 2AR antagonist treatment (Mateo, Pineda, & Meana, 1998). On the other hand, a dose of 3

mg/kg or higher resulted in an increase in cortical NE (Mateo et al., 1998) as a result of changed LC firing.

Surgically menopausal aged rhesus monkeys treated with EE in doses typical of oral contraceptive use were tested in cognitive paradigms equivalent to NOR and NOC.

Parentetically, there was no significant performance difference between the vehicle and EE-treated groups in a test analogous to the NOR test (Lacreuse et al., 2002). Yet, in the test similar to an NOC paradigm, the EE group outperformed the estrogen deficient control (Lacreuse et al., 2002). The EE benefit may be reflective of NE levels.

Diarylpropionitrile, an ER β -selective agonist, decreased NE activity (NE/MHPG) in the CA1 area of the hippocampus while improving the performance on a spatial NOR test (Jacome et al., 2010). In the same way, ovariectomized rats treated with 17 α and 17 β -estradiol, evinced a 49% statistical decrease in CA1 whole tissue NE (HPLC) when compared to controls (Inagaki et al., 2010). We saw a 43% decrease ($p=0.09$, Cohen $d = 1.06$) in NE level in the total hippocampus in the low dose EE group and a statistical 52% decrease ($p=0.05$, Cohen $d = 1.27$) in the EE 30 μ g-treated group vs. vehicle.

Simultaneously, compared to vehicle, a 31% decrease ($p = 0.13$, Cohen $d = 0.83$) in the NMN tissue level occurred in the EE 10 μ g, while a 61% decrease ($p < 0.01$, Cohen $d = 2.10$) occurred in the EE 30 μ g group. This loss of NE to extracellular metabolism rather than reuptake does not result in an increase in tyrosine hydroxylase activity but rather a decrease; thus the synthesis of NE is increased minimally (Eisenhofer et al., 2004). These authors postulate a new equilibrium is reached by compensation within the neuron by reduced vesicular leakage. Therefore, turn-over rate (NMN/NE ratio) will not differ between the groups despite the inhibitory action of EE on reuptake; as seen in this study.

Within the hippocampus, however, the dose of EE plays a functional part since the greater decrement in NE coincides with a loss of NOC learning.

For cognitive processing of novelty, the prefrontal cortex appears to be required in object recognition tests (Wallace, Frankfurt, Arellanos, Inagaki, & Luine, 2007). The prefrontal cortex manages episodic memories and coordinates with medial temporal lobe to complete the mnemonic process, and this connectivity is remarkably similar across mammalian species (Dickerson & Eichenbaum, 2010). Like humans with dorsolateral prefrontal cortex damage, rats with medial prefrontal lobe damage lost the recollection of new items but retained the identification of old items (familiarity) (Farovik, Dupont, Arce, & Eichenbaum, 2008). Optogenetic silencing of the LC in rodents resulted in decreased NE in the prefrontal cortex (Carter et al., 2010) concurrent with the impaired acquisition of memory that required attentional shift setting (Janitzky et al., 2015). While decreases in NE have been shown to impair cognitively driven tasks, increases have been shown to occur when the task involved novel stimuli (T. W. Robbins, 2002). Velley et al. 1991, found that acquisition of memory correlated with the stimulus of the LC. Low dose psychostimulants and NRIs all increase NE in the prefrontal cortex preferentially over other cortical and subcortical areas (Lapiz, Bondi, & Morilak, 2007; Schmeichel & Berridge, 2013). Lapiz et al. (2007), saw approximately a 300% increase in prefrontal cortex NE in the acute desipramine-treated (NRI) rats; we saw approximately a 250% increase in the EE 30 μ g treated rats. The increase seen in NE levels may have improved the processing of novel in the NOR test. However, since a dose-response was seen, there appears to be an optimal level of noradrenergic tone for ideal prefrontal cortex

functioning (Carter et al., 2010). The mechanism of action to obtain this optimal balance may be through NETs and $\alpha 2AR$.

NE released is rapidly removed to terminate transmission and this is accomplished predominantly by NET (Eisenhofer, 2001). NETs are found only on noradrenergic soma, axons, and dendrites and are prevalent in the prefrontal cortex axons (Schroeter et al., 2000). NETs outside synaptic membranes regulate the transmitter levels in the extracellular space (Zoli et al., 1999). In the LC, intracellular immunolabeling of NET protein suggests a significant synthesis of NET, and notably, it is the densest NET labeled region (Schroeter et al., 2000). In the hippocampus, there is a regional distribution of NETs, with the dentate having the most NET fibers, CA2/3 with fewer fibers and CA1 with sparse fibers (Schroeter et al., 2000). Additionally, there are regional changes that occur in NET with exposure to drugs or physiological factors like a change in hormones or stress (Jeannotte, McCarthy, Redei, & Sidhu, 2009). There is an upregulation of NETs mRNA with chronic aversive stress in estrogen-deficient rats in the hippocampus and prefrontal cortex, but not in the LC, (Charoenphandhu, Nuntapornsak, Wongdee, Krishnamra, & Charoenphandhu, 2013). Chronic treatment of Wistar rats with desipramine led to a decrease of NETs in frontal cortex and hippocampus but not in the LC (Jeannotte, McCarthy, & Sidhu, 2009). Whereas, chronic treatment with desipramine in Sprague -Dawley rats showed a decrease in NETs in the hippocampus but not in the cortex or LC (Bauer & Tejani-Butt, 1992). NE levels alter NETs; for example, a decrease in NE results in a decrease in NETs (Cheetham, Viggers, Butler, Prow, & Heal, 1996; Klimek et al., 1997). However, Erickson et al. 2011, demonstrated in rats treated with desipramine a decrease in tyrosine hydroxylase immunolabeling in the prefrontal cortex

terminals but did not demonstrate a decrease in NETs. They felt this indicated a post-translational rather than transmitter-induced mechanism for regulating NET activity (Erickson et al., 2011). In summary, there is considerable variability in NETs, and our findings of an increase in NETs in the LC and not in the hippocampus may be due to specific actions of EE as a hormone and/or as an NRI in a dose-responsive manner. In this experiment, there was insufficient prefrontal cortical tissue to perform the NET Elisa. However, plans are to further evaluate the dose-dependent effect of EE on NET mRNA/protein in the coeruleo-cortical noradrenergic system.

In the prefrontal cortex, the unusual competition between NE and dopamine for reuptake through NET creates a higher sensitivity to even moderate increases of NE (Berridge & Arnsten, 2015). The competition modulates the post-synaptic α 2AR in the prefrontal cortex to function optimally at moderate levels of NE (inverted U) (Berridge & Spencer, 2015) This may explain why the EE 30 μ g-treated rats performed better in the NOR non-spatial form of learning. Shackelford Jr et al. (1988), found that the density of different adrenergic receptors is dependent on the type of estrogen used to treat rats. Estradiol did not increase α 2AR in the prefrontal cortex following stress conditioning (Shansky, Bender, & Arnsten, 2009). The analog—EE, caused a significant decrease of the α 2AR in the LC and the frontal cortex; this was felt to be due to an increase in noradrenergic activity (Shackelford Jr et al., 1988). Therefore, future investigations will include evaluations of NE by microdialysis and α 2AR immunolabeling.

4.7 Conclusion

The aim of our study was to probe our preliminary data further, examining cognitive behavior with changes in NE and NET levels in intact rats exposed to two doses of EE,

which had never been studied before. The results suggest a dose-dependent beneficial effect of EE on spatial and non-spatial learning. This inverse-U response suggests an optimal lowest dose to EE supplementation, in combination contraceptives, to witness cognitive benefits. We will continue ongoing assessments since many avenues need to be explored. As 'no man is an island,' no hormone stands alone. Given the scale of contraceptive hormones usage, the cross-species relevance is significant.

