INVOLVEMENT OF THE AMYGDALA AND NUCLEUS ACCUMBENS IN ETHANOL-CONDITIONED REINFORCEMENT IN MICE

by

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Permissions

Work presented in this dissertation has been previously published.

The research presented in chapter 1: Roles of the nucleus accumbens and amygdala in the acquisition and expression of ethanol-conditioned behavior in mice, has been previously published (2008) in *The Journal of Neuroscience* by Christina M. Gremel and Christopher L. Cunningham as *"Roles of the nucleus accumbens and amygdala in the acquisition and expression of ethanol-conditioned behavior in mice"*. A letter of permission for publication in this thesis has been submitted to the journal.

The research presented in chapter 2: Involvement of amygdala dopamine and nucleus accumbens NMDA-receptors in ethanol-seeking behavior in mice, will be submitted to *Neuropsychopharmacology* as *"Involvement of amygdala dopamine and nucleus accumbens NMDA-receptors in ethanol-seeking behavior in mice"* by Christina M. Gremel and Christopher L. Cunningham. A letter of permission for publication will be obtained if the manuscript is accepted.

A letter of permission has been obtained from Elsevier Limited for reproductions of schematic diagrams representing the mouse brain as portrayed in *Paxinos & Franklin, 2001*, on January 17, 2008.

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Abbreviations

(Ac) anterior cingulated cortex
(Acb) nucleus accumbens
(AcbC) nucleus accumbens core
(AcbSh) nucleus accumbens shell
(aCSF) artificial cerebrospinal fluid
(AMPA) alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
(Amy) amygdala
(ANOVA) analysis of variance
(AP-5) 2-amino-5-phosphopentoanoic acid
(BLA) basolateral nuclei of the amygdala
(BM) basomedial nuclei of the amygdala
(BST) bed nucleus stria terminalis
(CE) central nuclei of the amygdala
(CPA) conditioned place aversion
(CPP) conditioned place preference
(CR) conditioned response
(CRF) corticotrophin releasing factor
(CS) conditioned stimulus
(D1) dopamine type 1 receptor
(D2) dopamine type 2 receptor
(D3) dopamine type 3 receptor
(D4) dopamine type 4 receptor
(DA) dopamine
(DARP-32) dopamine and adenosine 3'-5'-monophosphate-regulated phosphoprotein
(GABA) gamma-aminobutyric acid
(DG) dentate gyrus
(icv) intracereroventricular
(NMDA) N-methyl-D-aspartate
(ORL) nociceptin receptor
(PBS) phosphate buffered solution
(pcs) paracapsular intercalated cells
(PFC) prefrontal cortex
(Pre-L) prelimbic cortex
(PVN) paraventricular nucleus
(S^D) discriminative stimulus
(SN) substantia nigra
(VTA) ventral tegmental area
(Uni) unilateral amygdala
(UR) unconditioned response
(US) unconditioned stimulus

Abstract

Few studies have examined the neural areas mediating ethanol-conditioned reinforcement, which may be critically involved in ethanol seeking behaviors and relapse. Environmental or internal cues closely paired in space and time with a drug's effects can gain motivational value through an associative process known as Pavlovian conditioning, and in turn exert control over drug seeking and taking behaviors. Conditioned place preference (CPP) offers a way to investigate the learning mechanisms that underlie the ability of an ethanol-paired cue to elicit behavior in the absence of the drug. In CPP, a subject is passively administered a drug paired with a particular cue in the environment. After repeated pairings, preference for the previous drug-paired cue is assessed in the absence of the drug. If the subject spends more time with the previously drug-paired cue, the basic interpretation is the subject finds the drug be rewarding. Associative learning mechanisms that underlie the acquisition and expression of this behavior may control drug-seeking behaviors involved in drug abuse and dependence. Given the paucity of information on the functional neural mechanisms controlling cue-induced ethanol-seeking behavior, the following studies used the CPP procedure to gain a further understanding of the processes involved in acquisition and expression of this behavior.

Initial investigations have implicated the ventral tegmental area (VTA) in the expression of ethanol CPP (Bechtholt & Cunningham, 2005). However, the neural areas downstream mediating the acquisition and expression of this effect are unknown. In the first part of this thesis (Gremel & Cunningham, 2008), we examined the roles of the nucleus accumbens and amygdala in the acquisition and expression of ethanol CPP (chapter 2). We demonstrate that acquisition of ethanol CPP depends upon an intact

accumbens and amygdala, while expression seems to require an intact amygdala. However, lesions of the accumbens core facilitated a loss of this expression behavior. Contributions of dopamine receptors (implicated in reward signaling), and NMDA receptors (implicated in learning processes) in the amygdala and accumbens to expression behavior were examined in chapter 3. Dopamine receptor activation within the basolateral amygdala, but not central amygdala or accumbens, is necessary for cue-induced ethanol-seeking behavior. Further, expression of ethanol CPP was dependent upon NMDA receptor activation in accumbens (Gremel & Cunningham, *submitted*).

For the first time, these studies demonstrate that acquisition and subsequent expression of cue-induced ethanol seeking behavior is modulated via mechanisms within the amygdala and accumbens. These are the first studies to delineate neural areas involved in the acquisition versus expression of a drug-conditioned behavior in mice. Further, they provide the first evidence of amygdala dopamine receptor involvement in any ethanol behavior, the first NMDA receptor involvement in mouse accumbens, and the first to delineate contributions of specific mouse amygdala nuclei. The current results also suggest that conditioned reinforcement processes may be controlling the observed expression behavior, providing a possible neural mechanism underlying ethanol-conditioned reinforcement. In summary, the work presented in this thesis suggests expression of ethanol-seeking behaviors involved in relapse, may be dependent upon amygdala dopamine receptor activation and accumbens NMDA receptor activation.

Chapter 1: Neurobiological processes underlying ethanol-seeking behavior

Alcohol abuse and dependence are prevalent worldwide. To date, there are very few treatments, with those used having little effectiveness at maintaining abstinence, reducing craving, and preventing relapse (see Johnson, 2008). Alcohol use and abuse involve a complex group of behaviors that lead to an increase in seeking and consumption of alcohol (e.g., Koob, 2003). Associative learning processes are considered an extremely important component of the dependence pathway that starts with initial drug use and escalates to drug abuse and dependence (for review see Everitt, Dickinson, & Robbins, 2001; Franken, 2003; Weiss, 2005). As with most abused drugs, initial consumption of a drug that is reinforcing increases the frequency of drug self-administration. With continued use, environmental or internal cues that are closely associated in time and space with the drug's effect can gain incentive salience. When these cues are presented or encountered, they can alter behavior, produce, amplify, and/or predict a drug event or effect (e.g., Corbit & Janak, 2007; Cunningham, 1994, 1998; Di Ciano, Blaha, & Phillips, 2001; Everitt, Parkinson, Olmstead, Arroyo, Pobledo, & Robbins, 1999; Krank, 1989, 2003). Researchers have hypothesized the combination of these conditioned effects along with the subjective effects of a drug can drive behavior to become compulsive and lead to habitual use and dependency (e.g., Childress, Mozley, McElgin, Fitzgerald, Reivich, O'Brien, 1999; Everitt & Robbins, 2005). Understanding the neurobiological mechanisms of associative control over alcohol behaviors may offer insight into prevention, and possibly the development of new treatments effective at reducing craving and relapse.

Associative learning mechanisms involved in ethanol-seeking behaviors

Reinforcement leads to increases in frequency of drug use, as a subject learns that a response provides access to a reinforcing drug. This goal-directed response, where learned modulation of voluntary actions leads to a particular outcome (e.g., drug), depends on the knowledge concerning the relationship between the action and outcome, and on the knowledge about the value of the outcome (e.g., Dickinson & Balleine, 1994). Since drug use generally consists of individual actions or responses to gain access to or consume the drug of choice, this type of associative learning is fundamental for the development of addictive behaviors. However, instrumental responses are open to influences from other associative processes (e.g., Rescorla, 1987; 1988), which in turn may govern the transition from responding for a predicted outcome, to a habitual and/or compulsive pattern of responding independent of the obtained outcome as is often observed in drug addicts.

