# THE INFLUENCE OF ESTROGEN ON CONDITIONED CONTEXT AVERSION LEARNING

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## ABSTRACT

THE INFLUENCE OF ESTROGEN ON CONDITIONED CONTEXT AVERSION LEARNING

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Anticipatory nausea and vomiting (ANV) are distressing side effects of chemotherapy, hindering patients from adhering to treatment. ANV involves nausea in anticipation of chemotherapy, developed through classical conditioning. While ANV is more prevalent in women than in men, preclinical studies are scarce and focused on males. The limited literature on the sex differences in animal model of ANV, conditioned context aversion (CCA), also shows enhanced conditioned response in females, possibly explained by female gonadal hormones. The present study investigates the cause of sexual dimorphism observed in ANV through a CCA procedure in which female CD1 mice associate a distinctive context with LiCl. In Experiment 1, the influence of ovarian hormones is investigated by comparing the aversion levels of ovariectomized and sham-operated mice. The results show that ovariectomized mice extinguished faster than sham-operated mice. Experiment 2 examined the role of estradiol in prolongation of extinction in intact females. To this end, subjects were ovariectomized and half of them were administered estradiol while the remaining were administered oil to control. While estradiol-administered animals maintained aversion

for a single retention trial in Experiment 2, control animals did not exhibit an aversion. The results of Experiment 1 show that ovarian-secreted hormones facilitate CCA. On the other hand, estradiol administration in Experiment 2 partially rescued the impact of the ovariectomy either due to the inefficiency of estradiol itself, or the hormone treatment method. Overall, the experiments suggest that the ovarian-secreted hormones facilitate CCA in mice which can explain observed sex differences in ANV in humans.

**Keywords**: Anticipatory nausea, conditioned context aversion, mice, estrogen

# ÖZ

ÖSTROJENİN KOŞULLU ÇEVRE İTİNMESİ ÜZERİNE ETKİSİ

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Beklentisel bulantı (ANV) kemoterapinin rahatsız edici yan etkilerindendir ve tedavi devamlılığını zorlaştırabilir. Bu fenomen, hastane ortamına yönelik bulantı beklentisinin bir sonucudur ve klasik koşullanma yolu ile oluşur. ANV kadınlarda erkeklere kıyasla daha yaygın olmasına rağmen, dişi fareler ile yapılan ön klinik çalışmalar sınırlıdır. ANV'nin hayvan modeli olarak geliştirilen koşullu çevre itinmesi (KÇİ) üzerine iki cinsiyeti de içeren sınırlı literatür dişi kemirgenlerde daha güçlü itinme gözlemlemiştir. Bu durum dişi gonad hormonlarıyla açıklanabilir. Güncel çalışmada dişi CD1 farelerin karakteristik bir bağlam ve LiCl'ü eşleştirilerek KÇİ paradigması oluşturulması ve ANV'deki cinsiyet farklılıklarının sebebini anlamak hedeflenmiştir. Deney 1'de ovariektomi ve sahte operasyon geçirmiş fareler kıyaslanarak dişi gonad hormonlarının KÇİ öğrenmesine etkisi gözlemlenmiştir. Deney 1'de ovariektomi operasyonu geçiren farelerde sahte operasyon geçirenlere kıyasla sönmenin daha hızlı gerçekleştiği gözlemlenmiştir. Bu sonuçları takiben Deney 2 estradiolün sönme gecikmesi üzerindeki rolünü araştırmayı hedeflemiştir. Bu

amaçla ovariektomize farelerin yarısı estradiol, kalan yarısıysa kontrol grubu oluşturmak amacıyla yağ takviyesi almıştır. Deney 2'deki estradiol takviyesi almış fareler yalnızca ilk bellek testi sırasında itinme göstermiş, kontrol grubuysa bellek testleri sırasında anlamlı bir itinme göstermemiştir. Sonuçlar dişi gonad hormonlarının KÇİ öğrenmesi üzerinde indükleyici bir etki yarattığını göstermektedir. Bununla birlikte estradiol takviyesi ovariektomi prosedürünün etkilerini kısmi olarak kurtarmıştır. Bu durum estradiolün tek başına yetersiz olmasından ya da hormon takviyesi metodunun yetersizliğinden kaynaklı olabilir. Sonuç olarak overlerden salgılanan hormonlar KÇİ öğrenmesini indükleyerek ANV'de görülen cinsiyet farklılığını açıklayabilir.

**Anahtar Kelimeler**: Beklentisel bulantı, koşullu çevre itinmesi, fare, östrojen

*To my grandmother*

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Anticipatory nausea and vomiting (ANV)

Chemotherapy-induced nausea and vomiting (CINV)

Unconditioned stimulus (US)

Conditioned stimulus (CS)

Conditioned response (CR)

Conditioned context aversion (CCA)

Lithium chloride (LiCl)

Sodium chloride (NaCl)

Conditioned taste aversion (CTA)

Area postrema (AP)

N-methyl-D-aspartate (NMDA)

Nucleus tractus solitarius (NTS)

Central nucleus of the amygdala (CeA)

Supraoptic nucleus (SON)

Ventromedial prefrontal cortex (vmPFC)

Agranular insula (aIC)

Paraventricular nucleus (PVN)

# CHAPTER 1

# INTRODUCTION

The first chapter of the thesis aims to cover some general concepts and findings important concepts for our experiments including anticipatory nausea and vomiting, conditioned context aversion, sex differences in anticipatory nausea, conditioned context aversion, and other classical conditioning paradigms as well as estrogen-nausea and estrogen-brain relationship.

## 1.1. Anticipatory Nausea and Vomiting

Anticipatory nausea and vomiting (ANV) are side effects of the chemotherapy that cancer patients undergo as a part of their treatment (Boakes et al., 1993). The leading cause of ANV is chemotherapy-induced nausea and vomiting (CINV), which is one of the most severe symptoms (Aslam et al., 2014; Coates et al., 1983; Love et al., 1989) and refers to the experience of nausea and vomiting side effect followed by the administration of chemotherapy drugs (Kamen et al., 2014; Morrow & Rosenthal, 1996). Psychological processes cause the nausea and vomiting to take place in anticipation of the treatment, and strengthen after each chemotherapy session (Matteson et al., 2002; Molassiotis et al., 2016). ANV can be observed in patients as early as in anticipation of the second treatment and in 30% of cancer patients by the fourth chemotherapy cycle (Roscoe et al., 2010).

The development of ANV is a result of a psychological phenomenon called classical conditioning (Stockhorst et al., 1993) (see Figure 1). Classical conditioning occurs when a neutral stimulus, which does not generate a response by the organism, is associated with an unconditioned stimulus (US) which generates an unconditioned response (UR). As a result of the association between neutral and unconditioned stimuli, the neutral stimulus becomes a conditioned stimulus (CS) which generates a conditioned response (CR) pendant to the UR. In the case of ANV, the hospital is the

neutral stimulus consisting of a distinct smell, lightning, and noise, in which the cancer patient does not exhibit a response. The chemotherapy drug is the US which generates the UR of nausea and vomiting as a side effect of the treatment. By the time the patient receives the chemotherapy drug in this neutral environment, an association occurs between the hospital cues and the chemotherapy drug and the patient starts to generate CR of nausea and vomiting when they are presented with the hospital cues, in lack of the chemotherapy drug.

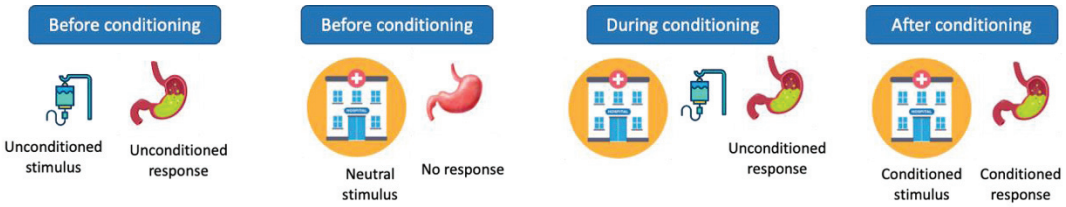


**Figure 1.** The chemotherapy drug that cancer patients start to receive during the first cycle (US) induces CINV (UR) as a side effect. The hospital environment does not induce such UR due to conditioning before the first session of chemotherapy. After exposure to the chemotherapy drug in the hospital environment enriched with specific visual, tactile, and auditory cues; the patient becomes conditioned to the hospital environment (CS) and starts to generate nausea and vomiting (UR) in anticipation of the chemotherapy treatment.

ANV generates anxiety in the patients and induces distress, which might lead to quitting the chemotherapy treatment consequently (Andrykowski, 1990; Jordan et al., 2005; Van Komen & Redd, 1985). Therefore, it is crucial to understand the nature of ANV and target the factors that enhance the susceptibility. The primary determinant is the CINV, which is crucial for the development of ANV is the emetogenicity levels induced by the specific chemotherapy drug. Furthermore, the presence of both CINV and ANV is strongly determined by the age and gender of the patient (NCI, 2024). Specifically, individuals under 50 and women are more susceptible to generating ANV. Detecting the vulnerable populations and examining their underlying reasons for this vulnerability will help to generate novel prevention and treatment methods.

### 1.2. Conditioned Context Aversion

One way to investigate ANV is to use animal models in preclinical studies to understand this phenomenon and generate novel treatments. Conditioned context aversion (CCA) is the animal model of the ANV observed in the human population

(Cloutier et al., 2017, 2018; Hall & Symonds, 2006; İlhan et al., 2023; Kislal & Blizard, 2016; Limebeer et al., 2008; Rodríguez et al., 2000). Conventionally, rodents are subjected to experiments that create analogous machinery to ANV with a novel environment to act as the hospital environment (neutral stimulus), an aversion-inducing agent that is administered to mimic the chemotherapy drug (US), and aversion-related response (UR) similar to nausea and vomiting in the human population. Following the conditioning, the neutral environment becomes the CS and the CR of the animals is measured to quantify the aversion levels in anticipation of the illness-inducing agent (see Figure 2).

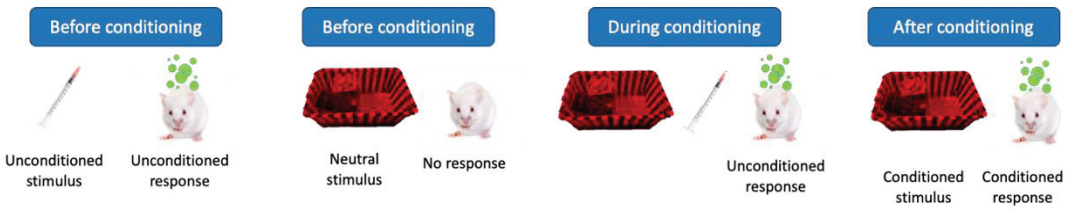


**Figure 2.** The illness-inducing lithium chloride (LiCl) injection (US) rodents receive intraperitoneally leads to an aversion response (UR). The animal does not generate any response to the novel context before conditioning. After experiencing the side effects of LiCl injection in the conditioning context enriched with specific visual, tactile, and auditory cues; the subject becomes conditioned to the context (CS) and starts to generate aversion (UR) in anticipation of the LiCl injection.

The hospital is the neutral stimulus in ANV and is composed of several different cues distinguishing it from other environments to which the patients are exposed. The neutral visual, auditory, and olfactive cues patients are exposed to during chemotherapy sessions in the hospital are distinct from everywhere else, inducing anticipation of treatment following conditioning (Nesse et al., 1980; Roscoe et al., 2010; Stockhorst et al., 2006). Rodent studies used different contextual cues to create a distinct environment for the rodents to act as neutral stimuli. Using a different cage from the home cage, using cat litter rather than wood shavings, illuminating the experimental room with a dim red light, and providing white noise and a specific odor cue are the ways to obtain such a distinct environment (İlhan et al., 2023; Rodríguez et al., 2000; Symonds & Hall, 1997). Furthermore, Kislal and Blizard (2016) showed that rodents have the ability to generate aversion in anticipation even with slight visual changes in the context. Specifically, the mice successfully distinguished the plastic bottles and glass bottles which they were served the water with and generated an aversion when the glass bottle was paired with LiCl injections.

Cancer patients receive chemotherapy or radiotherapy as a treatment method which induces emetogenic effects acting as a US for ANV (Rolia et al., 2010). While rodents do not have a vomiting reflex, they still exhibit an aversion response to the administration of certain procedures (Horn et al., 2013). LiCl injection to induce a nausea-like state in rodents is widely used in both CCA and conditioned taste aversion (CTA) paradigms (Bishnoi et al., 2023; Chan et al., 2009; Kislal & Blizard, 2017; Limebeer et al., 2008; Nachman & Ashe, 1973; Parker et al., 1984; Symonds & Hall, 1997; Symonds & Hall, 2000; Rodríguez et al., 2000; Wang et al., 2017). In CTA paradigms, the subjects pair a novel taste presented during the conditioning with the abrasive impact of the LiCl injection. On the other hand in CCA, a neutral stimulus is a distinct context and the US is the LiCl injections. Following the conditioning procedure, the rodents pair the context and aversive effects of LiCl injections leading them to evoke CR when they are exposed to the CS. The LiCl injections in such experiments are usually administered intraperitoneally for an acute effect and are expected to influence the rats within approximately 10 minutes with 1 to 3 mEq LiCl/kg and can change the cardiovascular function (O'Connor et al., 1987). The LiCl injections result in lying on belly as the predominant UR (Meachum & Bernstein, 1992; Tuerke et al., 2012) in addition to decreased locomotion and increased rearing (Limebeer et al., 2008) which can act as the UR in the CCA paradigms.

The US evokes a UR, which becomes the CR following the pairing. The CR is crucial for the measurement of aversion levels induced by anticipation during the testing phase. Measuring the consumption of a palatable solution is one way to operationalize the CR levels during the testing phase. To this end, the animal is served with a sucrose solution in the conditioning context during the testing phase which can be a consumption or a blocking test (Hall & Symonds, 2006). The procedure for the consumption test starts with water restriction, in which the water bottles are served to the animals for 30 minutes in morning and afternoon sessions (Rodríguez et al., 2000). Two different contexts were prepared for the conditioning. On the first day, the animals received LiCl injections in the first context leading them to get conditioned to Context A, and received NaCl injections in the second context (Context B) which do not generate an aversion, therefore conditioning towards Context B. After continuing this cycle for three more time, half of the animals were tested in the Context A

(experimental group) and the remaining were tested in the Context B (control group) through measuring the consumed sucrose amount. Since the animals in the experimental group develop an aversion (CR), they are expected to consume less water compared to the control. In the blocking test, a similar conditioning procedure to the consumption test is used, in which the animal receives a LiCl injection in Context A and a NaCl injection in Context B (Rodríguez et al., 2000; Symonds & Hall, 1997). Following that, the animals are divided into two groups, the experimental group is served sucrose solution together with LiCl injections in Context A and the control group in Context B. Sucrose consumption of each animal is measured in the home cage during testing. Because the cues in Context A block the sucrose-LiCl pairing, the experimental group is expected to consume more amount of liquid compared to the control group.

Other alternative ways that do not employ the consumption of a palatable solution to assess the CR are water consumption and observable behavior. For the water consumption test, the subjects undergo a water restriction procedure similar to the consumption and blocking tests (İlhan et al., 2023). After a conditioning trial in the novel context which LiCl is paired with to serve as the experimental and NaCl injected to serve as the control group, the subjects are tested for their water consumption in that specific context during the retention test days. In those test days, no injection is provided and the water consumption is expected to decrease in anticipation of the illness, therefore in the LiCl group. A way to quantify the CR of the rodents without the involvement of drinking is by using observable behavior. After the animal is conditioned with injections of LiCl, the rodents do not vomit, however, they generate gaping behavior in anticipation of the illness (Limebeer et al., 2006; Rock & Parker, 2013; Rock et al., 2016; Tuerke et al., 2012). Furthermore, the gaping response observed in rodents uses muscles similar to the ones activated during shrew retch which is a sign of nausea and vomiting (Travers & Norgren, 1986). This gaping behavior is used as a measurement of aversion as it is the CR in different CCA studies (Cloutier et al., 2012, Kavaliers et al., 2012; Limebeer et al., 2006; Limebeer et al., 2008; Ossenkopp et al., 2011).

