2022

BLOCKING THE ACQUISITION OF ETHANOL-INDUCED CONDITIONED PLACE PREFERENCE WITH 11, 21-BISPHENYL-19-NORPREGNANE (PT150) IN COTURNIX QUAIL

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Digital Object Identifier: https://doi.org/10.13023/etd.2022.201

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THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Arts and Sciences
at the University of Kentucky

By

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ABSTRACT OF THESIS

BLOCKING THE ACQUISITION OF ETHANOL-INDUCED CONDITIONED PLACE PREFERENCE WITH 11, 21-BISPHENYL-19-NORPREGNANE (PT150) IN COTURNIX QUAIL

Alcohol use disorder (AUD) has been associated with a dysregulated stress system. Therefore, regulating stress hormones has been investigated as a potential therapeutic target for AUDs. The purpose of the current study was to investigate whether a stress hormone receptor antagonist, PT150, would block the rewarding properties of ethanol. Quail were used as subjects because a conditioned place preference (CPP) apparatus that utilized visual cues was used, and quail readily attend to visual cues. Visual cues in the environment have been shown to become associated with alcohol effects and later induce craving. Starting on day one, quail were pretreated with vehicle or PT150 (20mg/kg). Thirty minutes later, quail received a treatment of either water or ethanol (0.75g/kg) and were placed in their initially least preferred side as determined by a preference test. On alternate days, all quail received pretreatment and treatment of water. Results revealed pretreatment of PT150 blocked the acquisition of a place preference in quail that were treated with ethanol. This further supports that PT150 is highly selective at blocking CORT without causing peripheral effects associated with ethanol consumption. These preliminary findings suggest that PT150 may reduce the rewarding properties of ethanol by blocking the stress hormone receptor.

KEYWORDS: Alcohol, Corticosterone, Glucocorticoid, Quail

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DEDICATION

To Christian Perrin.
The optimism to my pessimism. The motivation to my doubt. The light in my life.
Thank you for your infinite support.

ACKNOWLEDGMENTS

I would like to thank my Thesis Chair and mentor, Dr. Chana Akins, who has worked tirelessly to assist me and embodies all of the qualities as a researcher and mentor to which I aspire .

I want to thank my Thesis Committee for not only their insight that guided me and challenged me, but also their constant enthusiasm and support: Dr. Michael T. Bardo and Dr. Mark Prendergast.

I would also like to thank those that have helped me along the way. First, my passionate undergraduate lab members, Phillip Jewell, Adam Rosipal, and Jessica Marty. You have done a thankless job and played a pivotal role in not only the success of this research, but also my personal success.

Next, I would like to acknowledge Dr. Beth Ann Rice for aiding in my success as a graduate student and researcher. I am forever indebted to you.

Finally, a thank you to my friends, Morgan DeNardo and Marissa Cragle, who have encouraged me to pursue my dreams from a few hundred miles away.

Last, but certainly not least, a thank you to my family. Roger, Renee, Dimitri, Gianna, and Nikki Radevski, you have listened to me, you have read for me, you have challenged me. But most importantly, you have inspired me. With you on my side, I can truly accomplish anything.

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CHAPTER 1

Introduction

1.1. Background

Alcohol is one of the most frequently used recreational drugs across the world despite its negative consequences. In America, 85% of the population over the age of 18 have reported drinking alcohol in their lifetime (NIAAA, 2020). Excessive alcohol use increases the risk of developing other health issues including a range of cancers, liver disease, and diabetes (NCI, 2015; Singal, et al., 2013). The yearly average deaths due to excessive alcohol use averages around 95,000 (CDC, 2015).

Alcohol use disorder (AUD) is characterized by a range of symptoms including the urge to drink alcohol, dependence, adverse feelings during periods without alcohol, and chronic relapse of drinking alcohol (DSM V, American Psychiatric Association, 2017). In 2016, it was reported that 7.8% of men and 4.2% of women over the age of 18 in America were diagnosed with AUD (SAMHSA, 2018), but less than 9% of people that admitted to needing treatment for alcohol abuse sought out help (SAMHSA, 2015). Although some treatments are successful in treating AUD (Miller, et al., 2001), inconsistencies in the data show anywhere between 40-80% of those that receive treatment remain abstinent long term (Vera, 2021). These discrepancies in the data support the need for continued development of treatments for this disorder.

Ethanol is typically consumed orally and is used recreationally (Hendler et al., 2011; Martin et al., 1993). The most common method for producing ethanol is through a fermentation process (EIA, 2021). It works primarily on the nervous system as a depressant, but at low doses, it has stimulant effects (Hendler, et al., 2011; Oscar-

Berman, et al., 2007). Alcohol consumption results in a variety of neurobiological and behavioral effects that are based upon an individual's blood alcohol concentration (BAC). These effects include impaired memory, cognitive, and motor skills, increased impulsivity and sociability, mood elevation, and even sedation. Frequent and recurrent use of ethanol may lead to dependence, tolerance, and withdrawal during periods of abstinence (Hendler, et al., 2011).

1.2 Pharmacokinetics of Ethanol

Ethanol is a water and lipid-soluble substance that easily dissolves into all body tissues and crosses the blood-brain barrier (BBB) (Davies, 2003). After crossing the BBB, ethanol is equally distributed throughout the body and interacts with a range of systems, making it difficult to identify a specific mechanism of action for ethanol (Oscar-Berman, 2007). Approximately 90-95% of alcohol ingested is metabolized by the enzyme alcohol dehydrogenase (ADH), and 85% of this metabolism occurs in the liver. The metabolic process begins with ADH converting alcohol to acetaldehyde. Nicotinamide adenine dinucleotide (NAD+) is a coenzyme required for ADH activity. Acetaldehyde dehydrogenase (ALDH) converts acetaldehyde into acetic acid. Acetic acid is further broken down to H2O and CO2. The remaining alcohol not broken down by this process is excreted through the lungs unchanged (Advokat, et al., 2019).

