THE ROLE OF NUCLEAR FACTOR KAPPA B AND THE NEUROKININ-1 RECEPTOR IN ALCOHOL- AND STRESS-RELATED BEHAVIORS

by

SADIE ELIZABETH NENNIG

(Under the Direction of Jesse Schank)

ABSTRACT

Alcoholism is one of the most prevalent psychiatric disorders not only in the United States, but worldwide. Despite its prevalence, less than 10% of those diagnosed with alcohol use disorder (AUD) receive treatment each year. Alcoholism is a chronic, relapsing disorder that is vastly heterogenous in nature, with a combination of biological, environmental, and genetic factors all contributing to its development. As such, identifying treatments that are successful in subgroups of alcoholics, perhaps determined by their psychiatric comorbidities, may be an effective approach. In this report, we explore the transcription factor nuclear factor kappa light chain enhancer of activated B cells (NFkB) and the neurokinin-1 receptor (NK1R) in the processes underlying alcoholism and its comorbidity with depression. Using conditioned place preference and Daun02 selective inactivation, we report that NFkB is activated in the nucleus accumbens (NAC) shell during alcohol place conditioning, and that inactivation of NFkB-expressing cells in this region during conditioning attenuates alcohol CPP. This data indicates that NFkB has a functional role in the rewarding effects of alcohol. We then shifted our focus to processes underlying alcoholism and depression comorbidity. Our group has previously found that forced chronic alcohol exposure via intragastric gavage increases sensitivity to social defeat stress (SDS). However, it is unknown if voluntary intermittent ethanol access (IEA) has the same effect. We found that, like

alcohol gavage, voluntary consumption on an IEA schedule increases sensitivity to SDS. We also assessed effects of IEA and chronic alcohol gavage on components of the NFkB pathway and found that both of these alcohol exposures increase NFkB in the central nucleus of the amygdala (CEA). Lastly, we explored the role of the NK1R in the behavioral phenotypes induced by SDS, including decreased social interaction and increased alcohol consumption. We found that NK1R antagonism prior to defeat sessions protected against the decrease in social interaction, but not increased alcohol consumption, following chronic SDS. In addition, overexpression of NK1R in the NAC shell increased sensitivity to SDS. Together, these data support the further investigation of NFkB and NK1R as potential pharmacotherapeutic targets for alcoholism and its comorbidity with depression.

INDEX WORDS: Alcoholism, Depression, Comorbidity, NFkB, NK1R

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DEDICATION

This dissertation is dedicated to my family. Mom, Dad, Bailey, and Al, this would not have been possible without your constant support and I am so thankful for you all. I would especially like to dedicate this to my father, Tim, who was diagnosed with Stage 4 Pancreatic Cancer on October 28th, 2017. Dad, thank you for showing me what strength truly is and for teaching me to never give up no matter what life may throw at you.

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CHAPTER 1:

INTRODUCTION AND LITERATURE REVIEW

1.1 Alcoholism Overview

1.1.1 Alcohol Use Disorder (AUD)

Alcoholism is one of the most prevalent psychiatric disorders not only in the United States, but worldwide. In 2018, 14.4 million adults over the age of 18 in the United States were diagnosed with an alcohol use disorder (AUD)[1]. This is comprised of 10.1% of individuals between ages 18-26 and 5.1% of individuals 27 years or older[1]. Out of the 14.4 million adults diagnosed with an AUD, just 6.3% received treatment in the last year[1]. Alcohol misuse results in a substantial economic burden both in the country and worldwide[2]. Excessive alcohol use costs the United States nearly $250 billion each year, with 70% of these costs related to binge-drinking, more than 40% of which was paid by the government[2, 3]. Considering these statistics, both alcohol misuse and AUD results in an immense societal burden.

Alcoholism is a chronic, relapsing disorder that is vastly heterogenous in nature, with a combination of biological, environmental, and genetic factors all contributing to its development. Diagnostic criteria for this disorder are described in the Diagnostic and Statistical Manual of Mental Disorders (DSM). Historically, AUD was classified as two distinct diagnoses, alcohol dependence and alcohol abuse, depending on number of symptoms presented by an individual[4]. Released in 2013, the most recent version of the DSM (DSM-5) has integrated alcohol dependence and alcohol abuse into one spectrum diagnosis of AUD (for criteria, see Table 1.1)[5, 6]. Depending on the number

of criteria displayed, AUD diagnosis is further classified as mild, moderate, or severe. The diagnostic criteria for AUD encompass a variety of symptoms characterized by compulsivity to seek and consume alcohol, loss of control of consumption, continuation of consumption despite negative consequences, and signs of withdrawal and unpleasant symptoms in the absence of alcohol[5, 7, 8].

The negative consequences of alcoholism involve various facets of an individual's life, including health, economic, and social ramifications. Related to health, alcohol-related causes account for nearly 88,000 deaths each year, making it the third leading preventable cause of death behind tobacco use and physical inactivity/poor nutrition[9, 10]. In addition to the development of alcoholism, chronic alcohol use can contribute to the development of more than 200 different diseases including other psychiatric disorders, infectious diseases, various forms of cancer, diabetes, cardiovascular disease, and diseases of the liver and pancreas[2, 11-13]. In addition to these diseases, chronic alcohol use also increases risks of injury to oneself and others. Alcohol impairs psychomotor activity, increasing the likelihood of unintentional injuries such as car accidents[14]. Alcohol use also increases risk of intentional injuries, as AUDs associate with both suicidal ideation and attempt[15]. Further, alcohol use increases propensity to commit violent crimes, such as aggression and homicide, and therefore also increases risk of incarceration[16, 17]. Economically, individuals with AUD are more likely to miss work, be unemployed, and have financial struggles[13]. Socially, alcohol use can lead to family and relationship disruptions[13]. Together, the negative consequences resulting from alcohol use and misuse are vast and impact nearly every aspect of one's life.

1.1.2 Behavioral Models Used to Assess Alcohol-Related Behavior

Well-established, preclinical rodent models have been developed to assess alcohol-related behaviors. Behavioral paradigms that are used and discussed in this report include conditioned place preference (CPP), models of voluntary alcohol consumption, involuntary chronic alcohol exposure, and self-administration. These models will be explained here to establish a foundation for understanding and interpreting the previous findings and data presented in this report.

CPP is an assay used to assess the rewarding properties of drugs of abuse[18-21]. CPP is preformed using an apparatus that contains two chambers with visual and contextual differences (for example, black walls with grid flooring, and white walls with bar flooring). A guillotine door separates the chambers and can be opened or closed depending on the phase of the protocol. The CPP protocol consists of three phases: a pretest, a conditioning phase, and a test. During the pretest, the animal is free to roam the entirety of the chamber and time spent in either compartment is recorded. The conditioning phase involves the pairing of a drug with confinement in one of the distinct compartments of the apparatus, while the other compartment is paired with administration of a physiological inert vehicle such as saline. Following the conditioning phase, animals undergo a test session that is identical to the pretest and time spent on each side of the apparatus is recorded. The change in preference from the pretest to the test is the dependent variable. Administration of drugs with rewarding properties, such as alcohol, will result in more time spent in the drug-paired compartment compared to pretest and thus result in a positive change in preference. On the other hand, aversive drugs will result in less time in, or avoidance of, the drug-paired compartment and a negative change in preference. Many variations of this protocol exist, for example, the number of days spent in each phase of the protocol, the number of conditioning

sessions, and the length of each session. Additionally, some CPP apparatuses contain a third, neutral chamber in between the conditioning chambers that is only accessible during the pretest and test sessions. Despite these possible variations, the data is interpreted the same way and change is preference is compared between groups to provide insight on the rewarding effects of a particular drug.

Two-bottle choice (2BC) allows for the assessment of voluntary alcohol consumption. This paradigm involves the presentation of two bottles within the mouse's homecage, one containing water and one containing an ethanol solution, allowing for free access to consume alcohol. Access to the alcohol bottle can be continuous (24 hours every day) or intermittent (24 hours every other day)[22]. In 2BC, bottles are weighed at the same time each day (continuous access) or after each 24-hour alcohol access period (IEA), and gram per kilogram (g/kg) intake is calculated based on each mouse's body weight. This measurement of intake can then be compared between various treatment groups as a dependent variable. Conversely, access can be allowed for a predetermined amount of time, such as 4 weeks, in order to assess alterations following a fixed period of voluntary alcohol access. Continuous access results in consumption of sub-intoxicating amounts of alcohol[22]. On the other hand, intermittent ethanol access (IEA), which involves alcohol access on Monday, Wednesday, and Friday, induces higher levels of consumption compared to continuous access due to the alternating periods of consumption and deprivation[23-25]. IEA results in intoxicating levels of alcohol consumption with a fair amount of consistency, particularly in alcohol-preferring strains such as C57BL6/J mice[22]. In humans, binge-drinking is classified as reaching blood ethanol concentrations (BECs) over 0.08g/dl, which is also the legal limit to operate a motorized vehicle[26, 27]. While BECs obtained during continuous 2BC do not surpass 0.08g/dl, BECs near 0.1g/dl have been observed in IEA studies[25].In

addition to continuous and intermittent schedules, access to 2BC can also be restricted to a certain number of hours per day, such as 30 minutes[28] or 4 hours each day[27].

Outside of 2BC, other forms of voluntary alcohol consumption have been developed. This includes “Drinking-in-the-Dark” (DID), which involves placing a single tube with alcohol in an animal’s cage for 2 hours beginning 3 hours into the circadian dark cycle for three consecutive days[22]. Alcohol is introduced at this time because it has been shown that rodents ingest the majority of their daily food and water intake at the beginning of the dark cycle[29]. The fourth day involves a test session in which the bottle containing alcohol is available for 4 hours. This test session typically leads to intoxicating blood alcohol concentrations (BECs) over 0.1g/dl[22]. Thus, DID results in voluntary consumption of intoxicating amounts of alcohol. However, it is important to note that while DID is assessing voluntary consumption, there is only one bottle presented in the cage, and thus rodents only have one fluid to consume. When DID is performed with a bottle of water in addition to the alcohol bottle, comparable amounts of alcohol are ingested, but due to water also being consumed, BECs are lower than in the standard single-bottle test[30]. In addition to the single-bottle DID protocol, other consumption assays involve the presentation of multiple bottles of alcohol with the same[31] or increasing[32] concentrations in the animal’s homecage[27]. Interestingly, alcohol availability through multiple bottles results in higher overall consumption compared to standard 2BC.

In comparison to voluntary consumption, forced methods of alcohol administration allow for tight experimenter control over consumption levels. One method to deliver chronic, large doses of alcohol is via intragastric gavage[33]. Intragastric administration methods are preferred when using high alcohol doses in order to prevent irritation and damage of the abdominal cavity that can result from repeated

intraperitoneal injection of high doses of alcohol[34]. Chronic alcohol gavage induces high levels of intoxication comparable to that of human alcohol abusers[33]. Thus, this model of alcohol administration induces reliable BECs much higher than those obtained by voluntary 2BC and is preferred in studies assessing the effects of binge-like alcohol exposure. An additional method of chronic alcohol exposure exists and consists of placing a rodent in a vapor chamber in which ethanol is vaporized and breathed in by the subject[35-37]. Intermittent bouts of ethanol vapor exposure and alcohol consumption or self-administration sessions (discussed below) reliably induce dependence and withdrawal, thus allowing for assessment of behavior alterations during these states[22]. Following cycles of CIE, animals display escalated and increased levels of alcohol self-administration[38] and typically obtain BECs over 0.14g/dl after self-administration sessions[39]. In addition to assessing self-administration behavior after repeated vapor inhalation, another, well-established model of CIE involves 4 days of ethanol vapor or air exposure (16 hours/day, which leads to consistent BECs between 0.15-0.2g/dl), 72 hours of abstinence, then 5 days of limited access 2BC[40]. When exposed to this protocol, mice display increased alcohol consumption and consume alcohol faster than air-exposed controls after each repeated CIE cycle[40]. As a result of the increased amount and speed consumption, vapor-exposed mice display BECs nearly 2-fold higher than mice exposed to air[41]. Regardless of administration of chronic alcohol (intragastric gavage or vapor inhalation), these protocols result in higher BECs obtained from voluntary procedures and thus are relevant models of drinking patterns observed in individuals with AUDs. As such, various models of alcohol consumption have been developed, and the particular model used in a given study is selected based on the goals of the study and the type of behavior being assessed.

Self-administration is a behavioral paradigm that allows for the assessment of the reinforcing properties of and motivation to seek a drug of abuse[42]. In this operant conditioning paradigm, animals are trained to perform a response (such as a lever press or nose poke) under a certain schedule of reinforcement, meaning that responses will result in a delivery of a reward (such as alcohol) following a certain number of responses (ratio schedule) or length of time (interval ratio)[43]. Ethanol self-administration studies typically start with a period of training involving the delivery of saccharin, during which concentrations of saccharin decrease while concentrations of ethanol are introduced and increased over a certain number of days. Once the preferred concentration of ethanol is reached, animals continue self-administering until a stable level of responses is obtained. When stability is obtained, motivation for the drug can be assessed with a progressive ratio test[44]. In this test, the number of responses required to obtain a reward is increased with each reinforcer delivery. The maximum number of lever responses that an animal will exhibit to obtain a reinforcer is referred to as the breakpoint. In other words, this measurement describes how hard an animal is willing to "work" for the reward.

In addition to assessing motivation, relapse-like behavior can be assessed with the self-administration protocol[45]. After stable responding is obtained, extinction training begins in which completed responses no longer result in reward delivery. The lack of a reinforcer delivery following responses results in a decrease in responding. Once the behavioral response is extinguished, methods of reinstatement are used to induce relapse-like behavior, or the continuation of responding despite this response being previously extinguished and the lack of drug delivery. This includes drug-primed, cue-primed, and stress-induced reinstatement models, which represent various triggers of relapse in human alcoholics, such as consumption of alcohol, exposure to drug-

related environments or people, and stressful experiences[45]. Drug-primed reinstatement involves administration of a non-contingent drug injection prior to the start of the reinstatement session[46]. Early studies have determined that modest, dose-dependent reinstatement of drug seeking occurs after a priming dose of alcohol[46]. In cue-induced reinstatement models, discrete cues, such as a light or a tone, accompany drug delivery upon lever pressing during initial training[45]. Thus, the cue becomes a conditioned stimulus. During extinction, both the drug and the conditioned stimulus are removed. The cue-induced reinstatement session involves the reintroduction of the cue upon lever pressing, which has been found to increase responding in the absence of drug availability[47]. Stress-induced reinstatement involves the application of a stressor, such as intermittent footshock[46] or administration of the pharmacological stressor yohimbine[48], prior to the reinstatement session. Stress-induced reinstatement protocols reliably result in a robust increase in drug-seeking behavior following extinction[46]. Together, reinstatement models provide a platform to investigate mechanisms driving relapse-like behavior following experiences translatable to those observed in abstinent alcoholics.

1.2 The Addiction Cycle

1.2.1 The Neurocircuitry and Stages of Addiction

Addiction develops when an individual's patterns of alcohol use shifts from being impulsive to compulsive in a cycle of three stages, preoccupation/anticipation, binge/intoxication, and withdrawal/negative affect, with each stage being intensified after each complete cycle due to plasticity changes within the reward, stress, and executive function systems[7, 8, 49-52]. Drug use in the binge/intoxication stage is primarily motivated by a process called positive reinforcement, for example, drinking alcohol due to positive experiences in the past and a desire to achieve positive mood states induced

by intoxication[51]. As an individual progresses through the various stages of the cycle, drug use shifts from being impulsive and driven by positive reinforcement to compulsive and driven by negative reinforcement[51]. At this point of the addiction cycle, the individual consumes the drug to remove withdrawal effects and negative emotions associated with drug cessation. The negative affect involved in the withdrawal/negative affect stage of the addiction cycle involves dysphoria, or a depressed mood, anxiety, and irritability) and emerges as a direct result of drug abstinence[51]. Motivation to seek the drug is therefore increased to remove the unpleasant effects following drug removal, which is described as being driven by negative reinforcement. The individual then enters the preoccupation/anticipation stage, which is accompanied by the emergence of intense cravings. These cravings, in combination with the increased motivation to remove the negative side effects associated with withdrawal, are a key contributor to relapse[51, 53].

Each of the three stages of addiction are mediated by distinct brain regions and mechanisms. The binge/intoxication stage is driven by positive reinforcement from the rewarding properties of drugs of abuse[51]. At this stage, impulsive intake is mediated by the mesolimbic DA system, particularly the dopaminergic projection from the ventral tegmental area (VTA) to the nucleus accumbens (NAC)[51]. The mesolimbic DA system is the major target of addictive drugs[54, 55], which increase transmission of this pathway and release of DA in the NAC[56, 57]. DA release is also increased by exposure to stimuli associated with the drug, which motivates drug seeking and consumption[58]. This same pathway is involved in reward following natural reinforcers that are necessary for survival, such as food, sex, and social interaction[59, 60]. Compared to natural rewards, in which DA release ceases when the reinforcer is consumed or obtained, drugs of abuse increase DA release not only prior to consumption, but also during consumption, which further motivates the individual to

continue to take the drug[57]. This may explain why an individual is more likely to display compulsive use of drugs of abuse compared to natural reinforcers[57]. Therefore, drugs of abuse are known to co-opt this system designed to motivate the responses necessary for survival, and repeated consumption of a drug can result in a dampening of this circuitry[59].

The withdrawal/negative affect stage is governed primarily by the extended amygdala (AMY), which is comprised of the central nucleus of the amygdala (CEA), bed nucleus of the stria terminalis, and the medial subregion of the NAC shell[51]. During this stage, activity of the mesolimbic DA system is decreased, particularly within the accumbens[61]. During withdrawal, brain stress systems within the extended AMY, as well the hypothalamic-pituitary-adrenal (HPA) axis are activated[62, 63]. Further, the brain's anti-reward system, composed of the ventral striatum, extended AMY, and habenula, is recruited during this stage. Activation of these stress and anti-reward systems induces a negative affective state characterized by dysphoria, anxiety, emotional distress, sleep disturbance, and irritability[49].

The various symptoms of withdrawal emerge at characteristic timepoints after drug cessation[64] Three phases of withdrawal exist, called acute withdrawal, early abstinence, and protracted withdrawal, occurring for periods of 42-78 hours, 3-6 weeks, and more than three months, respectively[64]. During acute withdrawal, individuals experience hyperexcitability of the nervous system, which can lead to seizures and tremors that can be fatal[65]. In this phase, individuals seek alcohol to remove the intense physical symptoms that emerge shortly after drug consumption is stopped. In early abstinence, the physical symptoms of acute withdrawal dissipate, while the anxious, dysphoric mood and sleep disturbances occur and are experienced for a period of 3-6 weeks[64, 66]. Affective dysregulation continues throughout during protracted

withdrawal, in which individuals display strong emotional reactivity and negative affective, and experience drug cravings[67, 68]. Protracted withdrawal can last for months, even years. While studies have indicated that 70-80% of individuals relapse within the first year, the vast majority of these relapses occur after the physical symptoms of acute withdrawal have subsided[69]. However, relapse can also occur during the acute withdrawal phase, heavily driven by the physical symptoms. On the other hand, negative affect is a main contributor to relapse while an individual is experiencing early abstinence and protracted withdrawal[64].

The preoccupation/anticipation stage is characterized by an emergence of craving and a disruption of inhibitory control. During this stage, drug use transitions to being habit-like and compulsive due to conditioned drug reinforcement and altered incentive salience[51]. This shift to compulsive behavior involves NAC input to the dorsal striatum (DS), a region involved in habit formation[57]. In addition to the DS, additional regions contribute to craving, particularly the orbitofrontal cortex (OFC), prefrontal cortex (PFC), basolateral amygdala (BLA), hippocampus (HIP), and insula[51]. Components of the PFC, such as the OFC, are typically hypoactive in the absence of the drug or drug related cues, but become hyperactive following exposure to conditioned cues and as a result increases reward salience/incentive to consume the drug[70]. In addition to craving, this stage is characterized by reduced inhibitory control attributed to a disruption of cortical control circuit, composed of the cingulate gyrus, dorsolateral PFC, and inferior frontal cortex[51, 57, 71]. This system allows for the assessment of predicted and actual reward outcomes, conveying this information to control pathways and influencing the actions an individual makes[57]. Typically, activity of control circuitry is controlled by tonic DA signaling and by assessing reward outcomes, leads to proper decision-making and executive function[57]. However, signaling within this circuit is dysregulated in

addicted individuals, which leads to reduced inhibitory control and motivates drug consumption and relapse[51, 57].

Overall, progression through the various stages of addiction involves maladaptive neuroadaptations of the brain's reward, stress, and control systems, culminating in increased reactivity to drug-related cues, aversive withdrawal systems, a shift from impulsive to compulsive drug use, and loss of inhibitory control. Together, these factors work in concert to contribute to drug relapse and continuation through the cycle of addiction.

1.2.2 Neurotransmitter Responses to Acute and Chronic Alcohol

Unlike other drugs of abuse, alcohol does not act on a specific receptor or have a clearly defined mechanism of action. As mentioned in the previous section, the effects of alcohol exposure involve various sites of action and major neurotransmitter systems of the brain, including DA, serotonin, gamma-aminobutryic acid (GABA), and glutamate. Alcohol use leads to an imbalance of these systems and dysregulates the delicate balance of excitatory and inhibitory neurotransmission observed under basal conditions[52, 72, 73]. In addition, activation of the neurotransmitters corticotropin releasing factor (CRF) and norepinephrine induce the negative affect and anxiety associated with withdrawal. Together, these neurotransmitters are responsible for the effects observed at each stage of the addiction cycle discussed above.

The rewarding effects of alcohol result from both direct and indirect activation of the mesolimbic DA pathway[54, 55]. Alcohol activates this system by disinhibiting VTA dopaminergic projections to DA D_2 receptors on medium spiny neurons in the NAC[56, 57]. DA release not only increases following consumption of a drug (the reinforcer), but also following exposure to contextual or environmental stimuli associated with drug taking, which is a conditioned response resulting from a process called associative

learning[57, 74, 75]. As such, this system is also responsible for the evaluation of incentive to obtain the reinforcer and becomes dysregulated in addicted states[76, 77]. In addition to increased DA release in NAC, alcohol also increases the release of serotonin, opioid peptides, and gamma-amino butyric acid (GABA) and decreases glutamate transmission in the NAC, VTA, and AMY[51, 52, 78, 79]. The net effect of these signals indirectly increases DA release in the NAC and contribute to the reinforcing properties of drugs of abuse during the binge/intoxication stage of the addiction cycle[51, 52].

Acute alcohol exposure both increases inhibitory transmission and decreases excitatory inhibition[80]. The increased inhibitory neurotransmission is mediated by GABA, the major inhibitory neurotransmitter of the brain, particularly GABA's actions at the $GABA_A$ receptor[80]. Alcohol's actions as an agonist at the $GABA_A$ receptor[81] are responsible for the depressant effects, such as reduced anxiety and increased sedation, of alcohol consumption[72]. Additionally, a role of another inhibitory neurotransmitter glycine and the neuromodulator adenosine have also been indicated as modulators of the increased inhibitory neurotransmission following acute alcohol exposure[72]. Concurrently, alcohol dampens the activity of the major excitatory neurotransmitter, glutamate. Glutamate leads to neuronal excitation by activating both N-methyl-D-aspartate (NMDA) receptors and non-NMDA receptors, including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainite receptors[72]. Acute exposure to alcohol inhibits activity of both NMDA and non-NMDA receptors[72]. Related to the effects of alcohol exposure on memory, long-term potentiation (LTP) is fundamental process underlying memory formation and requires inhibition of $GABA_A$ receptors and activation of glutamate receptors[82]. Acute alcohol, by activating $GABA_A$ receptors and

inhibiting glutamate receptors specifically within the HIP, can also inhibit LTP and thus lead to memory impairments while under the influence of alcohol[83, 84].

Following chronic exposure to alcohol, opposing effects on GABA and glutamate transmission are observed. Chronic alcohol exposure decreases the function of $GABA_A$ receptors by decreasing expression of this receptor, while glutamate receptors adapt to the inhibitory effects by becoming hyperexcitable[72, 85, 86]. In addition to these effects, activity of adenosine is dampened, further contributing to the reduced inhibitory transmission during this state[72]. The desensitization of the $GABA_A$ receptor underlies the development of tolerance, in which increased amounts of alcohol are required to obtain a desired effect[72]. In addition to this desensitization, serotonin, norepinephrine, DA, and the hormone vasopressin all contribute to the development and maintenance of alcohol tolerance[72, 85]. The hyperexcitable state resulting from decreased $GABA_A$ function and increased NMDA signaling contributes to the physical and emotional components of withdrawal. Increased glutamate signaling leads to excess levels of calcium that can be taken up by neurons controlling movement, which in combination with decreased inhibition by GABA, contributes to withdrawal-induced seizures and physical symptoms experienced during acute withdrawal[72]. This hyperexcitable state may also contribute to the anxiety symptoms that emerge in early withdrawal[72]. During protracted withdrawal, symptoms related to negative affect emerge as a result of decreased activity of the mesolimbic DA pathway[87]. The hypodopaminergic state experienced during withdrawal results in an anhedonic state, paralleled by an increased reactivity to drug-related stimuli due to an increase in reward threshold and increased drug cravings[61, 71, 88, 89]. In addition to the effects on the DA system, CRF, norepinephrine, and dynorphin (an opioid peptide that activates the kappa-opioid receptor) activate brain stress systems within the extended AMY and the HPA axis (CRF

and norepinephrine) and anti-reward systems in the habenula (dynorphin)[62, 63, 90]. During this hypodopaminergic state, exposure to drug-related cues activates glutamatergic projections from the PFC, HIP, and BLA to the NAC and DS increases DA signaling and enhances craving for the drug[57, 73]. As such, the negative symptoms associated with the absence of alcohol, in addition to increased reactivity to drug related cues, results in increased motivation to consume the drug, thus promoting relapse and continuation through the addiction cycle.

1.2.3 Approved Treatments for AUD

Current treatments for alcoholism include both psychosocial and pharmacological therapies. Psychosocial therapies can occur in individual or group settings and are designed to help individuals with AUD learn how to recognize their alcohol-related cues, control cravings, and in some cases, reduce or maintain low levels of alcohol consumption[91, 92]. Various forms of psychosocial therapy exist, including cognitive behavioral therapy (CBT), contingency management (CM), and motivational interviewing (MI)[93-95]. The CBT approach involves teaching an individual how to assess their drug use and understand the consequences that arise from it[96]. In addition, CBT teaches important skills, such as recognizing relapse triggers, how to avoid situations where triggers may be present, and how to cope effectively if triggers are encountered[96]. This approach has strong empirical support for the treatment of AUD[97]. Another form of psychosocial treatment for AUD with strong empirical support includes CM, an approach which is based on operant conditioning and positive reinforcement[96]. CM is an incentive based approach that involves rewarding an individual for their abstinence, for example, with vouchers for money, goods, and services that are contingent on drug-free urine or breath samples[96, 98]. An additional approach, MI, involves guiding an individual to explore their personal motives for changing their behavior[99]. While this

approach has also been shown to be successful, there is stronger support for it to be used in combination with other psychosocial treatment strategies[96]. Overall, these behavioral approaches for AUD treatment have resulted in positive outcomes, but they do not work for every individual. As such, these strategies continued to be studied while new treatments are explored to help those who do not respond to the strategies discussed above. In addition to behavioral treatment, pharmacotherapies for AUD can be delivered alone, or in conjunction with psychosocial treatment.

Currently, four medications are approved by the Food and Drug Administration (FDA) for the treatment of AUD: disulfiram, naltrexone (oral and injectable formulation), and acamprosate[100]. These pharmacotherapies aim to reduce high levels of consumption in individuals with alcoholism that are unable to abstain from drinking, while also to prevent or reduce craving in order to decrease risk of relapse[101-103]. Disulfiram works to prevent alcohol drinking by impacting the metabolism of alcohol. Alcohol is primarily metabolized in the liver, where the enzyme alcohol dehydrogenase (ADH) converts alcohol to its metabolite acetaldehyde, which is in turn converted to acetate by the enzyme aldehyde dehydrogenase (ALDH)[104]. Disulfiram blocks the metabolism of acetaldehyde, resulting in accumulation of this metabolite which leads to unpleasant symptoms[105]. These symptoms include flushing, nausea, vomiting, increased heart rate, decreased blood pressure, dizziness, and headache[105], appear within 15 minutes of alcohol intake, and can last hours[106]. As these symptoms can be easily avoided by not taking disulfiram, a major issue with this medication is compliance. However, likelihood of abstinence on this treatment is increased with supervised or witnessed treatment. As such, disulfiram has been suggested as a second-line treatment for AUD behind the naltrexone and acamprosate.

Naltrexone is a nonselective opioid antagonist receptor antagonist that has been found to reduce mesolimbic opioid signaling, an effect which ultimately reduces DA levels and decreases the rewarding properties of alcohol[100, 107]. Thus, naltrexone results in decreased cravings for alcohol and decreased alcohol consumption[108, 109]. This is in contrast to acamprosate, as its therapeutic effects on consumption and relapse are due to a normalization of the dysregulated glutamatergic transmission associated with AUD via actions on NMDA receptors[100, 110]. It has also been proposed that the anti-relapse effects of acamprosate are mediated by mechanisms involving calcium, but there is not substantial evidence in support of this[111]. Clinically, both naltrexone and acamprosate have shown significant efficacy compared to placebo or abstinent groups, supporting the use of these drugs as first-line treatment for AUD[101]. Success rates of these pharmacological treatments have varied, which is likely explained by patient compliance and the negative side effects of these drugs[102].

In addition to the approved medications for AUD, several other off-label medications have been investigated for the treatment of AUD. Nalmefene, a mu- and delta-opioid antagonist used to reverse the effects of opioid drugs, has been approved for reducing alcohol consumption in Europe[100, 112]. However, studies evaluating the effectiveness of nalmefene were not comprehensive, only comparing it to placebo as opposed to an FDA AUD approved treatment[113]. Baclofen, an FDA approved GABA agonist used to treat spasticity related to multiple sclerosis, has been shown to prolong abstinence and increase time until relapse, particularly in heavy drinkers[100, 114]. Apart from those outcomes, baclofen has not been observed to decrease other outcomes such as binge-drinking. Gabapentin is an anti-epileptic medication that has received some clinical support in the treatment of AUD[100], specifically its ability to increase abstinence and decrease heavy drinking[115]. However, another study found

gabapentin to be ineffective on any additional study outcomes[116], highlighting that additional research is necessary to determine the effectiveness. Other studies have assessed the ability of topiramate, an anti-seizure, migraine, and weight loss medication, to reduce alcohol-related outcomes has been explored[100]. Topiramate treatment had a modest effect of increasing days abstinent and reducing binge-drinking days[117]. Lastly, varenicline, a partial agonist of the α4β2 nicotinic receptor that is used for the treatment of nicotine addiction, has been shown to reduce alcohol consumption in individuals that both drink and smoke heavily[110, 118]. As such, various medications have been explored for their off-label efficacy at treating alcohol-related outcome measures and have shown some promise, particualy in specific subsets of alcohlics.

Despite the available psychosocial and pharmacological treatments, the majority of individuals with AUD do not seek treatment, largely in part due to stigmatization of seeking treatment for alcoholism and psychiatric disorders in general, lack of health insurance coverage, and inconsistent insurance coverage for these treatments[119-121]. Considering the heterogenous nature of alcoholism, none of these treatments result in widespread efficacy. Thus, the efficacy of AUD treatments may improve using personalized approaches such as pharmacogenetics to tailor the treatment strategy of each individual[103, 122].

1.3 Comorbidity of Alcoholism and Depression

1.3.1 Prevalence of Alcoholism and Depression Comorbidity

Comorbidity between alcoholism and other psychiatric disorders is extremely common. According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA), a substantial portion of alcohol-dependent individuals also meet clinical criteria for depression (27.9%). Epidemiological studies have indicated that severity of AUD symptoms predicts onset of depressive disorders[123] and a history of alcohol

dependence can increase risk for depression by up to 4-fold[124]. In turn, a history of mood disorders increases risk of substance abuse[125], and severity of depression symptoms is associated with problematic alcohol use in undiagnosed patients[126]. Stressful experiences can increase motivation to consume alcohol and therefore trigger relapse in individuals with AUD[127]. The ability of stress to increase motivation to relapse can generally be explained by the anxiolytic, or anxiety-reducing, properties of alcohol, which is called the tension/stress-reducing hypothesis of relapse[72, 90, 127-132]. In addicted individuals, repeated periods of withdrawal dysregulate the HPA axis and CRF-activated stress systems, and these perturbations further contribute to the increased motivation to consume alcohol to alleviate the negative affect associated withdrawal states[62-64, 133]. Additionally, the self-medication hypothesis of alcohol, or consuming alcohol to alleviate dysphoria and negative affect, also provides a potential explanation for this relationship[130, 131, 134-136]. There are also overlapping genetic factors contributing to the development of these disorders[137, 138]. Genes that have been identified to associate with risk and/or diagnosis of both alcoholism and depression include those for alcohol dehydrogenase 1B (ADH1B) and aldehyde dehydrogenase 2 (ALDH2)[139], the β1 subunit of the $GABA_A$ receptor (GABRB1)[140], the dopamine D2 receptor (DRD2)[139], brain derived neurotrophic factor (BDNF)[141], mu-opioid receptor (OPRM1)[140], muscarinic acetylcholine receptor M2 (CHRM2)[142], and corticotropin releasing hormone binding protein (CRHBP)[140]. However, it is difficult to determine the direction of causality of the associated genes in individuals with both disorders and therefore this continues to be researched. This clinical, epidemiological, and genetic evidence suggests a bidirectional relationship between alcoholism and depression, both directions of which have been extensively studied at the preclinical level and will be discussed in the following sections.

