AN ANALYSIS OF NMDA AND BETA-ADRENERGIC RECEPTOR ANTAGONISTS ADMINISTRATION AND NEURAL C-FOS EXPRESSION FOLLOWING CONDITIONED CONTEXT AVERSION LEARNING IN MICE

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ABSTRACT

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Cancer patients develop anticipatory nausea and vomiting (ANV) following chemotherapy treatment as a result of classical conditioning learning. Although researchers have been conducting clinical studies to understand the characteristics of ANV, animal models can also be utilized to develop novel diagnostics and therapeutics. Conditioned context aversion (CCA) has been accepted to arise from classical conditioning and used as an animal model of ANV.

Although antiemetic agents were widely used to prevent nausea and vomiting, 25% of cancer patients still develop ANV. However, the formation of memories can be prevented during consolidation phase of memory formation. We conducted two experiments (Experiment 1 and Experiment 2) to investigate memory impairing effect of N-methyl-D-aspartate antagonist MK-801 and beta-adrenergic antagonist propranolol on CCA. We found that systemic administration of propranolol but not MK-801 prevented animals from developing CCA.

Although behavioral research mainly established the principles of CCA learning, it's neural substrate is yet to be investigated. Experiment 3 was conducted to identify the brain regions involved in CCA learning. We found elevated c-Fos expression in the prelimbic division of the medial prefrontal cortex, IC, basolateral nucleus of the amygdala, CA1-CA2 and dentate gyrus subregions of the hippocampus, indicating that these brain regions are activated following CCA.

To the best of our knowledge, ours is the first study finding memory impairments induced by beta-adrenergic receptor antagonist in CCA and showing neural correlates of CCA learning. Further research is necessary to unravel causal involvement of these brain regions in the development of CCA.

Keywords: Anticipatory nausea and nausea, cancer, conditioning, learning, mice

ÖZ

FARELERDE KOŞULLU ÇEVRE İTİNMESİ ÖĞRENMESİNİ TAKİBEN UYGULANAN NMDA VE BETA-ADRENERJİK RESEPTÖR ANTAGONİSTLERİNİN ETKİSİ VE NÖRAL C-FOS EKSPRESYONU ÜZERİNE BİR ANALİZ

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Kanser hastaları, klasik koşullanma öğreniminin bir sonucu olarak kemoterapi tedavisinin ardından beklentisel bulantı ve kusma (BBK) geliştirir. Araştırmacılar, BBK'nın altında yatan mekanizmaları anlamak için klinik çalışmalar yürütüyor olsalar da yeni teşhis ve terapötik ilaçlar geliştirmek için hayvan modelleri de kullanılmaktadır. Koşullu çevre itinmesi (KÇİ), klasik koşullama sonucu ortaya çıkan ve BBK'nın hayvan modeli olarak kullanılan bir paradigmadır.

Antiemetik ajanlar, kemoterapiye bağlı bulantı ve kusmayı engellemek için yaygın olarak kullanılsa da, kemoterapi tedavisi gören kişilerin yaklaşık %25'i, hastalık hali ve çevresel ipuçları arasındaki ilişkisel öğrenmenin bir sonucu olarak BBK geliştirmektedir. Ancak bellek oluşumunu, konsolidasyon aşamasına müdahale ile engellenebilir. Bu çalışmada, Deney 1 ve Deney 2 öğrenme ile ilgili olduğu bilinen iki farklı reseptör sistemi olan N-metil-D-aspartat ve beta-adrenerjik reseptörlerin aktivasyonunu konsolidasyon sürecinde MK-801 ve propranolol ile bloke etmenin

CD1 farelerde KÇİ belleği üzerindeki bozucu etkisini araştırmak için yapılmıştır. Propranolol KÇİ öğrenmesini bozarken bu etki MK-801'da görülmemiştir.

Araştırmalar temel olarak KÇİ öğreniminin ilkelerini açığa çıkarmış olsa da bu tür öğrenmede görevli beyin bölgeleri henüz incelenmemiştir. Deney 3'te, KÇİ öğrenimiyle ilgili beyin bölgelerini bulunması amaçlanmıştır. Medyal prefrontal korteksin prelimbik bölümünde, insular kortekste, amigdalanın bazolateral çekirdeğinde, hipokampusun CA1-CA2 ve dentat girus alt bölgelerinde, bu beyin bölgelerinin KÇİ öğreniminin ardından aktive olduğuna işaret eden c-Fos ekspresyonunda artış bulgulanmıştır.

Bu çalışma KÇİ öğrenmesinde beta-adrenerjik reseptör antagonisti propranolol'ün belleğin konsolidasyon sürecini bozduğunu ve KÇİ öğrenmesinde görevli beyin bölgelerini gösteren ilk çalışmadır. Bu beyin bölgelerinin KÇİ öğrenmesindeki rollerini ortaya çıkarmak için daha fazla araştırma gereklidir.

Anahtar Kelimeler: Beklentisel bulantı ve kusma, kanser, koşullama, öğrenme, fare

To my family

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LIST OF ABBREVIATIONS

Nausea and vomiting (NV)

Gastrointestinal (GI)

Vomiting center (VC)

Dorsal vagal complex (DVC)

Nucleus tractus solitarii (NTS)

Chemoreceptor trigger zone (CTZ)

5-hydroxytryptamine (5-HT3)

Neurokinin-1 (NK-1)

Enterochromaffin cells (EC)

Chemotherapy induced NV (CINV)

Substance P (SP)

Anticipatory Nausea and Vomiting (ANV)

Unconditioned stimulus (US)

Unconditioned response (UR)

Conditioned stimulus (CS)

Conditioned response (CR)

Conditioned taste aversion (CTA)

Conditioned context aversion (CCA)

Lithium chloride (LiCl)

Intraperitoneal (i.p.)

Sodium chloride (NaCl)

N-methyl-D-aspartate (NMDA)

[(+)-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]cyclo-hepten-5,10-imine-maleate] (MK-801)

Post-traumatic stress disorder (PTSD)

Water acclimation (WA)

[D(-)-2-amino-5-phosphonovaleric acid] (AP5)

[2R,4R,5S-2-amino-4,5-(1,2-cyclo hexyl)-7-phosphonoheptano acid] (NPC17742)

[5-amino-phosphonovaleric acid] (APV)

Long term potentiation (LTP)

Dentate gyrus (DG)

CHAPTER 1

INTRODUCTION

This chapter covers some of the general concepts that are of interest to our experiments. These concepts include nausea and vomiting, chemotherapy treatment, classical conditioning, anticipatory nausea and vomiting, and conditioned context aversion learning.

1.1. Nausea and Vomiting

Humans as well as other mammals developed a protective nausea and vomiting (NV) defense mechanisms to avoid digestion of toxic substances. (Zhong et al., 2021). The noxious sensation that precedes vomiting is called nausea, the expulsion of expulsion of the gastrointestinal contents via the mouth (Gelberg, 2018). Although vomiting usually follows nausea feeling, humans can experience just one of the conditions (Singh & Kuo, 2016). Nausea and vomiting are not considered as diseases but rather they are seen as symptoms of a wide variety of conditions (Chepyala & Olden, 2008). Toxins, bacteria, fungi, viruses, and some drugs can trigger NV (Zhong et al., 2021). Gastrointestinal (GI) tract pathologies, strong emotional and cognitive load, and motion sickness also can lead to NV experience (Cai et al., 2007; Cohen et al., 2019; Gagliuso et al., 2019).

GI axis plays a vital role in instigating and modulating NV induced by a variety of emetogenic agents or instances (Sanger & Lee, 2008). GI regulation is accomplished by a bidirectional network between neural and endocrine systems involved in the gut-brain axis (Cussotto et al., 2018). This axis consists of the central nervous system, that is the brain and spinal cord, the sympathetic and parasympathetic parts of the autonomic nervous system, the hypothalamic-pituitary-adrenal axis, and the enteric

nervous system (Carabotti et al., 2015). Brainstem structures that are involved in the GI axis and responsible for NV modulation are the vomiting center (VC) that contains muscarinic receptors, the vestibular nuclei that contains histamine and muscarinic receptors, and the dorsal vagal complex (DVC; Borison & Wang, 1953; MacDougall & Sharma 2022; Yates et al., 2014). DVC, comprised of the nucleus tractus solitarii (NTS), dorsal motor nucleus of the vagus, and chemoreceptor trigger zone (CTZ; also known as the area postrema) that lies on the floor of the fourth ventricle, is involved in regulating gastric motility and vomiting reflex via 5-hydroxytryptamine (serotonin, 5-HT3), neurokinin-1 (NK-1) and dopamine 2 receptors (Wickham et al., 2020). CTZ serves as an interface between the brain and cerebrospinal fluid (MacDougall & Sharma 2022). The lack of blood brain barrier makes this zone a circumventricular organ which samples the blood and cerebrospinal fluid for the presence of emetic agents (Miller & Leslie 1994). Epithelial enterochromaffin cells (EC) that are found in the mucosa of GI tract, enteric nervous system, the splanchnic nerves, and the vagus nerve are the peripheral sites that are indicated to have varying contributions to NV circuitry (Zhong et al., 2021). Higher cortical regions and limbic structures also play a role in this circuitry (Miller, 1999).

1.2. Chemotherapy induced Nausea and Vomiting

Chemotherapy induced NV (CINV) is the most common iatrogenic effect of cancer treatment (Rao & Faso, 2012). Neurotransmitter systems within the gut-brain axis have various roles in the pathophysiology of CINV (Hesketh, 2008). Serotonin and substance P (SP) are the neurotransmitters that initiate signaling cascades within the nervous system by binding to the 5-HT3 and NK-1 receptors which transmits information from the gut and vagus nerve to the NTS and the CTZ, respectively (Hesketh, 2008). Dopamine also plays a role in this circuitry; however, its exact function remains unrevealed clearly (Janelsins et al., 2013).

Chemotherapeutic agents induces NV because of the generation of free radicals that causes excessive amounts of serotonin release from the ECs lining of the GI mucosa (Zhong et al., 2021). The vagus nerve conducts this chemical information to the brain via the 5-HT3 receptors that are found on the terminal side of its axon (Blackshaw et al., 2007; Lesurtel et al., 2008). Another mechanism that chemotherapy initiates

emesis or nausea is via the NK-1 receptors that are located in the NTS, the area postrema and the gut (Borison & McCarthy, 1983). Chemotherapy drugs have been shown to cause SP release which binds to the NK-1 receptors that are found in these regions, and this signal is then sent to the CTZ and subsequently to the VC, causing NV (Diemunsch & Grélot, 2000)

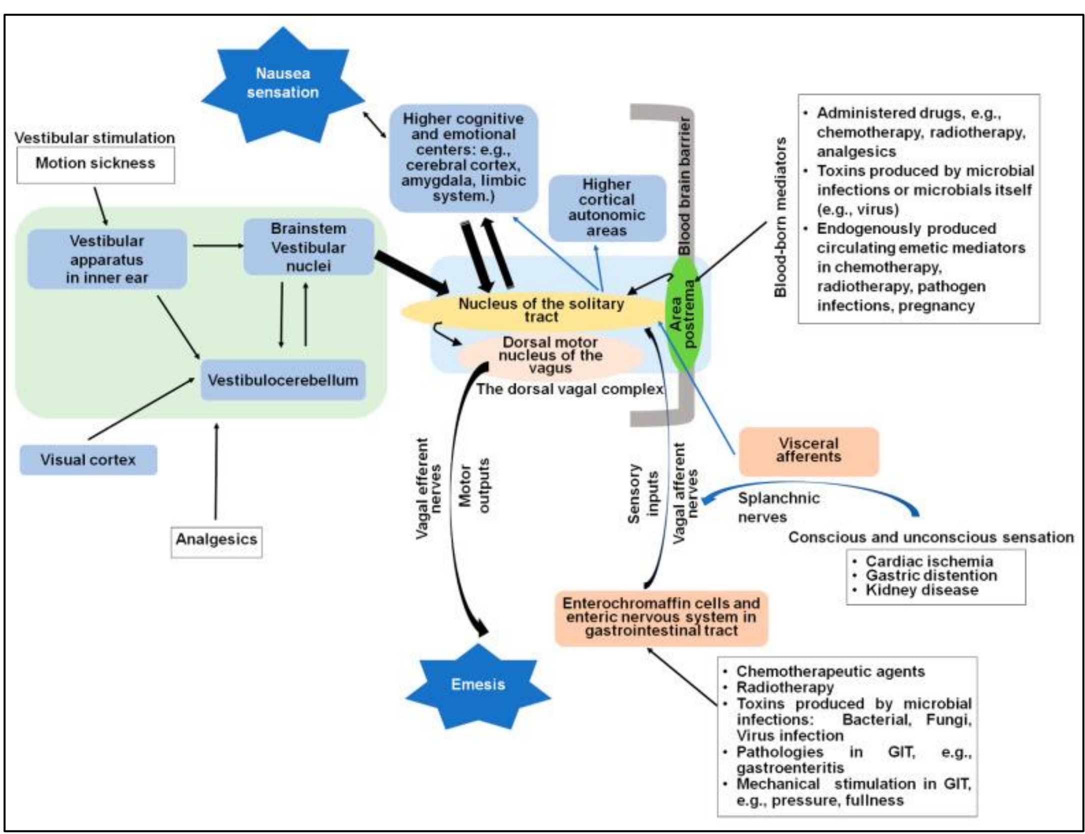

Figure 1. Diagram showing the brain structures, agents and other elements that are involved in NV (Zhong et al., 2021).

CINV is classified as acute, delayed, or anticipatory in terms of the onset of NV feelings that patients experience during chemotherapy treatment (Durand et al., 2009). The NV that starts within the 24 hours following treatment is called acute (Roila et al., 1991). If acute NV continues after this 24-hours period, it is now accepted as delayed which can last for a week (Roscoe et al., 2004). Anticipatory NV (ANV) is somewhat different from acute and delayed forms because it is not due to pathophysiological mechanisms that induce NV, but rather it rises from classical conditioning learning (Roscoe et al., 2004). We will analyze ANV further after reminding some of the basic concepts of classical conditioning in the next two sections.

1.3. Classical Conditioning

Classical conditioning is a type of associative learning in which a neutral stimulus comes to elicit a response after its pairing with another stimulus that have an innate or acquired psychological/physiological value for the organism. The stimulus is called neutral because it is incapable of inducing any response before the conditioning. A neutral stimulus is associated with an unconditioned stimulus (US) when they are presented with temporal contiguity during conditioning. USs are the stimuli that generate an autonomic response from the organism. This response that the US elicits is called unconditioned response (UR). Neutral stimulus becomes conditioned stimulus (CS) once it starts eliciting a response that is similar to UR which is called conditioned response (CR).

One of the classical conditioning paradigms that has been used predominantly in memory research is fear conditioning (Mahan & Ressler, 2012). Many species are able to associate either a cue or a context with a fear-inducing stimulus (Kim & Jung, 2006). Fear conditioning experiments measure freezing behavior as a CR that animals display when they encounter once a neutral stimulus that has been paired with an US, such as foot shock (Baldi et al., 2004). Fear conditioning is classified as contextual or cued depending on the CS modality (Wehner & Radcliffe, 2004) Contextual fear conditioning involves applying a foot-shock (US) after placing animal to a novel chamber (Curzon et al., 2009). Animal displays freezing behavior when re-exposed to the same chamber as a result of the association between the chamber and foot-shock (Curzon et al., 2009). Freezing is defined as the “absence of movement other than respiration” (Acevedo-Triana et al., 2020). This CR that animals show to fear-associated context may persist for months depending on the procedural applications utilized to establish US-CS pairings such as the intensity and the frequency of the shock, the number of conditioning trials, and the learning sensitivity of the animal (Curzon et al., 2009). Cued fear conditioning differs from contextual fear conditioning in that animals are repeatedly pre-exposed to a chamber preceding conditioning and then are given a foot-shock after a novel CS such as a sound (Wehner & Radcliffe, 2004). This allows animals to associate the US with not the chamber but with the novel stimulus (Curzon et al., 2009).

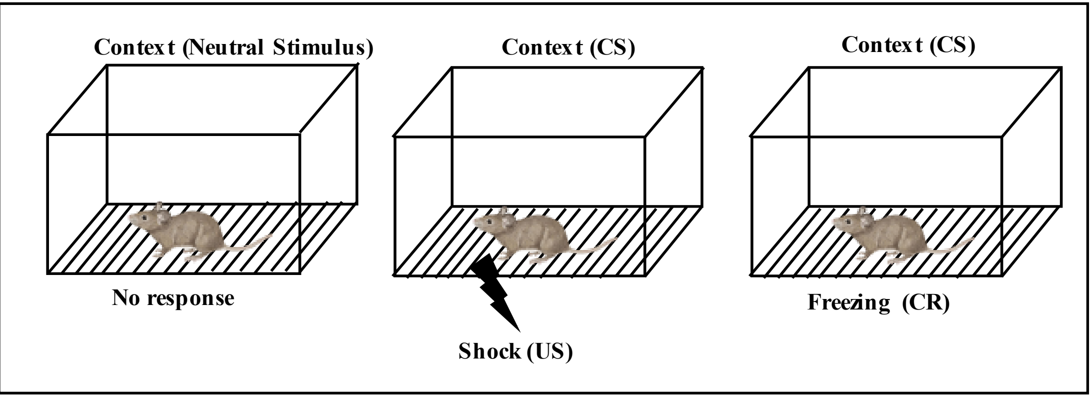

Figure 2.Contextual fear conditioning. Initially neutral stimulus (context) comes to elicit a CR (freezing) after it's pairing with US shock.

Another classical conditioning paradigm that has been studied in detail is called conditioned taste aversion (CTA; Welzl et al., 2001). Animals develop aversion to foods and liquids that have been associated with malaise previously (Bernstein, 1999). The capacity of developing CTA has survival value (as other learning types) since animals must learn to avoid different types of foods that are not safe to ingest (Lavi et al., 2018). In the laboratory, it is also possible to induce CTA to neutral or even to initially nutritious foods. This is accomplished by allowing animals to ingest a solution/food after or before the treatment of a nausea-inducing agent (Welzl et al., 2001). The most commonly used nausea-inducing agent in CTA studies is lithium chloride (LiCl; Yamamoto et al., 1995). A typical CTA study involves the injection of LiCl before or after animals are allowed to consume a flavored solution that is usually sucrose or saccharin (Eddy et al., 2012). The suppressed consumption of the malaise-paired solution during retention test is used as evidence of the development of CTA (Roman et al., 2009).

1.4. Anticipatory Nausea and Vomiting

ANV is accepted to arise from classical conditioning learning (Stockhorst et al., 1993). The highly nauseagenic and emetogenic chemotherapy treatment induces NV in patients, as described in the previous sections. In the terminology of classical conditioning model of ANV, chemotherapy treatment is the US, and CINV is the UR (Schnell, 2003). One or more of the environmental stimuli where chemotherapy treatment is received become CS as repeated chemotherapy cycles (conditioning)

endow these stimuli with conditioned properties (Roscoe et al., 2011). Chemotherapy equipment, smells and sounds in the clinic, even nurses can be the reminders of illness and act as a CS (Morrow & Rosenthal, 1996). Once the association between the US and CS is established as patients continue to receive chemotherapy, these reminders start to elicit NV (Roscoe et al., 2011). As it can be seen, ANV is not physiologically or pharmacologically induced, but rather it is a psychological response arising from classical conditioning learning (Kamen et al., 2014).