External or internal stimuli closely paired in space and time with a drug's effects can gain incentive salience through an associative process known as Pavlovian conditioning, and in turn exert control over drug behaviors. In Pavlovian learning, an unconditioned stimulus (US) (e.g., ethanol) elicits an unconditioned response (UR). After repeated pairings of the US with a conditioned stimulus (CS) (e.g., environmental cue) (stimulus-outcome learning), the CS alone can produce a conditioned response (CR) that may alter physiological/behavioral responses, motivational states (e.g., craving) or expectancies of drug-related behaviors (e.g., Corbit & Janak, 2007; Cunningham, 1994, 1998; Krank, 1989, 2003). In ethanol-seeking behaviors, if the previously ethanol-paired

cue has gained associative value, the conditioned response evoked by the cue may produce, amplify, or motivate ethanol behaviors.

Pavlovian approach behavior. Palovian approach behavior reflects the conditioned power of an environmental cue, where a predictive stimulus can elicit an approach response, even though the response action is not necessary to obtain the outcome, i.e., there is no contingency between the response and the outcome. Often described as a form of stimulus-outcome learning, it is more commonly labeled as "autoshaping" or "sign-tracking" (e.g., Everitt & Robbins, 2005; Hearst & Jenkins, 1974; Tomie et al., 1989). While this type of learning has readily been shown with natural reinforcers (e.g., Cardinal & Everitt, 2004; Kearns & Weiss, 2004), only more recently has it been reliably demonstrated in animal models of drug-seeking behavior (Cunningham & Patel, 2007; Uslaner, Acerbo, Jones, & Robinson, 2006). In a modified conditioned place preference (CPP) procedure, ethanol-treated mice demonstrated an increase in approach behavior to a spatially isolated visual stimulus (CS+) over acquisition trials. Further, mice displayed this same increase in approach behavior towards the cue predictive of ethanol's effects during drug-free testing (i.e., in the absence of ethanol) (Cunningham & Patel, 2007). An additional demonstration of approach behavior to a drug-paired stimulus was observed in rats that received infusions of cocaine paired with simultaneous insertion of a lever (Uslaner et al., 2006). Rats increased approach responses directed towards the lever even though administration of cocaine was not contingent upon any response. However, it was not reported whether after acquisition rats would approach the lever in the absence of a cocaine infusion. These studies are the first to suggest that drug-predictive environmental stimuli can elicit and

control approach responses that have been hypothesized to play a large role in directing and guiding drug-seeking behavior (Everitt & Robbins, 2005; Ludwig, 1988).

Incentive motivation. Predictive stimuli after gaining associative value may also motivate, enhance, and/or activate behavior. If stimuli have gained motivational value during conditioning, they may serve to energize or increase drug-related behaviors. Examples of this are observations of a stimulus increasing locomotor activity or enhancing instrumental responding (e.g., Berridge, 2006; Corbit & Janak, 2007; Cunningham, 1994; Stewart, 1992; Stewart et al., 1984; Robbins & Everitt, 2007; Robinson & Berridge, 1993). While there is debate about whether incentive motivation directs specific drug-seeking behaviors versus generally activating drug-related behaviors, the idea that drug-predictive stimuli can motivate and energize drug behaviors is generally well accepted (Berridge, 2006; Robbins & Everitt, 2006, 2005).

Conditioned reinforcement. Another example of Pavlovian control over responding is when a previously neutral stimulus acquires motivational value that can support reinforced responding and new learning (e.g., Cardinal & Everitt, 2004; Mackintosh, 1974). Conditioned reinforcers are thought to be extremely important in maintaining drug-seeking behavior because, in the absence of the drug, conditioned reinforcers can serve to bridge delays and maintain or increase the allocation of behavior aimed at procuring the drug (for review see Everitt & Robbins, 2005; Everitt et al., 1999). A conditioned reinforcer gains associative value through Pavlovian pairings with the reinforcing drug. In Mackintosh's (1974) definition of conditioned reinforcement, an increase in the frequency of the behavior directed towards the conditioned reinforcer is observed after it has gained associative value during acquisition. This behavior change

can be attributed to the associative value of the conditioned reinforcer, given that the behavior has not produced the primary reinforcer. Further, the underlying motivational value of the conditioned reinforcer is independent of changes in the value of the primary reinforcer. In other words, devaluing the primary reinforcer has no effect on the behavior that the conditioned reinforcer can support (Parkinson, Roberts, Everitt, & Di Ciano, 2005).

Given that conditioned reinforcers can control behavioral responding, they can support the acquisition of a new response requirement. For example, a stimulus (light cue) previously paired through Pavlovian processes with cocaine or heroin was able to support the acquisition of lever presses, when access to the conditioned reinforcer (light cue) was contingent upon a fixed ratio lever press response (Di Ciano & Everitt, 2004[a]). Responding on a lever that produced the conditioned reinforcer was interpreted as a drug-seeking behavior. Further, the increase in drug-seeking behavior was contingent on responding for the conditioned reinforcer, since the same effect was not observed when the CS was presented in a non-contingent manner (Di Ciano & Everitt, 2003, 2004[a]). Conditioned reinforcement is particularly interesting since the observed increases in behavior don't directly produce the drug. Instead, this behavior is solely supported by conditioned cues in the environment that provide predictive or motivational information concerning previous drug effects or upcoming drug events.

Use of self-administration models to examine associative control of cue-induced ethanol-seeking behavior. In contrast to other drugs of abuse, very little is known about the specific neural areas and mechanisms mediating associative control over cue-induced ethanol-seeking behavior. Research on appetitive cue-induced psychostimulant-seeking

behaviors has implicated many of the same neural mechanisms indentified in this thesis (for review see Everitt & Robbins, 2005; Everrit et al., 2001). However, in contrast to the procedure used in this thesis, many of the investigations into associative control over cue-induced psychostimulant-seeking behaviors have used self-administration procedures with second order schedules of reinforcement or acquisition of a new response (for review see Everitt & Robbins, 2005). In a second order schedule, rats are initially trained to press a lever that results in a simultaneous intra-venous infusion of cocaine and cue light presentation. After acquisition, subjects are then trained to fulfill a work requirement to gain access to a cue light. Responding is then maintained by the cue light until the session ends or the work requirement produces a cocaine infusion (e.g., Di Ciano & Everitt, 2005). However, when a similar procedure was attempted using oral ethanol self-administration, the stimulus barely supported responding and only slightly attenuated extinction of responding in the absence of ethanol when presented alone (Slawecki, Chappell, & Samson, 1999).

As stated above, acquisition of a new response has also been used to examine the neural areas underlying associative control over drug-seeking behavior, where the ability of a previously cocaine-paired stimulus to function as a conditioned reinforcer was assessed. After passive cocaine administration has been paired with a CS, the CS was able to support new lever responding (i.e. rats pressed a lever to gain access to the CS) (e.g., Di Ciano & Everitt, 2004[b]). When this type of procedure was used with ethanol as the reinforcer, the initial association made was between a stimulus and access to ethanol via oral self-administration (Slawecki, Samson, & Chappell, 1997). When they assessed the ability of the previously ethanol-paired cue to support the acquisition of a new lever

response, increases in responding directed towards the new lever were only observed after intra-Acb infusions of amphetamine. These findings suggest that the stimulus alone was insufficient as a conditioned reinforcer.

Use of CPP to examine associative control over cue-induced ethanol-seeking behaviors. Given the difficulty in employing an oral route of ethanol administration to measure acquisition of a new response or maintenance of a second order schedule of reinforcement, I propose that the CPP procedure provides an alternative means to assess associative processes controlling ethanol-seeking behaviors. CPP provides a model to investigate the environmental cues associated with a drug experience and the motivational influence they accrue (Carr, Fibiger, & Phillips, 1989). Although in CPP subjects are passively administered ethanol, and this may not provide the same face validity as seen in self-administration procedures, CPP does provide a means to examine Pavlovian associations that may be controlling ethanol-seeking behavior. CPP consists of a basic Pavlovian pairing, where a subject is passively administered the US (e.g., ethanol) that is then paired with a particular CS. After repeated pairings, preference for the CS (indirect conditioned response) is measured in the absence of ethanol. Preference is commonly compared to preference for a different stimulus that has been conditioned to a presumably neutral US. For example, if subjects spend more time in the presence of the CS previously paired with ethanol, the most basic interpretation has been that the subject previously found ethanol to be rewarding. By comparison, if the subject has a preference for the CS paired with the neutral stimulus, generally the interpretation is that ethanol was aversive since the subject avoids the ethanol-paired CS (i.e., conditioned place aversion (CPA)).

