Alcohol Cues, Craving, and Relapse: Insights from Animal Models

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Abstract

Alcoholism is a chronic relapsing and remitting disorder, where relapse to drinking is often triggered by an intense desire for alcohol (craving) and the consequent motivation to obtain alcohol (seeking). Environmental stimuli (cues) associated with past alcohol use are believed to strongly contribute to relapse, as exposure to these cues can trigger intense feelings of craving and drive alcohol seeking. Over the past several decades, much progress has been made in identifying the neurobiological correlates of alcohol seeking and relapse. Much of this progress is owed to the development of animal models and advanced techniques to manipulate neural activity. In this chapter, we describe some of the most commonly used rodent models of alcohol intake and seeking as well as the methods used to identify the neural structures and circuits involved in alcohol-mediated behavior. Several of the most routinely identified brain structures in alcohol seeking are also described.

Keywords: alcohol, ethanol, relapse, amygdala, accumbens, VTA

1. Introduction

Alcohol use disorders (AUDs) constitute a major global health concern. In 2013 alone, 5.9% of all deaths worldwide were attributed to alcohol intake (WHO, 2015). This statistic combined with the social, emotional, and other physical consequences of excessive alcohol use makes it is difficult to deny the ongoing need for preclinical research. Of greatest interest is identifying the treatments to promote and maintain abstinence in individuals diagnosed with an AUD. Remission, however, is often characterized by a chronic vulnerability to relapse, which is poorly understood. In fact, estimates of long-term relapse rates following remission are as high as 60%,

depending on the treatment sought [1]. Lack of information on the neurobiological antecedents and psychological determinates of relapse makes AUDs all the more problematic to address.

Further complicating our understanding of the persistent risk of relapse are the complex interactions between internal processes and the external environment. Most noted are the relationships that develop between environmental stimuli (cues), both contextual and discrete, and the internal states produced by alcohol. Over the course of alcohol use, specific cues become associated with the effects of alcohol through a Pavlovian learning process, whereby an associative (alcohol-cue) relationship is formed. Once the relationship has been acquired, these associative cues are able to autonomously produce psychological and physiological states that are powerful enough to elicit behavior responses. These responses have been suggested to play an important role in the development of AUDs and relapse.

Even after lengthy periods of abstinence, exposure to drug-associated cues can trigger intense feelings of craving and drive drug seeking [2–5], leading to relapse to drug use. When considering alcohol in particular, this lingering sensitivity to related cues is especially problematic given the omnipresent nature of alcohol and alcohol-related cues in society. Therefore, it is important that the neurobiology of this phenomenon be understood so that more effective and durable treatments for alcoholism can be designed.

In the following sections, we describe common methodologies used to probe the neurobiology underlying primary and conditioned ethanol reward. Specifically, these methodological sections detail several commonly used animal models and tools to manipulate the brain. We then discuss the neural substrates that have been identified in ethanol-seeking behavior using these models and tools.

2. Animal models

To gain an understanding of the neurobiological mechanisms underlying AUDs, numerous animal models have been developed. These models are designed to reflect various aspects of alcoholism. The most widely used procedures assess ethanol reward and reinforcement and include drinking, self-administration, and conditioned place preference (CPP). These animal models share significant homology to certain elements of AUDs in humans, such as the patterns of alcohol consumption, responses to alcohol-associated cues, and alcohol-seeking behavior. Note that, in this review, the terms reward and reinforcement are distinguished from one another. Although reward is used to refer to the appetitive nature of a stimulus as indicated by the ability of environmental stimuli to elicit approach behavior, reinforcement will refer to experimental contingencies that increase the likelihood of behavior(s) occurring [6, 7]. It should be noted that no animal model could fully emulate all aspects of human alcoholism. However, animal models allow for unparalleled access to the brain and thus provide a means to evaluate neural mechanisms involved in the aspects of alcohol reward and dependence. These models therefore represent invaluable preclinical tools for identifying potential biological correlates of and treatments for AUDs.

2.1. Drinking

For nearly a century, it has been known that rodents, like humans, will voluntarily consume alcohol [8, 9]. For this reason, rodents have long been used in drinking procedures that involve home-cage access to alcohol (ethanol). This represents the simplest way to gauge ethanol reward, through consumption, which is done simply by providing rodents with a bottle and measuring the amount they drink. Although alcohol is occasionally the only solution provided in drinking studies, two-bottle choice procedures are more commonly used in rodents and yield an additional measure of preference for alcohol. In a two-bottle choice drinking procedure, home-cage access to alcohol and another alcohol-free fluid (typically water) is provided continuously or at temporally controlled intervals. Evidence of ethanol reward is then indicated by the amount consumed and preference for the alcohol-containing solution over the other available fluid. Manipulations that affect alcohol consumption and/or preference but not water or total fluid intake are believed to have interfered with the rewarding properties of ethanol [10].

Although these studies have high face validity (as humans voluntarily consume alcohol orally), they are often limited by the fact that, like humans, rodents are sensitive to the aversive taste of ethanol. At higher concentrations, the aversive taste of ethanol makes it difficult for rodents to drink to the state of intoxication. Therefore, procedures requiring oral intake of ethanol may require water deprivation, slow increases in ethanol concentration, and/or the addition of a sweetener such as sucrose to the ethanol-containing solution to help rodents overcome the aversive taste [11, 12]. For instance, modified sucrose fading techniques [13] are a common strategy that has been used to achieve voluntary consumption of high concentration of ethanol in rodents. With this technique, sweeteners such as sucrose or saccharin are initially added to an ethanol solution then slowly faded out. However, this illustrates a pitfall of these procedures, which is that the underlying motivation for ethanol consumption is not always understood. For example, rodents may freely consume ethanol for the sweetened taste or for its caloric value. Therefore, it is not always evident that ethanol is being consumed in this procedure for its postabsorptive pharmacological effects. Additionally, intervention-induced decreases in alcohol intake in this procedure do not always indicate that a manipulation decreased the ethanol reward. It is possible that a reduced intake may reflect an enhancement of the pharmacological effects of ethanol, resulting in a leftward shift in the dose-response curve, which translates to an increased effect of ethanol at lower amounts. Furthermore, rodents tend to titrate their dose of ethanol consumption and often do not reach blood ethanol concentrations (BECs) of intoxication unless induced to consume greater volumes via sucrose fading or limited access to ethanol [14]. As a result, care must be taken when interpreting results in drinking studies, as the underlying reasons for decreased intake may not always be apparent.

2.2. Self-administration

In self-administration procedures, rodents must successfully perform an operant response (e.g., lever press or nose poke) to receive a small volume of an ethanol solution [15]. With this method, requisite responding is used to assess the reinforcing value of ethanol. A major

advantage of self-administration procedures is that they allow for the assessment of ethanol reinforcement at distinct phases. Methodological manipulations in these procedures allow for the evaluation of the development of and enhancement in ethanol responding, including in the absence of drug (extinction) and the reemergence of responding to various environmental stimuli after responding has been extinguished (cue-, stress-, and ethanol-induced reinstatement). Thus, this procedure can be successfully used as a model aspect of alcohol seeking (rate of responding or latency to bar press for alcohol). Also, self-administration studies can be used to assess the animal's motivation to receive ethanol by increasing the difficulty of the requisite responding (a progressive ratio schedule, in which responding requirements are increased after every the delivery of reinforcer) and thus evaluating the willingness of the animal to work for an ethanol reinforcer [16]. As with humans, rodents will exert effort to obtain ethanol and this effort or seeking behavior can be reduced by administration of therapeutic agents indicated for the treatment of AUDs, such as naltrexone [17]. As such, reinstatement procedures have been shown to be highly useful for the preclinical evaluation of pharmacotherapies aimed at reducing ethanol relapse in humans (reviewed in [18]).