4.8 Figures

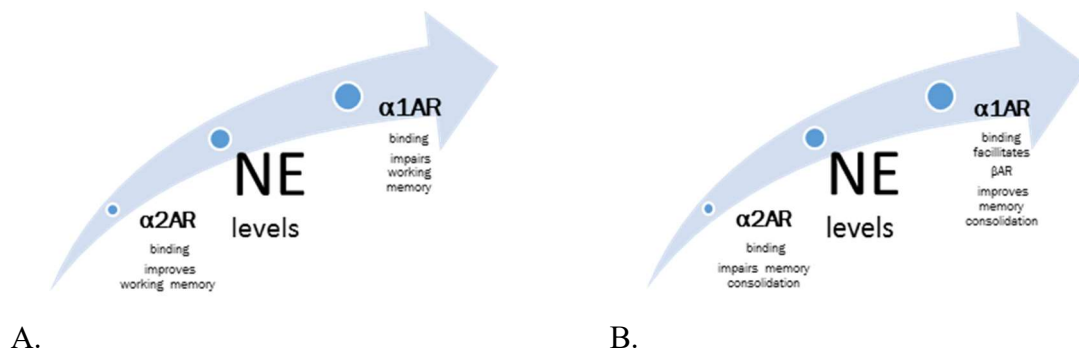
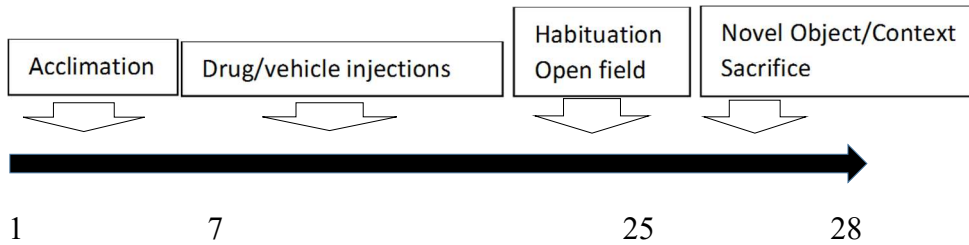


Figure 4.1 Norepinephrine and alpha-adrenergic receptors—gradations in response
A. Prefrontal Cortex: Lower norepinephrine (NE) bind preferentially to $\alpha 2$ adrenergic receptors ($\alpha 2AR$) due to greater affinity. Rising NE levels allow for binding to $\alpha 1$ adrenergic receptors ($\alpha 1AR$) and have been shown to impair working memory (Arnsten, Mathew, Ubriani, Taylor, & Li, 1999). B. Hippocampus: Reverse from the prefrontal cortex, in the hippocampus the preferential binding to $\alpha 2AR$ at low levels of NE results in impaired memory consolidation. Contrastingly, rising levels of NE in the hippocampus improve memory consolidation by binding to $\alpha 1AR$. (adapted from Arnsten, 2000).

Timeline for the experiment



Experiment timeline. The start date for the drug/vehicle 21-day regimen was staggered to allow small group testing in the behavioral paradigms and sacrifice each day.

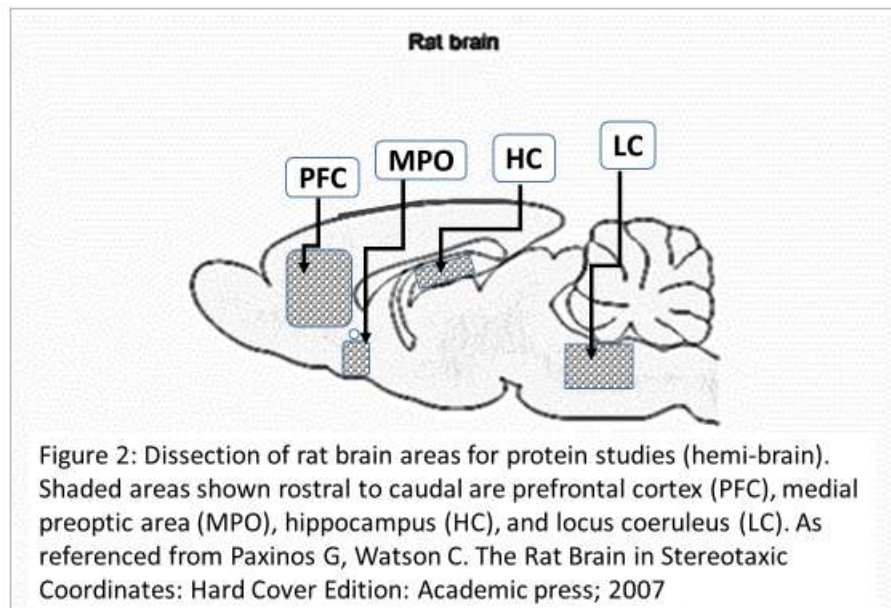


Figure 4.2 Diagram of brain dissections

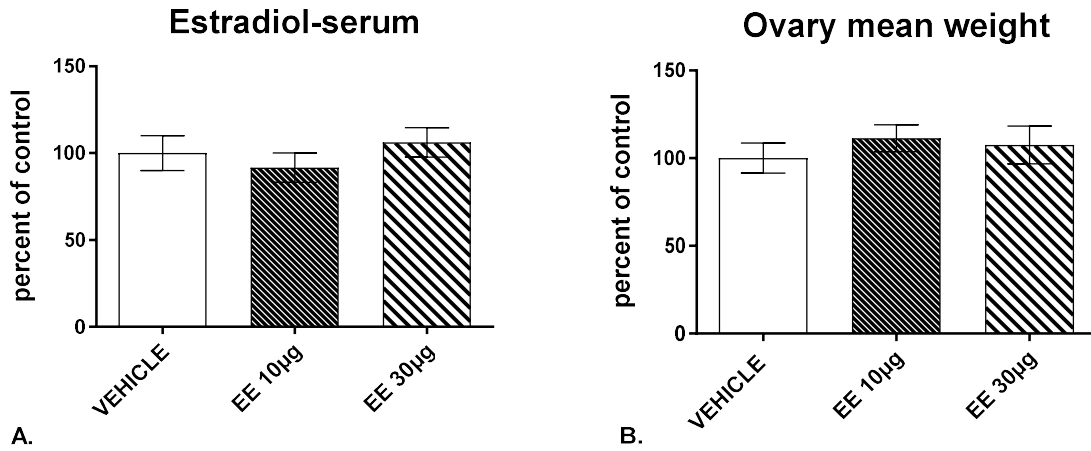


Figure 4.3 Biophysical response to ethinyl estradiol

A. Estradiol percent of control. The vehicle control groups fell into the metestrus-early diestrus range of serum 17β -estradiol levels. Similarly, both ethinyl estradiol groups evinced ovarian suppression by diminished estradiol levels. B. Ovary weight. No difference was seen between the groups in ovarian weight.