1.3 Neurotransmitters

A wide variety of behavioral and biological effects are a direct result of the interaction ethanol has on receptors and the second messenger system. There are two major types of receptors: metabotropic and ionotropic. Metabotropic receptors are also

referred to as g-protein-coupled receptors (Iversen, et al., 2009). When activated, these receptors release the corresponding intracellular G protein that either directly open or close an ion channel, or the G protein moves along the inside of the cell until it finds an enzyme and indirectly activates it. Both of these processes result in a widespread signal. For example, a released G protein can activate adenylyl cyclase, the sole enzyme responsible for the synthesis of cyclic AMP (cAMP). This is important because cAMP is a major second messenger that initiates several responses including, opening ion channels, altering enzyme activity, or gene activation changes (Iversen, et al., 2009). Ionotropic receptors result in a much faster response than metabotropic receptors. These are ion channels in the membrane of a neuron that produce a pore. These pores open or close when they are activated by endogenous neurotransmitters or drugs, and result in a flow of specific ions, such as chloride or sodium, into or out of the neuron (Iversen, et al., 2009).

1.3.1 Glutamate

Glutamate (Glu) is a non-essential amino-acid neurotransmitter, located in the central nervous system and is the main excitatory neurotransmitter. Glu is synthesized by glutamine and an enzyme, glutaminase, and occurs in the brain because glu cannot penetrate the blood-brain barrier. It is then stored in the presynaptic terminal vessels. It plays an important role in synaptic plasticity, long-term potentiation, and learning and memory functions (Riedel, et al., 2003).

The release of Glu occurs through depolarization (Iversen, et al., 2009). While in the synapse, Glu can bind to eight types of metabotropic and three types of ionotropic receptors (Afshari, et al., 2020). Termination occurs through reuptake into the

presynaptic terminal or through the nearby astrocytes. In the astrocytes, glutamine synthase facilitates the breakdown of Glu into glutamine. It is then released and may be taken into surrounding neurons to serve as a precursor for Glu or GABA (Schousboe, et al., 2014).

Glu metabotropic receptors are labeled mGluR1 through mGluR8 (Niswender, et al., 2010). These receptors are further categorized by their influence on the coupled G-proteins. mGluR1 and mGluR5 are coupled to second messengers which increase Ca2+. mGluR2 and mGluR3 are coupled to the second messengers that inhibit adenylyl cyclase. The other 4 mGluR receptors are classified together based on their varying agonist action.

The ionotropic Glu receptors are grouped into either NMDA or non-NMDA receptors (AMPA or kainic acid (KA)). In addition to allowing Na+ into the cells, NMDA receptors also allow Ca2+ to enter (Ozawa, et al., 1998). The NMDA receptors have two requirements to open ion channels: ligand activation and depolarization. High receptor density has been identified within the hippocampus and the cerebral cortex. AMPA and KA are associated with voltage-independent channels that facilitate depolarization through the influx of NA+ (Ozawa, et al., 1998)

1.3.2 γ-aminobutyric acid (GABA)

GABA is one of the primary inhibitory neurotransmitters in the central nervous system (CNS) (Kumar, et al., 2009). Glu is the precursor for GABA, and GABA is synthesized by glutamic acid decarboxylase (GAD). GABA receptors are found widely throughout the CNS and are found on most neurons. Once these receptors are activated, an influx of Cl- ions enter the postsynaptic cell resulting in hyperpolarization within the

neuron and an overall decrease in the depolarizing effects of any excitatory action. There are two major types of GABA receptors originally divided because of the pharmacological mechanisms they are involved in (Kumar, et al., 2009).

GABAA receptors are ionotropic receptors thought to be involved in mediating anxiolytic, sedative, and anticonvulsant activity (Davies, 2003). They are more commonly found on the postsynaptic membrane (for review, see Davies, 2003) and are the most prevalent GABA receptors in the mammalian CNS. Some astrocytes contain GABAA receptors and have been linked to assisting in Cl- channel regulation (for review, see Kumar, 2009).

GABAB receptors are metabotropic GABA receptors (Iversen, et al., 2009). They are located both on the pre- and the postsynaptic membranes and may play a role in GABA release regulation. Specifically, GABAB has been implicated in inhibiting postsynaptic potentials (PSP) in many brain regions (Benarroch, 2008). Unlike the GABAA receptors, GABAB receptors may be coupled with Ca2+ or K+ channels through second messenger systems (Benarroch, 2008). This second messenger influence appears to mediate the inhibitory hyperpolarizing action of GABAB receptors (Pinard, et al., 2010).

1.3.3 Serotonin (5-HT)

5-HT is an inhibitory, monoamine neurotransmitter that is linked to almost all human behavior (Berger, et al., 2009). Only 1-2% of all 5-HT is found in the brain (Iversen, et al., 2009) implicating it in an array of other bodily functions including, mood, memory, sleep, and appetite (Berger, et al., 2009). 5-HT is synthesized in the brain

because it cannot cross the BBB. The synthesis process begins with the uptake of tryptophan, an amino acid, primarily found in the diet. After uptake, tryptophan is hydroxylated via the enzyme tryptophan hydroxylase at the 5-position and converted to 5-hydroxytryptophan (5-HTP) which is very quickly decarboxylated to produce 5-HT. When 5-HT is released into the synaptic cleft, serotonin reuptake transporters (SERT) are the primary means of inactivation. SERT, a protein located predominantly in the presynaptic membrane, actively removes 5-HT from the synaptic cleft and draws it back into the presynaptic neuron. It is regulated by kinase-linked pathways, specifically protein-kinase C (PKC). Free 5-HT in the presynaptic membrane is broken down by monoamine oxidase-A (MAO) and is found in the membrane of the intracellular mitochondria.

There are 7 different families of 5-HT receptors made up of 14 total receptors (Iversen, 2009). Six of the receptor families are metabotropic (5-HT1-2, 5-HT4-7) and mediate both excitatory and inhibitory activity (Frazer & Hensler, 1999) while the other family is ionotropic (5-HT3) and is solely an excitatory neurotransmitter (Frazer & Hensler, 1999). Five receptors belong to the 5-HT1 (5-HT1A,5-HT1B, 5-HT1D, 5-HT1E, and 5-HT1F) family and three receptors (5-HT2A, 5-HT2B, and 5-HT2C) belong to the 5-HT2 family. 5-HT1 and 5-HT5 receptors decrease the intracellular levels of cAMP (Frazer & Hensler, 1999). 5-HT2 receptors are coupled with a Gq protein. When activated, increases of inositol triphosphate (IP3) and diacylglycerol (DAG) are observed intracellularly. Families 5-HT4, 5-HT6, and 5-HT7 are involved in increases in intracellular cAMP. Finally, once 5-HT3, the ionotropic receptor, is activated, the structure exhibits change that permits cations to enter and result in an excitatory response.