While acquisition of a CPP involves learning the Pavlovian relationship between the CS and ethanol, where the CS is endowed with motivational properties similar to that of ethanol it self, learning must also occur during the expression test. During testing, the retrieved memory of the Pavlovian association between ethanol and the CS may dictate new learning that controls behavior expressed towards the previously ethanol-paired cue. Multiple associative processes may influence the approach behavior to the previously drug-paired CS in ethanol CPP (e.g., Cunningham et al., 2006[a]; Robbins & Everitt, 2002; Uslaner et al., 2006). First, the ethanol-paired CS through Pavlovian processes may acquire the ability to elicit an approach response (i.e. sign-tracking). Further, conditioned reinforcement processes may control the approach response to the previously ethanol-paired CS. During the expression test, the subject for the first time learns that making the approach response brings reinforcement that is presumably obtained from the conditioned reinforcer (previously ethanol-paired CS). Another possibility is that the ethanol-paired CS has acquired incentive motivational properties that serve to enhance responding either by increasing Pavlovian approach behaviors to the CS or by increasing responding to gain access to the conditioned reinforcer. Through these associative processes, the CPP procedure allows for a way to investigate neural mechanisms underlying the ability of a cue endowed with motivational properties to elicit seeking behavior.

While different associative processes may control the learning in CPP, it should be noted that different stimulus and motivation properties of ethanol could underlie the goal of ethanol-seeking behavior. For example, ethanol has specific sensory and motivational effects that may enter separately into associations with environmental cues and directed responses. Whether ethanol-seeking behaviors are driven by the memory of

the direct sensory or motivational effects of ethanol is not clear (i.e. are seeking behaviors driven towards the attainment of specific ethanol sensory or motivational properties?). Further, it may be that cue-elicited ethanol-seeking behavior is no longer specific to ethanol itself. This idea is supported from conditioned reinforcement studies, where it appears that conditioned reinforcers are insensitive to devaluation of the original stimulus (Parkinson, Roberts, Everitt, & Di Ciano, 2005). Present data from our laboratory demonstrating that ethanol CPP is resistant to devaluation treatments (Cunningham & Young, unpublished data), suggests that ethanol-seeking behavior in CPP is not supported by a representation of ethanol itself. However, whether ethanol-seeking behavior in CPP is supported by accrued motivational value dependent or independent of ethanol's current value, the behaviors supported by both lead to drug-directed actions that contribute to abuse and dependence.

Neurobiological mechanisms of ethanol-seeking behavior

Although ethanol-seeking behaviors may be controlled through the above processes, the specific neural areas and mechanisms underlying the acquisition and expression of that control are largely unknown. In the following sections, involvement of possible neural circuits, as well as previous investigations implicating specific neural areas and/or mechanisms in ethanol reinforcement and ethanol-seeking behaviors will be presented and used to form the hypothesis behind the present thesis.

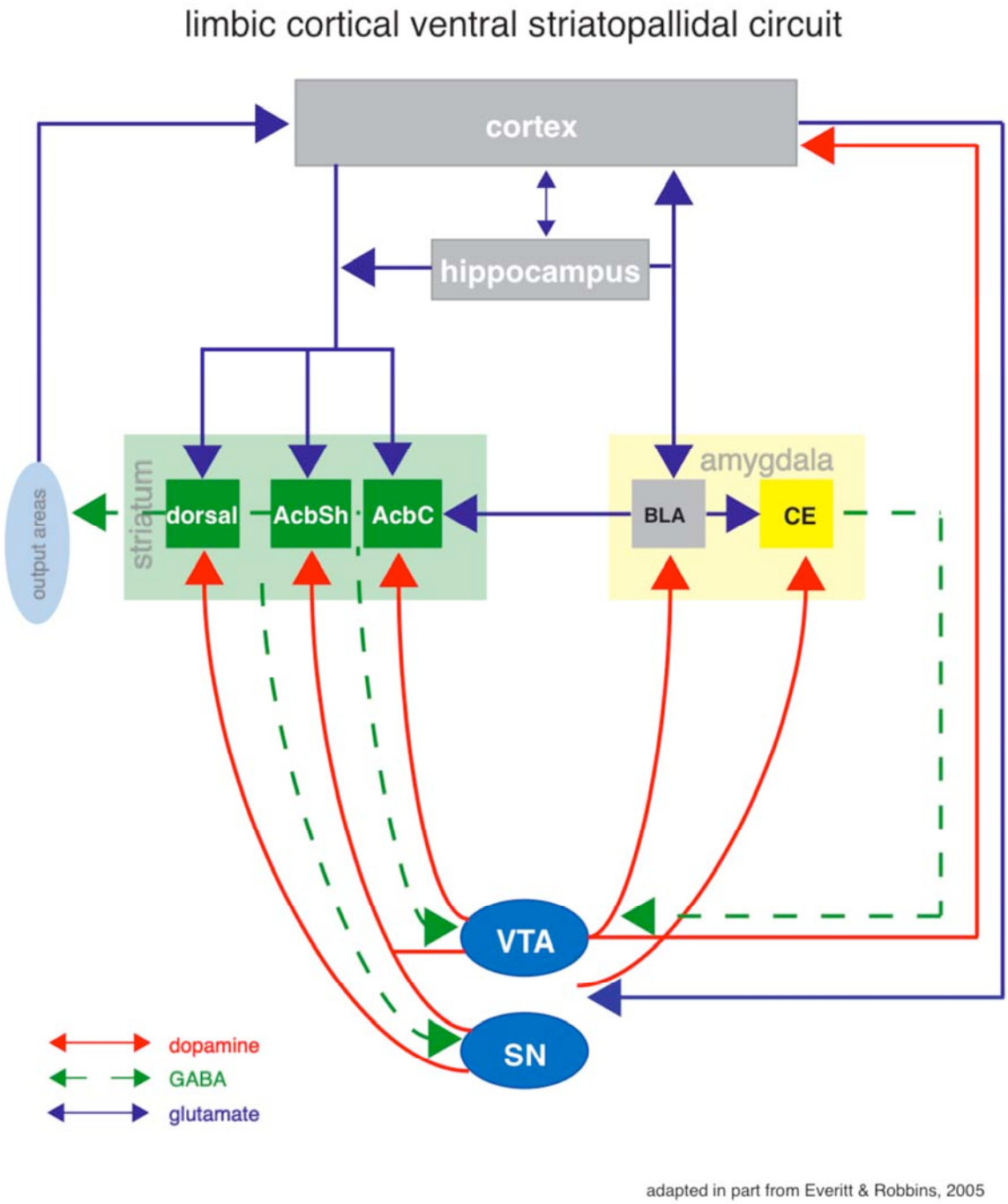
Involvement of the limbic cortical ventral striatopallidal circuit in drug-seeking behaviors. Connections between the mesolimbic dopamine system and cortical and striatal systems have been implicated in mediating not only the direct effects of many abused drugs (Koob & Le Moal, 2006), but also associative processes controlling drug

seeking and taking behaviors that can lead to compulsive and habitual drug use (for review see Everitt & Robbins, 2005; Day & Carelli, 2007). Connections between brain areas and the implicated neurotransmitter systems are summarized in Figure 1.

The mesolimbic dopamine system is comprised of a group of neuronal cell bodies that originate in the ventral tegmental area (VTA) in the midbrain. Dopamine cell bodies in the VTA not only send axons to the ventral striatum, synapsing in the nucleus accumbens (Acb), but also innervate cortical and cortical-like structures including the prelimbic cortex (Pre-L), anterior cingulate cortex (Ac), entorhinal cortex, agranular insular cortex, amygdala (Amy), and the hippocampus (e.g., Ford, Mark, & Williams, 2006; Gonzales, Job, & Doyon, 2004; Swanson, 1982).

Figure 1 Diagram of the limbic cortical ventral striatopallidal circuit. Connections between the VTA, cortical areas, amygdala, and striatum are represented via projection arrows. Dopamine projections are shown in solid red lines, while dashed green lines represent gamma-aminobutyric acid (GABA) projections, and glutamate projections are diagrammed in solid blue lines. Regions within specific areas of interest are separately labeled in order to show differences between afferent and efferent projections. This figure was in part adapted from Everitt & Robbins, 2005. The following regions are abbreviated as follows; basolateral amygdala (BLA), central nuclei of the amygdala (CE), nucleus accumbens core (AcbC), nucleus accumbens shell (AcbSh), ventral tegmental area (VTA), substantia nigra (SN), and globus pallidum (GP).