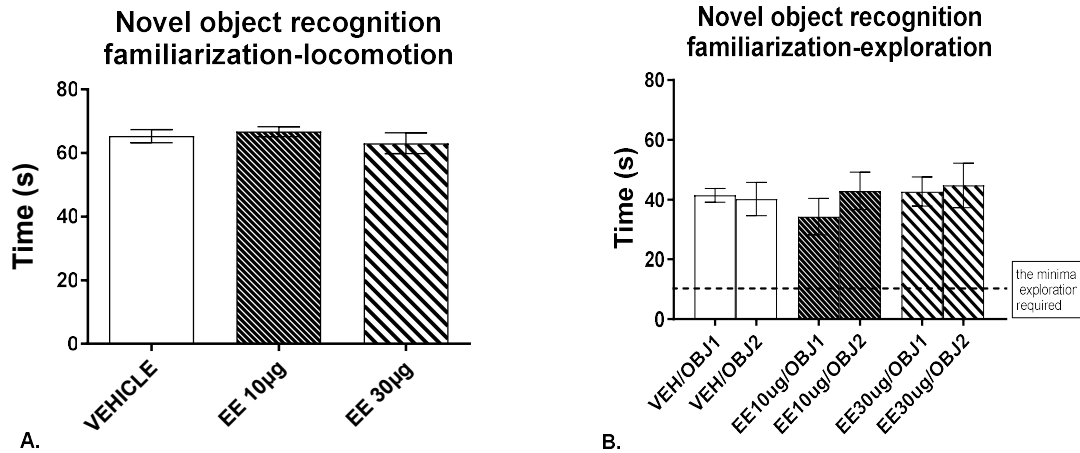


Figure 4.4 Behavioral tests of learning and memory: novel object recognition familiarization

A. In the familiarization phase of the novel object test (T1), there was no statistical difference in the locomotive activity between the control and the drug treated rats ($F(2, 18) = 0.514, p = 0.61, R^2 = 0.05$). Therefore, motility was not a confounding variable. B. All groups met the minimal requirement of 10 seconds of exploration of each object needed to assure a reliable discrimination between the objects in the testing phase of the NOR test (Akkerman et al., 2012). Additionally, there was no difference in the exploration of each object for each group (see results section). (Veh = vehicle, Obj1 = object 1, Obj2 = object 2)

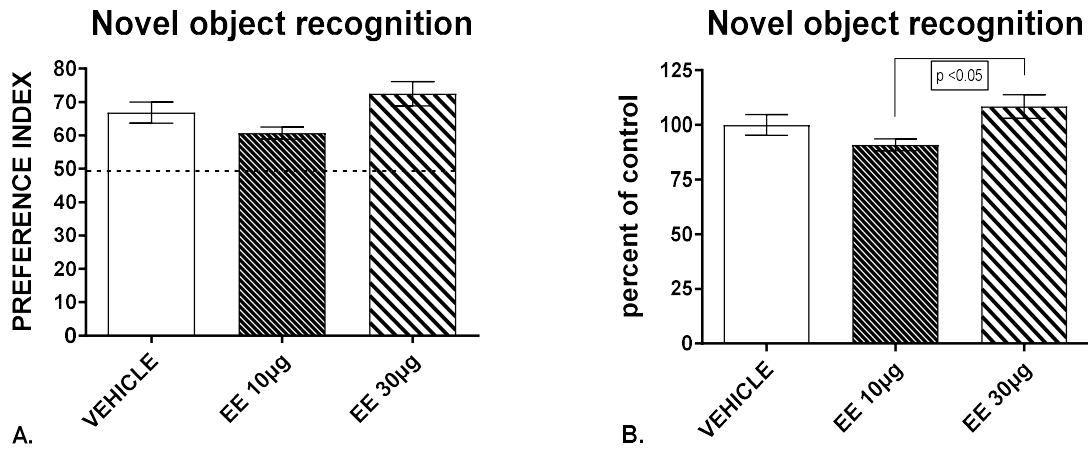


Figure 4.5 Behavioral tests of learning and memory: novel object recognition test

A. Preference index (PI) is the ratio of the novel object exploration versus the exploration of both objects, novel and same. A PI significantly greater than 50% is an indicator of learning and memory; this was seen to occur in all the rat groups. B. A notable increase in learning and memory was seen in the EE 30µg treated rats compared to the lower EE 10µg treated rats ($p < 0.05$). A clinically significant effect size was seen between the vehicle control (Cohen $d = -0.86$) and the lower dose EE 10µg group, suggesting the vehicle group learned better than the EE 10 µg treated rats.

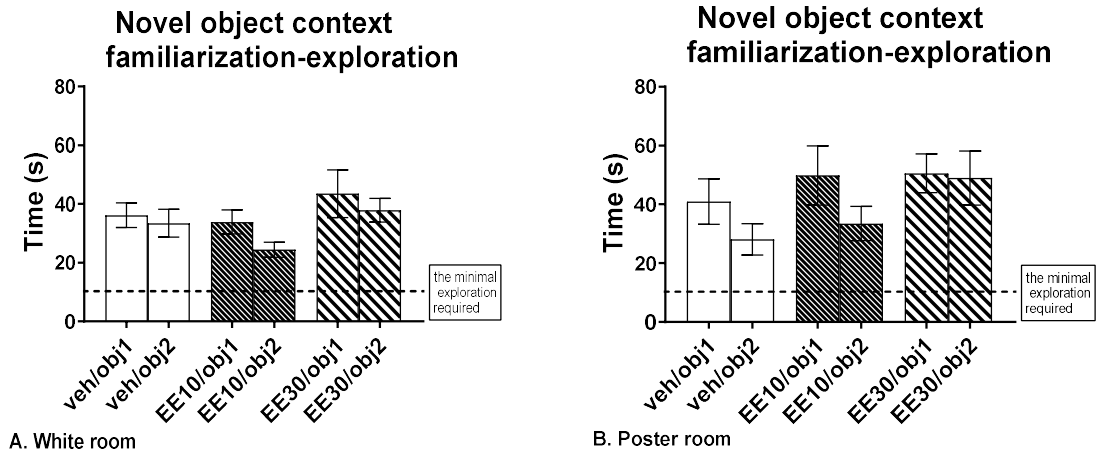


Figure 4.6 Behavioral tests of learning and memory: novel context recognition familiarization

Familiarization phase (T1) in the novel object context. A. White room. The minimal criteria were met for each object in each group so that the testing phase (T2) could be completed; no significant difference between the groups was seen in object exploration. B. Poster room. The rats were required to be familiarized in the second room for context testing. The room differed in the wall décor at eye level to the rats. Adequate exploration of objects was noted for all groups, and there were no statistical group differences. (Veh = vehicle, Obj1 = object 1, Obj2 = object 2)

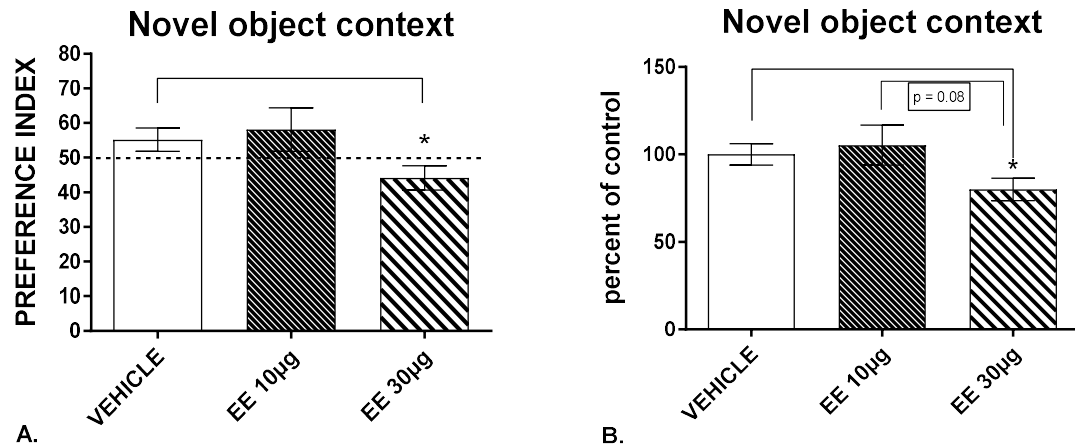


Figure 4.7 Behavioral tests of learning and memory: novel context recognition test Novel object context. A. The ability to prefer the novel over the same context object was accomplished by the vehicle and lower dose EE (10µg) drug-treated rats as demonstrated with >50% preference index (PI). On the other hand, the higher dose EE (30µg) drug-treated rats exhibited an inability to learn the context-determined novel object as demonstrated by the less than 50% PI. B. This inability to learn in the EE 30µg treated rats represents a clinically significant decrease from the vehicle control group (Cohen d = -3.18). A dose-dependent trend was seen between the two EE drug doses with the lower dose exhibiting a better ability to distinguish context-determined novel objects (p = 0.08, Cohen d = -2.74). (*p<0.05).