The examination of ANV using the CCA paradigm which involves pairing a distinct context with an aversion-inducing agent is widely used. Specifically, the injection of

LiCl as the US in rodents is prevalent to investigate the anticipated nausea response in humans created by the chemotherapy sessions used for cancer treatment. Preclinical studies serve as a useful tool to try different behavioral and pharmacological treatment methods before a safe application to a clinical setting as well as detecting the biological vulnerability factors.

### 1.3. Sex Differences in ANV and CCA

ANV can be observed in both women and men. However, like many other clinical conditions and treatment side effects, there is a sexual dimorphism observed in ANV (Agabio et al., 2016; Eid et al., 2019; Fillingim et al., 2005; Grossman et al., 2008; Lovejoy & Sainsbury, 2009; Queme & Jankowski, 2019; Vetvik & MacGregor, 2017; Yamamoto et al., 2008). In fact, gender is one of the major determinants of ANV susceptibility and women are more likely to experience this condition compared to men (Qureshi et al., 2016; Kamen et al., 2014; Fetting et al., 1983). Conditioned aversion studies conducted with humans, and CCA studies subjecting rodents also show sex differences which should be considered while understanding and treating ANV.

Conditioned nausea paradigms conducted with humans prevalently use a rotation paradigm which induces motion sickness for the participants (Arwas et al., 1989). For this, the healthy participants are rotated to create motion sickness which leads to nausea and vomiting as well as pallor and cold sweating which is triggered by the vestibular system (Money, 1970; Yates et al., 1998). While motion sickness is different from chemotherapy drugs by nature since one is a physical stimulation and the other is chemical respectively, the vestibular stimulation can be successfully used as the US in rodent models to create conditioned gaping (Limebeer et al., 2008). Similarly, the rotation paradigm is used with humans to stimulate the vestibular system and play a role as the US to create ANV (Stockhorst et al., 2014). Klosterhalfen et al. (2005) examined the impact of latent inhibition on ANV in humans using a chair rotation paradigm to induce motion sickness. Latent inhibition refers to a detainment of the CR as a result of pre-exposure to the stimulus which is going to be the CS during conditioning (Lubow, 1973). In this specific experiment, the CS was the chair where the rotation would take place and the conditioned nausea (CR) of the participants was

measured with a rating scale. The results show that women scored higher for anticipatory nausea in lack of latent inhibition, and latent inhibition was more effective on them compared to men. Another study by Stockhorst et al. (2014) investigated the disruption of ANV through using an overshadowing with rotation paradigm in humans. Overshadowing is the attenuation of CR towards a relatively weak stimulus when it is compounded with a stronger stimulus (CS) (Mackintosh, 1976). As a result, the weak stimulus becomes overshadowed by the strong stimulus which leads to the generation of CR. In the experiment, the overshadowing is obtained through the consumption of a beverage that has a salient taste and the context acted as the weaker stimulus. The participants in the experimental group consumed the beverage before the three consecutive rotation days during which conditioning took place while the control group consumed water instead of the beverage. The results revealed that the difference between the overshadowing and control group in female participants was greater than that of male participants. The researchers concluded that the overshadowing procedure was more effective in women compared to men.

The data from the human population suggests that women are more susceptible to experiencing ANV compared to men, however, as it is the norm for animal studies, mostly male rodents are subjected to CCA experiments (Beery & Zucker, 2011). As a result, sex differences in medical conditions and the biological underpinnings of those sex differences stay underinvestigated. Although limited, the current literature shows that the animal findings are in parallel with the humans in terms of sex differences. İlhan et al. (2023) examined the sex differences using a CCA paradigm with a single conditioning trial and found that the extinction is prolonged in female subjects compared to that of males. For this experiment, CD1 mice first underwent a water acclimation procedure to train them to drink when the water is served. The conditioning trial was carried out in a single trial in which the experimental group was injected with LiCl and the control group with NaCl in the conditioning context. During the retention test , the animals are presented to the conditioning context with water provided, and their water consumption is recorded to quantify the aversion levels. The consumed water amount remained small for more test days for female mice compared to males, showing that the male mice extinguished faster. In studies conducted with rats with a slightly different procedure, the females exhibited enhanced aversion responses compared to males (Cloutier et al., 2017; Cloutier et al., 2018). In those

studies, male and female Long-Evans rats received four conditioning trials in the conditioning context with either LiCl to form the experimental group or NaCl for control. A single testing day took place in which the animals were put in the conditioning context and their disgust-related behaviors were recorded. The results revealed that the female rats exhibited conditioned gaping behavior more frequently compared to male rats.

There is only one study that is conducted with female rodents to reveal the potential biological cause of female susceptibility to CCA. In this experiment, Cloutier et al. (2018) followed the estrous cycle of the female rats were followed. The rodents' estrous cycle is analogous to the human menstrual cycle and consists of proestrus, diestrus, estrus, metestrus, and diestrus, each continuing approximately for one day. The animals in proestrus, which is characterized by high estrogen levels; and diestrus with low estrogen constituted two groups in this experiment. Trials took place 96 hours apart with a four-trial conditioning procedure and a one-trial test to match all procedures to the same estrus cycle (proestrus or diestrus). During conditioning, each two group was subdivided into two groups, half of them received LiCl in the conditioning context intraperitoneally, and the other half were injected with NaCl. During the test, no injections were given, and the behavior of the animals was recorded. The gaping behavior was more frequent in LiCl-injected proestrus rats compared to the LiCl-injected diestrus rats. The NaCl-injected control group did not generate an aversion and thus, did not exhibit significant gaping behavior. The conclusion drawn from this study is the facilitatory impact of estrogen on CCA.

Previous studies that are conducted both in humans and rodents support the demographic finding of female susceptibility to ANV. On the other hand, the number of these studies is limited, and there is a lack of focus in the literature that examines the nature of CCA and different treatment methods. It is crucial to further investigate CCA, and other conditions that exhibit sex differences, to generalize the findings to not only men but also women.

### 1.4. Estrogen and Nausea

Nausea and vomiting are side effects of chemotherapy treatment that show sexual dimorphism in prevalence and etiology (Qureshi et al., 2016). It is important to

determine the susceptibility to nausea in different profiles to shed light on the observed sex differences in ANV. Since the UR is nausea for cancer patients who receive chemotherapy treatment, the intensity of this aversive experience can lead to stronger associations between CS and the US, the hospital environment, and aversion-inducing agent. The hormonal differences and genetic factors potentially contribute to sex differences in nausea and vomiting, and animal studies help to reveal the underlying mechanisms.

In studies conducted with humans, women exhibit enhanced levels of nausea and vomiting compared to men under many different conditions such as postoperative nausea (Koivuranta et al., 1997; Salazar-Parra et al., 2020; Stadler et al., 2003), symptoms of acute coronary syndromes (Goldberg et al., 2000; Patel et al., 2004), and motion sickness (Koslucher et al., 2015). Additionally, conditioned food aversion was correlated with the motion sickness of women but not men (Fessler & Arguello, 2004). The enhanced susceptibility to nausea in women might be due to ovarian-secreted hormones. Findings of women showing different levels of motion sickness during stages of the menstrual cycle suggest the influence of female gonadal hormones (Golding et al., 2005). Particularly, women tend to experience nausea more during menstruation and this tendency is the lowest during pre-menstruation and ovulation due to fluctuating levels of female gonadal hormones, estrogen in particular (Golding et al., 2005; Matchock et al., 2008). Women particularly experience nausea and vomiting during pregnancy, a period in which hormonal fluctuations are experienced. During this phase, the rise of human chorionic gonadotropin (hCG) (Dekkers et al., 2019), as well as estradiol (Lagiou, 2003), were found to be correlated to enhanced nausea. Moreover, progesterone disrupted the gastric slow-wave rhythm of non-pregnant women and induced nausea levels similar to pregnancy (Walsh et al., 1996). Administration of estradiol in combination amplified this effect indicating that the combination of these two hormones might be contributing to nausea during pregnancy. CINV is also observed more frequently in women than in men (Mosa et al., 2020). However, this sexual dimorphism was not observed before puberty (Zevy, 2017), and after menopause (Yokokawa et al., 2023) which is an indication of female gonadal hormones that play an important role.

While rodents do not have a vomiting reflex as indicated earlier, the analogous

discomfort created by LiCl (Andrews & Sanger, 2014) and other emetic agents might be facilitated through steroid hormones. The nucleus of the solitary tract combines the information coming from the area postrema (AP), vestibular system, cerebrum, and abdominal vagal afferents and creates an emetic circuitry in humans (Horn, 2008). AP is especially important for ANV since it is weak in the blood-brain barrier and houses chemosensitive receptors that respond to nauseant chemicals, including chemotherapy drugs (Miller & Leslie, 1994; Shinpo et al., 2012). Even though the rodents do not vomit, the aversion induced by LiCl was found to be correlated with AP activation since lesions to this area eliminated the discomfort created by LiCl injections (Bernstein et al., 1992; Wang et al., 1997; Spencer et al., 2012). This important brain region that plays a crucial role in emetic circuitry houses estrogen receptors with high density (Brăiloiu et al., 2007; Shughrue & Merchenthaler, 2001; Merchenthaler et al., 2004). The promoter of ERα, which is a type of estrogen receptor was higher in amount in female AP compared to male AP in a study that subjected transgenic rats with green fluorescent protein controlling ERα 0/B promoter (Zhang & Hamada, 2013). Additionally, estrogen can induce an aversion to creating aversion in rodents when administered in the US in high quantities (Miele et al., 1988). Therefore, the estrogen might be leading to female susceptibility to develop stronger CCA. On the other hand, a study that observed brain activation in AP towards LiCl injection found no difference in activation levels between sexes (Bernanke et al., 2022). While these results do not support the idea of an interaction between estrogen and emetogenicity, their behavioral results did not reveal a significant difference either despite using the same behavioral procedure with Cloutier et al. (2018). Yet, the strain differences of rats in these two experiments might have led to null findings, since Clouiter et al. (2018) subjected Long Evans and Bernanke et al. (2022) subjected Sprague Dawleys in their experiments.

### 1.5. Estrogen and Learning-Related Brain Regions

Sexual dimorphism observed in ANV can be potentially explained by gonadal hormones' influence on the central nervous system. The literature on CCA relies heavily on data obtained from behavioral studies on male rodents, thereby, the underlying biological causes of such behavioral differences are still unaccounted for. Identifying the neurobiological forces leading to sexual dimorphism can provide more effective solutions to prevent and reduce the development of AN with specialized

treatments for different sexes.

In rodent studies, estrogen has shown diverse influences on the brain, with a particular emphasis on the hippocampus, a region strongly involved in memory processes (Scoville & Milner, 1957; Eichenbaum et al., 1999; Olton et al., 1979; Eichenbaum et al., 1994; Ergorul & Eichenbaum, 2004; Bunsey & Eichenbaum, 1996; Burgess et al., 2002; Broadbent et al., 2004). This is particularly relevant in investigations exploring the connection between estrogen and learning because the hippocampus is dense in estrogen receptors (Almey et al., 2015). CCA is a learning paradigm dependent on associating the conditioning context with LiCl, a process involving the hippocampus due to its context-dependent nature (Bishnoi et al., 2023) and estrogen is believed to influence the hippocampus acting through the estrogen receptors located within this brain region (Foy et al., 2008; Liu et al., 2008; Spencer-Segal et al., 2012). The brain-estrogen interaction can involve slow or rapid pathways involving different mechanisms of action. The genomic pathway acts through classical estrogen receptors alpha and beta present in both male and female hippocampus and contributes to the synaptic plasticity (Spencer-Segal et al., 2012) as well as the non-genomic pathway with GPER that involves kinase networks (Hasegawa et al., 2015). Notably, studies have demonstrated that ovariectomy leads to a reduction in the density of dendritic spines in the CA1 region of female rats—an effect that can be reversed with estradiol administration within three days (Gould et al., 1990). Furthermore, spine density undergoes fluctuations over the estrous cycle, and mice are found to have higher spine density when their estradiol levels are higher (Woolley & McEwen, 1992). Estrogen is demonstrated to decrease the threshold for LTP activation, decrease the threshold for hippocampal seizures, and increase the excitability of neurons in this region accompanied by the effects on synaptic plasticity (Montoya & Carrer, 1997; Terasawa & Timiras, 1968). The alterations in hippocampus activity are potentially mediated through the interaction between estrogen and NMDA receptors (El-Bakri et al., 2004). In fact, estradiol is found to increase NMDA receptors through regulating the NMDAR subunit protein in CA1 and dentate gyrus (Gazzaley et al., 1996). The involvement of this receptor is especially important since the ANV possibly involves NMDA receptors whose activation is inhibited by APV (Hernández-Matias et al., 2021). In previous studies, NMDA has been found to mediate an increase in dendritic spine density in

CA1 pyramidal cells in relation to estradiol (Woolley & McEwen, 1994; Woolley et al., 1997). Synaptic plasticity is also demonstrated to increase in estradiol-treated rats with enhanced EPSP and LTP which is mediated via NMDA receptors (Foy et al., 1999). Despite the effects of estrogen secreted via the gonads on the hippocampus, it should be kept in mind that estrogen synthesis takes place also in the hippocampus and this local synthesis might influence the learning process through synaptic plasticity (Fester et al., 2011).

A previous study aiming to investigate the differential brain circuits activated by acute nausea and conditioned nausea investigated both male and female rats' behavior and c-Fos activation (Bernanke et al., 2022). C-fos is an indicator of neuronal activity and an immediate early gene (Herrera & Robertson, 1996). The behavioral test in the study of Bernanke et al. (2022) failed to find a significant difference between males and females in conditioned gaping responses. The identified brain regions activated for acute LiCl treatment AP, nucleus tractus solitarius (NTS), central nucleus of the amygdala (CeA), and supraoptic nucleus (SON). None of the areas were affected by the sex except SON which had increased expression for males compared to females. The ventromedial prefrontal cortex (vmPFC), exhibited overall increased interaction for females with no significant difference between NaCl and LiCl administration or acute and conditioned nausea. On the other hand, the males treated with NaCl had enhanced c-Fos expression while conditioning and LiCl-treated males exhibited more c-Fos expression. The overall agranular insula (aIC) activation was higher for females, however, only males exhibited a significant increase in activation for LiCl treated group during the testing of conditioned nausea. Paraventricular nucleus (PVN) activation was overall similar for both sexes. Only male rats had an enhanced activation for conditioned nausea in the LiCl-treated group. On the other hand, the nucleus accumbens core, granular insula, and basolateral nucleus of the amygdala were other brain regions that were investigated with no identified function on acute and conditioned nausea. Despite the lack of sexual dimorphism in a paradigm that previously created an increased conditioned gaping behavior in rats (Cloutier, 2017), the activated brain areas diverged as a function of sex, treated drug (LiCl or NaCl), and time of expression (during conditioning or testing). The dimorphism was present as a response to LiCl treatment during conditioning only for SON. Yet, the testing

phase created a difference in parts of the hypothalamic structure and cortical area accounted for contextual cues.