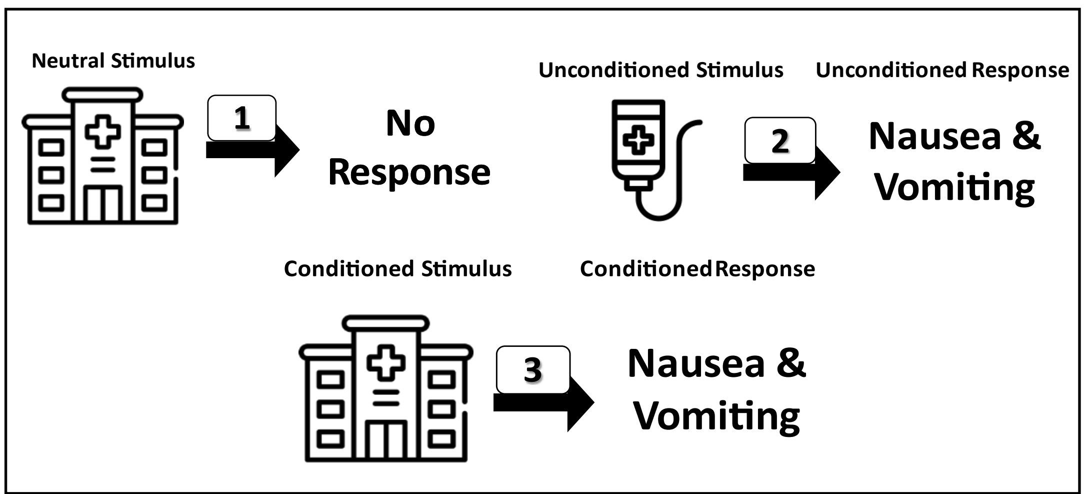

Figure 3. Pavlovian model of ANV. In this model, the US is the chemotherapy treatment that naturally produces an UR, that is NV. CINV is associated with the neutral stimuli in the hospital. These stimuli can be sights and smells of the clinic, the doctors, the chemotherapy room, etc. After one or more chemotherapy sessions, exposure to a CS alone is sufficient to elicit CR.

Current prophylaxis to alleviate and manage the iatrogenic NV is the prescription of anti-emetics (Tonato et al., 1994). However, epidemiological studies show that 25 to 30% of cancer patients develop ANV because of the difficulty of controlling CINV (Morrow et al., 1998). ANV causes a reduction in the patient's life quality, and also impair physical, cognitive and social functioning (Yoo et al., 2005). Patients who develop ANV report high levels of fatigue, insomnia, and dyspnea (Redeker et al., 2000). ANV is one of the leading factors for the discontinuation of chemotherapy treatment due to patient's fear and anxiety of experiencing further NV (Andrykowski, 1990). For these reasons, understanding the psychological and neurobiological underpinnings of this debilitating phenomenon has a substantial clinical relevance. Although clinical studies are effective ways to learn about the development of ANV, the findings of relevant animal models are also useful in establishing a fundamental

understanding of a disease. Additionally, preclinical models allow the investigation of the efficacy and the safety of a therapeutic intervention before it is used in humans. Conditioned context aversion (CCA) provides a useful murine model for the investigation of behavioral and neurobiological underpinnings of ANV. We will look at some of the basic concepts of CCA in the next section.

1.5. Conditioned Context Aversion

CCA has been used as a preclinical tool to recapitulate ANV in animals (Cloutier et al., 2017, 2018; Limebeer & Parker, 2000). CCA is established by pairing a novel context with illness during conditioning which results in the development of aversion to the context, evidenced by a CR that animals display during retention tests. Various CCA procedures are established to mimic ANV experience of cancer patients. Although some CCA studies used radiation, pharmacological agents are usually employed as an US to induce illness. One of the commonly used agents is LiCl. In rodents, the result of an intraperitoneal (i.p.) LiCl injection is abdominal malaise and diarrhea. Behavioral manifestations of LiCl treatment are hypophagia (McCann et al, 1989), gastric emptying (McCann et al, 1989), lying-on belly (Meachum & Bernstein, 1992) and the ingestion of non-nutritive substances (Fortin et al., 2016; Mitchell et al, 1976).

Various CRs are employed to investigate the development of CCA. In most of the studies, fluid consumption is measured as the CR that indicates the establishment of context aversion (Parker et al., 1984). A general experimental design of these studies usually consists of 5 phases including water deprivation, water acclimation, conditioning, recovery, and retention (Rodriguez et al., 2000). A classic example of a study employing fluid consumption as the CR is by Rodriguez et al. (2000). In this study, rats are injected with LiCl before being introduced to a novel context. Next day, the same rats receive sodium chloride (NaCl) injections before being introduced to a different novel context. The 2-day cycle of conditioning is repeated for 4 times. After 4 days of recovery period following the last conditioning cycle, animals are then tested for their sucrose consumption in one of these contexts in a drug-free state. They show that rats given access to the sucrose solution in reinforced context (paired with LiCl)

consume less than rats given access to the same solution in NaCl-paired context, arriving to the conclusion that contextual cues can be associated with gastrointestinal illness and gain the properties of a CS, making the procedure a valid animal model for ANV (Rodriguez et al., 2000).

Research by Grill and Norgren (1978) proves that rats show conditioned gaping reactions to intraoral infusion of a palatable solution that has been associated with an emetic drug previously (Grill & Norgren, 1978). They argue that this behavior is an example of nausea-induced behavior (Grill & Norgren, 1978). Researchers also have shown that conditioned gaping can be induced by emetic agents (Parker, 2003) and be prevented by anti-emetics (Limebeer & Parker, 2000), which supports Grill and Norgren's argument. Another animal model of ANV, employed by Limebeer et al., (2006,), makes use of gaping reaction (Limebeer et al., 2006, 2008). In their experiments, they digitally record orofacial and somatic responses of rats by means of a mirror positioned below the conditioning chamber during four 30-minute conditioning trials (separated by 3 days) in which one group of the animals injected with LiCl (paired) and the other with NaCl (non-paired). 3 days after the last conditioning trial animals are reintroduced to the conditioning context in a 15-minute retention test without any injection and orofacial and somatic responses are again recorded. They found that rats in the paired group express more conditioned gaping responses during retention. These results prove that rats associate LiCl-induced illness with a context and later they show conditioned gaping response in the same context even at the absence of any drug treatment (Limebeer et al., 2006; 2008). Another study by Cloutier et al., (2018) also utilized gaping reaction and another behavioral response, forelimb flailing as the indices of aversion (Cloutier et al., 2018). They found that LiCl-treated animals display CCA (they named as conditioned disgust behavior) as evidenced by higher recurrence of gaping reaction and forelimb flailing during a drug-free retention trial (Cloutier et al., 2018). Body-washing, scratching, limb flicks, and rearing activity were also utilized to measure conditioned aversion in another studies (Parker et al., 1984). In a very recent report, vertical activity (rearing) is measured in the LiCl-paired context as a sign of CCA learning (Doobay et al., 2021).

In these studies, a variety of exteroceptive cues (tactile, auditory, visual and odor) are employed in the conditioning context to create a novel environment. These exteroceptive stimuli usually include a white noise, odor, different types of bedding and lightning. The size of the conditioning cages also differs from home cages usually. However, few studies investigated the role of small alterations in the contextual environment (Revusky & Parker, 1976). One study showed that rats display aversion to drinking cups previously paired with toxicosis. Another study also evaluated the role of sensory cues in aversion learning by pairing only small visual cues with illness (Kislal & Blizard, 2016; 2018). This study proves that even a subtle change in the drinking cup (such as a piece of tape on the spout) was enough for animals to develop aversion following its pairing with illness (Kislal & Blizard, 2016; 2018). In this study, plain tap water was also utilized in retention tests to eliminate the confounding effects of flavored solutions used in other studies (Kislal & Blizard, 2016; 2018). The results of this study highlight the importance of visual cues in aversion learning (Kislal & Blizard, 2016; 2018).

CCA in animals is analogous to ANV experience of cancer patients (Rodriguez et al., 2000). In CCA experiments, a novel environment, equipped with various stimulus, is used as conditioning room. Animals are treated with illness inducing drug and introduced to this conditioning room. This causes animals to experience malaise in a novel environment. This procedure is parallel to the experience of cancer patients. During chemotherapy, cancer patients encounter a place that contains a wide variety of exteroceptive cues when they receive chemotherapy. They also experience NV in this place following treatment.

The similarities between CCA and ANV have prompted researchers to establish the principles of CCA to better understand the genesis of ANV. Studying CCA not only allows the investigation of the possible mechanisms behind the contextual and aversion learning, but it also gives insight into the development of ANV. Understanding both the behavioral and the neurobiological mechanisms of CCA can be beneficial in terms of creating novel therapeutic approaches for cancer patients.

CHAPTER 2

THE AMNESTIC AGENTS ADMINISTERED DURING THE CONSOLIDATION ATTENUATES THE ESTABLISHMENT OF CCA LEARNING

This chapter includes experiments that were conducted to determine whether the development of CCA can be impaired with the administration of amnestic agents.

2.1. Introduction

Historically, the term of consolidation has been used to describe two related but different processes (McClelland et al., 1995; Squire & Alvarez, 1995). In the biological sense, consolidation refers to a protein synthesis- and time-dependent synaptic/cellular process that causes the formation of new synapses and strengthening or weakening of the previously formed ones (Nader et al., 2000) To put it differently, synaptic consolidation is a time dependent process in which newly acquired information is transferred into long-term memory by means of structural and chemical changes in the brain. Recently learned memories become stable and long-lasting after consolidation (Squire et al., 2005). It has been hypothesized that synaptic consolidation takes place between 500 milliseconds to hours (Miller & Matzel, 2006). Another use of the term consolidation refers to systems consolidation which is defined as a reorganization of memory traces from the hippocampus to the neocortex, causing the establishment of a stable long-term memory (Squire & Alvarez, 1995). We will use the term consolidation to indicate the former description since our study involves the disruption of the memory formation immediately after the learning, and it is not related to system consolidation.

The theory of memory consolidation was first theorized by Muller and Pilzecker (1900) to explain the observation that newly acquired memories remain vulnerable to interventions for a limited period (Muller & Pilzecker, 1900; Lechner et al., 1999). This means that, before consolidation, learned materials are in a labile state and memory formation can be interrupted (Alberini, 2005). The consolidation theory was later supported by the findings that electroconvulsive shock or hippocampal injuries near the time of memory acquisition retroactively impairs memory in rats (Duncan, 1949; Russell & Nathan, 1946). The disruption in avoidance learning with a protein synthesis inhibitor puromycin made it clear that de-novo protein expression is necessary for memories to be consolidated (Flexner et al., 1962). The notion of the protein synthesis-dependent consolidation of the newly acquired memory is later supported by umpteen number of reports using different protein synthesis inhibitors in different learning tasks in different species (Flexner et al., 1965; Hernandez & Abel 2008) One of the examples of these studies found that protein-synthesis inhibitor puromycin prevented the consolidation of avoidance memory when injected over the brain immediately following training, however, the administration of the same dose one hour after the training did not cause any memory impairment in goldfish (Agranoff et al., 1965). Puromycin also caused the loss of avoidance memory in mice (Flexner et al., 1963). Another study showed that Y-maze memory was obliterated when the puromycin injected bitemporally into mice brains (Barondes & Cohen, 1966). A different protein synthesis inhibitor acetoxycycloheximide also impaired the long- but not short-term avoidance memory in mice (Barondes & Cohen, 1968). The same memory impairment pattern was also found with subcutaneous cycloheximide injection preceding the avoidance training (Berman et al., 1978). Addition to protein-synthesis inhibitors, some antagonist agents that block the activation of a receptor system that related to learning were also found to impair memory formation. Two of these receptors, N-methyl-D-aspartate (NMDA) and beta-adrenergic receptors are of interest to our experiments because of their role in learning and memory (Shimizu et al., 2000; O'Dell et al., 2015).

Animal research established that the neurobiological underpinnings of memory consolidation are mediated by NMDA and beta-adrenergic receptors. Animals treated with NMDA or beta-adrenergic receptor antagonists show impairments in their ability

to consolidate newly acquired information into long-term memory (Rezvani, 2006; Villain et al., 2016).

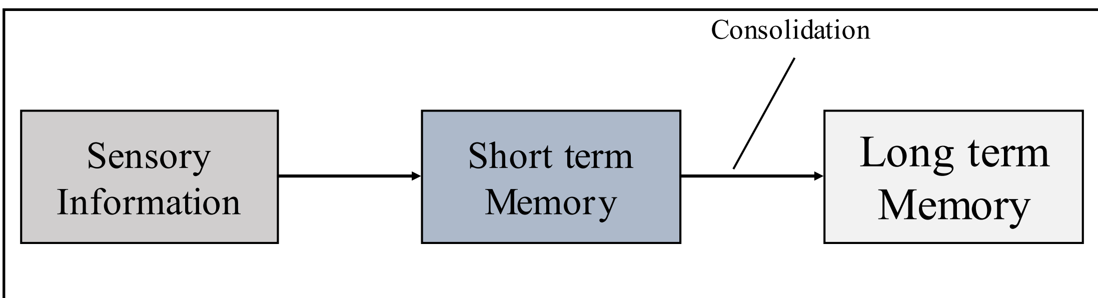

Figure 4. Consolidation phase of memory formation. Newly acquired information is stabilized from short-term memory to long-term memory via a process called consolidation.

A selective, noncompetitive NMDA receptor antagonist [(+)-5-methyl-10,11-dihydro-5H-dibenzo-[*a*,*d*]cyclo-hepten-5,10-imine-maleate] (MK-801) is frequently utilized in learning and memory research to investigate these receptors' role in the consolidation of various types of memory paradigms by means of systemic administration or intracerebral infusion of the drug (van der Staay et al., 2011). These memory paradigms include object recognition (Adriani et al., 1998; Mandillo et al., 2003), object location (Roullet et al., 1996; Nilsson et al., 2007), Morris water maze (Ahlander et al., 1993; Duda et al., 2016; Filliat and Blanchet, 1995), T-maze (Boess et al., 2004; Mackes & Willner, 2006), fear conditioning (Bardgett et al., 2003; Csernansky et al., 2005), radial arm maze (Caramanos & Shapiro, 1994; Huang et al., 2004; Nishiga et al., 2002), inhibitory avoidance (da Silva et al., 2009).

Propranolol, a nonselective competitive beta-adrenergic receptor antagonist, is another widely used pharmacological agent to disrupt consolidation of various memory tasks. Researchers have shown that the consolidation of contextual fear memories (Ji et al., 2003; Nasehi et al., 2017), spatial memories (Cahill et al., 2000), passive avoidance memories (Gallagher et al., 1977; Schneider et al., 2011), taste aversion memories (Bahar et al., 2003; Reyes-López et al., 2010; Miranda et al., 2008, Guzmán-Ramos et al., 2012), drug memories (Bernardi & Lattal, 2012), and olfactory fear memories (Kroon and Carobrez, 2008) are all disrupted by propranolol. The findings of preclinical studies have been successfully translated into clinical trials and post-traumatic stress disorder (PTSD) patients who suffer from maladaptive memories

show improvements in their symptoms after propranolol treatment. In the light of these previous research, we hypothesized that propranolol can also be utilized to prevent the development of ANV by impairing associative learning that underlies the conditioned nausea response. However, whether propranolol induces a memory impairment in the animal model of ANV had not been investigated.

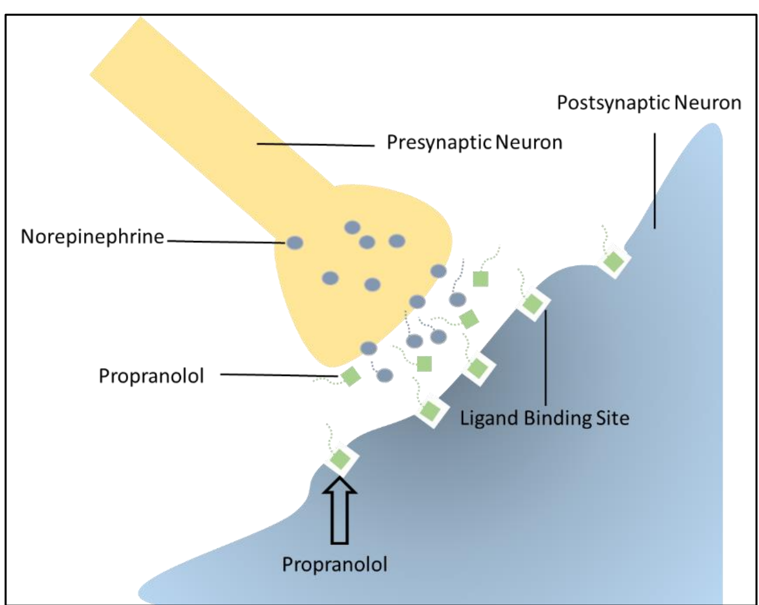

Figure 5. Propranolol's mechanism of action. Propranolol blocks norepinephrine from binding it's receptors which leads to impairments in learning when applied during consolidation or reconsolidation.

Building on extensive research that has established NMDA and beta-adrenergic receptors' role in neuronal plasticity (van der Staay et al., 2011), we designed a set of experiments to investigate the role of these receptors in the consolidation of CCA via the systemic injection of MK-801 and propranolol. We hypothesized that MK-801 and propranolol might prevent the consolidation of CCA learning.

2.2. Experiment 1: The analysis of systemic MK-801 administration on the consolidation of CCA memory

In our first experiment we investigated the role of NMDA receptors in CCA learning via the systemic MK-801 injection. As we mentioned before; NMDA receptors, especially in the hippocampus (Bliss & Collingridge, 1993, Maren & Baudry, 1995) and amygdala have been found to be vital for learning and memory (Huang & Kandel,

1998; Maren & Fanselow, 1995). Our hypothesis is CCA learning can be disrupted with MK-801 administered following conditioning. To test this hypothesis, we challenged animals with two different doses (low dose = 0.05 mg/kg; high dose = 0.2 mg/kg) of MK-801 right after conditioning trial to see whether the LiCl–context association was disrupted.

The following methods and procedures used in Experiments 1 and 2 were approved by the Animal Ethics Committee of Middle East Technical University (Protocol # 2021/01).

2.2.1. Method

2.2.1.1 Subjects

In our first experiment, forty-eight CD1 male mice each weighing between 19 and 25 g were used. Animals were housed individually in Eurostandard Type II long standard cages with transparent walls (365 x 207 x 140 mm) under a 12-h reverse light/dark schedule. They were given *ad libitum* access to food. However, water restriction was used during experiments. Each mouse's bodyweight was measured on the first and last days of habituation and at the end of the water acclimation period.

Table 1. Design of Experiment 1. The groups, number of animals and drug administrations are shown in the table.

Design	Group	Number of Animals	Injection During Conditioning	Targeting Consolidation (After Conditioning)
Control Groups	LiCl–NaCl	12	LiCl	NaCl
	NaCl – NaCl	12	NaCl	NaCl
Experimental Groups	LiCl–MK801 High Dose	12	LiCl	MK-801
	LiCl–MK801 Low Dose	12	LiCl	MK-801

The experimental design is provided in Table 1. Animals were allocated into two experimental and two control groups according to their initial bodyweight. The control groups were LiCl–NaCl (n = 12) and NaCl–NaCl (n = 12); the experimental groups were LiCl–MK801 High Dose (n = 12), and LiCl–MK801 Low Dose (n=12). Two intraperitoneal injections were given during a 20-minute conditioning trial. The first injection, administered 5 minutes after the onset of conditioning, was either LiCl or

NaCl to induce illness or as a sham treatment, respectively. While the LiCl–NaCl, LiCl–MK801 High Dose, and LiCl–MK801 Low Dose groups received LiCl injections, the NaCl–NaCl group received NaCl injections. Immediately after the conditioning, animals received their second injections either to impair memory consolidation (MK-801) or as a sham treatment (NaCl). The LiCl–MK801 High Dose and LiCl–MK801 Low Dose groups received MK-801 injections; the LiCl–NaCl and NaCl–NaCl groups received NaCl injections.

2.2.1.2. Drug Administration

NaCl, LiCl, and MK-801 were obtained from Sigma (St. Louis, MO, USA). LiCl was administered at a dose of 6 mEq/kg. MK-801 was administered at either a low (0.05 mg/kg) or high dose (0.2 mg/kg). NaCl injections were used at a concentration of 0.9%. The volume of the first LiCl and NaCl injections adjusted according to the bodyweights of animals which was 0.24 mL/kg. MK-801 and the second NaCl injections were administered at a volume of 0.2 mL/kg. The MK-801 was dissolved in 0.9% NaCl.