1.3.2 The Social Defeat Stress Model

To assess the relationship of alcohol and stress responses, our lab uses the social defeat stress (SDS) behavioral model. SDS is a major preclinical model of depression that results in depressive-like behavior in rodents, including anhedonia, social avoidance, weight loss, and immune suppression[143-145]. These behavioral and physiological outcomes mirror depressive symptoms at the clinical level, as three DSM-5 criteria for depression include anhedonia, weight alterations, and a marked reduction in normal social functioning[5]. The effects of SDS are reversed following chronic, but not acute, administration of antidepressants, supporting the predictive validity of this model[146-148]. The SDS model involves the use of aggressive male CD-1 retired breeder mice that are housed on one side of a large cage that is separated in half by a clear, perforated divider. SDS involves repeated exposure to brief physical defeat sessions by a novel CD-1 mouse each day of the protocol, after which defeated mice are moved to the other side of the divider and housed overnight to the aggressor it just encountered. This allows for visual, olfactory, and auditory cues to be exchanged between the C57BL6/J mouse and the aggressor overnight. As such, SDS consists of brief physical stress and prolonged emotional stress. To assess depressive-like behavior, mice are tested for social interaction (SI) 24 hours after the final defeat session[143]. Two phenotypic subpopulations arise following the SDS protocol in mice: those displaying minimal SI and are thus “susceptible” to SDS, and those “resilient” to this stressor that display typical levels of SI[143, 145]. Following this protocol, typically 70% of mice are of the susceptible phenotype, while 30% are considered resilient to this stressor and behavior similarly to nonstressed controls. In addition to the chronic form of SDS, a variation of the SDS protocol called subthreshold SDS can be used to study sensitivity to social stress. This subthreshold protocol involves a 1-day “microdefeat” that

does not typically induce depressive-like behavior[143]. Similar to chronic SDS, mice undergo the SI test 24 hours later. Thus, this subthreshold level of defeat allows for the assessment of increases in sensitivity to SDS following pro-depressant interventions prior to this protocol[149-152].

1.3.3 The Bidirectional Relationship of Alcohol and Stress

As discussed, long-term administration of alcohol and the sudden cessation of alcohol use can lead to withdrawal states that induce feelings of dysphoria and anxiety. This negative affective state can persistent past acute withdrawal and into abstinence and protracted withdrawal, increasing motivation to drink and risk of relapse[64, 68, 90, 153-156]. To examine this phenomenon preclinically, rodents are exposed to chronic, large amounts of alcohol, typically via intragastric gavage or vapor inhalation chambers, for a predetermined amount of time. Following alcohol exposure, a stressor can be applied and alterations in sensitivity to the stressor are examined. In general, chronic alcohol exposure leads to increased stress responsivity in various animal models assessing stress sensitivity and anxiety-like behavior, including elevated plus maze[157-159], social interaction[160], open field test[159], marble-burying[159], and fear suppression and extinction[161, 162]. Additionally, many studies have demonstrated that a history of chronic alcohol not only increases stress responsivity, but also enhances the ability of stress exposure to increase post-stress alcohol consumption and self-administration[35, 158, 161, 163-166]. Related to SDS, our lab has recently demonstrated that 10 days of chronic alcohol gavage increases sensitivity to subthreshold SDS, as evidenced by a decrease in SI displayed by alcohol-exposed mice[167].

Examining the opposite direction of this relationship, stress effects on alcohol-related behaviors, has proven to be difficult in the preclinical setting[127]. While it is well

established that stress plays a pivotal role in alcohol use and AUD, the relationship is complex and is not completely understood. Many studies have evaluated the impact of stress on subsequent alcohol consumption but have yielded inconsistent results, with stress exposure increasing, decreasing, or not changing consumption depending on the specific conditions of the study. This inconsistency of results has made studying the relationship between stress and alcohol considerably challenging[127, 131]. Numerous parameters, including the type and intensity of stressor, duration of the stressor (acute or chronic), timing of alcohol availability after stress (immediate or delayed), and controllability of the stressor influence effects on consumption and underlie the inconsistent results obtained in the literature[168-170]. In general, exposure to acute stress tends to decrease or not significantly alter alcohol consumption, while chronic exposure to stress typically induces increased alcohol consumption[127, 171].

Pertinent to this report, numerous studies have assessed the effects of SDS on alcohol-related behaviors. These studies have determined that exposure to chronic SDS typically increases alcohol consumption[167, 172-180]; however, some studies have reported that SDS decreases[35, 176, 181] or did not change alcohol consumption[182]. In addition to consumption, SDS exposure has also been found to increase sensitivity to the rewarding effects of alcohol[183], increase alcohol self-administration[184, 185], and motivation to seek alcohol[176, 184]. Two additional studies found SDS decreases alcohol self-administration, but alcohol seeking behavior was assessed after a shorter duration of SDS[181, 186]. As discussed above, particular experiment design, such as intensity and duration of defeat sessions and timepoint of alcohol availability are possible explanations for the observed decreases in alcohol-related behaviors following SDS. In addition, these studies treat SDS-exposed mice as one stressed cohort in comparison to nonstressed controls. Stratifying SDS-exposed animals allows for the assessment of

differences between susceptible and resilient subpopulations, which when combined, may wash out any observable effects. Our lab studies susceptible and resilient subpopulations independently, and by doing so, we have recently shown that SDS increases voluntary alcohol consumption in specifically in mice susceptible to this stressor compared to resilient mice and unstressed controls[167]. Together, the findings of our lab and others support the bidirectional relationship between alcohol exposure and stress-sensitivity.

1.4 NFkB as a Potential Mediator of Alcoholism and Depression Comorbidity

1.4.1 The Neuroimmune System and NFkB Pathway

The neuroimmune system serves to respond to, and protect the brain from, injury, disease, and toxins. Elements of the neuroimmune system not only mediate the innate immune response to insults such as infection and injury, but also play an integral role in synaptic transmission and plasticity, gene expression, neurogenesis, and homeostasis[187, 188]. Once activated in response to events such as infection, injury, or stress, activated microglia and astrocytes within the brain release pro-inflammatory cytokines (including interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNFα)) and chemokines (including C-C Motif Chemokine Ligand 2 (CCL2), CCL5, CXCL1)[189-191]. Stimulation of these factors contribute to the activation of transcription factors such as nuclear factor kappa light chain enhancer of activated B cells (NFkB). Activation of NFkB and activator-protein 1 (AP-1) in turn induces expression of additional cytokines, cytokine receptors, and expression of toll-like-receptors (TLRs) such as TLR4, thus promoting further inflammatory signaling. The level of immune response depends on the particular insult. While high levels of inflammation can result in the recruitment of additional immune cells, tissue damage, and cell death, lower levels of inflammation can promote protective factors such as tissue repair[189].

A primary focus of this report is NFkB and components of its signaling cascade[192, 193]. NFkB is comprised of dimers of various subunits, including p65, RelB, c-Rel, p50, and p52. These NFkB subunits form heterodimer and homodimer complexes, resulting in up to 15 dimer combinations, each with specific functions. The most common of these combinations is the p65 and p50 heterodimer complex, which is responsible for transcriptional activation. Under baseline conditions, NFkB subunit dimers are bound in the cytosol by an inhibitory protein known as inhibitor of NFkB (IkB). The IkB kinase (IKK) complex consists of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (NEMO). When activated, IKK phosphorylates IkB, tagging it for proteasomal degradation and freeing the NFkB subunit dimers. TLR4, TNF-α receptor (TNFR) and interleukin-1 receptor (IL-1R) are known to stimulate NFkB through this pathway, as activation of these receptors leads to phosphorylation of IKK[194]. After freeing from the IkB complex, NFkB dimers translocate to the nucleus where they bind to specific DNA sequences in the promoter region of a wide array of genes[195]. Most prominently, NFkB stimulation triggers the expression of inflammatory mediators including cytokines, chemokines and cell adhesion molecules, thus creating a positive feedback loop. However, NFkB also regulates genes not involved in inflammation and influences processes such as responses to drugs, learning and memory, and stress responses[192]. Drug use stimulates NFkB, and genes regulated by NFkB influence alcohol- and drug-related responses, indicating this transcription factor as a key mediator of the physiological and behavioral responses to drugs of abuse.

1.4.2 The Interaction Between the Neuroimmune System, NFkB, and Alcohol

In recent decades, the neuroimmune system has gained attention for its involvement in the physiological and behavioral responses following alcohol exposure[191, 196, 197]. Alcohol has been demonstrated to activate the TLR4[198], and

similar to insults such as infection and injury, increases release of proinflammatory cytokines[199]. Acute ethanol exposure decreases microglial gene expression during intoxication, while withdrawal increases gene expression of microglial markers and other neuroimmune-related genes such as TNFα and IL-1R[200]. In addition to its physiological response to alcohol exposure, the neuroimmune system has functionally been linked to alcohol consumption. Induction of an immune response via administration of lipopolysaccharide (LPS) results in a long-term increase in voluntary alcohol intake[201]. Furthermore, animals with genetic deletion of the IL-1R display attenuated consumption of high concentrations of alcohol, but display typical alcohol place preference and increased levels in consumption levels following stress exposure comparable to that of wildtype mice[178]. Interestingly, double knockout mice lacking IL-1R and TNF-1R display attenuated alcohol consumption both at baseline and in response to stress[178], indicating an involvement of both of these receptors in the processes governing alcohol consumption. Additionally, analysis of postmortem tissue has indicated that several of the components of neuroimmune signaling are dysregulated in alcoholics, as evidenced by increased levels of activated microglia[202], TLRs[203], and IL-1β[191].

Of specific interest to this report, the role of NFkB in the underlying mechanisms of alcoholism have been suggested by studies at both the clinical and preclinical level. Clinically, polymorphisms in the NFKB1 gene are associated with increased risk of alcoholism[204] and postmortem alcohol brains exhibit evidence of NFkB dysfunction[205]. Preclinically, NFkB activity increases following alcohol exposure in both human astroglial cells[206] and rodent hippocampal slice cultures[207]. In rodents, exposure to chronic alcohol increases brain levels of NFkB[199], and in turn, NFkB mediates the immune-related effects following long-term ethanol exposure[208]. A

functional role of NFkB in alcohol-related behaviors has also been identified. Administration of lipopolysaccharide (LPS), a potent activator of NFkB activity[209], results in an increase in voluntary intake that persists for months after LPS injection[201]. Further, pharmacological inhibition and viral knockdown of IKKβ decreases both continuous and restricted access of voluntary alcohol consumption[210]. While these data identify a role of NFkB specifically in voluntary alcohol consumption, the effect of NFkB on other alcohol-related responses remains unknown.

In addition to alcohol, the involvement of NFkB in response to other drugs of abuse has been examined. Administration of cocaine[211] and morphine[212, 213] increases NFkB activation *in vitro.* Functional roles of this NFkB activation have been examined for both cocaine and morphine. Chronic administration of cocaine upregulates NFkB in the NAC[214], which was later found to be responsible for neuronal spine formation following cocaine administration[215]. In addition, viral-mediated knockdown of NFkB activity within the NAC decreased the rewarding effects of cocaine and the reward-sensitizing effects of repeated cocaine injection[215]. Furthermore, inhibition of NFkB activation in the NAC was also found to decrease the rewarding effects of morphine[216]. While NFkB has a clear impact on the rewarding effects of both cocaine and morphine, the role of NFkB in alcohol reward has yet to be elucidated.

1.4.3 Interaction Between the Neuroimmune System, NFkB, and Stress/Depression

In addition to its involvement in alcoholism and alcohol-related responses, the neuroimmune system has also been linked to disorders such as stress and depression[217-219]. Clinically, patients affected by these conditions display elevated cytokines and inflammatory markers, including TNFα, IL-1β, and IL-6, compared to healthy controls[217, 220-225], and chronic exposure to an elevated inflammatory state can contribute to the development of depression[226, 227]. Human subjects undergoing

acute stress, such as psychosocial stress, exhibit elevated blood levels of components of the immune system, including IL-6, TNFα, and NFkB[228]. Systemically administered cytokines or endotoxin (LPS) induce a depressed mood[229, 230], while anti-inflammatory drugs have been found to have potential anti-depressant effects in patients with depression[231-233]. It has also been suggested that polymorphisms in the genes encoding IL-1β, IL-6, and TNFα impact development of depression and responsivity to anti-depressants[234, 235]. Moreover, analysis of postmortem brains of depressed patients also display elevated levels of cytokines and apoptotic factors[236].

Preclinical studies have corroborated the role of the neuroimmune system in stress-related and depressive-like behaviors in rodents[237]. Activation of an immune response via LPS administration or direct injection of TNF-α and IL-1β leads to depressive-like behavior[238, 239]. It was also recently determined that peripheral levels of IL-6 following acute stress or LPS administration can predict later susceptibility to chronic SDS, while antibody-mediated or genetic knockout of IL-6 promotes resilience to SDS[225]. Additionally, learned helplessness behavior induced by inescapable footshock is associated with an increase in IL-6 expression[240]. Similar to chronic SDS, this behavior can be reversed by chronic, but not acute, antidepressant treatment[241], indicating predictive validity of using learned helplessness as a preclinical model for depression. Numerous studies have examined the role of NFkB in these behaviors which will be discussed below.

The relationship between NFkB and stress-responses has been studied extensively. In general, exposure to various forms of stress increases levels of NFkB, mirroring the clinical data discussed above. NFkB suppresses neurogenesis in the HIP following acute restraint stress[242]. NFkB is also increased following exposure to various stress paradigms: predator-scent stress[243], cold-restraint stress[244], chronic

mild unpredictable stress[242, 245, 246], and chronic restraint stress[247]. LPS, a potent activator of NFkB in the brain[209], increases depressive-like behavior such as anhedonia[239]. Moreover, infusion of a virus expressing the constitutively active form of IKK, the enzyme responsible for phosphorylation IkB and subsequent release of NFkB, into the NAC increases responses to forced swim stress and promotes anhedonia following forced swim stress[151]. Outside of rodent studies, one study found gene expression of NFkB to be elevated following dexamethasone-induced early life stress in zebrafish[248].

Relevant to this report, the NFkB pathway mediates depressive-like behavior resulting from SDS. Exposure to social defeat increases elements of the NFkB signaling pathway, including protein levels of IKK, IkB, and phosphorylated IkB, in the NAC of susceptible, but not resilient, mice[149]. Infusion of an IKK dominant-negative virus into the NAC of susceptible mice results in a reversal of social avoidant behavior, while infusion of the constitutively active form of IKK increases sensitivity to subthreshold SDS and induces reduced social interaction[149, 151]. A role of NFkB was also determined in the maladaptive alterations in post synaptic density and synaptic plasticity following SDS[149]. Considering the involvement of NFkB in alcohol-related responses, stress-responsivity, and depressive-like behavior, targeting this transcription factor may be beneficial in the development of pharmacotherapeutics for patients displaying comorbid alcoholism and depression.

1.5 The NK1R as a Potential Mediator of Alcoholism and Depression Comorbidity

1.5.1 The Substance P and NK1R

Substance P (SP) is an 11-amino-acid neuropeptide that belongs to the tachykinin family of neuropeptides[249, 250]. In addition to SP, two other neuropeptides belong to the tachykinin family, neurokinin A (NKA) and neurokinin B (NKB). These

neuropeptides act at three neurokinin receptors: neurokinin-1 receptor (NK1R), neurokinin-2 receptor (NK2R), and neurokinin-3 receptor (NK3R). While SP, NKA, and NKB preferentially bind to NK1R, NK2R, and NK3R respectively, each of the neurokinin receptors has modest affinity for each neuropeptide. In this report, we will focus on the SP/NK1R receptor system, as it has been implicated in a variety of psychiatric disorders, including addiction, stress, anxiety, and depression[251]. SP and NK1R are heavily expressed in brain regions involved in affective behavior and reward circuitry, such as the AMY, NAC, DS, hypothalamus, HIP, dorsal raphe, habenula, and periaqueductal gray[251-259]. Due to its expression throughout these regions and involvement in a myriad of psychiatric disorders, NK1R may be a mechanistic mediator of comorbid alcoholism and depression.

Interestingly, activation of the NK1R can lead to activation of the NFkB pathway. The NK1R is a G-protein coupled receptor and signals through the $G\alpha_q$ pathway[260]. Activation of the NK1R by SP activates phospholipase C, which through the second messenger diacyl-glycerol (DAG), activates protein kinase C (PKC). PKC then signals mitogen-activated protein kinases (MEKs), which activate extracellular signal-related kinases 1/2 (ERK1/2) that regulate transcription of NFkB. Thus, NK1R regulates the expression of inflammatory mediators such as cytokines and chemokines via activation of the NFkB signaling pathway[260].

1.5.2 The Relationship of the NK1R and Alcohol

The role of the NK1R in alcohol-related behaviors have been extensively studied. Preclinical studies have determined a prominent role of this receptor in alcohol consumption and seeking behavior, particularly driving alterations in these behaviors following stress exposure[250, 251, 261-269]. NK1R knockout mice display decreased consumption, an effect which is replicated following NK1R antagonism[262, 264].

Another study using viral mediated silencing of NK1R observed similar reductions in voluntary alcohol consumption[263]. Furthermore, NK1R knockout mice display a reduction in alcohol reward and their alcohol consumption is unaffected by repeated periods of alcohol access and deprivation, which typically induces escalated intake in wildtype mice[262].

Administration of NK1R antagonists attenuate escalated alcohol consumption following intermittent ethanol access[261], stress-induced alcohol intake following administration of the pharmacological stressor yohimbine[268], and stress-induced reinstatement of alcohol seeking following intermittent footshock or yohimbine exposure[266-268, 270-272]. While NK1R antagonism does not impact normal consumption or self-administration in outbred strains of rats such as Wistars and Long-Evans, it does reduce these behaviors in two alcohol-preferring rat strains (alcohol-preferring (P) and the Marchigian-Sardinian alcohol-preferring (msP) rats[266, 269, 270, 272]. These alcohol-preferring rat strains display increased drinking behaviors, which can be explained by increased expression of the NK1R in regions such as the AMY and NAC.

Compared to alcohol-naïve Wistars, alcohol-naïve P-rats display increased TACR1, the gene for the NK1R, in the PFC and CEA; however, the role of NK1R in these alcohol-related behaviors is specifically driven by the CeA[270]. The CEA, but not the PFC, displayed increased NK1R binding in P-rats compared to Wistars, further supporting its role in these behaviors[270]. Further, intracranial infusion of a NK1R antagonist into the CEA decreases the escalated self-administration and increases sensitivity to stress-induced reinstatement observed in P rats[270, 271]. We have also recently found that viral-mediated overexpression of NK1R in the CeA of Wistar rats results in an increased sensitivity to yohimbine-induced reinstatement, resulting in

behavior similar to that of P rat[273]. In addition to the CEA, the NAC also plays an important role in the NK1R-mediated effects on stress-induced reinstatement and escalated self-administration. Reinstatement-induced staining of the neuronal activation marker cFos in the NAC shell was blocked by pretreatment with a NK1R antagonist, and intra-NAC shell infusion of a NK1R antagonist prevented footshock-induced reinstatement[267].

Clinically, single nucleotide polymorphisms in the TACR1 gene has been associated with diagnosis and susceptibility to alcohol dependence[274, 275], number of symptoms, and functional magnetic resonance imaging (fMRI) response to alcohol cues[276]. Additionally, administration of a NK1R antagonist to abstinent alcoholics results in reduced spontaneous alcohol cravings and stress-induced cravings, improved well-being, and reduced cortisol response to stress[264]. This study also demonstrated that the NK1R antagonist decreased insula activity in response to viewing negative images, and increased activation in the striatum in response to positive images[264]. Together, this preclinical and clinical data indicates an important role of the NK1R in the processes underlying alcohol-related behaviors, and in particular, alterations in these behaviors following stress exposure.

1.5.4 The Relationship of the NK1R and Stress/Depression

In addition to studies examining the NK1R in alcohol- and drug-related behaviors, the role of this receptor in the underlying mechanisms of stress, anxiety, and depression has also been closely examined[211, 250, 277, 278]. Both systemic injection of SP, NK1R agonists, or infusion of SP directly into the brain increases anxiety-like behavior in the elevated plus maze, indicated by a decrease in open arm entries and an increase in time spent in the closed arms of the maze[279, 280]. SP is increased in the medial AMY following immobilization stress and elevated platform exposure[281]. In this study,

immobilization stress increased anxiety-like behavior in the elevated plus maze, which was blocked by administration of a NK1R antagonist, which, in addition to genetic deletion of NK1R, generally reduces anxiety-like responses in this test[280, 282, 283]. In a study using another anxiety assay, novelty-suppressed feeding, genetic knockdown of NK1R also results in an anxiolytic effect and decreases time to approach the food pellet[282]. SP is elevated in the locus coeruleus[284] and lateral septum[285] following forced swim stress, and the former was attenuated by both systemic administration and infusion of a NK1R antagonist into the lateral septum. Additional evidence in support of anxiolytic effects of NK1R antagonist have been displayed in studies evaluating this receptor's involvement in alleviating anxiety-like behavior associated with maternal separation[273, 282, 286-288], exploratory behavior[282], marble burying[287], and social interaction[287, 289-292]. Similar to its role in anxiety-like behavior, this receptor is also involved in fear-related behavior, as it mediates fear potentiated startle[293], fear conditioning[294], and acoustic startle response[295].

Related to depressive-like behavior, systemic administration NK1R antagonists decrease immobility time and increase time climbing in the forced swim test, an assay for anti-depressant efficacy[296]. Additionally, our lab has recently shown that anhedonia resulting from LPS administration is prevented following NK1R antagonism[239]. Chronic mild stress, which leads to depressive-like behavior, increases SP in the lateral habenula[297], while systemic[298] and local infusion of a NK1R antagonist into the lateral habenula[297] prevented depressive-like behavior following this stressor. Furthermore, genetic deletion of NK1R reduces the behavioral outcomes of bulbectomy, which induces depressive-like behavior and physiological alterations in rodents[299]. Pertinent to this report, our lab has recently reported that mice susceptible to chronic SDS display increased expression of TACR1 in the NAC[167]. Together, these findings

provide strong evidence that NK1R is a key mediator of anxiety-like behavior, stress responses, and depressive-like behavior.

1.6 Summary

Based on the evidence described above, it is well-established that both NFkB and the NK1R are heavily involved in the processes underlying alcoholism, depression, and the comorbidity between the two. However, there are various gaps in the literature that we aim to fill with the data presented in this report. For example, while NFkB is known to mediate the rewarding properties of morphine and cocaine, the involvement of NFkB in alcohol reward is unknown (Chapter 2). Additionally, while the effects of chronic, forced alcohol administration on stress sensitivity are numerous, the impact of voluntary alcohol consumption on stress sensitivity has not been as extensively studied (Chapter 3). Finally, the NK1R is intricately involved in various alcohol- and stress-related responses, but the effects of NK1R antagonism on the behavioral alterations following chronic SDS have yet to be elucidated (Chapter 4). As such, this report contains three separate, but related, data chapters examining important gaps in the literature discussed above. The results of these studies will be related back to the therapeutic potential of targeting NFkB and NK1R to treat alcoholism, depression, and the comorbidity between the two.

Table 1.1 DSM-5 Criteria for Alcohol Use Disorder (AUD).

In the past year, have you:
• Drank more and for a longer period than you intended?
• More than once, wanted to decrease or stop drinking alcohol, or tried to cut back, but couldn't?
• Spent a significant amount of time drinking or recovering from drinking?
• Experienced cravings for alcohol?
• Noticed that drinking or recovering from drinking was interfering with important aspects of life, such as at home, work, or school?
• Continued drinking despite the negative consequences at home, work, or school?
• Decreased or stopped participating in activities that were previously important, interesting, and pleasurable to you in order to have more time to drink?
• Been in a situation at least once, during or after drinking, that increased risk of being harmed or injured?
• Continued to drink despite anxious and depressed feelings, negative effects on other medical conditions, or after a memory blackout?
• Developed tolerance, in that you need to drink more than you once needed to reach your desired effect, or your normal amount of alcohol is less effective than before?
• Experienced signs of physical signs of withdrawal?
2+ criteria indicates an Alcohol Use Disorder (AUD) diagnosis, further categorized as: Mild (2-3 criteria), Moderate (4-5 criteria), Severe: (6+ criteria)

CHAPTER 2:

SELECTIVE LESIONING OF NUCLEAR FACTOR KB ACTIVATED CELLS IN THE NUCLEUS ACCUMBENS SHELL ATTENUATES ALCOHOL PLACE PREFERENCE[1]

[1]Nennig, S.E., Fulenwider, H.D., Chimberoff, S.H., Smith, B.M., Eskew, J.E., Sequeira, M.K., Karlsson, C., Liang, C., Chen, J.F., Heilig, M., & Schank, J.R. Neuropsychopharmacology, 2018: **43**(5): p. 1032-1040.

Abstract

Nuclear factor κ light chain enhancer of activated B cells (NFkB) is a transcription factor commonly associated with innate immunity and is activated by infection and inflammation. NFkB has recently gained attention as a mediator of complex psychiatric phenomena like stress and addiction. In regard to alcohol, most research on NFkB has focused on neurotoxicity, and few studies have explored the role of NFkB in alcohol reward, reinforcement, or consumption. In these studies, we used conditioned place preference to assess the activity of NFkB in response to rewarding doses of alcohol. To measure NFkB activity we used a line of transgenic mice that express the LacZ gene under the control of an NFkB regulated promoter. In these animals, staining for β-galactosidase (β-gal) identifies cells in which NFkB has been activated. We then used the Daun02 inactivation method to specifically silence NFkB expressing cells during place preference conditioning. Daun02 is an inactive prodrug that is converted to the inhibitory molecule daunorubicin by β-gal. After alcohol place conditioning, we observed increased β-gal staining in the nucleus accumbens (NAC) shell and dorsal raphe nucleus (DR) and found that disruption of NFkB expressing cells using Daun02 attenuated the development of alcohol place preference when infused into the NAC shell following conditioning sessions. We found this effect to be regionally and temporally specific. These results suggest that, in addition to its role in alcohol-induced neurotoxicity, NFkB mediates the development of alcohol place preference via its actions in the NAC shell.

2.1 Introduction

Recently, the impact of the neuroimmune system in behavioral and neurophysiological responses to alcohol has gained much attention[191]. For example, the transcription factor nuclear factor kappa light chain enhancer of activated B cells (NFkB), best known for orchestrating the neuroimmune response[193], is activated by alcohol administration. Specifically, prolonged exposure to alcohol increases NFkB-DNA binding levels in conjunction with elevated cytokine expression[199, 207]. However, the majority of preclinical research on NFkB has examined its role in neurotoxicity[300, 301], particularly when induced by high concentrations of alcohol[207, 302-304]. Virtually nothing is known about the effect of moderate concentrations of alcohol on NFkB function, and whether NFkB activity contributes to the behavioral effects of alcohol at such doses.

Drugs of abuse such as opiates and psychostimulants activate NFkB in the brain, influence spine formation in response to chronic drug exposure, and mediate the rewarding properties of these drugs[212, 215, 216]. These effects are not likely to be due to drug-induced neurotoxicity and some of these effects have been localized to the nucleus accumbens (NAC). The NAC is part of the mesolimbic reward circuit that consists primarily of dopaminergic neurons projecting from the ventral tegmental area to the NAC, which is a major target of drugs of abuse including alcohol[305, 306]. Given the role of NFkB in reward for these other substances and the ability of alcohol to stimulate NFkB, we hypothesized that NFkB is involved in the rewarding properties of alcohol as well. Along these lines, a few studies have examined the role of NFkB in voluntary alcohol intake. For example, administration of lipopolysaccharide (LPS), a cell wall component of gram negative bacteria that stimulates an inflammatory response and

increases NFkB activity[209], results in a persistent increase alcohol consumption[201]. Conversely, alcohol intake is attenuated by inhibition of IKK, a kinase involved in NFkB activation[194, 210]. Taken together, these findings support the notion that NFkB positively modulates alcohol reward value and leads to increased consumption.

In these studies, we assess NFkB activity using transgenic reporter mice that express LacZ under the control of an NFkB regulated promoter. Thus, wherever NFkB is stimulated, activity-dependent β-galactosidase enzyme (β-gal) will be expressed, providing a method for visualization of NFkB activity[307]. To achieve highly targeted lesioning of cells in which NFkB is stimulated, we used the novel Daun02 inactivation method[308]. Daun02 functions as an inactive prodrug that is catalytically converted to its active state, daunorubicin, in the presence of β-gal[309]. Daunorubicin then permanently destroys the β-gal expressing cells through apoptotic mechanisms[310]. This method of inactivation has been used in a series of studies by Hope and colleagues using Fos-LacZ transgenic rats to study neuronal ensembles involved in drug-induced behaviors[310-316].

In the present study, we set out to characterize the functional involvement of NFkB within specific brain regions in alcohol conditioned place preference (CPP). In our initial experiments, we found that NFkB activity was specifically increased in the NAC shell and dorsal raphe nucleus (DR) after alcohol conditioning, but not following testing for place preference. This fits well with the literature demonstrating a role for NFkB in learning and memory formation[317]. Therefore, we targeted these regions using the Daun02 method following alcohol conditioning sessions in attempt to disrupt the development of alcohol CPP.

2.2 Materials and Methods

Animals

Male and female NFkB-LacZ mice[215, 307] on a C57BL6/J strain background ages 3-6 months were used. Mice were group housed on a normal light cycle (lights on 7:00 AM, off 7:00PM) in the UGA College of Veterinary Medicine vivarium. Testing took place during the light phase. Food and water was available *ad libitum*. All protocols were approved by Institutional Animal Care and Use Committee of the University of Georgia and experiments were performed in accordance to these guidelines.

Drugs

Ethanol was diluted to a 20% v/v solution in 0.9% saline and delivered intraperitoneal (IP) at a dose of 2.0g/kg or 1.5g/kg depending on experiment. Volume-matched saline injections were administered to control animals. Daun02 (ApexBio Technology, Houston TX) was prepared in 20% DMSO, 5% Tween 80, 75% 0.01M PBS to a final concentration of 3.3µg/µl.

CPP

CPP was used to assess the rewarding properties of specified doses of alcohol. The two compartment CPP chambers (Med Associates, Fairfax VT) had one side with white walls/grid flooring while the other side had black walls/bar flooring. CPP chambers were located within a testing suite in the vivarium. Mice were habituated to the testing room for an hour prior to the start of testing. During pretest, mice were allowed to roam freely in the apparatus for 15 minutes. The compartment in which the mice were initially placed was random and alternated between the white and black sides. Groups were formed using an unbiased and counterbalanced design. Specifically, half of the animals received alcohol on their preferred side and half on their non-preferred side, and half of

the animals received alcohol on the black side and half on the white side. For saline-saline controls, the afternoon conditioning side was balanced in the same way. Conditioning occurred for 3 consecutive days, with saline injections in the morning and ethanol injections (2g/kg or 1.5g/kg) 4 hours later. The test session was conducted 1 or 3 days following the third day of conditioning (see experimental timelines) and took place at a time of day halfway between conditioning sessions. During the test, mice were placed into the chambers without pretreatment in a randomized, alternating pattern (black vs. white). Mice were allowed to roam the apparatus freely for 15 minutes. If specified, an additional 15-minute retest was performed. Preference scores were calculated by subtracting the amount of time spent on the saline paired side from the amount of time spent on the alcohol paired side.

Immunohistochemistry

In these experiments, we used a line of NFkB-LacZ reporter mice which allows for the visualization of NFkB activation via staining for β-gal. Two hours following the third alcohol (2.0g/kg) conditioning session, mice were overdosed with ketamine/xylazine and were transcardially perfused with 4% paraformaldehyde. Brains were postfixed overnight in 4% paraformaldehyde, transferred to 30% sucrose until the tissue sank, then frozen quickly on powdered dry ice. Thirty-micron sections were collected in a freezing cryostat (Leica, Buffalo Grove IL) and floating sections were frozen in cryopreservant at -20°C. After washing, tissue was incubated for 48 hours with chicken anti-β gal (1:1000, Abcam; Cambridge MA) at 4˚C, followed by incubation with biotinylated goat anti-chicken secondary (1:500, Vector Labs, Burlingame CA), and visualization with DAB chromagen. Stained sections were then mounted onto glass microscope slides and cover-slipped. Images were taken on a Zeiss Axioscope A1 and

quantified using ImageJ. For NAC shell, images from 6 fields (3 per side) were taken at 40x magnification, and the average of these fields was used as the dependent measure. For DR, 1 field was imaged at 20x magnification and used for quantification. Cell counts were completed by an investigator blind to experimental conditions.

Immunofluorescence

To assess the activation of NFkB in neurons, we co-labeled for β-gal and the neuronal marker (NeuN) in the NAC shell and DR. After washing, fixed tissue was stained with both chicken anti-β gal (1:1000, Abcam, Cambridge MA) and rabbit anti-NeuN (1:500, Millipore, Temecula CA) for 48 hours at 4°C. Fluorescent secondary antibodies used were goat anti-chicken Alexafluor 488 and goat anti-rabbit Alexafluor 633 (1:500, Life Technologies, Carlsbad CA). Stained sections were then mounted onto glass microscope slides and cover-slipped. Images were taken on a Zeiss L710 confocal microscope (40x for NAC and 20x for DR, as above) and quantified using ImageJ.