Bernanke et al. (2022) found no sexual dimorphism in terms of behavior, thus, the differences in brain activity might not be conclusive. While there is a gap in the literature on the sex differences in brain circuits and hormonal influences for CCA, studies on gonadal hormones' influences on the brain areas related to learning and memory can shed light on the sexual dimorphism observed in AN.

# CHAPTER 2

# THE INFLUENCE OF ESTROGEN ON CCA

In this chapter of the thesis, two experiments that aim to investigate the impact of estrogen in CCA will be described.

## 2.1. Experiment 1

The first experiment investigated the impact of ovarian-secreted hormones on CCA learning and extinction in mice.

### 2.1.1. Method

#### 2.1.1.1. Subjects

44 female CD1 mice (10-12 weeks) were subjected to the current study. The impact of ovarian-secreted hormones was manipulated through ovariectomy, which involves the removal of the ovaries, thus lowering serum sex hormones. To this end, one group of animals was ovariectomized (n = 23) while the remaining underwent sham surgery (n = 21). Then, these two groups are subdivided into two more groups to form experimental (OVX-LiCl, n = 12) and control (OVX-NaCl, n = 11) groups. Half of the animals received LiCl injections during conditioning to induce discomfort so the animal will become conditioned to the context in which it received the injection. On the other hand, the control animals received NaCl in the same volume. The same procedure was applied to the sham-operated animals (SHAM-LiCl, n= 1 1; SHAM-NaCl, n = 10) and the experiment consisted of four groups in total.

| Group | Number of Animals | Injection During Conditioning | Surgery |
|---|---|---|---|
| OVX-LiCl | 12 | LiCl | Ovariectomy |

| | | | |
|---|---|---|---|
| OVX-NaCl | 11 | NaCl | Ovariectomy |
| SHAM-LiCl | 11 | LiCl | Sham surgery |
| SHAM-NaCl | 10 | NaCl | Sham surgery |

**Table 1.** The number of animals in each group, the injection type they received, and the surgery they underwent are indicated in the table.

#### 2.1.1.2. Colony Room

Each mouse was housed in transparent Eurostandard Type II cages. The bedding consisted of wood shavings and the water was served in standard plastic bottles. The colony room in which cages were placed had a 12/12h light/dark cycle and the temperature of the room was maintained at 21°C ±1. Food and tap water were available *ad libitum*, except for the water restriction periods described below. The experimental protocol was approved by the Animal Ethics Committee of Technical Universal Verification (protocol number: 0026/2023/01).

#### 2.1.1.3. Apparatus

A distinct environment from the home cages was obtained for the conditioning procedure to take place in a different context. To this end, a different room enriched with lemon oil scent, 75 dB white noise, and a 60 W red lamp was used. Cat litter was used as the bedding of Eurostandard Type II cages which was confined with black and white stripes. The water was served in green glass bottles with stainless steel spouts and ball bearings.

#### 2.1.1.4. Experimental procedure

*Surgery*. The surgeries were conducted 10 minutes after 80 mg/kg Ketamine and 8 mg/kg Xylazine cocktail injection intraperitoneally (Kawai et al., 2011). 23 of the animals underwent ovariectomy which involved bilateral removal of the ovaries through a 1-cm dorsal incision towards the caudal region. The ovaries on both sides were located and removed. The remaining 21 animals underwent the same procedure, however their ovaries were not removed and were located back without removal. The animals received subcutaneous antibiotic and analgesic injections and were placed in their cages following the surgery. Antibiotic and analgesic injections were provided

for 2 days following the surgery. The animals continued to receive food and water *ad libitum* for 4 more days of recovery.

*Habituation.* After the healing period, the animals were habituated to experimental handling to reduce stress. To this end, each animal was handled for 3 consecutive days, 3 minutes each day. The animals continued to receive food and water *ad libitum* during the first two days of the handling. On the third day, the plastic water bottles were removed from the cage at 17:30 and water restriction started.

*Training to drink.* Water training started the day after the restriction for animals to drink the water by the time they were served with it. To this end, their respective plastic bottles were provided to the animals for three days between periods of 10:00-10:30 and 17:00-17:30.

*Pre-exposure.* The mice were introduced to the conditioning context for 5 minutes on the third day of water training since it has been shown to facilitate conditioning (İlhan et al., 2023). Each animal was placed in the conditioning context starting at 12:30. In these 5 minutes, the water was provided to the animal in glass bottles located in the conditioning cage. After the pre-exposure was complete, the animals were located in their respective home cages in the colony room. On pre-exposure day, the water was accessible between periods of 10:00-10:30 and 17:00-17:30 in the colony room.

*Conditioning.* The current experimental procedure includes a single conditioning session which started 24 hours after pre-exposure. Therefore, the animals were placed in the conditioning context starting from 12:30 in which water was provided in green glass bottles for a 20-minute conditioning session. After the first 5 minutes spent in the conditioning cages, the animals in the experimental groups (OVX-LiCl and SHAM-LiCl) received LiCl injections (6 mEq/kg) while the animals in the control groups (OVX-NaCl and SHAM-NaCl) received 0.9% NaCl injections in an equivalent volume with respect to body weight of each animal in the conditioning room. After each injection, the mouse was placed back in its respective cage and allowed to spend 15 more minutes. The consumed water amount was measured during the conditioning. On the conditioning day, the water was not served in the morning session at 10:00-10:30 but was only provided in the afternoon session between 17:00-17:30 in the colony room.

*Recovery.* The animals were given two days to recover from the side effects of LiCl for two days. The mice were provided with their respective plastic bottles between periods of 10:00-10:30 and 17:00-17:30 during these two days.

*Retention.* 72 hours after conditioning, starting at 12:30, the animals were placed in the conditioning context in their respective cages for 15 minutes. The water amount consumed during the retention test was used to quantify the aversion levels of the mice toward the conditioning context. The water was provided only during the afternoon session between 17:00-17:30 on retention days. The retention days were followed by two days of recovery with morning and afternoon water sessions. The retention was repeated every 72 hours until the extinction was observed. The completion of extinction was decided based on the consumed water amounts during the retention. When all animals consumed statistically similar amounts of water, the behavioral test was finalized. This took 5 retention test for the Experiment 1.

*Body weight.* The animals were weighed at different times during the experiment on the day of surgery, conditioning, and second and third retention.

*Uterine weight.* The animals were sacrificed, and their uteri were removed and weighed promptly.

#### 2.1.1.5. Statistical analysis

All statistical analyses were conducted using GraphPad Prism (Version 9.0). A 2x2x5 mixed effect model matched by the trial was used for analyzing retention. The same analysis plan was conducted for body weight analysis which matched by time point at which the animal was weighed. Remaining of the data was analyzed by 2x2 factorial ANOVA. Fisher's LSD was used for post-hoc comparisons with a <.05 significance level.

### 2.1.2. Results

#### 2.1.2.1. Conditioning

The consumed water amount during 20-minute conditioning session did not significantly differ between groups (see Figure 3). 2 (Operation: OVX vs. SHAM) x 2 (Drug: LiCl vs. NaCl) factorial ANOVA was conducted and found no main effect (Operation: $F(1, 41) = 0.005$, $p = .944$; Drug $F(1,41) = 2.029$, $p = .162$) or interaction between operation and drug ($F(1, 41) = 0.066$, $p = .799$ ). The quantity consumed by

OVX-LiCl ($M$ = 0.718, $SD$ = 0.398), OVX-NaCl ($M$ = 0.928, $SD$ = 0.292), SHAM-LiCl ($M$ = 0.759, $SD$ = 0.451) and SHAM-NaCl ($M$ = 0.905, $SD$ = 0.508) groups were similar in conditioning trial.

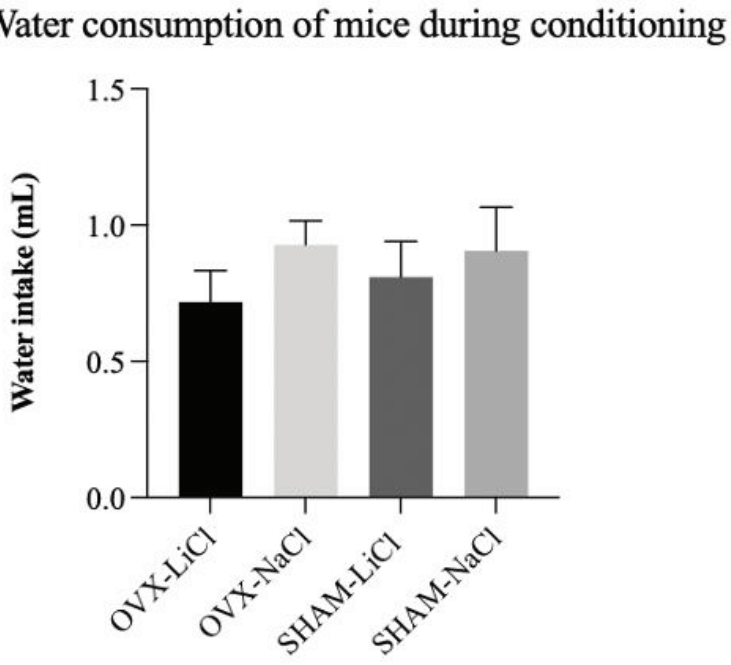


**Figure 3.** The amount of consumed water during 20-min conditioning. Female CD1 mice in all four groups consumed similar amount of water during conditioning trial. All data depicted are mean ± SEM.

#### 2.1.2.2. Retention Tests

Figure 4 illustrates the water quantities consumed by each group across five retention trials. The results of 2 (Operation: OVX vs. SHAM) x 2 (Drug: LiCl vs. NaCl) x 5 (Trial: Retention Test 1 vs. Retention Test 2 vs. Retention Test 3 vs. Retention Test 4 vs. Retention Test 5) mixed-effects model revealed a main effect of Trial F(3.347, 133.9) = 24.65, $p$ < .001 and Drug F (1,40) = $p$ < .001 and an interaction between Trial and Drug F(4, 160) = 2.724, p = .03. Fisher's LSD shows that water quantity consumed by SHAM-LiCl and SHAM-LiCl groups significantly differ during Retention Test 1 (SHAM-LiCl: $M$ = 0.266, $SD$ = 0.169 vs. SHAM-NaCl: $M$ = 0.803, $SD$ = 0.345, $p$ < .001), Retention Test 2 (SHAM-LiCl: $M$ = 0.444, $SD$ = 184 vs. SHAM-NaCl: $M$ = 1.006, $SD$ = 0.389, $p$ < .001), Retention Test 3 (SHAM-LiCl: $M$ = 0.538, $SD$ = 0.292 vs. SHAM-NaCl: $M$ = 1.104, $SD$ = 0.442, $p$ = .003), Retention Test 4 (SHAM-LiCl: $M$ = 0.708, $SD$ = 0.198 vs. SHAM-NaCl: $M$ = 1.081, $SD$ = 0.364, $p$ = .012). The consumed water amounts of SHAM-LiCl and SHAM-LiCl were equalized by the

Retention Test 5 (SHAM-LiCl: $M = 0.782$, $SD = 0.199$ vs. SHAM-NaCl: $M = 1.038$, $SD = 0.345$, $p = .058$). The difference between OVX-LiCl and OVX-NaCl groups was only significant during the Retention Test 1 (OVX-LiCl: $M = 0.331$, $SD = 0.187$ vs. OVX-NaCl: $M = 0.697$, $SD = 0.281$, $p = .002$ ) which diminished by the Retention Test 2. Therefore, extinction was observed faster in ovariectomized mice compared to gonadally intact mice.

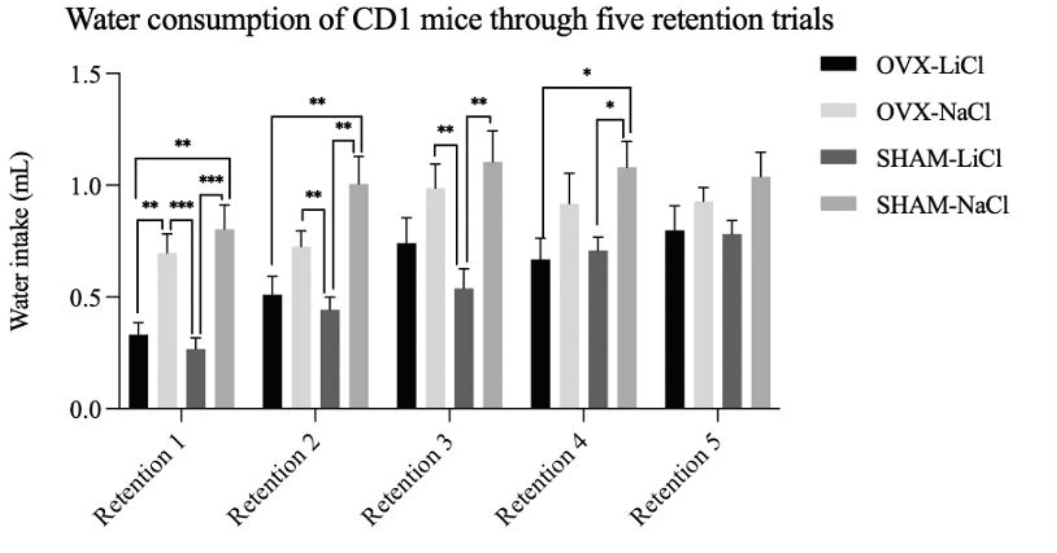


**Figure 4.** The amount of consumed water during 15-minute retention tests. Extinction in ovariectomized CD1 mice were observed by the Retention 2 while sham-operated mice extinguished by Retention 5. All data depicted are mean ± SEM. * $p < .05$, ** $p < .01$, *** $p < .001$

### 2.1.2.3. Body Weight

The animals in each group had similar body weights at the start of the experiment, however, the ovariectomized animals outgrew the sham-operated groups by the end of the experiment see Figure 5). 2 (Operation: OVX vs. SHAM) x 2 (Drug: LiCl vs. NaCl) x 4 (Time point: Day 1 vs. Day 13 vs. Day 19 vs. Day 24) mixed-effects model shows a main effect of Time point $F(2.278, 91.11) = 252.9$, $p < .001$ and interaction between Time point and Operation $F(3, 120) = 26.58$, $p < .001$. Fisher's LSD shows that there is no significant difference between the body weights of the groups during Day 1 (OVX-LiCl: $M = 27.559$, $SD = 2.603$; OVX-NaCl: $M = 28.385$, $SD = 1.895$; SHAM-LiCl: $M = 28.475$, $SD = 1.273$; SHAM-NaCl: $M = 27.635$, $SD = 2.654$). A significant difference between ovariectomized and sham-operated groups emerged by Day 24 (OVX-LiCl: $M = 25.892$, $SD = 2.906$ vs. SHAM-NaCl: $M = 24.965$, $SD = 2.584$, $p = .035$; OVX-NaCl: $M = 28.043$, $SD = 2.448$ vs. SHAM-LiCl: $M = 26.051$, $SD = 1.344$,

$p$ = .031; OVX-NaCl: $M$ = 28.043, $SD$ = 2.448, SHAM-NaCl: $M$ = 24.965, $SD$ = 2.584, $p$ = .012).