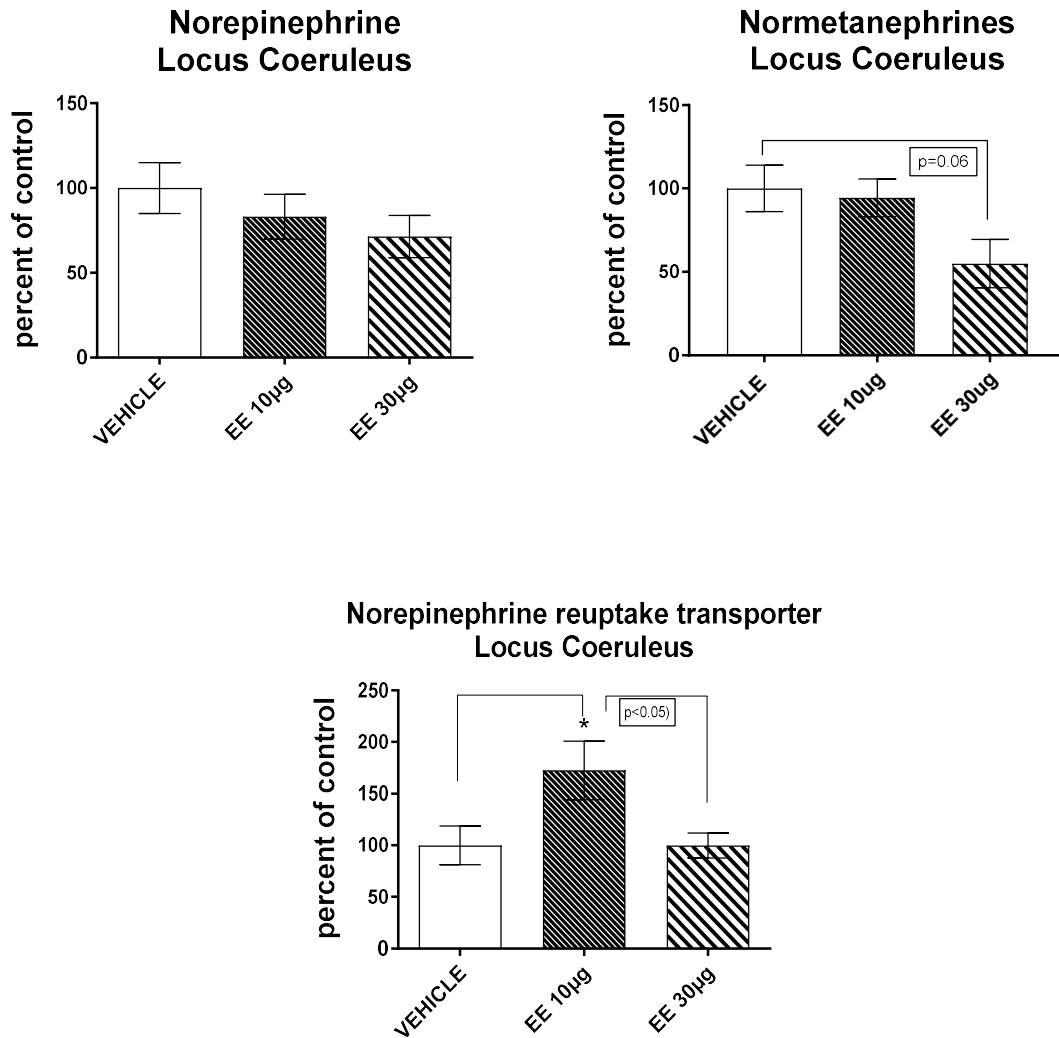


Figure 4.8 Noradrenergic response in the locus coeruleus

The norepinephrine levels did not reveal a statistical difference between the drug-treated rats and the vehicle control rats. There was a trend for the normetanephrines to decrease with rising ethinyl estradiol (EE) treatment. On the other hand, only the EE 10µg treated rats showed an increase in norepinephrine transporter protein. This may represent a dose-dependent response of EE or a transmitter driven response. (* $p < 0.05$).

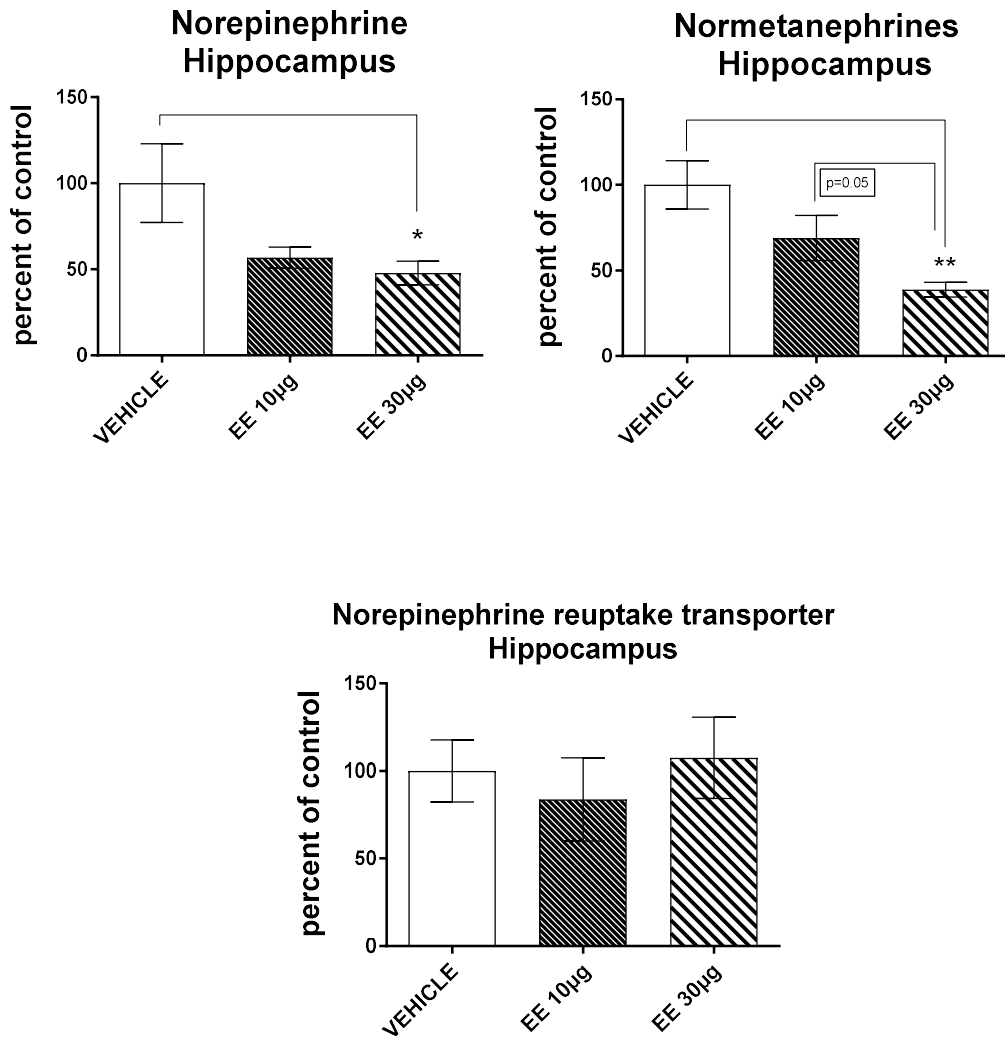


Figure 4.9 Noradrenergic response in the hippocampus

The norepinephrine levels significantly decreased in the high dose ethinyl estradiol (EE) group, and these were followed by the same decrease in normetanephrines. The transporters for reuptake of norepinephrine did not alter with EE drug therapy. (* $p < 0.05$, ** $p > 0.01$).