Cannula Implantation

In order to deliver Daun02 intracranially, mice underwent cannulation surgeries. Mice were anesthetized using a mixture of ketamine (100mg/kg) and xylazine (20 mg/kg). Cannulae (Plastics One, Roanoke VA) cut to 4mm in length were stereotaxically implanted (Stoelting, Wood Dale IL), aimed at coordinates (relative to bregma in flat skull position) for the NAC shell (bilateral, AP: +1.7 mm, ML: ± 2.3 mm, DV: -3.7 mm; 20º angle) or DR (unilateral, AP: -4.4 mm, ML: ± 1.2 mm, DV: -3.0 mm; 22º angle). Injectors were 5mm in length and extended 1mm past guide cannulae. Coordinates accounted for 1mm extension. Dummy caps were inserted to protect the cannulae. Mice were injected with 5mg/kg carprofen immediately following surgery and for 2 days thereafter. Mice were allowed 7 days to recover before behavioral testing started. At the completion of

each experiment, cresyl violet was delivered via the cannulae and brains were sectioned to ensure proper placement. Animals in the NAC shell experiments were excluded from the study if both cannulae were misplaced. Behavioral data for mice with one or two accurate NAC shell placements were compared statistically, and no significant differences were found. Cannula placements are shown in Figure 2.1A (NAC) and Figure 2.1B (DR).

Intracranial Infusions

Injector needles were connected via polyethylene tubing to a 10μL gastight Hamilton syringe (Reno, NV) and secured to a programmable syringe pump (New Era Pump Systems, Farmingdale NY). For Daun02 experiments, Daun02 (2μg) or vehicle was intracranially infused in a volume of 0.6 μl at a rate of 0.5μL/minute. Injectors stayed in place 1 minute following the completion of the infusion. Daun02 dose was based on the study by Koya and colleagues which resulted in selective disruption in fos-LacZ rats[316]. Mice were infused with Daun02 or vehicle 2 hours following each alcohol conditioning session due to our immunohistological results, which indicated a difference in NFkB activation 2 hours following the final alcohol or saline conditioning session.

TUNEL Assay

Finally, to confirm that Daun02 was resulting in apoptosis of NFkB-activated cells, a TUNEL assay was used. Brains were extracted and fixed in 4% paraformaldehyde at 4 degrees overnight. After washing with PBS three times, fixed brains were cryoprotected in 25% sucrose at 4 degrees for 2 days and then frozen on dry ice. Fourteen-micron sections were collected in a freezing cryostat. The apoptotic cells in situ were detected by the indirect TUNEL methods as described in the ApopTag Fluorescein In Situ Apoptosis Detection Kit (S7110, Millipore). All sections were counter

stained with 1ug/ml Hoechst at room temperature for 20 minutes to label nuclei. TUNEL positive cells were quantified with ImageJ software.

Statistics

Statistical analyses were performed using Statistica software. Tests for main effects were performed using t-test (if 2 groups across 1 factor) or two-way ANOVA. When appropriate, post-hoc comparisons were performed using Newman Keuls tests. For each experiment, data for males and females were compared and no statistical differences were found so data was combined.

2.3 Results

Alcohol CPP increases NFkB activity in the NAC shell and DR.

Mice were conditioned with 2.0 g/kg ethanol (n=19) and preference for the alcohol paired side was assessed (Figure 2.2A). Control animals received saline on both sides of the CPP apparatus (n=12). Two-way ANOVA revealed main effects of test session (pretest versus test; $F(1,29)=7.2$, $p=0.01$) and treatment (saline versus alcohol; $F(1,29)= 7.9$, $p=0.009$) as well as a test session x treatment interaction ($F(1,29)=6.6$, $p=0.02$). Mice conditioned with alcohol spent more time on the alcohol paired side when compared to their pretest as well as when compared to saline conditioned animals on test day ($p<0.01$ for all comparisons; Figure 2.2B).

In a parallel cohort of animals, mice (n=7/group) were conditioned with 2.0 g/kg ethanol or saline and sacrificed 2 hours following the final conditioning session (see Figure 2.2A). We chose this timepoint because NFkB has been shown to mediate learning and memory processes, and we hypothesized that this transcription factor would be involved in the formation of alcohol CPP. Tissue was stained for β-gal in several regions including the NAC shell, NAC core, DR, insula, lateral septum, central

amygdala (CEA), basolateral amygdala (BLA), and bed nucleus of the stria terminalis (BNST). When compared to animals conditioned with saline, animals receiving alcohol conditioning had significantly increased expression of β-gal in the NAC shell ($t(9)=2.3$, $p=0.04$; Figure 2.2C-D) and DR ($t(10)=3.9$, $p=0.003$; Figure 2.2E-F). There were no significant differences between saline and alcohol conditioned animals in the NAC core, insula, lateral septum, CEA, BLA, and BNST.

To ensure that the differences in NFkB activation observed during alcohol CPP were not solely due to the pharmacological effects of alcohol, mice (n=6/group) received the same injection protocol as CPP but remained in their homecages for the duration of the experiment. Animals received 3 days of injections of saline in the morning and alcohol (2.0g/kg, 20%, v/v) in the afternoon 4 hours apart and remained in their respective homecage throughout the protocol. Controls received saline at both injection time points. Two hours following the final injection, animals were perfused and tissue was stained for β-gal expression. No significant difference was found between alcohol and saline treated animals for NFkB-activated cells in the NAC shell (data not shown; mean ± SEM, saline: 24.7 ± 7.4, alcohol: 23.3 ± 3.1; $t(10)=0.17$, $p=0.87$). NFkB activity was not detected in the DR of the same subjects, suggesting that the activity seen is contingent upon exposure to the CPP apparatus.

Next, we further characterized NFkB activation via double-labeling immunohistochemistry (Figure 2.2G-H). Brain sections from the NAC shell and DR obtained from mice that underwent alcohol place conditioning were stained for β-gal and the neuronal marker NeuN (n=5-6/region). Most β-gal expression colocalized with the neuronal marker NeuN, with 60% overlap in the NAC shell (Figure 2.2G) and 88% overlap in the DR (Figure 2.2H). In the NAC shell, neurons colocalized with β-gal

accounted for 16% of the total neuronal population, indicating a relatively selective set of neurons expressing NFkB.

Specific lesioning of NFkB expressing cells in the NAC shell disrupts the strength of alcohol CPP.

Next, we aimed to selectively lesion NFkB expressing cells within the NAC shell and DR of NFkB-LacZ reporter mice via Daun02 inactivation (see Figure 2.3 for schematic of Daun02 inactivation method). Animals received Daun02 or vehicle into the NAC shell or DR 2 hours following each alcohol conditioning session. This time point was consistent with measurements of β-gal expression as shown in Figure 2.2. A delay of three days was included before assessing the change in preference to ensure the Daun02 had adequate time to lesion the cellular population of interest[310]. The day following this initial test session, an additional test session was performed to examine retention of the place preference (“retest” session; Figure 2.4A).

When infused into the NAC shell following each alcohol conditioning session, Daun02 did not have an effect on initial test day preference; however, preference towards the alcohol paired side during the retest session was reduced (n=14/group; Figure 2.4B). Two-way ANOVA revealed a main effect of test session ($F(2,52=31.3$, $p<0.001$) and a test session x treatment interaction ($F(2,52)=6.3$, $p=0.004$). Post-hoc tests indicated that there was a significant increase in preference for the alcohol-paired side on test day relative to pretest day for both treatment groups ($p<0.001$). This increased preference persisted on retest day for both control mice ($p<0.001$ for pretest versus retest) and the Daun02 infused group ($p=0.02$). There was a trend level decrease in preference score on retest day when compared to test day for the Daun02-treated group ($p=0.06$), but not for the vehicle-treated group ($p=0.16$). More permissive Fisher

LSD tests indicated that retest preference was significantly lower in the Daun02 group compared to their test preference ($p=0.02$) and also as compared to retest preference in vehicle-treated mice ($p=0.04$). In the vehicle group, preference did not decrease between test and retest ($p=0.07$). If anything, the preference was slightly increased in this group on the retest day.

No differences in test or retest preference were observed when Daun02 ($n=10$) or vehicle ($n=8$) was administered into the DR following conditioning sessions (Figure 2.4C). Two-way ANOVA revealed only a main effect of test session ($F(2,32)=12.5$, $p<0.001$). Post hoc tests across the factor of session indicated that preference was significantly higher on both test day ($p<0.001$) and retest day ($p<0.001$) when compared to pretest day. There was no difference between test and retest ($p=0.46$).

Given the results of the experiments above, we focused on the NAC shell for the remainder of the study. Next, we assessed if NFkB activity is induced during the initial test session and if disruption of these cells is sufficient to impact retention of alcohol CPP. NFkB-LacZ mice were conditioned with 2.0g/kg ethanol and assessed for place preference. Daun02 ($n=15$) or vehicle ($n=13$) was infused into the NAC shell 2 hours following the test session (Figure 2.5A). Daun02 did not significantly impact retest preference during the retest session three days later (Figure 2.5B). Two-way ANOVA detected only a main effect of test session ($F(2,54)=23.82$, $p<0.001$). Post hoc tests across the factor of session indicated that preference was significantly higher on both test day ($p<0.001$) and retest day ($p<0.001$) as compared to pretest. There was no difference between test day and retest day ($p=0.23$). To assess NFkB activity on the CPP test day NFkB-LacZ mice were conditioned with saline ($n=4$) or 2.0g/kg ethanol ($n=4$) and β-gal expression was assessed. No difference in NFkB activated cells in the

NAC shell was found (data not shown; mean ± SEM, saline: saline: 24.7 ± 12.0, alcohol: 23.5 ± 11.8; t(6)=0.07, p=0.9457). This suggests the role of NFkB in the NAC shell in alcohol CPP is temporally specific to the conditioning phase and is not activated during the CPP test session. Together, these findings suggest that NFkB in the NAC shell is not activated on the CPP test day.

Specific lesioning of NFkB expressing cells in the NAC shell attenuates CPP to moderate dose of alcohol.

The results above suggest that lesioning NFkB activated cells in the NAC shell during the development of alcohol CPP weakened the reward memory. However, this effect was only observed on the retest day and not the initial test day. We suspected that the CPP paradigm was not sensitive enough to pick up a decreased preference to this dose of alcohol on the test day, only when the mice were retested. To test this hypothesis, we conditioned mice with a lower dose of alcohol (1.5 g/kg) and infused them with Daun02 (n=7) or vehicle (n=5) in the NAC shell on either conditioning days (Figure 2.6A) or test day (n=14 Daun02 treated, n=11 vehicle treated; Figure 2.6B).

First, Daun02 or vehicle was infused into the NAC shell 2 hours following each conditioning session with 1.5 g/kg alcohol (Figure 2.6A). Three days later, animals underwent the test session. Animals receiving Daun02 displayed significantly attenuated preference towards the alcohol paired side on the test day compared to controls (Figure 2.6C). Two-way ANOVA revealed a main effect of test session ($F(1,10)=14.5$, $p=0.003$) and a test session x treatment interaction ($F(1,10)=5.3$, $p=0.04$), and a trend level main effect of treatment ($F(1,10)=3.1$, $p=0.11$). Posthoc tests indicated that test day preference was increased relative to pretest for controls ($p=0.004$) but not for Daun02 treated mice ($p=0.55$). Additionally, test day preference was significantly lower in the

Daun02 group when compared to the control group ($p=0.02$). Thus, selectively lesioning NFkB expressing cells in the NAC shell during the conditioning phase impairs the development of alcohol CPP.

To assess the role of NFkB during the test session, animals were conditioned with 1.5g/kg alcohol and CPP was assessed the next day. Daun02 was infused into the NAC shell 2 hours following the test session, and animals were allowed to recover for 3 days (Figure 2.6B). After the recovery period, animals were retested. No differences were observed between Daun02 and control groups (Figure 2.6D). Two-way ANOVA revealed only a main effect of test session ($F(2,23)=27.05$. $p<0.001$). Posthoc tests across the factor of test session indicated that preference for the alcohol paired side was higher than pretest preference on both test day ($p<0.001$) and retest day ($p<0.001$). There was no difference in preference between test day and retest day ($p=0.65$). These results further corroborate that NFkB in the NAC shell has a temporally specific role on preference formation, as inactivation of NFkB expressing cells during the conditioning phase, but not following the test session, attenuates alcohol CPP.

Daun02 inactivation results in apoptosis of NFkB expressing cells in the NAC shell.

Finally, to confirm the findings of Pfarr and colleagues[310] that Daun02 induces apoptosis of β-gal expressing cells, and that this occurs in the NAC shell with our treatment schedule, mice were conditioned with 1.5 g/kg ethanol and received Daun02 ($n=6$) or vehicle ($n=4$) into the NAC shell 2 hours following each alcohol conditioning session. Mice were sacrificed 3 days following the final conditioning session, the same time point at which preference was assessed above (Figure 2.6A). Tissue was collected and analyzed for apoptosis via TUNEL assay (Figure 5E-F). Daun02 treated animals displayed significantly more TUNEL positive cells in the NAC shell compared to controls

(p=0.026). This confirms that Daun02, when infused into the NAC shell of NFkB-LacZ mice following alcohol place conditioning, results in apoptosis of NFkB expressing cells and removal from the functional circuitry of alcohol CPP.

2.4 Discussion

The primary finding from these studies is that NFkB activity, specifically in the NAC shell, mediates the development of alcohol CPP. NFkB activity was increased in this region 2 hours following alcohol conditioning sessions, and specific lesioning of these cells attenuates the strength of alcohol CPP. When we used a high dose of alcohol, we did not detect blunted CPP on the initial test day, only on retest day when there was a longer delay between conditioning and testing. However, when we used a lower dose of alcohol, we saw effects on the initial test day, suggesting that this dose of alcohol was strong enough to induce CPP in controls, but was not so strong as to resist manipulation by disruption of NFkB expressing cells. However, we did not see any effect when we selectively lesioned these cells following the test session at either dose. This suggests that NFkB is required for the development of alcohol CPP, but is not likely to be reactivated during the expression of place preference, at least not in the NAC shell.

Previous research has demonstrated NFkB activation in response to very high doses of alcohol and suggests that NFkB is a critical mediator of neurotoxicity that results from such challenges[199, 207, 302]. In general, NFkB has a reputation for being associated with cellular stress and neurodegeneration. However, NFkB has many functions beyond these, including a role in learning and memory[317, 318], drug reward[216], and spine remodeling in response to chronic drug exposure[215]. Based on these functions of NFkB, we suspected that this transcription factor may have a role in the rewarding properties of alcohol. Consistent with our hypothesis, we found that NFkB

activity is in fact induced by moderate, rewarding doses of alcohol, and NFkB activated cells play a functional role in the development of alcohol CPP. To our knowledge, this is the first demonstration that NFkB plays a role in the positive, rewarding effects of alcohol. Additionally, this is the first study to use the Daun02 inactivation method in NFkB-LacZ reporter mice.

Our results indicate regionally localized NFkB activation during alcohol CPP. We analyzed several regions that are involved in reward processing and alcohol seeking, and only the NAC shell and DR showed significant NFkB activation relative to saline treated controls. Furthermore, this activation specifically depended on alcohol exposure *and* introduction to the CPP chamber/procedure. We found that, under the conditions we used for testing, NFkB activity in the DR did not play a functional role in the development of alcohol CPP. However, we cannot rule out that the DR plays some role in alcohol CPP, but was not revealed in the limited analysis performed.

It is intriguing that we observed such a strong activation of NFkB in neurons. Based on the extensive role that NFkB plays in neurological functions and behavioral processes[149, 151, 215, 242], we would fully expect some level of activation in neurons, but it was unexpected that this would be the majority of the NFkB activated cells in the DR and NAC shell. This is, however, consistent with some recently published findings[210]. It is possible that the proportion cell types (neuronal versus glial) where NFkB is activated by alcohol can be influenced by a complex set of variables including dose, exposure regimen, brain region, and homeostatic state of the animal.

Taken together, our experiments support a role of NFkB in alcohol CPP and localize this effect specifically to the NAC shell. This activation occurs primarily in neuronal cell types and selective disruption of NFkB expressing cells attenuates the

development of alcohol CPP. We speculate that NFkB activation during place conditioning triggers specific gene expression that contributes to the formation of the reward memory. However, by the time of testing, these cellular alterations have taken place, and NFkB is not reactivated. The specific genes that are targeted during alcohol CPP are unknown. NFkB has extremely diverse gene targets[192], and a major focus of future research will be the identification of the genes that link NFkB activation by alcohol to the behavioral output of CPP.

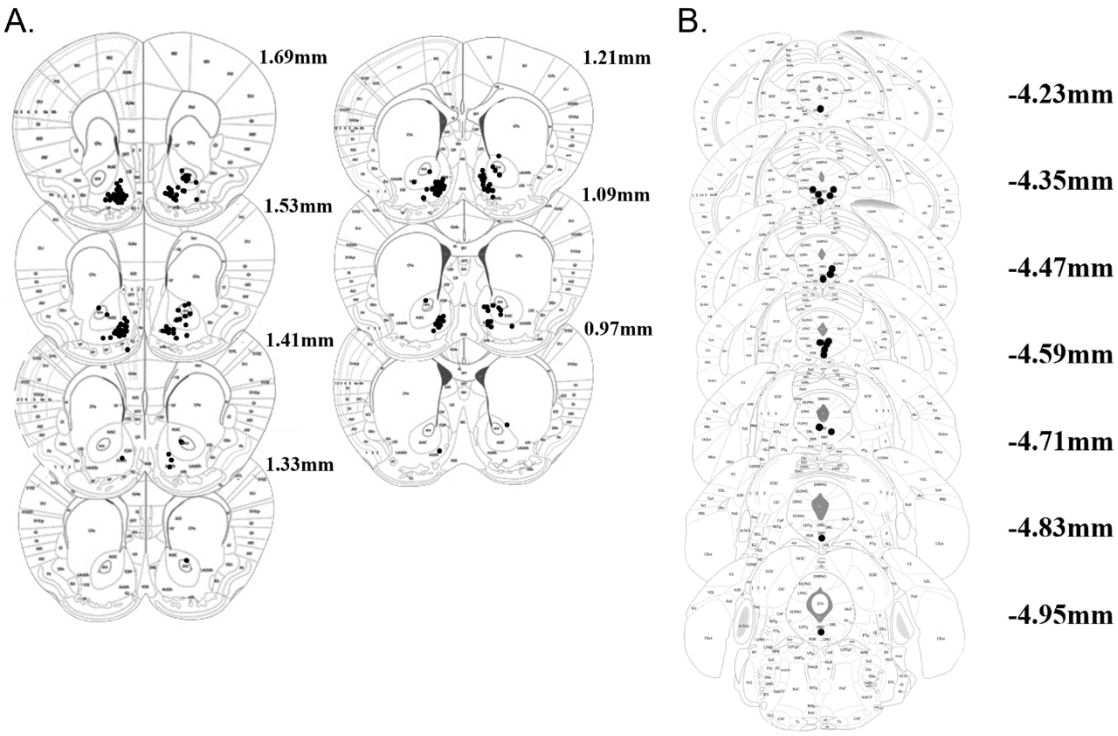

A.
1.69mm
1.53mm
1.41mm
1.33mm
1.21mm
1.09mm
0.97mm
B.
-4.23mm
-4.35mm
-4.47mm
-4.59mm
-4.71mm
-4.83mm
-4.95mm

Figure 2.1: Cannula placements for the NAC shell (A) and DR (B).

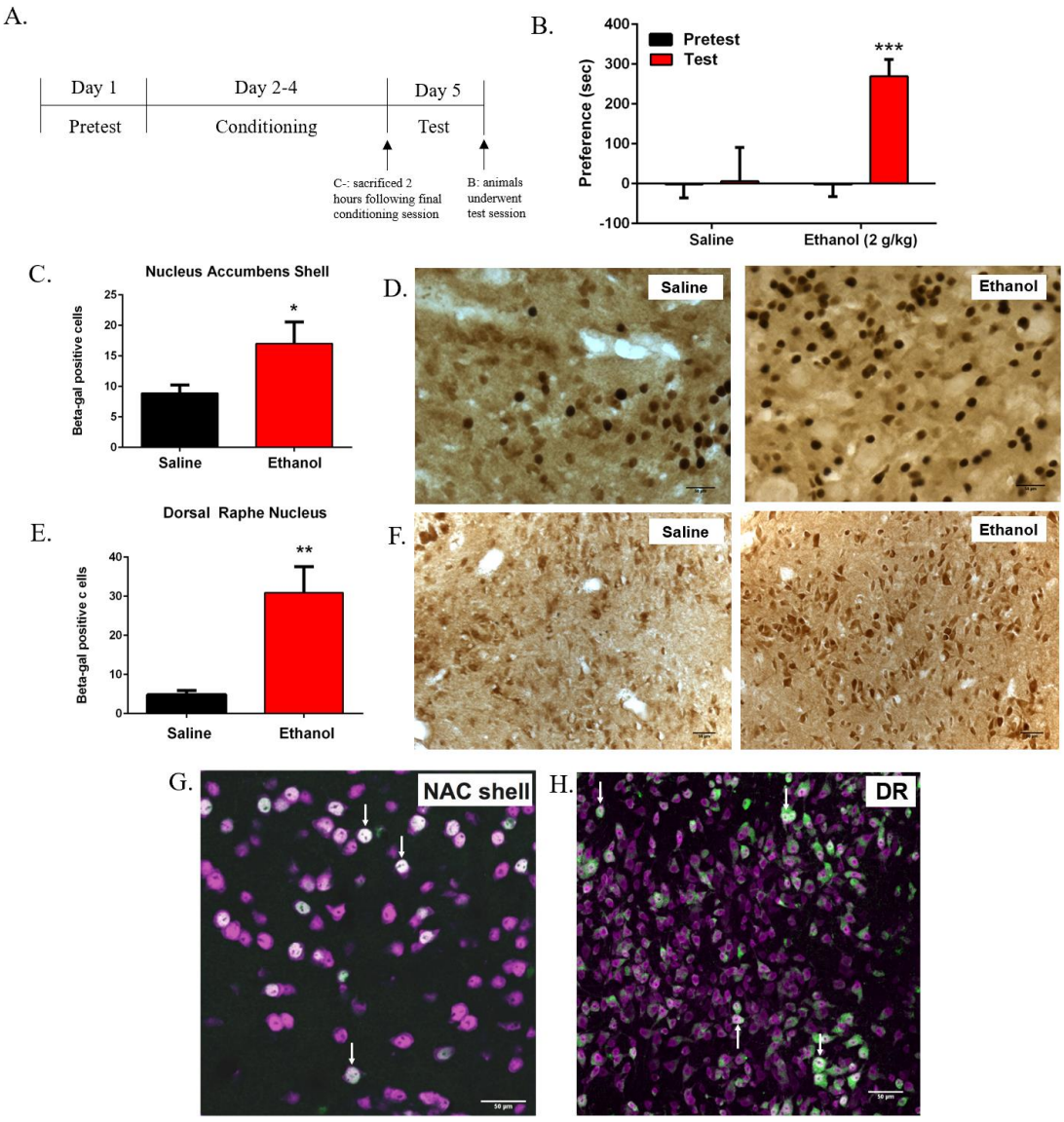

A.
Day 1
Day 2-4
Day 5
Pretest
Conditioning
Test
C-: sacrificed 2 hours following final conditioning session
B: animals underwent test session
B.
Pretest
Test
***
Preference (sec)
400
300
200
100
0
-100
Saline
Ethanol (2 g/kg)
C.
Nucleus Accumbens Shell
Beta-gal positive cells
25
20
15
10
5
0
*
Saline
Ethanol
D.
Saline
Ethanol
E.
Dorsal Raphe Nucleus
Beta-gal positive c ells
40
30
20
10
0
**
Saline
Ethanol
F.
Saline
Ethanol
G.
NAC shell
H.
DR

Figure 2.2: NFkB is activated by alcohol place conditioning. (A) Timeline of experimental treatments and behavioral measurements. **(B)** Ethanol (2 g/kg) induced a strong place preference on test day. No significant change from pretest preference was observed in saline controls. ***p<0.001 compared to all other groups. **(C, D)** Graphical representation and representative images of β-gal positive cell counts in NAC shell following place conditioning. 40x magnification. *p<0.05 compared to saline group. **(E, F)** Graphical representation and representative images of β-gal positive cell counts in DR following place conditioning. 20x magnification. **p<0.01 compared to saline group. **(G, H)** Representative images of NeuN (purple) and β-gal (green) double labeling in the NAC shell (40x) and DR (20x). White arrows indicate representative cells with NeuN and β-gal co-localization. Scale bars 50 μm.

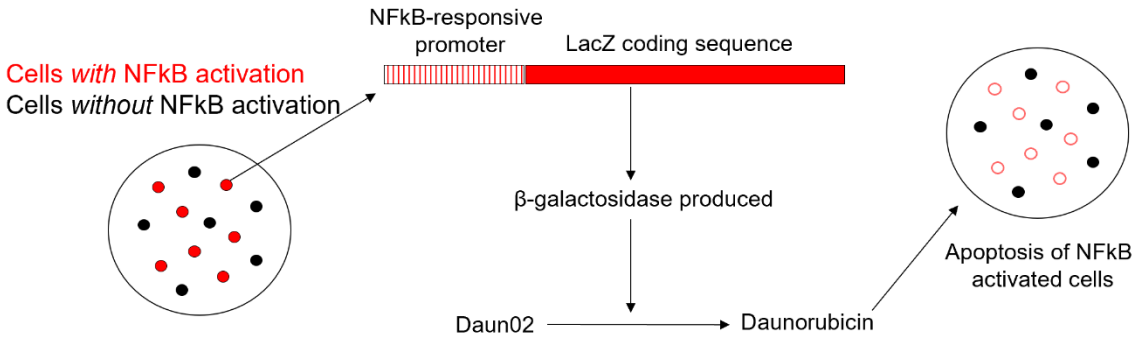
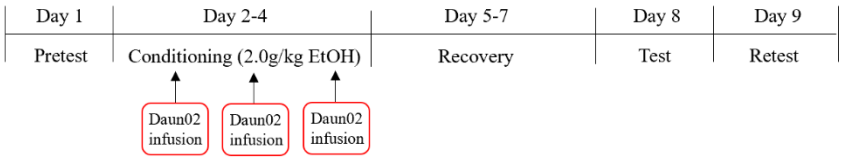
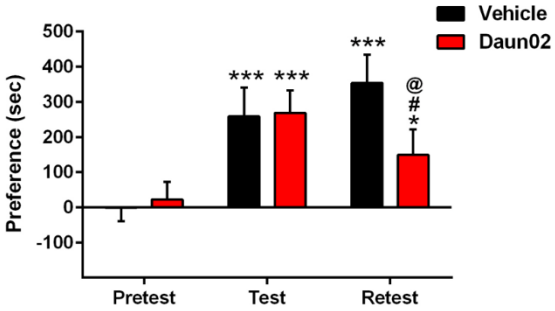

NFkB-responsive
promoter
LacZ coding sequence
Cells *with* NFkB activation
Cells *without* NFkB activation
β-galactosidase produced
Daun02
Daunorubicin
Apoptosis of NFkB
activated cells

Figure 2.3: Schematic of Daun02 lesioning method. Daun02 is a prodrug that is converted to the active form (daunorubicin) by β-gal enzyme. This leads to apoptosis specifically in cells in which β-gal is expressed. In our transgenic reporter mice, this would be cells in which NFkB is activated.

A.


Day 1
Day 2-4
Day 5-7
Day 8
Day 9
Pretest
Conditioning (2.0g/kg EtOH)
Recovery
Test
Retest
Daun02 infusion
Daun02 infusion
Daun02 infusion

B.


Nucleus Accumbens Shell
Vehicle
Daun02
Preference (sec)
500
400
300
200
100
0
-100
***
***
***
@
#
*
Pretest
Test
Retest

C.

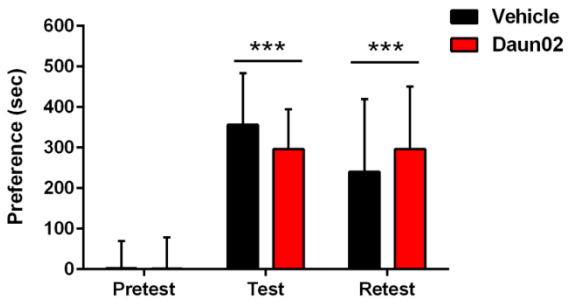
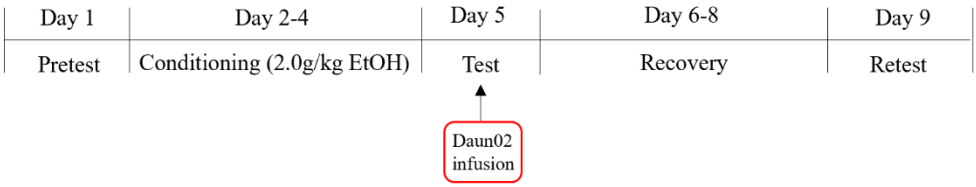

Dorsal Raphe
Vehicle
Daun02
Preference (sec)
600
500
400
300
200
100
0
***
***
Pretest
Test
Retest

Figure 2.4: Daun02 weakens alcohol CPP when infused into the NAC shell on conditioning days. (A) Timeline of experimental treatments and behavioral measurements. **(B)** Alcohol (2 g/kg) induced a significant CPP. When Daun02 was infused into the NAC shell following each alcohol conditioning session there was no effect on test day preference, but retest preference was weakened. **(C)** There was no effect of Daun02 infusion in the DR. *p<0.05, ***p<0.001 compared to pretest preference; #p=0.06 compared to test day preference.

A.


Day 1
Day 2-4
Day 5
Day 6-8
Day 9
Pretest
Conditioning (2.0g/kg EtOH)
Test
Recovery
Retest
Daun02 infusion

B.

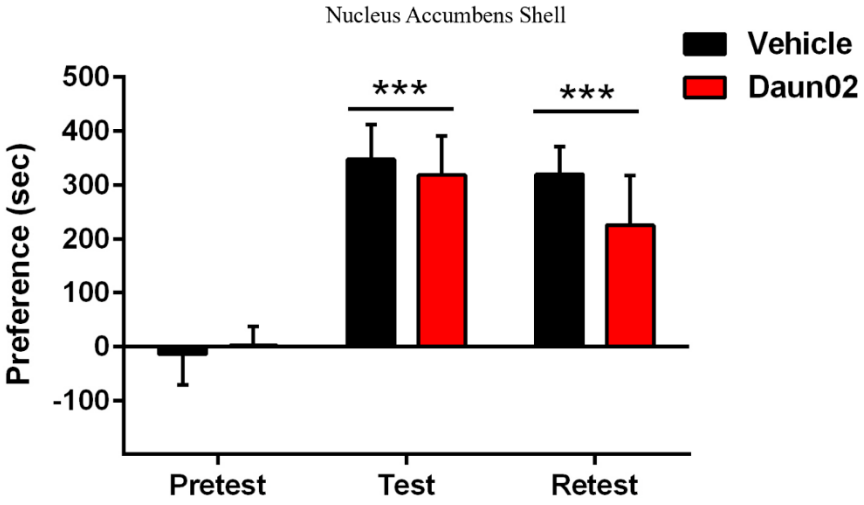

Nucleus Accumbens Shell
Vehicle
Daun02
Preference (sec)
500
400
300
200
100
0
-100
***
***
Pretest
Test
Retest

Figure 2.5: Daun02 does not affect retest preference when infused following the initial test day. (A) Timeline of experimental treatments and behavioral testing. **(B)** When Daun02 was infused into the NAC shell 2 hours after the initial place preference test for CPP, there was no significant change in place preference observed on the retest day. ***p<0.001 compared to pretest preference.

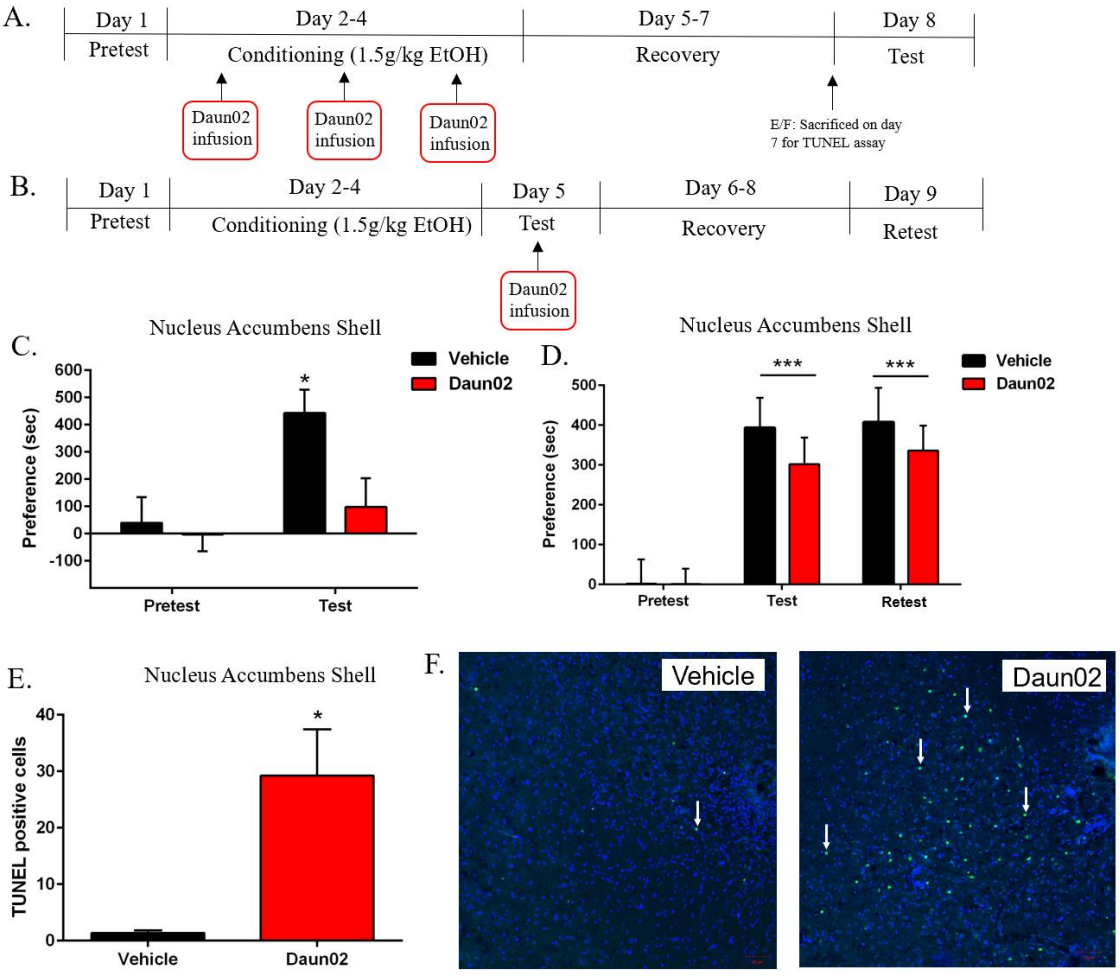

A.
Day 1
Pretest
Day 2-4
Conditioning (1.5g/kg EtOH)
Day 5-7
Recovery
Day 8
Test
Daun02 infusion
Daun02 infusion
Daun02 infusion
E/F: Sacrificed on day 7 for TUNEL assay
B.
Day 1
Pretest
Day 2-4
Conditioning (1.5g/kg EtOH)
Day 5
Test
Day 6-8
Recovery
Day 9
Retest
Daun02 infusion
C.
Nucleus Accumbens Shell
Preference (sec)
Vehicle
Daun02
Pretest
Test
D.
Nucleus Accumbens Shell
Preference (sec)
***
***
Vehicle
Daun02
Pretest
Test
Retest
E.
Nucleus Accumbens Shell
TUNEL positive cells
Vehicle
Daun02
F.
Vehicle
Daun02

Figure 2.6: Daun02 infusion into the NAC shell during the conditioning phase attenuates the rewarding properties of a moderate dose of alcohol. (A, B) Timeline of experimental treatments and behavioral testing. **(C)** When infused into the NAC shell following each conditioning session with 1.5 g/kg alcohol, Daun02 treatment attenuated alcohol place preference on the initial test day. *p<0.05 compared to all other groups. **(D)** When infused into the NAC shell following the initial test session, Daun02 treatment has no effect on alcohol place preference on the retest day. ***p<0.001 compared to pretest**. (E, F)** Graphical representation and representative images of Daun02-induced apoptosis in the NAC shell. White arrows indicate a representative TUNEL positive cells. *p<0.05 compared to vehicle group. Scale bars 100 µm.