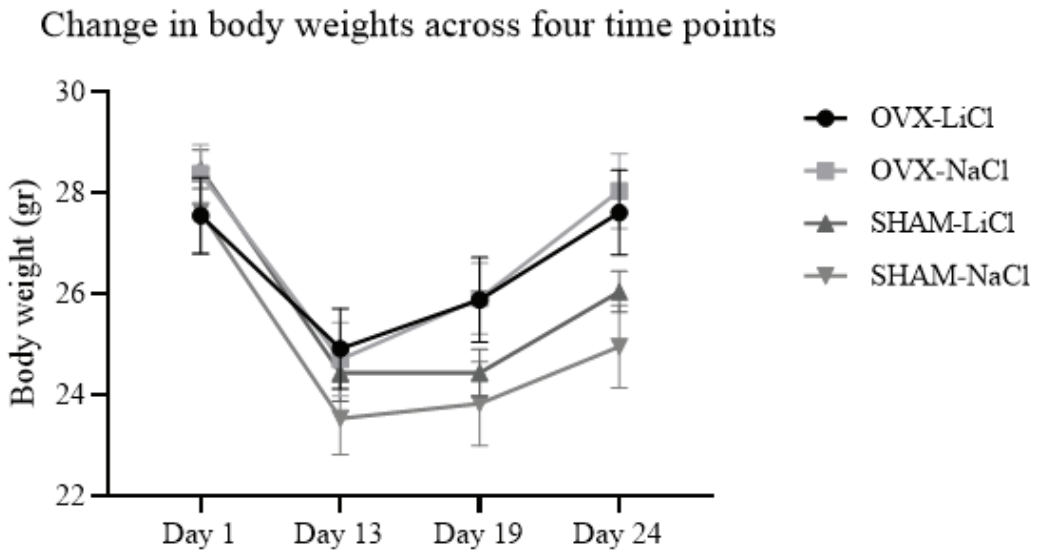


**Figure 5.** The body weights of animals across four different time points. All data is depicted as mean ± SEM.

#### 2.1.2.4. Uterine Weight

The relative uterine weights of the animals were calculated by dividing the uterine weight (g) of the animal by its body weight (g) multiplied by 100. The results across four different groups were analyzed by 2 (Operation: OVX vs. SHAM) x 2 (Drug: LiCl vs. NaCl) factorial ANOVA. There is a main effect of Operation $F(1, 40) = 68.01$, $p < .001$) and an interaction between Drug and Operation $F(1, 40) = 12.92$, $p < .001$. Post-hoc comparison using Fisher's LSD showed a significant difference between OVX-LiCl ($M$ = 0.163, $SD$ = 0.028) and SHAM-LiCl ($M$ = 0.523, $SD$ = 0.144, $p <$ .001), OVX-NaCl ($M$ = 0.218, $SD$ = 0.028) and SHAM-NaCl ($M$ = 0.359, $SD$ = 0.132, $p$ = .003), SHAM-LiCl ($M$ = 0.523, $SD$ = 0.144) and SHAM-NaCl ($M$ = 0.359, $SD$ = 0.132, $p < .001$), OVX-LiCl ($M$ = 0.163, $SD$ = 0.028) and SHAM-NaCl ($M$ = 0.359, $SD$ = 0.132, $p < .001$), and SHAM-LiCl ($M$ = 0.523, $SD$ = 0.144) and OVX-NaCl ($M$ = 0.218, $SD$ = 0.057, $p < .001$) mice. On the other hand, OVX-LiCl ($M$ = 0.162, $SD$ = 0.028) and OVX-NaCl ($M$ = 0.218, $SD$ = 0.057, $p$ = .202) groups did not significantly differ in terms of their relative uterine weights. Therefore, the ovariectomized animals had fewer relative uterine weight scores compared to the sham-operated mice.

Furthermore, the LiCl injection enhanced the uterine weight of the gonadally intact mice.

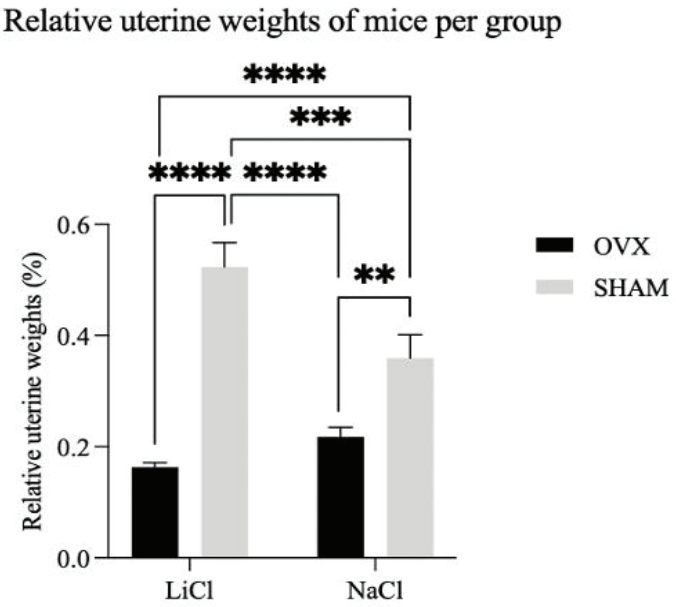


**Figure 6.** Relative uterine weights of animals across four different groups. The ovariectomized mice had significantly lower uterine weight compared to sham-operated mice. LiCl injection to sham-operated mice increased uterine weight while the uterine weight of the ovariectomized mice was not influenced by LiCl injection. All data is depicted as mean ± SEM. ** $p < .01$, *** $p < .001$, **** $p < .0001$

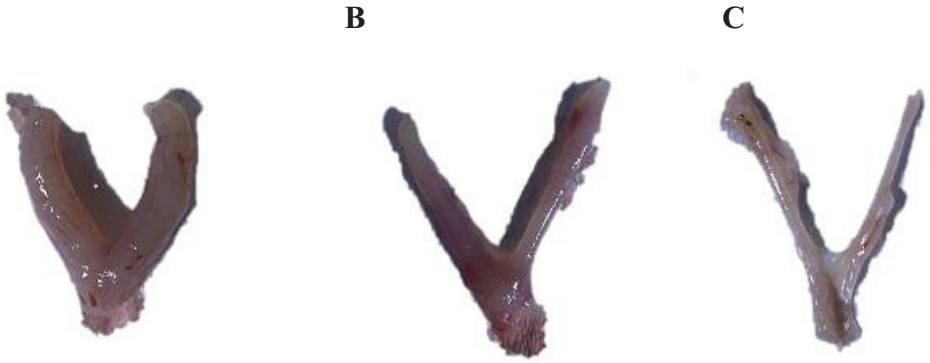


**Figure 7.** A. Uterus of the sham-operated mouse received a single injection of LiCl during conditioning. B. Uterus of the sham-operated mouse received NaCl injection during conditioning. C. Uterus of the ovariectomized mouse.

### 2.1.3. Discussion

The first experiment was conducted to investigate whether the hormones secreted through ovaries have an impact on CCA which an aversion-inducing agent is paired with a distinctive context during a single conditioning procedure. The sham-operated animals that were injected with LiCl consumed less amount of water compared to their

sham-operated NaCl counterparts due to the development of conditioned aversion. Similarly, ovariectomized mice injected with LiCl consumed less water compared to ovariectomized NaCl-injected animals since LiCl-injected animals developed an aversion. On the other hand, the sham-operated animals that received LiCl during conditioning maintained their conditioned aversion for more retention trials compared to the ovariectomized LiCl group. The results of this study have shown that removal of the ovaries accelerated retention, thus, the conditioned response was maintained for a shorter period of time when the gonadal hormone levels of female mice were lower.

## 2.2. Experiment 2

The first experiment revealed the impact of ovarian-secreted hormones on CCA. To further investigate the impact of specific hormones, 17β-estradiol (E2) was administered to ovariectomized mice to observe the impact of E2 on CCA learning and extinction in mice.

### 2.2.1. Method

#### 2.2.1.1. Subjects

39 female CD1 mice (10-12 weeks) were subjected to Experiment 2. The sex hormone levels of all subjects were lowered through ovariectomy. One group of animals received E2 treatment (n = 20) while the remaining received sesame oil (n = 19). Then, these two groups are subdivided into two more groups to form experimental and control groups. Half of the animals that received estrogen received LiCl injections (E-LiCl, n = 10) during conditioning to induce discomfort so the animal will become conditioned to the context in which it received the injection. On the other hand, the control animals received NaCl in the same volume (E-NaCl, n = 10). The same procedure was applied to the vehicle (sesame oil) treated animals (O-LiCl, n = 9; O-NaCl, n = 10) and the experiment consisted of four groups in total. The experimental protocol was approved by the Animal Ethics Committee of Technical Universal Verification (protocol number: 0035/2023/01).

| Group | Number of Animals | Injection During Conditioning | Surgery | Hormone Treatment |
|---|---|---|---|---|
| E-LiCl | 10 | LiCl | Ovariecomy | Estradiol |
| E-NaCl | 10 | NaCl | Ovariecomy | Estradiol |

| | | | | |
|---|---|---|---|---|
| O-LiCl | 9 | LiCl | Ovariecomy | Sesame oil |
| O-NaCl | 10 | NaCl | Ovariecomy | Sesame oil |

**Table 2.** The number of animals in each group, the injection type they received, and the surgery they underwent are indicated in the table.

#### 2.2.1.2. Colony Room

The housing condition in the colony room was the same as in Experiment 1.

#### 2.2.1.3. Apparatus

The apparatus was the same as in Experiment 1.

#### 2.2.1.4. Experimental Procedure

The experimental procedure was the same as Experiment 1 with an additional protocol for E2 administration proposed by Ström et al. (2012).

*Nutella acclimation.* The E2 administration was actualized through a mixture of E2 dissolved in sesame oil and Nutella. To ensure voluntary consumption immediately after mice are being served, an acclimation procedure was applied for four days prior to surgery. The animals were served 60 mg of Nutella per 30 gr animal on 5x5 cm white marble pieces daily between 9:30 and 17:30.

*Estrogen administration.* The E2 administration started the day following the ovariectomy surgery. The E2 administration protocol through the peroral method developed by Ström et al. (2012) was followed for hormone replacement. First, E2 was dissolved in sesame oil which is then mixed with Nutella. Each 30 gr animal received a 1.12 ug E2 dissolved in 0.312 uL sesame oil in 60 mg Nutella relative to their body weights. The control group received the same proportion of mixture similarly, without E2. The Nutella was served on the same 5x5 white marble pieces that were used during the Nutella acclimation period between 9:30-10:00. All animals finished their proportion of Nutella within 1 to 2 minutes.

#### 2.2.1.5. Statistical Analysis

The statistical analysis is conducted in the same way as in Experiment 1.

### 2.2.2. Results

#### 2.2.2.1. Conditioning

The water quantity consumed by mice of each group during 20 minutes of conditioning did not differ from each other (see Figure 9). 2 (Hormone: estrogen vs.

oil) x 2 (Drug:

**Figure 8.** The figure shows the marble pieces which the animals are served with Nutella.

LiCl vs. NaCl) factorial ANOVA was conducted for statistical analysis. The results revealed no significant main effect of Drug $F(1, 38) = 0.254$, $p = .617$, Hormone $F(1, 38) = 0.003$, $p = .957$) and interaction between Drug and Hormone $F(1, 38) = 0.050$, $p = 825$). The amount of water consumed during conditioning by E-LiCl (M = 0.666, SD = 0.313), E-NaCl (M = 0.737, SD = 0.266), O-LiCl (M = 0.683, SD = 0.361), O-NaCl (M = 0.710, SD = 0.305) groups were similar.

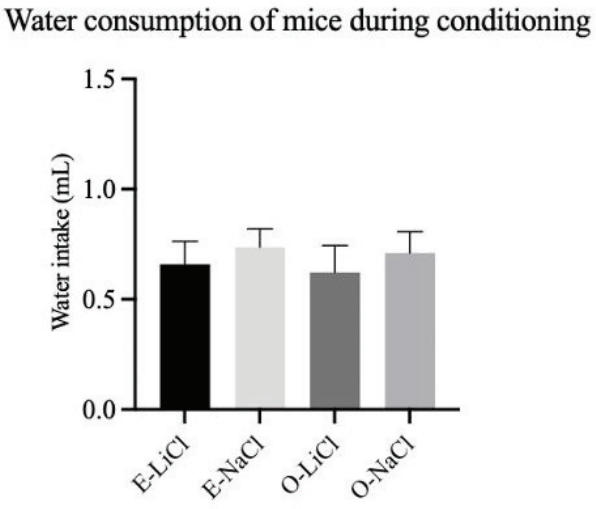


**Figure 9.** The amount of consumed water during 20-min conditioning. Female CD1 mice in all four groups consumed a similar amount of water during the conditioning trial. All data depicted are mean ± SEM.

**2.2.2.2. Retention Tests**

Figure 10 illustrates the water quantities consumed by each group across five retention

trials. During the first retention trial, the E-LiCl group consumed less amount of water compared to mice in the E-NaCl group. However, O-LiCl and O-NaCl animals did not significantly differ in their water consumption. 2 (Hormone: estrogen vs. oil) x 2 (Drug: LiCl vs. NaCl) x 5 (Trial: Retention Test 1 vs. Retention Test 2 vs. Retention Test 3 vs. Retention Test 4 vs. Retention Test 5) mixed-effects model results revealed a main effect of Trial $F(2.843, 98.78) = 32.28$, $p < .001$ and Drug $F(1,35) = 4.566$, $p = .040$, and interaction between Trial and Drug $F(4, 139) = 4.316$, $p = .003$. Fisher's LSD shows that E-LiCl and E-NaCl groups significantly differ in consumed water quantities during the Retention Test 1 (E-LiCl: M = 0.183, SD = 0.132 vs. E-NaCl: M = 0.620, SD = 0.329, $p = .002$). On the other hand, the E-LiCl and E-NaCl groups did not significantly differ in their water consumption by the Retention Test 2 (E-LiCl: M = 0.475, SD = 0.363 vs. E-NaCl: M = 0.803, SD = 0.334, $p = .050$). O-LiCl and O-NaCl groups consumed similar amounts of water in Retention Test 1 (O-LiCl: M = 0.248, SD = 0.389 vs. O-NaCl: M = 0.598, SD = 0.405, $p = .071$) and in the following trials.

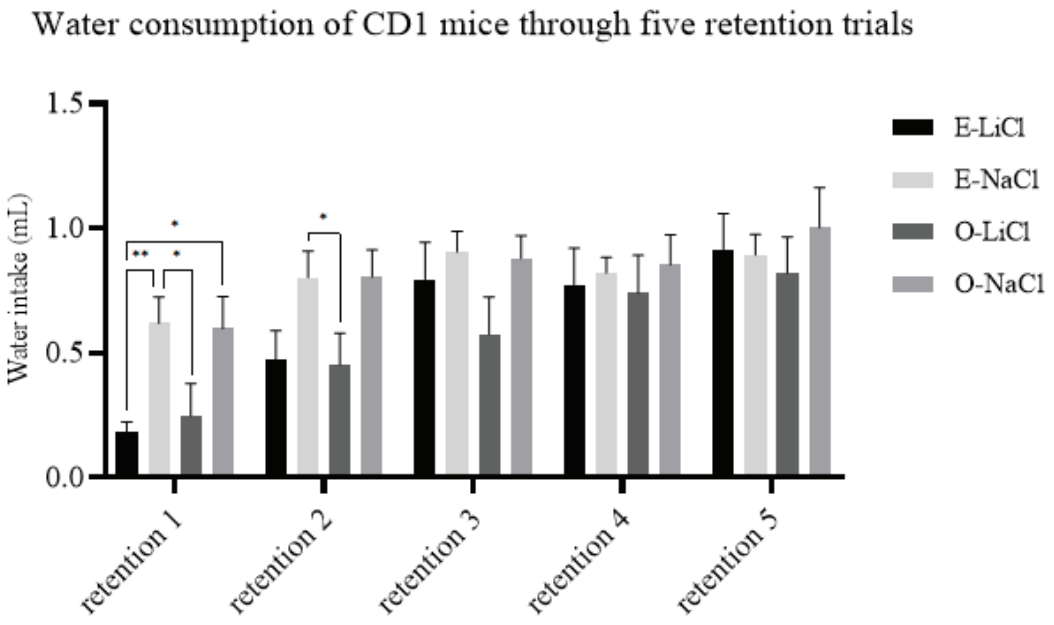


**Figure 10.** The amount of consumed water during 15-minute retention tests. Extinction in E2-treated CD1 mice was observed by Retention 2 while oil-treated mice did not exhibit a significant aversion during retention tests. All data depicted are mean ± SEM. * $p < .05$, ** $p < .01$

**2.2.2.3. Body weight**

The animals in each group had similar body weights at the start of and throughout the

experiment (see Figure 11). 2 (Hormone: estrogen vs. oil) x 2 (Drug: LiCl vs. NaCl) x 6 (Time point: Week 1 vs. Week 2 vs. Week 3 vs. Week 4 vs. Week 5 vs. Week 6) mixed-effects model revealed a main effect of Time point $F(2.030, 71.04) = 113.3$, $p < .001$ and interaction between Time point and Hormone $F(5, 175) = 6.146$, $p < .001$. Post-hoc pairwise comparison with Fisher's LSD shows no significant difference by the start (E-LiCl: M = 25.039, SD = 3.527, E-NaCl: M = 23.971, SD = 4.253, O-LiCl: M = 21.478, SD = 4.172, O-NaCl: M = 21.830, SD = 3.522) or at the end (E-LiCl: M = 29.006, SD = 2.547, E-NaCl: M = 27.371, SD = 3.763, O-LiCl: M = 27.476, SD = 2.447, O-NaCl: M =28.299, SD = 3.410) of the experiment.