Figure 1


limbic cortical ventral striatopallidal circuit
cortex
hippocampus
striatum
dorsal
AcbSh
AcbC
amygdala
BLA
CE
output areas
VTA
SN
dopamine
GABA
glutamate
adapted in part from Everitt & Robbins, 2005

Much focus has been on the Acb, which can be further divided into core- (AcbC) and shell- (AcbSh) regions (e.g., Heimer, Alheid, de Olmos, Groenewegen, Haber, Harlan, & Zahm, 1997) and their differential modulation by combined dopamine input from the VTA (Deutch & Cameron, 1992) and glutamate from cortical and cortical like afferents. The Acb, discussed in greater detail in a later section, receives the majority of its glutamate innervation from the cortical mantel (Heimer et al., 1997), but also receives a large amount of glutamatergic innervation from the basolateral amygdala (BLA) and from the hippocampus through the ventral subiculum (Carlsen & Heimer, 1986; Groenewegen, Berendse, Wolters, & Lohman, 1990) and a small GABA projection from the VTA (Van Bockstaele & Pickel, 1995). In turn, the Acb sends information through reciprocal GABAergic projections back to the VTA (Chang & Kitai, 1985), and via the thalamus and global pallidum, sends glutamate projections to the hypothalamus and cortical areas (e.g., Haber, Fudge, & McFarland, 2000).

An additional focus of the current work has been the functional role of the Amy in ethanol-seeking behavior, which is discussed in greater detail in later sections. Within the Amy, the BLA and central nucleus (CE) receive dense DA innervation from the VTA (Ford et al., 2006; Swanson, 1982). In turn the BLA modulates Acb and CE activity via glutamate projections (for review see: Swanson & Petrovich, 1998) that synapse on and mediate VTA GABA projection neurons (Chang & Kitai, 1985, Sah, Faber, Lopez De Armentia, & Power, 2003). An additional feature in the circuit described above, is a group of dopamine cell bodies located within the substantia nigra that project to the Acb and dorsal striatum that have been implicated in the display of habitual control over response-outcome learning (for review, see Costa, 2007). However, the remaining

discussion in this thesis will focus on the limbic-corticostriatal areas discussed above and their involvement in drug-seeking behaviors.

Effects of ethanol on the limbic cortical ventral striatopallidal circuit. Ethanol, like most other drugs of abuse, exerts actions on the mesolimbic dopaminergic system (e.g. Koob, Roberts, Schulteis, Parsons, Heyser, Hyytia, Merlo-Pich, & Weiss, 1998). Systemic administration of ethanol increases activity of ventral tegmental neurons (Gessa, Muntoni, Collu, Vargiu, & Mereu, 1985), increases the firing rate of VTA DA neurons (Brodie, Shefner, & Dunwiddie, 1990; Brodie, Trifunovic, & Shefner, 1995), enhances somatodendritic DA release in the VTA (Campbell, Kohl, & McBride, 1996; Kohl, Katner, Chernet, & McBride, 1998), and increases DA levels in the Acb (Di Chiara & Imperato, 1988) and Amy (Yoshimoto, Ueda, Kato, Takeuchi, Kawai, Noritake, & Yasuhara, 2000). Additionally rats will self-administer ethanol directly in the VTA (Gatto, McBride, Murphy, Lumeng, & Li, 1994; Rodd, Melendez, Roberto, Bell, Kuc, Zhang, Murphy, & McBride, 2004), raising an interesting hypothesis that there may be overlap between areas involved in the direct reinforcing, and associative processes that are involved in drug-seeking and drug-taking behaviors.

Evidence from rodent models of ethanol self-administration. In the rodent model of self-administration, the required response to obtain the reinforcer (ethanol) is thought to involve both ethanol-seeking and ethanol-taking behaviors (e.g., Self, 2004). In particular, this model is thought to provide face-validity in that humans generally also self-administer alcohol. In this task, the subject performs a certain work requirement such as a fixed ratio schedule of lever presses to obtain access to ethanol. While there have been a few rodent self-administration studies using intra-venous (e.g., Grahame, Low, &

Cunningham, 1998) or intra-gastric administration (e.g., Fidler, Crews, & Cunningham, 2006), ethanol is more routinely administered by oral intake, with ethanol presented via a sipper tube or in a dipper presentation (e.g., Samson & Hodge, 1996; Cunningham, Fidler, & Hill, 2001). Acquisition of ethanol self-administration to levels of intoxication was initially observed via use of schedule-induced polydipsia, where periodically small amounts of food or other fluids are available during ethanol sessions in fluid or food deprived rats (for review see, Meisch & Thompson, 1974). Interpretations made from these early studies were complicated by the suggestion that ethanol self-administration could be driven by the calories ethanol provided, instead of ethanol's reinforcing effects (see Meisch & Thompson, 1974; Samson, 1986). However, ethanol self-administration was later observed in free-feeding rats (e.g., Grant & Samson, 1985), suggesting caloric intake alone does not support ethanol reinforcement. While the model has the ability to assess reinforcement-related behaviors, multiple problems arise with the use of ethanol as a reinforcer. As discussed by Samson and colleagues (1988), one such issue is rodents generally seem to have an innate ethanol taste aversion. To achieve higher levels of ethanol responding, a method was developed by Samson and colleagues (1986) that used a sucrose fading procedure in the initiation of ethanol self-administration. This initially involved training animals to respond for access to 20% sucrose that served as the reinforcer. Over the period of a few weeks, the ethanol concentration of the solution was increased from 0% to 10% and then the sucrose was slowly removed. After this sucrose induction of ethanol responding and consumption, rats maintained relatively high levels of ethanol responding and consumption. Since then, acquisition of ethanol self-administration is commonly performed with some version of the sucrose/saccharine

fading procedure (e.g., Funk, O'Dell Crawford, & Koob, 2006; Hodge, Chappell, & Samson, 1996; Samson, Czachowski, Chapell, & Legg, 2003). However, the use of sucrose/saccharine during acquisition raises a theoretical issue about what is responsible for ethanol self-administration. Ethanol responding may be supported through a Pavlovian motivational process that develops between the contextual cues present in the environment, and the previously experienced effects of sucrose. This is in contrast to suggestions that ethanol can act as an oral reinforcer through its pharmacological effects.

The assertion that ethanol's pharmacological effect is driving responding raises a second issue with ethanol self-administration as a model of ethanol reward and reinforcement. Generally, a fulfilled response requirement allows access to a small quantity of the reinforcer. Whether response requirements can be completed and reinforcer consumed in a pattern and time course that would lead to pharmacologically relevant levels of ethanol in the blood to have effects within the central nervous system, is a major issue in the interpretation of these studies (e.g., Samson & Hodge, 1996; Samson, Tolliver, Pfeffer, Sadeghi, & Haraguchi, 1988; Fidler et al., 2006). With longer time courses of responding and the slow rise of blood ethanol concentration, the reinforcement from ethanol's actions in the brain may be delayed, and because of the delay, may not control associative relationships with cues and behaviors that initiated responding. It may be that other motivational or sensory (taste) processes support the level of responding observed. While most of the studies presented below have attempted to address these confounding issues of initiation and levels of ethanol oral self-administration responding in the examination of neural mechanisms underlying ethanol

reinforced behaviors, close examination of parameters should be used in interpreting the results.

Neurobiological mechanisms underlying the reinforcing effects of ethanol. Additional support for involvement of the VTA in ethanol's reinforcing effects was demonstrated in oral self-administration studies, where dopamine type-2 receptor (D2) antagonism within the VTA reduced responding (Hodge, Haraguchi, Erickson, & Samson, 1993). Further, modulation of $GABA_A$ receptors produced bidirectional effects depending on dose infused. Low doses of the $GABA_A$ agonist muscimol prolonged responding, while a high dose lead to an early termination of ethanol responding (Samson & Hodge, 1996). Opioid systems within the VTA have also been implicated in ethanol reward, with intra-VTA opioid antagonist administration decreasing responding for ethanol (June, Cumming, Eiler, Fostan, McKay, Seyaum, Garcia, McLow, Grey, Hawkins, & Mason, 2004). These findings suggest that modulation of the VTA and its efferent connections are involved in governing behaviors seen in self-administration procedures.