CHAPTER 3:

INTERMITTENT ETHANOL ACCESS INCREASES SENSITIVITY TO SOCIAL DEFEAT STRESS[2]

[2]Nennig, S.E., Fulenwider, H.D., Eskew, J.E., Whiting, K.E., Cotton, M.R., McGinty, G.E., & Schank, J.R. Submitted to: *Alcoholism: Clinical and Experimental Research,* 9/4/19.

Abstract

Comorbidity between alcoholism and depression is extremely common. Recent evidence supports a relationship between alcohol exposure and stress sensitivity, an underlying factor in the development of depression. Our lab has recently shown that chronic alcohol exposure, which increases brain levels of NFkB, increases sensitivity to social defeat stress (SDS). However, the effects of voluntary alcohol consumption, resulting from protocols such as intermittent ethanol access (IEA), on stress sensitivity and the NFkB pathway have yet to be elucidated. We first assessed the effects of 4 weeks of IEA to 20% on sensitivity to subthreshold SDS exposure. Next, we analyzed gene expression of inhibitor of NFkB (IkB) following IEA or chronic alcohol exposure (10 days of 3.0g/kg alcohol via intragastric gavage). Then, we quantified NFkB activation via β-galactosidase immunohistochemistry following IEA or chronic alcohol gavage in NFkB-LacZ mice. IEA-exposed mice displayed an increase in sensitivity to subthreshold SDS compared to water-drinking controls. We also found that IkB gene expression was decreased in the nucleus accumbens (NAC) and amygdala (AMY) following IEA but was not altered following chronic alcohol exposure. Finally, we observed increased NFkB activity in the central amygdala (CEA), basolateral amygdala (BLA), and medial amygdala (MEA) after IEA, and increased NFkB activity solely in the CEA following chronic alcohol exposure. These findings further corroborate that prior alcohol exposure, in this case intermittent voluntary consumption, can impact development of depressive-like behavior by altering stress sensitivity. Further, our results suggest the CEA as a potential mediator of alcohol's effects on stress sensitivity, as NFkB was activated here following both IEA and chronic alcohol gavage. Thus, this study provides novel insight

on alterations in the NFkB pathway and identifies specific regions to target future experiments assessing the functional role of NFkB on these effects.

3.1 Introduction

Comorbidity between alcoholism and other psychiatric disorders such as depression is extremely common. According to NIAAA, a substantial portion of alcohol-dependent individuals also meet clinical criteria for depression (27.9%) and anxiety (36.9%). Epidemiological data has indicated that the presence of alcohol use disorder (AUD) and severity of AUD symptoms predicts onset of depressive disorders[123, 319]. Previous alcohol misuse in young adulthood also associates with increased rates of major depressive disorder (MDD)[319, 320]. Comorbid depression in detoxified alcohol abusers has been linked to increased risk for relapse[321], as co-occurrence of these disorders can lead to increased symptom severity and impairment[322]. As such, comorbidity of AUDs and MDD presents a significant health burden within our society. The circuitry and cellular mechanisms intertwining these disorders must be further understood in order to develop effective therapeutics for patients displaying this comorbidity.

The relationship between alcohol exposure and subsequent alterations in stress sensitivity has been extensively studied. Clinically, alcohol withdrawal can induce a depressed mood and negative affect that can persist into abstinence and increase risk of relapse[64, 68, 90, 153-156]. In animal models, exposure to chronic alcohol results in an increased responsivity to various paradigms assessing stress sensitivity and anxiety-like behavior, including elevated fear suppression using a model of punished drinking[161], decreased open-arm exploration in the elevated plus maze[157-159], decreased social interaction (SI) following restraint stress[160], decreased time in the center in the open

field test[159], increased marble burying[159], and impairment of fear extinction[162]. In addition to increasing stress-sensitivity, a history of alcohol exposure also potentiates the ability of stress exposure to increase alcohol consumption and self-administration[35, 158, 161, 163-166].

Our lab and others use the social defeat stress (SDS) model to assess the bidirectional relationship of alcohol exposure and stress sensitivity. We have previously found that exposure to chronic alcohol gavage increases sensitivity to social stress[167]. This relationship is bidirectional, as exposure to chronic SDS can impact drinking behaviors, including consumption[167, 172-179], conditioned place preference[183], self-administration[184, 185], and motivation to seek alcohol[184]. While we have examined the effect of experimenter-delivered chronic alcohol on sensitivity to social stress, it is important to determine if voluntary alcohol consumption could have a similar effect in mice. To address this question, we used intermittent ethanol access (IEA), which is a limited two-bottle choice (2BC) access model that results in elevated alcohol consumption compared to that observed on a continuous access schedule[24, 25, 323, 324]. Throughout 3 weeks of IEA, C57BL6/J mice reach consumption levels stabilizing around 20g/kg/day, reach blood alcohol levels above 100mg/dl, and display higher ethanol preference compared to mice on continuous access[23]. As such, IEA results in consumption patterns which may be more similar to drinking behaviors observed in human alcohol abusers[323].

To begin to examine a mechanism that could underlie the relationship between alcohol exposure and stress sensitivity, we assessed the effects of alcohol exposure on activation of transcription factor nuclear factor light chain enhancer of activated B cells (NFkB). Under baseline conditions, NFkB subunit dimers are bound in the cytosol by

inhibitor of NFkB (IkB)[192]. A protein kinase known as IkB kinase (IKK) phosphorylates IkB, tagging it for proteasomal degradation and releasing the NFkB subunit dimers to translocate to the nucleus where they act as a transcription factor for a variety of genes involved in inflammation and various other processes[192]. Several studies have examined the influence of the NFkB pathway on alcohol-related behaviors[192] including continuous 2BC[210], drinking-in-the-dark[210], and conditioned place preference[325], as well as the immune-related effects following long-term ethanol exposure[208]. Clinically, a single nucleotide polymorphism in the NFKB1, a gene encoding one of the NFkB subunits, associates with alcohol dependence[204] This pathway has also been implicated in the behavioral and molecular outcomes following chronic SDS[149, 151]. More specifically, protein levels of IKK, IkB, and phosphorylated IkB were shown to be increased in the nucleus accumbens (NAC) of mice sensitive to SDS. Additionally, infusion of an IKK dominant-negative virus into the NAC of susceptible mice resulted in a reversal of defeat-induced behavioral phenotypes, whereas infusion of a constitutively active form of IKK into the accumbens increased sensitivity to SDS exposure[151].

In the present study we aimed to assess the effects of IEA on stress sensitivity and alterations in the NFkB pathway as a result of alcohol exposure. First, we examined the effects of IEA on sensitivity to subthreshold SDS. Then, we then analyzed gene expression of IkB and regionally specific NFkB activation following IEA. Last, we assessed gene expression of IkB and regionally specific NFkB activation following chronic alcohol exposure to determine if NFkB is activated in similar regions as following IEA. We hypothesized that IEA will increase sensitivity to social stress, and that activity of the NFkB pathway will be increased following both IEA and chronic alcohol gavage.

3.2 Materials & Methods

Animals

Male C57BL6/J mice (8 weeks of age, Jackson Laboratory, Bar Harbor, ME) were used in Experiments 1, 2, and 4. Male NFkB-LacZ mice (8 weeks of age, bred in house) bred on a C57BL6/J background were used for Experiments 3 and 5. These mice express the lacZ transcript under the direction of an NFkB-regulated promoter. Thus, wherever NFkB is activated, activity-dependent β-galactosidase (β-gal) will be expressed in that cellular population and can be stained for a visual readout of NFkB activity[215, 307, 325]. Retired breeder male CD-1 mice (4-5 months of age, Charles River, Wilmington, MA) were used as SDS aggressors in Experiment 1. Due to sex specific differences in territorial aggression and the SDS protocol being replicated and highly validated using male C57BL6/J mice, only male subjects were used. Mice were allowed 1 week of habituation to the UGA College of Veterinary Medicine vivarium before experiments began. Mice were housed in a 12hr light cycle (on at 1:00 and off at 13:00). Food and water were available *ad libitum*. All procedures were in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Georgia.

Drugs

For continuous access 2-bottle choice (2BC) in Experiments 2 and 3, 95% ethanol (Decon Labs, Inc., King of Prussia, PA) was diluted to a 20% v/v solution in tap water. For chronic alcohol gavage in Experiments 4 and 5, 95% ethanol was diluted to a 25% v/v solution in tap water.

IEA

One week prior to the start of IEA, mice were singly housed. A week later, mice were presented with two bottles in their homecage, one containing water and one containing a 20% ethanol solution for 24 hours on Monday, Wednesday, and Friday for a total of 4 weeks. On the remaining days of the week (Tuesday, Thursday, Saturday, and Sunday), the alcohol bottle was removed and replaced with a bottle containing water. Control mice were also singly housed but had access to water only throughout the 4-week period. Bottles were weighed at the same time each day and g/kg consumption was calculated based on each mouse's body weight.

Subthreshold SDS

Subthreshold SDS is a modification of the chronic SDS protocol described by Golden et al. 2011. Whereas chronic SDS is a 10-day stress protocol that reliably results in depressive-like behavior (such as reduced social interaction and anhedonia), the 1-day subthreshold SDS protocol does not induce these symptoms, thus allowing for the assessment of interventions, such as IEA or chronic alcohol gavage, on sensitivity to SDS[143]. Prior to subthreshold SDS, CD-1 mice were screened for aggressive behavior by placing a screener C57BL6/J mouse (not used in any of the experiments) into the home cage of the CD-1 mouse for 180 seconds for 4 consecutive days. Aggressors were selected based on the following criteria: the CD-1 mouse must initiate an attack in at least two consecutive sessions and the latency to first display aggressive behavior must be less than 60 seconds. For subthreshold SDS, mice were exposed to 3 5-minute defeat sessions separated by 15 minutes each. During each defeat session, mice were placed into the homecage of a novel male CD-1 mouse that passed aggression screening. Between defeat sessions, mice were returned to their homecage to recover

until the next defeat session started 15 minutes later. Defeats occurred at the end of the light cycle in order to visualize any injuries. If blood was drawn, the defeat session ended early and the time at which the session ended was noted.

SI Test

The SI test occurred at the beginning of the dark cycle approximately 24 hours after subthreshold SDS exposure. This test consisted of 2 150-second trials separated by 30 seconds, the first without a novel CD-1 target mouse present, and the second with a target mouse present in an enclosure in the predetermined interaction zone. Time spent in the interaction zone during each trial is recorded and divided (time in the interaction zone with the target mouse present divided by time when the target was absent) to obtain the SI ratio. SI tests were scored by an observer blind to the experimental group of each mouse.

Chronic EtOH Gavage

Mice were intragastrically gavaged with 3.0g/kg ethanol (25% ethanol in tap water) for a total of 10 days. Gavages occurred at the same time each day throughout the exposure period. This method of exposure was chosen because it allows for tight experimenter control and it induces exposure levels comparable to that of human alcohol abusers.

qPCR

Mice were sacrificed 48-hours after the last IEA drinking session or chronic alcohol treatment via rapid decapitation and gross dissections were taken of the amygdala (AMY) and NAC. Tissue dissections were snap frozen on isopentane and stored at -80°C in RNAase free tubes. Total RNA was extracted and reversed transcribed using a first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA).

qPCR reactions were ran in triplicate using specific FAM-labeled TaqMan probes (Gapdh Mm99999915_g1, IKB Mm00456853_m1; Applied Biosystems, Foster City, CA, USA) and ran on an Applied Biosystems QuantStudio6 machine. Reactions were normalized to endogenous control Gapdh and expressed as fold change from control. For IEA, 1 IEA and 2 water NAC samples and 1 IEA AMY sample were excluded due to insufficient sample quality to conduct qPCR. For chronic alcohol, 1 water NAC sample and 2 IEA AMY and 1 water AMY samples were excluded for the same reason.

Immunohistochemistry

NFkB-LacZ reporter mice were used for immunohistochemistry. Forty-eight hours after the last IEA drinking session or chronic alcohol treatment, mice were overdosed with ketamine/xylazine and were transcardially perfused with 4% paraformaldehyde. Brains were postfixed overnight in 4% paraformaldehyde, transferred to 10% sucrose for one hour and then 30% sucrose until the tissue sank, and then frozen quickly on powdered dry ice. Thirty-micron sections were collected in a freezing cryostat (Leica, Buffalo Grove, IL) and floating sections were frozen in cryopreservant at -20°C. After washing, β-galactosidase expression was visualized using X-gal (Roche Diagnostics, Germany) immunohistochemistry to examine NFkB activation. Briefly, after 3 10-minute 1xPBS washes, sections were incubated overnight at 37° in X-gal reaction buffer (X-gal diluted 1:25 in warmed X-gal dilution buffer containing 100mM sodium phosphate dibasic (Fisher Scientific, Fair Lawn, NJ, USA), 100mM sodium chloride (Fisher Scientific, Fair Lawn, NJ, USA), 5mM ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid (EGTA) (Sigma-Aldrich, St. Louis, MO, USA), 2mM magnesium chloride (Sigma-Aldrich, St. Louis, MO, USA), 5mM potassium hexacyanoferrate(III) (Sigma-Aldrich, St. Louis, MO, USA), 5mM potassium hexacyanoferrate(II) trihydrate (Sigma-Aldrich, St. Louis,

MO, USA) in 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA)). The next day, sections are washed in 1xPBS 3 times for 10 minutes after which sections were mounted on gelatin slides. Neutral red solution (Sigma-Aldrich, St. Louis, MO, USA) was used prior to the ethanol dehydration series as a counter stain to improve visualization of landmarks for imaging. Slides were coverslipped and images were taken on a Zeiss Axioscope A1. For NAC shell, images from 6 fields (3 per side) were taken at 40x magnification. For NAC core, images from 4 fields (2 per side) were taken at 40x magnification. For the central amygdala (CeA), basolateral amygdala (BLA), and medial amygdala (MEA), images from 2 fields (1 per side) were taken at 20x. X-gal positive cell counts were quantified using ImageJ software and were performed by an investigator blind to the experimental conditions. For each region, the average cell count across the total number of frames taken was used as the dependent variable for comparisons.

Experimental timelines

A summary timeline for each experiment in this study can be found in Figure 3.1 and specific protocols are described below. In Experiment 1, mice were exposed to 4 weeks of IEA. Forty-eight hours after the last drinking session, mice were exposed to subthreshold SDS and assessed for SI 24 hours later. In Experiments 2 and 4, mice were exposed to 4 weeks of IEA (Experiment 2) or chronic alcohol exposure (Experiment 4) and were sacrificed 48 hours after the last drinking session for qPCR. In Experiments 3 and 5, NFkB-LacZ mice were exposed to 4 weeks of IEA (Experiment 3) or chronic alcohol exposure (Experiment 5) and were perfused 48 hours after the last drinking session for immunohistochemistry.

Statistical Analyses

Statistical analyses were preformed using Prism GraphPad software. SI ratio, gene expression, and cell count analyses were compared using an unpaired t-test.

3.3 Results

IEA increases sensitivity to subthreshold SDS

As we have previously determined that experimenter-delivered chronic alcohol gavage decreases SI behavior following exposure to subthreshold SDS, we first assessed the effects of voluntary alcohol access via IEA on stress sensitivity. Mice (IEA: n=14, water: n=13) were allowed 4 weeks of IEA to 20% ethanol (Figure 3.2A) and were exposed to subthreshold SDS 48 hours after the final drinking period. We chose this timepoint of stress exposure based on our previous findings using chronic alcohol exposure[167]. Control mice had access to water only throughout the 4 weeks. IEA-exposed mice displayed significantly decreased SI following subthreshold SDS compared to water-drinking controls ($t(25)=2.53$, $p=0.018$, Figure 3.2B).

IEA decreases IkB expression in the AMY and NAC

Next, we were interested in how components of the NFkB signaling pathway, particularly IkB, are altered following IEA. Mice (n=12/group) were exposed to IEA or water for 4 weeks and were sacrificed 48 hours following the final alcohol access period, at which time the NAC and AMY were dissected and prepared for qPCR. Following 4 weeks of IEA, IkB gene expression was significantly decreased in the NAC ($t(19)=2.87$, $p=0.0098$, Figure 3.3A) and the AMY ($t(21)=3.35$, $p=0.003$, Figure 3.3B).

IEA increases NFkB expression in specific subregions of the amygdala

While assessing IkB gene expression can provide indirect insight on how NFkB regulation is altered following these alcohol exposure paradigms, this was performed in

homogenates encompassing the entire NAC and AMY. As this method lacks regional specificity, we next assessed NFkB activation in our NFkB-LacZ reporter mice by assessing β-gal expression via x-gal immunohistochemistry. This transgenic mouse provides a direct, functional readout of NFkB activation. Mice (IEA: n=6, water: n=4) were exposed to 4 weeks of IEA or water and were sacrificed 48-hours following the final drinking session. While no impact of IEA on NFkB activation in the NAC shell ($t(8)=1.29$, $p=0.23$, Figure 3.4A-B) or NAC core ($t(7)=0.95$, $p=0.38$, Figure 3.4C-D) was observed, a significant increase in x-gal positive cells was observed in the CEA ($t(6)=8.946$, $p=0.0001$, Figure 3.4E-F) and BLA ($t(7)=2.88$, $p=0.024$, Figure 3.4G-H). The MEA showed a similar pattern of x-gal positive cells, as it was nearly significant compared to water controls ($t(8)=2.28$, $p=0.052$, Figure 3.4I-J).

Chronic EtOH does not alter IkB expression in the AMY or NAC

Due to similar effects of IEA and chronic alcohol gavage on stress sensitivity, as shown in Experiment 1 above and our previously published work[213], respectively, we were also interested in how IkB gene expression was altered following chronic alcohol gavage. Mice (n=9/group) were exposed to 10 days of chronic alcohol (3.0g/kg) via intragastric gavage. Forty-eight hours after the final alcohol gavage, mice were sacrificed and the NAC and AMY were dissected. Chronic alcohol exposure did not alter IkB gene expression in the NAC ($t(15)=1.40$), $p=0.18$, Figure 3.5A) or AMY ($t(13)=0.18$, $p=0.86$, Figure 3.5B).

Chronic EtOH increases NFkB expression specifically in the CEA

Lastly, we assessed the effects of chronic alcohol exposure on NFkB activation once again using x-gal immunoreactivity in our NFkB-LacZ mice. Mice (EtOH: n=6, water: n=4) were exposed to 10 days of chronic alcohol gavage and were sacrificed 48-

hours following the final alcohol treatment. Chronic alcohol gavage did not impact the number of x-gal positive cells in the NAC shell ($t(9)=0.87$, $p=0.41$, Figure 3.6A-B) or NAC core ($t(9)=1.17$, $p=0.27$, Figure 3.6C-D). However, it did significantly increase the number of x-gal positive cells in the CEA ($t(8)=2.31$, $p=0.0497$, Figure 3.6E-F). This effect was specific to this subregion of the amygdala, as no differences were observed in the BLA ($t(8)=0.13$, $p=0.90$, Figure 3.6G-H), or MEA ($t(8)=0.08$, $p=0.94$, Figure 3.6I-J).

3.4 Discussion

The primary findings from these studies are that voluntary alcohol consumption via IEA increases sensitivity to subthreshold SDS, replicating our previous findings using chronic alcohol gavage, and increases NFkB activity, specifically in the amygdala. Specifically, IEA decreases IkB gene expression in the NAC and AMY. NFkB activation in our transgenic NFkB-LacZ reporter mice show increased NFkB activity in the CEA, BLA, and MEA, but not the NAC shell or NAC core. In contrast, chronic alcohol gavage exposure did not alter IkB gene expression in the NAC or AMY, but did selectively increase NFkB in the CEA subregion on the AMY.

Here, we demonstrate that voluntary alcohol consumption via IEA impacts stress sensitivity. Similar to other studies using the IEA model of consumption[23], we observed consistent consumption levels around 20g/kg in our IEA-exposed mice. Examining the effects of voluntary consumption on stress responsivity is an important, clinically relevant approach. IEA is a highly validated model that results in clinically relevant levels of consumption[326]. Considering alcohol misuse associates with increased rates of MDD[319, 320], the intermittent drinking bouts and high blood alcohol levels obtained by IEA make this model ideal for examining this relationship in a preclinical setting. This finding parallels the effects of chronic alcohol exposure on stress sensitivity we have

previously observed[167] and further corroborates that voluntary alcohol consumption can impact the development of depressive-like behavior by altering stress sensitivity, an underlying factor of development of depression[327].

The neuroimmune system has recently gained attention for its involvement in both the behavioral and molecular responses to alcohol exposure[191, 196] and the pathophysiology of stress and depression[217-219, 225, 237]. As such, components of the NFkB signaling cascade are intriguing candidates to target in studies examining the underlying mechanisms of comorbidity between these disorders. In our study, we found a decrease in IkB expression following 4 weeks of IEA that, in the amygdala specifically, paralleled an increase in NFkB activity with the CEA, BLA, and MEA. Our data suggests that 48 hours after stress, downregulated expression of IkB potentially results in less NFkB inhibition and more freed NFkB subunit dimers to translocate to the nucleus and act as a transcription factor. In agreement with this, NFkB activity in reporter mice was increased at the same timepoint at which IkB downregulation occurs. We predict that if we assessed NFkB activation in IEA-exposed mice exposed to stress, NFkB expression would be increased even further relative to unstressed IEA mice, as the pathway would be primed due to the decreased expression of IkB. While IkB expression and NFkB activity in our transgenic mice did not always show parallel effects, it is imperative to consider that the IkB expression analysis used whole region homogenates of the NAC and AMY, whereas the immunohistochemistry allowed for the assessment of NFkB in specific subregions of the NAC and AMY, and thus a higher degree of specificity.

It is interesting to note that exposure to chronic SDS was found to increase protein levels of IKK, IkB, and phosphorylated-IkB in mice sensitive to SDS 48 hours after the final defeat session[151]. In our study, we assessed IkB gene expression 48

hours after alcohol cessation, indicating critical differences in stimulation of NFkB (stress- vs. alcohol-exposure) and level of analysis (protein vs. gene expression) that may underlie these conflicting results. Considering these results, it appears an increase in IkB expression occurs following stress, while a decrease in its expression is observed following alcohol exposure and withdrawal. Our results also indicate a more prominent role of NFkB in the amygdala following alcohol exposure, while its activity in the nucleus accumbens, where NFkB mediates depressive-like behavior, is largely unaffected.

Both IEA and chronic alcohol exposure increased NFkB activity in the CEA, but only IEA increased NFkB in the BLA and MEA. Together, the BLA and CEA are involved in the development of alcohol-seeking behavior in rodents[328]. The CEA is the major output nucleus of the amygdala and has been heavily implicated in stress- and drug-related responsivity[329]. The CEA, along with other regions in the extended amygdala, regulates negative affect related to stress and alcohol use disorder[330, 331]. This study provides novel insight on not only the involvement of NFkB in response to various alcohol exposure paradigms, but also provides a specific region to target future experiments aimed at inhibiting NFkB activation following IEA or chronic alcohol exposure and the effects of this inhibition on stress sensitivity. Interestingly, only IEA increased NFkB activity in the BLA. The BLA is implicated in affective disorders and addiction due to its role in stress-responses, motivation, and cognition[332]. A projection from the BLA to the NAC shell was recently determined to control voluntary alcohol consumption[333], supporting the role of this region in both affect and voluntary intake. As chronic alcohol exposure is not a voluntary form of consumption, this may explain why no differences in activation were observed in the BLA after chronic alcohol exposure. IEA also increased NFkB activity in the MEA. Chronic alcohol exposure and

alcohol withdrawal dysregulate important mediators of stress circuitry such as corticotropin releasing hormone (CRH)[62, 161, 165]. Transcript expression of Crhr1, the gene encoding the CRH receptor 1 (CRH-R1), is upregulated in the BLA and MEA, and mRNA level of Crh, the gene encoding CRH, is increased in the CEA following alcohol dependence[161]. Interestingly, CRF-R1 activation can directly induce NFkB activity[334], perhaps indicating a potential mechanism of IEA's ability to increase NFkB activity within these three subregions.

Overall, our results indicate a complex relationship between alcohol-exposure, stress responsivity, and activation of the NFkB pathway. This study determined that intermittent, voluntary alcohol consumption increases sensitivity to social stress. This study also identifies that IkB gene expression is decreased in the NAC and AMY following IEA but not chronic alcohol gavage. We observed increased NFkB activity in multiple subregions of IEA-exposed mice, but only the CEA of chronic alcohol gavage exposed mice. Future experiments will focus on intra-AMY inhibition of NFkB during exposure to IEA or chronic alcohol gavage to assess alterations in stress sensitivity when NFkB is inhibited in a regionally specific manner prior to stress exposure. Identifying molecular targets involved in the pathophysiology of both alcohol abuse and depression, such as NFkB, may be extremely beneficial for development of novel pharmacotherapeutics for patients displaying comorbid alcoholism and depression.

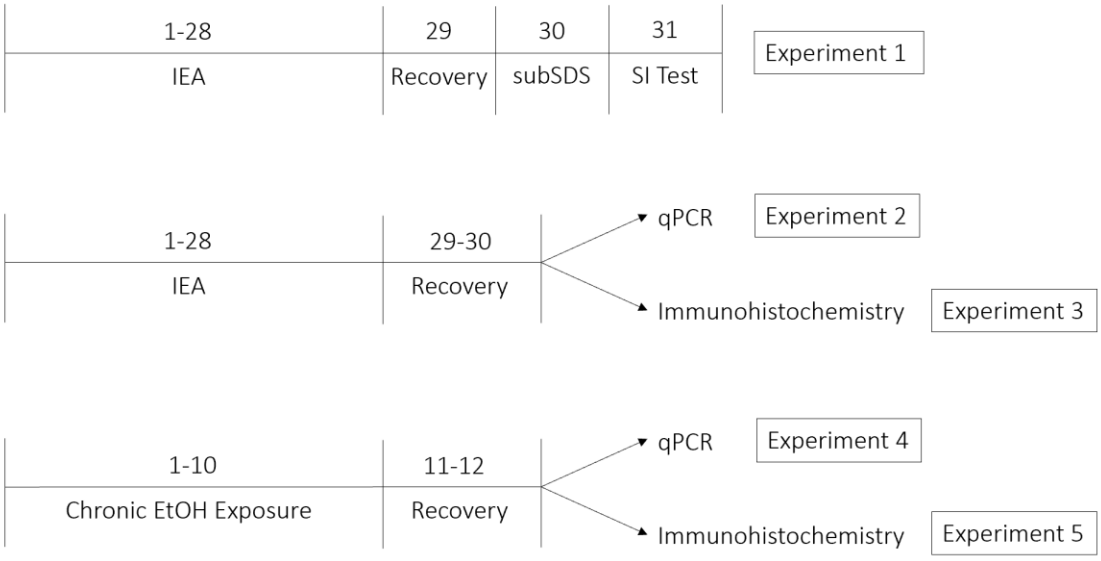

1-28
IEA
29
Recovery
30
subSDS
31
SI Test
Experiment 1
1-28
IEA
29-30
Recovery
qPCR
Experiment 2
Immunohistochemistry
Experiment 3
1-10
Chronic EtOH Exposure
11-12
Recovery
qPCR
Experiment 4
Immunohistochemistry
Experiment 5

Figure 3.1: Experimental timelines for Experiments 1-5.

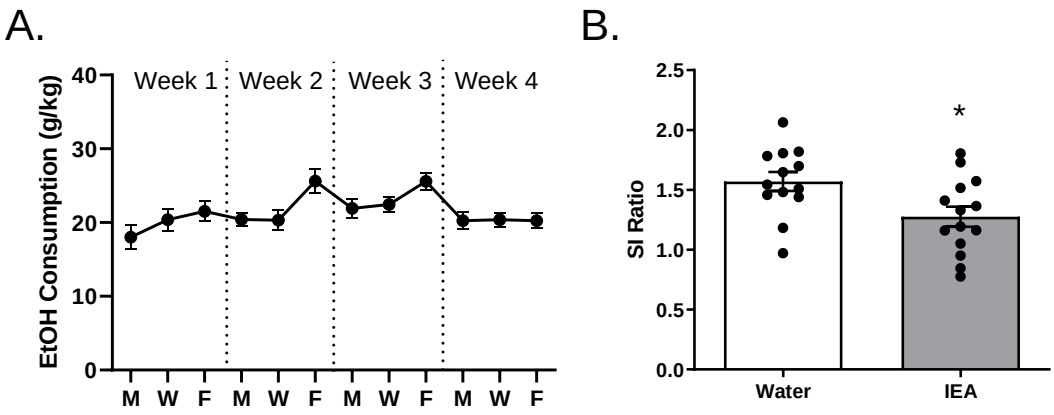

A.
B.
Week 1
Week 2
Week 3
Week 4
EtOH Consumption (g/kg)
40
30
20
10
0
M W F M W F M W F M W F
SI Ratio
2.5
2.0
1.5
1.0
0.5
0.0
*
Water
IEA

Figure 3.2: IEA increases sensitivity to subthreshold SDS. (A) Mice were allowed 4 weeks of IEA to 20% ethanol or water only, with IEA mice displayed elevated levels of consumption. Forty-eight hours after the last drinking period, mice were exposed to subthreshold SDS and SI was tested 24 hours later. **(B)** IEA-exposed mice displayed decrease social interaction compared water drinking controls. *$p<0.05$ compared to control group.

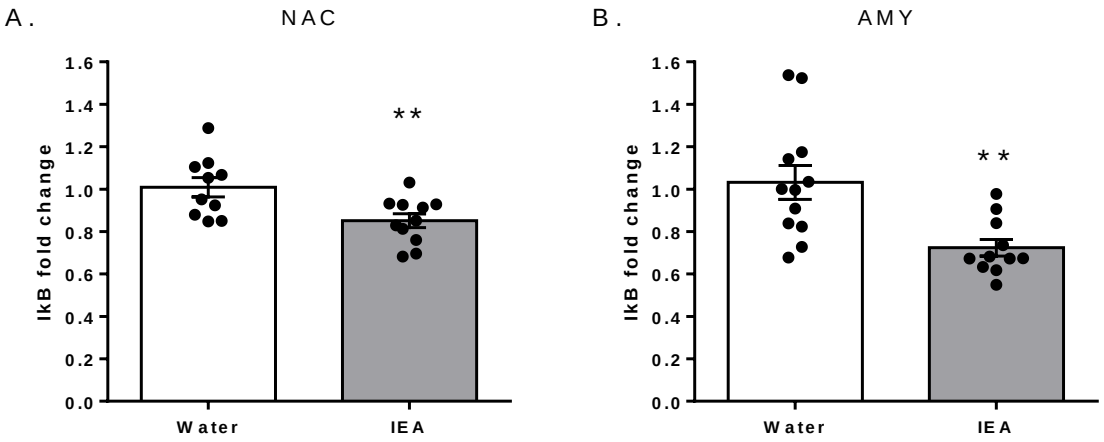

A.
NAC
IkB fold change
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Water
IEA
B.
AMY
IkB fold change
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Water
IEA

Figure 3.3: IEA decreases IkB expression in the AMY and NAC. Mice were allowed 4 weeks of IEA to 20% ethanol or water only. Forty-eight hours after the last drinking period, mice were sacrificed and the NAC and AMY were dissected and processed for qPCR. IEA-exposed mice displayed decreased IkB gene expression in both the NAC **(A)** and AMY **(B)** compared to water drinking controls. **p<0.01 compared to control group.