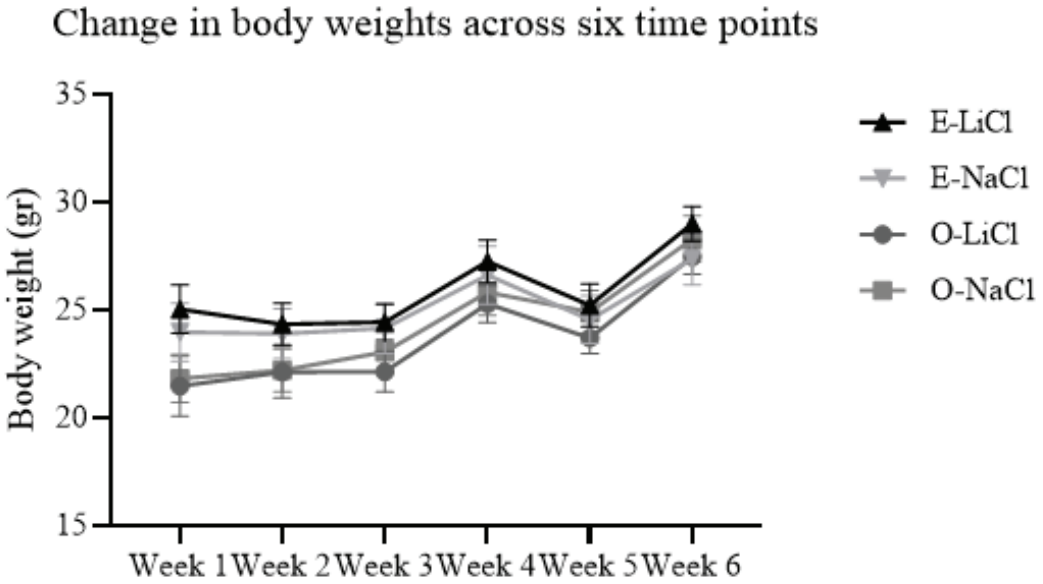


**Figure 11.** The body weights of animals across four different time points. All data is depicted as mean ± SEM.

#### 2.2.2.4. Uterine weight

The relative uterine weights of the animals were calculated by dividing the uterine weight (g) of the animal by its body weight (g) multiplied by 100. The results across four different groups were analyzed by 2 (Hormone: estrogen vs. oil) x 2 (Drug: LiCl vs. NaCl) factorial ANOVA. Only a main effect of Hormone $F(1, 35) = 6.018$, $p = .019$) was found. Post-hoc comparison using Fisher's LSD showed a significant difference between O-LiCl and E-NaCl groups (O-LiCl: M = 0.190, SD = 0.057 vs.

E-NaCl: M = 0.243, SD = 0.030, $p$ = .027) and E-NaCl and O-NaCl groups (E-NaCl: M = 0.243, SD = 0.030 vs. O-NaCl: M = 0.197, SD = 0.009, $p$ = .049).

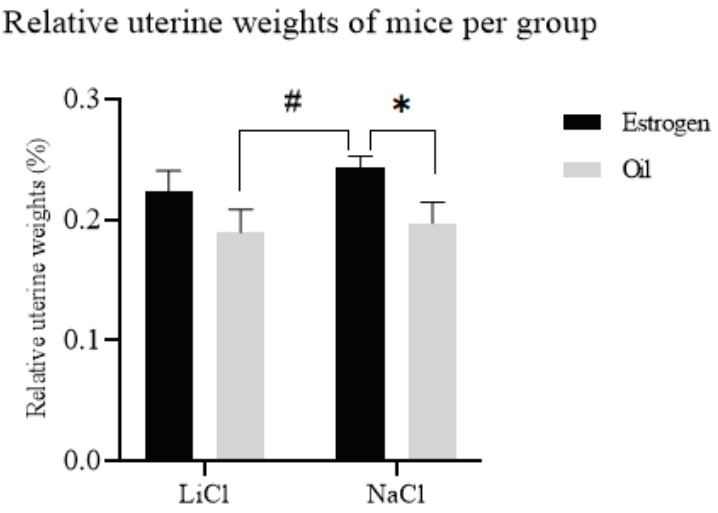


**Figure 12.** Relative uterine weights of animals across four different groups. Only NaCl-injected and E2-treated female CD1 mice had significantly higher uterine weight compared to NaCl-injected oil-treated mice and LiCl-injected oil-treated mice. All data is depicted as mean ± SEM. * $p < .05$, # $p < .05$

### 2.2.3. Discussion

The first experiment revealed a facilitatory impact of ovarian-secreted hormones on CCA. Therefore, a second experiment was conducted to investigate which specific hormone is responsible for this facilitatory effect. To this end, female mice were ovariectomized to reduce ovarian-secreted hormone levels and half of them received estradiol treatment while the remaining of the animals received oil to constitute a control group. The conditioned responses of E2-treated and control animals developed through a pairing of a novel context and LiCl were compared. There was only a significant difference between the LiCl and NaCl injected groups of E2-treated animals which was diminished by the second retention trial. Specifically, E2-treated and LiCl-injected animals consumed less water during the first retention trial compared to E2-treated NaCl-injected counterparts. On the other hand, such a significant difference between LiCl and NaCl-injected animals of the oil-treated group was not observed in any of the trials.

# CHAPTER 3

## GENERAL DISCUSSION

Two studies were conducted to examine the impact of estradiol on CCA which is a potential explanation for sex differences observed in preclinical studies. After finding an impact of ovarian-secreted hormones on CCA extinction in the first experiment, a second experiment was conducted to find out if estradiol is the responsible hormone for behavioral differences between sexes.

The gonadal hormone levels of female mice in Experiment 1 were manipulated through ovariectomy surgery which consists of the removal of the ovaries leading to a decrease in female gonadal hormones. In this experiment, conditioned aversion levels of ovariectomized and sham-operated animals were compared. Ovariectomized and sham-operated mice that received LiCl injections during conditioning did not exhibit a significant difference during any of the retention tests. On the other hand, the extinction was observed later in sham-operated mice while the ovariectomy procedure accelerated the retention. Those findings are in parallel with our previous data which shows that male mice extinguish faster compared to female mice (İlhan et al., 2023).

While Experiment 1 showed that the hormones that are secreted through the ovaries facilitate CCA learning, the specific hormone that is causing this facilitation was not clear. Experiment 2 was conducted to further investigate if estradiol was the cause of a longer retention period in female mice. The target hormone estradiol was selected based on the previous finding of enhanced conditioned response in the CCA paradigm during the period of the estrus cycle which is characterized by elevated estrogen levels (Clouiter et al. 2018). None of the groups in Experiment 2 retained their conditioned aversion for four retention trials which was observed for sham-operated mice in Experiment 1. The difference between LiCl and NaCl animals in each group was only significant for E2-treated animals for one retention. The oil-treated animals of LiCl and NaCl groups did not differ significantly in their conditioned aversion levels during any of the retention trials. The conditioned aversion of E2-treated animals was only

present for the first retention and was diminished by the second trial. These results show that E2 administration partially rescued the effect of ovariectomy on CCA learning.

Only a slight impact of E2 treatment in conditioned aversion was observed in Experiment 2. This might be due to the inefficacy of the route of administration that is selected for this experiment. The E2 administration in the second experiment was actualized through the peroral method which the hormone is mixed with Nutella. As a result, the rodents consume the hormone that is served with Nutella voluntarily reducing the stress element which is present during injection and subcutaneous implantation which requires surgery (Frick et al., 2002; Moran et al., 2007; Morgan & Pfaff, 2001; Tang et al., 2005). Behavior is sensitive to stress and can have a sex-specific impact on the organism which might create a confound in the experimental procedure (Luine et al., 2017). Through using the peroral administration method of Ström et al. (2012), such confound is eliminated. Furthermore, this method provides experimental flexibility and if proven to affect the behavior, the action of hormones at different phases of learning can be investigated without concerns of prolonged stress in injections and the influence of operation conducted in between ongoing behavioral tests. The influence of perorally administered E2 did not rescue the ovariectomy procedure in our second experiment is proposed by Ström et al. (2012). E2 induced cell proliferation in the uterine leads to a decreased uterine weight when the animal is ovariectomized which is rescued by exogenous administration of this hormone (Ingberg et al., 2012; Medlock et al., 1991; Rabin et al., 1990). However, in Experiment 2, the uterine weight of animals in the E2 and oil-administered groups did not exhibit a meaningful difference, as observed by Ström et al. (2012) which peaks with high E2 concentrations in the bloodstream. In our second experiment, neither the uterine weights did not exhibit a profound difference due to hormone treatment, and the impact of E2 on behavioral results was small. In Experiment 1, the uterine weights of the ovariectomized and sham-operated mice were different significantly. Furthermore, an interaction between LiCl and female gonadal hormones was evident in the uteri. The sham-operated animals that received a single LiCl injection during conditioning had significantly heavier uterine compared to sham-operated mice that were injected with NaCl. Ovariectomized mice had overall lighter uterine weights and exhibited no difference between LiCl and NaCl-injected groups. The interaction

between estradiol and LiCl was evident in previous findings indicating that estradiol induces cell proliferation in the uterus (Gunin et al., 2004).

Apart from the uterine weight, the body weight of the animals is also influenced by the serum-gonadal hormone levels. Ovariectomy surgery, therefore a decrease in ovarian-secreted hormones, results in enhanced body weight in females (Cao & Grégoire, 2016; Hong et al., 2009). The influence of ovariectomy is rescued by the administration of estradiol preventing weight gain, and building a link between estradiol and body weight (Ding et al., 2017; Mann et al., 2020). In Experiment 1, the weights of the animals did not significantly differ across the four groups. The difference between groups reached a significance by the last time point at which weights were measured, with ovariectomized mice gaining weight in consistency with the literature. However, the weights of the animals did not differ in Experiment 2 at any of the time points where body weights were assessed. Therefore, the expected long-term outcome of estradiol, preventing weight gain was not observed in the second study. The serum-estradiol levels were not measured, however, body weight as the indirect measure of E2 effectiveness indicates that the hormone administration might not have been as effective as it was proposed.

The E2 treatment did not fully rescue the impact of the ovariectomy procedure in terms of body weight, uterine weight, and behavioral results in Experiment 2. On the other hand, the mice in Experiment 1 exhibited decreased uterine weight, increased body weight, and accelerated extinction. The current results do not support the facilitation of CCA learning via estradiol, however, it still remains elusive to reject such a hypothesis. The findings of Cloutier et al. (2018) suggest that estrogen increases the strength of conditioned response in CCA by following the estrus cycles of the animals in which estrogen levels fluctuate. Our first experiment also supports this finding by demonstrating the importance of ovarian-secreted hormones in CCA learning, regardless of the estrus cycle each animal is in. The removal of the ovaries, the primary supplier of estrogen in the female body, influenced the uterine weight and body weight as well as the behavioral outcomes in the expected direction indirectly implying decreased estrogen levels. Therefore, the current results still suggest that estrogen and estradiol remain one of the suspects that facilitate CCA.

Estrogen receptors are present in different parts of the body including endometrium

(Couse et al., 1997; Plante et al., 2012), ovaries (Sar & Welsch, 1999), prostate (Zhang et al., 2001), testes (Van Pelt et al., 1999), breast (Speirs et al., 2002), bone (Bord et al., 2001; Heino et al., 2008; Nakamura et al., 2007), liver (Alvaro et al., 2000; Gao et al., 2008; Villa et al., 2002), adipose tissue (Davis et al., 2013), skin (Campbell et al., 2010), skeletal muscles (Ikeda et al., 2019), vasculature (Stirone et al., 2003), as well as the central nervous system (Shughrue et al., 1999; Warfvinge et al., 2020). In the central nervous system, hippocampal formation is especially dense in estrogen receptors (González et al., 2007; Liu et al., 2008; Shughrue & Merchenthaler, 2000) and due to the strong relationship between learning and hippocampus, previous studies have focused on the estrogen receptors located in the hippocampus (Li et al., 2010). Previous findings demonstrate the enhancement of synaptic plasticity via estrogen, including in function of dendritic spine density (Woolley & McEwen, 1992), electrical excitability (Kubo et al., 1975; Scharfman et al., 2003), LTP (Gureviciene et al., 2003; Montoya & Carrer, 1997), and LTD (Murakami et al., 2015), neurogenesis and neuroprotection (Barker & Galea, 2008; Li et al., 2010). Moreover, the changes in the brain due to estrogen led to changes in behavior in many studies showing a strong relationship between estrogen, brain, and behavior (Bean et al., 2014; Zurkovsky et al., 2006).

The influence of estrogen has been found in various classical conditioning paradigms subjecting rodents as model animals. The impact of estrogen on fear conditioning paradigms is a widely investigated topic. Even though the findings vary depending on the task properties, estrogen was found to influence the outcomes in different ways demonstrating the effect of this hormone on behavior (Colón et al., 2023; Gupta et al., 2001; Markus & Zečević, 1997). Another classical conditioning paradigm is eyeblink conditioning, which exhibits a more robust pattern with female outperformance in adult rodents (Waddell et al., 2010; Dalla et al., 2009; Bangasser & Shors, 2007; Rapp et al., 2021; Leuner et al., 2004). The sexual dimorphism in eyeblink conditioning starts to emerge after puberty indicating the role played by the gonadal hormones in this learning paradigm showing the importance of gonadal hormones in observed sex differences (Hodes & Shors, 2005). Estrogen's facilitation can be also observed through results showing that removal of the ovaries aggravates the acquisition (Rapp et al., 2021), and estrogen treatment rescues the impact of ovariectomy through enhancing conditioned response frequency (Leuner et al., 2004). Examining the impact

of estrogen in CTA paradigms is also important since the conditioning procedures of CCA and CTA are similar in the sense that both paradigms prevalently use LiCl as their US. CTA is also prone to be influenced by the gonadal hormones, and exhibits sexual dimorphism in its experimental results (Chambers, 2018). Foy and Foy (2003) found that CRs generated by male rats were enhanced as opposed to female rats. Removal of the gonads in male and female subjects alleviated the observed sexual dimorphism, which was rescued when the gonadectomized females were introduced to 17β-estradiol and males were introduced to 5α-DHT highlighting the impact of estradiol. In addition to the strength of CRs, the extinction in CTA is also influenced by estradiol, in a time-dependent manner (Yuan & Chambers, 1999). In the experiment conducted by Yuan and Chambers (1999), when estradiol was administered throughout the experiment, extinction was accelerated and observed in the earlier retention trials. The extinction was prolonged when the administration of this hormone was present during the acquisition phase which conditioning took place. The results might be due to the aversive properties of the estradiol which might strengthen the US during the acquisition since it has been used as the US in previous CTA experiments (De Beun et al., 1991; Flanagan- Cato et al., 2001).