In addition to the rate and pattern of responding, self-administration procedures also yield an additional measure of amount of alcohol consumed. However, similar to drinking studies, the aversive taste of ethanol may be difficult to overcome in self-administration procedures. Therefore, liquid deprivation and fading strategies have also been used to establish operant responding for and consumption of ethanol. This similarly compromises straightforward interpretations of the underlying purpose for the behavior. Interpretation of studies using operant oral paradigms may also be complicated because it is difficult to distinguish between the phases of intake. As training procedures are necessary to establish ethanol self-administration and responding or intake serves as the primary dependent variable, it can be challenging to separate acquisition and learning from seeking, for example.

2.3. Place conditioning

Another approach to modeling reward in rodents is the CPP procedure. With this Pavlovian (classical) conditioning procedure, a distinct environmental stimulus [conditioned stimulus (CS)] can acquire incentive salience after being paired with a motivationally significant stimulus [unconditioned stimulus (US)]. Ultimately, the previously neutral stimulus (CS) develops the ability to elicit a conditioned motivational response similar to the response elicited by the US. This mimics the ability of cues or contexts associated with alcohol (e.g., alcohol containers, odors, advertisements, and drinking establishments) to elicit craving and seeking for alcohol in humans with AUDs. In addition, drug-induced CPP can be established in humans in a laboratory setting, further validating this model [19]. As such, the CPP procedure is widely used to study the motivational properties of many abused drugs, and given its numerous benefits, the popularity of this procedure continues to grow. In the last decade alone, there has been a greater than two-fold increase in the total number of publications reporting its use (**Figure 1**). Thus, this procedure is considered one of the most popular models of drug reward [20, 21].
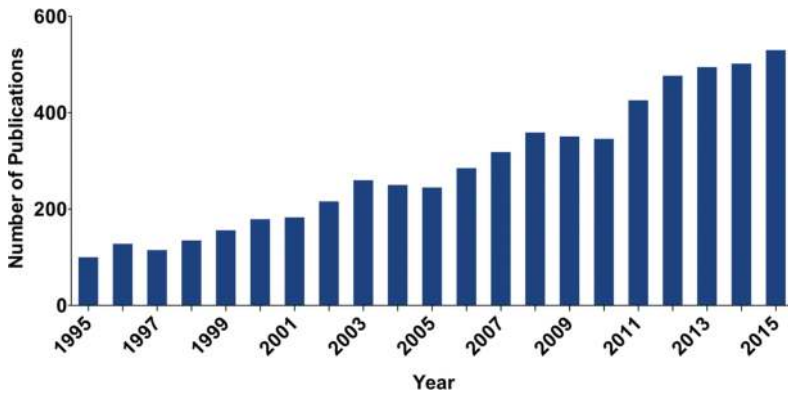
Figure 1. Annual number of CPP studies published over the last 20 years. Values were obtained from PubMed using the search term "CPP" OR "place preference" OR "place conditioning". Adapted from Tzschentke [20, 21].

In a standard ethanol CPP procedure, a discrete cue [e.g., visual or tactile stimulus; referred to as the positive CS (CS+)] presented in one spatial location is repeatedly paired with ethanol, usually administered by the investigator (i.e., noncontingently). On alternating sessions, a different stimulus not paired with ethanol [typically paired with saline, the negative CS (CS-)] is presented in a location adjacent to where the CS+ was presented. During this acquisition phase, an association develops between the CS+ and the subjective effects of ethanol (US). In the subsequent drug-free expression phase, animals are given access to the entire conditioning apparatus and thus to both cues (CS+ and CS-). When given the choice between the CS+ and CS-, animals assess the memory formed in the acquisition phase and will generally approach and maintain contact with (i.e., prefer) the CS+ when a US is rewarding. In other words, if an animal spends a greater amount of time with the ethanol-paired stimulus (CS+) in relation to the nondrug-paired stimulus (CS-), this is taken as an indication of the positive motivational effects of alcohol. Conversely, a greater amount of time spent with the saline-paired stimulus compared to the ethanol-paired stimulus would be considered conditioned place aversion (CPA) and taken to indicate a negative motivational effect of ethanol. The ability of alcohol to produce CPP or CPA depends on many factors, such as past history of ethanol exposure, route of administration, injection timing, and dose (e.g., [22–26]). A result of conditioned reward or aversion can vary by species and strain. Although conditioned ethanol responses have been reported in some strains of rat (e.g., [27–29]), studies overall have shown conflicting results ranging from lack of CPP [30] to CPA [31, 32]. However, ethanol CPP has been found in a wide array of mouse strains [33] and thus has much utility as a model of ethanol reward in this species.

Unlike self-administration, CPP does not require a lengthy training phase. In fact, in an inbred st rain of mouse (DBA/2J) commonly used in ethanol CPP, a significant place preference can be conditioned after only two ethanol-cue pairings [34–36]. Another advantage to this procedure is that it does not involve oral intake of ethanol, which is required in drinking and self-administration procedures. This is highly beneficial in cases where manipulations, such

as pharmacology agents, reduce general consummatory behavior in addition to ethanol reward or reinforcement. For example, erroneous conclusions may be made when a drug with anorectic liability reduces the oral intake of ethanol. However, because CPP involves noncontingent ethanol administration, the effect of anorectic drugs on ethanol reward can be assessed more accurately [35]. The place preference paradigm also permits for the evaluation of manipulations on the various phases of learning an ethanol-paired cue that are often difficult to isolate in other procedures, and an understanding of these various phases of learning is important for understanding the progression of alcohol addiction. These phases include the acquisition, conditioned expression, extinction, and reinstatement of the conditioned effects of ethanol. Acquisition and conditioned expression of CPP are described in the paragraph above. Extinction occurs following repeated exposures to the CS+ unpaired with the US (ethanol), which results in a loss of conditioned responding as the animal learns that the CS+ and US are no longer associated and the CS+ loses its rewarding value. Reinstatement, the phase most comparable to relapse in the human condition of addiction, is the reemergence of ethanol-seeking behavior following extinction, usually elicited by a priming injection of a low dose of ethanol (US), stress, or ethanol cues before being placed within the conditioning apparatus. Specifically, when a manipulation disrupts the expression of ethanol CPP, this is generally taken to indicate that it interfered with the conditioned rewarding effect of ethanol or ethanol seeking. Indeed, because CPP can be used to gauge the conditioned rewarding or motivational value of stimuli, it also serves as an effective method to measure cue-induced ethanol-seeking behavior. On the other hand, manipulations that disrupt the development (acquisition) of CPP are thought to impact either associative learning or the primary rewarding effect of ethanol. To distinguish between these two possibilities, this procedure can also be used to assess whether the manipulation also disrupts the acquisition of other associations such as CPA induced by ethanol or other drugs (e.g., [34]). However, if a manipulation specifically affects the extinction of CPP, it is thought to impair the formation of an inhibitory memory, which relies on different neural structures than those required for the acquisition of CPP. If a manipulation impairs the reinstatement of CPP, it is thought to either impair mechanisms that allow for evaluation updating or reemergence of behavior, but manipulations could also prevent access to the original acquisition memory and manipulations must be assessed for specificity to reinstatement.