This hypothesis was further supported by studies demonstrating that downstream VTA dopamine release and modulation of the Acb were involved in supporting self-administration behavior. Microinjections into the Acb of dopamine type 1 (D1) and type 2 (D2) receptor antagonist flupenthixol attenuated ethanol self-administration (Rassnick, Pulvirenti, & Koob, 1992), while non-selective dopamine agonists increased responding for ethanol (Hodge, Samson, & Haraguchi, 1992; Samson, Hodge, Tolliver, & Haraguchi, 1993). Selective D1 and D2 receptor antagonists SCH 23390 and raclopride demonstrated that the reductions in ethanol responding were due to effects at both receptor types

(Hodge, Samson, & Chappelle, 1997, Samson et al., 1993). However, dopamine is not the sole mediator of activity within the Acb that regulates ethanol self-administration behavior. The N-methyl-D-aspartate (NMDA) receptor competitive antagonist 2-amino-5-phosphopentoanoic acid (AP-5) (Rassnick et al., 1992) or various opioid antagonists (Froehlich, O'Malley, Hyytiä, Davidson, & Farren, 2003; June et al., 2004) also reduce responding for ethanol when infused into the Acb.

Of particular interest given numerous reports of Amy and cortical involvement in appetitive conditioning (e.g., Holland & Gallagher, 1999, 2004), limbic areas and ventral striatum are not the only areas that have been shown to modulate ethanol self-administration behavior. Specifically, within the CE, multiple neurotransmitter systems have been implicated in modulating responding. Initial experiments showed intra-CE infusions of the $GABA_A$ antagonist SR 95531 reduced responding for ethanol (Hyytia & Koob, 1995). Further support was provided for the involvement of GABA systems in ethanol self-administration when infusions of the $GABA_A$ agonist muscimol into the CE decreased responding in rats subjected to a chronic ethanol exposure paradigm (Roberts, Cole, & Koob, 1996). In addition to $GABA_A$ receptors, findings suggest serotonin receptors are involved, since a $5\text{-}HT_3$ receptor antagonist infused into the CE also decreased moderate responding for ethanol (Dyr & Kostowski, 1995). Recent work by Funk et al., (2006) has also implicated corticotropin releasing factor (CRF) receptor activation within the CE in the modulation of ethanol self-administration. By combining site-specific neural manipulations with an ethanol or air vapor exposure paradigm, the authors were able to assess contributions of the CE CRF system to ethanol self-administration in dependent versus non-dependent rats. CRF non-specific type receptor

antagonist administered into the CE reduced ethanol reinforced responding in withdrawn ethanol-dependent rats, but had no effect in non-dependent rats (Funk et al., 2006). Further, infusion of the selective CRF 2 receptor agonist urocortin 3 decreased ethanol self-administration in withdrawn ethanol dependent rats, while increasing responding in non-dependent rats (Funk & Koob, 2007). Together, these findings suggest that the CE CRF system influences dependence driven ethanol self-administration. However, GABA and serotonin receptor regulation of ethanol self-administration in dependent versus non-dependent animals have not been examined, leaving open the question as whether the CE is a critical area for the roles played by these neurotransmitters in dependence-induced alterations in ethanol reinforcement.

In addition to Amy systems and Acb systems, evidence suggests dopamine modulation of the prefrontal cortex (PFC) arising from VTA projections plays a role in ethanol self-administration. Raclopride (D2 receptor antagonist) injected into the PFC decreased responding for ethanol, while PFC D2 type receptor agonism with quinpirole delayed responding for ethanol (Hodge et al., 1996). In addition to dopamine, GABAergic transmission in the PFC has been implicated in the rewarding effects of ethanol. Muscimol ($GABA_A$ agonist) injected into the PFC decreased ethanol self-administration (Samson & Chappell, 2001), suggesting a role for GABA as well as dopamine in supporting the direct reinforcing effects of ethanol.

Neurobiological mechanisms involved in ethanol-seeking behaviors: use of appetitive and extinction responding. While the studies discussed in the preceding sections examined the neural areas and mechanisms thought to influence the maintenance of ethanol self-administration, it is difficult to separate the neural mechanisms involved

in reinforcer seeking (appetitive) from those influencing reinforcer taking (consummatory) behaviors. In ethanol self-administration, initial responding is performed in the absence of any ethanol effects. However, subsequent responding after the first reinforced work requirement is performed under the influence of ethanol's US effects. For example, ethanol consumption could influence later seeking behaviors within the same session through satiation, taste, or pharmacological processes (for discussion see: Czachowski, Santini, Legg, & Samson, 2002). To bypass this issue and isolate reinforcer-seeking, from reinforcer-taking processes in self-administration procedures, Samson and colleagues procedurally separated appetitive and consummatory responding by having a fulfilled work requirement lead to unrestricted access to a sipper tube within each session (Czachowski & Samson, 1999; Samson, Slawecki Sharpe, & Chappell, 1998). Intra-cranial infusions are performed before the start of the test session, and one can examine infusion-induced effects on the initial work requirement (appetitive responding) separately from infusion effects on ethanol drinking (consummatory behavior). Using this procedure, the authors found that blockade of D2 receptors by intra-Acb infusions of raclopride significantly reduced the onset and total number of appetitive responses for ethanol without effecting ethanol consumption onset, and had only slight effects on total ethanol consumption (Czachowski, Chappell, & Samson, 2001). It was hypothesized that D2 receptors within the Acb were involved in the stimulus processing that initiates ethanol-seeking behavior. Additional evidence implicated the Acb in ethanol seeking behaviors, since intra-Acb infusions of a serotonin 1B agonist, but not serotonin 1A agonist, decreased appetitive ethanol responding before the onset of ethanol consumption (Czachowski, 2005).

However, a problem with the interpretation of the findings of Czachowski et al. (2001) that Acb D2 receptors influence ethanol-seeking behaviors arose because in intra-Acb raclopride sessions, responding was reinforced with ethanol. Even though only small effects on ethanol drinking were observed, the reduction in ethanol-seeking observed could have been due to raclopride's effect on ethanol's stimulus properties experienced post consumption (i.e., decreased the reinforcing effect of ethanol), that in turn reduced seeking behavior in later sessions. To circumvent this problem, Samson and Chappell (2004) used extinction trials to examine the effects of raclopride on ethanol-seeking behavior. During extinction trials, fulfillment of the work requirement did not result in access to ethanol, hence they avoided the possibility that raclopride affected post-consumatory stimulus effects. By interspersing an extinction trial between days of reinforced responding, they found that intra-Acb D2 antagonism with raclopride did decrease ethanol-seeking behaviors, without affecting responding in the absence of the antagonist (Samson & Chappell, 2004). An alternative interpretation suggests that raclopride may have served as an inhibitory discriminative stimulus which reduced responding, since raclopride was only given in the absence of reinforcement. However, the same pattern of effects were observed during the first raclopride-infusion trial, suggesting that behavior displayed was due to Acb raclopride D2 receptor blockade effects on ethanol-seeking behavior. This finding in combination with those presented above, suggests that at least aspects of ethanol-seeking behavior as measured in an ethanol self-administration procedure depend upon D2 receptors within the Acb.

Use of cue-induced reinstatement to examine neurobiological mechanisms of ethanol-seeking behaviors. After responding in ethanol self-administration has been

extinguished by removing ethanol reinforcement, reinstatement of ethanol-seeking behaviors has been induced by ethanol-predictive stimuli (e.g., Katner, Magalong, & Weiss, 1999; Katner & Weiss, 1999), stress, and ethanol itself (e.g., Lê, Quan, Juzytch, Fletcher, Joharchi, & Shaham, 1998). The following section will focus solely on the neural areas and mechanisms implicated in cue-induced reinstatement of ethanol self-administration. Cue-induced reinstatement procedures generally use a discriminative stimulus which serves to set the occasion for drug reinforcement, and also identifies contexts where responding will produce reinforcement. Generally, in these procedures, a discriminative stimulus (e.g., light, tone, odor) signals availability of ethanol following lever responses (S^+), while a different discriminative stimulus signals that lever presses either have no consequence or will produce a water solution (S^-). After initiation of ethanol self-administration and discriminative stimulus conditioning, extinction is performed where responses on the ethanol lever in the absence of the S^+ do not produce ethanol. With cessation of responding on the ethanol-associated lever, the discriminative stimulus is presented and responding is measured.