While estradiol might possibly influence the learning process through interaction with the hippocampus, the facilitation of CCA might be also due to the additive effect of estrogen on nausea. Women experience nausea more frequently in comparison to men under various health-related conditions (Goldberg et al., 2000; Koslucher et al., 2015; Patel et al., 2004; Salazar-Parra et al., 2020). Furthermore, the motion sickness levels fluctuate through menstrual cycle in women, which indicates the impact of gonadal hormones on nausea (Golding et al., 2005). CINV, which is observed in each chemotherapy cycle and is the US in ANV, also more prevalent in women, and such sex differences emerge after puberty and diminish with menopause. The rodents might also be prone to nausea due to the distribution of estrogen receptors in AP, which is a fundamental brain region for nausea, especially when induced by chemicals. Therefore, both ANV and CCA might be more prevalent or manifested stronger in women and in female rodents due to the additive effect of estrogen on nausea.

In conclusion, the current studies provide insights into the impact of gonadal hormones in ANV by investigating biological mechanisms that might explain female

susceptibility in CCA. While the impact of estradiol is not clear, female gonadal hormones play an important role in CCA learning and maintenance in our study. The following studies might enhance our understanding of sexual dimorphism not only in ANV but also in other medical conditions through the inclusion of both sexes in preclinical research, which will pave the way for novel treatment and prevention methods tailored to the specific needs of individuals.

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# APPENDICES

## A. APPROVAL OF THE METU HUMAN SUBJECTS ETHICS COMMITTEE

| | Technical Universal Verification Belgelendirme Laboratuvar Eğitim Ve Sağlık Hizmetleri San. Tic. Ltd. Şti. | Doküman Kodu/ Document Code | FR.94 |
|---|---|---|---|
| | | Yürürlük Tarihi/ Effective date | 05.05.2022 |
| | | Revizyon No/ Revision No | 01 |
| | | Revizyon Tarihi/ Revision Date | 26.01.2023 |
| | | Sayfa/ Page | 1/1 |

HAYVAN DENEYLERI YEREL ETIK KURUL KARARI ONAY FORMU/ ANIMAL EXPERIMENTS LOCAL ETHICS COMMITTEE DECISION PERMISSION FORM

Technical Universal Verification Yerel Etik Kurulu Yönergesi'ne göre aşağıda belirtilen kapsamda bahsi geçen çalışmanın yapılmasına oy çokluğu / oy birliği ile karar verilmiştir.

| | |
|---|---|
| **Toplantı Tarihi/** Meeting Date | 30.03.2023 |
| **Toplantı No/** Meeting No | 0015/2023 |
| **Etik Kurul No/** Ethical Committee No | EKN.0026/2023/01 |
| **Karar No/** Decision No | KN.0026/2023/01 |
| **Çalışmanın Adı/** Name of The Research | Östrojenlerin koşullu çevre itinmesi üzerindeki etkilerinin incelenmesi |
| **Çalışmanın Yürütücüsü/** Coordinator of The Research | Dr. Öğr. Üyesi Sezen KIŞLAL |
| **Çalışmanın Talep Edildiği Kurum-Kuruluş/** Institution-Organization Where The Research is Requested | Orta Doğu Teknik Üniversitesi Fen Edebiyat Fakültesi Psikoloji Anabilim Dalı |
| **Çalışmada Kullanılacak Hayvanın Türü/** The Species of The Animal To Be Used in The Research | Fare (CD1) |
| **Çalışmada Kullanılacak Hayvanın Cinsiyeti/** Gender of The Animal To Be Used in The Research | Dişi (10-12 Haftalık) |
| **Çalışmada Kullanılacak Hayvanın Sayısı/** Number of The Animals To Be Used in The Research | 48 |
| **Geçerlilik Süresi/** Period of Availability | 1 yıl |

| ETİK KURUL ÜYELERİ / MEMBERS OF ETHIC COMMITTEE | | | |
|---|---|---|---|
| **Adı-Soyadı/** Name-Surname | **Unvanı/** Title | **Görevi/** Position | **İmzası/** Signature |
| Gizem ARALAN | Dr. Veteriner Hekim | Etik Kurul Başkanı | |
| Serdar AYYILDIZ | Genel Müdür | Etik Kurul Başkan Yrd. | |
| Ahmet Çevik TUFAN | Prof. Dr. | Etik Kurul Başkan Yrd. | |
| Gülben AKCAN | Dr. Biyolog | In Vivo Sorumlusu | |
| Betül ÇAĞLIOĞLU | Genel Koordinatör/Biyolog | Üye | |
| Tuğba ŞİMŞİR | Biyolog | Üye | |
| Murat Ekin ERDOĞAN | İç Mimar | STK Üyesi | |

| | Technical Universal Verification Belgelendirme Laboratuvar Eğitim Ve Sağlık Hizmetleri San. Tic. Ltd. Şti. | Doküman Kodu/ Document Code | FR.94 |
|---|---|---|---|
| | | Yürürlük Tarihi/ Effective date | 05.05.2022 |
| | | Revizyon No/ Revision No | 02 |
| | | Revizyon Tarihi/ Revision Date | 31.05.2023 |
| | | Sayfa/ Page | 1/1 |
| HAYVAN DENEYLERI YEREL ETIK KURUL KARARI ONAY FORMU/ ANIMAL EXPERIMENTS LOCAL ETHICS COMMITTEE DECISION PERMISSION FORM | | | |

Technical Universal Verification Yerel Etik Kurulu Yönergesi' ne göre aşağıda belirtilen kapsamda bahsi geçen çalışmanın yapılmasına oy çokluğu/ oy birliği ile karar verilmiştir.

| | |
|---|---|
| **Toplantı Tarihi/ Meeting Date** | 25.08.2023 |
| **Toplantı No/ Meeting No** | 0022/2023 |
| **Etik Kurul No/ Ethical Committee No** | EKN.0035/2023/01 |
| **Karar No/ Decision No** | KN.0035/2023/01 |
| **Çalışmanın Adı/ Name of The Research** | Estradiol'ün Koşullu Çevre İtinmesi Üzerindeki Etkilerinin İncelenmesi |
| **Çalışmanın Yürütücüsü/ Coordinator of The Research** | Dr. Öğr. Üyesi Sezen Kışlal |
| **Çalışmanın Talep Edildiği Kurum-Kuruluş/ Institution-Organization Where The Research is Requested** | ODTÜ- Fen Edebiyat Fakültesi Psikoloji ABD |
| **Çalışmada Kullanılacak Hayvanın Türü/ The Species of The Animal To Be Used in The Research** | CD1 |
| **Çalışmada Kullanılacak Hayvanın Cinsiyeti/ Gender of The Animal To Be Used in The Research** | Dişi |
| **Çalışmada Kullanılacak Hayvanın Sayısı/ Number of The Animals To Be Used in The Research** | 48 |
| **Geçerlilik Süresi/ Period of Availability** | 1 yıl |

| ETİK KURUL ÜYELERİ/ MEMBERS OF ETHIC COMMITTEE | | | |
|---|---|---|---|
| **Adı-Soyadı/ Name-Surname** | **Unvanı/ Title** | **Görevi/ Position** | **İmzası/ Signature** |
| Gizem ARALAN | Dr. Veteriner Hekim | Etik Kurul Başkanı | |
| Serdar AYYILDIZ | Genel Müdür | Etik Kurul Başkan Yrd. | |
| A. Çevik TUFAN | Prof. Dr. | Etik Kurul Başkan Yrd. | |
| Gülben AKCAN | Dr. Biyolog | İn Vivo Sorumlusu | |
| Betül ÇAĞLIOĞLU | Genel Koordinatör/ Biyolog | Üye / Genel Sekreter | |
| Tuğba ŞİMŞİR | Biyolog | Üye | |
| Murat Ekin ERDOĞAN | İç Mimar | STK Üyesi | |

# B. CURRICULUM VITAE


## Cemile Ceren Akgül

Ankara, Turkey •


### Education

**Middle East Technical University** — Ankara, Turkey
Master's Degree in Cognitive Psychology — 03/2023 - Present
Thesis: "The influence of estrogen on conditioned context aversion learning"
Advisor: Dr. Sezen Kışlal

**Bilkent University** — Ankara, Turkey
Bachelor in Psychology (50% scholarship) — 08/2017 – 06/2022

**İzmir Atatürk High School** — İzmir, Turkey
09/2013 – 06/2017

### Skills

**Surgeries:** Transcardial perfusion, ovariectomy, brain extraction
**Technical skills:** Brain sectioning, immunohistochemistry, dosing (intraperitoneal and subcutaneous), cell counting
**Behavioral assays on rodents:** Classical conditioning, Y-Maze
**Programs:** Python, MATLAB, GraphPad Prism, SPSS, JASP, ImageJ

### Publications

Akgul, C. C., Kıslal, S., Blizard, D. (2023). *The influence of estrogen on conditioned context aversion learning* [Manuscript in preparation]. Department of Psychology, Middle East Technical University.

### Poster and Oral Presentations

Akgul, C. C., & Kıslal, S. (2023, September 28-29). Sex differences in conditioned context aversion [Oral presentation]. International Cankaya Scientific Studies Congress, Ankara, Turkey.

Akgul, C. C., & Kıslal, S. (2023, September 22-24). The influence of female gonadal hormones on conditioned context aversion learning [Oral presentation]. 9th International Conference on Social Sciences, İzmir, Turkey.

### Experience

**Dr. Sezen Kışlal** (METU Neuroscience Lab) — Ankara, Turkey

**Master's degree student** — 03/2023 – Present

- Guided the project "*The influence of estrogen on conditioned context aversion learning*" with full responsibility and organized the lab team
- Conducted ovariectomy and provided post-operative care to mice including subcutaneous injections of analgesics and antibiotics, conditioned the animals using intraperitoneal injections, and conducted behavioral tests
- Analyzed the data using GraphPad Prism and wrote a report
- Found an impact of ovarian-secreted hormones on conditioned context aversion learning in mice

**Intern** — 09/2022 – 03/2023

- Conducted behavioral assays, transcardial perfusion, brain extraction, and sectioning, provided post-operative care, and participated in immunohistochemistry process to examine c-Fos expression in mice

**Dr. Devin Terhune** (Goldsmiths Timing, Awareness, and Suggestion Lab) Remote
**Intern** 05/2021 – 07/2022

- Built experiments for the project "*Modulation of Bayesian temporal prior weighing by context salience*" using Psychopy
- Organized and cleaned data for further analysis using MATLAB

**Bilkent University** Ankara, Turkey
**Tutor** 09/2019 – 01/2020

- Tutored a Python course for social science students; assisted and graded the lab assignments, and provided feedback

**Dr. Hüseyin Boyacı** (Bilkent Computational and Biological Vision Group) Ankara, Turkey
**Undergraduate research assistant** 02/2021 – 05/2022

- Collected psychophysical data from human participants using Psychtoolbox for the study *"Investigating the effects of perceptual learning on the function and microstructure of visual cortex"*

**Dr. Hande Ilgaz** (Bilkent Bil-Ge Development Lab) Ankara, Turkey
**Undergraduate research assistant** 09/2019 – 01/2020

- Conducted behavioral data coding, transcription, and data entry using Excel

## Certificates

**Teilnahmebestätigung A2.1** (Goethe-Institut) 05/2023

**Training of Experimental Animal Use** 12/2022

**IELTS** (score: 7.5) 11/2022

**The Addicted Brain** (Coursera) 06/2020

## Extracurricular Activities

**Team Member** Ankara, Turkey
METU Sailing Club 09/2023 – Present

**Member** Ankara, Turkey
Bilkent Archeology Club 09/2021 – 05/2022

**Exchange Student and Volunteer** Minnesota, USA
Lions Youth Exchange Program 07/2018 – 08/2018

**Member** Ankara, Turkey
Bilkent Outdoor Sports Society 08/2017 – 06/2019

# C. TURKISH SUMMARY / TÜRKÇE ÖZET

## BÖLÜM 1

## GİRİŞ

Tezin ilk bölümünde deneylerin anlamlandırılması için önemli olan beklentisel bulantı ve kusma, koşullu çevre itinmesi, beklentisel bulantı, koşullu çevre itinmesi ve diğer klasik koşullanma prosedürlerindeki cinsiyet farklılıkları ve östrojen-bulantı ve östrojen-beyin ilişkisi gibi genel konseptlere ve buluntulara değinilmiştir.

### 1.1. Beklentisel Bulantı ve Kusma

Beklentisel bulantı ve kusma (BBK) kemoterapinin yan etkilerinden biridir ve kemoterapiye bağlı bulantı ve kusma (KBBK) tarafından tetiklenmektedir (Boakes et al., 1993; Coates et al., 1983). BBK klasik koşullanmanın bir sonucu olup hastanedeki nötral uyaranların kemoterapi tedavisinin rahatsız edici yan etkisiyle eşleşmesi ile tedavi beklentisinde bulantı ve kusma olarak kendini göstermektedir (Stockhorst et al., 1993). Bu psikolojik süreç her kemoterapi seansı sonrasında yoğunlaşma eğilimindedir ve ikinci seanstan itibaren hastaları etkileyebileceği gibi dördüncü döngüden itibaren hastaların yaklaşık %30'unda yaygın hale gelir (Roscoe et al., 2010). BBK'nin psikolojik yükü kaygı ve stresi arttırarak tedavinin devamlılığına engel olabilir (Andrykowski, 1990; Jordan et al., 2005; Van Komen & Redd, 1985). BBK yaygınlığı kullanılan kemoterapi ilacının bulantı etkisi, yaş, cinsiyet gibi faktörlere bağlıdır ve özellikle kadınlar BBK göstermeye daha yatkındır (NCI, 2024). Bu yatkınlığa sebep olan faktörleri saptamak ve anlamak önlem ve tedavi yöntemleri geliştirmek açısından önem arz etmektedir.

### 1.2. Koşullu Çevre İtinmesi

BBK'yi anlamakta sıklıkla koşullu çevre itinmesi (KÇİ) adı verilen hayvan modeli kullanılmaktadır (Cloutier et al., 2017, 2018; Hall & Symonds, 2006; İlhan et al., 2023;

Kislal & Blizard, 2016; Limebeer et al., 2008; Rodríguez et al., 2000). Bu model kemirgenlerin hastane ortamındakine benzer nötr bir bağlam ve lityum klorür (LiCl) gibi itinme yaratan bir ajanın eşleştirmesiyle oluşturulur (Bishnoi et al., 2023; Chan et al., 2009; Kislal & Blizard, 2017; Limebeer et al., 2008; Nachman & Ashe, 1973; Parker et al., 1984; Symonds & Hall, 1997; Symonds & Hall, 2000; Rodríguez et al., 2000; Wang et al., 2017). Klasik koşullanma sayesinde kemirgenler bağlam ve LiCl tarafından oluşan itinmeyi eşleştirerek BBK'de olduğu gibi bağlamla karşılaşıldığında beklenti sonucu bir itinme gösterirler. KÇİ prosedürü geliştirilirken çevresel uyaranlar değiştirilerek farklı bağlamlar oluşturulur ve sükroz tüketimi, su tüketimi, ve gözlemlenebilir davranışlar gibi farklı davranış testleriyle beklentisel itinme ölçülebilir (Cloutier et al., 2012; Hall & Symonds, 2006; İlhan et al., 2023; Kavaliers, et al., 2012; Limebeer et al., 2006; Limebeer et al., 2008; Ossenkopp et al., 2011; Rodríguez et al., 2000). Bu preklinik çalışmalar BBK'nin altında yatan mekanizmayı anlayabilmeye yardımcı olmakla birlikte yeni tedavi yöntemlerinin geliştirilmesine ve bu rahatsız edici yan etkiyle ilişkili biyolojik hassasiyet faktörlerinin tanımlanmasına da yardımcı olarak kanser hastalarının tedavi sırasında yaşam kalitesini iyileştirmede yarar sağlayabilmektedir.