2.2.1.3. Apparatus

Two different contexts were utilized in all experiments. Context A included normal housing conditions in the colony room (Table 2 & Figure 7).

Animals stayed in the Eurostandard Type II standard home cages with transparent wall throughout the experiments except stated otherwise. Wood shaving was used as bedding and water was presented with the standard plastic bottles. The room contained 12/12 h light/dark cycle with no natural lighting. Context B was created in a separate room located far from the colony room. This room was equipped with several novel exteroceptive stimuli, including a red lightning produced by 60W ceiling lamp, the scent of a highly odoriferous lemon oil, and a constant 75db white noise. Also, the conditioning cages were covered with vertical black and white stripes using vinyl tape. Cat litter was used in these cages as bedding. Green color glass bottles with ball-bearings in the spouts were used to water-bottles.

Table 2. Exteroceptive cues that were used to differentiate Context B from Context A are shown in the table.

Context A	Context B
• Eurostandard Type II Long standard cages with transparent walls • ood shavings as bedding • Standard plastic water bottles • Normal lightning • No odor • No sound	• Eurostandard Type II Long standard cages with black and white tapes • Cat litter as bedding • Green colored glass bottles with stainless steel ball bearing tipped spouts • Single 0 red lamp • Lemon oil odor • hite noise at an intensity of 5 dB

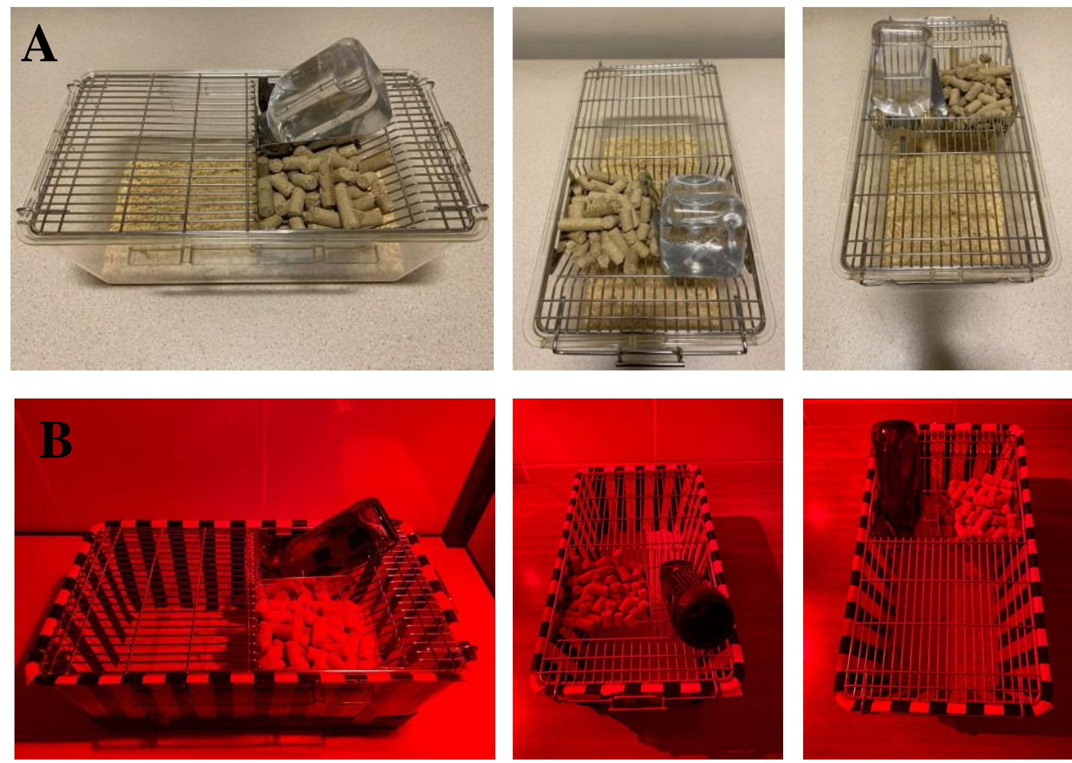

Figure 6. Pictures of the home (A) and conditioning cages (B). Eurostandard Type II cages were used for both cages. Home cages (Context A) had transparent walls. These cages were covered with wood shavings as bedding. Plastic water bottles were used in these cages. Conditioning cages (Context B) were created by vertically striping black and white vinyl tapes onto the standard cages. Unscented cat litter was used as bedding. Green glass water bottles with ball-bearing spouts were used.

2.2.1.4. Procedure

The experimental procedure is described in Table 2. The procedure entailed five phases: habituation, water acclimation, conditioning, recovery, and retention.

Habituation. An experimenter picked up each mouse with a cupped hand and held it for 3 minutes per day for 4 consecutive days to make them accustomed to being handled. Tail picking was never used through the experiments to avoid causing stress and anxiety in animals. Animals were given NaCl injection on the last day of handling to reduce the stress and novelty caused by injection. Water restriction was started subsequently at 5:30 p.m.

Water acclimation (WA). Three WA sessions were conducted on consecutive days. At the each day of WA, mice were only allowed to drink water twice: during the intervals of 10:00–10:30 a.m. and 5:00–5:30 p.m. The water was presented with standard plastic bottles (400 mL) while mice stayed in their home cages. On the last WA day, the animals were pre-exposed to the conditioning context for 5 minutes to prevent neophobic responses.

Conditioning. The completion of all WA sessions was followed by a 20-minute conditioning trial which started at 12:30 p.m. During conditioning, an experimenter introduced each mouse to its conditioning cage with planned time intervals, and these cages were carried to the conditioning room by another experimenter. After 5 minutes of context exposure, another experimenter in the conditioning room gently removed each mouse from its conditioning cage and gave injection of either LiCl or NaCl depending on the animal's group and placed the animal back into its cage where it stayed for another 15 minutes. Immediately after the conditioning, an experimenter injected the animals with either an amnestic drug or NaCl. Water bottles of each mouse were weighed before and after the conditioning to measure water consumption.

Recovery. The following two days of the conditioning were the recovery period. During this time animals were given access to water at the same time intervals, 10:00–10:30 a.m. and 5:00–5:30 p.m.

Retention. After the completion of 2-day recovery period, an experimenter re-introduced animals to their conditioning cages and another experimenter carried them to the conditioning rom. During retention, animals stayed in the conditioning room for 15 minutes and no injections were given. Water consumption was measured as an index of aversion.

Table 3. Procedural steps of Experiment 1. Habituation, WA, Conditioning, Recovery and Retention phases are shown in the table.

Days 1–4	Days 5–7	Day 8	Day 9–10	Day 11
Handling & Saline Injection	**Water Acclimation & Pre-exposure**	**Conditioning & Targeting Consolidation**	**Recovery**	**Retention**
3 min/day handling for 4 days Saline injection	Access to water, 10:00–10:30 a.m. and 5:00–5:30 p.m. 5-min pre-exposure period, 12:30–12:35 p.m.	20-min conditioning trial, 12:30–12:50 p.m. MK-801 or propranolol injection immediately after conditioning	Access to water, 10:00–10:30 a.m. and 5:00–5:30 p.m.	15-min retention trial, 12:30–12:45 p.m.

2.2.1.5. Data Analysis

Statistical analyses were conducted with Prism GraphPad Version 9 (GraphPad Software Inc., San Diego, CA, USA). One-way ANOVA was employed to analyze the animals' water intake during the conditioning and retention trials. Fisher's LSD test was employed for post-hoc comparisons. The confidence level was set to 95% ($p < .05$).

2.2.2. Results

2.2.2.1. Conditioning Results

Figure 7 depicts mean water intakes of groups during conditioning. LiCl-treated animals had lower water intake than NaCl-treated animals. As revealed by one-way ANOVA, there was a significant difference among groups in water intake, F (3, 44) =

4.11, $p = .012$. Fisher's LSD test indicated that NaCl–NaCl (M = 0.682, SD = 0.11) group drank significantly more water than LiCl–NaCl (*M* = 0.46, *SD* = 0.206; $p = .008$), LiCl–MK801 High Dose (*M* = 0.424, *SD* = 0.257; $p = .002$), and LiCl–MK801 Low Dose (*M* = 0.512, *SD* = 0.177; $p = .038$) groups. No significant difference was observed between the other groups.

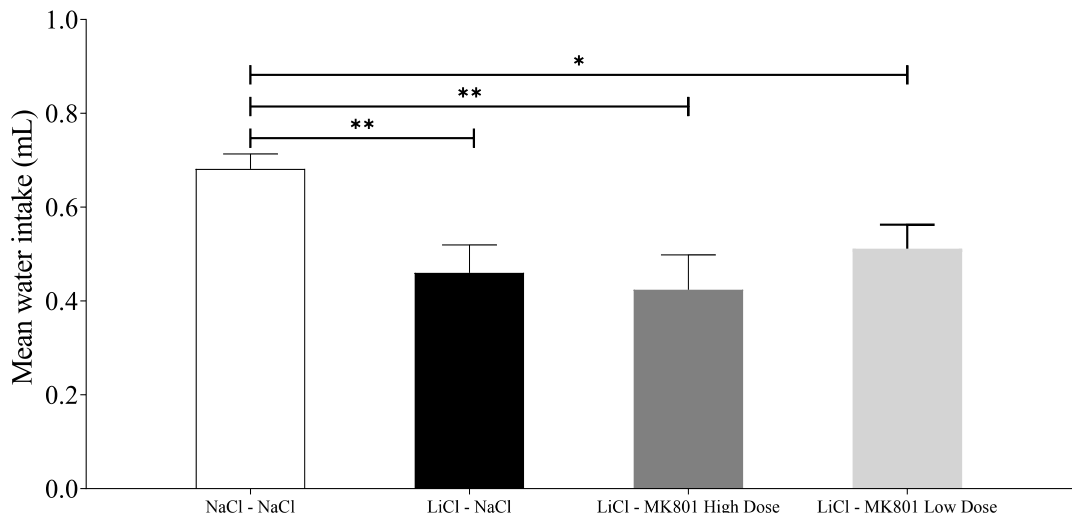

Figure 7. The mean water intake of each group during conditioning. NaCl injected animals had higher water consumption than LiCl injected animals. All data depicted as mean ± SEM. * $p < .05$, ** $p < .01$.

2.2.2.2. Retention Results

Figure 8 depicts mean water intakes of groups during retention. In retention test, LiCl-treated animals had lower water intake than NaCl-treated animals during conditioning. As revealed by one-way ANOVA, there was a significant difference among groups in water intake, F (3, 44) = 31.96, $p < .001$. Fisher's LSD test displayed that the NaCl–NaCl (M = 0.677, SD = 0.113) group drank significantly more water than the LiCl–NaCl (*M* = 0.36, *SD* = 0.125; $p < .001$), LiCl–MK801 High Dose (*M* = 0.299, *SD* = 0.112; $p < .001$), and LiCl–MK801 Low Dose (*M* = 0.367, *SD* = 0.053; $p < .001$) groups.

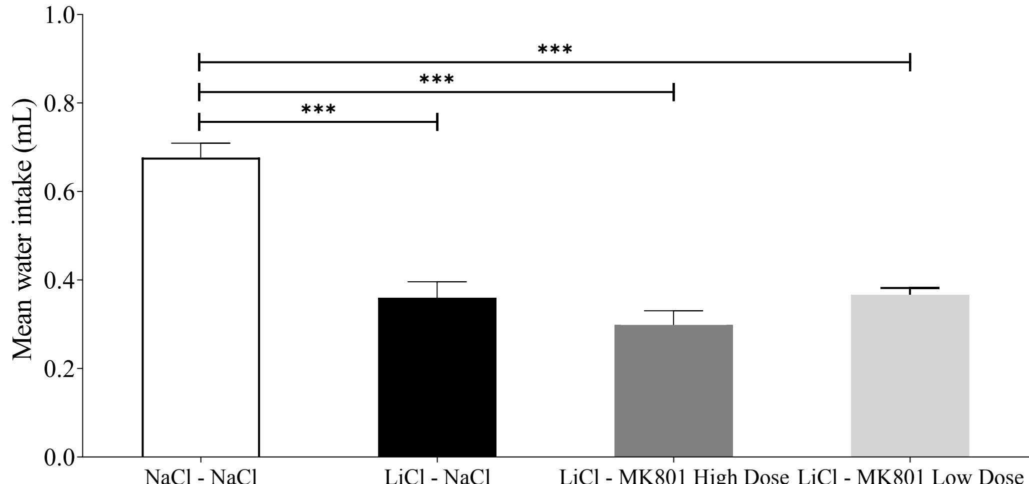

Figure 8. The mean water intake of each group during retention test. NaCl injected animals during conditioning had higher water consumption than LiCl injected animals. High or low dose MK-801 injections failed to impair learning, as evidenced by low consumption levels of LiCl-treated animals independent of second injections. All data depicted as mean ± SEM. *** $p < .001$.

2.2.3. Discussion

In our first experiment, we aimed to test whether MK-801 treatment prevents the consolidation of CCA learning. Our results indicates that MK-801 injection have no effect on the retention of CCA memory when it is given immediately after the conditioning trial since LiCl-treated animals during conditioning displayed suppressed water consumption independent of the second MK-801 or NaCl injections. This data can be interpreted as that NMDA receptor activation following conditioning is not vital for animals to learn CCA.

2.3. Experiment 2: The analysis of systemic propranolol administration on the consolidation of CCA

In our second experiment we investigated whether blocking the activation of the beta-adrenergic receptors with propranolol prevents animals from developing a CCA. To do so, we injected mice with propranolol (10 mg/kg) following the conditioning trial. Three days after the conditioning we tested CCA memory by reintroducing animals to the reinforced context in a drug-free state.

2.3.1. Method

2.3.1.1. Subjects

In our second experiment the subjects were thirty-four CD1 male mice each weighing between 19 and 25 g. The home cages and housing conditions were the same as our first experiment. Bodyweights were also measured on the first and last habituation days, and on the end of WA.

Table 4. Design of Experiment 2. The groups, number of animals and drug administrations are shown in the table.

Design	Group	Number of Animals	Injection During Conditioning	Targeting Consolidation (After Conditioning)
Control Groups	LiCl–NaCl	12	LiCl	NaCl
	NaCl–NaCl	11	NaCl	NaCl
Experimental Group	LiCl–Propranolol	11	LiCl	Propranolol

The experimental design is provided in Table 3. Animals were divided into two control groups and one experimental group according to their bodyweight at the start of the experiment. The control groups were LiCl–NaCl (n = 12) and NaCl–NaCl (n = 11); the experimental group was LiCl–Propranolol (n = 11). We injected animals twice during conditioning. The first injections, administered 5 minutes after the onset of conditioning, was either LiCl or NaCl to induce illness or as a sham treatment, respectively. While the LiCl–NaCl and LiCl–Propranolol groups received LiCl injections, the NaCl–NaCl group received NaCl injections. Immediately after the conditioning, animals received their second injections either to impair memory consolidation (propranolol) or as a sham treatment (NaCl). The LiCl–Propranolol group received propranolol injections; the LiCl–NaCl and NaCl–NaCl groups received NaCl injections.

2.3.1.2. Drug Administration

NaCl, LiCl, and propranolol were purchased from Sigma (St. Louis, MO, USA). The propranolol was dissolved in the NaCl (0.9%). LiCl was administered at a dose of 6

mEq/kg; propranolol administered at a dose of 10 mg/kg. The systemic NaCl (0.9%) administration served as sham treatment. All drugs were administered at a volume of 0.24 mL/kg.

2.3.1.3. Apparatus

We used the same apparatus from the first experiment.

2.3.1.4. Procedure

We used the same CCA protocol and experimental design from the first experiment.

2.3.1.5 Data Analysis

Data analysis was similar to the first experiment.

2.3.2. Results

2.3.2.1. Conditioning Results

Figure 9 depicts mean water intakes of groups during conditioning. Two outliers were excluded from the data. Water intake during conditioning was similar for the animals that had been injected with LiCl and those that had been injected with NaCl. One-way ANOVA analysis revealed no significant difference in water intake among groups, F (2, 29) = 4.11, p = .949.

2.3.2.2. Retention Results

Figure 10 depicts mean water intakes during retention. One-way ANOVA analysis revealed a significant difference in water intake among groups, F (2, 29) = 4.45, p = .021. Fisher's LSD test showed that the LiCl–NaCl (M = 0.084, SD = 0.054) group drank significantly less water than the NaCl–NaCl (M = 0.377, SD = 0.306; p = .017) and LiCl–Propranolol (M = 0.404, SD = 0.305; p = .011) groups. However, there was no significant difference in water intake between the NaCl–NaCl and LiCl–Propranolol groups (p = .8).

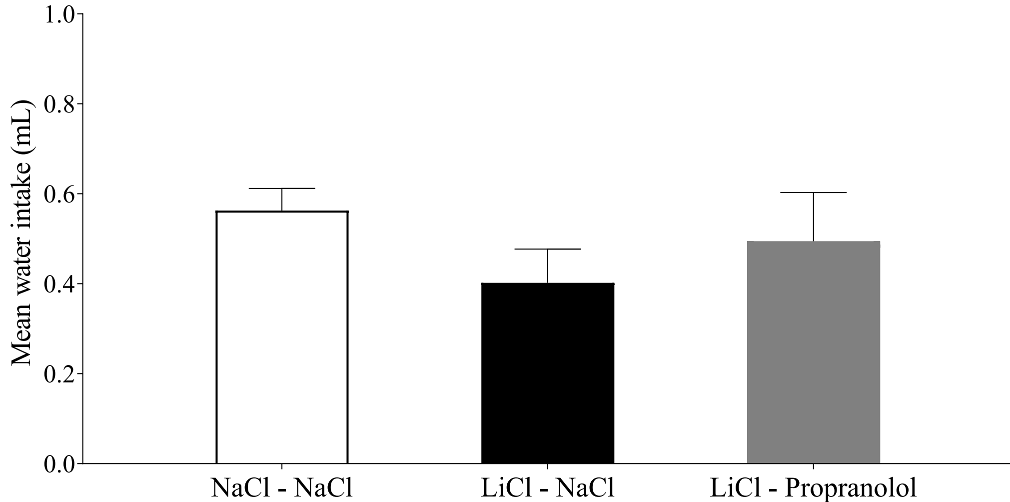

Figure 9. The mean water intake of each group during conditioning. Although, it did not reach statistical significance, NaCl injected animals had higher water consumption than LiCl injected animals. All data depicted as mean ± SEM.

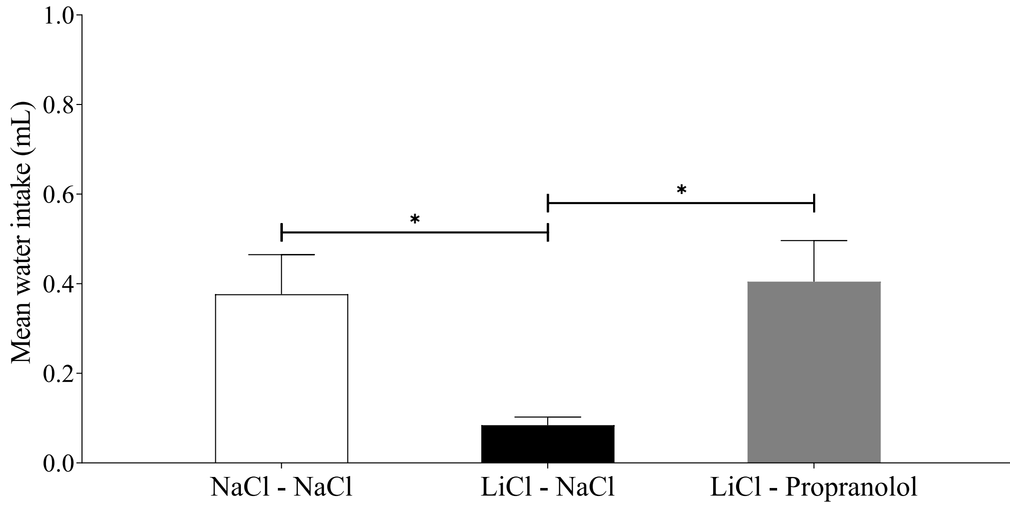

Figure 10. The mean water intake of each group during retention test. NaCl injected animals during conditioning had higher water consumption than LiCl injected animals when it is followed by NaCl injection. However, propranolol injection following LiCl-treatment impaired CCA learning, as evidenced by the LiCl–Propranolol group's high-water intake. All data depicted as mean ± SEM. * $p < .05$.