One disadvantage of the CPP procedure is that the drug is administered by the investigator and therefore delivered noncontingently. Although this may be considered an advantage given the control over the dose it provides, it reduces the face validity of this model. Unlike humans, in this procedure, rodents do not consume alcohol of their own volition. Similarly, humans do not take alcohol via intraperitoneal injections, as is used in this animal model. Moreover, this procedure does not typically involve an escalation in intake, as is usually observed in humans. Comparable to self-administration procedures, manipulations that affect locomotor activity may also nonspecifically impact CPP expression. It has been previously demonstrated that increases in activity may disrupt ethanol CPP expression, thereby obscuring its detection [37]. Hence, results obtained by manipulations that increased or decreased preference test activity must be cautiously interpreted. Despite these drawbacks, this model

presents a rapid and efficient method to evaluate the primary and conditioned motivational effects of ethanol in rodents.

2.4. Summary

In summary, animal models have been used extensively in alcohol research, with several models designed to study the various aspects of ethanol-mediated behavior. The most standard used procedures to model ethanol seeking are self-administration and CPP. Although it should be noted that no one procedure is able to mimic all features of human alcohol use, these models allow for the investigation of the underlying neural mechanisms involved in distinct alcohol-related behaviors. Furthermore, the paradigms described here do not encompass all models of AUDs available. Additional models include operant runway models, vapor chamber exposure, intracranial self-stimulation, and locomotor sensitization. In the following sections, several commonly used techniques to probe the neural structures and circuits involved in rodent behavior and their application to animal models of ethanol seeking are described.

3. Tools to manipulate neural structures

Many techniques have been developed to evaluate distinct brain structures. These methods allow for the direct manipulation of a defined brain area to manipulate their activity during behavior. Thus, they require intracranial access typically gained through stereotaxic surgery. Of these methods, the most widely used are lesions and microinjection. However, more modern tools have been developed that harness the capabilities of viral gene transfer to more precisely control cells and circuits. Each of these techniques, classic and contemporary, possesses inherent benefits and drawbacks that are discussed in detail below and summarized in **Table 1**.

3.1. Classical tools

3.1.1. Lesions

A classical method used to study brain function involves the removal or destruction of neural tissue. With this method, experimental lesions are made to defined brain structures through manual, chemical, or electrical means and can also be neurotransmitter specific. Popular neurotransmitter-specific lesioning agents include 6-hydroxydopamine (dopaminergic and noradrenergic neurons), 5,7-dihydroxytryptamine (serotonergic neurons), ibotenic acid [N-methyl-D-aspartate (NMDA) receptor-containing neurons], kainic acid (kainate receptor-containing neurons), and many others. Behavior is then examined in the absence of this tissue, thus providing insight into the involvement of the lesioned structure. Histology, such as simple cresyl violet stains or for markers of neuronal damage, is performed on neural tissue after behavior to confirm the location of damage. One issue that arises is the propensity for other brain structures to compensate for the damaged region. This may severely compromise the

interpretation of results obtained from studies using a lesion procedure. Another issue is the difficulty encountered when using lesions to assess the effects at distinct phases of a behavior, as lesions cause irreversible damage to the region. Thus, with many animal models, it is difficult to determine whether ablation of a structure impacted the development (acquisition), performance (expression), extinction, or reinstatement of behavior. To affect a distinct phase of behavior, lesions can be made at specific times in the model. However, phase-specific lesions can unintentionally alter later behavior and can even impair the memory of earlier phases.

Criteria	Classical tools			Contemporary tools	
	Lesion	Microinjection	Tracing	Optogenetic	Chemogenetic
Spatial resolution	1 Depends on lesion type	1 Spread difficult to determine	2 Labeled spread; targeted to cells	2 Labeled spread; targeted to cells	2 Labeled spread; targeted to cells
Temporal resolution	0 Permanent	1 Minutes to hours depending on drug	0 Permanent	2 Milliseconds	1 Minutes to hours depending on actuator
Non invasive	1 Single i.c. entry but involves intentional tissue damage	0 Permanent hardware, repeated i.c. access	1 Single i.c. entry, but tracers may be toxic	0 Permanent hardware, repeated i.c. access	2 Single i.c. entry, activation by peripheral injection
Cell specificity	0 Marginally possible with chemical lesions	0 Receptors targeted across cell types	1 Increased with viral tracers and transgenic strains	2 Using transgenic strains and viral promoters	2 Using transgenic strains and viral promoters
Minimal need for specialized equipment	0 Current-generating device and electrodes for electrolytic	0 Surgical stainless steel cannula, injectors custom gauge and length	2 Requires micro injectors only	0 Cannula and fiber optics of custom length, multichannel light source	2 Requires microinjectors only
Circuit specificity	0 Indirect with disconnection procedure	0 Indirect with disconnection procedure	1 Labeling of defined projections	2 With axonal light excitation	2 With i.c. actuator infusion or anterograde/retrograde viruses
Bidirectional Modulation Ability	0 Inactivation only	2 Inhibition and activation depend on drug	n/a	2 Depends on opsins and wavelengths	2 Depends on receptors and actuators

0, low; 1, moderate; 2, high; i.c., intracranial.

Table 1. Comparison of commonly used classical and contemporary tools in behavioral neuroscience.

3.1.2. Intracranial microinjections

The local administration of pharmacological agents into discrete brain targets is another strategy to control neural activity. This technique typically requires permanent surgical placement of guide cannula to allow for later access to otherwise inaccessible brain structures while animals are awake and behaving. Small volumes of drug solutions are then administered directly into the brain by threading a smaller gauge injector through the guide cannula. These solutions typically contain drugs that bind to distinct membrane proteins (receptors) expressed within the target brain region to enhance or inhibit local cellular activity during behavior. Similar to lesions, histology is performed afterwards to verify the site of microinjection. This procedure has several major advantages compared to lesions, most of which relate to its ability to produce more temporally specific effects. Unlike lesions, the effects of most pharmacological antagonists and agonists are temporary and can therefore be more precisely controlled and administered during distinct phases of behavioral procedures. This allows for more straight-forward interpretation of the effects of this manipulation on behavior. Additionally, this technique can provide insight into the neurochemical signals involved, as agents selective for distinct receptor types can be infused. To a lesser extent than lesions, microinjections also produce damage resulting in reactive gliosis from cannula installation and injector placement. Finally, it is difficult to ascertain the exact extent of diffusion of the administered solution. As diffusion may depend on a variety of factors, such as the volume injected and the nature of the solution (polarity, hydrophobicity), it is difficult to predict. Thus, it is not always clear that the site of infusion is the region directing the observed behavior. For this reason, it is often necessary to include additional groups that receive drug injections in locations proximal to the target structure.

3.2. Contemporary tools

In recent years, there has been a rapid emergence of novel tools engineered to control neuronal activity. Of benefit to these tools have been the advancements in recombinant viruses that are capable of gene transfer in the central nervous system (CNS). For example, viruses with low immunogenicity and cytotoxicity such as adeno-associated virus (AAV) can be delivered directly into the brain to safely and efficiently express recombinant genes [38]. This provides a means to site-specifically express proteins in the CNS that can be used to modulate the activity of cells in target brain tissue. Optogenetics and chemogenetics represent the two most widely used contemporary tools in behavioral neuroscience, as they can be applied in vivo to modulate neural activity in awake behaving mice and rats. As with the classical tools described above, these modern methods have inherent advantages and disadvantages (**Table 1**), which are detailed below.