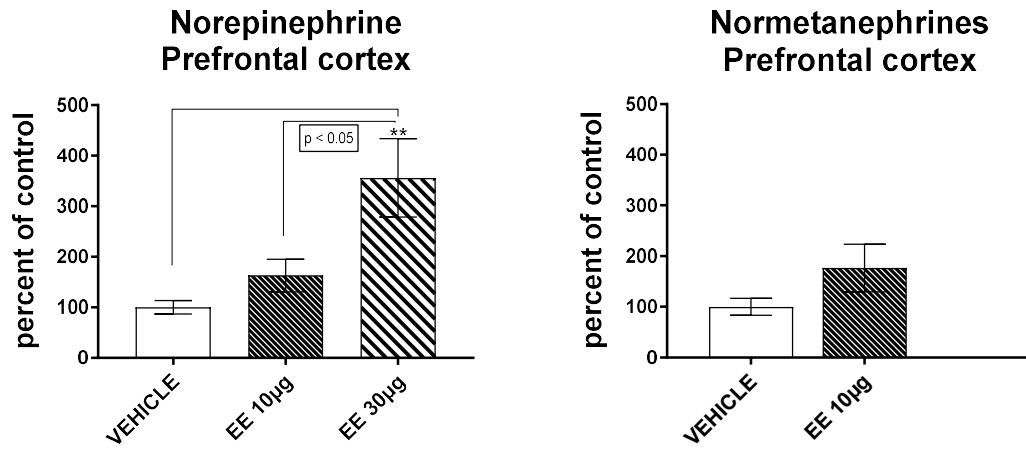


Figure 4.10 Noradrenergic response in the prefrontal cortex

The level of norepinephrine rose more than three-fold in the high dose ethinyl estradiol (EE) treated rats. The high dose EE normetanephrines loss statistical power for analysis due to values in an outlier range. (** p>0.01).

4.9 References

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CHAPTER 5

Summary

5.1 Discussion

Contraceptive hormones are prescribed to women over a wide range of ages, as young as 14 years old and as old as 45. Their inhibition of reproductive potential occurs during a women's life when peak gonadal hormone production exists. Estrogen establishes the brain prenatally, calibrates the brain at puberty and perpetually regulates the brain for life. Contraceptive hormones disrupt production of the usual magnitude of ovarian steroids. Gonadal hormones play an integral part in cognitive processing (Spencer et al., 2008). Estrogen receptors are ubiquitous, occurring in and on every major cell group in the brain. Hippocampal conversion of the testosterone to the neurosteroid estradiol, with its neuromodulatory capabilities, can essentially be characterized as a neurotransmitter (Balthazart & Ball, 2006). Aided by estrogen and neurotrophins such as BDNF (Tanapat et al., 1999), hippocampal neurogenesis underlies the formation of memory by incorporating into existing networks (Aimone, Wiles, & Gage, 2006). Essentially, learning and memory are best the first half of a women's life span, for neural progenitor cells decline with age (Manganas et al., 2007). Hence, the impact of moderating endogenous hormones and adding exogenous analogs on cognitive behavior begs to be explored. This dissertation looked at a rat model of learning and two-dose treatments of conceptive hormones alone and in combinations.

We saw ovulation suppression occur in both drug doses resulting in lowered endogenous 17β -estradiol levels; allowing us to compare drug treatments to controls in a low estrogen state—met-diestrus. Few rodent studies assess ovulatory suppressed females, nevertheless, this allows for the physiological and direct action of the exogenous synthetic hormones themselves to be better defined. Ovariectomy in rats with delayed supplementation greater than a few weeks results in diminished responsiveness to estrogen and progesterone (Tanapat, Hastings, & Gould, 2005), making studies of intact females a better translational model of reproductive age women.

In study one (chapter 3), the range of the drugs brought out a dose-dependent response in anxiety and learning paradigms. In both the elevated plus-maze and the shock probe defensive burying tasks, an anxiolytic-like behavior was seen with the low dose of ethinyl estradiol (EE) and levonorgestrel (LNG) treatments. In the low dose drug treatments, a significant decrease from the controls was seen in the object recognition task of exploring novel over previously seen objects. Consistent with these behavioral changes was a reduction in tyrosine hydroxylase mRNA (in the locus coeruleus) and brain-derived neurotrophic factor (in the hippocampus) in these same low EE groups. In contrast, the higher dose EE showed statistically greater learning in the novel object recognition test and a significant increase in tyrosine hydroxylase mRNA (EE: $t_{(40)} = 2.102$, $p < 0.05$). There was a notable difference in hippocampal BDNF between the two doses of EE (CA2: $t_{(27)} = 2.94$, $p < 0.01$; CA3: $t_{(28)} = 2.34$, $p < 0.05$) concordant with the decrease of BDNF in the low dose treatments. Overall, this suggested a dose-dependent change in noradrenergic tone prompted by the contraceptive hormone treatments.

A possible explanation for the biphasic results may be rooted in the drugs acting as concentration-dependent norepinephrine reuptake inhibitors (Ghraf et al., 1983; Iversen & Salt, 1970) with regional variances. In behavioral tests, EE shows a synergistic antidepressant response in the forced swim test, in rats on an acute desipramine dose (a norepinephrine reuptake inhibitor) (Estrada-Camarena, Fernández-Guasti, & López-Rubalcava, 2004). Idazoxan (a α 2-AR antagonist) blocked this antidepressant effect of EE in the forced swim task (Vega-Rivera Nelly et al., 2013). A synergistic sedative response occurred in women taking contraceptive hormones and clonidine (a α 2-AR agonist) and was attributed to a change in noradrenergic tone (Chalmers, Fulli-Lemaire, & Cowen, 1985). Norepinephrine levels increased in the cortex of rats after an acute dose of EE, but it decreased in the midbrain tissue that included the hippocampus (Tonge & Greengrass, 1971).

An increase in extracellular norepinephrine resulting from reuptake inhibition could at the lower dose activate presynaptic alpha-2 receptors—somatodendritic autoreceptors—of the locus coeruleus (LC), an area known for volume transmission (Callado & Stamford, 2000). Thus, low doses would reduce cellular firing and cause a net reduction in noradrenergic activity in downstream brain areas (Figure 5.1). A desipramine dose response curve identified a dose-dependent inhibition of the LC basal firing rate, with complete inhibition of firing occurring at a 0.8mg/kg dose (Alba-Delgado et al., 2012). A dose of 1mg/kg decreased extracellular norepinephrine in the cingulate cortex, an LC projection area (Mateo et al., 1998). The authors added a α 2-AR antagonist and saw an increase in norepinephrine in the cortex in the 1mg/kg treated rats, suggesting the initial decrease in cortical norepinephrine was secondary to LC autoreceptor stimulus. The

3mg/kg and 10mg/kg IP doses increased cortical norepinephrine (Mateo et al., 1998). In our study, the EE 10ug group's decreased learning is suggestive of a dose-dependent (low) inhibition of firing of the LC. Moreover, higher doses of reuptake inhibitor drugs could result in binding of pre-and post-synaptic alpha-2-receptors (α 2AR) and have an opposite net cognitive effect (Chamberlain & Robbins, 2013) as seen in our EE 30 μ g group.