2.3.3. Discussion

We conducted our second experiment to investigate whether consolidation of CCA learning critically depends on the activation of beta-adrenergic receptors by means of

systemic propranolol injection after the conditioning trial in which LiCl-induced illness is paired with a novel context. Retention results show that when LiCl-treated animals injected with propranolol, they displayed higher water intake than those injected with NaCl. The water intake of propranolol injected group was similar to those mice that had not experienced illness during conditioning. Our findings indicate that blocking the beta-adrenergic receptor activation with propranolol impaired animals CCA learning.

2.4. General Discussion

We conducted two experiments to determine whether consolidation of CCA learning is disrupted by NMDA or beta-adrenergic receptor antagonism. In our first experiment we used NMDA receptor antagonist MK-801. We injected three groups of animals with LiCl and one group of animals with NaCl during conditioning. After the conditioning trial, LiCl-treated animals are given their second injections; low dose (0.05 mg/kg) or high dose (0.2 mg/kg) of MK-801 or NaCl. NaCl-treated animals were again injected with NaCl. We found that when LiCl-treated animals reintroduced to the conditioning context without injection, they showed suppressed water consumption independent of the second injections, indicating that systemic MK-801 administration did not impair memory consolidation of CCA. It is surprising that we found no memory impairment with MK-801 since previous research shows that NMDA receptor activation initiate and regulate intracellular events that cause plasticity-related gene expression and synaptic strengthening, a vital mechanism for learning and memory (Elgersma & Silva, 1999; Malenka & Bear, 2004). One explanation for our results is that MK-801 did not impair the formation of CCA because NMDA receptor-independent plasticity mechanisms allowed animals to learn context-illness association. Several studies have shown that memory impairments in spatial (Bannerman et al., 1995; Saucier and Cain, 1995) and fear learning (Hardt et al., 2009; Sanders and Fanselow, 2003; Wiltgen et al., 2010) caused by NMDA receptor antagonism can be prevented with pre-training. Bannerman et al. (1995) reported that NMDA receptor antagonist AP5 [D(-)-2-amino-5-phosphonovaleric acid] induced learning deficits were completely erased when rats were pre-trained in water maze (Bannerman et al., 1995). In a different study although NMDA receptor

antagonist NPC17742 [2R,4R,5S-2-amino-4,5-(1,2-cyclo hexyl)-7-phosphonoheptano acid] prevented long term potentiation (LTP) in the dentate gyrus (DG), it failed to impair spatial memory when rats previously learned the general task requirements of water-maze training (Saucier and Cain, 1995). Similar findings to these observations also have been reported for fear learning. Pre-training mitigated memory impairments caused with NMDA receptor antagonist APV [5-amino-phosphonovaleric acid] injection in fear conditioning evidenced by the continuation of freezing response to a context paired with shock (Sanders and Fanselow, 2003). Researchers also have found evidence for fear learning mechanisms independent of NMDA receptor activation using knockout and transgenic mice models (Tayler et al., 2011; Wiltgen et al., 2010). The findings of these studies indicate that NMDA receptor activation is not always essential for animals to learn. The hallmark of the studies in which NMDA-receptor independent mechanisms have been found to play a role in learning is the procedural utilization of pre-exposure/pre-training. It appears that pre-training/pre-exposure alleviates the learning deficits induced by NMDA receptor antagonists. Our procedural design also includes a pre-exposure phase in which animals are introduced to the conditioning context prior to conditioning trial. In the light of this previous research, the lack of memory impairing effect of MK-801 in our study can be explained as that pre-exposing animals to the conditioning context induced the activation of NMDA receptor-independent plasticity mechanisms and blocking the activation of NMDA receptors with MK-801 failed to impair learning because the activation of these receptors was not necessary for animals to learn a task when they have prior experience. However, this explanation is based on previous research and our study was not designed to elucidate the mechanism behind our observations. The relationship between NMDA receptors and the novelty of the learning experience warrants further exploration.

Another explanation for the lack of memory impairing effect of MK-801 could be due to the injection timing. We chose to inject MK-801 immediately following conditioning based on the extensive research showing memory impairments induced by MK-801 following the acquisition of a task (Castellano et al., 1999; de Lima et al., 2005). However, studies also have found MK-801 induced memory impairments when it is injected not after, but before the initial learning (Nilsson et al., 2007; Venable and

Kelly, 1990). Therefore, it is also possible that NMDA receptors are activated in the initial phase of the consolidation of CCA learning, but not later phases. This could also explain why animals developed CCA even though they are injected with MK-801. Further research is necessary to elucidate NMDA receptors' role in early stages of consolidation of CCA memory.

In our second experiment, our aim was to investigate whether beta-adrenergic receptor signaling is vitally important for the consolidation of CCA memory. We have found evidence for their involvement since animals did not display suppressed water consumption in a context previously paired with illness when they are given propranolol injection following conditioning. The LiCl–Propranolol group drank similar amount of water as NaCl–NaCl group, the group that had not experience illness-context pairing. However, the LiCl–Propranolol and NaCl–NaCl groups had higher water consumption than LiCl–NaCl group, the group that experienced illness during novel context exposure but not injected with amnestic agent. These results indicate that CCA learning is disrupted by propranolol injection. A huge amount of literature found memory impairments induced by propranolol during reconsolidation but not consolidation (Villain et al., 2016). However, our results indicated that beta-adrenergic receptors play a vital role in the consolidation of CCA. To the best of our knowledge, this is the first report of propranolol producing memory impairing effect during consolidation of CCA learning. Although our results provide evidence for the involvement of beta-adrenergic receptor system in CCA learning, our experiment was not designed specifically to elucidate through which brain structure propranolol induces memory impairments. However, amygdala have been found to be responsible for the formation of US-CS association (McGaugh, 2004). Intra-amygdala infusion of propranolol causes retrograde amnesia in passive avoidance (Gallagher et al., 1977), taste aversion (Bahar et al., 2003; Guzmán-Ramos et al., 2012), and water maze (Hatfield and McGaugh, 1999). Additional research will help clarify through which brain structures propranolol is inducing its memory impairing effect in CCA learning.

Although, propranolol impaired consolidation of CCA, we did not investigate its effect during reconsolidation. Other preclinical studies mostly used propranolol during fear memory reconsolidation (Zhu et al., 2018). These studies have observed that

propranolol injection during the fear learning reconsolidation causes impairments in memory (Abrari et al., 2008; Taherian et al., 2014; Villain et al., 2018). Preclinical studies laid the foundation for the human clinical trials utilizing propranolol intervention following traumatic memory reactivation to cause a reduction in PTSD patients emotional response to the traumatic event. Clinical studies have found that after PTSD patients receive propranolol intervention almost 70% of them no longer met the diagnostic criteria (Brunet et al., 2011, 2014; Young and Butcher, 2020). Our findings in mice indicate that including propranolol to the prophylaxis of ANV might be beneficial in terms of reliving some of the stress and challenges that cancer patients experience. Clinical studies should investigate the protective role of propranolol intervention on the development of ANV for cancer patients.

2.5. Conclusion

Our basic research has substantial clinical relevance since one of the major factors causing patients to discontinue chemotherapy stems from aversive learning and memories. Interventions with propranolol might be a novel therapeutic approach to prevent patients from developing ANV. Our findings also give insight into NMDA receptor-independent learning mechanism in CCA.

CHAPTER 3

C-FOS EXPRESSION IN THE BRAIN FOLLOWING CONDITIONED CONTEXT AVERSION

This chapter covers an experiment conducted to find the neural substrate of CCA learning by investigating c-Fos expression in the brain.

3.1. Introduction

Although behavioral research mainly established the principles of CCA learning, its neural substrate has not been investigated (Best et al., 1973; Hall et al., 1997; Parker et al., 1984; Symonds et al., 1998). In our third experiment we aimed to identify the brain regions involved in CCA learning. To do so, we employed c-Fos expression to indirectly measure neural activation.

Voltage-gated calcium entry into neurons causes the expression of c-Fos proto-oncogene (Morgan and Curran, 1986). This finding prompted researchers to use the expression of c-Fos protein as an indirect measure of neuronal activation using immunohistochemistry (Perrin-Terrin et al., 2016). Immunohistochemistry is a staining method that permits the quantification and localization of protein expression. Studies show that protein product of c-Fos gene can be identified within the 20-90 minutes following neuronal excitation with immunohistochemistry (Mugnaini et al., 1989). In our study, we investigated c-fos expression 60 minutes after the conditioning since its expression peaks around this time. (Mugnaini et al., 1989).

We chose a priori four brain regions to quantify c-Fos expression based on previous research: medial prefrontal cortex (mPFC) with two subregions, insular cortex (IC), hippocampus with three subregions and amygdala with four nuclei. Studies indicate

that c-Fos expression in the nervous system plays a vital role in learning-related plasticity (Filipkowski et al., 2006). For example, Campeau et al. (1991) have shown that contextual cues associated with fear induces c-Fos expression in the amygdala (Campeau et al., 1991). Also, CTA memory acquisition is found to be dependent on c-Fos expression in the same region (Koh & Bernstein, 2005; Lamprecht & Dudai, 1996). As addition to the lateral and basolateral nuclei, the medial division of the central nucleus of the amygdala has been found to express c-Fos protein after fear learning renewal (Knapska & Maren 2009). Memory retrieval and extinction learning increases c-Fos expression in the CA1 and DG subregions of the hippocampus, respectively (Strekalova et al., 2003; Knapska & Maren 2009). Novel context exposure also induces CA1 c-Fos expression (Murawski et al., 2012). Extinction learning causes high levels of c-Fos in the infralimbic (IL) division of the (mPFC). On the other hand, renewal of contextual fear learning is found to be associated with c-Fos expression in the prelimbic (PL) division of the mPFC (Knapska & Maren 2009). IC has been suggested to play a role in the acquisition of CTA as evidenced by increased c-Fos expression in this region after illness-taste pairing (Koh & Bernstein, 2005). Our regions of interest were chosen based on their role in conditioning, aversion, and fear learning as shown by the aforementioned studies. Our aim was to investigate if these brain regions are also involved in CCA learning. We quantify c-Fos expression in the PL and IL divisions of the mPFC; the IC; medial, cortical, basolateral, and central nuclei of the amygdala; CA1-CA2, CA3, and DG subregions of the hippocampus. We used the same CCA procedure as in our previous experiments. We hypothesized that c-Fos expression will be increased in the brains regions that are activated after CCA learning. Since c-Fos expression is downregulated with repeated same stimulus exposure, our single-trial conditioning procedure allowed us to analyze c-Fos expression induced by CCA learning.

3.2. Methods

3.2.1. Subjects

In our third experiment the subjects were 54 male 12-week-old CD1 outbred male mice weighing between 19 and 25 g each at the start of the experiment. The home cages and housing conditions were the same as our previous experiments.

Bodyweights were measured on the first and last habituation days, and on the end of WA. Experimental procedures were approved by the Ethics Committee of the Middle East Technical University, Ankara, Turkey (Protocol # 2021/01).

Table 5. Design of Experiment 3. The groups, exposure to CS and US, number of animals are shown in the table.

Design	Group	Exposure	Number of animals for behavioral test	Number of animals for c-Fos
Control groups	LiCl–Context A	Exposed to US only	12	6
	NaCl–Context B	Exposed to CS only	12	6
Experimental group	LiCl–Context B	Exposed to both US and CS	12	6

We allocated CD1 male mice into two control and one experimental groups as following: NaCl–Context B (n = 18), LiCl–Context A (n = 18) and LiCl–Context B (n = 18). While Context A referred to the home cages, Context B referred to the conditioning context. During conditioning, we injected animals in the LiCl–Context A group with LiCl while they were in their home cages. We injected animals in the NaCl–Context B and LiCl–Context B groups with NaCl and LiCl, respectively, in the conditioning context. Six animals of each group were perfused exactly 1 hour after conditioning to investigate c-Fos protein expression. The remaining 12 animals in each group were used in a 15-minute retention test, conducted 72 hours after conditioning to see whether animals developed CCA.

We designed this group such that US or CS exposure induced c-Fos expression can be differentiated from learning-induced c-Fos expression. NaCl–Context B group was only exposed to the CS but not the US, however LiCl–Context A group was only exposed to the US but not the CS; therefore, in these groups, any c-Fos expression in our regions of interest was the result of only CS and only US exposure, respectively. To put it differently, these two groups did not experience the US and CS concurrently, therefore, we did not expect to see development of CCA, and to find c-Fos expression as a result of CCA learning. Our experimental group, LiCl–Context B, however, experienced both the US and the CS during conditioning. Therefore, we expected animals in this group to develop CCA, and to find increased c-Fos expression in regions responsible for CCA learning.

3.2.2. Behavioral Procedure

Similar to our first experiment, our second experiment also consisted of five phases: habituation, WA, conditioning, recovery, and retention. Except for the conditioning trial, our procedural design was the same as our previous experiments. Please see "Behavioral Procedure" section in Experiment 1 for details. See below for the details regarding conditioning trial.

The conditioning cages and room was the same as those used in our first two experiments. Briefly, the conditioning room contained a red light (60W lamp), lemon oil scent, and constant white noise (75 decibels). The cages used for conditioning were furnished with vertical white and black bands and, cat litter was used as bedding inside of these cages. Green glass bottles were used to supply water. The conditioning trial started at 12:30 p.m. During conditioning, an experimenter introduced animals in the Context B groups to their conditioning cages and another experimenter transported these cages to the conditioning room. Animals in the Context A group continued to stay in their home cages. Animals were gently removed from their home or conditioning cages 5 minutes after the onset of conditioning and injected with either LiCl or NaCl (i.p.) before being reintroduced to their cages. Six animals from each group were perfused exactly 1 hour after the completion of the conditioning and their brains were extracted to investigate c-Fos expression.

Table 6. Procedural steps of Experiment 3. Habituation, WA, Conditioning, Recovery and Retention phases are shown in the table.

Days 1 – 4	Days 5 – 7	Day 8	Days 9 – 10	Day 11
Handling	Water Acclimation & Pre-exposure	Conditioning & Brain Extraction	Recovery	Retention
Mice were handled for 3 minutes per day for 4 days Mice were injected with saline on the final day of habituation Water deprivation started at 5:30 p.m.	Mice were allowed to drink water between 10:00 and 10:30 a.m. and 5:00 and 5:30 p.m. Mice were introduced to the conditioning context and given access to water for 5 minutes starting at 12:30 p.m.	Mice were injected with LiCl or NaCl 5 minutes after they were exposed to the conditioning context starting at 12:30 p.m. One hour after the conditioning, 6 animals per group were perfused and their brains were	Mice were allowed to drink water between 10:00 and 10:30 a.m. and 5:00 and 5:30 p.m.	Mice were tested for water consumption between 12:30 and 12:45 p.m.

on the final day of habituation		extracted for c-Fos staining		

3.2.3. Immunohistochemistry

Six mice from each group were used for c-Fos evaluation. One hour after conditioning, each animal was deeply anesthetized using ketamine (130 mg/gm) and xylazine (13 mg/gm) cocktail. Animals were perfused transcardially with 0.1 Molar ice-cold phosphate buffer followed by 4% paraformaldehyde to clear blood and preserve brain for immunohistochemistry. Each brain was extracted after perfusion, post-fixed in 4% paraformaldehyde for 24 hours, and then cryoprotected in 30% sucrose in 0.1 Molar phosphate buffer for 48 hours. Specimens were stored in -80°C using isopentane until sectioning. The brains were cut into 40 µm coronal sections and collected in polyvinylpyrrolidone. c-Fos immunohistochemistry protocol was applied. First, sections were preincubated in 10% normal goat serum for 60 minutes to prevent non-specific binding, then placed in 3% hydrogen peroxide for 30 minutes to inactivate endogenous peroxidase activity. This is followed by 24 hours anti-c-Fos antibody (1:2000 dilutions; Cell Signaling Technologies) incubation. Next day, sections were incubated in biotinylated goat anti-rabbit antibody (1:250; Vector Labs) for 1 hour. ABC complex (ABC Kit; Vector Labs) was applied to sections to amplify the signal. SG HRP substrate (Vector Labs) was used as chromogen. After the staining was done, sections were gently mounted on positively charged slides, air-dried overnight, dehydrated, and covered with glass slide covers.

3.2.4. c-Fos analysis

We selected a priori four brain regions; mPFC, IC, amygdala, and hippocampus to quantify c-Fos expression based on previous research that establish a role for these regions in aversive and contextual learning. These regions were identified on c-Fos-stained sections. Corresponding cresyl violet-stained sections were also used to verify the structure borders. Images were captured under x10 magnification using a digital

camera mounted to a light microscope. An experimenter blind to the experimental groups manually counted c-Fos-positive cells by using ImageJ software (National Institutes of Health). The ratio of the number of c-Fos positive cells to the area of 0.1 mm^2 occupied by the structure was analyzed. The stereotaxic mouse brain atlas was used to determine anteroposterior coordinates of the regions of interest relative to bregma (Paxinos & Franklin, 2013). The coordinates of the regions were as follows: PL cortex, AP +2.45 mm to +1.97 mm; IL cortex, AP +1.97 mm to +1.53 mm; IC, AP +1.4 mm to +0.6 mm; dorsal hippocampus; and amygdala, AP -1.21 mm to AP -2.03 mm.

3.2.5. Data Analysis

Water intake of groups during conditioning trial and retention test, and c-Fos-positive cell nuclei were statistically analyzed using GraphPad Prism. The ROUT method with Q = 1% was employed to detect outliers. One-way ANOVA test was conducted to investigate the statistical difference among groups, and pairwise comparisons were made using Fisher's LSD test. Statistical significance was defined as $p < .05$.

3.3. Behavioral Test Results

3.3.1. Conditioning Results

Figure 11 depicts the mean water intakes of the animals perfused following conditioning for c-Fos immunohistochemistry. LiCl-treated animals in the conditioning context had lower water consumption than LiCl-treated animals in their home cages or NaCl-treated animals in the conditioning context. As revealed by one-way ANOVA, there was a significant difference among groups in water intake, F (2, 15) = 12.42, $p < .001$. Fisher's LSD test displayed that LiCl–Context B (M = 0.298, SD = 0.17) group drank significantly less water than LiCl–Context A (M = 1.165, SD = 0.447) or NaCl–Context B (M = 1.12, SD = 0.342) groups (p values < .001). No significant difference was observed between the LiCl–Context A and NaCl–Context B (p = .821) groups.

The mean water intakes of the animals used for the evaluation of CCA learning are shown in Figure 12. One outlier in the NaCl–Context B group was excluded from the

data. LiCl-treated animals in the conditioning context had lower water consumption than LiCl-treated animals in their home cages or NaCl-treated animals in the conditioning context. As revealed by one-way ANOVA, there was a significant difference among groups in water intake, F (2, 32) = 26.06, $p < .001$. Fisher's LSD test displayed that LiCl–Context B (M = 0.347, SD = 0.167) group drank significantly less water than LiCl–Context A (M = 1.423, SD = 0.574) or the NaCl –Context B (M = 0.664, SD = 0.234) groups (p values < .001). No significant difference was observed between the LiCl–Context A and NaCl–Context B groups (p = .050).