3.2.1. Optogenetics

In this technique, neurons are genetically modified through intracranial injection of a viral vector to express photosensitive proteins. The most commonly used photosensitive receptors are channelrhodopsin (ChR; excitatory ion channel), halorhodopsin (NpHR; inhibitory ion pump), and archaerhodopsin (ArchT; inhibitory proton pump; reviewed in [39]). These light-

gated proteins are activated by targeted illumination, causing rapid (millisecond timescale) depolarization or hyperpolarization of neurons (reviewed in [40]). By evoking or inhibiting spike activity with this light-protein interaction, the activity of distinct brain regions and cell types can be experimentally controlled, including during the performance of behavioral tasks [39]. Because these engineered opsins can be controlled by different light wavelengths, neural activity and behavior can be modulated bidirectionally (i.e., multiplexed), offering a major advantage to this technique. Moreover, the high temporal resolution afforded by this tool makes it ideal to examine the discrete phases of behavior. However, a major issue posed by optogenetics is the possibility of desensitization of the opsin, which can occur within seconds of photoactivation [41]. Thus, this is especially problematic for studies that require inhibition or activation of longer durations, such as is required in certain behavioral tasks that occur on the order of minutes. Repeated stimulation leading to the desensitization of the opsin may even produce opposing results. This is especially problematic in the case of the excitatory opsin ChR, as the desensitization of this receptor and repeated stimulation of the cell may result in a net inhibition, the opposite effect of what is initially intended. The extent of viral diffusion and resulting protein expression is easily measurable with this technique, as most viral constructs contain a fluorescent tag. However, similar to microinjections, implantable hardware is necessary to allow for intracranial insertion of fiber-optic probes. This technique also requires specialized equipment such as fiber-optic probes and programmable light sources, which can be costly. Tethering the animal to the external light source is also necessary, which may restrict the range of apparatuses that can be used and behaviors that can be assessed (although for recent developments in wireless technologies; see [42–46]). Recently, questions regarding the effect of illumination in brain structures have arisen, specifically in regards its thermal effects on neural tissue. It has been suggested that focal illumination, especially when intense and prolonged, can result in phototoxicity, heat-induced cell damage, and oxidative stress that independently alters cellular activity [47]. Even more problematic is evidence indicating that heat alone can increase neuronal firing rates [48]. In fact, even at commonly used intensities, the thermal effect of illumination is sufficient to increase cell firing rates [49]. Overall, optogenetics provides a unique tool to control neuronal activity with high spatiotemporal resolution. However, the required implantable hardware, specialized equipment, tethering, risk of desensitization, and light induction may render this tool less than ideal given the experimental question and design.

3.2.2. Chemogenetics

This relatively new technique involves the engineering of G protein-coupled receptors (GPCRs) to interact exclusively with small molecules that were otherwise unrecognized by the GPCR [50]. The most common of these mutated GPCRs are designer receptors exclusively activated by designer drugs (DREADDs) [51]. The engineered GPCRs possess no detectible constitutive activity and are robustly activated at nanomolar concentrations of otherwise pharmacologically inert compounds. The first established DREADDs were based on excitatory G_q-coupled and inhibitory G_i-coupled human muscarinic receptors M3 (hM3Dq) and M4 (hM4Di), respectively [51–53]. Receptors hM3Dq and hM4Di possess no affinity for the endogenous ligand acetylcholine and are robustly activated by the drug clozapine-N-oxide

(CNO), a pharmacologically inert metabolite of clozapine, which is highly bioavailable and produces no pharmacological effect in rodents [51, 54]. Since their inception, other DREADDs have been engineered, which include a G_s-coupled muscarinic-based (rM3Ds) receptor and G_i-coupled KOR-based DREADD (KORD) [55–57]. Notably, the development of KORD with actuator salvinorin B allows for the bidirectional control of behavior when used in combination with hM3Dq receptors and CNO [57].

Comparable to optogenetics, DREADDs can be ectopically expressed in the CNS by focal infusion of a vector encoding for these receptors. A major advantage of chemogenetics over optogenetics, however, is the lack of required specialized equipment and need for permanently implanted hardware. In fact, DREADDs require just one initial intracranial entry to infuse the viral vector carrying the DREADD-encoding gene. The receptors can then be activated by a relatively noninvasive peripheral injection of an actuator. This is highly advantageous when performing sensitive behavioral procedures that are affected by excessive handling (e.g., CPP and self-administration) [58]. Similar to optogenetics, a more precise detection of viral spread and DREADD expression are possible with this technique, as they are designed to encode for DREADDs as well as a fluorescent marker.

Unlike optogenetics, DREADDs signal through canonical G-protein pathways. Once activated, the duration of the inhibition or activation produced by the DREADD can be long lasting. The duration of effect is also determined by the half-life of actuators, which may remain in central tissue and activate DREADDs for minutes to hours. In some cases, this low temporal resolution may serve as a major shortcoming of this technique. However, a protracted effect is often highly valued in studies where behavioral tasks are of longer duration and long-lasting effects of manipulations are desired. Another issue is that the presence of the receptor does not always indicate that it is a functionality. Additional measures may be necessary to demonstrate the function of these receptors in target tissue. Although it is theoretically possible, no studies have reported DREADD desensitization. However, this presents another reason for including some form of functional confirmation of DREADD effects.

In summary, DREADDs are a useful technique to control neuronal signaling in vivo. Considering the sensitivity and duration of many behavioral tasks, the noninvasive nature of DREADD activation (i.e., peripheral drug injection) and longer time course of inhibition/activation make chemogenetic strategies highly desirable in behavioral research.

3.3. Summary

The above-described tools provide a means through which to target and manipulate brain regions. These tools offer variable degrees of selectivity, with contemporary techniques typically being associated with higher precision in terms of spatial and neuronal targeting. The tools mentioned above do not encapsulate all available methods of discovering the neurobiology behind behaviors. Other commonly used methods not described in this chapter are intracranial electrical or self-stimulation, intracranial microdialysis, electrophysiology, immunohistochemistry (IHC), genetic knockout rodents, and many others that similarly assess the importance of a brain region and specific neurotransmitter systems to behavior.

4. Tools to manipulate specific neural pathways

Historically, the direct manipulation of neural circuits has been a challenging task, with much of the difficulty due to limited methodologies. In the past, many of the tools used possessed relatively low selectivity and provided indirect manipulation. Several strategies, however, have been designed using both classical and contemporary tools to probe the neural circuitry underlying behavior. This section describes several commonly used strategies and includes a discussion of their merits and weaknesses.