1.3.4 Dopamine (DA)

Dopamine (DA) is a catecholamine neurotransmitter most commonly associated with reward processing (Luo, et al., 2016). The majority of DA synthesis takes place in the ventral tegmental area (VTA) and the substantia nigra (see Kalivas, 1993 for review). The synthesis starts with the enzyme tyrosine hydroxylase (TH). Tyrosine is converted to L-dihydroxyphenylalanine (L-DOPA). L-DOPA is then converted into DA by L-aromatic amino acid decarboxylase. DA release from the nerve terminal is calcium-dependent. The main mechanism of DA inactivation occurs through reuptake by the dopamine transporter (DAT). There are two main classes of enzymes responsible for the metabolism of DA, monoamine oxidases (MAO) and catechol methyltransferases (COMT) (Luo, et al., 2016). MAO-As are preferentially located in the DA neurons, specifically in the striatum. COMT activity is thought to have a more important role in DA regulation in the frontal cortex than in other brain regions.

There are two types of postsynaptic DA receptors, D1 or D2 that are further broken down into 5 subtypes (for review, see Vallone et al., 2000). D2, D3, and D4 are in the D2 class, and the D1 and D5 are in the D1 class. The D1 class is coupled to a second messenger system that increases adenylyl cyclase, and the D2 class is coupled to a second messenger system that decreases, or inhibits, adenylyl cyclase.

DA is located in four major pathways: mesolimbic, nigrostriatal, mesocortical, and tuberoinfundibular (Luo, 2016). Both the mesolimbic and mesocortical pathways originate in the ventral tegmental area, but they project to different areas. The mesolimbic pathway, associated with reward and drug-taking, projects to the nucleus accumbens (NAc), and the mesocortical pathway, associated with regulation of motivation, projects

to the prefrontal cortex (for review, see Koob, 1992). The nigrostriatal pathway, responsible for motor movement, begins in the substantia nigra and ends in the striatum. And finally, the tuberoinfundibular pathway, involved in hormone regulation, projects from the hypothalamus to the posterior pituitary (Bjorklund et al., 1973; Swanson, 1982).

1.3.5 Opioid Peptides

Opioid peptides are the endogenous opioid system that have been linked to the rewarding effects of drugs of abuse and ethanol (Hughes, et al., 1983). There are over 20 known opioid peptides, also known as endorphins (Akil, et al., 1998). There are three primary opioid receptors, mu (μ; MOP), kappa (κ; KOP), and delta (δ; DOP). All opioid receptors are G-protein-coupled, but have different effects when activated (Williams, et al., 2001). MOP receptors are found in the mesolimbic and mesocortical pathways and when activated in the VTA, GABA is inhibited, resulting in inhibited DA (Waldhoer, et al., 2004). The MOP receptors are often associated with the euphoria of drug-taking. The DOP receptors is found in the limbic system and when activated, intercellular communication is initiated. Finally, the KOP receptors are found throughout the brain and are considered to be the opposite of MOP receptors because of their role in producing unpleasant and dysphoric feelings associated with drug-taking (Waldhoer, et al., 2004).

1.4 Neurotransmitters and Ethanol

1.4.1 Glutamate and Ethanol

Ethanol is a potent inhibitor of the NMDA receptors. NMDA receptors have been linked to adaptation to chronic ethanol use, ethanol withdrawal effects, and may be a potential site for treating ethanol dependence (Prendergast, et al., 2012). Specifically,

NMDA antagonists have been used to reduce ethanol intake, prevent the development of tolerance, and mitigate withdrawal symptoms and neurotoxicity (Holter et al., 1997; Khanna, Morato and Kalant 1993; Prendergast et al., 2004; Veatch and Becker, 2005). Finally, NMDA receptors increase in sensitivity after prolonged ethanol use. This upregulation may result in seizures during withdrawal from ethanol (Mann, et al., 2008).

1.4.2 GABA and Ethanol

GABAA plays a role in both acute and chronic effects of ethanol in the CNS. It has been demonstrated that at low doses, GABA turnover increases**,** but at high doses, GABA turnover remains unchanged (Hunt, et al., 1983). Turnover refers to the rate of accumulation of GABA in the synapse after inhibition of its metabolism mechanism (Hunt, et al., 1983). There is evidence that acute exposure to ethanol results in potentiated currents at the GABAA receptor (Davies, 2003).

1.4.3 Serotonin and Ethanol

Serotonin plays an important role in developing a preference for ethanol, dependence, and eliciting craving (Sari, et al., 2011). It has also been linked to dysregulated impulsivity (Kirby, et al., 2011), a contributing factor in addiction and relapse. Specifically, the dorsal striatum, an area highly dependent on 5-HT transmission, has been linked to the obsessive tendencies related to addiction (Mukherjee, et al., 2008). Ethanol has been shown to inhibit the elimination and alter the synthesis of 5-HT (Daws et al., 2006). Furthermore, 5-HT3 receptors may play an important role in the rewarding effects of ethanol, regulation of use, and the development of dependence (Sari, et al., 2011). For example, when ethanol is administered, it directly targets the excitatory 5-

HT3 receptors which has been found on GABA interneurons (Vengeliene, et al., 2008; Zhang, et al., 1997).

1.4.4 Dopamine and Ethanol

Ethanol affects dopamine in many ways. Ethanol administration *in vivo* has been shown to initiate DA firing in the VTA (Gessa, et al., 1985). It has also been demonstrated that after intraperitoneal (ip) ethanol injection, DA metabolism increases in the nucleus accumbens (Khatib, et al., 1988). Rodent research suggests that DA levels in the NAc increase as a result of ethanol's direct effects on the GABAergic and opioidergic systems (Cowen, et al., 1999). An anticipatory-like response has also been observed in rodents. After rodents were given repeated ethanol administration, DA levels increased with an injection of vehicle alone (Philpot, et al., 1998). Thus, the dopaminergic system may play a role in the rewarding effects of ethanol.