In study 2 (Chapter 4), we were unable to demonstrate the increase of norepinephrine in the LC by the norepinephrine reuptake inhibition action of EE. Nevertheless, in the prefrontal cortex, we did evince a norepinephrine level significantly less in the EE 10 μ g group than the EE 30 μ g treatment, which in itself was significantly greater than the control. The α 2A-AR subtype is the most prevalent in the prefrontal cortex as well as in the LC (Aoki, Venkatesan, Go, Forman, & Kurose, 1998). Post-synaptic α 2-AR cognitive enhancing properties are distinctive to the prefrontal cortex and working memory (Arnsten, Steere, & Hunt, 1996) and are dose-dependent. Treatment of ovariectomized rats with 17 β -estradiol for 48 hours reduced the density of the post-synaptic α -2A receptors (suggestive of an increase in norepinephrine) in the prefrontal cortex but not in the hypothalamus (Karkanias et al., 1997). Treatment of male rats with high dose (10mg/kg) norepinephrine reuptake inhibitor—desipramine—decreased the cortical α 2-AR yet no change occurred in the hippocampus (Subhash et al., 2003) (suggestive of regional differences in noradrenergic activity). The α 2-AR are inhibitory and in the prefrontal cortex are found postsynaptic on inhibitory interneurons; stimulation of them by an increase in extracellular norepinephrine results in disinhibition of the cortical neurons, thus an excitatory effect (Andrews & Lavin, 2006) (Figure 5.2).

Contrastingly, in the hippocampus, α 2-AR binding impairs memory consolidation at low levels of norepinephrine (Arnsten, 2000). Notably, the hippocampal norepinephrine level in study 2 (Chapter 4) was decreased compared to controls, most significantly in the EE 30 μ g dose and this was associated with a significant impairment of spatial learning in the novel context recognition task. The LC is the singular source of noradrenergic input to the hippocampus (Samuels & Szabadi, 2008). Norepinephrine is more important than serotonin for hippocampal long-term potentiation (LTP) and a decrease in norepinephrine results in marked reduction of LTP (Stanton & Sarvey, 1985). The α 2-AR in the hippocampus are predominantly found presynaptically (Milner, Lee, Aicher, & Rosin, 1998), and may be a contributing factor to the decrease in norepinephrine levels seen in the drug groups. Also, hippocampal norepinephrine dose-dependently (high levels) binds presynaptic alpha-1-adrenoreceptors, reducing the number of inhibitory postsynaptic potentials, which results in more excitatory postsynaptic potentials and summates in an increased firing rate (Madison & Nicoll, 1988). It would follow that a decrease in norepinephrine results in the opposite, which is an increase of inhibitory potentials, less excitatory potentials, fewer action potentials, and less spatial learning (Figure 5.3). Like our context recognition task, specific hippocampal-based place learning by referencing context of the surroundings was impaired when norepinephrine was depleted by 6-hydroxydopamine (Roschlau & Hauber, 2017). More specifically, these norepinephrine depleted rats learned using a different cognitive strategy to succeed on their second probe test. It is possible, both in the prefrontal cortex and the hippocampus, norepinephrine level-dependently acts via a disinhibitory effect as seen in other noradrenergic targets such as the lateral geniculate nucleus (Rogawski & Aghajanian, 1980).

Nevertheless, simplifying the noradrenergic effect to a receptor would be ignoring the interplay of receptors and neurotransmitters (Zhang, Cordeiro Matos, Jago, Adamantidis, & Seguela, 2013). Alpha-1-receptors demonstrate regional estrogen-dependent efficacy in generating a noradrenergic response, with the cortex much more efficient than the hippocampus (Favit, Fiore, Nicoletti, & Canonico, 1991). Yet, in both areas, alpha-1-receptor density does not change in response to less than 25% pharmacological depletion of norepinephrine; instead ligand affinity for the α 2-AR increases (Dooley, Bittiger, Hauser, Bischoff, & Waldmeier, 1983). Chronic treatment of rats with an analog of EE resulted in a decrease in α 2-AR density in the frontal cortex and LC, but not the hypothalamus, reflective of a region-specific increase of noradrenergic processing (Shackelford Jr et al., 1988). Three weeks of $\approx 10\mu\text{g}$ EE reduced cortical but not hippocampal beta-adrenergic receptor density, possibly related to the level of noradrenergic activity (Wagner, Crutcher, & Davis, 1979). Suffice it to say, regulation of the alpha-2-autoreceptors can cause fluctuations in levels of the neurotransmitter in projection regions of the LC (Flugge, 2000), so future investigations into the underlying neurochemical mechanisms of EE on adrenergic region-specific density/binding are planned.

Another means of neuromodulation is the adaptive abilities of norepinephrine transporters (NET) density. Cortical NET binding sites are positively correlated with the norepinephrine levels (Cheetham et al., 1996). In the LC, where NET binding sites are most dense (Biegon & Rainbow, 1983) we did see a statistical increase in NET in the low dose EE group, despite not seeing the expected increase in norepinephrine. It is

suggestive of a compensatory mechanism seen with norepinephrine reuptake inhibitors, as is the decrease in tyrosine hydroxylase mRNA in this group (Eisenhofer et al., 2004). The prefrontal cortex receives both norepinephrine and dopamine input; despite that, there is a low expression of dopamine transporters (Moron, Brockington, Wise, Rocha, & Hope, 2002). Hence in the prefrontal cortex, both transmitters compete for the norepinephrine transporter (NET) (Pozzi, Invernizzi, Cervo, Vallebuona, & Samanin, 1994). Other norepinephrine re-uptake inhibitors such as amphetamine have resulted in cognition-enhancing effects as a result of drug-induced increases in prefrontal cortex catecholamines (Berridge & Arnsten, 2015). One rationale for this increase in cognition is extracellular norepinephrine competition with dopamine for NET (Berridge & Arnsten, 2015) and thus it is not possible to ascribe their effects on cognition to one transmitter. Biphasic behavioral and cell molecular responses are typical for estrogen receptor driven neuromodulation (Calabrese & Baldwin, 2003). Our biphasic results could be attributed to binding to estrogen receptors since EE has twice the affinity of estradiol to estrogen receptor alpha (Blair et al., 2000). Other reasons could include, GABA receptor changes have been associated with contraceptive hormone treatments (Follesa et al., 2002) and the interneurons of the prefrontal cortex are GABAergic (Markram et al., 2004), as well as alterations in the neurosteroids production within the central nervous system secondary to ovulation suppression by the contraceptive hormones. Since the concentration of *de novo* neurosteroid estradiol is synchronized by gonadotrophic releasing hormone (GnRH) at the level of the hippocampus (Brandt, N., Vierk, R., & Rune, G. M. 2013), it follows that with diminished GnRH pulsitivity secondary to contraceptive drugs (Gaspard et al., 1984), hippocampal estradiol might be significantly reduced.