4.1. Classical tools

Historically, disconnection procedures involving lesions and pharmacological microinjections have been used to evaluate neural circuitry. This strategy involves the disruption of two directly connected brain regions to assess whether their interaction is involved in behavior (e.g., [59–62]). Typically, a unilateral lesion or inactivating microinjection [e.g., γ-aminobutyric acid (GABA) agonists or channel blocker] is made in source regions and another lesion or microinjection is made in the contralateral hemisphere of its terminal target. Thus, if a behavior is dependent on a source-target interchange, then their contralateral disconnection should be more disruptive to behavior than ipsilateral disconnection or unilateral manipulation of each region individually. However, a major weakness of this strategy is the indirect nature of the manipulation on the circuit. Indeed, the imprecision of this method has at times been proven problematic resulting in significant reductions in behavior with ipsilateral and unilateral manipulations alone (e.g., [60]). This is likely due to the inability of this technique to directly target distinct yet intermixed populations of target-projecting neurons within source regions. Instead, each region is broadly manipulated leading to the inhibition of their overall activity and output throughout the brain.

To help visualize and identify the circuit, neuronal tracing has sometimes been used in conjunction with these classical tools. In these studies, tracing agents are injected into the brain to label neurons in a manner that is retrograde (axon terminal back to the soma), anterograde (soma to axon terminal), or transsynaptic (to adjacent neurons retrogradely or anterogradely). In studies of behavior, circuit involvement is inferred by colabeling of neuronal activity markers such as c-Fos with the tracer through IHC (e.g., [63]). Cells that are immunopositive for both the tracer and activity marker are then used to identify afferent or efferent projection neurons that were activated during behavior. When used in tandem with disconnection procedures, this provides a means to visually assess the impact of disconnection on circuit activity (e.g., [62]). Although these procedures help to label neurons within the circuit and gauge their activity, they still fall short of allowing isolated modulation of the circuit. Overall, this remains a major weakness of classical techniques, as results only offer an indirect measure of neural circuit involvement in behavior.

4.2. Contemporary tools

Optogenetic and chemogenetic strategies have provided a refined and more selective means to directly manipulate neural circuits. This is principally due to viral transduction, as proteins

(opsins and DREADDs) are trafficked downstream from soma to axon terminals (anterograde) and therefore expressed on presynaptic boutons as well as to cell bodies [64]. In the case of optogenetics, illumination can then be targeted to axon terminals, which results in the depolarization or hyperpolarization of the neuron. This strategy has been successfully implemented in many behavior studies (reviewed in [64]). However, a concern that arises when using this method is the possibility of antidromic stimulation of the cell. The stimulation of terminals may result in the back-propagation of an action potential that activates the neuron and its collateral inputs to other regions outside the circuit of interest. Therefore, with this strategy, there is a potential for the activation of multiple circuits, which diminishes the selectivity of the manipulation.

Several chemogenetic-based strategies have been used to modulate neural circuit activity. First, a functional disconnection procedure methodologically similar to that used with lesions or microinjections has been reported by Mahler et al. [65]. In this study, hM4Di receptors were unilaterally expressed in ventral tegmental area (VTA) dopamine (DA) cells and contralaterally in rostral ventral pallidum (RVP) cells. The contralateral disconnection of RVP-VTA DA reduced cued reinstatement of cocaine seeking relative to both unilateral RVP inhibition and unilateral VTA DA inhibition. Despite these positive results, it is unclear whether the simultaneous inhibition of RVP and VTA DA, regardless of hemisphere, would have been sufficient to produce a similar effect. Thus, with this technique, it is important to include ipsilateral as well as unilateral controls. In summary, the lack of precision and necessary inclusion of numerous controls renders this a less desirable strategy for the targeted modulation of neural circuits.

Another DREADD-based method that has been used involves the intracranial injection of CNO. The principle behind this strategy is similar to that of the optogenetic circuit-selective method outlined above. Here, like illumination, CNO is targeted to the terminal region of DREADD-expressing cells [65, 66]. The focal infusion of CNO therefore serves to activate/ inhibit DREADD-expressing cells and/or presynaptic neurotransmitter release from DREADD-expressing nerve terminals [66]. In this manner, the activity of defined neural circuits can be more precisely controlled. However, given that this strategy requires intracranial microinjections, it also carries with it the disadvantage of requiring permanently indwelling hardware (i.e., guide cannula and obturators), repeated intracranial entries, and a resulting increased risk of tissue damage.

An alternative approach to this involves the intersection of multiple viruses that are injected into serially connected nuclei. Typically, a retrograde virus encoding for cre recombinase is injected in a target region and a cre-inducible virus encoding for DREADD is injected into the source region. In this manner, the activity of a specific source's inputs to the target region (i.e., projection neurons) can be controlled by the systemic injection of CNO. This approach has been successfully implemented using canine adenovirus (CAV-2) to retrogradely infect source region cells and selectively express DREADDs in a specific neural projection [67–69]. Notably, this strategy provides an ideal way to control circuits, especially during behavior. Not only does this method provide a high degree of selectivity, it does not require implantable hardware and can be robustly activated by a simple peripheral injection of an actuator. Theoretically, it

is possible for axon collaterals from source region cells to express DREADDs, which given the systemic nature of DREADD activation may lead to activity in sites outside the circuit. Hence, studies using this method should be careful to address this potential caveat.

4.3. Summary

Overall, several techniques involving classical and contemporary tools have been used to manipulate defined neural circuits. On one hand, classical tools provide indirect manipulation and generally require the use of multiple controls to carefully and appropriately interpret results derived using these strategies. Conversely, contemporary tools involving viral-mediated gene transfer confer greater circuit selectivity and have been successfully used to manipulate serial projections form source to target region.

5. Neural structures involved in ethanol-seeking behavior

In the following sections, several neural structures and circuits involved in ethanol-seeking behavior are discussed. Building off of earlier sections, the studies that are described below involved several different animal models and tools to identify the underlying neurobiology of ethanol seeking. Specifically, this chapter will focus on the neural structures involved in ethanol self-administration and CPP, as these models specifically assess ethanol-seeking behavior. A proposed circuit of the key neural structures implicated in cue-induced ethanol seeking as indexed by self-administration and CPP procedures is included in **Figure 2**.

5.1. Findings from ethanol self-administration studies

Studies using ethanol self-administration procedures to evaluate the neural mechanisms underlying ethanol seeking have by and large employed intracranial microinfusions. Most of the neural regions that have been evaluated in self-administration studies are part of the mesocorticolimbic system [70] and are typically situated downstream of the VTA. As with many drugs of abuse, acutely administered ethanol excites DA neurons within the VTA through direct and indirect mechanisms [71–74]. Furthermore, the VTA is robustly activated by ethanol-associated cue exposure [75]. Downstream, the nucleus accumbens (NAc) and amygdala receive dopaminergic input from the VTA [76] and considerable evidence suggests that this dopaminergic input to each region underlies associative learning and motivated behavior [77–81]. As such, NAc and amygdala are two of the most well-characterized structures in terms of their involvement in ethanol seeking and several of these studies are described below.