Using discriminative stimulus-induced reinstatement procedures, researchers have investigated neural areas activated by a S^+ presentation (Dayas, Simms, & Weiss, 2007; Radwanska, Wrobel, Korkosz, Rogowski, Kostowski, Bienowskim & Kaczmarek, 2007; Zhao, Dayas, Aufila, Baptista, Martin-Fardon, & Weiss, 2006). The neural areas activated by a S^+ in comparison to extinction level responding are listed in Table 1. In summary, studies that used an S^+ have shown cFos activation (a neuronal marker of early transcription factor activation) within areas of the cortices, hippocampus, regions of the Amy, ventral striatum, bed nucleus stria terminalis (BST), VTA, and paraventricular

nucleus (PVN) (Dayas et al., 2007; Radwanska et al., 2008; Zhao et al., 2007). Further, treatment with naloxone, a putative theraputic broad opioid receptor antagonist used in the treatment of alcoholism (e.g., O'Malley, Jaffe, Chang, Schottenfield, Meyer, & Rounsaville, 1992; Volpicelli, Alterman, Hayashida, & O'Brien, 1992), that decreased S^+ induced reinstatement responding, also decreased this cue-induced cFos activation within regions of the hippocampus (CA1, CA3), striatum (AcbSh and AcbC), and PVN (Dayas et al., 2007), implicating these areas in cue-induced reinstated responding. However, treatment with naloxone had mixed effects, increasing cFos activation within the infra-limbic cortices (Dayas et al., 2007), suggesting that cue-induced ethanol-seeking behaviors may induce inhibition of activity within certain areas of the brain.

Table 1: *Effect of S^+ induced reinstatement of ethanol self-administration on cFos activation*

Neural areas activated by ethanol S^+	S^+ modality	treatment	effect on cFos activation	reference
cortical areas				
mPFC	odor	-	-	Zhao et al., 2006
Pre-L	odor	-	-	
Infra-L	odor	NTX	NTX↑ S^+ compared to NTX S^-	Dayas et al., 2007
Ac	odor	-	-	
hippocampus				
CA1	odor	LY379268	LY379268↓ S^+ compared to VEH S^+	Zhao et al., 2006
		NTX	NTX↓ S^+ compared to NTX S^- and NTX controls	Dayas et al., 2007
CA3	odor		NTX↓ S^+ compared to NTX S^- and VEH S^{D+}	
DG	odor	LY379268	LY379268↓ S^+ compared to VEH S^+	Zhao et al., 2006
amygdala				
BLA	odor	-	-	Zhao et al., 2006
	light/noise	-	-	Radwanska et al., 2007
CE	odor	LY379286	LY379268↑ S^+ compared to VEH S^+ /extinguished controls	Zhao et al., 2006
	light/noise	-	-	Radwanska et al., 2007
limbic and striatum				
VTA	odor	-	-	Zhao et al., 2006
AcbC	odor		-	
		NTX	NTX↓ S^+ compared to NTX S^-	Dayas et al., 2007
AcbSh	odor		NTX↓ S^+ compared to NTX S^-	
	odor	-	-	Zhao et al., 2006
BST	odor	LY379268	LY379268↑ S^{D+} compared to VEH S^{D+}	
PVN	odor	NTX	NTX↓	Dayas et al., 2007
		LY379268	LY379268↑ S^+ compared to VEH S^+ and extinguished controls	Zhao et al., 2006

(NTX)- Naltrexone, a nonselective opioid antagonist; LY379268, selective mGluR2/3agonist
↑ = increase in cFos activation observed, ↓ = decrease in cFos activation observed, - = no effect

Another set of studies investigated the effects of the group II metabotropic glutamate receptor antagonist LY379268 on S^+ induced reinstated responding for ethanol and on cFos activation within the brain (Zhao et al., 2007). This compound was used given the strategic location of group II metabotropic receptor expression within the limbic cortical ventral striatopallidal circuit (Ohsishi et al., 1993[a]; 1993[b]). Antagonist treatment dose-dependently reduced S^+ induced ethanol reinstatement, as well as decreased cFos activation within the CA1 and DG (dentate gyrus) regions of the hippocampus. However, like naloxone, LY379268 also increased cFos activation in some neural areas. In this study, the antagonist blocked S^+ induced inhibition of the CE, BST, and PVN, implicating group II metabotropic receptors in inhibiting activity within these areas during cue-induced reinstatement.

Interpretational issues with findings from neural activation and self-administration findings. Although activation studies have implicated the involvement of particular neural areas within the limbic cortical ventral striatopallidal circuit in cue-elicited ethanol seeking behaviors, they offer no information on the functional contribution of these brain areas. Further, the use of self-administration often confounds interpretation of differences between conditioned cue effects, versus instrumental effects in the neural activation observed. Since activation was examined after reinstated responding, the effects of cue-induced increases or decreases in cFos following exposure to an ethanol S^+ could be due to effects of increases or decreases in instrumental responding and not specific to the conditioned cue.

While the behavior underlying self-administration models may be classified as involving both drug-seeking and drug-taking behavior (e.g., Self, 2004), understanding

the dissociation of these two behaviors and the neural mechanisms underlying each is difficult (Bardo & Bevins, 2001). The drug-seeking behavior observed in self-administration may be under control of discriminative stimuli (e.g., insertion of the lever into the operant chamber) that have been used to signal the availability of the drug. However, as discussed in the preceding section, dissociation of mechanisms regulating seeking behaviors using ethanol self-administration procedures is difficult, since the measured response has also been the action the subject performs to obtain the outcome. For example, in Samson et al. (2004) the decreased responding observed during extinction trials could have been due to raclopride's non-specific effects on conditioned contextual or instrumental responses, and not on the motivational processes driving ethanol-seeking behavior.

Use of CPP to examine neural mechanisms underlying cue effects on ethanol-seeking behaviors.

The CPP procedure allows for investigation into the acquisition of an association between the drug's rewarding effect and the predictive cue, as well as the expression behavior that is elicited when the subject encounters the previous drug-paired cue. While acquisition of CPP may very well offer a reflection of an US's hedonic properties (i.e. reward), it is also dependent on associative processes that guide learning about stimuli predictive of US effects (e.g., Bardo & Bevins, 2001; Cunningham, 1998). Acquisition of CPP may be altered if the subjects are unable to process the subjective effects (e.g., Font, Aragon, & Miquel, 2006), if the hedonic value of the US is altered, or if the learning processes recruited are either disrupted or enhanced (see Cunningham, Gremel, Grobleweski et al., 2006).

Additionally, the response observed during expression testing is not solely a result of the previously paired US's hedonic effects. The behavior measured is approach and maintenance of contact with the previously drug-paired cue, which is an indirect measure of US hedonic properties. Further dissection of the behavior observed during testing reveals multiple processes that may be controlling the measured responding and multiple possible explanations for disruptions in the measured behavior. First, the subject needs to retrieve the association between the drug and the conditioned stimulus based on sensory information in the environment. For instance, one could interfere with retrieval of a conditioned association by altering sensory input pathways (e.g., Bammer & Chesher, 1982). Secondly, the ability of the retrieved association to motivate behavior needs to be assessed. Thirdly, if the association is qualified as motivationally sufficient it may direct new learning that allows the subject to gain access to the cue. For example, previous studies have altered the underlying motivational value of the retrieved association (e.g., Cunningham, Henderson, & Bormann, 1998) or interfered with the subject's ability to learn the appropriate response (e.g., Cole & McNally, 2007). Lastly, a subject must be able to perform for behavior to be measured. We have previously observed that interference with the performance of the approach and maintenance behavior by significantly altering the locomotor activity in a subject could alter the magnitude of preference expressed (see Gremel & Cunningham, 2007).

The following sections will focus on possible neural mechanisms examined via pharmacological manipulations in the acquisition and expression of ethanol CPP in mice. Findings from studies using knockout mice to examine neural mechanisms of conditioned reward that offer information relevant to the hypotheses of this thesis have also been

included (for review see; Cunningham & Phillips, 2003). However, data from rat studies reporting ethanol CPP in rats have been excluded. Multiple studies have demonstrated substantial difficulty in establishing a reliable ethanol CPP in rats (e.g., Fidler, Bakner, & Cunningham, 2004; Tzschentke, 2007), which may reflect a reduced sensitivity to ethanol's rewarding effect as indexed by this procedure (Cunningham, Niehus, & Noble, 1993). We generally interpret the reported display of significant ethanol CPP in rats as an alleviation of an aversive state (for discussion see; Fidler et al., 2004).