### 1.3. BBK ve KÇİ'de Cinsiyet Farklılıkları

BBK yaygınlıkta kayda değer cinsiyet farklılıkları gösterir ve kadınlar erkeklere kıyasla BBK geliştirmeye daha yüksek duyarlılık sergilerler (Qureshi et al., 2016; Kamen et al., 2014; Fetting et al., 1983). Bu bulgular koşullu mide bulantısı paradigmalarını kullanan insan çalışmaları ve KÇİ modelini kullanan kemirgen deneyleri ile desteklenmektedir. BBK'yi simüle etmek amacıyla devinim sayrılığı yaratan rotasyon paradigmaları kullanılmış olup kadınlarda engelleme ve gölgeleme tekniklerinin erkeklere oranla daha etkili olduğu gözlemlenmiştir (Klosterhalfen et al., 2005; Stockhorst et al., 2014). Benzer şekilde, kemirgenlerle yürütülen KÇİ çalışmaları dişi kemirgenlerin erkeklere oranla daha fazla koşullu tepki ve daha geç sönme göstermiştir (Cloutier et al., 2017; Cloutier et al., 2018; İlhan et al., 2012). Ayrıca geçmiş bir çalışmada sıçanların östrus döngülerini takip ederek yüksek östrojen seviyeleri görülen proöstrus evresinde koşullu tepkinin arttığı saptanmıştır (Cloutier et al., 2018). Bu bulgulara rağmen BBK'de görülen cinsiyet farklılıklarına sebep olan mekanizma üzerine yapılan araştırmalar eksikliğini devam ettirmektedir. Bu nedenle,

bu mekanizmaları kapsamlı bir şekilde aydınlatmak iki cinsiyeti de kapsayan hedefe yönelik tedavi stratejileri geliştirmeye yardım edecek ve dolayısıyla kanser tedavisi gören bireylere yarar sağlayacaktır.

### 1.4. Östrojen ve Bulantı

Araştırmalar kemoterapinin yaygın yan etkileri olan mide bulantısı ve kusmanın cinsel dimorfizm sergilediğini; kadınların genellikle kemoterapinin neden olduğu mide bulantısı ve kusma (Qureshi et al., 2016), ameliyat sonrası mide bulantısı (Koivuranta et al., 1997; Salazar-Parra et al., 2020; Stadler et al., 2003), taşıt tutması (Koslucher et al., 2015) ve akut koroner sendrom (Goldberg et al., 2000; Patel et al., 2004) gibi çeşitli durumları erkeklere oranla daha yoğun olarak yaşadığını göstermektedir. Kadınlardaki bu belirgin duyarlılığın yumurtalıklardan salgılanan östrojen ve progesteron gibi hormonların etkilediği düşünülmektedir (Golding et al., 2005; Matchock et al., 2008; Walsh et al., 1996). Menstrual döngü ve hamilelik sırasında dalgalı bir seyir izleyen bu hormonlar geçmiş araştırmalarda artan mide bulantısı ile ilişkilendirilmiştir (Dekkers et al., 2019; Lagiou et al., 2003). Kemirgenler üzerine yapılan araştırmalar kusma refleksi olmamasına rağmen insandaki mide bulantısını taklit etmek için LiCl enjeksiyonu kullanmıştır (Andrews & Sanger, 2014). Özellikle sıçanlarda mide bulantısı ile homolog etkiler uyandıran durumlarda AP bölgesinin aktive olduğu görülmüş (Bernanke et al., 2022), ve bu gölgenin dişi ve erkeklerde farklı miktarlarda östrojen reseptörleri içerip cinsiyet farklılıklarına sebep olabileceği vurgulanmıştır (Zhang & Hamada, 2013).

### 1.5. Östrojen ve Beyindeki Öğrenme ile İlişkili Bölgeler

BBK'de görülen cinsiyet farklılıkları potansiyel olarak merkezi sinir sistemi, östrojenin özellikle de öğrenme ile ilişkilendirilen hipokampüs gibi östrojen açısından zengin beyin bölgeleri üzerindeki etkileri ile açıklanabilir. Hipokampüs, BBK'nin hayvan modeli olan KÇİ paradigması için de bağlamsal doğası sebebiyle fazlasıyla önem taşımaktadır (Broadbent et al., 2004; Bunsey & Eichenbaum; Burgess et al., 2002; Eichenbaum et al., 1999; Ergorul & Eichenbaum; Olton et al., 1979; Scoville & Milner, 1957). Öğrenme ve cinsiyet arasındaki etkileşim, östrojenin hipokampal reseptörlerle etkileşime girmesi sonucu nöral plastisite ve uyarılabilirliğin artmasıyla açıklanabilir (Gould et al., 1990; Montoya & Carrer; Spencer-Segal et al., 2012;

Terasawa & Timiras, 1968; Woolley & McEwen, 1992). Geçmiş çalışmalar östrojenin dendritik omurga yoğunluğu, LTP aktivasyon eşiği, sinaptik plastisite ve öğrenme için kritik olan NMDA reseptör modülasyonundaki rolünü göstermektedir (Foy et al., 1999; Montoya & Carrer; Woolley & McEwen, 1994; Woolley et al., 1997). Dolayısıyla gonad hormonlarının öğrenme ile alakalı beyin bölgeleri üzerine etkisini anlamak KÇİ ve BBK'deki cinsel dimorfizmin sebebini açıklığa kavuşturabilir.

# BÖLÜM 2

# ÖSTROJENİN KOŞULLU ÇEVRE İTİNMESİNE ETKİSİ

Tezin bu bölümünde östrojenin öğrenme üzerindeki etkisini görmeyi amaçlayan iki deney açıklanmıştır.

## 2.1. Deney 1

İlk deneyde yumurtalıklardan salgılanan hormonların farelerde KÇİ öğrenmesi ve sönmesinin etkisi araştırılmıştır.

### 2.1.1. Yöntem

#### 2.1.1.1. Denekler

Mevcut çalışmaya 10-12 haftalık 44 dişi CD1 fare dahil edildi. Deneyde yumurtalıklardan salgılanan hormonların KÇİ üzerindeki etkisinin incelenmesi için ovariektomi prosedürü gerçekleştirilerek kandaki eşey hormon miktarının azaltılması sağlanmıştır. Bu hedeflenerek bir grup hayvan ovariektomi edilmiş (n = 23) ve kalan hayvanlar kontrol amacıyla sahte operasyon geçirmiştir (n = 21). Sonrasında bu iki grup tekrar alt gruplara bölünerek deney ve kontrol gruplarını oluşturmuştur. Ovariektomi operasyonu geçirmiş grubun bir kısmı LiCl alarak deney grubunu (OVX-LiCl, n = 12) ve kalanıysa NaCl enjeksiyonuyla kontrol grubunu oluşturmuştur (OVX-NaCl, n = 11). Sahte operasyon geçirmiş hayvanlara da aynı prosedür uygulanmıştır

(SHAM-LiCl, n = 11; SHAM-NaCl, n = 10) ve dolayısıyla deneyde toplam dört grup yer almaktadır. Deney protokolü Technical Universal Verification hayvan etiği komitesi tarafından onaylanmıştır (protokol numarası: 0026/2023/01).

#### 2.1.1.2. Koloni Odası

Fareler şeffaf Avrupa Standart Tip II kafeslere yerleştirilmiştir. Yataklık talaş kullanılarak oluşturulmuş ve su standart plastik şişelerde servis edilmiştir. Kafeslerin bulunduğu koloni odasında 12/12 saat aydınlık/karanlık döngüsü sağlanmıştır ve oda sıcaklığı 21°C±1'de tutulmuştur. Aşağıda belirtilen su kısıtlama dönemleri dışında yiyecek ve musluk suyuna sınırsız erişim sağlanmıştır.

#### 2.1.1.3. Araçlar

Koşullama koloni odasından farklı bir bağlamda gerçekleştirilmiştir. Farklı bir bağlam yaratmak amacıyla limon yağı kokusuyla zenginleştirilmiş farklı bir odada 75 dB beyaz gürültü sağlanmış ve oda 60 Watt kırmızı lamba ile aydınlatılmıştır. Siyah ve beyaz çizgilerle çevrelenen Standart Tip II kafeslerde yataklık olarak kedi kumu kullanılmıştır. Koşullandırma bağlamında su paslanmaz çelik ağızlı ve bilye ağızlıklı yeşil cam şişelerde sunulmuştur.

#### 2.1.1.4. Deney Prosedürü

*Ameliyat.* Ameliyatlar intraperitoneal olarak 80 mg/kg Ketamin ve 8 mg/kg Xylazine kokteyli enjeksiyonundan 10 dakika sonra gerçekleştirilmiştir. Hayvanların 23'üne kaudal bölgeye doğru 1 cm'lik sırt kesisinden yumurtalıkların iki taraflı çıkarılmasını içeren ovariektomi prosedürü uygulanmıştır. Kalan 21 hayvana da sahte operasyon için aynı işlem uygulanmıştır ancak yumurtalıklar çıkarılmamış, yalnızca yerleri belirlenip geri yerleştirilmiştir. Ameliyatların ardından hayvanlara subkutan analjezik ve antibiyotik enjeksiyonu yapılmıştır ve kafeslerine geri yerleştirilmiştir. Ameliyatın devamındaki iki gün analjezik ve antibiyotik enjeksiyonlarına devam edilmiştir. Hayvanlara dört günlük iyileşme süresi boyunca sınırsız yiyecek ve su sağlanmıştır.

*Alışma.* İyileşme periyodunun ardından hayvanlar uygulama esnasındaki stresi azaltmak amacıyla alıştırma prosedürüne tabi tutulmuştur. Bu amaçla hayvanlar birbirini takip eden üç gün boyunca her gün üç dakika süreyle elde tutulmuşlardır.

Farelere alıştırmanın ilk iki günü kısıtlama olmadan yiyecek ve su verilmiştir. Alıştırmanın üçüncü günü saat 17:30'dan itibaren su şişeleri kafeslerden çıkarılarak su kısıtlamasına başlanmıştır.

*Su eğitimi.* Su eğitimi uygulaması su kısıtlamasını takip eden günde başlamıştır. Bu amaçla hayvanlara üç gün boyunca 10:00-10:30 ve 17:00-17:30 saatleri arasında plastik şişelerle su verilmiştir.

*Önceden maruz bırakma.* Geçmiş çalışmalarda koşullandırmayı kolaylaştırdığı görüldüğü için su eğitiminin üçüncü gününde fareler 5 dakika boyunca koşullandırma bağlamına maruz bırakılmıştır. Her hayvan saat 12:30'dan itibaren şartlandırma bağlamına yerleştirildi. Bu 5 dakika içerisinde hayvana şartlandırma kafesindeki cam şişelerde su verilmiştir. Ön maruziyet tamamlandıktan sonra hayvanlar koloni odasındaki kafeslerine yerleştirilmiştir. Ön maruz bırakmanın gerçekleştiği gün farelere 10:00-10:30 ve 17:00-17:30 saatleri arasında plastik şişelerle su verildi.

*Koşullanma.* Mevcut deneysel prosedür, ön maruziyetten 24 saat sonra başlayan tek bir koşullandırma seansını içermektedir. Bu nedenle hayvanlar, saat 12:30'dan itibaren, 20 dakikalık bir şartlandırma seansı için yeşil cam şişelerde suyun sağlandığı şartlandırma bağlamına yerleştirilmiştir. Şartlandırma kafeslerinde geçirilen ilk 5 dakikanın ardından deney gruplarındaki (OVX-LiCl ve SHAM-LiCl) hayvanlara LiCl enjeksiyonu (6mEq/kg), kontrol gruplarındaki (OVX-NaCl ve SHAM-NaCl) hayvanlara ise her hayvanın vücut ağırlığına göre eşdeğer hacimde %0,9 NaCl enjeksiyonu yapılmıştır. Her enjeksiyondan sonra fareler kendi kafesine geri yerleştirilmiş ve farelerin 15 dakika daha kalmasına izin verilmiştir. Şartlandırma sırasında tüketilen su miktarı ölçülmüştür. Şartlandırma gününde koloni odasına sabah oturumunda saat 10:00-10:30 arasında, öğleden sonra oturumunda ise saat 17:00-17:30 arasında su verilmiştir.

*İyileşme.* Hayvanlara iki gün boyunca LiCl'nin yan etkilerinden kurtulmaları için iki gün süre verilmiştir. Bu süre boyunca farelere 10:00-10:30 ve 17:00-17:30 saatleri arasında plastik şişeler ile su verilmiştir.

*Bellek testi.* Şartlandırmadan 72 saat sonra, saat 12:30'dan itibaren hayvanlar, 15 dakika süreyle şartlandırma bağlamına yerleştirilmiştir. Bellek testi sırasında tüketilen

su miktarı, farelerin koşullandırma bağlamına yönelik kaçınma seviyelerini ölçmek için kullanılmıştır. Su, test günlerinde sadece 17:00-17:30 saatleri arasında verilmiştir. Test günlerini, sabah ve öğleden sonra su seanslarıyla iki günlük iyileşme süreci izlemiştir. Bellek testi, sönme gözlemlenene kadar her 72 saatte bir tekrarlanmıştır. Sönmenin tamamlanmasına, test sırasında tüketilen su miktarlarına göre karar verilmiştir. Tüm hayvanlar istatistiksel olarak benzer miktarda su tükettiğinde davranış testi sonlandırılmıştır. Bellek testi Deney 1 için 5 kez tekrarlanmıştır.

*Vücut ağırlığı.* Hayvanlar deney sırasında ameliyat günü, şartlandırma ve ikinci ve üçüncü bellek testi olmak üzere farklı günlerde tartılmıştır.

*Rahim ağırlığı.* Hayvanlar sakrifiye edilip ve rahimleri çıkarıldıktan sonra rahim ağırlıkları tartılmıştır.

#### 2.1.1.5. İstatistiksel Analiz

Tüm istatistiksel analizler GraphPad Prism (Sürüm 9.0) kullanılarak yapılmıştır. Bellek testini analiz etmek için denemeyle 2x2x5 son faktörde tekrar ölçümlü karma ANOVA kullanılmıştır. Aynı analiz planı, hayvanın tartıldığı zaman noktasına göre eşleşen vücut ağırlığı analizi için de yürütülmüştür. Verilerin geri kalanı 2x2 faktöriyel ANOVA ile analiz edilmiştir. Fisher'in LSD'si, <.05 anlamlılık düzeyiyle post-hoc karşılaştırmalar için kullanılmıştır.

### 2.1.2. Sonuçlar

#### 2.1.2.1. Koşullanma

20 dakikalık şartlandırma seansı sırasında gruplarda tüketilen su miktarları arasında anlamlı bir farklılık bulunamamıştır. 2 (Operasyon: OVX vs. SHAM) x 2 (İlaç: LiCl vs. NaCl) faktöriyel ANOVA yürütülmüştür ve hiçbir temel etki (Operasyon, $p = .944$; İlaç, $p = .162$) ya da operasyon ile ilaç arasındaki etkileşimde anlamlı bir fark gözlemlenmemiştir ($p = .799$). OVX-LiCl, OVX-NaCl, SHAM-LiCl ve SHAM-NaCl grupları tarafından tüketilen miktarları koşullanma sırasında benzer bulunmuştur.