Behavioral outcomes of cognitive processing have amazing intricacy driven by many noradrenergic factors including phasic and tonic firing, differences in the varicosity of terminal fields, and the location, type, affinity, and density of neurotransmitter receptors. The complexity of the interactions of hormonal analogs within the central nervous system cannot be overstated given the sexual dimorphism of cognitive areas of the brain (Table 2). The behavioral tasks in these studies need further elucidation of the dose parameters of the inverted-U responses. Future research should investigate the cognitive effects of doses between the 10 and 30 μg of EE as well as doses above the 30 μg dose to focus on alternative means for improving cognitive deficits created by endogenous estradiol loss.

5.2 Conclusion

In the future, as in the past, the choice of the drug and the dose used in contraceptive formulations should be based on safety, efficacy and clinical trials examining the undesirable side effects. Despite fifty plus years of existence, there is still room for improvement. A continuous reduction in the dose of EE to minimize adverse side effects has been prevalent. The results of our studies suggest that EE has a noradrenergic impact on cognitive strategies and a dose-response curve suggestive of a baseline below which the benefits of supplementary EE are not seen. Women over the age of 35 taking 20 μg EE oral contraceptive hormones have greater ovarian suppression than their younger counterparts, exemplified by significantly low serum estradiol levels (Fitzgerald, Elstein, & Spona, 1999). Given the evidence that premature loss of ovarian function increases the risk of dementia (Shuster, Gostout, Grossardt, & Rocca, 2008), it is imperative a risk-benefit ratio calculating the lowest dose of EE in birth control pills should be explored (Figure 5.4). These two studies are a start toward investigating the cognitive benefits of

EE as an offset to the loss in endogenous estrogens; further research is needed before definitive conclusions can be drawn.

5.3 Figures/Table

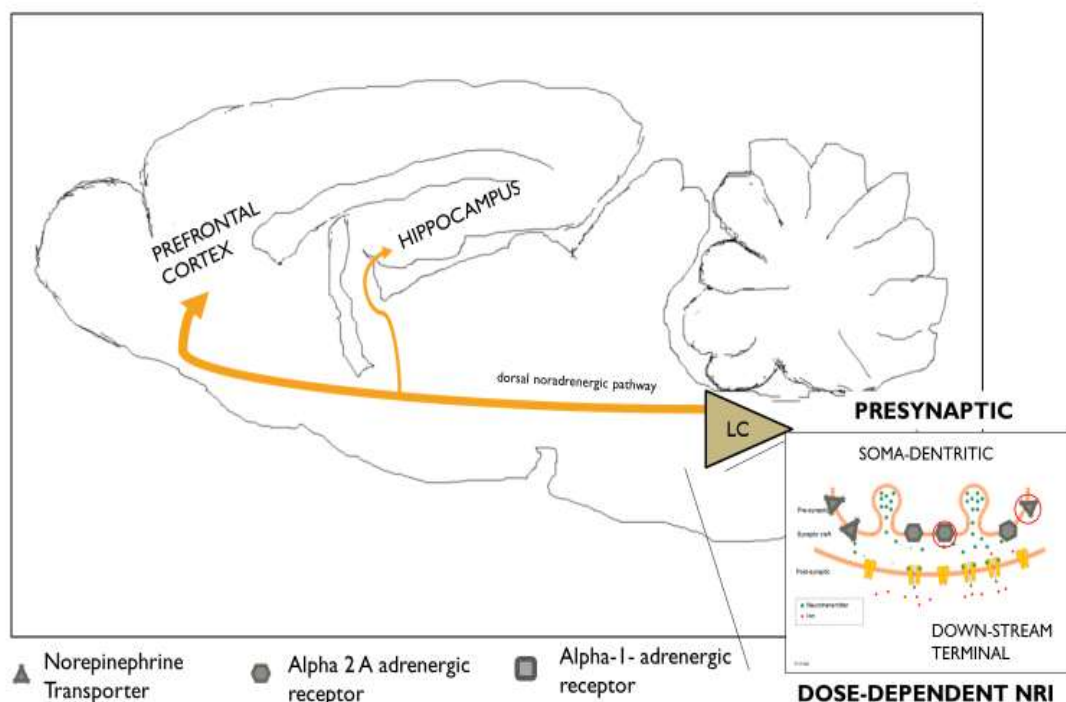


Figure 5.1 Sagittal section of rat brain. Hypothetical mechanism of action of ethinyl estradiol in the locus coeruleus

Ethinyl estradiol (EE) acts as a norepinephrine reuptake inhibitor (NRI) with a dose-dependent response. EE 10 μ g binds to the norepinephrine transporters (NET) and inhibits the reuptake of norepinephrine. The NET was significantly increased over control in the low dose EE group. The increase in extracellular norepinephrine activates presynaptic alpha-2 receptors—somatodendritic autoreceptors reduces the firing rate of the locus coeruleus. This reduced firing results in a decreased release of norepinephrine in regional terminal synapses. The EE 30 μ g dose does not down regulate the locus coeruleus firing

like the low dose EE and results in more norepinephrine released in the prefrontal cortex. A similar dose-dependent response is seen with desipramine (a norepinephrine reuptake inhibitor). (LC = locus coeruleus)

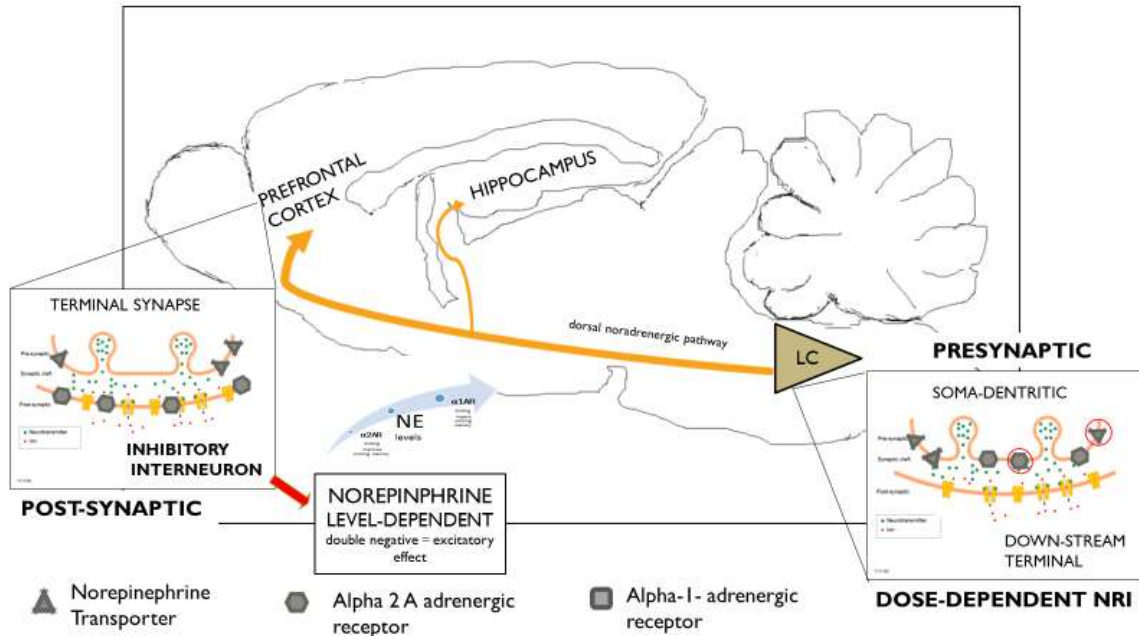


Figure 5.2 Sagittal section of rat brain. Hypothetical mechanism of action of ethinyl estradiol in the prefrontal cortex