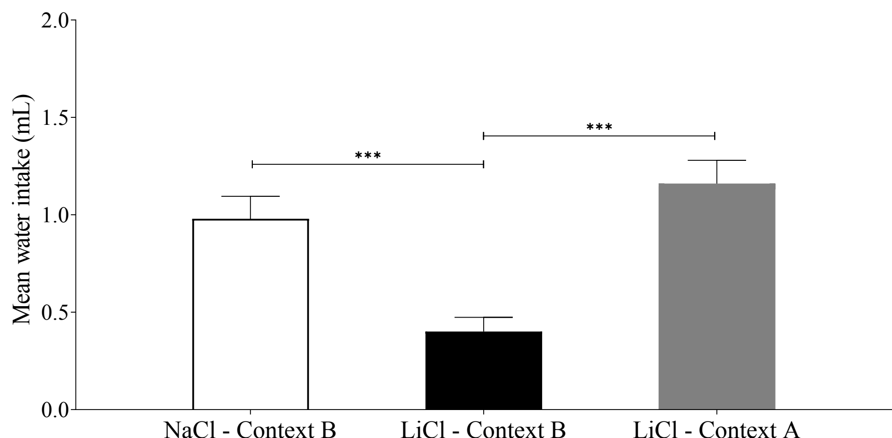

Figure 11. The mean water intake of each group used for c-Fos immunohistochemistry. Animals that were injected with LiCl during novel context exposure showed decreased water consumption during conditioning. However, NaCl injected animals during novel context exposure or LiCl injected animals in their home cages had high levels of water consumption. All data depicted as mean ± SEM ***$p < .001$.

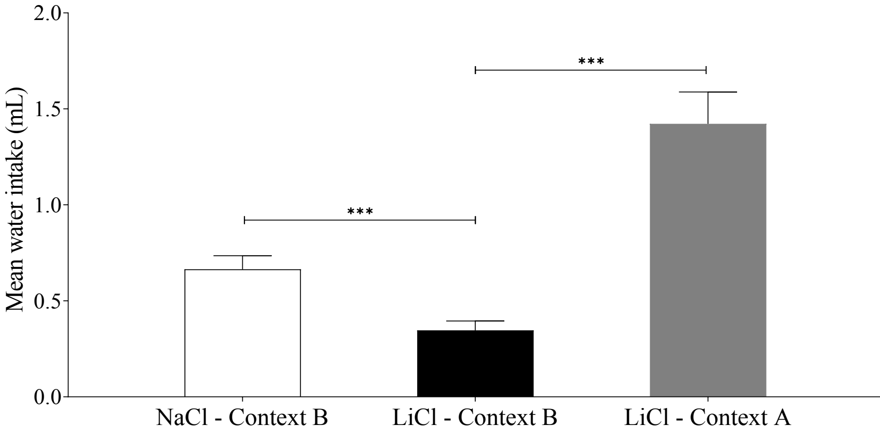

Figure 12. The mean water intake of each group used for behavioral evaluation of CCA learning. Animals that were injected with LiCl during novel context exposure showed decreased water consumption during conditioning. However, NaCl injected animals during novel context exposure and LiCl injected animals in their home cages had high levels of water consumption. All data depicted as mean ± SEM ***$p < .001$.

3.3.2. Retention Results

Figure 13 depicts mean water intake of each group during the retention test. Animals who were injected with LiCl in conditioning context had low levels of water consumption compared to animals that were injected with LiCl in their home cages or NaCl in the conditioning context. As revealed by one-way ANOVA, there was a significant difference among groups in water intake, $F(2, 32) = 41.7, p < .001$. Fisher's LSD test displayed that the LiCl–Context B (M = 0.191, SD = 0.178) group drank significantly less water than the LiCl–Context A (M = 1.726, SD = 0.539) or NaCl–Context B (M = 0.919, SD = 0.434) groups ($p < .001$). The NaCl–Context B group also drank significantly less water relative to the LiCl–Context A group ($p < .001$).

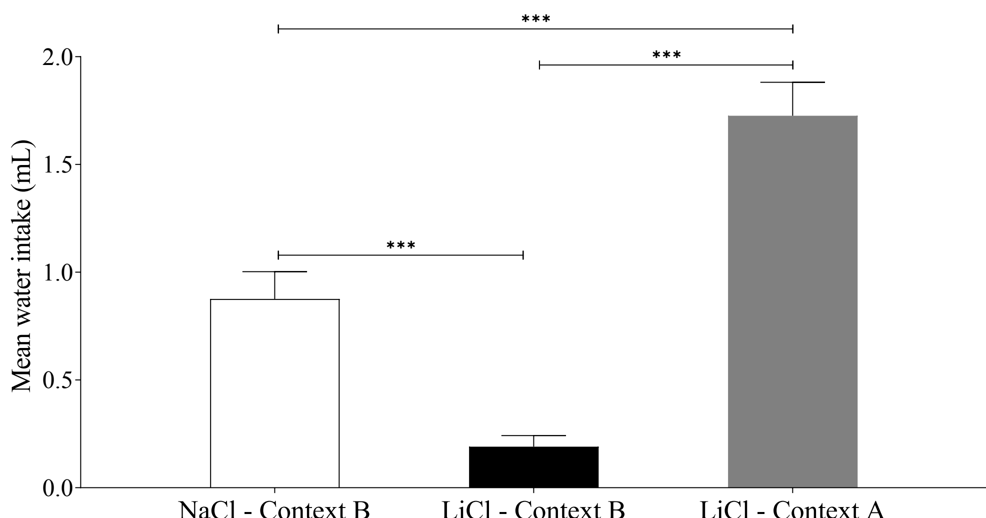

Figure 13. The mean water intake of each group during retention test. LiCl treatment in conditioning context induced suppressed water consumption. However, LiCl treatment in home cages or NaCl treatment in conditioning context did not induce suppressed water consumption. All data depicted as mean ± SEM ***$p < .001$.

3.4. Discussion

We injected animals with LiCl or NaCl during a single conditioning trial to induce illness or as a sham treatment. One of our control groups, LiCl–Context A received LiCl injections while staying in their home cages, as this group was created to see the effect of only US exposure. Another control group, NaCl–Context B received NaCl injections in the conditioning context. This group was created so that animals would only experience CS in the absence of US. Our experimental group injected with LiCl

in the conditioning context; therefore, this group experienced both the US and the CS in temporal contiguity. Our hypothesis was that animals in this group would show CCA when they are tested in a retention trial conducted 72 hours after the conditioning. Supporting our notion, the results of retention test revealed that LiCl–Context B group showed evidence of CCA, that is suppressed water consumption in the reinforced-context. However, high water intakes of LiCl–Context A and NaCl–Context B indicate that these animals did not develop CCA. Therefore, we expected to see learning induced c-Fos expression in the LiCl–Context B group but not in the LiCl–Context A and NaCl–Context B groups, in brain regions responsible for the development of CCA.

3.5. Immunohistochemistry Results

3.5.1. Medial Prefrontal Cortex

PL cortex. Figure 14(A) depicts the mean c-Fos positive nuclei per 0.1 mm^2 in the PL division. The c-Fos expression was higher in the PL cortex of LiCl-treated animals in the conditioning context relative to the LiCl-treated animals in their home cages or NaCl-treated animals in the conditioning context. As revealed by one-way ANOVA, there was a significant difference among groups, F (2, 15) = 5.413, p = .01 . Fisher's LSD test displayed that in the PL division, LiCl–Context B (M = 150.09, SD = 47.77) group had significantly more c-Fos positive nuclei per 0.1 mm^2 than the LiCl–Context A (M = 81.74, SD = 30.91; p = .011) or the NaCl –Context B (M = 85.21, SD = 42.57; p = .014) group. No significant difference was found between the NaCl–Context B and LiCl–Context A groups (p = .885).

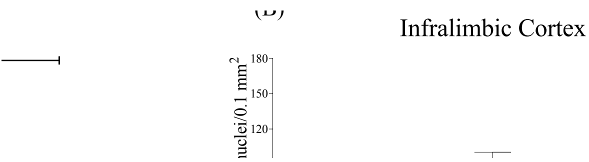

Figure 14. c-Fos expression in the PL and IL divisions of the mPFC: (A) c-Fos positive nuclei per 0.1 mm^2 in the PL cortex; and (B) c-Fos positive nuclei per 0.1 mm^2 in the IL cortex. Statistical analyses revealed increased c-Fos expression in the PL, but not IL division of the

mPFC in the group that were expected to develop CCA. All data depicted as mean ± SEM. *p < .05.

IL cortex. Figure 14(B) depicts the mean c-Fos positive nuclei per 0.1 mm^2 in the IL division. The c-Fos expression in the IL division was similar among the three groups. ANOVA analysis revealed no significant difference among the three groups, F (2, 15) = 1.844, p = .192.

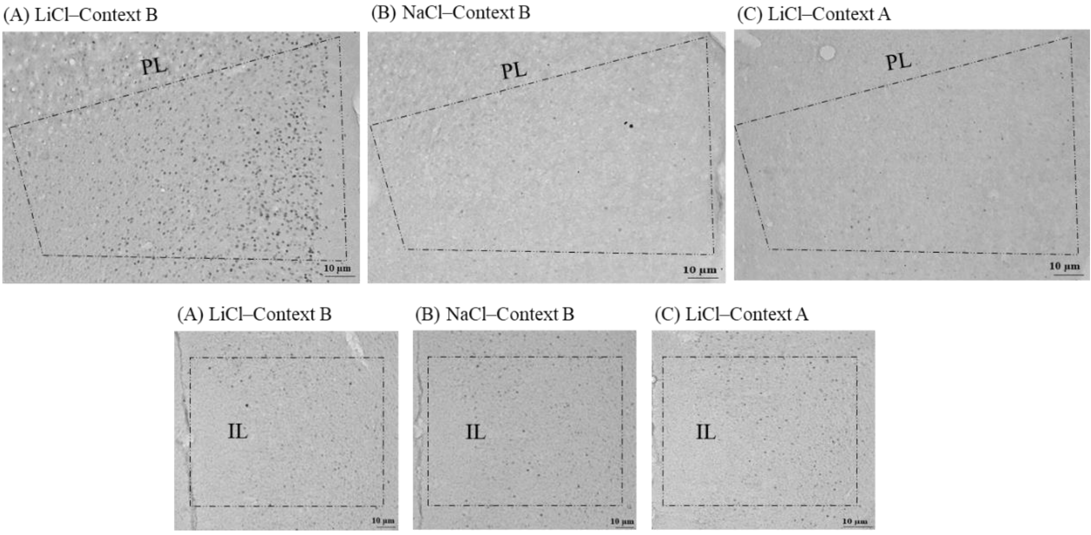

Figure 15. Representative photomicrographs of the subdivisions of the mPFC. (A) is from the LiCl–Context B group; (B) is from the NaCl–Context B group; (C) is from the LiCl–Context A group. c-Fos expression is quantified by manually counting the round or oval shape structures that are darkly stained. Note that the PL, but not IL cortex is more densely stained in experimental group compared to that of the control groups. mPFC: medial prefrontal cortex, PL: prelimbic division, IL: infralimbic division.

3.5.2. Insular Cortex

Insular cortex. Figure 16 depicts the mean c-Fos positive nuclei per 0.1 mm^2 in the IC. The c-Fos expression was higher in the IC cortex of the LiCl-treated animals in the conditioning context relative to the LiCl-treated animals in their home cages or NaCl-treated animals in the conditioning context. As revealed by one-way ANOVA, there was a significant difference among groups, F (2, 15) = 5.341, p = .018. Fisher's LSD test displayed that in the IC, LiCl–Context B (M = 55.89, SD = 14.13) group had significantly more c-Fos positive nuclei per 0.1 mm^2 than the LiCl–Context A (M = 38.26, SD = 9.501; p = .024) or NaCl–Context B (M = 34.25, SD = 12.48; p = .008)

group. No significant difference was revealed between the NaCl–Context B and LiCl–Context A groups (p = .577).

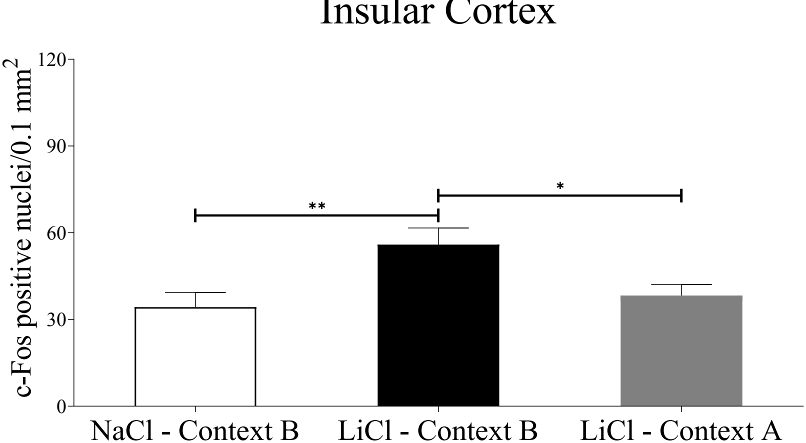

Figure 16. c-Fos expression in the IC. The graph shows c-Fos positive nuclei per 0.1 mm^2 in the IC. Statistical analyses revealed increased c-Fos expression in the IC of animals that were expected to develop CCA. All data depicted as mean ± SEM. *p < .05, **p < .01.

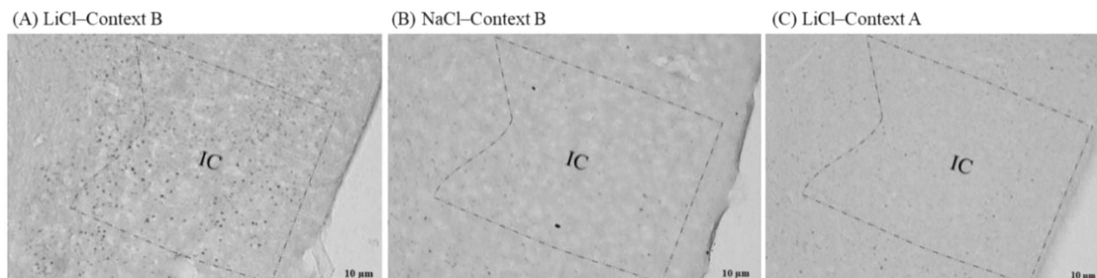

Figure 17. Representative photomicrographs of the IC. (A) is from the LiCl–Context B group; (B) is from the NaCl–Context B group; (C) is from the LiCl–Context A group. c-Fos expression is quantified by manually counting the round or oval shape structures that are darkly stained. Note that the IC is more densely stained in experimental group compared to that of the control groups. IC: insular cortex

3.5.3. Hippocampus

CA1-CA2. Figure 18(A) depicts the mean c-Fos positive nuclei per 0.1 mm^2 in the CA1-CA2 subregions of the hippocampus. The c-Fos expression in the CA1-CA2 subregions of LiCl-treated animals in the conditioning context was higher relative to the LiCl-treated animals in their home cages or NaCl-treated animals in the conditioning context. As revealed by one-way ANOVA, there was a significant difference among groups, F (2, 15) = 21.3, p < .001. Fisher's LSD test displayed that the LiCl–Context B (M = 82.33, SD = 25.7) group had significantly more c-Fos

positive nuclei per 0.1 mm^2 than LiCl–Context A (*M* = 36.67, *SD* = 2.582; $p < .001$) or NaCl–Context B (*M* = 19.33, *SD* = 15.1; $p < .001$) group. No significant difference was observed between the NaCl–Context B and LiCl–Context A groups ($p = .103$).

CA3. Figure 18(B) depicts the mean c-Fos positive nuclei per 0.1 mm^2 in the CA3 subregion of the hippocampus. The c-Fos expression in the CA3 subregion of the LiCl-treated animals in the conditioning context was higher relative to the LiCl-treated animals in their home cages or NaCl-treated animals in the conditioning context. As revealed by one-way ANOVA, there was a significant difference among groups, F (2, 15) = 4.644, $p = .02$. Fisher's LSD test displayed that LiCl–Context B (*M* = 59.5, *SD* = 22.33) group had significantly more c-Fos positive nuclei per 0.1 mm^2 than NaCl–Context B (*M* = 29.5, *SD* = 17.73; $p = .008$), but not than the LiCl–Context A (*M* = 41.5, *SD* = 8.408; $p = .089$) group. No significant difference was revealed between the NaCl–Context B and LiCl– Context A groups ($p = .245$).

DG. Figure 18(C) depicts the mean c-Fos positive nuclei per 0.1 mm^2 in the DG subregion of the hippocampus. The c-Fos expression in the DG subregion of the LiCl-treated animals in the conditioning context was higher relative to the LiCl-treated animals in their home cages or NaCl-treated animals in the conditioning context. As revealed by one-way ANOVA, there was a significant difference among groups, F (2, 15) = 5.46, $p = .01$. Fisher's LSD test displayed that LiCl–Context B (*M* = 57.67, *SD* = 10.67) group had significantly more c-Fos positive nuclei per 0.1 mm^2 than the LiCl–Context A (*M* = 34, *SD* = 6.325; $p = .006$) or NaCl–Context B (*M* = 40, *SD* = 18.58; $p = .031$) group. No significant difference was revealed between the NaCl–Context B and LiCl–Context A groups ($p = .433$).

Figure 18. c-Fos expression in the hippocampus: (A) c-Fos positive nuclei per 0.1 mm^2 in the CA1-CA2 subregions; (B) c-Fos positive nuclei per 0.1 mm^2 in the CA3 subregion; and (C) c-Fos positive nuclei per 0.1 mm^2 in the DG subregion. Statistical analyses revealed increased c-Fos expression in the CA1-CA2 and DG, but not CA3 subregion of the hippocampus in the

group that were expected to develop CCA. All data depicted as mean ± SEM. *$p < .05$, **$p < .01$, ***$p < .001$.

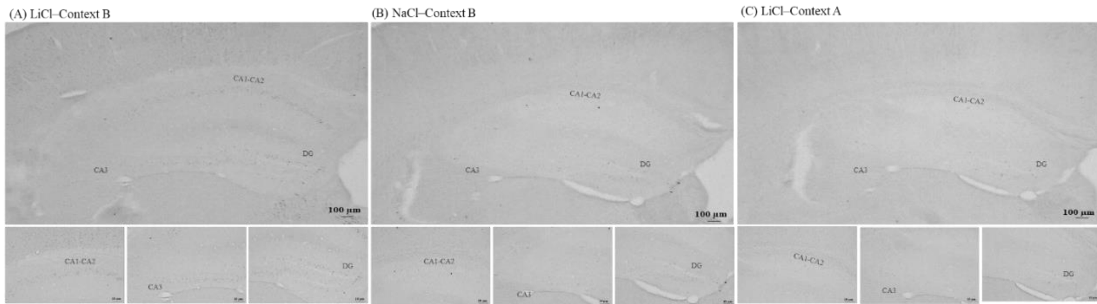

Figure 19. Representative photomicrographs of the hippocampus. (A) is from the LiCl–Context B group; (B) is from the NaCl–Context B group; (C) is from the LiCl–Context A group. Higher-magnification photomicrographs of the same sections are shown below. c-Fos expression is quantified by manually counting the round or oval shape structures that are darkly stained. Note that the CA1-CA2 and DG subregions are more densely stained the experimental group than that of the control group. DG: dentate gyrus

3.5.4. Amygdala

Basolateral Nucleus. Figure 20(A) depicts the mean c-Fos positive nuclei per 0.1 mm^2 in the basolateral nucleus of the amygdala. The c-Fos expression in the basolateral nucleus of those amygdala was higher in LiCl-treated animals in the conditioning context relative to the LiCl-treated animals in their home cages or NaCl-treated animals in the conditioning context. As revealed by one-way ANOVA, there was a significant difference among groups, F (2, 15) = 6.324, p = .010. Fisher's LSD test displayed that the LiCl–Context B (M = 80.07, SD = 38.65) group had significantly more c-Fos positive nuclei per 0.1 mm^2 than the LiCl–Context A (M = 38.91, SD = 14.55; p = .011) or NaCl–Context B (M = 34.26, SD = 10.22; p = .006) group. No significant difference was found between the NaCl–Context B and LiCl–Context A groups (p = .747).

Central Nucleus. Figure 20(B) depicts the mean c-Fos positive nuclei per 0.1 mm^2 in the central nucleus of the amygdala. The c-Fos expression in the central nucleus of the amygdala was similar among the three groups. No significant difference was revealed by one-way ANOVA among the three groups, F (2, 15) = 0.882, p = .434.