The NAc has been routinely implicated in the drug-seeking behavior [82, 83]. Many studies have found a differential involvement of core and shell subdivisions in drug seeking depending on the drug of abuse, phase of self-administration, and nature of the procedure and/or stimuli used [84–86]. As with other drugs of abuse, the NAc core and shell have been shown to be differentially involved in cue-induced ethanol seeking. For example, ethanol self-administration studies have shown that transient inactivation of the NAc core but not shell
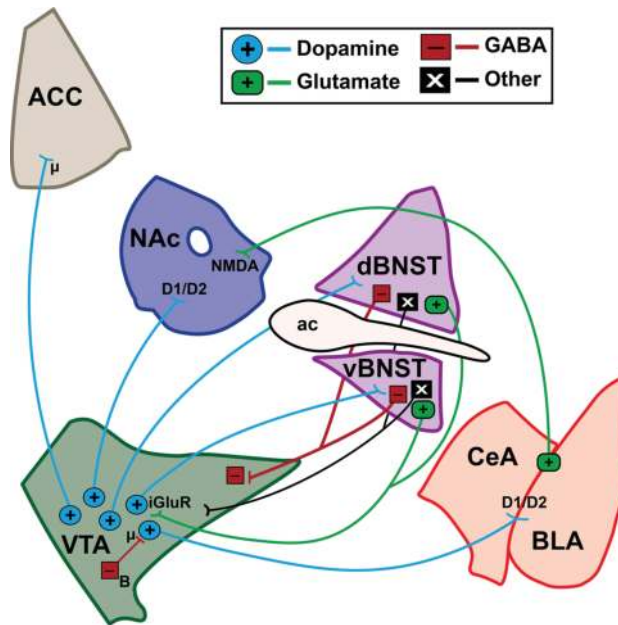
Figure 2. Diagram of the neural circuitry involved in ethanol CPP. Previous studies have demonstrated the involve-
ment of cortex (ACC), amygdala (BLA/CeA), striatum (NAc), and midbrain (VTA) in ethanol CPP. The present work
now demonstrates the involvement of the dorsal and ventral BNST (dBNST and vBNST, respectively) and a direct
BNST-VTA circuit. Each of these regions receives dopaminergic input from the VTA (indicated by blue arrows) and is
part of a broader mesocorticolimbic dopamine system that underlies reward and motivation. Some of the neurochemi-
cal signals underlying the involvement of each region in ethanol CPP have also been identified. +, excitatory; -, inhibi-
tory; blue circles, dopamine cells; green rectangles, glutamate cells; red squares, GABA cells; black boxes, unknown
neurochemical signal; green arrows, glutamate projections; μ, μ-opioid receptors; B, GABA$_B$ receptor; D1, dopamine
D1-like receptor; D2, dopamine D2-like receptor; iGluR, ionotropic glutamate receptors, NMDA, iGluR subtype.

reduces cue-induced reinstatement [87] and context-induced renewal [88] of ethanol seeking.
Other studies, however, have demonstrated that NAc shell modulates the expression of cue-
induced ethanol seeking [89] and that blockade of DA D1 receptors in the NAc shell but not
core reduces the spontaneous recovery of ethanol-seeking behavior following prolonged
abstinence [90]. In addition, DA D1 receptor antagonism in NAc core and shell has been
reported to block context-induced renewal of extinguished ethanol seeking [91]. These studies
vary in their findings of core versus shell involvement in ethanol seeking, likely indicating that
the role of NAc subdivisions varies by different phases of self-administration (e.g., acquisition,
expression, and reinstatement) and by the use of cues or context. However, this literature
overall supports a role for the NAc in general ethanol-seeking behavior and suggests that DA
input may underlie its involvement.

The amygdala has also been strongly implicated in drug-seeking behavior, specifically the
basolateral amygdala (BLA) and central nucleus of the amygdala (CeA) subdivisions as well
as the bed nucleus of the stria terminalis (BNST) of the extended amygdala [92–94]. In ethanol

self-administration studies, the inactivation of the BLA has been shown to reduce context-induced renewal of previously extinguished ethanol seeking [95]. The involvement of the BLA in ethanol seeking may involve a glutamatergic mechanism, as intra-BLA ionotropic glutamate receptor antagonism reduced the expression of ethanol seeking elicited by a discrete cue and invigorated by an ethanol-associated context [96]. Notably, the excitatory transmission from the amygdala to the NAc is believed to underlie reward seeking and suggests that an amygdala-NAc circuit may be involved in the expression of reward-seeking behavior. For example, the optical stimulation of BLA glutamate to NAc has been shown to be reinforcing, as mice worked to earn additional stimulation of BLA-NAc synaptic inputs [97]. Conversely, the inhibition of BLA-NAc inputs reduced cue-induced responding for sucrose [97]. In addition, pharmacological disconnection has revealed that an amygdala-NAc interaction underlies a stimulus-controlled expression or maintenance of cocaine seeking [59].

The CeA and BNST have generally been implicated in stress-induced ethanol-seeking reinstatement but may also play a role in cue-induced ethanol reinstatement. For instance, intra-CeA but not intra-BLA infusion of mifepristone, a glucocorticoid receptor antagonist, has been reported to suppress reinstated ethanol seeking induced by the pharmacological stressor yohimbine [98]. Activation of group II metabotropic glutamate receptors blocks stress- and cue-induced reinstatement of ethanol seeking presumably through CeA and BNST actions [99].

Lastly, additional regions that have been implicated in ethanol seeking using self-administration procedures include the dorsomedial striatum [100, 101], medial prefrontal cortex (mPFC) [75], prelimbic cortex, and VTA [102]. Importantly, the VTA, which is the chief source of DA input to NAc, amygdala, and cortical regions, has been shown to be explicitly involved in context- and cue-induced ethanol seeking [103–105]. Overall, self-administration studies have been important in identifying several key neural substrates involved in ethanol-seeking behavior. Several of these structures have also been implicated in cue-induced ethanol seeking through ethanol-induced CPP procedures and thus are discussed in the next section.

5.2. Findings from ethanol CPP studies

Studies on the neural mechanism of ethanol CPP can be grouped into several categories that include acquisition, expression, extinction, and reinstatement. Acquisition studies are those that assess the development of ethanol place preference. These typically include procedures where manipulations occurred during the conditioning or training phase, where animals learn to associate contextual cues with ethanol reward. Conversely, expression studies involve manipulations that occur after the conditioning phase and before preference testing. Expression studies, in particular, are useful in assessing ethanol-seeking behavior and conditioned reward. Below, findings from each of these types of ethanol CPP studies are discussed. Of note, relatively few laboratories study the primary and conditioned rewarding properties of ethanol using a CPP procedure. This is partly due to the difficult and unreliable nature of ethanol place conditioning in rats [21, 106] and the relatively weak ethanol CPP obtained with commonly used inbred mouse strains, such as C57BL/6J [107, 108]. In view of this, many of the studies described below have used male DBA/2J mice. This inbred strain will rapidly and reliably develop and ethanol CPP, even with a minimal amount of conditioning sessions [34, 36, 108].

In this section, we provide an overview of studies that have directly examined the neural areas underlying the acquisition and expression of ethanol place preference.

5.2.1. Acquisition

As with self-administration studies, the NAc and amygdala are the two most evaluated regions in terms of their involvement in ethanol CPP acquisition. Previously, the involvement of the NAc in ethanol place preference acquisition has been investigated. In one study, bilateral electrolytic lesions of the NAc before CPP training disrupted the acquisition of CPP [109]. In a later study, bilateral NAc infusions of the D1-like receptor antagonist SCH-23390 disrupted the development of ethanol CPP [110]. This finding is similar to a finding reported in rats showing that nonselective DA antagonism prevented CPP induced by intracerebroventricular (icv) infusions of ethanol [111]. Combined, these studies indicate that the NAc is necessary for establishing associative relationships between ethanol reward and environmental cues likely through a DA D1-like receptor-dependent mechanism. Other work has looked at the role of the amygdala in ethanol CPP acquisition [109]. Using electrolytic lesions, the amygdala was ablated bilaterally before CPP training. Amygdala lesions disrupted acquisition (and/or expression) of ethanol place preference, suggesting that this region is also involved in ethanol cue learning.