1.4.5 Opioids and Ethanol

Chronic ethanol use may impact the synthesis, and release of endogenous opioid neurotransmitters (Gianolaukis, et al., 1996). These effects appear to be region- and dose-dependent. In rodents, MOP knockout mice do not self-administer ethanol and quickly develop an aversion to ethanol (Roberts, et al., 2000). Further, in rodent models, MOP antagonists decrease responding for ethanol and overall ethanol consumption (Samson & Doyle,1985; Ripley et al., 2015). Equally, in DOP knockout mice, ethanol administration is increased (Van Rijn, et al., 2009). Finally, genetic variations in genes associated with KOP have been linked to an increase in ethanol dependence (Xuei, et al., 2006).

1.5 Stress

The biological stress response is highly individualistic (Ebner, et al., 2017). Stressors are initiators of the stress response because they are perceived as unknown, aversive, and potentially harmful (Shaham, et al., 2003). Activation of the sympathetic nervous system and hypothalamic-pituitary-adrenal (HPA) axis is most commonly associated with the stress response (for review, see Turnbull and Rivier, 1997). The paraventricular nucleus (PVN) within the hypothalamus initiates the release of corticotropin release factor (CRF). This results in activation of the pituitary gland and the release of adrenocorticotrophin hormone (ACTH) which acts on the adrenal cortex to release glucocorticoid hormones (GCs), the primary stress hormones. GCs are synthesized through a number of enzymatic reactions and are catalyzed by 11β-hydroxysteroid dehydrogenase-1 (11β-HDS1) into their active form: cortisol (humans) or corticosterone (animals). There are two types of GC receptors in the brain. Mineralocorticoid receptors (MRs) are localized in the limbic system and glucocorticoid receptors (GRs) are ubiquitously found throughout the rat brain (Reul & de Kloet, 1985, 1986). GRs are activated by corticosterone (CORT), the primary stress hormone found in animals. When CORT binds to the GRs, an acute stress response is observed.

There is a complex relationship between stress and ethanol. Ethanol use is known to activate the HPA axis in humans and rodents (Prendergast, et al., 2012). Initial ethanol consumption results in GABA neuron activation within the VTA, DA neurons within the NAc, and activation in the HPA-axis (Lee, et al., 1997). This alcohol-induced signaling may be similar to other stressors and may be involved in increasing the motivational properties of alcohol (Lee, et al., 1997). Preclinical and clinical models have also demonstrated the influence of acute stressors on the increased motivation to drink ethanol

(Chaplin, et al., 2008). Dysregulation in the HPA-axis during ethanol consumption has been associated with compulsive ethanol consumption in rodents (Richardson, et al., 2008; Vendroscolo, et al., 2012).

1.5.1 RU486

A non-specific GR antagonist, mifepristone (RU486), has previously been used as a possible treatment for addiction. RU486 competes with CORT to bind to GRs in the cytoplasm (Peeters, et al., 2004). For example, a significant reduction in the severity of ethanol withdrawal symptoms was observed in rats that were administered RU486 before a 4-day binge-like regimen (Sharrett-Field, 2013). Another experiment investigated the effects RU486 had on ethanol-dependent mice. It successfully reduced cognitive deficits (i.e., memory) measured in a repeated T-maze procedure and reduced hyperexcitability often associated with ethanol withdrawal (Jacquot, et al., 2008). Together, the research on RU486 supports the likelihood that GRs may play a role in alcohol dependence.

RU486 is no longer used as a treatment because it is non-selective. In addition to acting on the GR receptors, it also acts as a progesterone receptor blocker (Vegeto, et al., 1992). Progesterone is a necessary hormone for pregnancies to be viable (FDA, 2021). As a result of the non-selectivity, RU486 causes spontaneous abortions (Vegeto, et al., 1992). Therefore, a more selective GR antagonist that acts only on the GR receptors may be a better option for treating ethanol dependence.

1.5.2 PT150

A more selective GR antagonist, 11, 21-bisphenyl-19-norpregnane (PT150, formerly called ORG 34517) is a pure competitive antagonist. It also has an affinity for

the progesterone receptors sixfold lower than RU486 (Peeters, et al., 2004). PT150 has been used as an experimental treatment in mood disorders and PTSD (Morice et al., 2021). These mood disorders and alcohol and substance use disorders have been linked to a dysregulated HPA-axes, leading to believe PT150 may be a therapeutic target for addiction (for review, see Peeters, et al. 2004). For example, a recent experiment was conducted to test the safety of PT150 administered in conjunction with alcohol. There were no significantly different adverse effects in participants that received ethanol with PT150 versus placebo deeming it safe (Morice et al., 2021).

In recent years, pre-clinical models of addiction have demonstrated the usefulness of PT150 as a potential inhibitor of drug seeking behaviors. For example, fentanyl-treated rats that received oral doses of PT150 showed a decrease in stress-induced relapse-like behavior (Hammerslag, et al., 2021). In this study, rats pretreated with PT150 and exposed to a stressor (i.e., foot shock) showed a decrease in lever pressing when compared to the placebo group (Hammerslag, et al., 2021). Additionally, quail have been used as a model to test the effects of PT150 on drug-seeking behavior using visual cues (i.e., sign tracking). Sign tracking is a well-established model that has been used to demonstrate individual differences in subjects and their likelihood to develop compulsive drug-seeking behaviors (Flagel, et al., 2009; Tomie, et al., 2008). A 20mg/kg dose of PT150 has been shown to decrease sign tracking behavior (Rice, et al., 2019).

1.6 Conditioned Place Preference and Conditioned Place Aversion (CPP/CPA)

Conditioned place preference is a Pavlovian conditioning paradigm that has been used to assess the rewarding and aversive properties of specific stimuli (Bardo & Bevins, 2000; Tzschentke, 2007). The basis of Pavlovian conditioning is to establish a predictive

relationship between a neutral stimulus and a naturally significant stimulus (i.e., food) (Pavlov, 1927). In CPP, a naturally significant stimulus that elicits a response with no prior training is an unconditioned stimulus (UCS). This UCS is paired with a neutral stimulus (NS) that acquires the motivational characteristics of the UCS through conditioning and becomes the conditioned stimulus (CS) (for review see, Bardo & Bevins, 2000; Tzschentke, 2007).