Medial Nucleus. Figure 20(C) depicts the mean c-Fos positive nuclei per 0.1 mm^2 in the medial nucleus of the amygdala. The c-Fos expression in the medial nucleus of the amygdala was similar among the three groups. No significant difference was revealed by one-way ANOVA among the three groups, F (2, 15) = 2.135, p = .153.

Cortical Nucleus. Figure 20(D) depicts the mean c-Fos positive nuclei per 0.1 mm^2 in the cortical nucleus of the amygdala are shown. The c-Fos expression in the cortical nucleus of the amygdala was similar among the three groups. No significant difference was revealed by one-way ANOVA among the three groups, F (2, 15) = 1.297, p = .302.

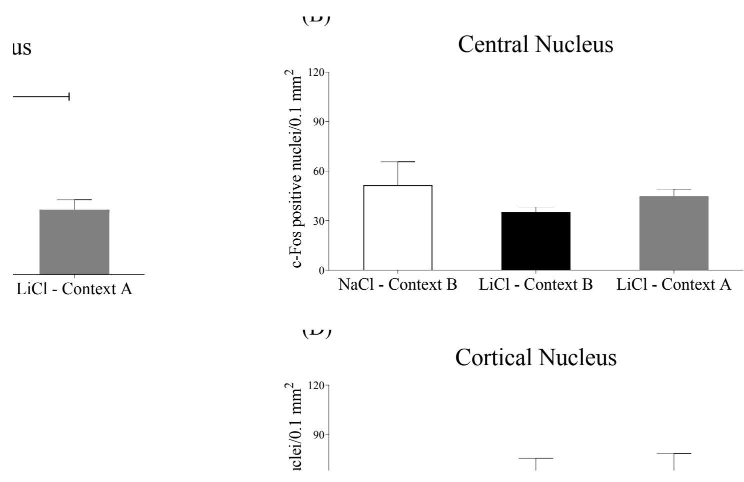

Figure 20. c-Fos expression in the amygdala (A) c-Fos positive nuclei per 0.1 mm^2 in the basolateral nucleus; (B) c-Fos positive nuclei per 0.1 mm^2 in the central nucleus; (C) c-Fos positive nuclei per 0.1 mm^2 in the medial nucleus; and (D) c-Fos positive nuclei per 0.1 mm^2 in the cortical nucleus. Statistical analyses revealed increased c-Fos expression in the basolateral nucleus of amygdala in the group that were expected to develop CCA. However, this was not the case for central, medial, and cortical nuclei. All data depicted as mean ± SEM. $^*p < .05$, $^{**}p < .01$.

3.6. Discussion

We aimed to find brain regions responsible for CCA learning using c-Fos expression as a marker for neural activation. To do so, we sacrificed 6 animals from three different groups 1 hour after the conditioning trial during which animals were injected with LiCl

or NaCl either in the conditioning context or in their home cages. c-Fos immunoreactivity was quantified in four brain regions, namely in mPFC, IC, amygdala, and hippocampus. We found that control animals did not develop CCA when they have received NaCl in Context B (NaCl–Context B) or LiCl in Context A (LiCl–Context A); therefore, we did not expect to see increased c-Fos expression in these groups as a result of CCA learning. However, when LiCl injections were given to the animals when they are introduced to Context B (LiCl–Context B), they developed CCA. Therefore, we expected to see increased c-Fos expression in brain regions responsible for CCA learning in this group. We found c-Fos positive nuclei per 0.1 mm^2 were higher in the CA1-CA2 and DG subregions of the dorsal hippocampus, basolateral nucleus of the amygdala, and PL divisions of the mPFC and IC in the LiCl–Context B group compared to the NaCl–Context B and LiCl–Context A groups indicating a role for these regions in CCA learning.

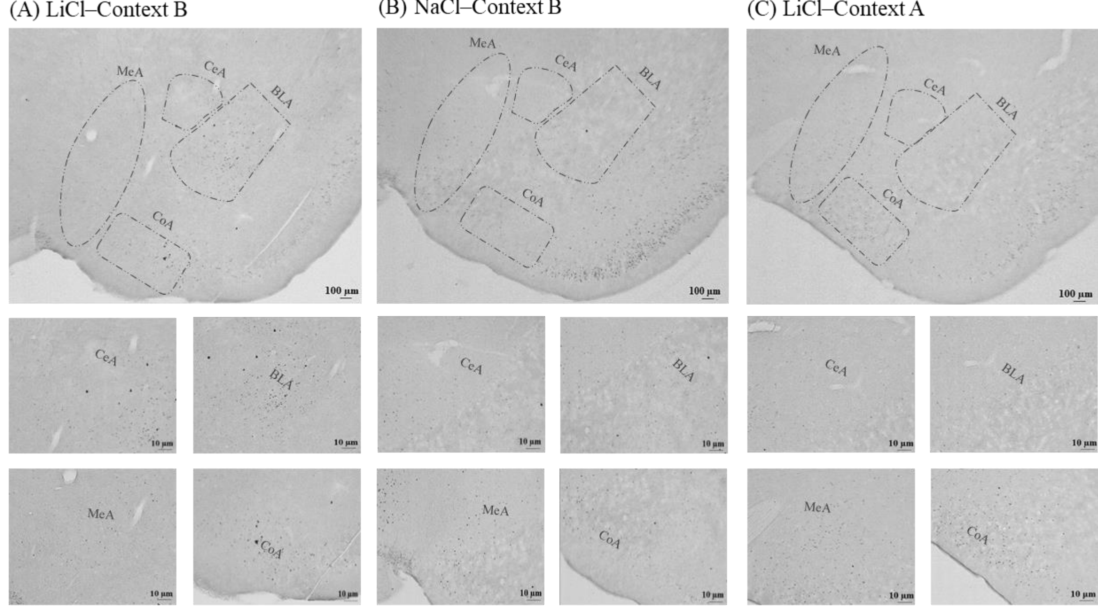

Figure 21. Representative photomicrographs of the amygdala. (A) is from the LiCl–Context B; (B) is from the NaCl–Context A; (C) is from the LiCl–Context A. Higher-power photomicrographs of the same sections are shown below. Higher-magnification photomicrographs of the same sections are shown below. c-Fos expression is quantified by manually counting the round or oval shape structures that are darkly stained. Note that BLA, but not the MeA, CoA, and CeA, is more densely stained in the experimental group compared to that of the controls. BLA: basolateral nucleus of amygdala, MeA: medial nucleus of amygdala, CoA: cortical nucleus of amygdala, CeA: central nucleus of amygdala.

3.7. General Discussion

The animals injected with LiCl in the conditioning context (LiCl–Context B) have developed strong CCA as evidenced by suppressed water consumption in retention test. However, animals who were only exposed to the CS (NaCl–Context B) or to the US (LiCl–Context A) did not show suppressed consumption indicating they have not developed CCA.

We also observed increased c-Fos expression in the CA1-CA2 and DG subregions of the hippocampus, basolateral nucleus of the amygdala, and PL division of the mPFC and IC one hour after the conditioning trial in the LiCl–Context B group, a group that has developed CCA. However, our control groups did not show increased c-Fos expression in these brain regions or develop CCA. This indicates that the induction of c-Fos expression in our experimental group is not solely in response to exposure to the CS or US since we did not observe same increased c-Fos pattern in these brain regions in animals that were only exposed to the CS or US. Rather, our findings indicate that CCA learning induced c-Fos expression in these brain regions. The widespread c-Fos expression implies that there is a brain circuitry responsible for CCA learning.

Researchers have shown the involvement of mPFC in various phases of memory formation using lesions, pharmacological treatments, microstimulation, single-unit recording, optogenetics, and chemogenetics. The mPFC has been found to be responsible for the acquisition of new memories, memory suppression, and extinction learning, especially in fear conditioning (Giustino and Maren, 2015). Neural activation in the mPFC was also investigated with c-Fos staining (Davis et al., 2003; Herry and Mons, 2004). The studies have found increased c-Fos expression in the PL and IL divisions of mPFC following fear learning (Morrow et al., 1999). c-Fos immunoreactivity was also found to be increased in the same region as a result of extinction learning, albeit at lower levels than new learning (Morrow et al., 1999). These results indicate that mPFC plays a role in the acquisition of new memories, and to a lesser degree, in extinction learning (Morrow et al., 1999). Although these studies found increased c-Fos expression in both subregions of the mPFC, in our experiment, we only found a significant increase in the PL, but not IL division.

Previous research established that the hippocampus, a complex brain structure mainly responsible for contextual learning, sends excitatory projections to the mPFC (Knapska and Maren, 2009). The neurons within the CA1 subregion of the hippocampus have shown to be express c-Fos protein after fear conditioning (Milanovic et al., 1998). The CA1-CA2, CA3 and DG subregions of hippocampus were also implicated to have a role in the acquisition of contextual memory, with various contributions at different temporal stages (Lee and Kesner, 2004). It also has been found that contextual fear memory is dependent on the activity of ventral CA1 hippocampal neurons that project to the amygdala (Kim and Cho, 2020). We have also observed increased c-Fos expression within the CA1-CA2 and DG subregions following conditioning, corroborating these earlier findings. Although it did not reach statistical significance, we also observed increased c-Fos expression in the CA3 subregion in our experimental group compared to the controls.

Synaptic plasticity in the basolateral amygdala has been found to be vital for learning US-CS associations (Sun et al., 2020). CTA memory acquisition is also correlated with amygdalar c-Fos expression (Lamprecht and Dudai, 1996). Increased c-Fos mRNA in the amygdala is observed after unconditioned and conditioned fear learning (Campeau et al., 1991). Parallel to previous research we also have found increased c-Fos expression in the basolateral nucleus of the amygdala following CCA learning.

Researchers have found that projections from IC to the lateral and central amygdala is vital for threat and fear learning (Berret et al., 2019). Activity in the IC, amygdala and mPFC is linked to CTA learning (Yiannakas and Rosenblum, 2017). IC neurons show increased c-Fos expression following CTA conditioning (Soto et al., 2017). Additionally, c-Fos expression is increased within the deep layers of the IC after novel taste learning. (Doron and Rosenblum, 2010). It also has been found that rats with bilateral electrolytic lesions of the IC show impaired CTA learning and IC-dependent c-Fos activity in other brain regions decreased in these animals after conditioning (Schafe and Bernstein, 1998). Our results provide further evidence for the involvement of IC in CCA learning.

3.8. Conclusion

We assessed the behavior of mice following illness-context pairing and c-Fos protein induced by CCA learning in brain regions that are implicated to have a role in contextual fear and CTA learning to elucidate the principles of CCA and its neurobiological underpinnings. To do so, we employed a CCA procedure in which illness induced by intraperitoneal LiCl injection is paired with the experience of novel context consisting of various exteroceptive cues. Our procedure induced robust CCA in animals as evidenced by suppressed consumption in the reinforced context. We also found elevated c-Fos expression in the PL division of the mPFC, IC, basolateral nucleus of the amygdala, CA1-CA2 and DG subregions of the hippocampus indicating that these brain regions are activated following CCA learning. To the best of our knowledge, ours is the first study elucidating the neural correlates of CCA learning. Further research is necessary to unravel causal involvement of these brain regions in the development of CCA.

CHAPTER 4

FINAL SUMMARY, OVERALL CONCLUSIONS AND LIMITATIONS

In a set of experiments, we investigated memory impairing effect of propranolol and MK-801 administration during the consolidation of CCA learning. CCA is a paradigm of classical conditioning that is used as a preclinical model of ANV and it is established by inducing an illness state after exposing animals to a novel context. As a result, animals show evidence of CCA such as suppressed consumption of water in the reinforced context. In our propranolol experiment, we allocated animals to three different groups. Our experimental group injected with LiCl during conditioning and with propranolol afterwards. Our two control groups injected with LiCl or NaCl during conditioning and with NaCl afterwards. We found that, when LiCl-treated mice are given propranolol following conditioning trial, their water consumption did not reduce during retention test as opposed to the mice that had been given NaCl. In fact, propranolol injected animals displayed similar water intake as the animals that had been injected with NaCl during conditioning. The findings of our experiment indicate that propranolol treatment prevented animals from developing CCA probably by impairing memory consolidation. However, our experiment was not designed specifically to elucidate the mechanism behind the observed effect of propranolol. Whether propranolol treatment prevents the consolidation of new information into long-term memory by inhibiting signaling cascades within the nervous system, or whether it causes a change in the emotional valence of the memory remains to be investigated. Another limitation of our study is the lack of any experiment investigating the drugs mechanism of action. Beta-adrenergic receptors are found mainly in the nervous system and in the periphery. We chose to use systemic injection because it is more relevant to translational research with current methods. However, this disallows us to decide whether the effect of propranolol was due to the antagonism

in the brain or in the body. Further research is necessary to elucidate the mechanism of action of propranolol in CCA learning.

We also conducted another experiment designed as our first experiment, but this time two groups of mice were given different doses of MK-801 injections instead of propranolol following conditioning. Two similar control groups were also used. We did not find any effect of MK-801 injection on the development of CCA as LiCl-treated animals continued to display suppressed water consumption in the reinforced-context.

We also found the brain regions that might be involved in CCA learning using c-Fos expression as a neural activation marker. We observed significantly increased c-Fos expression in the PL division of the mPFC, but not in the IL division, following conditioning only in animals that were expected to develop CCA. We also provided corroborating evidence for the research establishing vital role of IC in CTA learning by showing increased c-Fos expression in this region. Another brain region that was of interest to our c-Fos study was the hippocampus. We found neurons within the CA1-CA2 and DG subregions of the hippocampus, but not CA3 subregion, expressed c-Fos protein following CCA indicating that these subregions were also activated. Lastly, we quantified c-Fos expression in the four nuclei of the amygdala and found that only neurons in the basolateral nucleus but not neurons in the central, cortical, and medial nuclei expressed high levels of c-Fos protein. To our knowledge, our research is the first to elucidate brain regions involved in CCA learning. However, the c-Fos technique has its limitations. It only gives a snapshot of activated neurons in a specific region and does not allow to investigate the interconnection between neurons. Therefore, causal involvement and the role of interconnection of these brain regions in CCA learning should be investigated.

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APPENDICES

A. APPROVAL OF THE METU HUMAN SUBJECTS ETHICS COMMITTEE

ORTA DOĞU TEKNİK ÜNİVERSİTESİ
HAYVAN DENEYLERİ ETİK KURUL KARARLARI

KARAR SAYISI : 01	**KARAR TARİHİ: 01.02.2021**
ODTÜ Psikoloji Bölümü Dr. Öğretim Üyesi Sezen Kışlal'ın Etik-2021/01 no'lu "Bellek Konsolidasyon Sürecine Müdahale İle Beklentisel Bulantının Önlenmesi" başlıklı araştırma projesinin etik başvurusu değerlendirilmiştir. Etik Kurul'un sayı ve kullanım amaçlarının ve projenin deney hayvanlarına ilişkin yönlerinin O.D.T.Ü. Hayvan Deneyleri Yerel Etik Kurul Yönergesinde belirtilen "Hayvan Deneyleri ile İlgili Etik İlkeler" dikkate alınarak hazırlandığı saptanmıştır. Bu değerlendirme sonucu çalışmanın yapılmasının hayvan etiği açısından uygun olduğu oy çokluğu/oy birliği ile onaylanmıştır.	
Doç. Dr. Tülin Yanık Başkan	Doç. Dr. Sreeparna Banerjee Başkan Yardımcısı
Prof. Dr. Orhan Adalı Üye	Prof. Dr. Gülay Özcengiz Üye
Prof. Dr. Ayşen Tezcaner Üye	Prof. Dr. Mayda Gürsel Üye (invivo çalışan)
Prof. Dr. C. Can Bilgin Üye	Doç. Dr. Barış Parkan Üye
Süleyman Ekşioğlu STK Üye	Şakir Yıldıran Vet. Hekim, Üye
Nilüfer Golar Sivil Üye	

09.02.2021

ODTÜ Hayvan Deneyleri Yerel Etik Kuruluna

ANKARA

Ankara dışında olmam sebebiyle imzalayamadığım belgeleri benim adıma imzalaması için Hayvan Deneyleri Yerel Etik Kurulu üyelerimizden Prof.Dr. C. Can Bilgin'i yetkilendirdiğimi bildirmek isterim. Saygılarımla

Süleyman Elezioğlu

O.D.T.Ü

HAYVAN DENEYLERİ

YEREL ETİK KURULU

BAŞKANLIĞI'NA

11/02/2021

Üyesi bulunduğum Etik Kurul'un karar defterlerini imzalamaya Prof Dr Sreeparna BANERJEE yetkilidir.

Saygılarımla,

Nilüfer GÜLAR (Tc. 22982102466)

B. CURRICULUM VITAE

Personal Information

Date of Birth: 02/04/1993
Address: Ankara/Turkey
Mobile Phone: +905442795482
E-mail: cnarilhan@gmail.com

Professional Summary

A master's student majored in psychology with experience in preclinical studies. Sensitive to the animals' health and well-being, well trained in behavioral tests (rotarod performance test, horizontal ladder rung walking test, water maze, etc.) and highly motivated and diligent in performing his duties and responsibilities. Have extensive knowledge of immunohistochemistry, immunofluorescence, and histological staining techniques; skilled in stereotaxic neurosurgery in rodents, fully capable of performing brain, spinal cord, and muscle dissection. Also proficient in using ImageJ, Stereo Investigator, SPSS, GraphPad, MATLAB and R Studio software for stereological, morphometric, and statistical analyses.

Skills

- Animal Handling
- Behavioral Tests
- Immunohistochemistry
- Perfusion
- Euthanasia Techniques
- Stereotaxic Surgery
- Histology
- Sectioning
- Stereological Methods

Education

Master's Degree: Psychology – Current GPA: 3.68
Graduate School of Social Science
Middle East Technical University, Ankara/Turkey
Expected graduation: February, 2023

Master's Degree: Interdisciplinary Neuroscience – GPA: 3.76
Graduate School of Health Science
Eskisehir Osmangazi University, Eskisehir/TURKEY
Incomplete

Bachelor's Degree: Psychology – GPA: 2.74
Faculty of Sciences and Letters
Istanbul University, Istanbul/ TURKEY

Graduation: July, 2017

Research Experience

METU, Department of Psychology, Ankara, Turkey
Advisor: Dr. Sezen Kışlal Kislal

I was part of a TUBITAK 2232 project called "Impairing memory consolidation to prevent the development of anticipatory nausea." I helped to investigate the effect of the systemic and intracerebral administration of propranolol and MK-801 on the animal model of anticipatory nausea, a debilitating phenomenon that cancer patients experience. I found the opportunity to practice my immunohistochemistry, stereotaxic surgery, and stereology skills.

During this time, with the help of my advisor Dr. Sezen Kislal, we wrote a grant proposal which received funding from TUBITAK. In this project, called "Impairing Effect of Behavioral and Pharmacological Interventions on Fear Memory After CS or US Reminder", the behavioral and pharmacological interventions on fear memory will be investigated.

ESOGU, Department of Interdisciplinary Neuroscience, Eskisehir, Turkey
Advisor: Prof. Dr. Emel Ulupinar

I actively worked in a TUBITAK 1001 project "Developing a new animal model causing proteinopatic damage in cortical motor neurons" conducted by Professor Dr. Emel Ulupinar, focusing on creating a novel animal model for the most common motor neuron disease, amyotrophic lateral sclerosis using viral vectors. As a part of the project, I worked in the laboratory and acquired significant experience on a myriad of behavioral tests such as rotarod, modified grip test, horizontal ladder rung walking test, water maze, etc.; intracardiac perfusion; brain, spinal cord, and muscle dissection; stereotaxic operations; frozen and paraffin-embedded tissue sectioning; immunohistochemistry, immunofluorescence, and histological staining techniques such as methylene blue, cresyl violet, and hematoxylin-eosin. I also performed stereological, morphometric, and statistical analyses.