Table 2 Effects of pharmacological treatment during acquisition or expression on ethanol CPP in mice

Experiment & system	mechanism	treatment	dose mg/kg	outcome	reference
Acquisition					
opioid	nonselective opioid antagonist	naloxone	0.15, 1.5, 3.0, 10	-	Cunningham et al., 1995
	nonselective opioid antagonist	naloxone	0.1, 1.0	-	Kuzmin et al., 2003
	nonselective opioid antagonist	naloxone	10	↓ CPP	Kuzmin et al., 2003
	ORL1 agonist	Ro 64-6198	0.1, 0.3, 1.0	↓ CPP	
dopamine	D3 antagonist	U-99194A	20	↑ CPP	Boyce & Risinger, 2000, 2002
	D2, D3, D4 antagonist	haloperidol	0.1	-	Risinger et al., 1992
	D4 antagonist	clozapine	1	-	Thrasher et al., 1999
GABA	$GABA_A$ antagonist	picrotoxin	2	↑ CPP	Chester & Cunningham, 1999[b]
	$GABA_A$ antagonist	bicuculline	1	↑CPP	
		bicuculline	3, 5	-	
	$GABA_B$ agonist	baclofen	2.5, 5, 7.5	-	Chester & Cunningham, 1999[a]
	$GABA_A$ inverse agonist	Ro15-4513	3	-	Risinger et al., 1992
Glutamate	NMDA glutamate binding site antagonist	CGP-37849	5	-	Boyce-Rustay & Cunningham, 2004
		CGP-37849	10, 15	↓ CPP	
	NMDA channel blocker	MK-801	0.05, 0.2	-	
	NMDA channel blocker	ketamine	5, 10, 20	-	
	NMDA NR2B subunit antagonist	ifenprodil	5, 10, 20	-	
	NMDA NR2B subunit antagonist	CP-101,606	5, 10, 25	-	
	NMDA $Glycine_B$ binding site antagonist	(+)-HA966	5, 15, 30	-	
	mGluR5 glutamate receptor antagonist	MPEP	1, 5, 20	-	McGeehan & Olive, 2003[a]
	NMDA/mGluR5 glutamate receptor antagonist	acamprosate	30, 100	-	McGeehan & Olive, 2003[b]
		acamprosate	300	↓ CPP	
steroids	neurosteroid	allopregnanol-one	3.2, 10, 17	-	Gabriel et al., 2004
	5α-reductase inhibitor	finasteride	50, 100	-	
	steroid synthesis inhibitor	aminoglutethi-mide	50	-	Chester & Cunningham, 1998
Other	5-HT1A antagonist	pindobind-5HT1A	2.5	↑CPP	Risinger & Boyce, 2002

Table 2 cont.

Experiment & system	mechanism	treatment	dose mg/kg	outcome	reference
	σ1-antagonist	BD1047	3	-	Maurice et al., 2003
		BD1047	10	↓ CPP	
	σ1-agonist	PRE-084	1	-	
		PRE-084	3	↑ CPP	
	nNOS inhibitor	7-nitroindazole	25	↓ CPP	Itzhak & Martin, 2000
	acetylaldehyde inactivator	D-penicillamine	50, 75	↓ CPP	Font et al., 2006
	catalase inhibitor	3-amino-1,2,4-triazole	1000	↓ CPP	Font et al., 2007
	anti-convulsant	topiramate	5, 10, 20, 50	-	Gremel et al., 2006
Expression					
opioid	nonselective opioid antagonist	naloxone	0.15	-	Cunningham et al., 1995
		naloxone	1.5, 3.0, 10	↓ CPP	
	nonselective opioid antagonist	naloxone	10	↓ CPP last 30	Cunningham et al., 1998
	nonselective opioid antagonist	naloxone	0.1 1,10	- ↓ CPP	Kuzmin et al., 2003
	nonselective opioid antagonist	naltrexone	0.3, 3 0.6, 1	- ↓ CPP	Middaugh & Bandy, 2000
	ORL1 agonist	Ro 64-6198	0.1, 0.3, 1	↓ CPP	
dopamine	D2, D3, D4 antagonist	haloperidol	0.5, 1.0	-	Cunningham et al., 1992
	D2 antagonist	Raclopride	0.3, 0.6	-	Dickinson et al., 2003
	D3 antagonist	U99194A	10, 20	-	
	D1 antagonist	SCH23390	0.015, 0.03	-	
glutamate	mGluR5 glutamate receptor antagonist	MPEP	10	↓ CPP	Lominac et al., 2006
	mGluR1 antagonist	CPCC)Et	10	↓ CPP	
other	neurosteroid	allopregnanol-one	3.2, 10, 17	-	Gabriel et al., 2004
	steroid hormone synthesis inhibitor	aminoglutethi-mide	50	-	Chester & Cunningham, 1998
	anti-convulsant	topiramate	10, 50, 100	-	Gremel et al., 2006
	aversive agent	lithium cholride	(3 mEq/kg)	-	Cunningham et al., 1998
	ethanol	ethanol	2000	↓ CPP in DBA/2J mice, -in NZBB1NJ mice	Gremel & Cunningham, 2006

↓ = decrease in CPP, ↑ = enhanced CPP, - = no effect

Neural mechanisms underlying acquisition of ethanol CPP. Investigations into contributions of various receptor and neuromodulatory systems in the acquisition of ethanol CPP have examined opioid, dopamine, GABA, glutamate, and neurosteroid receptor systems as well as other drug treatments (see Table 2 for summary of acquisition ethanol CPP experiments) (for review see Tzschentke, 2007). Generally, blockade of opioid receptors during conditioning (i.e., antagonist given before CS+ trials) with low doses of naloxone (a non-selective broad opioid receptor antagonist) has not been effective at disrupting acquisition of ethanol CPP (Cunningham, Dickinson, & Okorn, 1995; Kuzmin, Sandin, Terenius, & Ogren, 2003). However, mixed effects of high naloxone doses have been observed. In one study, a naloxone dose of 10 mg/kg did not attenuate acquisition of ethanol CPP (Cunningham et al., 1995), while in a separate study a significant CPA was observed when a high dose of naloxone (10 mg/kg) was co-administered with ethanol (Kuzmin et al., 2003). In both studies, naloxone (10 mg/kg) given alone as the US resulted in CPA (Cunningham et al., 1995; Kuzmin et al., 2003). These findings suggest that while the opioid system is not involved in the acquisition of an ethanol CPP, blockade of opioid receptors may produce a conditioned aversion independent of ethanol's effects. Although opioid receptors do not seem to be involved, blockade of the related nociceptin receptor ORL1 by Ro 64-6198 did block the acquisition of ethanol CPP (Kuzmin et al., 2003), suggesting a possible role for the nociceptin system in the conditioned effects of ethanol.

Of particular interest to the current thesis, systemic investigations into the neuromechanisms underlying acquisition of ethanol CPP in mice have shown mixed effects of dopamine receptor antagonism depending upon the receptor subtype blocked.

For example, blockade of dopamine type 3 (D3) receptor with U-99194A enhanced the acquisition of ethanol CPP (Boyce & Risinger, 2000; 2002). In contrast, blockade of either the dopamine type 4 (D4) receptor with clozapine (Thrasher, Freeman, & Risinger, 1999) or the dopamine type 2 (D2)/D3/D4 receptors with haloperidol (Risinger, Dickinson, & Cunningham, 1992) had no effect on acquisition of ethanol CPP, suggesting that specific subtypes of dopamine receptors may be recruited during acquisition of cue-induced ethanol seeking behaviors.

Similar to effects observed with dopamine antagonists, GABA receptor antagonists had mixed effects depending on specific receptor subtypes. Blockade of $GABA_A$ receptors during conditioning trials with picrotoxin or bicuculline enhanced acquisition of ethanol CPP (Chester & Cunningham, 1999[b]), while activation of $GABA_B$ receptors with baclofen was without effect (Chester & Cunningham, 1999[a]). These findings suggest that $GABA_A$, not $GABA_B$ receptors, normally attenuate the acquisition of ethanol CPP. However, Ro 15-4513 (an inverse agonist at the benzodiazepine binding site of the $GABA_A$ receptor) was without effect on acquisition (Risinger, Malott, Riley, & Cunningham, 1992) suggesting that various binding sites between the different subunits of the $GABA_A$ receptor may differentially influence ethanol CPP.