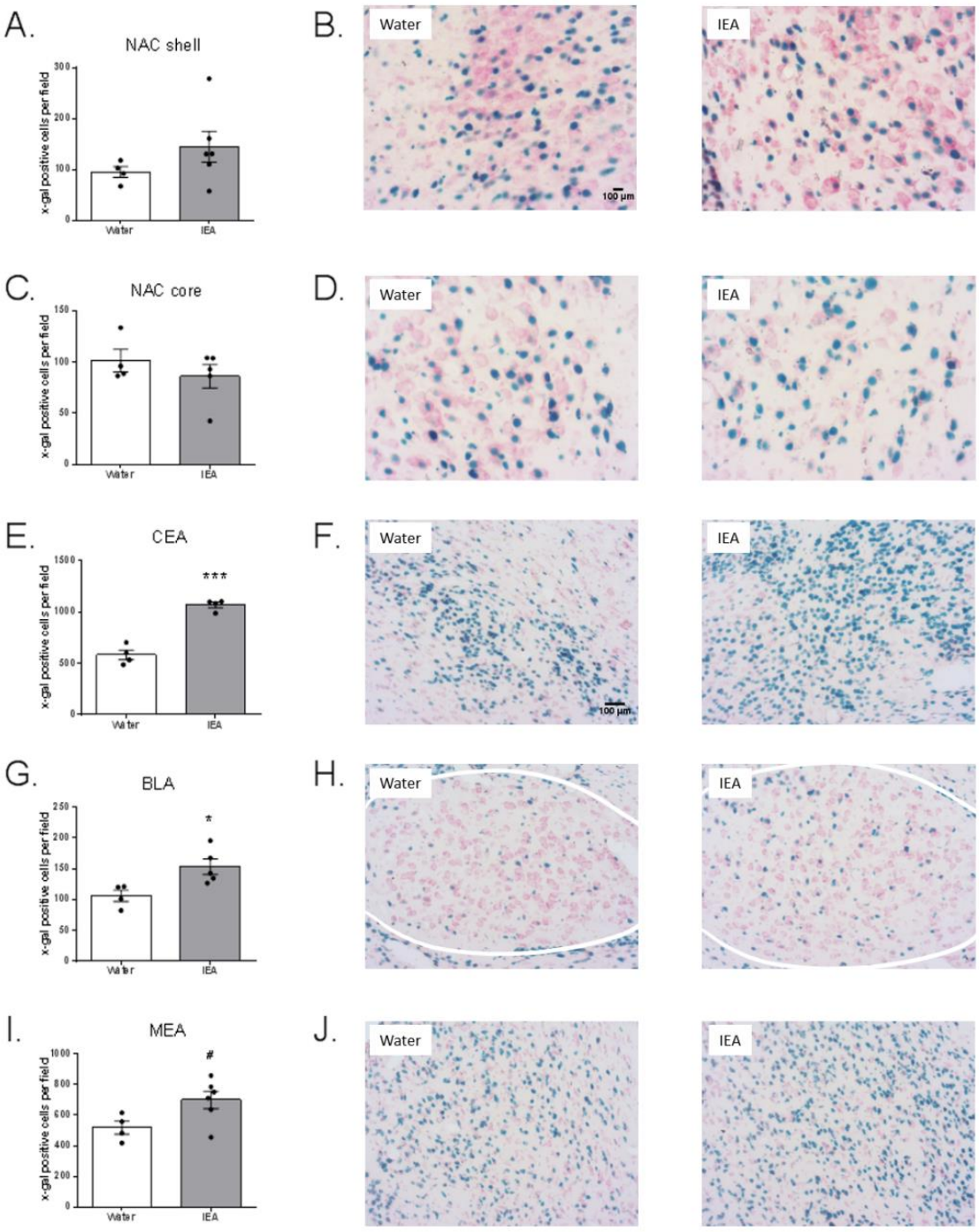

A.
NAC shell
x-gal positive cells per field
300
200
100
0
Water
IEA
B.
Water
100 µm
IEA
C.
NAC core
x-gal positive cells per field
150
100
50
0
Water
IEA
D.
Water
IEA
E.
CEA
x-gal positive cells per field
1500
1000
500
0
***
Water
IEA
F.
Water
100 µm
IEA
G.
BLA
x-gal positive cells per field
250
200
150
100
50
0
*
Water
IEA
H.
Water
IEA
I.
MEA
x-gal positive cells per field
1000
800
600
400
200
0
#
Water
IEA
J.
Water
IEA

Figure 3.4: IEA increases NFkB expression in subregions of the amygdala. Mice were allowed 4 weeks of IEA to 20% ethanol or water only. Forty-eight hours after the last drinking period, mice were perfused and stained for β-gal expression as a readout of NFkB activity. While IEA-exposed mice did not display differences from water drinking controls in the NAC shell **(A/B)** or NAC core **(C/D)**, an increase was observed in the CEA **(E/F)**, BLA **(G/H)**, and MEA **(I/J)**. ***$p<0.001$, * $p<0.05$, and $^{\#}p=0.052$ compared to control group. Scale bars at 100µm.

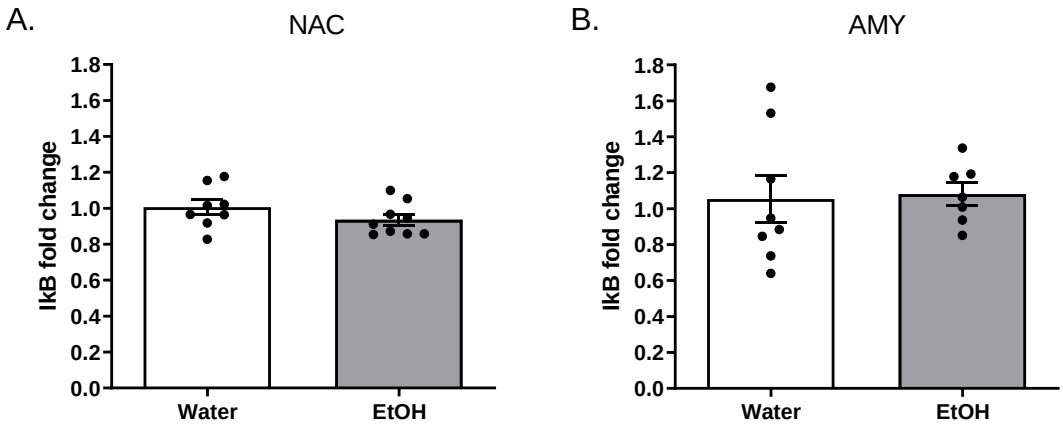

A.
NAC
IkB fold change
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Water
EtOH
B.
AMY
IkB fold change
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Water
EtOH

Figure 3.5: Chronic EtOH does not alter IkB expression in the AMY or NAC. Mice were intragastrically gavaged with 3.0g/kg ethanol or water for 10 days. Forty-eight hours after the gavage, mice were sacrificed and the NAC and AMY were dissected and processed for qPCR. No differences in IkB expression was observed in the NAC **(A)** or AMY **(B)** compared to water-gavaged controls.

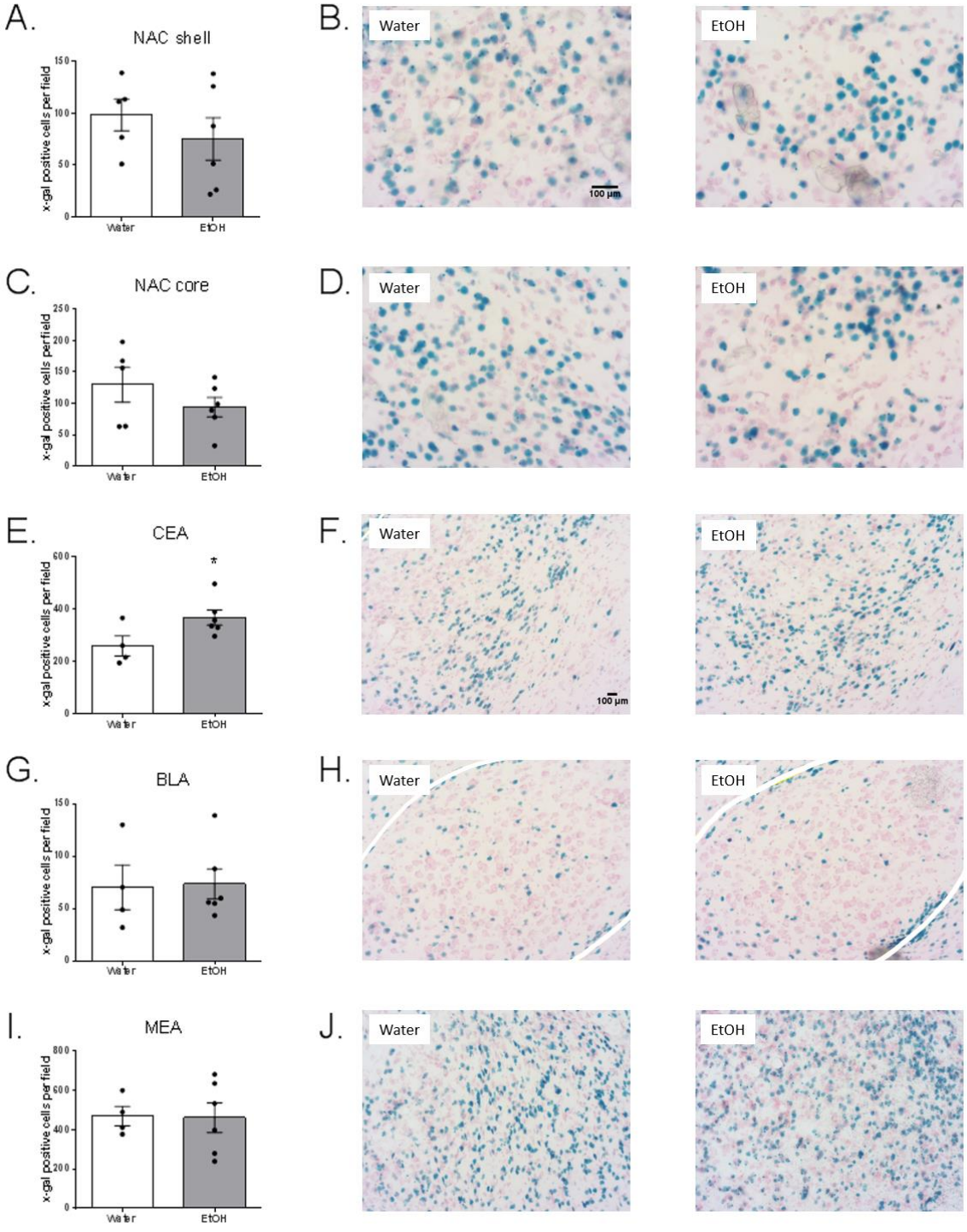

A.
NAC shell
x-gal positive cells per field
150
100
50
0
Water
EtOH
B.
Water
100 µm
EtOH
C.
NAC core
x-gal positive cells per field
250
200
150
100
50
0
Water
EtOH
D.
Water
EtOH
E.
CEA
x-gal positive cells per field
600
400
200
0
*
Water
EtOH
F.
Water
100 µm
EtOH
G.
BLA
x-gal positive cells per field
150
100
50
0
Water
EtOH
H.
Water
EtOH
I.
MEA
x-gal positive cells per field
800
600
400
200
0
Water
EtOH
J.
Water
EtOH

Figure 3.6: Chronic EtOH increases NFkB expression in the CEA. Forty-eight hours after the last alcohol or water gavage, mice were perfused and stained for β-gal expression as a readout of NFkB activity. No differences in x-gal positive cells were observed in the NAC shell **(A/B)** or NAC core **(C/D)**. In the amygdala, chronic alcohol-exposed mice displayed increased x-gal positive cells in the CEA **(E/F)**, but not the BLA **(G/H)** or MEA **(I/J)**. *$p<0.05$ compared to control group. Scale bars at 100μm.

CHAPTER 4:

THE ROLE OF THE NEUROKININ-1 RECEPTOR IN SOCIAL INTERACTION AND ALCOHOL CONSUMPTION FOLLOWING SOCIAL DEFEAT[3]

[3]Nennig, S.E., Fulenwider, H.D., Whiting, K.E., Eskew, J.E., & Schank, J.R. To be submitted to: *Psychopharmacology.*

Abstract

Comorbidity between alcoholism and depression is extremely common. The relationship between the effects of stress on alcohol consumption has been extensively studied. To examine this relationship, our lab and others have used the social defeat stress model (SDS). We have previously shown that mice susceptible to social stress voluntarily consume more alcohol compared to resilient and control mice. Susceptible mice also display increased gene expression of TACR1, the gene encoding the neurokinin-1 receptor (NK1R) in the nucleus accumbens (NAC). In this study, we assess the effects of genetic and pharmacological blockade of the NK1R on alterations in social interaction (SI) and alcohol consumption following chronic SDS. We also examine the effects of NK1R overexpression in the NAC shell on stress sensitivity. To accomplish this, we exposed NK1R knockout (KO) mice and wildtype (WT) littermate controls to chronic SDS and assessed SI and alcohol consumption following stress exposure. We then pretreated WT mice with the NK1R antagonist L703-606 or vehicle prior to each defeat to assess effects on SDS-induced alterations on SI and alcohol consumption. Lastly, we infused a virus overexpressing the NK1R receptor in the NAC shell of WT mice and assessed sensitivity to subthreshold SDS. Genetic deletion of NK1R did not alter the effects of chronic SDS on SI or alcohol consumption and preference, as both SDS-exposed KO and WT mice display a decrease in SI and an increase in alcohol intake and preference. L703-606 pretreatment prevented the decrease in SI following chronic SDS but did not decrease subsequent alcohol consumption. Finally, NK1R overexpression in the NAC shell increased stress sensitivity. The results obtained in this study suggest a functional role of NK1R during SDS exposure in the depressive-like behavior but not the increased alcohol consumption following stress exposure.

4.1 Introduction

Comorbidity between alcoholism and depression is extremely common. According to NIAAA, a substantial portion of alcohol-dependent individuals also meet clinical criteria for depression (27.9%). A bidirectional relationship between these disorders has been identified at the clinical and preclinical level. Clinically, epidemiological studies have indicated that severity of alcohol use disorder symptoms predicts onset of depressive disorders[123], while a history of mood disorders in turn increases risk of substance abuse[125].

The relationship between alcohol exposure and depressive-like behavior has been studied preclinically using the social defeat stress (SDS) model[143, 167, 173-176, 335]. SDS is a major preclinical model of depression that results in depressive-like behavior in rodents, including anhedonia, social avoidance, weight loss, and immune suppression[143, 144]. Two phenotypic stratifications result from chronic SDS: those “susceptible” that display depressive-like behaviors such as social avoidance and reduced sucrose preference, and mice that are “resilient” to these behavioral alterations[143, 145, 152] Chronic SDS has been demonstrated to increase alcohol consumption[167, 172-178], conditioned place preference[183], self-administration[184], and motivation to seek alcohol[184]. Our lab has further investigated these effects on the phenotypic subpopulations that arise following chronic SDS exposure. We have found that mice susceptible to SDS voluntarily consume more alcohol compared to resilient and control counterparts[167]. Additionally, susceptible mice display increased TACR1, the gene encoding the neurokinin-1 receptor (NK1R), in the nucleus accumbens (NAC) compared to resilient and control mice, suggesting NK1R as a potential mediator of this bidirectional relationship[167].

The NK1R receptor system and its endogenous ligand substance P (SP) have been implicated in numerous psychiatric disorders including stress, anxiety, depression, and alcoholism[251]. This receptor is heavily expressed in brain regions involved in affective behavior and reward circuitry, such as the amygdala, NAC, hypothalamus, hippocampus, and periaqueductal gray[251, 252]. The involvement of NK1R and SP has been extensively studied, indicating a role of this system in response to many preclinical stress- and anxiety-related paradigms in rodents[250, 277, 282], including immobilization stress[281], fear potentiated startle[293], fear conditioning[294], acoustic startle response[295], forced swim stress[282, 284, 285, 287, 297], maternal separation[273, 282, 286-288], chronic mild stress[297, 298], novelty-suppressed feeing[282], social interaction (SI)[287, 289-292], open field[282], and elevated plus maze[280-283, 336]. Specific to depressive-like behavior, our lab has recently determined NK1R mediates the anhedonic effects resulting from administration of lipopolysaccharide (LPS)[239]. The involvement of NK1R in anhedonic-behavior was displayed in another study assessing sucrose preference following bulbectomy, which induces depressive-like behavior and physiological alterations in rodents[299]. In this study, bulbectomy resulted in reduced sucrose preference in WT mice but not NK1R KO mice.

In addition to its role in stress and depression, NK1R and SP has also been studied as a mediator of alcohol-related behaviors[251, 337]. Clinically, a single nucleotide polymorphism in the TACR1 gene has been associated with alcohol dependence[274], and administration of a NK1R antagonist to abstinent alcoholics results in reduced alcohol cravings[264]. A role of this receptor system has also been identified in various preclinical models of alcohol consumption[261-265] and self-administration[266-269]. Related to consumption, NK1R antagonists attenuate escalated

alcohol consumption following intermittent ethanol access[261] and stress-induced alcohol intake following administration of the pharmacological stressor yohimbine[268]. NK1R is also involved in stress-induced relapse-like behavior, as antagonism of this receptor blocks reinstatement of alcohol seeking following intermittent footshock or yohimbine exposure[266-268, 270, 271]. Recent evidence has indicated a selective role of NK1R in the central amygdala (CeA) and NAC shell on these behaviors. For example, NK1R antagonism specifically within either the CeA or the NAC shell decreases sensitivity to stress-induced reinstatement[267, 270, 271], while NK1R overexpression in the CeA increases sensitivity to stress-induced reinstatement[271]. In addition, neuronal activation within the NAC shell following reinstatement is blocked with administration of a NK1R antagonist[267], further implicating NK1R in the circuitry of stress-induced behavioral alterations. Considering the vast amount of literature supporting a role of NK1R in stress responses, depressive-like behaviors, and alcohol-related behaviors, targeting this receptor system may be beneficial for the treatment of patients with comorbid alcoholism and depression.

In this study, we explored the involvement of the NK1R receptor in the effects of SDS. We hypothesized that NK1R would mediate the depressive- and alcohol-related responses following chronic SDS, and that overexpression of this receptor would increase sensitivity to social stress exposure. To test this hypothesis, we first determined the consequences of genetic deletion of NK1R on chronic SDS-induced alterations in SI and voluntary alcohol consumption. Next, we assessed the effects of pharmacological NK1R antagonism with administration of L703-606 on these same behaviors. Last, we examined the effects of viral-mediated NK1R overexpression in the NAC shell on stress sensitivity following subthreshold SDS.

4.2 Materials and Methods

Animals

Male NK1R KO mice bred on a C57BL6/J background and WT littermate controls (7 weeks of age, bred in house) were used in Experiment 1. Male C57BL6/J mice (7 weeks of age, Jackson Laboratory, Bar Harbor, ME) were used in Experiments 2 and 3. Retired breeder male CD-1 mice (4-5 months of age, Charles River, Wilmington, MA) were used as SDS aggressors. Due to sex specific differences in territorial aggression and the SDS protocol being replicated and highly validated using male C57BL6/J mice, only male subjects were used in this study. Mice were allowed 1 week of habituation to the UGA College of Veterinary Medicine vivarium before experiments began. Mice were housed in a 12hr light cycle (on at 1:00 and off at 13:00). Food and water were available *ad libitum*. All procedures were in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Georgia.

Drugs

For continuous access 2-bottle choice (2BC) in Experiments 1 and 2, 95% ethanol (Decon Labs, Inc., King of Prussia, PA) was diluted to a 20% v/v solution in tap water. In Experiment 2, NK1R antagonist L703-606 (Sigma-Aldrich, St. Louis, MO) was dissolved in 45% w/v 2-hydroxypropyl-beta-cyclodextrin (Sigma-Aldrich, St. Louis, Missouri) to a concentration of 1mg/ml and was injected at 10ml/kg, resulting in a final dose of 10mg/kg. Vehicle-treated mice were injected with 10ml/kg of 2-hydroxypropyl-beta-cyclodextrin.

Chronic SDS

SDS was performed as previously described[167] and was based off of the protocol described in Golden et al. 2011[143]. Prior to SDS, male CD-1 mice were screened for aggressive behavior by placing a screener C57BL/6J mouse (not used in the study) into the home cage of the CD-1 mouse for 180 seconds for 4 consecutive days. Aggressors were selected based on the following criteria: the CD-1 mouse must initiate an attack in at least two consecutive sessions and the latency to first display aggressive behavior must be less than 60 seconds. Aggressors that passed aggression criteria were placed into a large cage (26.7cm (w) x 48.3cm (d) x 15.2cm (h); product number: N40, Ancare, Bellmore, NY) with a clear, perforated divider 72 hours prior to the start of SDS. Defeat sessions consisted of placing C57BL6/J mice into the homecage of the CD-1 mouse for five minutes, after which the defeated mice were placed on the opposite side of the partition for the remainder of the 24 hours. This non-physical defeat phase allows for olfactory, visual, and auditory cues to be exchanged between the C57BL6/J mouse and the aggressive CD-1 it just encountered. This process then repeats for 10 consecutive days with the C57BL6/J mice encountering a novel aggressor each day. Control mice were housed in pairs in identical hamster cages with a mouse on either side of the perforated divider. Mice were rotated each day to mimic the novel housing conditions experienced by the SDS mice but did not get physically defeated nor encountered any CD-1 mice throughout the 10 days. Defeats occurred at the end of the light cycle (as close to the beginning of the dark cycle as possible) in order to visualize any injuries. If blood was drawn, the mice were separated and the time at which the session ended was noted.

SI Test

Immediately following the final defeat session, mice were singly housed in regular mouse cages with food and water available *ad libitum.* Approximately 24 hours following the last defeat exposure (at the beginning of the dark cycle), mice underwent the SI test. This test consisted of 2 150-second trials separated by 30 seconds, the first without a novel CD-1 target mouse present, and the second with a target mouse present in an enclosure in the predetermined interaction zone. Time spent in the interaction zone during each trial is recorded and divided, time in the interaction zone with the target mouse present by the time when the target was absent, to obtain the SI ratio. SI tests were scored by an observer blind to the treatment group of each mouse.

2BC

Two weeks following the final defeat session, mice were presented with two bottles in their homecage, one containing water and one containing a 20% ethanol solution on a continuous access schedule. Bottles were weighed at the same time each day and g/kg consumption was calculated based on each mouse's body weight. Mice were allowed access until stable (group variability under 25% for three consecutive days for all groups within the cohort). Consumption levels are presented as g/kg on average over the last 3 days of drinking access. Ethanol preference was calculated by dividing the volume of ethanol consumed by the total volume (ethanol plus water) consumed and is presented as a percent (%) of total volume consumed.

Intracranial virus infusion

AAV1-NK1R-GFP was produced by PCR amplification of the NK1R (TACR1) cDNA from a lentiviral vector containing rat TACR1 (GeneCopoeia; # CS-Rn10116-Lv156–01) and subsequent insertion upstream of the IRES-EGFP element within pAAV

CMV-IE MCS IRES EGFP (Addgene #102936). The resulting construct pAAV CMV-IE NKR1 IRES GFP (Addgene #102935) was packaged as an AAV serotype 1 and purified by affinity chromatography as previously described[338]. The resulting vector is referred to as “AAV1-NK1R-GFP”. AAV1-NK1R-GFP or control AAV1-GFP (Vector Biolabs) virus was bilaterally infused into the NAC shell (bilateral, AP: +1.7mm, ML: ±2.3mm, DV: -4.7mm, 20° angle) of C57BL6/J mice using a stereotaxic instrument (Stoelting, Wood Dale, IL). Microinfusions were given through a blunt end Hamilton Neuros Syringe (Reno, NV) mounted to the stereotax and controlled by a Precision syringe pump (World Precision Instruments, Sarasota, FL). Infusions were given in a volume of 0.5ul at a rate of 0.1ul/minute. Syringes were left in place for 5 minutes following infusion to prevent aspiration along the needle tract. After 4 weeks of recovery to allow for full viral vector expression, mice were exposed to subthreshold SDS and then assessed for SI. Following the SI test, mice were sacrificed, and placements of viral infusion were checked via fluorescent microscopy.

Subthreshold SDS

Mice were exposed to 3 5-minute defeat sessions separated by 15 minutes each. During each defeat session, mice were placed into the homecage of a novel male CD-1 mouse previously screened for aggressive behavior. Between defeat sessions, mice were returned to their homecage to recover until the next defeat session started. Defeats occurred at the end of the light cycle (as close to the beginning of the dark cycle as possible) in order to visualize any injuries. If blood was drawn, the defeat session ended early and the time at which the session ended was noted. SI was tested 24 hours after subthreshold SDS.

Experimental timelines

A summary timeline for each experiment in this study can be found in Figure 4.1 and specific protocols are described in the following sections. Briefly, in Experiment 1, male NK1R KO mice or WT littermate controls were exposed to chronic SDS (or were non-stressed controls) and tested for SI 24 hours later. This resulted in four groups: SDS-exposed KO mice, SDS-exposed WT mice, non-stressed KO mice, and non-stressed WT mice. In Experiment 2, male C57BL6/J mice were exposed to chronic SDS (or were non-stressed controls) and received either 10mg/kg L703-606 or vehicle (i.p.) 30 minutes prior to each defeat. This resulted in four groups: SDS/antagonist mice, SDS/vehicle mice, non-stressed/antagonist mice, and non-stressed/vehicle mice. In both Experiments 1 and 2, mice were allowed continuous access to 20% alcohol via 2BC 2 weeks after the last defeat session. In Experiment 3, mice were injected with an AAV virus overexpressing the NK1R receptor or a GFP control virus into the NAC shell. After a month recovery, mice were exposed to subthreshold SDS and SI was assessed 24 hours after.

Statistical Analyses

Statistical analyses were preformed using GraphPad Prism software. Statistica software was used to preform Newman-Keuls posthoc tests. In Experiment 1, SI ratio, alcohol consumption, and alcohol preference were analyzed by a two-way ANOVA with factors of genotype (NK1R KO vs. WT) and SDS (SDS vs. control). In Experiment 2, SI ratio, alcohol consumption, and alcohol preference were analyzed by a two-way ANOVA with main factors of treatment (L703-606 vs. vehicle) and SDS (SDS vs. control). In the stratified comparisons, a one-way ANOVA was used with a factor of group. Newman-Keuls posthoc tests were performed to assess individual group differences if a significant

interaction or two significant main effects were obtained for two-way ANOVA analyses or if a significant main effect was obtained for the one-way ANOVA analyses.

4.3 Results

Chronic SDS reduces SI regardless of genotype

Due to previous findings implicating NK1R in depressive-like behavior, we first explored the effects of genetic deletion of NK1R on SI following 10 days of chronic SDS. NK1R KOs and WT littermate controls were exposed to chronic SDS (KO: n=4, WT: n=5) or were non-stressed controls (KO: n=4, WT: n=4). SI was assessed 24 hours after the final defeat session. A two-way ANOVA revealed a significant effect of SDS ($F(1,13)=18.69$, $p=0.00080$) but no interaction ($F(1,13)=0.046$, $p=0.83$) and no main effect of genotype ($F(1,13)=0.47$, $p=0.50$; Figure 4.2).

Chronic SDS increases alcohol consumption and preference regardless of genotype

Next, we assessed alcohol consumption via continuous access 2BC to 20% ethanol. Mice were allowed access to 2BC 2 weeks following the last defeat session for at least 2 weeks and until a stable level of consumption was obtained. We have previously shown that mice susceptible to chronic SDS display increased consumption compared to resilient and control counterparts. However, SI testing determined only one mouse out of both NK1R KOs and WTs exposed to SDS were of the resilient phenotype, and so we did not phenotypically stratify this data set. When comparing average consumption over the last three days, a two-way ANOVA revealed a significant main effect of SDS ($F(1,9)=8.47$, $p=0.017$), but no interaction ($F(1,9)=0.070$, $p=0.80$) and no main effect of genotype ($F(1,9)=0.46$, $p=0.53$; Figure 4.3A). Similar effects on alcohol preference were observed, as a two-way ANOVA revealed a main effect of SDS

(F(1,9)=5.35, p=0.046), but no interaction (F(1,9)=0.15), p=0.71) and no main effect of genotype (F(1,9)=0.0093, p=0.93; Figure 4.3B).

NK1R antagonism prevents the decrease in SI following chronic SDS

As the NK1R KO mice displayed SI behavior and alcohol consumption similar to that of WTs following chronic SDS, we next wanted to assess the effects of systemic administration of the NK1R antagonist L703-606 on these behaviors. This will allow for more temporally specific inhibition of NK1R as opposed to a using a KO line that has lacked this receptor since early development and may exhibit compensatory neuroadaptations. C57BL6/J mice were exposed to chronic SDS or were non-stressed controls and received either 10mg/kg L703-606 (i.p.) (SDS: n=4, control: n=9) or vehicle (SDS: n=6, control: n=6) 30 minutes prior to each defeat. SI was assessed 24 hours after the final defeat session. A two-way ANOVA revealed a significant interaction (F(1,21)=5.41, p=0.03) and a significant main effect of SDS (F(1,21)=4.51, p=0.046), but no main effect of treatment (F(1,21)=0.40, p=0.54; Figure 4.4A). Newman-Keuls posthoc comparisons indicated that the SDS/vehicle group was significantly lower than the non-stressed/vehicle group (p=0.023), but the SDS/antagonist group was not significantly different from the non-stressed/antagonist group (p=0.89) and the non-stressed/vehicle group was not statistically different from the non-stressed/antagonist group (p=0.30).

To stratify the SDS-exposed mice, we used a median split to determine our susceptible/resilient cutoff. This determined a SI ratio cutoff value of 1.2, whereas mice below 1.2 were considered susceptible, and mice over 1.2 were considered resilient. While all mice in the SDS/antagonist and non-stressed/vehicle groups were classified as resilient based on this criteria, one mouse in the non-stressed/antagonist group had a SI ratio under 1.2 and thus was not used in this comparison. A one-way ANOVA revealed a

significant group effect (F(4,19)=6.25, p=0.0022, Figure 4.4B). Newman-Keuls posthoc comparisons indicated that the susceptible SDS/vehicle mice were significantly different than every other group. More specifically, susceptible SDS/vehicle mice were significantly lower than resilient SDS/antagonist mice (p=0.011), resilient SDS/vehicle mice (p=0.003), resilient non-stressed/antagonist mice (p=0.009), and resilient non-stressed/vehicle mice (p=0.003).

NK1R antagonism during chronic SDS does not alter subsequent alcohol consumption

We have demonstrated that L703-606 administration prior to each defeat session prevents the decrease in SI typically observed following chronic SDS. We were also interested in assessing the effects of L703-606 treatment during SDS on subsequent alcohol consumption. Two weeks after the final SDS session, the mice were allowed continuous access 2BC to 20% ethanol until a stable level of consumption was obtained. A two-way ANOVA indicated a main effect of SDS exposure (F(1,21)=5.00, p=0.036), but no interaction (F(1,21)=1.98, p=0.17) or main effect of L703-606 (F(1,21)=1.32, p=0.26) (Figure 4.5A). Similar effects were observed when comparing alcohol preference, as a two-way ANOVA revealed a significant effect of SDS (F(1,21)=4.78, p=0.04) but no interaction (F(1,21)=0.77), p=0.39) and no main effect of L703-606 (F(1,21)=0.39, p=0.54) (Figure 4.5B).

We then assessed differences in consumption within our stratified populations. Elevated levels of alcohol consumption and preference were observed in resilient SDS/antagonist mice and susceptible SDS/vehicle mice compared to resilient SDS/vehicle mice, non-stressed/vehicle mice, and non-stressed/antagonist mice. A one-way ANOVA revealed a potential trend towards significance for consumption (F(4,19)=2.12, p=0.12, Figure 4.5C) and preference (F(4,19)=1.77, p=0.18, Figure 4.5D).

This pattern of data suggests that the alcohol-related behaviors of resilient SDS/antagonist mice are comparable to that of susceptible SDS/vehicle mice, and as the SDS/antagonist mice still display increased drinking behaviors despite their resilient phenotype in the SI test. While not statistically significant at this point, we believe this is an issue of power that will be resolved by including larger group sizes.

NK1R overexpression in the NAC shell increases sensitivity to subthreshold SDS

We have previously observed that mice susceptible to chronic SDS display increased expression of TACR1, the gene for the neurokinin-1 receptor, in the NAC[167]. Thus, we wanted to investigate the effects of NK1R overexpression in the NAC shell on sensitivity to social stress. To accomplish this, we used the subthreshold SDS protocol[143, 167]. This one-day stress exposure does not typically induce depressive-like behavior in mice, thus is allows for the investigation of pro-depressant interventions, such as chronic alcohol exposure, on stress sensitivity[143]. C57BL6/J mice were stereotaxically injected with either AAV1-NK1R-GFP (n=7) or AA1-GFP (n=7) in the NAC shell and were allowed to recover for 4 weeks. Following this recovery period, mice were exposed to subthreshold SDS and tested for SI 24 hours later. Mice infused with the NK1R overexpression virus in the NAC shell displayed an increase in stress sensitivity as evidenced by a decrease in SI following subthreshold SDS compared to mice infused with the GFP control virus ($t(12)=2.49$, $p=0.028$, Figure 4.6A). Placements of virus infusion can be observed in Figure 4.6B.

4.4 Discussion

In this study, we demonstrate selective effects of NK1R antagonism during chronic SDS on depressive-like behavior, but not alcohol-related behaviors, following stress exposure. First, we show that genetic deletion of NK1R does not affect the

reduced SI and increased alcohol consumption and preference observed in WT mice. We hypothesized that this was due to constitutive nature of the NK1R genetic deletion, thus we tested the effects of temporally specific antagonism of NK1R in WT mice. Then, we demonstrate that antagonism of NK1R by L703-606 prior to each defeat session prevents the decreased SI resulting from chronic SDS. However, L703-606 pretreatment during SDS did not impact later drinking behaviors, as mice receiving the NK1R antagonist during the defeats showed increased alcohol consumption and preference despite their resilient phenotype determined by the SI test. Last, we found that viral-mediated overexpression of the NK1R receptor in the NAC shell of WT mice increases sensitivity to subthreshold SDS. Together, these findings further support a functional role of NK1R at the time of SDS exposure on depressive-like behavior, but not drinking behaviors, following stress exposure.