At the terminal noradrenergic synapse in the prefrontal cortex, a norepinephrine level-dependent response occurs. The postsynaptic alpha 2A-adrenergic receptors (α 2A-AR) are found on inhibitory interneurons. The tissue norepinephrine level differed statistically between the low and high doses of ethinyl estradiol(EE), with the EE 30 μ g significantly higher than control. The released norepinephrine binds to the α 2A-AR preferentially, and the inhibitory action of these adrenergic receptors results in disinhibition of the cortical neurons, thus an excitatory effect and improved learning. (LC = locus coeruleus, NRI = norepinephrine reuptake inhibitor)

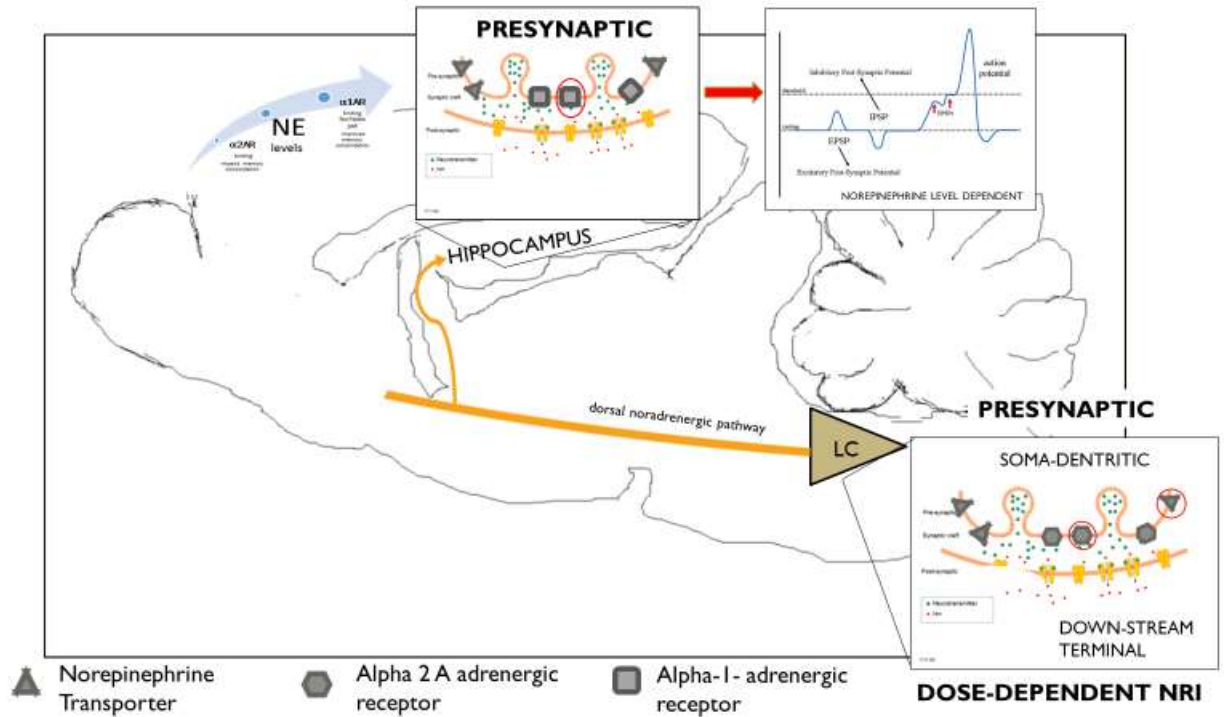


Figure 5.3 Sagittal section of rat brain. Hypothetical mechanism of action of ethinyl estradiol in the hippocampus

EE 30 μ g dose was associated with a significant impairment of spatial learning in the novel context recognition task, and the tissue norepinephrine level was statistically decreased from the control. The $\alpha 2$ -AR in the hippocampus are predominantly found presynaptic with the alpha-1-adrenergic receptors ($\alpha 1$ -AR). The norepinephrine binds preferentially to the $\alpha 2A$ -AR and decreases the norepinephrine release. Hippocampal norepinephrine binds to presynaptic alpha-1-adrenoreceptors reducing the amount of inhibitory synaptic potentials, resulting in more excitatory postsynaptic potentials and increased firing (Madison & Nicoll, 1988). But with the decrease in norepinephrine release and a decrease in binding to $\alpha 1$ -AR, there is an increase in the inhibitory postsynaptic potentials and fewer action potentials.

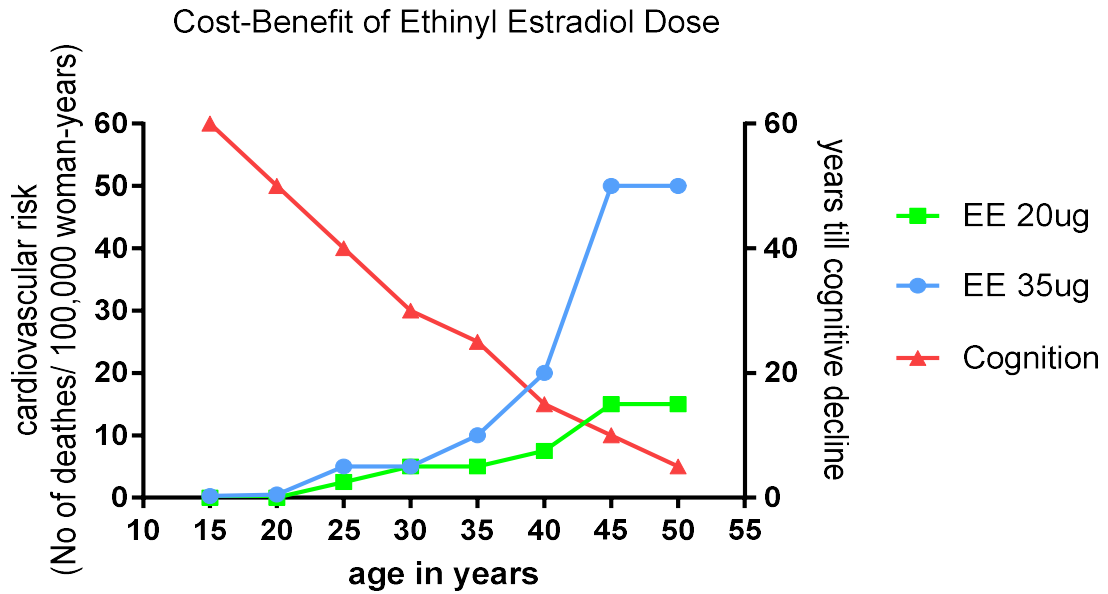


Figure 5.4 A Hypothetical Risk-Benefit graph

Depending on the ethinyl estradiol (EE) dose, the risk of death from a cardiovascular event per 100,000 woman-years is plotted against the risk of loss in cognitive powers. At some age point, the healthcare risk of a higher dose of EE would outweigh the cognitive benefit of the higher dose of EE.

Table 2. Sex differences in cognitive brain areas

Difference	Locus Coeruleus	Hippocampus	Prefrontal Cortex
Morphology	Yes	Yes	Yes
Neurochemistry	Yes	Yes	Yes
Volume loss with age	Yes	Yes	Yes
Cognitive Tasks	----	Yes	Yes
Stress-induced dysfunction	Yes	Yes	Yes
Neuropsychiatric diseases	Yes	Yes	Yes

Source: Gur, R. C., Gunning-Dixon, F., Bilker, W. B., & Gur, R. E. (2002). Cosgrove, K. P., Mazure, C. M., & Staley, J. K. (2007). Shors, T. J., Chua, C., & Falduto, J. (2001). Juraska, J. M. (1991). McCarthy, M. M. (2016).

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