Publications

Ilhan C.F., Kislal S., "Memory Impairing Effect of Propranolol on Consolidation and Reconsolidation for Various Learning Tasks", Archives of Neuropsychiatry
Date of Acceptance: 06.07.2022 - DOI: 10.29399/npa.28203

Presentations in Scientific Meetings

1. **Çınar F. Ilhan**, Esra Ülke, Gonzalo Urcelay, Sezen Kışlal, "c-Fos Expression in the Hippocampus Following Conditioned Context Aversion

Learning in Mice" *20. National Neuroscience Conference, NST, 19-23 December 2022*

2. **Çınar F. Ilhan**, Esra Ülke, Gonzalo Urcelay, Sezen Kışlal, "The β-adrenergic Receptor Antagonist Propranolol Attenuates the Establishment of Conditioned Context Aversion in Laboratory Mice", *FENS Forum, 9-13 July 2022*

3. **Çınar F. Ilhan**, Esra Ülke, Ayşe Seza Şahin, Gonzalo Urcelay, Sezen Kışlal, "Conditioned Context Aversion Learning in Inbred Mice", *4th East Mediterranean Congress of Laboratory Animal Science, 9-11 December 2021*

4. **Çınar F. Ilhan**, I. Buyukguduk, E. Polat Corumlu, H. A. Kapkac, M. Arslanyolu, E. Ulupinar, "Testing Fine Motor Skills of Rats Following Alterations in The Expression of TARDBP Gene Using Viral Vectors", *FENS Virtual Forum, 11-15 July 2020*

5. **Çınar F. Ilhan**, I. Buyukguduk, E. Polat Corumlu, H. A. Kapkac, M. Arslanyolu, E. Ulupinar, "Corticospinal Motor Neuron Specific Transduction of the TARDBP Gene via Viral Vectors", *18. National Neuroscience Conference, NST, 6-9 November 2020*

6. **Çınar F. Ilhan**, I. Buyukguduk, E. Polat Corumlu, H. A. Kapkac, M. Arslanyolu, E. Ulupinar, "Neuroinflammatory changes in a novel motor neuron disease model created by viral vector-mediated gene transfer method in rats", *21. National Anatomy Conference, Turkish Society of Anatomy and Clinical Anatomy, 28-29 November 2020*

7. Esra Ülke, **Çınar F. Ilhan**, Sezen Kışlal, "Conditioned context aversion in laboratory animals" *20. National Psychology Conference, NST, 13-16 December 2022*

8. Ayşe Seza Şahin, **Çınar F. Ilhan**, Gonzalo Urcelay, Sezen Kışlal, "Effects of stimulus pre-exposure on conditioned context aversion in CD1 outbred and C5 B L/ J inbred mice" *19. National Neuroscience Conference, NST, 21-23 November 2021*

9. I. Buyukguduk, E. Polat Corumlu, **Çınar F. Ilhan**, H. A. Kapkac, M. Arslanyolu, E. Ulupinar, "Effects Of The Nuclear Localization Signal Deletion In TDP-43 Protein On The Lower Motor Neurons Of Rats", *FENS Virtual Forum, 11-15 July, 2020*

10. I. Buyukguduk, E. Polat Corumlu, **Çınar F. Ilhan**, H. A. Kapkac, M. Arslanyolu, E. Ulupinar, "Lower Motor Neuron Deficits Caused by Systemic Delivery of Adeno- Assosiated Viral Vectors in Rats", *18. National Neuroscience Conference, NST, 6- 9 November 2020*

11. E. Polat Corumlu, I. Buyukguduk, Çınar F. Ilhan, H. A. Kapkac, M. Arslanyolu, E. Ulupinar, "Dose-Dependent Impairment in Motor Functions After Systemic Delivery of Transactive Response DNA Binding Protein-43 (TDP-43) Using AAV9 in Rats", *FENS Virtual Forum, 11-15 July 2020*

12. E. Polat Corumlu, I. Buyukguduk, **Çınar F. Ilhan**, H. A. Kapkac, M. Arslanyolu, E. Ulupinar, "Alterations in Motor Functioning After Systemic Delivery Of Native And Mutant Transactive Response DNA-Binding Protein-43 (TDP-43) Using AAV9 in Rats" *18. National Neuroscience Conference, NST, 6-9 November 2020*

13. E. Polat Corumlu, I. Buyukguduk, **Çınar F. Ilhan**, H. A. Kapkac, M. Arslanyolu, E. Ulupinar, "Characterization of changes in the motor innervation of somatic muscles in a novel animal model created by viral mediated gene transfer", *21. National Anatomy Conference, Turkish Society of Anatomy and Clinical Anatomy, 28-29 November, 2020*

14. E. Ulupinar, I. Buyukguduk, E. Polat Corumlu, **Çınar F. Ilhan**, H. A. Kapkac, M. Arslanyolu, "Selective Transduction Of The Rat Corticospinal Motor Neurons Via TDP-43 Expressing Viral Vectors", *FENS Virtual Forum, 11-15 July, 2020*

Programs & Softwares

- MS Office Package
- IBM SPSS
- GraphPad Prism
- MATLAB
- ImageJ
- Jamovi
- R Studio
- Neurostar Drill and Injection Robot
- MBF Neurolucida Software
- MBF Stereo Investigator Software

Memberships

Neuroscience Society of Turkey (NST)

Federation of European Neuroscience Societies (FENS)

Certificates

Certificate of Use of Experimental Animals – Medical and Surgical Experimental Animals Application and Research Center, Eskisehir Osmangazi University/Eskisehir, November 2019

C. TURKISH SUMMARY / TÜRKÇE ÖZET

BÖLÜM 1

GİRİŞ

Bu bölümde bulantı ve kusma (BK), kemoterapiye bağlı bulantı ve kusma (KBBK), klasik koşullamayı, beklentisel bulantı ve kusma (BBK) ve koşullu çevresel itinme (KÇİ) gibi kavramlar ele alınacaktır.

1.1. Bulantı ve Kusma

İnsanlar ve diğer memeliler, toksik maddelerin sindiriminden kaçınmak için bulantı ve kusma gibi koruyucu bir savunma mekanizması geliştirmiştir (Zhong vd., 2021). Kusmadan önceki hissedilen duyum mide bulantısı olarak adlandırılırken, kusma mide-bağırsak içeriğinin ağız yoluyla dışarı atılmasıdır (Gelberg, 2018). BK hastalık olarak kabul edilmemekte, birçok farklı hastalıkta ortaya çıkan bir belirti olarak görülmektedir (Chepyala ve Olden, 2008). Toksinler, bakteriler, mantarlar, virüsler ve bazı ilaçlar BK oluşumunu tetikleyebilir (Zhong vd., 2021). Gastrointestinal (GI) sistem patolojileri, güçlü duygusal ve bilişsel deneyimler, taşıt tutması BK oluşumuna neden olabilecek diğer durumlardır (Cai vd., 2007; Cohen vd., 2019; Gagliuso vd., 2019).

1.2. Kemoterapiye Bağlı Bulantı ve Kusma

KBBK, kanser tedavisinin en yaygın olarak gözlenen iatrojenik bir sonucudur (Rao ve Faso, 2012). KBBK, hastaların kemoterapi tedavisi sırasında yaşadıkları BK deneyimlerinin başlangıcına göre akut, gecikmiş veya beklentisel olarak sınıflandırılır (Durand vd., 2009). Tedaviyi takip eden 24 saat içinde başlayan BK akut olarak adlandırılır (Roila vd., 1991). Akut BK bu 24 saatlik sürenin ardından devam ederse gecikmiş olarak kabul edilmektedir (Roscoe vd., 2004). BBK, akut ve gecikmiş

formlardan biraz farklıdır, çünkü BK oluşumunu indükleyen patofizyolojik mekanizmalardan kaynaklanmaz. BBK gelişiminin klasik koşullanma öğrenmesinden kaynaklandığı kabul edilmiştir (Roscoe vd., 2004).

1.3. Klasik Koşullama

Klasik koşullanma, nötr bir uyaranın, organizma için doğuştan veya kazanılmış psikolojik/fizyolojik değeri olan başka bir uyaranla eşleştirilmesine dayanan ilişkisel bir öğrenme türüdür. Nötr bir uyaran, koşullama sırasında koşulsuz bir uyaranla (US) ilişkilendirilir. US, organizma otonomik bir tepki oluşturan uyaranlardır. US sunumunun ortaya çıkardığı yanıta koşulsuz tepki (UR) denir. Nötr uyaran, UR benzeri bir yanıt ortaya çıkarmaya başladığında koşullu uyaran (CS) haline gelir ve bu tepki koşullu tepki (CR) olarak adlandırılır.

1.4. Beklentisel Bulantı ve Kusma

BBK oluşumunun klasik koşullamaya bağlı olarak ortaya çıktığı edilmektedir (Stockhorst vd., 1993). Emetojenik kemoterapi tedavisi, önceki bölümlerde açıklandığı gibi hastalarda BK oluşumunu indükler. Klasik koşullama terminolojisinde kemoterapi tedavisi US, BK ise UR olarak kabul edilir (Schnell, 2003). Kemoterapi tedavisinin alındığı çevresel uyaranlardan bir veya birkaçı hastalık hali ile ilişkilendirildiğinde CS haline gelir (Roscoe vd., 2011).

1.5 Koşullu Çevresel İtinme

KÇİ, hayvanlarda, BBK olgusunun preklinik modeli olarak kullanılmıştır (Cloutier vd., 2017, 2018; Limebeer ve Parker, 2000). KÇİ, koşullama sırasında çeşitli uyaranlardan oluşan bir çevrenin hastalık hali ile eşleştirilmesine dayanır. Bazı KÇİ çalışmalarında radyasyon kullanılsa da US olarak genellikle farmakolojik ajanlar kullanılmaktadır. Lityum klorür (LiCl), bu çalışmalarda en yaygın kullanılan ajanlardan biridir.

BÖLÜM 2

KONSOLİDASYON SIRASINDA UYGULANAN AMNESTİK AJANLARIN, LABORATUVAR FARELERİNDE KOŞULLU ÇEVRESEL İTİNME OLUŞUMUNA ETKİSİ

Bu bölüm, amnestik ajanların uygulanmasıyla KÇİ öğrenmesinin oluşumu üzerine etkisini değerlendirmek için yapılan deneyleri içermektedir.

2.1. Giriş

Konsolidasyon, yeni sinapsların oluşumu ve öncekilerin güçlenmesi veya zayıflamasına neden olan, protein sentezi ve zamana bağlı sinaptik/hücresel süreçlere denir (Nader vd., 2000).

Konsolidasyon öncesinde, öğrenilen bilgiler kararsız durumdadır ve bellek oluşumu engellenebilir (Alberini, 2005). Konsolidasyon teorisi, bellek edinimi sırasında elektrokonvülsif şok uygulamalarının veya hipokampal lezyonların sıçanlarda hafızayı geriye dönük olarak bozduğunun gözlenmesi ile desteklenmiştir (Duncan, 1949; Russell ve Nathan, 194) . Yeni öğrenilen bilgilerin belleğe konsolidasyonu sırasında *de-novo* protein sentezi gereklidir. Bu durum protein sentezi inhibitörü olan puromisin verilen hayvanların kaçınma öğrenmelerinde bozulma gözlenmesi ile açıkça ortaya konmuştur (Flexner vd., 19 2) . Öğrenme ile ilgili bir reseptörün aktivasyonunu bloke eden bazı antagonist ajanlar da bellek oluşumunu bozabilir. N-metil-D-aspartat (NMDA) ve beta-adrenerjik reseptörlerin öğrenme ve bellek süreçlerinde öbemli rolleri olduğu bilinmektedir (Shimizu vd., 2000; O'Dell vd., 2015).

NMDA ve beta-adrenerjik reseptörlerin nöronal plastisite, öğrenme ve hafızadaki rolünü gösteren araştırmalara dayanarak, çalışmamızda bu reseptörlerin konsolidasyon sürecindeki rolleri araştırılmıştır. KÇİ öğrenmesinin NMDA reseptör antagonisti MK-801 ve beta-adrenerjik reseptör antagonisti propranolol enjeksiyonu yoluyla konsolidasyon sürecine bozucu etkisi olup olmayacağı incelenmesi amacıyla 2 deney yürütülmüştür.

2.2. Deney 1: Sistemik MK-801 uygulamasının CCA belleğinin konsolidasyonu üzerindeki etkisi

Bu deney sistemik olarak enjekte edilen MK-801'in CCA öğrenmesini engelleyip engellemeyeceğinin araştırılması için yapılmıştır.

2.2.1. Yöntem

2.2.1.1 Denekler

Bu deneyde 19 ila 25 gram arasında değişen kırk sekiz CD1 erkek fare kullanıldı. Hayvanlar başlangıçtaki vücut ağırlıklarına göre iki deney ve iki kontrol grubuna ayrıldı. Kontrol grupları LiCl–NaCl (n = 12) ve NaCl–NaCl (n = 12); deney grupları ise LiCl–MK801 Yüksek Doz (n = 12) ve LiCl–MK801 Düşük Doz (n=12) olarak adlandırıldı.
20 dakikalık koşullama sırasında farelere iki kere intraperitoneal enjeksiyon yapıldı. Koşullamanın başlamasından 5 dakika sonra hayvanlara ya hastalık indüklemek için LiCl, ya da kontrol uygulama olarak NaCl enjeksiyonları yapıldı. LiCl–NaCl, LiCl–MK801 Yüksek Doz ve LiCl–MK801 Düşük Doz gruplarının ilk enjeksiyonları LiCl iken, NaCl–NaCl grubuna NaCl enjeksiyonu yapıldı. Koşullamadan hemen sonra, hayvanlara konsolidasyon sürecini bozmak için ya MK-801 ya da kontrol uygulama olarak NaCl enjeksiyonları yapıldı. LiCl–MK801 Yüksek Doz ve LiCl–MK801 Düşük Doz gruplarına MK-801 enjeksiyonları yapılırken; LiCl–NaCl ve NaCl–NaCl gruplarına NaCl enjeksiyonları yapıldı.

2.2.1.2. İlaç Uygulaması

LiCl mEq/kg dozunda uygulandı. MK-801 düşük (0.05 mg/kg) veya yüksek dozda (0.2 mg/kg) uygulandı. NaCl enjeksiyonları %0.9 konsantrasyonda kullanıldı. İlk LiCl ve NaCl enjeksiyonlarının hacmi hayvanların vücut ağırlıklarına göre 0.24 mL/kg olacak şekilde ayarlandı. MK-801 ve ikinci NaCl enjeksiyonları yine vücut ağırlığına göre 0.2 mL/kg hacimde uygulandı. MK-801, %0.9 NaCl içinde çözüldü.

2.2.1.3. Aparatlar

Tüm deneylerde iki farklı bağlam kullanılmıştır. Bağlam A, koloni odasındaki normal barınma koşullarını ifade etmektedir. Koloni odasında, doğal aydınlatma olmaksızın 12/12 saat aydınlık/karanlık döngüsü uygulanmıştır. Hayvanlar, deneyler boyunca Avrupa Standard Tip II kafeslerde kalmıştır. Yataklık olarak talaş kullanılmış ve standart plastik şişelerle su verilmiştir. Bağlam B için koloni odasından uzakta bulunan ayrı bir oda kullanılmıştır. Bu oda, 0 wattlık tavan lambasının ürettiği kırmızı ışık, limon yağı kokusu ve 5 desibellik beyaz gürültü olmak üzere çeşitli uyaranlarla donatılmıştır. Ayrıca koşullama kafesleri vinil siyah be beyaz bantlarla vertikal olarak kaplanmıştır. Bu kafeslerde altlık olarak kedi kumu kullanılmıştır. Su şişeleri için bilyalı ağızları olan yeşil cam şişeler kullanılmıştır.

2.2.1.4. Prosedür

Deneysel prosedür beş aşamayı içermektedir: alıştırma, su eğitimi, koşullama, iyileşme ve bellek testi.

Alışma. Fareleri ele alınmaya alıştırmak için her biri 4 gün boyunca günde 3 dakika tutuldu. Hayvanlara, enjeksiyonun neden olduğu stresi azaltmak için alışmanın son gününde NaCl enjeksiyonu yapıldı. Son gün saat 1 .30'da su kısı tlaması başlatıldı.

Su Eğitimi. Alışmayı takip eden üç gün su eğitimini oluşturmaktadır. Bu günlerde farelere yalnızca 10:00–10:30 ve 17:00–1 :30 saatleri arasında su verildi. Son gün, neofobik tepkileri önlemek için hayvanlar 5 dakika süreyle koşullama bağlamına (Bağlam B) maruz bırakıldı.

Koşullama. Koşullama bağlamına (Bağlam B) konulan farelere 5 dakika sonrasında LiCl veya NaCl enjeksiyonu yapıldı ve fareler 15 dakika daha koşullama bağlamında kaldı. Koşullamadan hemen sonra, hayvanlara amnestik ilaç veya NaCl enjekte edildi. Her farenin su şişesi, su tüketimini ölçmek için koşullamadan önce ve sonra tartıldı.

İyileşme. Koşullamanın sonrasındaki iki gün iyileşme dönemiydi. Bu süre zarfında hayvanlara sadece 10:00–10:30 ve 17:00–1 :30 s aatleri arasında su verildi.

Bellek Testi. 2 günlük iyileşme süresinin ardından hayvanlar tekrar koşullama bağlamına (Bağlam B) 15 dakika süresince tekrar koyuldu. Bellek testi sırasında hayvanların su tüketimi ölçüldü.

2.2.1.5. Data Analizi

Koşullandırma ve bellek testi sırasında hayvanların su alımını analiz etmek için tek yönlü ANOVA kullanıldı. Post-hoc karşılaştırmalar Fisher'in LSD testi kullanılarak yapıldı. Farklılıkların anlamlı kabul edilmesi için $p < .05$ olarak ayarlandı.

2.2.2. Sonuçlar

2.2.2.1. Koşullama Sonucu

Koşullama sırasında, LiCl enjeksiyonu yapılan hayvanların su tüketimi, NaCl enjeksiyonu yapılan hayvanlara göre daha düşüktü ($p = .012$).

2.2.2.2. Bellek Testi Sonucu

Bellek testinde, LiCl enjeksiyonu yapılan hayvanların su tüketimi, NaCl enjeksiyonu yapılan hayvanlara göre daha düşüktü ($p <.001$). Fisher LSD analizi, NaCl–NaCl grubunun su tüketimi, LiCl–NaCl ($p <.001$), LiCl–MK801 Yüksek Doz ($p <.001$) ve LiCl–MK801 Düşük Doz ($p <.001$) gruplarınkinden fazla olduğunu gösterdi. Diğer gruplar arasında istatiksel olarak anlamlı bir fark bulunmadı.

2.2.3. Tartışma

Sonuçlarımız koşullama sonrasında MK-801 CCA belleği üzerinde etkisinin olmadığını göstermektedir.

2.3. Deney 2: Sistemik propranolol uygulamasının CCA belleğinin konsolidasyonu üzerindeki etkisi

İkinci deneyimizde, beta-adrenerjik reseptör aktivasyonunun propranolol ile bloke edilmesinin hayvanların KÇİ belleği üzerindeki etkisi araştırılmıştır. Bu amaçla koşullamanın hemen ardından farelere 10 mg/kg'lık dozda propranolol enjeksiyonları yapıldı.

2.3.1. Yöntem

2.3.1.1. Denekler

İkinci deneyde ağırlıkları 19 ila 25 gram arasında değişen otuz dört CD1 erkek fare kullanıldı. Deney başlangıcında hayvanlar vücut ağırlıklarına göre iki kontrol ve bir deney grubuna ayrıldı. Kontrol grupları LiCl–NaCl (n = 12) ve NaCl–NaCl (n = 11); deney grubu LiCl–Propranolol (n = 11) olarak adlandırıldı. Koşullama başlangıcından 5 dakika sonra LiCl–NaCl ve LiCl–Propranolol gruplarına LiCl enjeksiyonu yapılırken, NaCl–NaCl grubuna NaCl enjeksiyonu yapıldı. Koşullamadan hemen sonra, LiCl–Propranolol grubuna propranolol; LiCl–NaCl ve NaCl–NaCl gruplarına NaCl enjeksiyonları yapıldı.

2.3.1.2. İlaç Uygulaması

LiCl 6 mEq/kg, propranolol 10 mg/kg dozunda uygulandı. Tüm ilaçlar vücut ağırlığına göre 0.24 mL/kg olacak şekilde ayarlandı.