We have demonstrated in Experiment 1 that NK1R KO and WT mice display similar effects on SI and voluntary consumption following exposure to chronic SDS. The NK1R KO mice used in this study have a constitutive KO of the TACR1 gene, meaning that these mice have lacked this receptor throughout all periods of development. As such, expression of other receptors within the tachykinin family such as neurokinin-2 (NK2) and neurokinin-3 (NK3) receptors may be upregulated in a compensatory fashion. This effect has been observed in mice lacking the serotonin(1A) receptor ($5HT_{1A}$)[277] in that mice with a genetic deletion of this receptor are observed to have increased sensitivity of the serotonin(2A) receptor ($5HT_{2A}$)[277, 339]. Both NK2R and NK3R are expressed in regions involved in affective disorders and emotional processing and have been implicated in depressive-like and anxiety-like behavior in rodents[340-343]. While NK2R and NK3R preferentially bind to neurokinin A (NKA) and neurokinin B (NKB)

respectively, these receptors have modest affinity to bind SP[250]. As such, alterations in the expression of NK2R and NK3R in KOs compared to the basal expression of WTs will be examined in future studies.

NK1R KO mice also displayed increased drinking following exposure to chronic SDS. A previous study assessing drinking behavior of NK1R KO reported that KOs voluntarily consume less alcohol compared to WTs[262], an effect which was corroborated by another study demonstrating that viral mediated silencing of NK1R similarly reduced voluntary consumption[263]. In the latter study, the age of the mice used was not given, but the mice weighed between 20-30g indicating they were most likely in adulthood, thus the NK1R receptor was functional during development[263]. It is important to note that neither of these studies examine the effects of genetic deletion/silencing of NK1R on stress-induced consumption, let alone exposure to a stressor that robustly increases voluntary consumption in mice as chronic SDS does[167, 172-176, 178]. However, it must also be noted that we did not see a difference between our non-stressed KOs and non-stressed WTs, as has been previous demonstrated[262]. In our study, we used 20% ethanol for the entirety of the 2BC protocol, while Thorsell et al. 2010 started 2BC at 3% ethanol, and increased by 2% increments up until 15% ethanol which was used for the remainder of the 2BC protocol[262]; thus, stark variations in the 2BC protocol may explain the differences observed in our study. The robust nature of chronic SDS to increase alcohol consumption, in combination with the potential alterations in the expression of NK2R and NK3R, which may be another possible explanation as to why we observed increased consumption in the NK1R KO mice in addition to the potential upregulation of NK2R and NK3R.

In Experiment 2, we observed antidepressant effects of NK1R antagonism by L703-606 prior to defeat stress exposure as it prevented the decrease in SI observed in SDS/vehicle mice. However, we did not see an attenuation of alcohol consumption following stress, despite the resilient phenotype observed in the SDS/antagonist mice. This result could be explained by the timing of the antagonist treatment. Mice received L703-606 treatment during the SDS portion of the experiment, which was 2 weeks prior to the start of 2BC which lasted for at least another 2 weeks. Therefore, consumption was assessed for stability at least a month after the mice received the antagonist. While it has been reported that systemic NK1R antagonism reduces voluntary alcohol consumption in both stress-naïve[262] and stressed rodents[261], these effects were observed when the NK1R antagonist was administered during the drinking phase of the experiment once a stable level of consumption was obtained. To assess this in future studies, we will assess the effects of NK1R antagonism on the stress-induced increase in alcohol consumption observed in susceptible mice following chronic SDS by administering L703-606 once consumption levels have stabilized.

In our previous work, we found that mice susceptible to SDS display increased gene expression of TACR1 in the NAC. Here, we found that viral-mediated overexpression of NK1R in the NAC shell increases sensitivity to subthreshold SDS. The NAC shell plays a pivotal role in the response to SDS, particularly driven by the transcription factor nuclear factor kappa light chain enhancer of activated B cells (NFkB)[149, 151]. NK1R has been shown to activate NFkB[344-346] and the TACR1 gene has NFkB-responsive elements within its promotor[347], demonstrating a bidirectional relationship between NK1R and NFkB[192]. As mice exposed to social stress display increased activity of components of the NFkB pathway in the NAC

shell[149, 151], the increased sensitivity to social stress following NK1R overexpression we observed may be explained by an increase in NFkB activity within this region and will be assessed in future studies.

In this study, we demonstrate that manipulation of NK1R activity, specifically during stress exposure, influences the depressive-like behavioral sequelae, but not alcohol-related effects, of chronic SDS exposure. While NK1R KO mice display behavior similar to that of WT mice, temporal antagonism of NK1R during stress exposure prevented the decrease in SI observed in chronic SDS-exposed mice treated with vehicle. This effect was bidirectional, as overexpression of NK1R increased sensitivity to subthreshold SDS. While we did not obtain effects of NK1R antagonism during stress on later alcohol consumption or preference, this is likely explained by the antagonist treatment being at least a month prior to consumption stabilization. Future studies will examine NK2R and NK3R expression alterations as a consequence of NK1R genetic deletion and NK1R antagonism during alcohol consumption following exposure to chronic SDS. To our knowledge, our results are the first to report a functional role of NK1R in the SDS-induced behaviors and further support the role of NK1R in the underlying circuitry of depression.

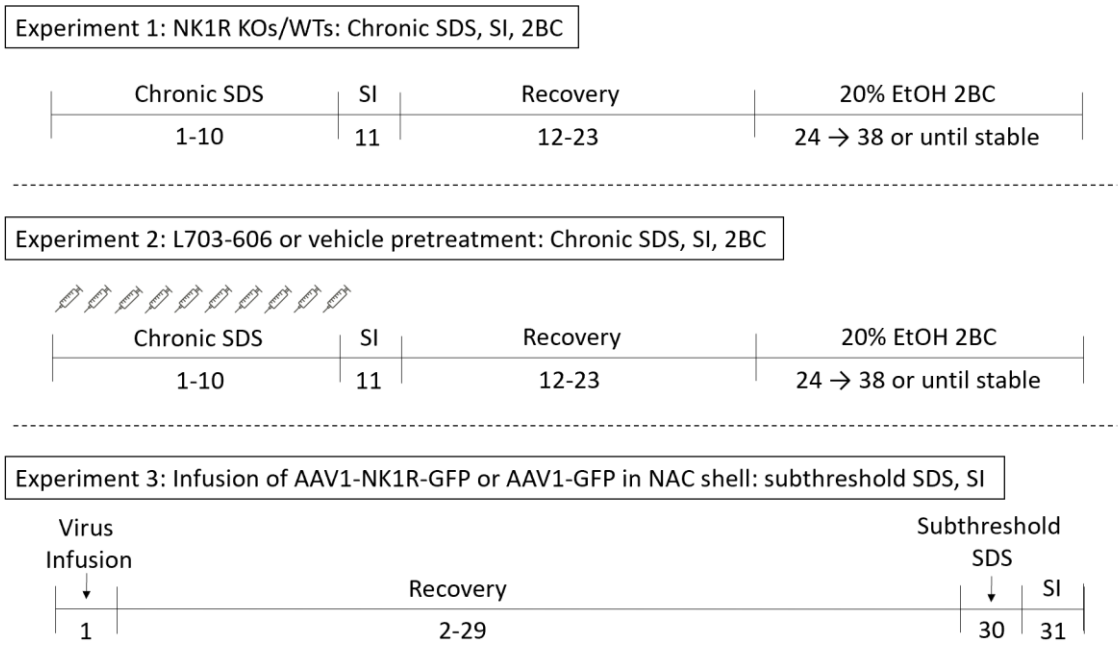

Experiment 1: NK1R KOs/WTs: Chronic SDS, SI, 2BC
Chronic SDS
1-10
SI
11
Recovery
12-23
20% EtOH 2BC
24 → 38 or until stable
Experiment 2: L703-606 or vehicle pretreatment: Chronic SDS, SI, 2BC
Chronic SDS
1-10
SI
11
Recovery
12-23
20% EtOH 2BC
24 → 38 or until stable
Experiment 3: Infusion of AAV1-NK1R-GFP or AAV1-GFP in NAC shell: subthreshold SDS, SI
Virus Infusion
1
Recovery
2-29
Subthreshold SDS
30
SI
31

Figure 4.1: Timelines for Experiments 1, 2, and 3.

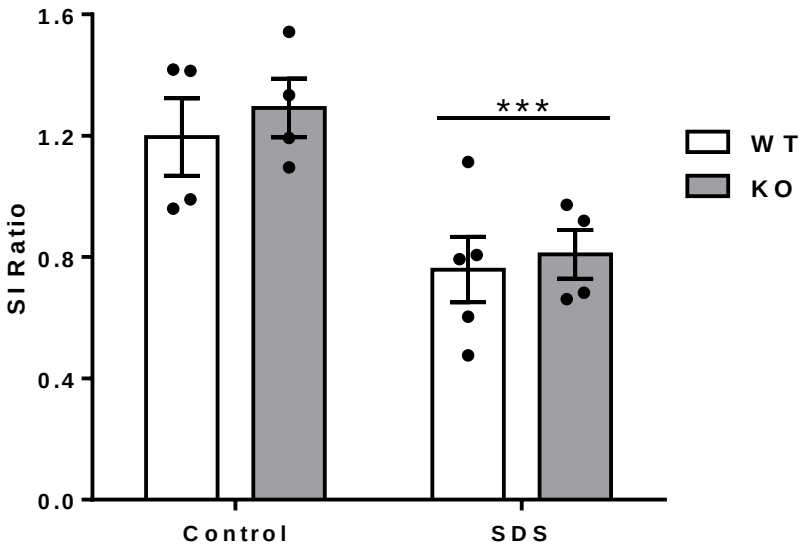

1.6
1.2
0.8
0.4
0.0
SI Ratio
***
WT
KO
Control
SDS

Figure 4.2: Chronic SDS reduces SI regardless of genotype. Chronic SDS significantly reduced SI in NK1R KO and WT mice. No differences between genotype were observed. ***p<0.001 compared to non-stressed controls.

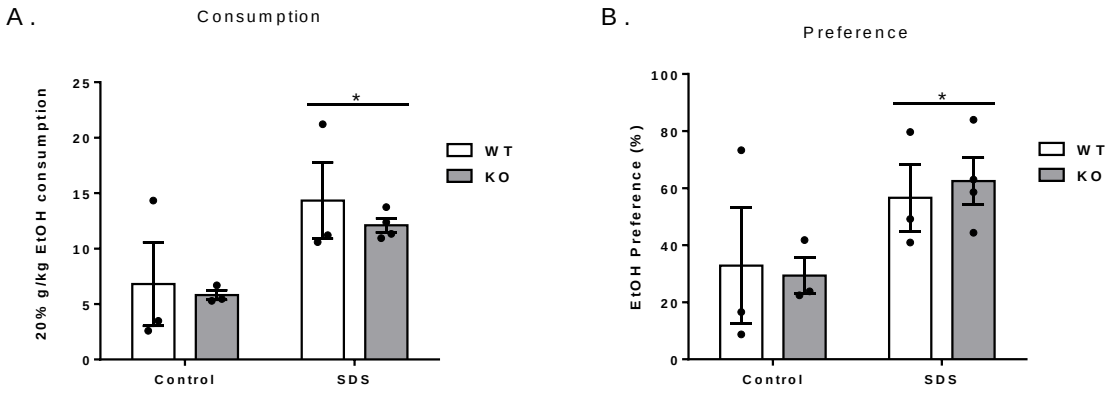
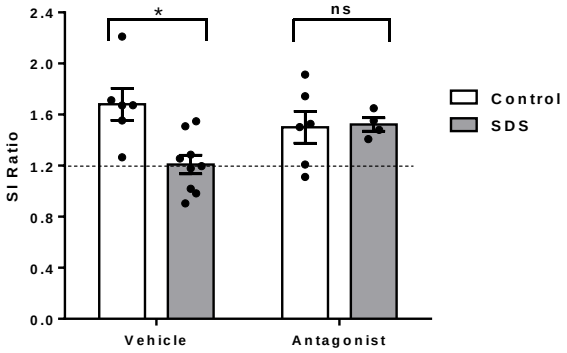

A.
Consumption
20% g/kg EtOH consumption
25
20
15
10
5
0
*
Control
SDS
WT
KO
B.
Preference
EtOH Preference (%)
100
80
60
40
20
0
*
Control
SDS
WT
KO

Figure 4.3: Chronic SDS increases alcohol consumption and preference regardless of genotype. (A) Following chronic SDS, both NK1R KO and WT mice display an increase in alcohol consumption compared to control mice. No differences were observed between genotypes. **(B)** Similarly, NK1R KO and WT mice exposed to SDS display an increase in alcohol preference compared to control mice, with no effect of genotype observed. *$p<0.05$ compared to non-stressed controls.

A.


*
ns
2.4
2.0
1.6
1.2
0.8
0.4
0.0
SI Ratio
Vehicle
Antagonist
Control
SDS

B.

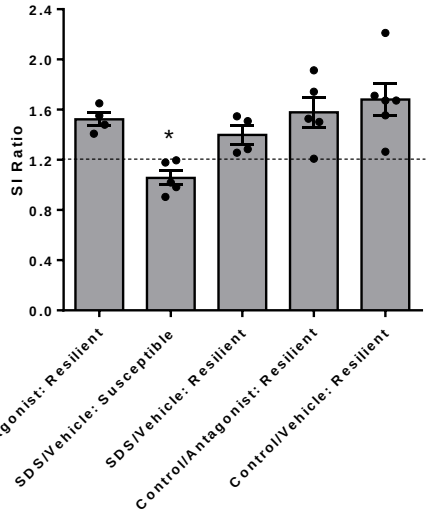

2.4
2.0
1.6
1.2
0.8
0.4
0.0
SI Ratio
*
SDS/Antagonist: Resilient
SDS/Vehicle: Susceptible
SDS/Vehicle: Resilient
Control/Antagonist: Resilient
Control/Vehicle: Resilient

Figure 4.4: Pretreatment with NK1R antagonist L703-606 prevents the decrease in SI observed in SDS-exposed mice. (A) While vehicle-treated mice displayed a decrease in SI following chronic SDS compared to the non-stressed/vehicle group, this effect was not observed in SDS/antagonist mice. Administration of L703-606 prior to defeat exposure attenuated this difference, resulting in SI behavior comparable to the non-stressed control groups. **(B)** A median split was used to separate the SDS/vehicle group into susceptible and resilient phenotypes for further analysis, indicated by the dotted line at 1.2. SDS/vehicle mice susceptible to SDS displayed an SI ratio significantly lower than all other groups. *$p<0.05$ compared to non-stressed/vehicle group **(A)** or compared to all other groups **(B)**.

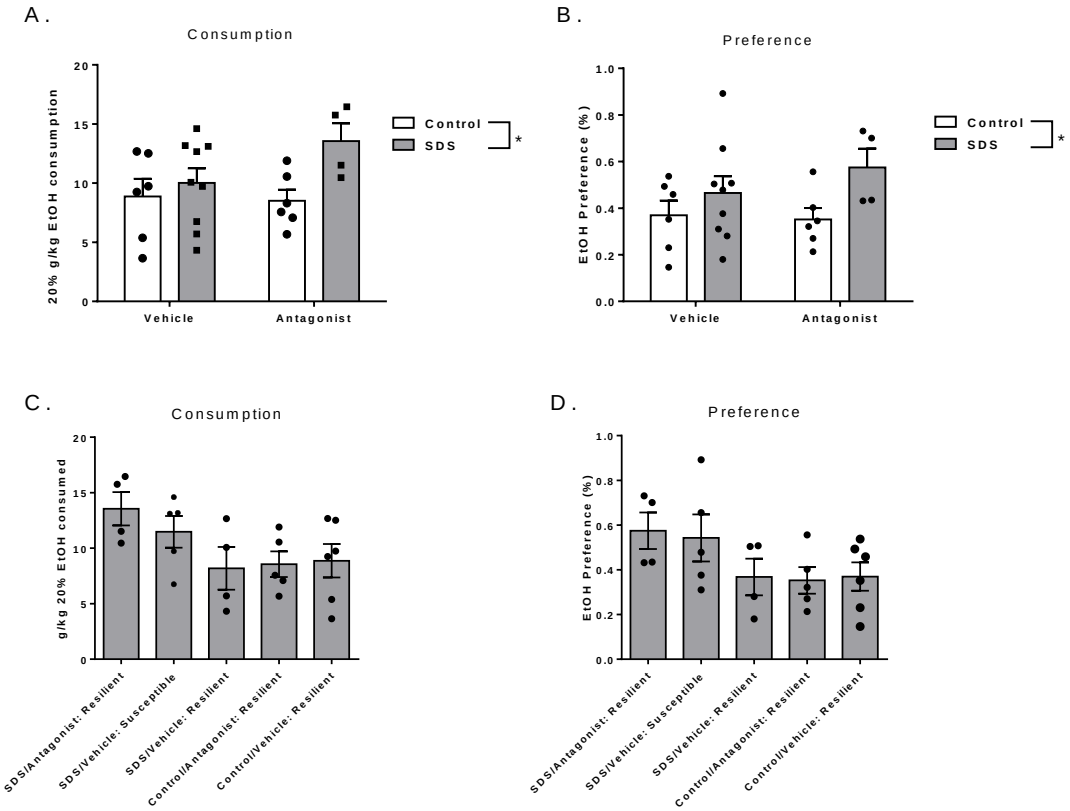
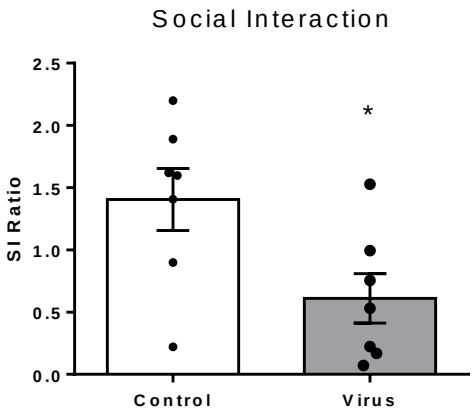

A.
Consumption
20% g/kg EtOH consumption
Control
SDS
*
Vehicle
Antagonist
B.
Preference
EtOH Preference (%)
Control
SDS
*
Vehicle
Antagonist
C.
Consumption
g/kg 20% EtOH consumed
SDS/Antagonist: Resilient
SDS/Vehicle: Susceptible
SDS/Vehicle: Resilient
Control/Antagonist: Resilient
Control/Vehicle: Resilient
D.
Preference
EtOH Preference (%)
SDS/Antagonist: Resilient
SDS/Vehicle: Susceptible
SDS/Vehicle: Resilient
Control/Antagonist: Resilient
Control/Vehicle: Resilient

Figure 4.5: NK1R antagonism during chronic SDS exposure does not impact SDS-induced alterations in alcohol consumption. (A) Mice exposed to chronic SDS display a significant increase in alcohol consumption compared to non-stressed controls, while no effect of antagonist treatment was observed. (B) Similar to consumption, mice exposed to chronic SDS display a significant increase in alcohol preference compared to non-stressed controls (C) Resilient SDS/antagonist mice and susceptible SDS/vehicle mice display a trend towards increased alcohol consumption and preference (D) compared to all other groups. *$p<0.05$ compared to non-stressed controls.

A.


Social Interaction
SI Ratio
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Virus

B.

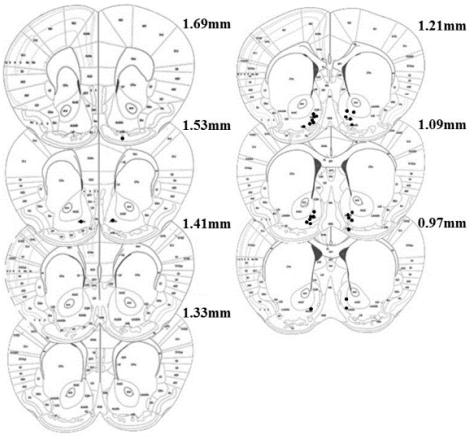

1.69mm
1.53mm
1.41mm
1.33mm
1.21mm
1.09mm
0.97mm

Figure 4.6: Overexpression of the neurokinin-1 receptor in the NAC shell increases sensitivity to subthreshold SDS. **(A)** Virus-infused mice display decreased SI following subthreshold SDS compared to control mice. **(B)** Placement of virus infusions. *$p<0.05$ compared to control group.

CHAPTER 5:

SUMMARY AND CONCLUSIONS

5.1 The Role of NFkB in Alcohol-Related Responses

In this report, we demonstrated a relationship between NFkB and the behavioral and physiological responses to alcohol. In Chapter 2, we determined a functional role of NFkB, specifically within the NAC shell, in the circuitry of alcohol reward. To do so, we used NFkB-LacZ mice, a transgenic mouse strain that expresses LacZ under the direction of an NFkB-regulated promoter[215]. Thus, wherever NFkB is activated, β-galactosidase will be present within that cellular population. The presence of β-galactosidase not only allows for a visual readout of NFkB activity using staining methods such as immunohistochemistry and immunofluorescence, but also allowed us to use the Daun02 selective inactivation method to permanently silence NFkB-expressing cells within a region of interest[316]. This is the first study using the Daun02 inactivation method in NFkB-LacZ mice, indicating an exciting degree of novelty of these experiments.

Using this method, we determined that NFkB in the NAC shell has an important role in the development of alcohol reward. The role of NFkB in alcohol reward was specific to the conditioning phase of CPP, as NFkB inactivation following the expression of alcohol CPP during the test session did not impact retest preference. Classically, NFkB is thought of in terms of its activation to large, neurotoxic doses of alcohol[199, 207, 302]. However, while NFkB indeed influences the transcription of genes associated with inflammatory processes, NFkB is also involved in various other processes including

learning and memory[317, 318], drug reward[216], and spine remodeling in response to chronic drug exposure[192, 215]. Here we provide further support for the involvement of NFkB in rewarding effects of moderate doses of alcohol.

As NFkB is a transcription factor, it is our hypothesis that silencing of NFkB-expressing cells in the NAC is preventing specific alterations in gene expression that are essential for the formation of alcohol reward. A potential way to assess these gene expression alterations in the future would be to preform CHIP-sequencing (CHIP-seq) on tissue from mice conditioned with either alcohol or saline. Tissue from the NAC would be assessed 2 hours after the final alcohol or saline conditioning session, which is the timepoint when we observed the initial increase in NFkB activity in mice conditioned with alcohol.

While the number of genes targeted by NFkB is vast, it is interesting to note that TACR1, the gene for the NK1R, has an NFkB binding site within its promoter[347, 348]. Genetic deletion of NK1R results in decreases alcohol consumption[262-264] and alcohol CPP[262]. Similarly, pharmacological and viral inhibition of IKK, the enzyme that frees NFkB from its inhibition by IkB, also decreases voluntary alcohol consumption[210]. If gene expression of TACR1 is increased in the NAC shell following alcohol place conditioning, this may indicate a specific mechanism through which NFkB mediates alcohol reward and consumption. This particular mechanism would be supported if infusion of a constitutively active form of IKK into the NAC shell of NK1R knockout mice prevents the decreased alcohol reward and consumption typically observed in knockouts. The results of such a study would provide novel insight on the particular mechanism by which NFkB and NK1R influence alcohol reward.

In Chapter 3, we shifted our focus to assess the effects of voluntary consumption on stress-sensitivity and alterations in the NFkB pathway that result following alcohol exposure. To accomplish this, we assessed sensitivity to subthreshold SDS following 4 weeks of IEA. We have previously demonstrated that involuntary, binge-like consumption via chronic alcohol gavage increases sensitivity to social stress[167]. In this study, we were curious if similar effects would be observed following voluntary alcohol consumption. To achieve high levels of consumption, we exposed mice to alcohol on an intermittent schedule. Due to alternating periods of alcohol access and deprivation, IEA results in elevated alcohol consumption levels compared to that observed on a continuous access schedule[24, 25, 323, 324]. Here, we found that exposure to 4 weeks of IEA increases sensitivity to social stress, similar to chronic alcohol gavage administration. We also used quantitative polymerase chain reaction (qPCR) to assess changes in IkB expression in wildtype mice and immunohistochemistry to assess β-galactosidase expression in NFkB-LacZ mice following both IEA and chronic alcohol gavage. We found that mice exposed to IEA show decreased expression of IkB in the NAC and AMY and increased NFkB activity in various subregions of the amygdala including the CEA, BLA, and MEA. This is in comparison to chronic alcohol gavage, which didn't impact IkB gene expression but did increase NFkB activity specifically in the CEA.

As both of these alcohol exposure methods increased sensitivity to subthreshold SDS and increased NFkB activity in the CEA, this suggests the CEA as a potential mediator of alcohol-induced increases in stress sensitivity. As such, a future direction of this study would be to inhibit NFkB activity, perhaps via Daun02 inactivation in our NFkB-LacZ mice, to prevent the increased stress-sensitivity resulting from these alcohol

exposure models. This would not only confirm the role of the CEA as an important mediator of alcohol-induced effects on stress-sensitivity, but also in the specific involvement of NFkB in these effects. While there is a plethora of data implicating NFkB in alcohol-related behaviors and stress-responses in separate studies, there is a current gap in the literature connecting the role of NFkB in both of these processes. These results would indicate the involvement of NFkB in the processes underlying comorbid alcoholism and depression and support further investigation of this transcription factor as a potential therapeutic target for individuals diagnosed with both of these disorders.

5.2 The Role of NK1R in Depressive-like Behavior and Alcohol Consumption

In Chapter 4 we introduced an additional, but related, potential therapeutic target for comorbid alcoholism and depression, the NK1R. In this study, we assessed the effects of genetic and pharmacological blockade of the NK1R, using a NK1R knockout line and systemic NK1R antagonism, respectively, on the depressive-like behavior and increased consumption that arises following exposure to chronic SDS. We have previously observed decreased SI and increased alcohol consumption in susceptible, but not resilient mice, following chronic SDS[167]. Here, we found that NK1R knockout mice display decreased SI and increased alcohol intake similar to wildtype mice. This result was unexpected and is a bit perplexing. However, NK1R knockout mice have been lacking the NK1R throughout development, thus upregulation of other neurokinin receptors, such as NK2R and NK3R, in a compensatory fashion is possible. While these receptors preferentially bind to other neuropeptides, they have modest affinity to bind SP[250], and consequently may be driving the decreased SI and increased consumption following chronic SDS in NK1R knockout mice.

Following this result, we displayed a protective effect of systemic NK1R antagonism in wildtype mice, as mice that were exposed to chronic SDS but received the NK1R antagonist prior to each defeat did not display a reduction in SI behavior and were of the resilient phenotype. However, despite their resiliency, the antagonist-treated SDS-exposed mice consumed similar amounts of alcohol to their susceptible vehicle-treated SDS-exposed counterparts. We have previously found that mice susceptible to chronic SDS display increased TACR1 expression in the NAC. In our study, we found that viral-mediated overexpression of TACR1 in the NAC increased sensitivity to subthreshold SDS, further supporting the role of TACR1 in stress-sensitivity. In future studies, we will use qPCR to determine expression levels of NK2R and NK3R in NK1R knockout mice and compare these levels to those observed in wildtype mice. If one of these receptors is indeed upregulated in knockout mice, perhaps antagonism of that particular receptor in NK1R knockouts prior to defeat sessions will successfully prevent the decreased SI and increased consumption observed in these mice.

In the systemic antagonist experiment, while NK1R antagonism attenuated the decrease in SI following chronic SDS, these mice still displayed elevated levels of alcohol consumption following stress. Treatment of the NK1R antagonist occurred during the chronic SDS phase of the experiment, which was two weeks prior to the beginning on 2BC. Thus, by the time the mice reached stability two weeks later, a month had passed since the antagonist treatment. In future studies, we will assess the effects of NK1R antagonism during the drinking phase of the experiment on the increased consumption observed in susceptible mice. We hypothesize that antagonist treatment will reduce consumption specifically in susceptible mice. In this experiment, resilient and control mice may also decrease consumption levels, but if this occurs, we predict it to be

to a much lower extent than observed in the susceptible mice. These studies would also implicate NK1R in the processes underlying comorbid alcoholism and depression, and targeting this receptor may have beneficial results in individuals displaying these disorders.

While NK1R has yet to be explored at the clinical level for the treatment of comorbid alcoholism and depression, NK1R antagonists has been evaluated in clinical trials for the treatment of major depressive disorder (MDD)[349]. While initial clinical trial results appeared promising, the investigation of NK1R antagonists for the treatment of MDD was abandoned in the early 2000s due to suboptimal results in a Phase III clinical trial[349]. However, further studies have determined potential explanations for the Phase III trial results, particularly issues regarding the formulation and the insufficient receptor occupancy of the antagonist tested at this level[349, 350]. Due to these conclusions, our group and others have continued to explore the role of this receptor in depression circuitry, particularly in relation to the relationship between depressive-like behavior and alcohol exposure. It is our hope that results from our studies examining the involvement of NK1R in the processes underlying comorbid alcoholism and depression will change the perspective of those who think targeting NK1R for psychiatric disorders is a lost cause.

5.3 Lack of Generalizability of SDS Studies

One drawback to the SDS model is that it can only be used in male mice due to sex specific differences in territorial aggression. Thus, the data presented in Chapters 2 and 3 were solely performed using male mice. This is of major concern due to the fact that depression is nearly twice as prevalent in females than in males[351]. Therefore, while the results of these studies are important for identifying potential mediators of

comorbid alcoholism and depression, they cannot be generalized to both sexes. Interestingly, the increased prevalence of depression observed in females is a global phenomenon, suggesting that the increased risk for depression in females is attributed to biological differences between the two sexes as opposed to race, cultural, and environmental factors[352].

One method by which the involvement of NFkB and NK1R in alcohol-related and depressive-like behavioral can be assessed in females is by vicarious exposure to SDS. This witness stress exposure, achieved by housing a test mouse directly next to the enclosure where another mouse is physically defeated, is sufficient to induce depressive-like behavioral phenotypes that are similar to physical defeat[353]. It was recently shown that this model can be used to induce depressive-like behavior in both male and female C57BL6/J mice[354], thus allowing for the direct assessment of the mechanisms that mediate depressive-like behavior in females and how this compares to the processes that govern this behavior in male animals. Therefore, in the future, the experiments in Chapters 3 and 4 should be repeated with female mice using the vicarious SDS protocol in order to compare the behavioral and physiological responses to alcohol and stress between the sexes. These studies may provide important insight on the mechanisms driving the increased prevalence of depressive disorders in females and may help determine if different treatment strategies may be beneficial for males and females with depression.

5.4 Concluding Thoughts

Overall, the data obtained in this report support a role of NFkB and NK1R in the processes underlying alcoholism and its comorbidity with depression. It is intriguing to think of how interconnected NFkB and NK1R are, and how the effects we have observed

assessing NFkB may be influenced by the NK1R and vice versa. Current treatment options have displayed a moderate degree of success, but there is a need for additional treatment options and for ways to predict how a particular individual will respond to a particular drug, such as through pharmacogenetic approaches. Focusing on treating specific subsets of alcoholics, such as alcoholics with depression, may be an extremely beneficial approach to not only treat one, but both of these debilitating disorders in patients displaying this comorbidity. These studies, in addition to future studies based off of the findings included in this report, have immense potential to impact the approach by which these disorders treated

REFERENCES

1. SAMHSA, *2018 National Survey on Drug Use and Health.* 2018.
2. Rehm, J., et al., *Global burden of disease and injury and economic cost attributable to alcohol use and alcohol-use disorders.* Lancet, 2009. **373**(9682): p. 2223-33.
3. Sacks, J.J., et al., *2010 National and State Costs of Excessive Alcohol Consumption.* Am J Prev Med, 2015. **49**(5): p. e73-e79.
4. APA, *Diagnostic and Statistical Manual of Mental Disorders*. 4th ed. 1994, Washington, DC.
5. APA, *Diagnostic and Statistical Manual of Mental Disorders*. 5th ed. 2013, Washington, DC.
6. Takahashi, T., et al., *Comparison of DSM-IV and DSM-5 criteria for alcohol use disorders in VA primary care patients with frequent heavy drinking enrolled in a trial.* Addict Sci Clin Pract, 2017. **12**(1): p. 17.
7. George, O. and G.F. Koob, *Individual differences in the neuropsychopathology of addiction.* Dialogues Clin Neurosci, 2017. **19**(3): p. 217-229.
8. Koob, G.F. and M. Le Moal, *Drug abuse: hedonic homeostatic dysregulation.* Science, 1997. **278**(5335): p. 52-8.
9. CDC. *Alcohol and Public Health: Alcohol-Related Disease Impact (ARDI).* Average for United States 2006-2010 Alcohol-Attributable Deaths Due to Excessive Alcohol Use; Available from: https://nccd.cdc.gov/DPH_ARDI/Default/Report.aspx?T=AAM&P=f6d7eda7-

036e-4553-9968-9b17ffad620e&R=d7a9b303-48e9-4440-bf47-070a4827e1fd&M=8E1C5233-5640-4EE8-9247-1ECA7DA325B9&F=&D=.