1.6.1 CPP Parameters

In CPP experiments, the NS that is paired with the UCS can take a variety of forms such as visual, olfactory, tactile, or auditory. The species is usually the determining factor as to which kind of NS is used to pair with the UCS. Rodents typically have poor vision which results in most CPP experiments with rodents using tactile or olfactory manipulations (Tzchentke, 2007). In some studies, the pattern of the floor is different to distinguish between the paired and unpaired contexts of the apparatus. For example, Cunningham (2003) used one flooring similar to chicken wire and another flooring with metal rods going in one direction. Each flooring was either paired with ethanol or water. On the other hand, birds can see color and have high visual acuity allowing for the use and manipulation of visual cues during CPP experiments. Previous research has established that quail can distinguish between yellow and green and develop a CPP for psychostimulant paired contexts (Akins et al., 2004; Bolin et al., 2012; Gill et al., 2016).

CPP experiments can be described as having an unbiased or biased design. An unbiased design uses a random assignment of paired context after subjects show no initial preference during a test session after habituation and before conditioning (Cunningham, et al., 2003). On the other hand, biased design is typically described as untrained animals

showing a consistent preference for one stimulus or context over another during a test session after habituation and before conditioning (Cunningham et al., 2003). It is important to establish which design to use because of the influence it has on the interpretation of results.

Unbiased designs are complex as they are defined as random assignment to a conditioning compartment or context as a result of the subject having no initial preference (Tzchentke, 2007). The term unbiased design can also be used to describe a CPP procedure in which subjects show a preference for one context over another, and the experimenter assigns half of each group to their preferred context and the other half to their nonpreferred context. This definition has also been called counterbalancing to distinguish between a true unbiased design and random assignment regardless of preference. Therefore, an unbiased design should only be used when animals do not innately prefer one context over the other.

Several studies have demonstrated that achieving unbiased designs may be dependent on factors such as cue manipulation and drug dose. For example, one study used tactile floor manipulation for each compartment in a two-chamber test box (Roma, et al., 2005). They further tested animals in either a brightly lit room or the dark. They found that rats tested in the dark with only the tactile manipulation had no initial preference, whereas the rats tested in a brightly lit room with the tactile cues resulted in all rats showing a clear preference for one context over the other (Roma, et al., 2005). By eliminating the white light, tactile stimulation was sufficient to demonstrate CPP. Because unbiased designs depend on the individual animals' innate non-preference and

may be influenced by outside factors such as visual cues, a biased design is recommended (Cunningham, 2003; Tzchentke, 2008).

On the contrary, using a biased design may influence the results of an experiment. There are two core explanations for how using a biased design might affect the results. First, the rewarding properties of the UCS may interact with an unconditioned motivational state that is reflected in the initial bias (Cunningham, et al., 2003). For example, if the UCS is paired with an initially aversive or nonpreferred context, a place preference may be observed, but the subject may be expressing a preference because of a reduction in fear rather than for the rewarding effects of the UCS (Carr, et al., 1989; Swerdlow, 1989). Another explanation for how a biased design may affect the results is that pairing the UCS with the initial non-preferred context may be used to maximize the potential shift in preference (Bozarth, 1987; Cunningham, et al., 2003). If the UCS is paired with the initially preferred stimulus or context, a ceiling effect may occur. This measurement error is accounted for by using the initial least preferred stimulus or context for the UCS pairing (Bozarth, 1987).

1.7 Thesis Statement

Irresponsible alcohol use and abuse is a growing problem and can be a precursor for a range of health risks, economic burden, and further impact family members and future generations. Rodent models commonly used to understand the rewarding properties of drugs have mixed results that depend heavily on the strain and ultimately remain inconclusive (Brabant, et al., 2014). Because birds actively overconsume fermented fruits in the wild, they may be an appropriate model to examine the effects of ethanol and preventative measures for alcohol abuse. With previous research establishing

both a preference for ethanol attenuation of drug-seeking behaviors with PT150 in quail, it is expected that PT150 may block the acquisition of ethanol preference.

CHAPTER TWO

Blocking The Acquisition of Ethanol-Induced Conditioned Place Preference With Pt150 In Coturnix Quail

2.1 Introduction

Alcohol is one of the most frequently used recreational drugs across the world despite its negative consequences. Few treatments have been proven successful in treating alcohol use disorder (AUD) and maintaining abstinence (Vera, 2021; Miller, et al., 2001). AUD has been associated with a dysregulated stress system which may be a possible therapeutic target (Morice, et al., 2021).

Cues that have been associated with rewards such as drugs or alcohol, may take on the rewarding properties themselves (Robinson, et al., 2013). Visual cues in the environment are highly salient and are known to facilitate drug-taking, craving, and relapse (Childress et al., 1999). Most preclinical models of addiction use rodents, but because of rodents' limited visual capacity, important factors that influence drug taking may not be represented. As an alternative species, quail are primarily visually oriented with the ability to see in color and with similar vision as humans (Fidura & Gray, 1966; Mills, et al., 1997). Their well-developed visual system allows for the use of visual cues to be manipulated in experimental paradigms.

There is little research investigating the properties of ethanol in a predominately visual species such as quail. In the wild, a range of avian species have been shown to actively consume ethanol in the form of fermented fruits (Kinde et al., 2012). In a laboratory setting, a variety of bird species, including quail, passerines, and zebra finches, also consume ethanol (Eriksson & Nummi, 1982; Olson et al., 2014). A more recent

study (Eaton et al., in press) demonstrated a dose-dependent effect of ethanol, such that a relatively low dose (0.75g/kg) of ethanol resulted in a CPP, and a relatively high dose (2g/kg) of ethanol resulted in a CPA. Thus, quail may be a useful model for investigating the relationship between visual cues and the rewarding effects of ethanol.