2.3.1.3. Aparatlar

Birinci deney ile aynı aparatlar kullanıldı.

2.3.1.4. Prosedür

Birinci deney ile aynı KÇİ protokolü ve deney tasarımı kullanıldı.

2.3.1.5 Data Analizi

Data analizi birinci deney ile aynıydı.

2.3.2. Sonuçlar

2.3.2.1. Koşullama Sonuçları

Grupların su tüketimi arasında fark gözlenmedi (p = .949).

2.3.2.2. Bellek Testi Sonuçları

Tek yönlü ANOVA, gruplar arasında su tüketiminde fark olduğunu gösterdi (p = .021). Fisher LSD analizi, LiCl–NaCl grubundaki hayvanların, NaCl–NaCl (p = .017) ve LiCl–Propranolol (p = .011) grubundaki hayvanlara göre daha az su tükettiğini ortaya koydu. Ancak, NaCl–NaCl ve LiCl–Propranolol grupları arasında su tüketiminde anlamlı bir fark yoktu (p = .8).

2.3.3. Tartışma

Koşullama sırasında LiCl enjekte edilen hayvanlara propranolol verildiğinde, NaCl verilen hayvanlara göre daha yüksek su tüketimi sergiledikleri bulunmuştur. Propranolol enjekte edilen grubun su tüketiminin, koşullama sırasında hastalık oluşturulmamış grubunkine bezer olduğu gözlenmiştir. Bulgularımız propranolol ile beta-adrenerjik reseptör aktivasyonunu bloke etmenin hayvanların KÇİ öğrenmesini bozduğunu göstermektedir.

2.4. Genel Tartışma

KÇİ öğreniminin, konsolidasyon sürecinde uygulanan NMDA ve beta-adrenerjik reseptör antagonistleri tarafından bozulup bozulmadığını belirlemek amacıyla iki deney yürütülmüştür. İlk deneyde NMDA reseptör antagonisti MK-801 kullanılmıştır. Bu deneyde sistemik MK-801 uygulamasının KÇİ belleğinin konsolidasyonunu bozmadığını bulunmuştur.

İkinci deney, KÇİ belleğinin konsolidasyonu için beta-adrenerjik reseptör sinyalinin gerekli olup olmadığını araştırmak amacıyla yürütülmüştür. Hayvanlara koşullama ardından propranolol enjekte edildiğinde KÇİ öğrenmesinin bozulduğuna işaret eden sonuçlar gözlenmiştir.

2.5. Sonuç

KÇİ öğrenmesinde NMDA reseptör antagonisti MK-801 herhangi bir bozucu etki göstermezken, propranolol konsolidasyon sürecini bozarak hayvanların öğrenmesini engellemiştir.

BÖLÜM 3

KOŞULLU ÇEVRE İTİNMESİNİ TAKİBEN BEYİNDE C-FOS EKSPRESYONU

Bu bölüm, beyindeki c-Fos ekspresyonunu araştırarak KÇİ öğrenmesinde görevli beyin bölgelerini bulmak için yürütülen deneyi kapsamaktadır.

3.1. Giriş

Çalışmalar temel olarak KÇİ öğrenmenin ilkelerini ortaya koysa da, bu tür öğrenmede görevli beyin bölgeleri henüz araştırılmamıştır (Best vd., 1973; Hall vd., 1997; Parker vd., 1984; Symonds vd., 1998). Bu deney, KÇİ öğreniminde görevli beyin bölgelerini belirlemek için yürütülmüştür. Bu amaçla beyinde nöronal aktivasyonun dolaylı olarak ölçmek için c-Fos ekspresyonu incelenmiştir.
Önceki araştırmaya dayanarak dört beyin bölgesinde c-Fos ekspresyonu dört beyin bölgesinde incelenmiştir. Bu bölgeler şunlardır: medyal prefrontal korteksin prelimbik ve infralimbik bölümleri, insular korteks, amigdalanın medyal, kortikal, bazolateral ve santral çekirdekleri ile hipokampusun CA1-CA2, CA3 ve dentat girus bölümleri.

3.2. Yöntem

3.2.1. Denekler

Her biri 19 ila 25 gram arasında değişen 12 haftalık 54 erkek CD1 fare kullanıldı. Fareler iki kontrol ve bir deney grubuna ayırıldı: NaCl–Bağlam B (n = 18), LiCl–Bağlam A (n = 18) ve LiCl–Bağlam B (n = 18). Koşullama sırasında, LiCl–Bağlam A grubundaki hayvanlara koloni odasındaki kafeslerinde LiCl enjeksiyonu yapıldı.

NaCl–Bağlam B ve LiCl–Bağlam B gruplarındaki hayvanlara koşullama bağlamında sırasıyla NaCl ve LiCl enjeksiyonları yapıldı. Her bir gruptan altı hayvan, c-Fos proteininin ekspresyonunu araştırmak için koşullamadan tam olarak 1 saat sonra perfüze edildi. Her grupta kalan 12 hayvan, koşullamanın başarılı olup olmadığını araştırmak için koşullamadan 72 saat sonra 15 dakikalık bir bellek testinde kullanıldı.

3.2.2. Davranışsal Prosedür

Davranışsal prosedür koşullama aşaması dışında birinci deney ile aynıydı. Koşullama sırasında Bağlam B grubundaki fareler koşullama kafesine koyularak koşullama odasına taşındı. Bağlam A grubundaki fareler koloni odasındaki kafeslerinde kalmaya devam etti. Koşullamanın başlamasından 5 dakika hayvanlar kafeslerinden alınarak LiCl veya NaCl (i.p.) enjeksiyonları yapıldı ve 15 dakika daha geçirmek üzere kafeslerine tekrar koyuldu. Her gruptan altı hayvan, koşullamanın tamamlanmasından tam olarak 1 saat sonra perfüze edildi ve beyinleri çıkarıldı.

3.2.3. İmmünohistokimya

Her bir grupta bulunan hayvanın beyinlerinde 40 mikron kalınlığında kesitler alınarak c-Fos proteini için immünohistokimyasal boyamalar uygulandı.

3.2.4. c-Fos analizi

c-Fos-pozitif hücreler ImageJ yazılımı kullanılarak manuel olarak sayıldı. Her beyin bölgesi için c-Fos pozitif hücre sayısı o beyin bölgesinde 0.1 mm^2 alana oranı hesaplandı ve analiz edildi.

3.2.5. Data Analizi

Koşullama ve bellek testi sırasında grupların su tüketim miktarları ve c-Fos-pozitif hücre çekirdekleri, GraphPad Prism programı kullanılarak istatistiksel olarak analiz edildi. Gruplar arasındaki istatistiksel farkı araştırmak için tek yönlü ANOVA testi

yapıldı ve ikili karşılaştırmalar için Fisher LSD testi kullanıldı. İstatistiksel anlamlılık $p < .05$ olarak tanımlandı.

3.3. Davranış Deneyi Sonuçları

3.3.1. Koşullama Sonuçları

c-Fos immünohistokimyası için perfüze edilen hayvanların koşullama sonuçları, tek yönlü ANOVA'nın ortaya koyduğu gibi, su tüketim miktarlarında gruplar arasında anlamlı bir fark olduğunu gösterdi ($p < .001$). Fisher LSD analizi, LiCl–Bağlam B grubunun, LiCl–Bağlam A ve NaCl–Bağlam B'den önemli ölçüde daha az su içtiğini gösterdi (p değerleri < .001). LiCl–Bağlam A ve NaCl–Bağlam B ($p = .821$) grupları arasında anlamlı bir fark gözlenmedi.

KÇİ öğreniminin değerlendirilmesi için kullanılan hayvanların koşullama sonuçları, tek yönlü ANOVA'nın ortaya koyduğu gibi, su tüketim miktarlarında gruplar arasında anlamlı bir fark olduğunu gösterdi ($p < .001$). Fisher LSD analizi, LiCl–Bağlam B grubunun, LiCl–Bağlam A ve NaCl–Bağlam B'den anlamlı ölçüde daha az su içtiğini gösterdi (p değerleri < .001). LiCl–Bağlam A ve NaCl–Bağlam B grupları arasında anlamlı bir fark gözlenmedi ($p = .050$).

3.3.2. Bellek Testi Sonuçları

Tek yönlü ANOVA analizi, su alımında gruplar arasında anlamlı bir fark olduğunu gösterdi ($p < .001$). Fisher LSD analizi, LiCl–Bağlam B grubunun, LiCl–Bağlam A ve NaCl–Bağlam B'den anlamlı ölçüde daha az su içtiğini gösterdi (p değerleri < .001). NaCl–Bağlam B grubu ayrıca LiCl–Bağlam A grubuna göre önemli ölçüde daha az su içti ($p < .001$).

3.4. Tartışma

Koşullama sırasında yeni bir bağlamda LiCl enjeksiyonu yapılan hayvanlar (LiCl–Bağlam B) KÇİ öğrenmesinin bir göstergesi olarak düşük sıvı tüketimi göstermişlerdir. Ancak bu durum koloni odasında LiCl (LiCl–Bağlam A) ve yeni bir bağlamda NaCl (NaCl–Bağlam B) enjeksiyonu alan hayvanlarda gözlenmemiştir. Bu

nedenle, LiCl–Bağlam B grubunda KÇİ öğrenmesinin gelişiminden sorumlu beyin bölgelerinde, öğrenme kaynaklı c-Fos ekspresyonunda artışını beklerken, LiCl–Bağlam A ve NaCl–Bağlam B gruplarında c-Fos ekspresyonunda artış olmayacağını düşündük.

3.5. Immunohistokimya Sonuçları

3.5.1. Medyal Prefrontal Korteks Sonuçları

Prelimbik korteks. Tek yönlü ANOVA sonuçları gruplar arasında anlamlı bir fark olduğunu gösterdi ($p = .017$). Fisher LSD analizi, LiCl–Bağlam B grubunda, LiCl–Bağlam A ($p = .011$) ve NaCl–Bağlam B ($p = .014$) grupları ile kıyaslandığında daha yüksek c-Fos ekspresyonu olduğunu ortaya çıkardı. NaCl–Bağlam B ve LiCl–Bağlam A grupları arasında anlamlı bir fark bulunmadı ($p = .885$).

Infralimbik korteks. Tek yönlü ANOVA sonuçları gruplar arasında anlamlı bir fark olmadığını gösterdi ($p = .192$).

3.5.2. İnsular Korteks Sonuçları

Tek yönlü ANOVA sonuçları gruplar arasında anlamlı bir fark olduğunu gösterdi ($p = .018$). Fisher'in LSD analizi, LiCl–Bağlam B grubunda, LiCl–Bağlam A ($p = .024$) ve NaCl–Bağlam B ($p = .008$) grupları ile kıyaslandığında daha yüksek c-Fos ekspresyonu olduğunu ortaya çıkardı. NaCl–Bağlam B ve LiCl–Bağlam A grupları arasında anlamlı bir fark gözlenmedi ($p = .577$).

3.5.3. Hipokampüs Sonuçları

CA1-CA2. Tek yönlü ANOVA sonuçları gruplar arasında anlamlı fark olduğunu gösterdi ($p < .001$). Fisher LSD analizi, LiCl–Bağlam B grubunda, LiCl–Bağlam A ($p < .001$) ve NaCl–Bağlam B ($p < .001$) grupları ile kıyaslandığında daha yüksek c-Fos ekspresyonu olduğunu ortaya çıkardı. NaCl–Bağlam B ve LiCl–Bağlam A grupları arasında anlamlı bir fark gözlenmedi ($p = .103$).

CA3. Tek yönlü ANOVA sonuçları gruplar arasında anlamlı fark olduğunu gösterdi ($p = .027$). Fisher LSD analizi, LiCl–Bağlam B grubunda NaCl–Bağlam B ile kıyaslandığında daha yüksek c-Fos ekspresyonu olduğunu ortaya çıkardı ($p = .008$).

LiCl–Bağlam A grubunda, LiCl–Bağlam B (p = .089) ve LiCl– Bağlam A (p = .245) grupları arasında anlamlı bir fark gözlenmedi.

Dentat Girus. Tek yönlü ANOVA sonuçları gruplar arasında anlamlı fark olduğunu gösterdi (p = .017). Fisher LSD analizi, LiCl-Bağlam B grubunda, LiCl-Bağlam A (p = .006) ve NaCl–Bağlam B (p = .031) grupları ile kıyaslandığında daha yüksek c-Fos ekspresyonu olduğunu ortaya çıkardı. NaCl–Bağlam B ve LiCl–Bağlam A grupları arasında anlamlı bir fark gözlenmedi (p = .433).

3.5.4. Amigdala Sonuçları

Bazolateral Çekirdek. Tek yönlü ANOVA sonuçları gruplar arasında anlamlı fark olduğunu gösterdi (p = .010). Fisher'in LSD analizi, LiCl-Bağlam B grubunda, LiCl-Bağlam A (p = .011) ve NaCl–Bağlam B (p = .00) grupları ile kıyaslandığında daha yüksek c-Fos ekspresyonu olduğunu ortaya çıkardı. NaCl–Bağlam B ve LiCl–Bağlam A grupları arasında anlamlı bir fark bulunmadı (p = .747).

Santral Çekirdek. Tek yönlü ANOVA sonuçları gruplar arasında anlamlı bir fark olmadığını gösterdi (p = .434).

Medyal Çekirdek. Tek yönlü ANOVA sonuçları gruplar arasında anlamlı bir fark olmadığını gösterdi (p = .153).

Kortikal Çekirdek. Tek yönlü ANOVA sonuçları gruplar arasında anlamlı bir fark olmadığını gösterdi (p = .302).

3.6. Tartışma

Bu deneyde KÇİ öğrenmesinden sorumlu beyin bölgelerini bulmak amacıyla nöral aktivasyon belirteci olarak kullanılan c-Fos ekspresyonu analiz edilmiştir. c-Fos immünoreaktivitesi, öğrenmenin gerçekleştiği deney grubunda medyal prefrontal korteksin prelimbik bölümünde, insular kortekste, hipokampüsün CA1-CA2 ve dentat girus bölümlerinde ve amigdalanın bazolateral çekirdeğinde bu proteinin ekspresyonunda artış olduğunu göstermiştir.

3.7. Genel Tartışma

Koşullama bağlamında LiCl enjekte edilen hayvanlar (LiCl–Bağlam B), bellek testinde gözlemlenen su tüketimlerindeki azalmanın kanıtlandığı gibi KÇİ öğrenmesi geliştirmiştir. Bununla birlikte, yalnızca bağlama maruz bırakılan (NaCl–Bağlam B) veya sadece LiCl enjeksiyonu yapılan hayvanlarda (LiCl–Bağlam A) KÇİ öğrenmesinin gerçekleşmediği gözlenmiştir. Öğrenmenin gerçekleştiği LiCl–Bağlam B grubunda medyal prefrontal korteksin prelimbik bölümünde, insular kortekste, hipokampüsün CA1-CA2 ve dentat girus bölümlerinde ve amigdalanın bazolateral çekirdeğinde c-Fos proteinin ekspresyonunda artış olduğu bulgulanmıştır. Ancak öğrenmenin gerçekleşmediği kontrol gruplarında bu beyin bölgelerinde c-Fos ekspresyonunda artış gözlenmemiştir. Bulgularımız KÇİ öğrenmesinde bu beyin bölgelerinin görevli olduğunu düşündürmektedir. Beyinde yaygın olarak bir çok bölgede görülen c-Fos ifadesindeki artış, KÇİ öğrenmesinden sorumlu bir beyin devresi olduğuna işaret etmektedir.

3.8. Sonuç

Bildiğimiz kadarıyla, bu çalışma KÇİ öğrenmesinin nöral bağıntılarını araştıran ilk çalışmadır. Yukarıda bahsi geçen beyin bölgelerinin KÇİ gelişiminde nedensel rolünü ortaya çıkarmak için daha fazla araştırma gerekmektedir.

BÖLÜM 4

SON ÖZET, GENEL SONUÇLAR VE ÇALIŞMANIN SINIRLILIKLARI

Bu çalışma kapsamında gerçekleştirilen bir dizi deneyde, KÇİ öğrenmesinin konsolidasyonu sırasında beta-adrenerjik reseptör antagonisti propranolol ve NMDA reseptörü antagonisti MK-801 uygulamasının bellek üzerindeki etkisi araştırılmıştır. KÇİ, BBK olgusunun preklinik modeli olarak kullanılan bir klasik koşullama şeklidir. KÇİ, hayvanları yeni bir bağlama maruz bıraktıktan sonra hayvanlara hastalık oluşturan ilaçların enjeksiyonu ile oluşturulur. Daha sonra hayvanlar aynı bağlama tekrar maruz bırakıldıklarında KÇİ öğrenmesinin bir kanıtı olarak su tüketimlerinde

azalma görülmektedir. İlk deneyimizin bulguları, KÇİ öğrenmesinden sonra NMDA reseptör aktivasyonunu MK-801 ile bloklamanın bellek üzerinde herhangi bir bozucu etkisi olmadığını göstermiştir. Ancak ikinci deneyimizin sonuçları beta-adrenerjik reseptör antagonisti propranolol'ün muhtemelen bellek konsolidasyonunu bozarak hayvanların KÇİ geliştirmesini engellediğini göstermektedir.

Propranolol'ün bellek bozucu etkisinin ardındaki mekanizmayı araştırmak için çalışmamızda herhangi bir deney yapılmamıştır. Propranolol'ün sinir sisteminde sinyalleşme yolaklarını engelleyerek yeni bilgilerin uzun süreli belleğe konsolidasyonunu önleyip önlemediği veya belleğin duygusal değerinde bir değişikliğe neden olup olmadığının araştırılması gerekmektedir. Çalışmamızın bir diğer kısıtlılığı da ilaçların etki mekanizmasını araştıran herhangi bir deneyin olmamasıdır. Beta-adrenerjik reseptörler, sinir sisteminde ve periferde yaygın olarak bulunmaktadır. Bu durum propranolol'ün yarattığı bellek bozukluğunun beyindeki reseptörleri bloklayarak mı yoksa vücuttakiler üzerindeki etkisinden mi kaynaklandığının analizini engellemektedir. KÇİ öğrenmesinde propranolol'ün etki mekanizmasının aydınlatılması gereklidir.

Bunlara ek olarak çalışmamızda bir nöral aktivasyon belirteci olan c-Fos ekspresyonu kullanılarak KÇİ öğreniminde görevli olabilecek bazı beyin bölgeleri bulunmuştur. KÇİ öğrenmesi geliştirmesi beklenen hayvanlarda koşullamanın ardından, medyal prefrontal korteksin prelimbik bölgesinde c-Fos ekspresyonunda önemli ölçüde artış olduğu gözlemlenmiştir. Ancak bu artış infralimbik bölgesinde gözlemlenmemiştir. Ayrıca, c-Fos ekspresyonunun insular kortekste de arttığı bulgulanmıştır. Bu bölgelere ek olarak hipokampüsün CA1-CA2 ve dentat girus bölümlerinde de KÇİ öğrenmesini takiben c-Fos ekspresyonunda artış bulunmuştur. Bu durum CA3 bölgesinde gözlenmemiştir. Son olarak, amigdalanın dört çekirdeğindeki c-Fos ifadesini ölçülmüştür ve yalnızca bazolateral çekirdekteki nöronların yüksek seviyelerde c-Fos proteini eksprese ettiği bulunmuştur. Bu artış santral, kortikal ve medyal çekirdeklerdeki nöronlarında gözlenmemiştir.

Bildiğimiz kadarıyla, araştırmamız CCA öğreniminde yer alan beyin bölgelerini inceleyen ilk çalışmadır. Ancak, c-Fos tekniğinin bazı sınırlılıkları vardır. c-Fos

analizi, yalnızca belirli bir bölgedeki aktif nöronların anlık görüntüsünü verir ve nöronlar arasındaki bağlantının araştırılmasında kullanılamamaktadır. Bu nedenle, bu beyin bölgelerinin ve birbirleriyle olan bağlantılarının KÇİ öğrenmesinde nedensel yerinin rolü araştırılmalıdır.

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