#### 2.1.2.2. Bellek Testi

Şekil 4, beş bellek testi boyunca her grup tarafından tüketilen su miktarlarını

göstermektedir. 2 (Operasyon: OVX vs. SHAM) x 2 (İlaç: LiCl vs. NaCl) x 5 (Test: Bellek Testi 1 vs. Bellek Testi 2 vs. Bellek Testi 3 vs. Bellek Testi 4 vs. Bellek Testi 5) karma etki modeli sonuçları Test ($p < .001$) ve İlaç ($p < .001$) için bir temel etki ve Bellek Testi ile İlaç arasında bir etkileşimi ortaya çıkarmıştır ($p = .03$). Fisher LSD'si, SHAM-LiCl ve SHAM-NaCl grupları tarafından tüketilen su miktarının Bellek Testi 1 ($p < .001$), Bellek Testi 2 ($p < .001$), Bellek Testi 3 ($p = .003$), ve Bellek Testi 4 ($p = .012$) sırasında anlamlı bir fark olduğunu göstermektedir. SHAM-LiCl ve SHAM-NaCl'nin tüketilen su miktarları, Bellek Testi 5 ile eşitlenmiştir ($p = .058$) . OVX-LiCl ve OVX-NaCl grupları arasındaki fark yalnızca Bellek Testi 1 sırasında anlamlı bulunmuştur ($p = .002$). Bu fark Bellek Testi 2 ile azalıp son bulmuştur.

#### 2.1.2.3. Vücut Ağırlığı

Her bir gruptaki hayvan deneyin başlangıcında benzer vücut ağırlıklarına sahiptir, ancak yumurtalıkları alınmış hayvanlar sahte ameliyat geçiren gruplara göre deney sonunda anlamlı olarak daha ağır bulunmuştur. 2 (Operasyon: OVX vs. SHAM) x 2 (İlaç: LiCl vs. NaCl) x 4 (Zaman: 1. Gün vs. 13. Gün vs. 19. Gün vs. 24. Gün) karma etki modeli, Zaman'ın ana etkisi ($p < .001$) ve Zaman ile Operasyon arasında bir etkileşim ($p < .001$) olduğunu göstermiştir. Fisher LSD'si, 1. Gün grupların vücut ağırlıkları arasında anlamlı bir fark olmadığını, ve bu farkın 24. Gün ortaya çıktığını göstermektedir (OVX-LiCl vs. SHAM-NaCl, $p = .35$; OVX-NaCl vs. SHAM-LiCl, $p = .031$; OVX-NaCl vs. SHAM-NaCl, $p = .012$).

#### 2.1.2.4. Rahim Ağırlığı

Hayvanların nispi rahim ağırlıkları, hayvanların rahim ağırlığının (g) vücut ağırlığına (g) bölünmesi ve 100 ile çarpılmasıyla hesaplanmıştır. Dört farklı grubun sonuçları 2 (Operasyon: OVX vs. SHAM) x 2 (İlaç: LiCl vs. NaCl) faktöriyel ANOVA kullanılarak analiz edilmiştir. Sonuçlar Operasyon ana etkisi ($p < .001$) ve İlaç ile Operasyon arasında bir etkileşim ($p < .001$) olduğunu ortaya çıkarmıştır. Fisher LSD'si kullanılarak yapılan post-hoc karşılaştırması, OVX-LiCl ve SHAM-LiCl ($p < .001$), OVX-NaCl ve SHAM-NaCl ($p = .003$), SHAM-LiCl ve SHAM-NaCl ($p < .003$) arasında anlamlı bir fark olduğunu göstermiştir.

### 2.1.3. Tartışma

İlk deneyde dişi farelerin yumurtalıklarından salgılanan hormonların KÇİ öğrenmesi üzerine etkisi araştırılmıştır. LiCl'yi yeni bir bağlam ile eşleştirdikten sonra normal hormon seviyelerini koruyan sahte operasyonlu fareler, yumurtalıkları alınmış farelere kıyasla daha uzun süre boyunca koşullu yanıt sergilemişlerdir. Spesifik olarak, LiCl enjekte edilmiş fareler NaCl enjekte edilmiş farelere oranla su tüketiminde bir azalma sergilemiştir ve bu da itinmenin bir göstergesidir. Sahte operasyonlu farelerde bu itinme daha fazla hafıza testi boyunca sürdürülmüş, dolayısıyla da bu hayvanlarda sönme daha geç gerçekleşmiştir.

Bu sonuçlar KÇİ'de koşullu tepkinin sürdürülmesinde dişi gonad hormonlarının rolünü göstermektedir.

## 2.2. Deney 2

İlk deneyde yumurtalıklardan salgılanan hormonların KÇİ sönmesi üzerinde zorlaştırıcı etkisi bulunmuştur ve bunu takiben hangi spesifik hormonun bu KÇİ'deki etkiye sebep olduğunu anlamak için ovariektomi operasyonu geçirmiş hayvanlara estradiol tedavisi uygulanmıştır.

### 2.2.1. Yöntem

#### 2.2.1.1. Denekler

Deney 2'ye 10-12 haftalık 39 dişi CD1 fare dahil edilmiştir. Bu deneyde bütün deneklerin dişi gonad hormonları ovariektomi yoluyla düşürülmüştür. Bir grup hayvan estradiol tedavisine tabi tutulurken (n = 20) kalan hayvanlara susam yağı verilmiştir (n = 19). Daha sonra bu iki grup tekrar alt gruplara ayrılarak LiCl deney ve kontrol grupları oluşturulmuştur. Estradiol alan hayvanların yarısına itinme oluşturmak amacıyla koşullanma sırasında LiCl enjekte edilmiş (E-LiCl, n = 10), ve böylece hayvanın yeni ortama koşullanması sağlanmıştır. Kalan estradiol almış hayvanlara ise kontrol amaçlı NaCl enjeksiyonu uygulanmıştır (E-NaCl, n = 10). Aynı prosedür susam yağı alan hayvanlara da uygulanmıştır (O-LiCl, n = 9; O-NaCl, n = 10) ve deney toplamda dört gruptan oluşmuştur. Deney protokolü Technical Universal Verification hayvan etiği komitesi tarafından onaylanmıştır (protokol numarası: 0035/2023/01).

#### 2.2.1.2. Koloni Odası

Koloni odasındaki barınma koşulları Deney 1'deki ile aynıdır.

#### 2.2.1.3. Aparatlar

Aparatlar Deney 1'deki ile aynıdır.

#### 2.2.1.4. Deney Prosedürü

Deney prosedürü, ek bir estradiol uygulaması protokolü dışında Deney 1'deki ile aynıdır.

*Nutella alıştırması.* Estradiol uygulaması susam yağında çözülmüş estradiol ve Nutella karışımı ile gerçekleştirilmiştir. Farelere Nutella servisi yapıldıktan hemen sonra gönüllü tüketimi sağlayabilmek için ameliyattan dört gün önce bir alıştırma prosedürü başlatılmıştır. Hayvanlara her gün 9:30-17:30 saatleri arasında 5x5 cm beyaz mermer parçaları üzerinde 30 gr hayvan başına 60 mg olmak üzere Nutella verilmiştir. *Östrojen uygulaması.* Estradiol uygulaması ameliyatın ertesi günü başlamıştır ve Ström ve arkadaşları (2012) tarafından geliştirilen peroral yöntem ile gerçekleştirilmiştir. Bunun için öncelikle estradiol susam yağında eritilmiş ve ardından Nutella ile karıştırılmıştır. Her 30 gramlık hayvana vücut ağırlığına göre 60 mg Nutella içinde 0.312 uL susam yağı içerisinde çözünmüş 1.12 ug estradiol verilmiştir. Kontrol grubu da aynı orandaki karışımı benzer şekilde, fakat estradiol olmadan almıştır. Nutella, 9:30-10:00 saatleri arasında Nutella alıştırması sırasında kullanılan 5x5 cm mermer parçaları üzerinde servis edilmiştir. Tüm hayvanlar kendi payına düşen Nutella'yı 1 ila 2 dakika içerisinde bitirmiştir.

#### 2.2.1.5. İstatistiksel Analiz

İstatistiksel analiz Deney 1'deki ile aynıdır.

### 2.2.2. Sonuçlar

#### 2.2.2.1. Koşullanma

İstatistiksel analiz için 2 (Hormon: estradiol vs. yağ) x 2 (İlaç: LiCl vs. NaCl) faktöriyel ANOVA kullanılmış olup 20 dakikalık şartlandırma seansı sırasında

gruplarda tüketilen su miktarları arasında anlamlı bir farklılık bulunamamıştır. Sonuçlar İlaç ($p$ = .617), Hormon ($p$ = .957) ana etkisi olmadığını ve İlaç ile Hormon arasında bir etkileşimin bulunmadığını ($p$ = .827) ortaya çıkarmıştır.

#### 2.2.2.2. Bellek Testi

Koşullanmanın ardından uygulanan ilk bellek testi sırasında E-LiCl grubu, E-NaCl grubundaki farelere kıyasla daha az miktarda su tüketmiştir. Ancak O-LiCl ve O-NaCl hayvanları su tüketimlerinde anlamlı bir fark göstermemiştir. 2 (Hormon: estradiol vs. susam yağı) x 2 (İlaç: LiCl vs. NaCl) x 5 (Test: Bellek Testi 1 vs. Bellek Testi 2 vs. Bellek Testi 3 vs. Bellek Testi 4 vs. Bellek Testi 5) karma etki modeli sonuçları Test ($p$ < .001) ve İlaç ($p$ = .04) temel etkisini ve Deneme ile İlaç arasındaki etkileşimi ($p$ = .003) ortaya çıkarmıştır. Fisher LSD'si, Bellek Testi 1 sırasında E-LiCl ve E-NaCl gruplarının anlamlı bir fark gösterdiğini vurgulamıştır ($p$ = .002). Öte yandan, E-LiCl ve E-NaCl grupları, Bellek Testi sırasında su tüketimlerinde anlamlı bir fark göstermemiştir ($p$ = .05). O-LiCl ve O-NaCl grupları, Bellek Testi 1 ($p$ = .071) ve sonraki testlerde benzer miktarlarda su tüketmiştir.

#### 2.2.2.3. Vücut Ağırlığı

Her bir gruptaki hayvan deneyin başlangıcında ve sonunda benzer vücut ağırlıklarına sahipti. 2 (Hormon: estradiol vs. susam yağı) x 2 (İlaç: LiCl vs. NaCl) x 6 (Zaman: 1. Hafta vs. 2. Hafta vs. 3. Hafta vs. 4. Hafta vs. 5. Hafta vs. 6. Hafta) karma etki modeli, Zaman ($p$ < .001) temel etkisi ve Zaman ile Hormon arasında bir etkileşim ($p$ < .001) ortaya çıkarmıştır. Fisher LSD testi gruplar arası herhangi bir anlamlı fark göstermemiştir.

#### 2.2.2.4. Rahim Ağırlığı

Hayvanların nispi rahim ağırlıkları hayvanın rahim ağırlığının (g) vücut ağırlığına (g) gölünmesi ve 100 ile çarpılmasıyla hesaplanmıştır. Dört farklı gruptaki sonuçlar 2 (Hormon: estradiol vs. susam yağı) x 2 (İlaç: LiCl vs. NaCl) faktöriyel ANOVA kullanılarak analiz edilmiştir. Sadece Hormon temel etkisi bulunmuştur ($p$ = .019). Fisher LSD'si kullanılarak yapılan post-hoc karşılaştırma, O-LiCl ve E-NaCl grupları ($p$ = .027) ile E-NaCl ve O-NaCl grupları ($p$ = .049) arasında anlamlı bir fark göstermiştir.

### 2.2.3. Tartışma

Yumurtalıklardan salgılanan hormonların dişi farelerin KÇİ öğrenmesine sönmeyi zorlaştırıcı etkisinin gözlemlenmesini takiben bu etkiden sorumlu spesifik hormonu tanımlamak amacıyla ikinci bir deney yürütülmüştür. Bu amaçla yumurtalıkları alınmış dişi farelerin bir kısmına estradiol veya yağ uygulanmıştır ve bu hayvanların bir kısmı koşullu tepki oluşturmak adına yeni bir bağlamda LiCl enjeksiyonuna, kalan kısmı ise kontrol amacıyla NaCl enjeksiyonuna maruz bırakılmıştır. Estradiol uygulaması gören ve LiCl enjekte edilen fareler ilk hafıza testinde estradiol uygulaması görüp NaCl enjekte edilen farelere oranla daha az su tüketmiştir. Fakat bu etki kalan bellek testlerinde sona ermiş ve sönme gözlemlenmiştir. Bununla birlikte susam yağı uygulaması alan LiCl ve NaCl enjekte edilmiş hayvanlar bütün bellek testleri süresince benzer miktarda su tüketmiştir ve bu da estradiolün sınırlı da olsa bir etkisinin olduğunu göstermektedir.

## BÖLÜM 3

## GENEL TARTIŞMA

Mevcut iki deneysel çalışmada klinik öncesi araştırmalarda gözlemlenen cinsiyet farklılıklarının sebeplerini ortaya çıkarmak için estradiolün KÇİ üzerindeki etkisini araştırmaktadır. Deney 1'de dişi farelerde gonad hormonlarının düzeylerini manipüle etmek amacıyla ovariektomi ameliyatı gerçekleştirilmiş ve yumurtalıklardan salgılanan hormonların KÇİ sönmesini zorlaştırdığı ortaya çıkarılmıştır. Bu deneyin sonucunda yumurtalıkları alınmış fareler sahte operasyona tabi tutulan farelere kıyasla koşullu yanıtta daha hızlı sönme gerçekleştirmiştir; bu da itinmenin sürdürülmesinde gonad hormonların rolünün altını çizmektedir. Gonad hormonlarının etkisini gözlemledikten sonra hangi spesifik hormonun buna sebep olabileceği araştırılmıştır. Bu amaçla Deney 2'de estradiolün KÇİ sırasında dişi farelerde sönmeyi gecikmesine katkıda bulunup bulunmadığına bakılmıştır. Ancak potansiyel olarak estradiolün

uygulama metodu sebebiyle Deney 2'den kesin bir sonuç alınamamıştır ve dolayısıyla estradiolün KÇİ öğrenmesindeki etkisinin gözlemlenmesini sınırlamıştır. Deney 2'de gözlemlenen sınırlı etkiye rağmen geçmişteki araştırma bulguları ve hormonların karmaşık etkileşimi, estradiolün KÇİ öğrenme sürecinde dahil olabileceğini göstermektedir. Bu bakış açısı östrojenin çeşitli klasik koşullanma paradigmalarına etkisi ve beyinde öğrenme ile alakalı bölgelerle etkileşimiyle desteklenmektedir (Bean et al., 2014; Colón et al., 2023; Zurkovsky et al., 2006). Dahası, kadınlarda gonad hormonlarla ilintili olarak mide bulantısının artması östrojenin itinmeyi arttırmaktaki rolünü vurgulamaktadır (Goldberg et al., 2000; Koslucher et al., 2015; Patel et al., 2004; Salazar-Parra et al., 2020). Bu bulgular, BBK'nin preklinik çalışmalarında cinsiyete bağlı farklılıkların göz önünde bulundurulmasının ve kişilerin ihtiyaçlarına göre şekillendirilmiş tıbbi stratejiler geliştirmenin önemini göstermektedir.

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