In preclinical models, CPP is widely used to examine the rewarding and aversive properties of ethanol, but there are inconsistencies in the findings of rodent studies. For example, DBA/2J mice show a preference for ethanol doses between 2-4g/kg ip, but this was not demonstrated in other strains (C57BL/6J) (Cunningham, et al., 2018). Additionally, the route of administration may be an important factor in the rewarding effects of ethanol. For example, DBA/2j mice show an aversion to the taste of ethanol when orally consumed whereas C57BL/6J mice that readily consumed large amounts of ethanol, showed an ethanol preference, and reached blood alcohol concentrations that produced visible physiological effects (i.e., motor incoordination) (Blizard, 2007; Rhodes, et al., 2005). Therefore, it appears that in rodents, evidence of CPP may depend on strain and route of administration.

Previous research has established quail as a suitable model for studying CPP with visual cues. For example, Levens et al. demonstrated that quail showed a CPP after repeated cocaine administration (2001). Further, a place preference was established in quail treated with nicotine using a biased CPP design (Bolin, et al., 2012). CPP procedures that use quail also take advantage of their well-developed visual system by utilizing different colored walls for the pairings (Bolin, et al., 2012; Levens, et al., 2001; Eaton, et al., in press) further supporting the importance of using a visual species when studying the rewarding effects of drugs of abuse.

A selective glucocorticoid antagonist, 11, 21-bisphenyl-19-norpregnane (PT150, formerly called ORG 34517), has been used to block the glucocorticoid receptors (GR) and decrease the effects of corticosterone (CORT) (Peeters, et al., 2004). Decreasing the amount of CORT that binds to the receptors has been shown to attenuate drug-seeking (i.e., sign tracking) behaviors in both rodents (Thomas & Papini, 2001) and quail (e.g., Rice et al., 2018). Specifically, in Japanese quail, both oral and subcutaneous administration of PT150 have been shown to decrease sign-tracking behavior (Rice, et al., 2018; Rice, et al., 2019), a behavior that has been shown to be predictive of drug taking and seeking (Morrow & Tomie, 2018). However, subcutaneous administration may be more effective because it does not go through first-pass metabolism (Verma, et al., 2010).

Egg laying in female quail may be affected by a number of stress-related factors, including elevated levels of CORT produced by the HPA-axis (Langen, et al., 2018). For example, Japanese quail bred for a high stress response showed a negative relationship between stress levels and egg production. And when doses of CORT were administered, an even greater negative relationship was revealed (Schmidt, et al., 2009). Because ethanol has been shown to activate the HPA-axis (Prendergast, et al., 2012), a decrease in egg production during ethanol administration may be indicative of increased stress in female quail.

The purpose of the current study was to further investigate visual CPP with a low dose of ethanol in quail, determine whether PT150 would block the ethanol-induced CPP, and to determine whether PT150 would block ethanol-induced reduction of egg laying. We hypothesized that subjects receiving ethanol gavage would develop a place

preference, quail treated with PT150 before ethanol gavage would not establish a preference, and that PT150 would attenuate ethanol-induced reduction of egg laying.

2.2 Methods

2.2.1 Subjects

Seventy-three adult Coturnix male (N=38) and female (N=35) quail were housed under 16:8 L:D throughout the experiment. Quail were purchased from Myshire Farm (Miamisburg, OH) and housed at the University of Kentucky between 4 and 5 weeks old. Quail were housed individually in wire mesh cages. All quail had free access to food and water until the start of the experiment. During the experiment, food was removed 1 hour before the start of each conditioning day to limit food in the crop prior to gavage. All procedures were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky.

2.2.2 Drugs

Ethanol was diluted in tap water (25% w/v alcohol) and gavaged at a dose of 0.75g/kg. This dose was selected based on previous work done in our lab that indicated quail show a conditioned place preference at 0.75g/kg ethanol (Eaton, et al., 2022) and that peak BEC was achieved at approximately 1 hour after administration (Eaton, unpublished). Water was used as a vehicle for ethanol-treated quail and will be given as a control. All dose volumes were calculated based on their weight taken prior to each conditioning trial.

Similar to previously established methods (Johnson, et al., 2007; Rice, et al., 2019), PT150 was dissolved in 30% dimethyl sulfoxide/70% polyethylene glycol (PEG 300) and was administered subcutaneously (SC) 30-minutes before the start of each ethanol conditioning day. A 20mg/kg dose was used based on previous work conducted in our laboratory that demonstrated a decrease in sign tracking behavior in male quail (Rice, et al., 2019). Half of the quail were subcutaneously administered vehicle in lieu of a water injection before receiving a water gavage (VW; n=7) and ethanol gavage (VE=9) on odd days.

2.2.3 Apparatus

The test apparatus consisted of three chambers (ENV-013; Med Associates Inc., St. Albans, VT). Each end chamber (28.6 cm long × 21.2 cm wide × 21.2 cm deep) had colored red or green walls with gray walls in the center chamber (10.8 cm long × 21.2 cm wide × 21.2 cm deep). Each test apparatus was separated by two removable doors. Green and red were chosen based on previous work that demonstrated that quail prefer wavelengths in the green and yellow range over wavelengths of red or blue (Dueker, et al., 1977). The floor of the entire apparatus was covered in white textured paper. Each test apparatus also had a camera placed 3' overhead for recording behavioral data. Each end chamber was equipped with 6 photo beams located 6.4 cm and 3.2 cm from the apparatus floor.

2.2.4 Procedure

Quail were randomly assigned to one of four treatment groups: PE, WE, PW, WW (see Figure 1). Similar to previous CPP studies with quail, a biased conditioned place preference design was used for the current experiment (Akins, et al., 2004; Gill, et

al., 2016; Levens & Akins, 2001). A biased design uses a pretest to determine initial chamber preference and assigns the least preferred chamber to be paired with the ethanol. Quail were habituated, tested, and conditioned in the same CPP chamber. A white noise generator was used to minimize peripheral noise.

Table 1.1 Experimental Design

		Pretreatment	
		PT150	Vehicle
Treatment	Ethanol	PE (n=19)	VE (n=19)
	Water	PW (n=18)	VW (n=17)

Habituation

Before the start of the experiment, quail were habituated to the entire CPP box. During habituation, quail were allowed access to the middle chamber and one end of the apparatus (i.e., red) for 60 minutes. On alternating days, quail had access to the middle chamber and the opposite outside chamber (i.e., green) for 60 minutes. This pattern took place once a day for a total of 4 days. This method was used in previous research conducted in our laboratory (Gill et al., 2016; Eaton et al., 2022).