10. Mokdad, A.H., et al., *Actual causes of death in the United States, 2000.* JAMA, 2004. **291**(10): p. 1238-45.
11. WHO, *Global Status Report on Alcohol and Health.* 2014: p. p. 57.
12. Rehm, J., et al., *The relation between different dimensions of alcohol consumption and burden of disease: an overview.* Addiction, 2010. **105**(5): p. 817-43.
13. Rehm, J., *The risks associated with alcohol use and alcoholism.* Alcohol Res Health, 2011. **34**(2): p. 135-43.
14. Taylor, B., et al., *The more you drink, the harder you fall: a systematic review and meta-analysis of how acute alcohol consumption and injury or collision risk increase together.* Drug Alcohol Depend, 2010. **110**(1-2): p. 108-16.
15. Borges, G. and C.R. Loera, *Alcohol and drug use in suicidal behaviour.* Curr Opin Psychiatry, 2010. **23**(3): p. 195-204.
16. Rehm, J., et al., *The relationship of average volume of alcohol consumption and patterns of drinking to burden of disease: an overview.* Addiction, 2003. **98**(9): p. 1209-28.
17. Tsai, J., et al., *Risk of incarceration and clinical characteristics of incarcerated veterans by race/ethnicity.* Soc Psychiatry Psychiatr Epidemiol, 2013. **48**(11): p. 1777-86.
18. Cunningham, C.L., C.M. Gremel, and P.A. Groblewski, *Drug-induced conditioned place preference and aversion in mice.* Nat Protoc, 2006. **1**(4): p. 1662-70.
19. Hoffman, D.C., *The use of place conditioning in studying the neuropharmacology of drug reinforcement.* Brain Res Bull, 1989. **23**(4-5): p. 373-87.

20. Bardo, M.T. and R.A. Bevins, *Conditioned place preference: what does it add to our preclinical understanding of drug reward?* Psychopharmacology (Berl), 2000. **153**(1): p. 31-43.

21. Tzschentke, T.M., *Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade.* Addict Biol, 2007. **12**(3-4): p. 227-462.

22. Crabbe, J.C., R.A. Harris, and G.F. Koob, *Preclinical studies of alcohol binge drinking.* Ann N Y Acad Sci, 2011. **1216**: p. 24-40.

23. Hwa, L.S., et al., *Persistent escalation of alcohol drinking in C57BL/6J mice with intermittent access to 20% ethanol.* Alcohol Clin Exp Res, 2011. **35**(11): p. 1938-47.

24. Wise, R.A., *Voluntary ethanol intake in rats following exposure to ethanol on various schedules.* Psychopharmacologia, 1973. **29**(3): p. 203-10.

25. Simms, J.A., et al., *Intermittent access to 20% ethanol induces high ethanol consumption in Long-Evans and Wistar rats.* Alcohol Clin Exp Res, 2008. **32**(10): p. 1816-23.

26. NIAAA, *Drinking levels defined.*

27. Crabbe, J.C., et al., *Intermittent availability of ethanol does not always lead to elevated drinking in mice.* Alcohol Alcohol, 2012. **47**(5): p. 509-17.

28. Ryabinin, A.E., et al., *High alcohol/sucrose consumption during dark circadian phase in C57BL/6J mice: involvement of hippocampus, lateral septum and urocortin-positive cells of the Edinger-Westphal nucleus.* Psychopharmacology (Berl), 2003. **165**(3): p. 296-305.

29. Armstrong, S., *A chronometric approach to the study of feeding behavior.* Neurosci Biobehav Rev, 1980. **4**(1): p. 27-53.

30. Rhodes, J.S., et al., *Mouse inbred strain differences in ethanol drinking to intoxication.* Genes Brain Behav, 2007. **6**(1): p. 1-18.

31. Tordoff, M.G. and A.A. Bachmanov, *Influence of the number of alcohol and water bottles on murine alcohol intake.* Alcohol Clin Exp Res, 2003. **27**(4): p. 600-6.

32. Rodd-Henricks, Z.A., et al., *Effects of concurrent access to multiple ethanol concentrations and repeated deprivations on alcohol intake of alcohol-preferring rats.* Alcohol Clin Exp Res, 2001. **25**(8): p. 1140-50.

33. Livy, D.J., S.E. Parnell, and J.R. West, *Blood ethanol concentration profiles: a comparison between rats and mice.* Alcohol, 2003. **29**(3): p. 165-71.

34. Turner, P.V., et al., *Administration of substances to laboratory animals: routes of administration and factors to consider.* J Am Assoc Lab Anim Sci, 2011. **50**(5): p. 600-13.

35. Lopez, M.F., R.I. Anderson, and H.C. Becker, *Effect of different stressors on voluntary ethanol intake in ethanol-dependent and nondependent C57BL/6J mice.* Alcohol, 2016. **51**: p. 17-23.

36. Avegno, E.M. and N.W. Gilpin, *Inducing Alcohol Dependence in Rats Using Chronic Intermittent Exposure to Alcohol Vapor.* Bio Protoc, 2019. **9**(9).

37. Gilpin, N.W., et al., *Vapor inhalation of alcohol in rats.* Curr Protoc Neurosci, 2008. **Chapter 9**: p. Unit 9 29.

38. O'Dell, L.E., et al., *Enhanced alcohol self-administration after intermittent versus continuous alcohol vapor exposure.* Alcohol Clin Exp Res, 2004. **28**(11): p. 1676-82.

39. Richardson, H.N., et al., *Alcohol self-administration acutely stimulates the hypothalamic-pituitary-adrenal axis, but alcohol dependence leads to a dampened neuroendocrine state.* Eur J Neurosci, 2008. **28**(8): p. 1641-53.

40. Griffin, W.C., 3rd, et al., *Repeated cycles of chronic intermittent ethanol exposure in mice increases voluntary ethanol drinking and ethanol concentrations in the nucleus accumbens.* Psychopharmacology (Berl), 2009. **201**(4): p. 569-80.

41. Becker, H.C. and M.F. Lopez, *Increased ethanol drinking after repeated chronic ethanol exposure and withdrawal experience in C57BL/6 mice.* Alcohol Clin Exp Res, 2004. **28**(12): p. 1829-38.

42. Tabakoff, B. and P.L. Hoffman, *Animal models in alcohol research.* Alcohol Res Health, 2000. **24**(2): p. 77-84.

43. Lopez, M.F. and H.C. Becker, *Operant ethanol self-administration in ethanol dependent mice.* Alcohol, 2014. **48**(3): p. 295-9.

44. Richardson, N.R. and D.C. Roberts, *Progressive ratio schedules in drug self-administration studies in rats: a method to evaluate reinforcing efficacy.* J Neurosci Methods, 1996. **66**(1): p. 1-11.

45. Shalev, U., J.W. Grimm, and Y. Shaham, *Neurobiology of relapse to heroin and cocaine seeking: a review.* Pharmacol Rev, 2002. **54**(1): p. 1-42.

46. Le, A.D., et al., *Reinstatement of alcohol-seeking by priming injections of alcohol and exposure to stress in rats.* Psychopharmacology (Berl), 1998. **135**(2): p. 169-74.

47. Davis, W.M. and S.G. Smith, *Role of conditioned reinforcers in the initiation, maintenance and extinction of drug-seeking behavior.* Pavlov J Biol Sci, 1976. **11**(4): p. 222-36.

48. Shepard, J.D., et al., *The anxiogenic drug yohimbine reinstates methamphetamine seeking in a rat model of drug relapse.* Biol Psychiatry, 2004. **55**(11): p. 1082-9.

49. Koob, G.F. and M. Le Moal, *Addiction and the brain antireward system.* Annu Rev Psychol, 2008. **59**: p. 29-53.

50. Koob, G.F., *Neurobiological substrates for the dark side of compulsivity in addiction.* Neuropharmacology, 2009. **56 Suppl 1**: p. 18-31.

51. Koob, G.F. and N.D. Volkow, *Neurocircuitry of addiction.* Neuropsychopharmacology, 2010. **35**(1): p. 217-38.

52. Koob, G.F. and N.D. Volkow, *Neurobiology of addiction: a neurocircuitry analysis.* Lancet Psychiatry, 2016. **3**(8): p. 760-73.

53. Koob, G.F., *Dynamics of neuronal circuits in addiction: reward, antireward, and emotional memory.* Pharmacopsychiatry, 2009. **42 Suppl 1**: p. S32-41.

54. Wise, R.A. and M.A. Bozarth, *A psychomotor stimulant theory of addiction.* Psychol Rev, 1987. **94**(4): p. 469-92.

55. Wise, R.A., *Dopamine and reward: the anhedonia hypothesis 30 years on.* Neurotox Res, 2008. **14**(2-3): p. 169-83.

56. Di Chiara, G., *Nucleus accumbens shell and core dopamine: differential role in behavior and addiction.* Behav Brain Res, 2002. **137**(1-2): p. 75-114.

57. Volkow, N.D. and M. Morales, *The Brain on Drugs: From Reward to Addiction.* Cell, 2015. **162**(4): p. 712-725.

58. Salamone, J.D., *Functions of mesolimbic dopamine: changing concepts and shifting paradigms.* Psychopharmacology (Berl), 2007. **191**(3): p. 389.

59. MacNicol, B., *The biology of addiction.* Can J Anaesth, 2017. **64**(2): p. 141-148.

60. Schultz, W., P. Dayan, and P.R. Montague, *A neural substrate of prediction and reward.* Science, 1997. **275**(5306): p. 1593-9.

61. Weiss, F., et al., *Ethanol self-administration restores withdrawal-associated deficiencies in accumbal dopamine and 5-hydroxytryptamine release in dependent rats.* J Neurosci, 1996. **16**(10): p. 3474-85.

62. Koob, G. and M.J. Kreek, *Stress, dysregulation of drug reward pathways, and the transition to drug dependence.* Am J Psychiatry, 2007. **164**(8): p. 1149-59.

63. Wee, S., et al., *Inhibition of kappa opioid receptors attenuated increased cocaine intake in rats with extended access to cocaine.* Psychopharmacology (Berl), 2009. **205**(4): p. 565-75.

64. Heilig, M., et al., *Acute withdrawal, protracted abstinence and negative affect in alcoholism: are they linked?* Addict Biol, 2010. **15**(2): p. 169-84.

65. Mayo-Smith, M.F., *Pharmacological management of alcohol withdrawal. A meta-analysis and evidence-based practice guideline. American Society of Addiction Medicine Working Group on Pharmacological Management of Alcohol Withdrawal.* JAMA, 1997. **278**(2): p. 144-51.

66. Schuckit, M.A. and V. Hesselbrock, *Alcohol dependence and anxiety disorders: what is the relationship?* Am J Psychiatry, 1994. **151**(12): p. 1723-34.

67. Sinha, R. and C.S. Li, *Imaging stress- and cue-induced drug and alcohol craving: association with relapse and clinical implications.* Drug Alcohol Rev, 2007. **26**(1): p. 25-31.

68. Heilig, M. and G.F. Koob, *A key role for corticotropin-releasing factor in alcohol dependence.* Trends Neurosci, 2007. **30**(8): p. 399-406.

69. Hunt, W.A., L.W. Barnett, and L.G. Branch, *Relapse rates in addiction programs.* J Clin Psychol, 1971. **27**(4): p. 455-6.

70. Volkow, N.D., et al., *Brain glucose metabolism in chronic marijuana users at baseline and during marijuana intoxication.* Psychiatry Res, 1996. **67**(1): p. 29-38.

71. Volkow, N.D., et al., *Profound decreases in dopamine release in striatum in detoxified alcoholics: possible orbitofrontal involvement.* J Neurosci, 2007. **27**(46): p. 12700-6.

72. Valenzuela, C.F., *Alcohol and neurotransmitter interactions.* Alcohol Health Res World, 1997. **21**(2): p. 144-8.

73. Seo, D. and R. Sinha, *The neurobiology of alcohol craving and relapse.* Handb Clin Neurol, 2014. **125**: p. 355-68.

74. Zweifel, L.S., et al., *Disruption of NMDAR-dependent burst firing by dopamine neurons provides selective assessment of phasic dopamine-dependent behavior.* Proc Natl Acad Sci U S A, 2009. **106**(18): p. 7281-8.

75. Weiss, F., et al., *Oral alcohol self-administration stimulates dopamine release in the rat nucleus accumbens: genetic and motivational determinants.* J Pharmacol Exp Ther, 1993. **267**(1): p. 250-8.

76. Robinson, T.E. and K.C. Berridge, *The neural basis of drug craving: an incentive-sensitization theory of addiction.* Brain Res Brain Res Rev, 1993. **18**(3): p. 247-91.

77. Berridge, K.C., *The debate over dopamine's role in reward: the case for incentive salience.* Psychopharmacology (Berl), 2007. **191**(3): p. 391-431.

78. Davidson, M., B. Shanley, and P. Wilce, *Increased NMDA-induced excitability during ethanol withdrawal: a behavioural and histological study.* Brain Res, 1995. **674**(1): p. 91-6.

79. Cunningham, K.A. and N.C. Anastasio, *Serotonin at the nexus of impulsivity and cue reactivity in cocaine addiction.* Neuropharmacology, 2014. **76 Pt B**: p. 460-78.

80. Banerjee, N., *Neurotransmitters in alcoholism: A review of neurobiological and genetic studies.* Indian J Hum Genet, 2014. **20**(1): p. 20-31.

81. Olsen, R.W., *GABAA receptor: Positive and negative allosteric modulators.* Neuropharmacology, 2018. **136**(Pt A): p. 10-22.

82. Bliss, T.V. and G.L. Collingridge, *A synaptic model of memory: long-term potentiation in the hippocampus.* Nature, 1993. **361**(6407): p. 31-9.

83. Lovinger, D.M., G. White, and F.F. Weight, *NMDA receptor-mediated synaptic excitation selectively inhibited by ethanol in hippocampal slice from adult rat.* J Neurosci, 1990. **10**(4): p. 1372-9.

84. Weiner, J.L., L. Zhang, and P.L. Carlen, *Potentiation of GABAA-mediated synaptic current by ethanol in hippocampal CA1 neurons: possible role of protein kinase C.* J Pharmacol Exp Ther, 1994. **268**(3): p. 1388-95.

85. Tabakoff, B. and P.L. Hoffman, *Alcohol addiction: an enigma among us.* Neuron, 1996. **16**(5): p. 909-12.

86. Mihic, S.J. and R.A. Harris, *GABA and the GABAA receptor.* Alcohol Health Res World, 1997. **21**(2): p. 127-31.

87. Diana, M., et al., *Profound decrement of mesolimbic dopaminergic neuronal activity during ethanol withdrawal syndrome in rats: electrophysiological and biochemical evidence.* Proc Natl Acad Sci U S A, 1993. **90**(17): p. 7966-9.

88. Melis, M., S. Spiga, and M. Diana, *The dopamine hypothesis of drug addiction: hypodopaminergic state.* Int Rev Neurobiol, 2005. **63**: p. 101-54.

89. Koob, G.F., *Brain stress systems in the amygdala and addiction.* Brain Res, 2009. **1293**: p. 61-75.

90. Koob, G.F., *The dark side of emotion: the addiction perspective.* Eur J Pharmacol, 2015. **753**: p. 73-87.

91. Martin, G.W. and J. Rehm, *The effectiveness of psychosocial modalities in the treatment of alcohol problems in adults: a review of the evidence.* Can J Psychiatry, 2012. **57**(6): p. 350-8.

92. Peacock, A., et al., *Effectiveness of community psychosocial and pharmacological treatments for alcohol use disorder: A national observational cohort study in England.* Drug Alcohol Depend, 2018. **186**: p. 60-67.

93. Prendergast, M., et al., *Contingency management for treatment of substance use disorders: a meta-analysis.* Addiction, 2006. **101**(11): p. 1546-60.

94. Carroll, K.M. and B.D. Kiluk, *Cognitive behavioral interventions for alcohol and drug use disorders: Through the stage model and back again.* Psychol Addict Behav, 2017. **31**(8): p. 847-861.

95. Coriale, G., et al., *Treatment of alcohol use disorder from a psychological point of view.* Riv Psichiatr, 2018. **53**(3): p. 141-148.

96. Carroll, K.M. and L.S. Onken, *Behavioral therapies for drug abuse.* Am J Psychiatry, 2005. **162**(8): p. 1452-60.

97. Miller, W.R. and P.L. Wilbourne, *Mesa Grande: a methodological analysis of clinical trials of treatments for alcohol use disorders.* Addiction, 2002. **97**(3): p. 265-77.

98. Barnett, N.P., et al., *A preliminary randomized controlled trial of contingency management for alcohol use reduction using a transdermal alcohol sensor.* Addiction, 2017. **112**(6): p. 1025-1035.

99. Dunn, C., L. Deroo, and F.P. Rivara, *The use of brief interventions adapted from motivational interviewing across behavioral domains: a systematic review.* Addiction, 2001. **96**(12): p. 1725-42.

100. Kranzler, H.R. and M. Soyka, *Diagnosis and Pharmacotherapy of Alcohol Use Disorder: A Review.* JAMA, 2018. **320**(8): p. 815-824.

101. Enoch, M.A., *Genetic influences on response to alcohol and response to pharmacotherapies for alcoholism.* Pharmacol Biochem Behav, 2014. **123**: p. 17-24.

102. Heilig, M. and M. Egli, *Pharmacological treatment of alcohol dependence: target symptoms and target mechanisms.* Pharmacol Ther, 2006. **111**(3): p. 855-76.

103. Litten, R.Z., et al., *Medications development to treat alcohol dependence: a vision for the next decade.* Addict Biol, 2012. **17**(3): p. 513-27.

104. Edenberg, H.J., *The genetics of alcohol metabolism: role of alcohol dehydrogenase and aldehyde dehydrogenase variants.* Alcohol Res Health, 2007. **30**(1): p. 5-13.

105. Fuller, R.K., et al., *Disulfiram treatment of alcoholism. A Veterans Administration cooperative study.* JAMA, 1986. **256**(11): p. 1449-55.

106. Goh, E.T. and M.Y. Morgan, *Review article: pharmacotherapy for alcohol dependence - the why, the what and the wherefore.* Aliment Pharmacol Ther, 2017. **45**(7): p. 865-882.

107. Benjamin, D., E.R. Grant, and L.A. Pohorecky, *Naltrexone reverses ethanol-induced dopamine release in the nucleus accumbens in awake, freely moving rats.* Brain Res, 1993. **621**(1): p. 137-40.

108. Anton, R.F., et al., *Naltrexone effects on alcohol consumption in a clinical laboratory paradigm: temporal effects of drinking.* Psychopharmacology (Berl), 2004. **173**(1-2): p. 32-40.

109. O'Malley, S.S., et al., *Naltrexone decreases craving and alcohol self-administration in alcohol-dependent subjects and activates the hypothalamo-pituitary-adrenocortical axis.* Psychopharmacology (Berl), 2002. **160**(1): p. 19-29.

110. Attilia, F., et al., *Pharmacological treatment of alcohol use disorder. Scientific evidence.* Riv Psichiatr, 2018. **53**(3): p. 123-127.

111. Spanagel, R., et al., *Acamprosate produces its anti-relapse effects via calcium.* Neuropsychopharmacology, 2014. **39**(4): p. 783-91.

112. Palpacuer, C., et al., *Risks and Benefits of Nalmefene in the Treatment of Adult Alcohol Dependence: A Systematic Literature Review and Meta-Analysis of Published and Unpublished Double-Blind Randomized Controlled Trials.* PLoS Med, 2015. **12**(12): p. e1001924.

113. Fitzgerald, N., et al., *Weak evidence on nalmefene creates dilemmas for clinicians and poses questions for regulators and researchers.* Addiction, 2016. **111**(8): p. 1477-87.

114. Pierce, M., et al., *Efficacy, tolerability, and safety of low-dose and high-dose baclofen in the treatment of alcohol dependence: A systematic review and meta-analysis.* Eur Neuropsychopharmacol, 2018. **28**(7): p. 795-806.

115. Mason, B.J., et al., *Gabapentin treatment for alcohol dependence: a randomized clinical trial.* JAMA Intern Med, 2014. **174**(1): p. 70-7.

116. Falk, D.E., et al., *Gabapentin Enacarbil Extended-Release for Alcohol Use Disorder: A Randomized, Double-Blind, Placebo-Controlled, Multisite Trial Assessing Efficacy and Safety.* Alcohol Clin Exp Res, 2019. **43**(1): p. 158-169.

117. Blodgett, J.C., et al., *A meta-analysis of topiramate's effects for individuals with alcohol use disorders.* Alcohol Clin Exp Res, 2014. **38**(6): p. 1481-8.

118. McKee, S.A., et al., *Varenicline reduces alcohol self-administration in heavy-drinking smokers.* Biol Psychiatry, 2009. **66**(2): p. 185-90.

119. Keyes, K.M., et al., *Stigma and treatment for alcohol disorders in the United States.* Am J Epidemiol, 2010. **172**(12): p. 1364-72.

120. Grant, B.F., et al., *Epidemiology of DSM-5 Alcohol Use Disorder: Results From the National Epidemiologic Survey on Alcohol and Related Conditions III.* JAMA Psychiatry, 2015. **72**(8): p. 757-66.

121. Olfson, M., et al., *Treatment of Common Mental Disorders in the United States: Results From the National Epidemiologic Survey on Alcohol and Related Conditions-III.* J Clin Psychiatry, 2019. **80**(3).

122. Jones, J.D., S.D. Comer, and H.R. Kranzler, *The pharmacogenetics of alcohol use disorder.* Alcohol Clin Exp Res, 2015. **39**(3): p. 391-402.

123. Boschloo, L., et al., *Alcohol-use disorder severity predicts first-incidence of depressive disorders.* Psychol Med, 2012. **42**(4): p. 695-703.

124. Hasin, D.S. and B.F. Grant, *Major depression in 6050 former drinkers: association with past alcohol dependence.* Arch Gen Psychiatry, 2002. **59**(9): p. 794-800.

125. Compton, W.M., et al., *Prevalence, correlates, disability, and comorbidity of DSM-IV drug abuse and dependence in the United States: results from the national epidemiologic survey on alcohol and related conditions.* Arch Gen Psychiatry, 2007. **64**(5): p. 566-76.

126. Pavkovic, B., et al., *Double screening for dual disorder, alcoholism and depression.* Psychiatry Res, 2018. **270**: p. 483-489.

127. Becker, H.C., M.F. Lopez, and T.L. Doremus-Fitzwater, *Effects of stress on alcohol drinking: a review of animal studies.* Psychopharmacology (Berl), 2011. **218**(1): p. 131-56.

128. Sayette, M.A., *Does drinking reduce stress?* Alcohol Res Health, 1999. **23**(4): p. 250-5.

129. Harrison, N.L., et al., *Effects of acute alcohol on excitability in the CNS.* Neuropharmacology, 2017. **122**: p. 36-45.

130. Brady, K.T. and S.C. Sonne, *The role of stress in alcohol use, alcoholism treatment, and relapse.* Alcohol Res Health, 1999. **23**(4): p. 263-71.

131. Spanagel, R., H.R. Noori, and M. Heilig, *Stress and alcohol interactions: animal studies and clinical significance.* Trends Neurosci, 2014. **37**(4): p. 219-27.

132. Muller, C.P. and G. Schumann, *Drugs as instruments: a new framework for non-addictive psychoactive drug use.* Behav Brain Sci, 2011. **34**(6): p. 293-310.

133. Koob, G.F. and M. Le Moal, *Drug addiction, dysregulation of reward, and allostasis.* Neuropsychopharmacology, 2001. **24**(2): p. 97-129.

134. Carrigan, M.H. and C.L. Randall, *Self-medication in social phobia: a review of the alcohol literature.* Addict Behav, 2003. **28**(2): p. 269-84.

135. Thomas, S.E., C.L. Randall, and M.H. Carrigan, *Drinking to cope in socially anxious individuals: a controlled study.* Alcohol Clin Exp Res, 2003. **27**(12): p. 1937-43.

136. Bolton, J.M., J. Robinson, and J. Sareen, *Self-medication of mood disorders with alcohol and drugs in the National Epidemiologic Survey on Alcohol and Related Conditions.* J Affect Disord, 2009. **115**(3): p. 367-75.

137. Helton, S.G. and F.W. Lohoff, *Pharmacogenetics of alcohol use disorders and comorbid psychiatric disorders.* Psychiatry Res, 2015. **230**(2): p. 121-9.

138. Andersen, A.M., et al., *Polygenic Scores for Major Depressive Disorder and Risk of Alcohol Dependence.* JAMA Psychiatry, 2017. **74**(11): p. 1153-1160.

139. Huang, S.Y., et al., *Possible interaction of alcohol dehydrogenase and aldehyde dehydrogenase genes with the dopamine D2 receptor gene in anxiety-depressive alcohol dependence.* Alcohol Clin Exp Res, 2004. **28**(3): p. 374-84.

140. Kertes, D.A., et al., *Neurotransmitter and neuromodulator genes associated with a history of depressive symptoms in individuals with alcohol dependence.* Alcohol Clin Exp Res, 2011. **35**(3): p. 496-505.

141. Su, N., et al., *The brain-derived neurotrophic factor is associated with alcohol dependence-related depression and antidepressant response.* Brain Res, 2011. **1415**: p. 119-26.

142. Wang, J.C., et al., *Evidence of common and specific genetic effects: association of the muscarinic acetylcholine receptor M2 (CHRM2) gene with alcohol dependence and major depressive syndrome.* Hum Mol Genet, 2004. **13**(17): p. 1903-11.

143. Golden, S.A., et al., *A standardized protocol for repeated social defeat stress in mice.* Nat Protoc, 2011. **6**(8): p. 1183-91.

144. Jasnow, A.M., et al., *Acute and chronic social defeat suppresses humoral immunity of male Syrian hamsters (Mesocricetus auratus).* Horm Behav, 2001. **40**(3): p. 428-33.

145. Krishnan, V., et al., *Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions.* Cell, 2007. **131**(2): p. 391-404.

146. Berton, O., et al., *Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress.* Science, 2006. **311**(5762): p. 864-8.

147. Rygula, R., et al., *Citalopram counteracts depressive-like symptoms evoked by chronic social stress in rats.* Behav Pharmacol, 2006. **17**(1): p. 19-29.

148. Rygula, R., et al., *Effects of fluoxetine on behavioral deficits evoked by chronic social stress in rats.* Behav Brain Res, 2006. **174**(1): p. 188-92.

149. Christoffel, D.J., et al., *IkappaB kinase regulates social defeat stress-induced synaptic and behavioral plasticity.* J Neurosci, 2011. **31**(1): p. 314-21.

150. Christoffel, D.J., et al., *Excitatory transmission at thalamo-striatal synapses mediates susceptibility to social stress.* Nat Neurosci, 2015. **18**(7): p. 962-4.

151. Christoffel, D.J., et al., *Effects of inhibitor of kappaB kinase activity in the nucleus accumbens on emotional behavior.* Neuropsychopharmacology, 2012. **37**(12): p. 2615-23.

152. Chaudhury, D., et al., *Rapid regulation of depression-related behaviours by control of midbrain dopamine neurons.* Nature, 2013. **493**(7433): p. 532-6.

153. Sinha, R., et al., *Enhanced negative emotion and alcohol craving, and altered physiological responses following stress and cue exposure in alcohol dependent individuals.* Neuropsychopharmacology, 2009. **34**(5): p. 1198-208.

154. Koob, G.F., *Neuroadaptive mechanisms of addiction: studies on the extended amygdala.* Eur Neuropsychopharmacol, 2003. **13**(6): p. 442-52.

155. Greenfield, S.F., et al., *The effect of depression on return to drinking: a prospective study.* Arch Gen Psychiatry, 1998. **55**(3): p. 259-65.

156. Witkiewitz, K. and N.A. Villarroel, *Dynamic association between negative affect and alcohol lapses following alcohol treatment.* J Consult Clin Psychol, 2009. **77**(4): p. 633-44.

157. Valdez, G.R., et al., *Antagonism of corticotropin-releasing factor attenuates the enhanced responsiveness to stress observed during protracted ethanol abstinence.* Alcohol, 2003. **29**(2): p. 55-60.

158. Valdez, G.R., et al., *Increased ethanol self-administration and anxiety-like behavior during acute ethanol withdrawal and protracted abstinence: regulation by corticotropin-releasing factor.* Alcohol Clin Exp Res, 2002. **26**(10): p. 1494-501.

159. Perez, E.E. and M. De Biasi, *Assessment of affective and somatic signs of ethanol withdrawal in C57BL/6J mice using a short-term ethanol treatment.* Alcohol, 2015. **49**(3): p. 237-43.

160. Breese, G.R., et al., *Prior multiple ethanol withdrawals enhance stress-induced anxiety-like behavior: inhibition by CRF1- and benzodiazepine-receptor antagonists and a 5-HT1a-receptor agonist.* Neuropsychopharmacology, 2005. **30**(9): p. 1662-9.

161. Sommer, W.H., et al., *Upregulation of voluntary alcohol intake, behavioral sensitivity to stress, and amygdala crhr1 expression following a history of dependence.* Biol Psychiatry, 2008. **63**(2): p. 139-45.

162. Holmes, A., et al., *Chronic alcohol remodels prefrontal neurons and disrupts NMDAR-mediated fear extinction encoding.* Nat Neurosci, 2012. **15**(10): p. 1359-61.

163. Anderson, R.I., M.F. Lopez, and H.C. Becker, *Forced swim stress increases ethanol consumption in C57BL/6J mice with a history of chronic intermittent ethanol exposure.* Psychopharmacology (Berl), 2016. **233**(11): p. 2035-43.

164. Anderson, R.I., M.F. Lopez, and H.C. Becker, *Stress-Induced Enhancement of Ethanol Intake in C57BL/6J Mice with a History of Chronic Ethanol Exposure: Involvement of Kappa Opioid Receptors.* Front Cell Neurosci, 2016. **10**: p. 45.

165. Becker, H.C., *Effects of alcohol dependence and withdrawal on stress responsiveness and alcohol consumption.* Alcohol Res, 2012. **34**(4): p. 448-58.

166. Griffin, W.C., 3rd, et al., *Increased extracellular glutamate in the nucleus accumbens promotes excessive ethanol drinking in ethanol dependent mice.* Neuropsychopharmacology, 2014. **39**(3): p. 707-17.

167. Nelson, B.S., M.K. Sequeira, and J.R. Schank, *Bidirectional relationship between alcohol intake and sensitivity to social defeat: association with Tacr1 and Avp expression.* Addict Biol, 2018. **23**(1): p. 142-153.

168. Sillaber, I. and M.S. Henniger, *Stress and alcohol drinking.* Ann Med, 2004. **36**(8): p. 596-605.

169. Caplan, M.A. and K. Puglisi, *Stress and conflict conditions leading to and maintaining voluntary alcohol consumption in rats.* Pharmacol Biochem Behav, 1986. **24**(2): p. 271-80.

170. Volpicelli, J.R. and R.R. Ulm, *The influence of control over appetitive and aversive events on alcohol preference in rats.* Alcohol, 1990. **7**(2): p. 133-6.

171. Noori, H.R., S. Helinski, and R. Spanagel, *Cluster and meta-analyses on factors influencing stress-induced alcohol drinking and relapse in rodents.* Addict Biol, 2014. **19**(2): p. 225-32.

172. Hwa, L.S., et al., *Social stress-escalated intermittent alcohol drinking: modulation by CRF-R1 in the ventral tegmental area and accumbal dopamine in mice.* Psychopharmacology (Berl), 2016. **233**(4): p. 681-90.

173. Newman, E.L., et al., *Persistent escalation of alcohol consumption by mice exposed to brief episodes of social defeat stress: suppression by CRF-R1 antagonism.* Psychopharmacology, 2018. **235**(6): p. 1807-1820.

174. Newman, E.L., et al., *Social Defeat Stress and Escalated Ethanol Drinking by C57bl/6j Mice: Suppression by Dopamine D3 Receptor Antagonism.* Alcoholism-Clinical and Experimental Research, 2018. **42**: p. 89a-89a.

175. Newman, E.L., et al., *Social defeat stress and escalation of cocaine and alcohol consumption: Focus on CRF.* Neurobiology of Stress, 2018. **9**: p. 151-165.

176. Norman, K.J., et al., *Social stress and escalated drug self-administration in mice I. Alcohol and corticosterone.* Psychopharmacology (Berl), 2015. **232**(6): p. 991-1001.

177. Croft, A.P., et al., *Social defeat increases alcohol preference of C57BL/10 strain mice; effect prevented by a CCKB antagonist.* Psychopharmacology (Berl), 2005. **183**(2): p. 163-70.

178. Karlsson, C., et al., *Proinflammatory signaling regulates voluntary alcohol intake and stress-induced consumption after exposure to social defeat stress in mice.* Addict Biol, 2017. **22**(5): p. 1279-1288.

179. Dong, L., et al., *Effects of the circadian rhythm gene period 1 (per1) on psychosocial stress-induced alcohol drinking.* Am J Psychiatry, 2011. **168**(10): p. 1090-8.

180. Molander, A., et al., *Brain-specific inactivation of the Crhr1 gene inhibits post-dependent and stress-induced alcohol intake, but does not affect relapse-like drinking.* Neuropsychopharmacology, 2012. **37**(4): p. 1047-56.

181. van Erp, A.M., N. Tachi, and K.A. Miczek, *Short or continuous social stress: suppression of continuously available ethanol intake in subordinate rats.* Behav Pharmacol, 2001. **12**(5): p. 335-42.

182. Sillaber, I., et al., *Enhanced and delayed stress-induced alcohol drinking in mice lacking functional CRH1 receptors.* Science, 2002. **296**(5569): p. 931-3.

183. Macedo, G.C., et al., *Consequences of continuous social defeat stress on anxiety- and depressive-like behaviors and ethanol reward in mice.* Horm Behav, 2018. **97**: p. 154-161.

184. Rodriguez-Arias, M., et al., *Social defeat in adolescent mice increases vulnerability to alcohol consumption.* Addict Biol, 2016. **21**(1): p. 87-97.

185. Caldwell, E.E. and D.C. Riccio, *Alcohol self-administration in rats: Modulation by temporal parameters related to repeated mild social defeat stress.* Alcohol, 2010. **44**(3): p. 265-74.

186. Funk, D., et al., *Effects of unconditioned and conditioned social defeat on alcohol self-administration and reinstatement of alcohol seeking in rats.* Psychopharmacology (Berl), 2005. **183**(3): p. 341-9.