Together, these findings demonstrate that these structures downstream the VTA are necessary for the development of ethanol CPP. Moreover, NAc involvement in ethanol CPP is directly attributed to dopaminergic innervation, as activity at D1-like receptors in this region is necessary for acquisition. One consideration of these results is that acquisition studies involving microinjections can be problematic, as the additional handling required to focally administer a drug can interfere with ethanol CPP [110]. Although they do not require added handling prior, lesions are also problematic when administered preconditioning, as it unclear whether they affected the acquisition or expression phase.

5.2.2. Expression

Over the last decade, studies have investigated the involvement of several brain areas in ethanol place preference expression. These include the VTA, NAc, amygdala, and anterior cingulate cortex (ACC). Each of these structures is thought to be involved in reward and motivation partly through DA mechanisms, and as such, each is part of a broader mesocorticolimbic DA system.

Although VTA DA cells are initially activated by rewarding stimuli, this activation diminishes over time with repeated reward exposures and subsequent learning [112]. Eventually, DA cells are no longer activated by the reward itself and instead become robustly activated by environmental stimuli that have become associated with the reward and predict its delivery [113, 114]. Thus, the involvement of this conditioned DA response in ethanol CPP expression can be supported by studies focusing on the VTA or downstream sites as are described below.

One pharmacological microinjection study separately infused the nonselective opioid receptor antagonist methylnaloxonium and the $GABA_B$ receptor agonist baclofen into the VTA to assess

the participation of the VTA in ethanol CPP expression. Methylnaloxonium decreased the magnitude of ethanol CPP, whereas baclofen blocked preference expression entirely. These findings suggested that the VTA activity is necessary for the expression of ethanol place preference. Moreover, GABAergic and opioidergic activity appear to underlie the involvement of the VTA in ethanol CPP expression, presumably through the local modulation of DA cell activity.

Although involvement of the NAc in ethanol place preference acquisition is clear, its involvement in expression is more complicated. In early work, an intra-NAc infusion of methylnaloxonium failed to impact ethanol preference expression, suggesting a lack of NAc opioid receptor involvement in the conditioned ethanol reward [115]. A later study directed bilateral electrolytic lesions at the NAc after ethanol CPP conditioning and before testing to isolate the involvement of this structure in expression [109]. Overall, lesions made at this time point did not affect ethanol place preference, suggesting that the NAc may not be critical in ethanol CPP expression.

However, additional pharmacological procedures have shown a more specific role for the NAc in CPP expression. In one study, intra-NAc antagonism of D1- and D2-like receptors prevented the expression of CPP induced by icv ethanol [111]. In another study, NAc DA (D1- and D2-like) and glutamate (NMDA) receptors were blocked during the CPP expression test using either flupenthixol or AP-5, respectively [116]. Although DA receptor antagonism did not affect ethanol place preference, NMDA receptor antagonism reduced it, suggesting that the involvement of the NAc in expression is specific to activity at NMDA receptors. This effect was reproduced in another study using only unilaterally administered AP5, further indicating the importance of NAc NMDA receptor involvement in ethanol place preference expression [60]. Notably, this study was designed to examine glutamate input to NAc from amygdala. Although findings appeared to demonstrate that amygdala disconnection from NAc blocked ethanol CPP expression, reduced CPP in mice unilaterally infused with AP5 in NAc prevented this interpretation. Overall, these findings have established a role for NAc NMDA, but not DA receptors, in ethanol place preference expression and suggest that glutamatergic input from amygdala may also be involved. Although DA input from VTA to NAc is a hypothesized mechanism underlying drug seeking, these results suggest that it does not underlie ethanol seeking, at least as indexed by CPP. Finally, these studies serve to demonstrate that manipulations more selective than global inactivation or deletion of a structure may be necessary to appropriately gauge that the importance of structure in behavior.

Accordingly, the role of the amygdala in ethanol CPP expression has also been addressed. Like lesions made before conditioning, the bilateral electrolytic lesion of the amygdala made before the test phase blocked ethanol place preference expression [109]. In addition, when bilaterally infused into the amygdala, the D1- and D2-like DA receptor agonist flupenthixol blocked ethanol CPP expression [116]. Together, these results illustrate the role of the amygdala in ethanol-seeking behavior and indicate the importance of dopaminergic input to this structure for ethanol CPP expression.

The BNST also has a role in modulating the expression of ethanol reward. Exposure to ethanol causes changes in glutamate synaptic plasticity [117], increases extracellular DA levels in the

BNST [118], and activates BNST cells [119]. In particular, the inhibition of the BNST during CPP expression using electrolytic lesions, coinfusion of GABA$_A$ and GABA$_B$ receptor agonists muscimol and baclofen, and activation of inhibitory DREADDs (hM4Di-DREADD) lead to a loss or reduction of the expression of ethanol CPP. All of these studies suggest the importance of the BNST to ethanol seeking.

Finally, ACC involvement in expression was assessed by bilaterally infusing the nonspecific opioid receptor antagonist methylnaloxonium into the ACC before the ethanol CPP test [120]. The intra-ACC infusion of methylnaloxonium disrupted ethanol place preference expression, reducing its magnitude at the lowest dose and abolishing it at the highest dose. Hence, the ACC appears to modulate ethanol CPP expression through an opioidergic mechanism.

In summary, findings from expression studies have demonstrated that the VTA, NAc, amygdala, BNST, and ACC are all structures involved in ethanol place preference expression. Infusion of a mixed opioid receptor antagonist into VTA and ACC but not NAc interfered with ethanol CPP expression. Similarly, lesions and mixed DA receptor antagonism disrupted expression when targeted to the amygdala and not NAc. Involvement of the NAc in ethanol CPP expression appeared to be confined to activity NMDA receptors only, suggesting that a more explicit neurochemical mechanism underlies its involvement in ethanol place preference expression.

5.2.3. Extinction and reinstatement

Unlike the acquisition and expression of ethanol CPP, the neural correlates of extinction of ethanol CPP is relatively understudied. The only region consistently studied for its role in ethanol CPP is the mPFC.

The mPFC is known to be involved in the acquisition and extinction of Pavlovian conditioned fear and drug memories [121]; thus, it is not surprising that disruption of its activity impairs the extinction of ethanol CPP. Several studies found that lesions or inhibition of the mPFC, but not the ACC, following acquisition blocks the extinction of CPP [122, 123]. These studies suggest the importance of this mPFC in the formation of inhibitory ethanol seeking memories. Additionally, one study links the NAc core with extinction of ethanol seeking. Lesions of the NAc core were found to have no effect on the expression of ethanol CPP but caused a rapid loss of responding during extinction [109], which suggest that the NAc core is associated with inhibitory ethanol memory, but further research will be needed to confirm the role of the NAc in extinction.