Pre-Test

After habituation and before the start of conditioning, quail were given a pre-test to determine their initial preference (i.e., biased design). Quail were placed in the center chamber and given 15 minutes to freely explore the CPP apparatus. Time spent in each chamber was recorded. Initial preference was based on the outside chamber in which quail spend more time.

Conditioning

Quail were weighed once daily before each conditioning trial to determine the appropriate volume of ethanol, water, vehicle, or PT 150 to administer. On the odd number conditioning days starting with day one, quail assigned to the PE and PW groups received a subcutaneous injection of 20mg/kg PT150 as their pretreatment. Quail assigned to the VE and VW groups received 20mg/kg water as their pretreatment. Thirty minutes later quail in the PE and VE groups received a treatment of 0.75g/kg of ethanol via gavage and quail assigned to the PW and VW groups received a treatment of 0.75g/kg of water via gavage. All doses were converted to volume as determined by their weight at the start of each day. Subjects were placed into their least preferred chamber (determined via pretest) for 30 minutes.

On alternate days (even), all quail received a pretreatment injection of water and 30-minutes later, a gavage treatment of water. They were placed in the opposite (preferred) chamber for 30 minutes. A total of eight conditioning days took place consisting of 4 ethanol and PT150 days and 4 water and vehicle days. Photobeam breaks were collected in 5-minute time bins and total time spent in the outside chamber versus the middle chamber was taken each day via Anymaze. Eggs were collected daily when food was taken 1-hour prior to testing and when food was put back at the end of testing.

Post Test

A posttest took place 24-hours after the last conditioning day. It was conducted using the same procedure as the pre-test.

2.3 Statistical Analysis

Place preference was assessed using a difference score (Gill et al., 2016). A difference score was calculated by subtracting the time spent (sec) in the least preferred

chamber during the pre-test from the time spent (sec) in the drug paired chamber during the post-test. Initially, a 2 x 2 x 2 ANOVA with sex (male and female), pretreatment of vehicle or PT150 (20mg/kg) and treatment of water or ethanol (0.75g/kg) as between-subject factors was performed. However, because this analysis resulted in no significant effect or interaction with sex (Fs ranged from .002 to 2.749), data were collapsed across sex. Additionally, treatment groups which quail received vehicle instead of water were collapsed because the analysis resulted in no significant differences in difference scores between the VW and WW (p=.531) and the VE and WE treatment groups (p=.446). Thus, a 2 x 2 ANOVA with pretreatment (vehicle or PT150) and treatment (water or ethanol) as between-subjects factors was conducted on the difference score.

Locomotor activity (via photobeam breaks) was analyzed on the first and last ethanol conditioning days using a 2x2x2 (pretreatment x treatment x day) repeated measures ANOVA (RM ANOVA) with pretreatment of vehicle or PT150 and treatment of water or ethanol as between-subjects-factors and day as a within-subject factor.

During conditioning trials, eggs were collected from female birds and the total number of eggs was analyzed using a one-way ANOVA with pretreatment (vehicle or PT150) and treatment (water or ethanol) as between-subjects-factors. Further post-hoc comparisons were conducted where appropriate.

2.4 Results

2.4.1 Difference Score Results

Figure 1 shows the mean difference score in subjects who received pre-treatment of vehicle or PT150 followed by ethanol or water. A two-factor ANOVA revealed a significant pre-treatment x treatment interaction, $F(1, 69)=5.839$, p=.018 partial $\eta 2$ =

.078. Simple main effect analyses showed that there was a significant main effect of pre-treatment (vehicle versus PT150) in subjects treated with ethanol [$F(1, 36)=7.588$, p=.009 partial η2 = .174] but not in subjects treated with water [$F(1, 33)=.324$, p=.573 partial η2 = .010]. Quail that received vehicle prior to ethanol (VE) (M=282.89, SEM=39.28) had a significantly greater mean difference score than quail that received PT150 followed by ethanol (PE) (M=4.85, SEM=12.54) and quail that received vehicle followed by water (VW) (M=73.34, SEM=20.78) and ps<.05.

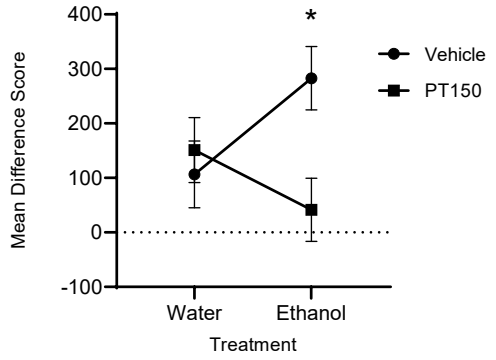

Figure1.1. Mean difference score. *indicates a significant main effect of pretreatment for ethanol treatment

2.4.2 Locomotor Activity

Figure 2 shows the mean number of photobeam breaks for each treatment groups on the first and last ethanol conditioning days (1 and 7). The RM ANOVA did not reveal a significant three-way pretreatment x treatment x day interaction, [$F(3, 69=2.562$, p=.114, partial η^2=.036). There were no significant 2-way interactions (F *'s* ranged from .007 to .913) or significant main effects of day, pretreatment, or treatment.

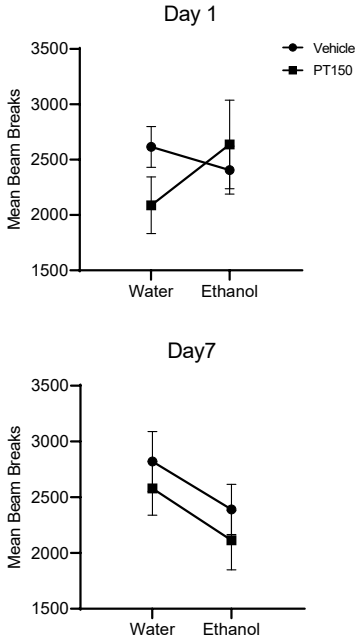

Figure 1.2 Mean number of photobeam breaks on day 1 and day 7

2.4.3 Egg Production

Figure 3 shows the mean number of eggs produced on all conditioning days for each treatment group. A two-way ANOVA did not reveal a significant ethanol dose x PT150 interaction on the number of eggs produced [$F(1, 31)=3.619$, $p=.066$, partial $\eta^2=.105$]. There was a main effect of pre-treatment (vehicle versus PT150), [$F(1, 31)=5.912$, $p=.021$, partial $\eta^2=.160$). Post hoc analyses revealed subjects pretreated with vehicle prior to ethanol treatment ($M=3.6$, $SEM=.751$) laid significantly fewer eggs than subjects pretreated with PT150 prior to ethanol treatment ($M=7.11$, $SEM=.792$), [$F(1, 17)=11.12$, $p=.004$, partial $\eta^2=.395$). Pretreatment did not have a significant effect on subjects treated with water ($p=.736$).