187. Boulanger, L.M., *Immune proteins in brain development and synaptic plasticity.* Neuron, 2009. **64**(1): p. 93-109.

188. Pribiag, H. and D. Stellwagen, *Neuroimmune regulation of homeostatic synaptic plasticity.* Neuropharmacology, 2014. **78**: p. 13-22.

189. DiSabato, D.J., N. Quan, and J.P. Godbout, *Neuroinflammation: the devil is in the details.* J Neurochem, 2016. **139 Suppl 2**: p. 136-153.

190. Norden, D.M., et al., *Sequential activation of microglia and astrocyte cytokine expression precedes increased Iba-1 or GFAP immunoreactivity following systemic immune challenge.* Glia, 2016. **64**(2): p. 300-16.

191. Crews, F.T., et al., *Neuroimmune Function and the Consequences of Alcohol Exposure.* Alcohol Res, 2015. **37**(2): p. 331-41, 344-51.

192. Nennig, S.E. and J.R. Schank, *The Role of NFkB in Drug Addiction: Beyond Inflammation.* Alcohol Alcohol, 2017. **52**(2): p. 172-179.

193. Oeckinghaus, A. and S. Ghosh, *The NF-kappaB family of transcription factors and its regulation.* Cold Spring Harb Perspect Biol, 2009. **1**(4): p. a000034.

194. Gerondakis, S., et al., *NF-kappaB control of T cell development.* Nat Immunol, 2014. **15**(1): p. 15-25.

195. Oeckinghaus, A., M.S. Hayden, and S. Ghosh, *Crosstalk in NF-kappaB signaling pathways.* Nat Immunol, 2011. **12**(8): p. 695-708.

196. Mayfield, J., L. Ferguson, and R.A. Harris, *Neuroimmune signaling: a key component of alcohol abuse.* Curr Opin Neurobiol, 2013. **23**(4): p. 513-20.

197. Cui, C., D. Shurtleff, and R.A. Harris, *Neuroimmune mechanisms of alcohol and drug addiction.* Int Rev Neurobiol, 2014. **118**: p. 1-12.

198. Crews, F.T., J. Zou, and L. Qin, *Induction of innate immune genes in brain create the neurobiology of addiction.* Brain Behav Immun, 2011. **25 Suppl 1**: p. S4-S12.

199. Qin, L., et al., *Increased systemic and brain cytokine production and neuroinflammation by endotoxin following ethanol treatment.* J Neuroinflammation, 2008. **5**: p. 10.

200. Walter, T.J. and F.T. Crews, *Microglial depletion alters the brain neuroimmune response to acute binge ethanol withdrawal.* J Neuroinflammation, 2017. **14**(1): p. 86.

201. Blednov, Y.A., et al., *Activation of inflammatory signaling by lipopolysaccharide produces a prolonged increase of voluntary alcohol intake in mice.* Brain Behav Immun, 2011. **25 Suppl 1**: p. S92-S105.

202. He, J. and F.T. Crews, *Increased MCP-1 and microglia in various regions of the human alcoholic brain.* Exp Neurol, 2008. **210**(2): p. 349-58.

203. Crews, F.T., et al., *High mobility group box 1/Toll-like receptor danger signaling increases brain neuroimmune activation in alcohol dependence.* Biol Psychiatry, 2013. **73**(7): p. 602-12.

204. Edenberg, H.J., et al., *Association of NFKB1, which encodes a subunit of the transcription factor NF-kappaB, with alcohol dependence.* Hum Mol Genet, 2008. **17**(7): p. 963-70.

205. Okvist, A., et al., *Neuroadaptations in human chronic alcoholics: dysregulation of the NF-kappaB system.* PLoS One, 2007. **2**(9): p. e930.

206. Davis, R.L. and P.J. Syapin, *Chronic ethanol inhibits CXC chemokine ligand 10 production in human A172 astroglia and astroglial-mediated leukocyte chemotaxis.* Neurosci Lett, 2004. **362**(3): p. 220-5.

207. Zou, J. and F. Crews, *Induction of innate immune gene expression cascades in brain slice cultures by ethanol: key role of NF-kappaB and proinflammatory cytokines.* Alcohol Clin Exp Res, 2010. **34**(5): p. 777-89.

208. Helms, C.M., et al., *A longitudinal analysis of circulating stress-related proteins and chronic ethanol self-administration in cynomolgus macaques.* Alcohol Clin Exp Res, 2012. **36**(6): p. 995-1003.

209. Qin, L., et al., *Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration.* Glia, 2007. **55**(5): p. 453-62.

210. Truitt, J.M., et al., *Inhibition of IKKbeta Reduces Ethanol Consumption in C57BL/6J Mice.* eNeuro, 2016. **3**(5).

211. Lepsch, L.B., et al., *Cocaine induces cell death and activates the transcription nuclear factor kappa-B in PC12 cells.* Mol Brain, 2009. **2**: p. 3.

212. Wang, X., et al., *A non-peptide substance P antagonist (CP-96,345) inhibits morphine-induced NF-kappa B promoter activation in human NT2-N neurons.* J Neurosci Res, 2004. **75**(4): p. 544-53.

213. Sawaya, B.E., et al., *TNF alpha production in morphine-treated human neural cells is NF-kappaB-dependent.* J Neuroimmune Pharmacol, 2009. **4**(1): p. 140-9.

214. Ang, E., et al., *Induction of nuclear factor-kappaB in nucleus accumbens by chronic cocaine administration.* J Neurochem, 2001. **79**(1): p. 221-4.

215. Russo, S.J., et al., *Nuclear factor kappa B signaling regulates neuronal morphology and cocaine reward.* J Neurosci, 2009. **29**(11): p. 3529-37.

216. Zhang, X., et al., *Involvement of p38/NF-kappaB signaling pathway in the nucleus accumbens in the rewarding effects of morphine in rats.* Behav Brain Res, 2011. **218**(1): p. 184-9.

217. Raison, C.L., L. Capuron, and A.H. Miller, *Cytokines sing the blues: inflammation and the pathogenesis of depression.* Trends Immunol, 2006. **27**(1): p. 24-31.

218. Menard, C., et al., *Immune and Neuroendocrine Mechanisms of Stress Vulnerability and Resilience.* Neuropsychopharmacology, 2017. **42**(1): p. 62-80.

219. Menard, C., G.E. Hodes, and S.J. Russo, *Pathogenesis of depression: Insights from human and rodent studies.* Neuroscience, 2016. **321**: p. 138-162.

220. Miller, G.E., et al., *Clinical depression and inflammatory risk markers for coronary heart disease.* Am J Cardiol, 2002. **90**(12): p. 1279-83.

221. Miller, G.E., S. Cohen, and A.K. Ritchey, *Chronic psychological stress and the regulation of pro-inflammatory cytokines: a glucocorticoid-resistance model.* Health Psychol, 2002. **21**(6): p. 531-41.

222. Dowlati, Y., et al., *A meta-analysis of cytokines in major depression.* Biol Psychiatry, 2010. **67**(5): p. 446-57.

223. Maes, M., et al., *Increased serum IL-6 and IL-1 receptor antagonist concentrations in major depression and treatment resistant depression.* Cytokine, 1997. **9**(11): p. 853-8.

224. Maes, M., et al., *Increased plasma concentrations of interleukin-6, soluble interleukin-6, soluble interleukin-2 and transferrin receptor in major depression.* J Affect Disord, 1995. **34**(4): p. 301-9.

225. Hodes, G.E., et al., *Individual differences in the peripheral immune system promote resilience versus susceptibility to social stress.* Proc Natl Acad Sci U S A, 2014. **111**(45): p. 16136-41.

226. Felger, J.C. and F.E. Lotrich, *Inflammatory cytokines in depression: neurobiological mechanisms and therapeutic implications.* Neuroscience, 2013. **246**: p. 199-229.

227. Haroon, E., C.L. Raison, and A.H. Miller, *Psychoneuroimmunology meets neuropsychopharmacology: translational implications of the impact of inflammation on behavior.* Neuropsychopharmacology, 2012. **37**(1): p. 137-62.

228. Bierhaus, A., et al., *A mechanism converting psychosocial stress into mononuclear cell activation.* Proc Natl Acad Sci U S A, 2003. **100**(4): p. 1920-5.

229. Eisenberger, N.I., et al., *Inflammation-induced anhedonia: endotoxin reduces ventral striatum responses to reward.* Biol Psychiatry, 2010. **68**(8): p. 748-54.

230. Dantzer, R., *Cytokine-induced sickness behavior: mechanisms and implications.* Ann N Y Acad Sci, 2001. **933**: p. 222-34.

231. Muller, N., et al., *The cyclooxygenase-2 inhibitor celecoxib has therapeutic effects in major depression: results of a double-blind, randomized, placebo controlled, add-on pilot study to reboxetine.* Mol Psychiatry, 2006. **11**(7): p. 680-4.

232. Raison, C.L., et al., *A randomized controlled trial of the tumor necrosis factor antagonist infliximab for treatment-resistant depression: the role of baseline inflammatory biomarkers.* JAMA Psychiatry, 2013. **70**(1): p. 31-41.

233. Kohler, O., et al., *Effect of anti-inflammatory treatment on depression, depressive symptoms, and adverse effects: a systematic review and meta-analysis of randomized clinical trials.* JAMA Psychiatry, 2014. **71**(12): p. 1381-91.

234. Bufalino, C., et al., *The role of immune genes in the association between depression and inflammation: a review of recent clinical studies.* Brain Behav Immun, 2013. **31**: p. 31-47.

235. Wong, M.L., et al., *Polymorphisms in inflammation-related genes are associated with susceptibility to major depression and antidepressant response.* Mol Psychiatry, 2008. **13**(8): p. 800-12.

236. Shelton, R.C., et al., *Altered expression of genes involved in inflammation and apoptosis in frontal cortex in major depression.* Mol Psychiatry, 2011. **16**(7): p. 751-62.

237. Hodes, G.E., et al., *Neuroimmune mechanisms of depression.* Nat Neurosci, 2015. **18**(10): p. 1386-93.

238. Dantzer, R., et al., *From inflammation to sickness and depression: when the immune system subjugates the brain.* Nat Rev Neurosci, 2008. **9**(1): p. 46-56.

239. Fulenwider, H.D., et al., *Cellular and behavioral effects of lipopolysaccharide treatment are dependent upon neurokinin-1 receptor activation.* Journal of Neuroinflammation, 2018. **15**.

240. Yang, C., et al., *Peripheral interleukin-6 promotes resilience versus susceptibility to inescapable electric stress.* Acta Neuropsychiatr, 2015. **27**(5): p. 312-6.

241. Pryce, C.R., et al., *Helplessness: a systematic translational review of theory and evidence for its relevance to understanding and treating depression.* Pharmacol Ther, 2011. **132**(3): p. 242-67.

242. Koo, J.W., et al., *Nuclear factor-kappaB is a critical mediator of stress-impaired neurogenesis and depressive behavior.* Proc Natl Acad Sci U S A, 2010. **107**(6): p. 2669-74.

243. Cohen, H., et al., *The characteristic long-term upregulation of hippocampal NF-kappaB complex in PTSD-like behavioral stress response is normalized by high-dose corticosterone and pyrrolidine dithiocarbamate administered immediately after exposure.* Neuropsychopharmacology, 2011. **36**(11): p. 2286-302.

244. Garabadu, D., B.C. Reddy, and S. Krishnamurthy, *Citalopram protects against cold-restraint stress-induced activation of brain-derived neurotrophic factor and expression of nuclear factor kappa-light-chain-enhancer of activated B cells in rats.* J Mol Neurosci, 2015. **55**(2): p. 355-66.

245. Orso, R., et al., *NFkappaB1 and NFkappaB2 gene expression in the prefrontal cortex and hippocampus of early life stressed mice exposed to cocaine-induced conditioned place preference during adolescence.* Neurosci Lett, 2017. **658**: p. 27-31.

246. Liu, Y.M., et al., *Berberine attenuates depressive-like behaviors by suppressing neuro-inflammation in stressed mice.* Brain Res Bull, 2017. **134**: p. 220-227.

247. Choubey, P., et al., *Ameliorative effect of fisetin against lipopolysaccharide and restraint stress-induced behavioral deficits via modulation of NF-kappaB and IDO-1.* Psychopharmacology (Berl), 2019. **236**(2): p. 741-752.

248. Kumari, Y., et al., *Melatonin receptor agonist Piper betle L. ameliorates dexamethasone-induced early life stress in adult zebrafish.* Exp Ther Med, 2019. **18**(2): p. 1407-1416.

249. Pennefather, J.N., et al., *Tachykinins and tachykinin receptors: a growing family.* Life Sci, 2004. **74**(12): p. 1445-63.

250. Schank, J.R. and M. Heilig, *Substance P and the Neurokinin-1 Receptor: The New CRF.* Int Rev Neurobiol, 2017. **136**: p. 151-175.

251. Schank, J.R., *The Neurokinin-1 Receptor in Addictive Processes.* Journal of Pharmacology and Experimental Therapeutics, 2014. **351**(1): p. 2-8.

252. Holmes, A., et al., *Neuropeptide systems as novel therapeutic targets for depression and anxiety disorders.* Trends Pharmacol Sci, 2003. **24**(11): p. 580-8.

253. Mantyh, P.W., *Neurobiology of substance P and the NK1 receptor.* J Clin Psychiatry, 2002. **63 Suppl 11**: p. 6-10.

254. Mantyh, P.W., S.P. Hunt, and J.E. Maggio, *Substance P receptors: localization by light microscopic autoradiography in rat brain using [3H]SP as the radioligand.* Brain Res, 1984. **307**(1-2): p. 147-65.

255. Quirion, R., et al., *Autoradiographic distribution of substance P receptors in rat central nervous system.* Nature, 1983. **303**(5919): p. 714-6.

256. Yip, J. and L.A. Chahl, *Localization of tachykinin receptors and Fos-like immunoreactivity induced by substance P in guinea-pig brain.* Clin Exp Pharmacol Physiol, 2000. **27**(11): p. 943-6.

257. Yip, J. and L.A. Chahl, *Localization of NK1 and NK3 receptors in guinea-pig brain.* Regul Pept, 2001. **98**(1-2): p. 55-62.

258. Hurd, Y.L., et al., *Preprotachykinin-A mRNA expression in the human and monkey brain: An in situ hybridization study.* J Comp Neurol, 1999. **411**(1): p. 56-72.

259. Wolf, S.S., et al., *Autoradiographic visualization of substance P receptors in rat brain.* Eur J Pharmacol, 1983. **91**(1): p. 157-8.

260. Mashaghi, A., et al., *Neuropeptide substance P and the immune response.* Cell Mol Life Sci, 2016. **73**(22): p. 4249-4264.

261. Sequeira, M.K., et al., *Neurokinin-1 Receptor Antagonism in the Dorsolateral Striatum Attenuates Escalated Ethanol Consumption Induced by Intermittent Access.* Alcoholism-Clinical and Experimental Research, 2017. **41**: p. 116a-116a.

262. Thorsell, A., et al., *Neurokinin-1 receptors (NK1R:s), alcohol consumption, and alcohol reward in mice.* Psychopharmacology (Berl), 2010. **209**(1): p. 103-11.

263. Baek, M.N., et al., *Artificial microRNA-based neurokinin-1 receptor gene silencing reduces alcohol consumption in mice.* Neurosci Lett, 2010. **475**(3): p. 124-8.

264. George, D.T., et al., *Neurokinin 1 receptor antagonism as a possible therapy for alcoholism.* Science, 2008. **319**(5869): p. 1536-9.

265. Barson, J.R., et al., *Substance P in the anterior thalamic paraventricular nucleus: promotion of ethanol drinking in response to orexin from the hypothalamus.* Addict Biol, 2015.

266. Schank, J.R., et al., *Stress-induced reinstatement of alcohol-seeking in rats is selectively suppressed by the neurokinin 1 (NK1) antagonist L822429.* Psychopharmacology (Berl), 2011. **218**(1): p. 111-9.

267. Schank, J.R., et al., *Neurokinin-1 receptor antagonism attenuates neuronal activity triggered by stress-induced reinstatement of alcohol seeking.* Neuropharmacology, 2015. **99**: p. 106-14.

268. Schank, J.R., et al., *The Role of the Neurokinin-I Receptor in Stress-Induced Reinstatement of Alcohol and Cocaine Seeking.* Neuropsychopharmacology, 2014. **39**(5): p. 1093-1101.

269. Ayanwuyi, L.O., et al., *Neurokinin 1 receptor blockade in the medial amygdala attenuates alcohol drinking in rats with innate anxiety but not in Wistar rats.* Br J Pharmacol, 2015. **172**(21): p. 5136-46.

270. Schank, J.R., et al., *Tacr1 gene variation and neurokinin 1 receptor expression is associated with antagonist efficacy in genetically selected alcohol-preferring rats.* Biol Psychiatry, 2013. **73**(8): p. 774-81.

271. Nelson, B.S., et al., *Escalated Alcohol Self-Administration and Sensitivity to Yohimbine-Induced Reinstatement in Alcohol Preferring Rats: Potential Role of Neurokinin-1 Receptors in the Amygdala.* Neuroscience, 2019. **413**: p. 77-85.

272. Augier, E., et al., *Wistar rats acquire and maintain self-administration of 20 % ethanol without water deprivation, saccharin/sucrose fading, or extended access training.* Psychopharmacology (Berl), 2014. **231**(23): p. 4561-8.

273. Kramer, M.S., et al., *Distinct mechanism for antidepressant activity by blockade of central substance P receptors.* Science, 1998. **281**(5383): p. 1640-5.

274. Sharp, S.I., et al., *Genetic association of the tachykinin receptor 1 TACR1 gene in bipolar disorder, attention deficit hyperactivity disorder, and the alcohol dependence syndrome.* Am J Med Genet B Neuropsychiatr Genet, 2014. **165B**(4): p. 373-80.

275. Seneviratne, C., et al., *Susceptibility locus in neurokinin-1 receptor gene associated with alcohol dependence.* Neuropsychopharmacology, 2009. **34**(11): p. 2442-9.

276. Blaine, S., et al., *TACR1 genotypes predict fMRI response to alcohol cues and level of alcohol dependence.* Alcohol Clin Exp Res, 2013. **37 Suppl 1**: p. E125-30.

277. Lesch, K.P., *Mouse anxiety: the power of knockout.* Pharmacogenomics J, 2001. **1**(3): p. 187-92.

278. Ebner, K. and N. Singewald, *The role of substance P in stress and anxiety responses.* Amino Acids, 2006. **31**(3): p. 251-72.

279. Duarte, F.S., R. Testolin, and T.C. De Lima, *Further evidence on the anxiogenic-like effect of substance P evaluated in the elevated plus-maze in rats.* Behav Brain Res, 2004. **154**(2): p. 501-10.

280. Teixeira, R.M., et al., *Effects of central administration of tachykinin receptor agonists and antagonists on plus-maze behavior in mice.* Eur J Pharmacol, 1996. **311**(1): p. 7-14.

281. Ebner, K., et al., *Substance P in the medial amygdala: emotional stress-sensitive release and modulation of anxiety-related behavior in rats.* Proc Natl Acad Sci U S A, 2004. **101**(12): p. 4280-5.

282. Santarelli, L., et al., *Genetic and pharmacological disruption of neurokinin 1 receptor function decreases anxiety-related behaviors and increases serotonergic function.* Proc Natl Acad Sci U S A, 2001. **98**(4): p. 1912-7.

283. Varty, G.B., et al., *The gerbil elevated plus-maze II: anxiolytic-like effects of selective neurokinin NK1 receptor antagonists.* Neuropsychopharmacology, 2002. **27**(3): p. 371-9.

284. Ebner, K. and N. Singewald, *Stress-induced release of substance P in the locus coeruleus modulates cortical noradrenaline release.* Naunyn Schmiedebergs Arch Pharmacol, 2007. **376**(1-2): p. 73-82.

285. Ebner, K., et al., *Neurokinin 1 receptor antagonism promotes active stress coping via enhanced septal 5-HT transmission.* Neuropsychopharmacology, 2008. **33**(8): p. 1929-41.

286. Rupniak, N.M., et al., *Pharmacological blockade or genetic deletion of substance P (NK(1)) receptors attenuates neonatal vocalisation in guinea-pigs and mice.* Neuropharmacology, 2000. **39**(8): p. 1413-21.

287. Brocco, M., et al., *Cellular and behavioural profile of the novel, selective neurokinin1 receptor antagonist, vestipitant: a comparison to other agents.* Eur Neuropsychopharmacol, 2008. **18**(10): p. 729-50.

288. Boyce, S., et al., *Intra-amygdala injection of the substance P [NK(1) receptor] antagonist L-760735 inhibits neonatal vocalisations in guinea-pigs.* Neuropharmacology, 2001. **41**(1): p. 130-7.

289. File, S.E., *Anxiolytic action of a neurokinin1 receptor antagonist in the social interaction test.* Pharmacol Biochem Behav, 1997. **58**(3): p. 747-52.

290. File, S.E., *NKP608, an NK1 receptor antagonist, has an anxiolytic action in the social interaction test in rats.* Psychopharmacology (Berl), 2000. **152**(1): p. 105-9.

291. Vassout, A., et al., *NKP608: a selective NK-1 receptor antagonist with anxiolytic-like effects in the social interaction and social exploration test in rats.* Regul Pept, 2000. **96**(1-2): p. 7-16.

292. Gentsch, C., et al., *Anxiolytic effect of NKP608, a NK1-receptor antagonist, in the social investigation test in gerbils.* Behav Brain Res, 2002. **133**(2): p. 363-8.

293. Zhao, Z., et al., *Effects of substance P in the amygdala, ventromedial hypothalamus, and periaqueductal gray on fear-potentiated startle.* Neuropsychopharmacology, 2009. **34**(2): p. 331-40.

294. Rupniak, N.M., et al., *The substance P (NK1) receptor antagonist L-760735 inhibits fear conditioning in gerbils.* Neuropharmacology, 2003. **44**(4): p. 516-23.

295. Krase, W., M. Koch, and H.U. Schnitzler, *Substance P is involved in the sensitization of the acoustic startle response by footshocks in rats.* Behav Brain Res, 1994. **63**(1): p. 81-8.

296. Dableh, L.J., et al., *Antidepressant-like effects of neurokinin receptor antagonists in the forced swim test in the rat.* Eur J Pharmacol, 2005. **507**(1-3): p. 99-105.

297. Yang, L.M., et al., *Substance P receptor antagonist in lateral habenula improves rat depression-like behavior.* Brain Res Bull, 2014. **100**: p. 22-8.

298. Papp, M., A. Vassout, and C. Gentsch, *The NK1-receptor antagonist NKP608 has an antidepressant-like effect in the chronic mild stress model of depression in rats.* Behav Brain Res, 2000. **115**(1): p. 19-23.

299. Frisch, P., et al., *Modulation of the CRH system by substance P/NKA in an animal model of depression.* Behav Brain Res, 2010. **213**(1): p. 103-8.

300. Zou, J. and F. Crews, *CREB and NF-kappaB transcription factors regulate sensitivity to excitotoxic and oxidative stress induced neuronal cell death.* Cell Mol Neurobiol, 2006. **26**(4-6): p. 385-405.

301. Zou, J.Y. and F.T. Crews, *TNF alpha potentiates glutamate neurotoxicity by inhibiting glutamate uptake in organotypic brain slice cultures: neuroprotection by NF kappa B inhibition.* Brain Res, 2005. **1034**(1-2): p. 11-24.

302. Crews, F., et al., *BHT blocks NF-kappaB activation and ethanol-induced brain damage.* Alcohol Clin Exp Res, 2006. **30**(11): p. 1938-49.

303. Qin, L. and F.T. Crews, *NADPH oxidase and reactive oxygen species contribute to alcohol-induced microglial activation and neurodegeneration.* J Neuroinflammation, 2012. **9**: p. 5.

304. Crews, F.T. and K. Nixon, *Mechanisms of neurodegeneration and regeneration in alcoholism.* Alcohol Alcohol, 2009. **44**(2): p. 115-27.

305. Salgado, S. and M.G. Kaplitt, *The Nucleus Accumbens: A Comprehensive Review.* Stereotact Funct Neurosurg, 2015. **93**(2): p. 75-93.

306. Boileau, I., et al., *Alcohol promotes dopamine release in the human nucleus accumbens.* Synapse, 2003. **49**(4): p. 226-31.

307. Bhakar, A.L., et al., *Constitutive nuclear factor-kappa B activity is required for central neuron survival.* J Neurosci, 2002. **22**(19): p. 8466-75.

308. Cruz, F.C., et al., *New technologies for examining the role of neuronal ensembles in drug addiction and fear.* Nat Rev Neurosci, 2013. **14**(11): p. 743-54.

309. Farquhar, D., et al., *Suicide gene therapy using E. coli beta-galactosidase.* Cancer Chemother Pharmacol, 2002. **50**(1): p. 65-70.

310. Pfarr, S., et al., *Losing Control: Excessive Alcohol Seeking after Selective Inactivation of Cue-Responsive Neurons in the Infralimbic Cortex.* J Neurosci, 2015. **35**(30): p. 10750-61.

311. Bossert, J.M., et al., *Ventral medial prefrontal cortex neuronal ensembles mediate context-induced relapse to heroin.* Nat Neurosci, 2011. **14**(4): p. 420-2.

312. Cruz, F.C., et al., *Role of nucleus accumbens shell neuronal ensembles in context-induced reinstatement of cocaine-seeking.* J Neurosci, 2014. **34**(22): p. 7437-46.

313. de Guglielmo, G., et al., *Recruitment of a Neuronal Ensemble in the Central Nucleus of the Amygdala Is Required for Alcohol Dependence.* J Neurosci, 2016. **36**(36): p. 9446-53.

314. Fanous, S., et al., *Role of orbitofrontal cortex neuronal ensembles in the expression of incubation of heroin craving.* J Neurosci, 2012. **32**(34): p. 11600-9.

315. Funk, D., et al., *Role of Central Amygdala Neuronal Ensembles in Incubation of Nicotine Craving.* J Neurosci, 2016. **36**(33): p. 8612-23.

316. Koya, E., et al., *Targeted disruption of cocaine-activated nucleus accumbens neurons prevents context-specific sensitization.* Nat Neurosci, 2009. **12**(8): p. 1069-73.

317. Snow, W.M., et al., *Roles for NF-kappaB and gene targets of NF-kappaB in synaptic plasticity, memory, and navigation.* Mol Neurobiol, 2014. **49**(2): p. 757-70.

318. Kaltschmidt, B., et al., *NF-kappaB regulates spatial memory formation and synaptic plasticity through protein kinase A/CREB signaling.* Mol Cell Biol, 2006. **26**(8): p. 2936-46.

319. Boden, J.M. and D.M. Fergusson, *Alcohol and depression.* Addiction, 2011. **106**(5): p. 906-14.

320. Fergusson, D.M., J.M. Boden, and L.J. Horwood, *Alcohol misuse and psychosocial outcomes in young adulthood: results from a longitudinal birth cohort studied to age 30.* Drug Alcohol Depend, 2013. **133**(2): p. 513-9.

321. Driessen, M., et al., *The course of anxiety, depression and drinking behaviours after completed detoxification in alcoholics with and without comorbid anxiety and depressive disorders.* Alcohol Alcohol, 2001. **36**(3): p. 249-55.

322. Prior, K., et al., *Substance use disorders comorbid with mood and anxiety disorders in the Australian general population.* Drug Alcohol Rev, 2017. **36**(3): p. 317-324.

323. Rosenwasser, A.M., et al., *Escalation of intake under intermittent ethanol access in diverse mouse genotypes.* Addiction Biology, 2013. **18**(3): p. 496-507.

324. Crabbe, J.C., et al., *Alcohol preference drinking in a mouse line selectively bred for high drinking in the dark.* Alcohol, 2011. **45**(5): p. 427-40.

325. Nennig, S.E., et al., *Selective Lesioning of Nuclear Factor kB Activated Cells in the Nucleus Accumbens Shell Attenuates Alcohol Place Preference.* Neuropsychopharmacology, 2017.

326. Carnicella, S., D. Ron, and S. Barak, *Intermittent ethanol access schedule in rats as a preclinical model of alcohol abuse.* Alcohol, 2014. **48**(3): p. 243-52.

327. Bale, T.L., *Stress sensitivity and the development of affective disorders.* Horm Behav, 2006. **50**(4): p. 529-33.

328. Janak, P.H. and N. Chaudhri, *The Potent Effect of Environmental Context on Relapse to Alcohol-Seeking After Extinction.* Open Addict J, 2010. **3**: p. 76-87.

329. Gilpin, N.W., M.A. Herman, and M. Roberto, *The central amygdala as an integrative hub for anxiety and alcohol use disorders.* Biol Psychiatry, 2015. **77**(10): p. 859-69.

330. Koob, G.F., *The role of the striatopallidal and extended amygdala systems in drug addiction.* Ann N Y Acad Sci, 1999. **877**: p. 445-60.

331. Gilpin, N.W. and M. Roberto, *Neuropeptide modulation of central amygdala neuroplasticity is a key mediator of alcohol dependence.* Neurosci Biobehav Rev, 2012. **36**(2): p. 873-88.

332. Sharp, B.M., *Basolateral amygdala and stress-induced hyperexcitability affect motivated behaviors and addiction.* Transl Psychiatry, 2017. **7**(8): p. e1194.

333. Millan, E.Z., H.A. Kim, and P.H. Janak, *Optogenetic activation of amygdala projections to nucleus accumbens can arrest conditioned and unconditioned alcohol consummatory behavior.* Neuroscience, 2017. **360**: p. 106-117.

334. Smith, E.M., et al., *Corticotropin Releasing Factor (CRF) activation of NF-kappaB-directed transcription in leukocytes.* Cell Mol Neurobiol, 2006. **26**(4-6): p. 1021-36.

335. Albrechet-Souza, L., et al., *Corticotropin Releasing Factor in the Bed Nucleus of the Stria Terminalis in Socially Defeated and Non-stressed Mice with a History of Chronic Alcohol Intake.* Front Pharmacol, 2017. **8**: p. 762.

336. Singewald, N., et al., *Modulation of basal and stress-induced amygdaloid substance P release by the potent and selective NK1 receptor antagonist L-822429.* J Neurochem, 2008. **106**(6): p. 2476-88.

337. Schank, J.R., et al., *Stress-Related Neuropeptides and Addictive Behaviors: Beyond the Usual Suspects.* Neuron, 2012. **76**(1): p. 192-208.

338. Howard, D.B. and B.K. Harvey, *Assaying the Stability and Inactivation of AAV Serotype 1 Vectors.* Hum Gene Ther Methods, 2017. **28**(1): p. 39-48.

339. Sibille, E., et al., *Genetic inactivation of the Serotonin(1A) receptor in mice results in downregulation of major GABA(A) receptor alpha subunits, reduction of GABA(A) receptor binding, and benzodiazepine-resistant anxiety.* J Neurosci, 2000. **20**(8): p. 2758-65.

340. Griebel, G. and S. Beeske, *Is there still a future for neurokinin 3 receptor antagonists as potential drugs for the treatment of psychiatric diseases?* Pharmacol Ther, 2012. **133**(1): p. 116-23.

341. Borelli, K.G., et al., *Effects of intra-hippocampal injections of the NK2 receptor antagonist saredutant on the elevated plus maze, and the mouse defense test battery.* Neurosci Lett, 2010. **485**(3): p. 241-5.

342. Ebner, K., S.B. Sartori, and N. Singewald, *Tachykinin receptors as therapeutic targets in stress-related disorders.* Curr Pharm Des, 2009. **15**(14): p. 1647-74.

343. Massi, M., I. Panocka, and G. de Caro, *The psychopharmacology of tachykinin NK-3 receptors in laboratory animals.* Peptides, 2000. **21**(11): p. 1597-609.

344. Burmeister, A.R., et al., *Human microglia and astrocytes constitutively express the neurokinin-1 receptor and functionally respond to substance P.* J Neuroinflammation, 2017. **14**(1): p. 245.

345. Rasley, A., et al., *Expression of functional NK-1 receptors in murine microglia.* Glia, 2002. **37**(3): p. 258-67.

346. Sun, J., et al., *Substance P enhances NF-kappaB transactivation and chemokine response in murine macrophages via ERK1/2 and p38 MAPK signaling pathways.* Am J Physiol Cell Physiol, 2008. **294**(6): p. C1586-96.

347. Simeonidis, S., et al., *Regulation of the NK-1 receptor gene expression in human macrophage cells via an NF-kappa B site on its promoter.* Proc Natl Acad Sci U S A, 2003. **100**(5): p. 2957-62.

348. Ramkissoon, S.H., et al., *Nuclear factor-kappaB is central to the expression of truncated neurokinin-1 receptor in breast cancer: implication for breast cancer cell quiescence within bone marrow stroma.* Cancer Res, 2007. **67**(4): p. 1653-9.

349. Rupniak, N.M.J. and M.S. Kramer, *NK1 receptor antagonists for depression: Why a validated concept was abandoned.* J Affect Disord, 2017. **223**: p. 121-125.

350. Keller, M., et al., *Lack of efficacy of the substance p (neurokinin1 receptor) antagonist aprepitant in the treatment of major depressive disorder.* Biol Psychiatry, 2006. **59**(3): p. 216-23.

351. Kuehner, C., *Why is depression more common among women than among men?* Lancet Psychiatry, 2017. **4**(2): p. 146-158.

352. Albert, P.R., *Why is depression more prevalent in women?* J Psychiatry Neurosci, 2015. **40**(4): p. 219-21.

353. Warren, B.L., et al., *Neurobiological sequelae of witnessing stressful events in adult mice.* Biol Psychiatry, 2013. **73**(1): p. 7-14.

354. Iniguez, S.D., et al., *Vicarious Social Defeat Stress Induces Depression-Related Outcomes in Female Mice.* Biol Psychiatry, 2017.