Like extinction, the brain regions involved in the reinstatement of ethanol CPP are not well studied, but there are several studies that implicate particular brain regions. One such study found an increase in c-Fos activity in the BLA of rats following cued reinstatement of ethanol seeking in a discriminative stimulus operant paradigm [75]. Although this is not a direct proof of the necessity of the BLA in ethanol seeking in CPP, another study found that the direct manipulation of the BLA alters cued reinstatement of cocaine seeking [59]. Together, these studies suggest the importance of the BLA in ethanol reinstatement and likely in the reinstatement of ethanol CPP. Other regions, such as the VTA, amygdala, and BNST, are likely involved

in the extinction and reinstatement of ethanol CPP, as they are important for the extinction and reinstatement of CPP of other addictive substances. Investigating the involvement of these regions in extinction and reinstatement of ethanol CPP will be an important step for understanding the circuitry of relapse.

5.3. Summary

In summary, these studies show the importance of several brain regions heavily implicated in drug reward to ethanol seeking as well. Most of these studies evaluated the structures situated downstream the VTA that have been implicated in drug seeking based on their efferent dopaminergic input. In addition to these studies demonstrating that downstream sites are involved, findings from Bechtholt and Cunningham [115] further illustrate the importance of the VTA in ethanol-seeking behavior. As opioid receptors are situated presynaptically on local GABAergic inputs to DA cells, the authors hypothesized that methylnaloxonium disinhibited VTA GABAergic interneurons. This likely inhibited VTA DA activity, thereby reducing ethanol place preference expression. Conversely, baclofen presumably reduced CPP by acting directly on VTA DA cells, as they express $GABA_B$ receptors. Thus, the VTA is a critical structure for drug and, specifically, ethanol cue associations. Although these findings and proposed mechanisms underscore the importance of VTA DA in ethanol CPP expression, they provide little information on the inputs to the VTA that modulate DA cell activity. Thus, the origins and neurochemical sources of VTA DA cell innervation involved in ethanol-seeking behavior remain unknown. Accordingly, the next section discusses the involvement of VTA input in reward and identifies several inputs that may be of importance and deserve further study for their role in ethanol CPP.

5.4. Inputs to the VTA

Excitatory (glutamatergic) afferents of the VTA arise from virtually all structures to which this region projects, with the exception of the NAc and lateral septum (LS), which provide strong GABAergic inputs [124]. This suggests that there is a broad network of excitatory reciprocal projections to and from the VTA, with much of the glutamatergic input to VTA neurons arising from subcortical regions that include but are not limited to amygdala, mesopontine nuclei, lateral habenula, and hypothalamus [125]. Although this reciprocal flow of neurotransmission is also found between the VTA and cortex, the PFC serves as the only cortical source of glutamate to the VTA [126]. Importantly, these glutamatergic afferents play a critical role in regulating VTA neuron firing. Specifically, glutamatergic input to the VTA appears to be critical for behaviorally relevant burst firing of VTA DA neurons [127]. The resulting phasic release of DA from the VTA is intimately associated with goal-directed behaviors and drug reward [128].

In addition to the contemporary methods described earlier, recent advances in immunohistological techniques have facilitated a more precise mapping of afferent and efferent projections of the VTA. As a result, an updated view on the role of broad neural circuit activity (including that of the VTA) in relation to behavior has been formed. In this current view, the net result of cell-specific projections onto cell-specific targets is accounted for and the subsequent plotting

of these circuits suggests a complex topographical map. This map indicates the existence of an intricate network of connectivity designed to tightly regulate the activity of neuronal ensembles, which in turn orchestrate complex and divergent behaviors, even from within the same circuits. In the VTA specifically, not all inputs are alike in their behavioral consequences. Here, DA activity is governed by a complex network of cell-type-specific neuron-to-neuron connections, the net effect of which can result in vastly different motivational states [129, 130].

Several lines of evidence suggest the importance of monosynaptic inputs to VTA DA neurons (one-step inputs) in governing motivational behaviors. For example, investigation of the laterodorsal tegmental (LDT) nucleus has revealed that 80% of its glutamatergic afferents synapse onto VTA DA neurons directly [131]. The importance of these glutamatergic inputs have been corroborated through electrophysiological analysis, which has shown that this region is essential to VTA DA cell burst firing [132]. Moreover, in vivo stimulation of LDT glutamate afferents has been reported to selectively terminate on and stimulate a distinct population of VTA DA neurons, which thereby generate reward [130].

The BNST is another region upstream the VTA that has been identified as critically involved in regulating the activity of DA cell activity [133–135]. Specifically, the BNST positively modulates VTA DA activity putatively through two primary and distinct mechanisms: (1) a direct glutamate projection to VTA DA and (2) a direct GABA projection to VTA GABA [129, 135–137]. Of note, more recent evidence derived from studies using more advanced and selective tools suggests that a BNST GABA input to VTA GABA is the predominant source of the modulation of the BNST of the VTA [129]. Of relevance, behavior in rodents has demonstrated that the BNST underlies cue-elicited drug seeking. For example, transient inactivation of the BNST has been shown to prevent cue-induced reinstatement of cocaine seeking [138]. Direct projections from BNST to VTA appear to be important for cocaine-related behaviors. For example, disconnection of the BNST-VTA pathway has been shown to reduce expression of cocaine CPP [62], but the role of that projection in ethanol seeking still remains unknown and further study of this projection and other VTA inputs will advance our understanding of the larger neural network driving ethanol seeking.

6. Conclusion

Alcohol is a widely used legal intoxicant that produces a huge financial toll ($223.5 billion in 2006 alone) on the United States (Centers for Disease Control and Prevention, 2014) due to the workplace, healthcare, legal, and criminal consequences of excessive consumption of alcohol. Despite the prevalent societal knowledge of the detriment of excessive alcohol use, AUDs continue to be a common disorder that is difficult to treat. The primary challenge of providing lasting treatments for AUDs is the high propensity for those with AUDs to relapse into alcohol use. Relapse is often caused when a cue associated with alcohol (i.e., a bar, alcohol advertisement) or alcohol itself is presented to a person with an AUD. This cue recalls the associative effects of ethanol and induces a craving and an internal drive to seek and consume alcohol. Thus, it is critical to understand how alcohol cue associations are formed and maintained

despite receiving treatment for alcohol abuse. This chapter has outlined the animal models that are being used by preclinical researchers to better understand the formation, expression, extinction, and reinstatement (relapse) of alcohol cue associations that promote ethanol seeking and has summarized each of their advantages and disadvantages. Of particular use is the CPP paradigm. CPP allows the experimenter to separate distinct phases of acquiring, expressing, extinguishing, and reexpressing conditioned ethanol seeking and thus can easily study the neural mechanisms involved in each. This chapter also presented the classical and contemporary tools that can be used separately and in conjunction to probe the exact neural structures and circuitry involved in alcohol cues and seeking. Although classical tools have given us the greatest insight into the neurobiology of ethanol seeking thus far, contemporary tools have been and will allow for a much clearer and specific understanding of the structures involved in animal models of alcohol seeking. Finally, this chapter presented evidence from ethanol self-administration and ethanol CPP studies of the modulation of ethanol seeking by the mesolimbic structures (VTA, NAc), the limbic system (amygdala, BNST, ACC), and cortical structures (mPFC). Of particular importance is the VTA that sends vast dopaminergic input to many of these structures. The challenge of future research is to identify more structures critical for the acquisition and expression, and especially the extinction and reinstatement, of ethanol CPP and the inputs of the VTA that modulate dopaminergic tone and thus ethanol-seeking behavior (such as the BNST-VTA projection). A better understanding of the whole circuit driving every aspect of ethanol seeking will improve our knowledge of AUDs and treatment options.

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