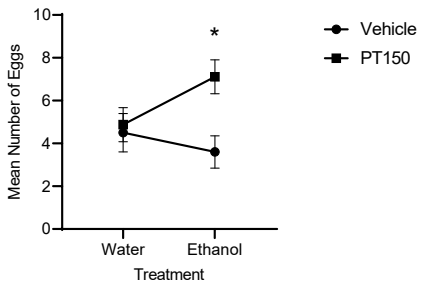

Figure 1.3. Mean number of eggs laid over the course of conditioning. *indicates a significant main effect of pretreatment on qual treated with ethanol

2.5 Conclusions

The current study investigated whether a glucocorticoid antagonist, PT150, would block the acquisition of ethanol-induced CPP, whether ethanol and/or PT150 would have an effect on locomotor activity, and whether PT150 would attenuate ethanol-induced reduction of egg production in females. The findings indicated that quail pretreated with PT150 prior to ethanol treatment (PE) showed less ethanol induced CPP than the other groups. Additionally, there were no significant changes in locomotor activity across days or as a result of pretreatment, treatment, or the interaction thereof. Finally, female quail that received pretreatment of vehicle prior to ethanol treatment (VE) laid significantly fewer eggs across conditioning days compared to the female quail received pretreatment of PT150 prior to ethanol (PE).

Similar to previous ethanol CPP research in quail, the current findings demonstrated a conditioned place preference at a relatively low dose (Eaton, et al., 2022). In addition, the current findings extend the literature on ethanol CPP by demonstrating that pretreatment with a glucocorticoid antagonist, blocked the acquisition of the ethanol CPP. The current study is the first of its kind, to date, to investigate the effects of PT150 on the rewarding effects of ethanol. PT150 has been shown to decrease stress-induced

fentanyl seeking behaviors in rats (Hammerslag, et al., 2021). It has also been shown to decrease sign tracking behavior in quail (Rice, et al., 2019). Sign tracking behavior has been associated with an increased likelihood of drug-taking (Morrow & Tomie, 2018). The results of the current study support the hypothesis that PT150 may reduce the rewarding effects of ethanol in a visual model and that the stress system may be a useful target for future treatments.

Visual cues in the environment are highly salient and are known to facilitate drug-taking, craving, and relapse (Childress, et al., 1999). Most preclinical models of addiction use rodents, but because of their limited visual capacity, important factors that influence drug seeking may not be represented. Rodents primarily rely on olfactory cues whereas avian species, such as quail, have a visual system that is more similar to humans. Previous studies with quail on drug addiction utilized different colored walls as the visual cues (Bolin, et al., 2012; Levens, et al., 2001; Eaton, et al., in press). In combination with the current study, this supports the important role visual cues play in drug and alcohol seeking (Childress et al., 1999) and justifies the use of a primarily visual species to assess the motivational properties of ethanol.

Pre-clinical models of alcohol use and relapse remain inconsistent when discussing the relationship between locomotion and ethanol. The findings of the current experiment align with previous research such that administration of a low dose of ethanol did not significantly change locomotor activity across days and did not differ from water treatment. For example, in rats, a 0.7g/kg dose of ethanol did not change locomotor activity, but a 1.5g/kg dose significantly reduced activity (Fidler, et al., 2004). Similarly in quail, a 2g/kg dose caused suppressing effects on locomotor activity, but these same

effects were not observed at the 0.75g/kg dose, which did not differ significantly from the water control group (Eaton, et al., 2022). Thus, a low dose of ethanol does not appear to be effective at reducing locomotor activity.

Previous research has shown that exposure to intermittent and prolonged stressors may impact the quantity and quality of eggs produced by female quail (Alagawany, et al., 2017; Sahin, et al., 2004; Vercese, et al., 2012). Typically, female quail lay one egg a day (Orcutt & Orcutt, 1976). In the current study, the frequency of egg laying was monitored during conditioning days to observe effects of ethanol and PT150. The current study found that when quail were pretreated with PT150 prior to treatment of ethanol, female quail produced significantly more eggs than quail pretreated with vehicle prior to ethanol treatment. Although speculative, the results suggest that intermittent ethanol exposure may reduce egg production similar to other stressors such as heat exposure and inconsistent housing and/or social contexts (Alagawany, et al., 2017; Rutkowska, et al., 2011). Thus, PT150 may be protecting the egg laying process in female quail from the effects of ethanol-induced CORT activation.

In summary, the findings of the current study suggest that repeated pairings of alcohol with visual cues may become associated and later, in the absence of ethanol, the visual cues may elicit ethanol-seeking behaviors. PT150 may serve as a treatment to block the acquisition of ethanol seeking behavior. These novel findings support both the use of a visual species as a preclinical model and targeting the stress system as a potential treatment option for alcohol seeking behaviors. Future studies might include investigating the effects of PT150 pharmacokinetically to further support the behavioral changes observed in the current study.

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Publications

Radevski, M.E. & Rice, B.A. (In press) Characterizing Sign Tracking Behavior in Female Japanese Quail (*Coturnix Japonica*).

Eaton, S. E., Dzhala, S., Robinson, L. E., Radevski, M. E., & Akins, C. K. (2022). Ethanol induces a dose-dependent conditioned place preference and conditioned place aversion in Japanese quail. *Experimental and clinical psychopharmacology*, 10.1037/pha0000548. Advance online publication. https://doi.org/10.1037/pha0000548

Fellowships, Honors, and Awards

2022	Research Society on Alcoholism Student Merit Travel Award
2021-2022	University of Kentucky Substance Use Priority Research Area (SUPRA) Graduate/Professional Student Grant