Investigations into the contributions of the glutamatergic receptor system to the acquisition of ethanol CPP have implicated both ionotropic and metabotropic glutamate receptors. In a series of experiments examining the role of the NMDA receptor, Boyce-Rustay and Cunningham (2004) found that blockade of the glutamate binding site, but not blockade of the channel, NR2B subunit, or glycine binding site, disrupted acquisition of ethanol CPP. Most likely the observed effect was due to a disruption in the subjects'

ability to learn the association and not a result of a decrease in ethanol reward, since blockade of the glutamate binding site with CGP-37849 also blocked the development of ethanol- and lithium chloride (LiCl)-induced CPA (Boyce-Rustay & Cunningham, 2004). Additional evidence suggests knockout mice that lack the NR2A subunit of the NMDA receptor do not condition an ethanol CPP (Boyce-Rustay & Holmes, 2006). Further, acamprosate, a NMDA and mGluR5 antagonist, blocked CPP (McGeehan & Olive, 2003[b]), suggesting a possible role for mGluR5 receptors. While it appears the NMDA receptor is involved in acquisition, the little evidence available suggests that the metabotropic receptor mGluR5 is not involved since blockade with MPEP was without effect (McGeehan & Olive, 2003[a]). However, the role of other metabotropic glutamate receptors in the acquisition of ethanol CPP is unknown.

Given the similarities between ethanol's acute actions, and those of the neurosteroid allopregnanolone (for review see Purdy, Valenzuela, Janak, Finn, Biggio, & Backstrom, 2005), it was hypothesized that neurosteroids might modulate the acquisition of ethanol CPP. However, in a series of studies, allopregnanolone itself had no effects on the development of ethanol CPP, and conditioning trial pretreatment with the allopregnanolone synthesis inhibitor finasteride did not alter acquisition of ethanol CPP (Gabriel, Cunningham, & Finn, 2004). To examine the contributions of another steroid, corticosterone, the steroid synthesis inhibitor aminoglutethimide was administered immediately before conditioning trials (Chester & Cunningham, 1998). The lack of effects on ethanol CPP by the synthesis inhibitor suggested that the neural processes regulating the acquisition of an ethanol place preference are not mediated by steroids.

Lastly, a number of studies have investigated a variety of possible neural mechanisms that may contribute to acquisition of ethanol CPP. It appears that the serotonin system is involved, since the 5HT1A receptor antagonist Pindobind enhanced acquisition (Risinger & Boyce, 2002). Additionally, the sigma 1 receptor, suggested to be a potent modulator of other neurotransmitter systems by affecting intracellular second messenger signaling systems (Hayashi, Maurice, & Su, 2000), has been implicated in acquisition, with a high dose of the agonist PRE-084 enhancing—while the antagonist BD1047 blocks ethanol CPP (Maurice, Casalino, Lacroix, & Romieu, 2003). Further, blockade of either nitrous oxide synthase, acetaldehyde (metabolite of ethanol), or disruption of ethanol metabolism by the catalase inhibitor 3-amino-1,2,4-triazole, disrupts acquisition of ethanol CPP (Itzhak & Martin, 2000; Font, Aragon Miquel, 2006; Font, Miquel, & Aragon, 2007). Lastly, topiramate, a potential therapeutic agent in the treatment of alcoholism (Johnson, Ait-Daoud, Bowden, DiClemente, Roache, Lawson, Javors, & Ma, 2003), was found to be ineffective at reducing or disrupting the acquisition of cue-induced ethanol-seeking behavior, since administration did not alter acquisition of ethanol CPP (Gremel, Gabriel, & Cunningham, 2006).

Possible neural mechanisms underlying expression of ethanol CPP. Investigation into the neural mechanisms underlying expression of ethanol CPP may offer insight into developing pharmacotherapies and strategies aimed at reducing cue-induced ethanol-seeking behaviors (see Table 2 for summary of expression ethanol CPP experiments). In contrast to the acquisition, the expression of ethanol CPP does depend upon opioid receptor activation (e.g., Cunningham et al., 1995, 1998; Kuzmin et al., 2003; Middaugh & Bandy, 2000). The opioid receptor system appears to modulate the motivation guiding

the observed behaviors in expression of ethanol CPP (e.g., Cunningham et al., 1995; 1998, Kuzmin et al., 2003, Middaugh & Bandy, 2000). A clear example of this effect is observed when naloxone is given before test sessions. Although initially naloxone-treated subjects displayed a preference similar in magnitude to that of controls, that preference extinguished over the duration of the first test (Cunningham et al., 1995; 1998). Further, Cunningham and colleagues (1998) demonstrated that during subsequent naloxone-pretreatment tests, subjects displayed a significant CPA.

Although naloxone can condition a CPA when used as the unconditioned stimulus (Cunningham et al., 1995; Kuzmin et al, 2003), the effect observed with treatment before expression testing was not due to direct conditioning of an aversive effect by naloxone (i.e., counter conditioning), since a lithium chloride group (an aversive unconditioned stimulus) displayed a similar level of preference compared to that of controls (Cunningham et al., 1998). Nor can this effect be attributed to an increase in the rate of extinction learning (i.e., facilitated extinction), since the original preference did not simply extinguish, but instead developed into an aversion. Instead, Cunningham et al. (1998) hypothesized that opioid receptors mediate the underlying motivation produced by the retrieved associated between the cue and ethanol's effects. Further, by altering that motivation, naloxone reduced expression of cue-induced ethanol-seeking behavior. While the opioid system appears to play a major role in modulated expression of ethanol CPP, similar to acquisition studies, the nociceptin and the ORL 1 agonist Ro 64-6198 also blocked expression (Kuzmin et al., 2003) suggesting that in general opioid and opioid-like systems are involved in the expression of cue-induced ethanol-seeking behaviors.

Given that ethanol seeking and taking behaviors, as well as mechanisms underlying other drug-seeking behaviors, have implicated the dopamine system (Koob & Le Moal, 2006; Hodge & Samson, 1996), previous findings suggesting that dopamine receptors are not involved in expression of cue-induced ethanol-seeking behaviors are surprising. Using CPP as a model of ethanol-seeking behavior in mice, antagonism of D2/D3/D4 type receptors with haloperidol did not alter expression of preference (Cunningham, Malott, Dickinson, & Risinger, 1992). This lack of involvement of dopamine autoreceptors was again observed with the use of more selective D2 and D3 receptor subtype antagonists, raclopride and U99194A respectively (Dickinson, Lee, Rindal, & Cunningham, 2003). Further, when post-synaptic dopamine receptors were investigated, with the use of SCH23390, D1 receptor blockade had no effect on expression of ethanol CPP (Dickinson et al., 2003). However, some contradictory evidence does come from the use of knockout mice. Both D2 (Cunningham, Howard, Gill, Rubinstein, Low, & Grandy, 2001) and DARP-32 (dopamine and adenosine 3',5'-monophosphate-regulated phosphoprotein, 32 kDa) knockouts displayed a reduced ethanol CPP (Risinger, Freeman, Greengard, & Fienberg, 2001). However, as noted earlier, these gene deletions were present at conception, so compensational changes could possibly explain the differences between pharmacological and knockout manipulations.

Even though glutamatergic systems, specifically NMDA receptors, have been implicated in learning, very few studies have examined the contributions of glutamatergic mechanisms to the learning and performance of ethanol CPP. Those studies focused on the contributions of metabotropic receptors mGluR5 and mGluR1. Administration of MPEP (mGluR5 receptor antagonist) or CPCCOEt (mGluR1 receptor antagonist)

blocked expression of ethanol CPP (Lominac, Kapasova, Hannun, Patterson, Middaugh, & Szumlinski, 2006). However, in contrast to acquisition investigations (Boyce-Rustay & Cunningham, 2004), the contribution of the NMDA receptor to expression is unknown.

While there have not been systemic investigations into the role of GABA, or serotonin receptor systems in expression of CPP, a variety of other possible neural mechanisms have been examined. In keeping with the lack of effects observed during acquisition manipulations, neither allopregnanolone ror the steroid hormone synthesis inhibitor aminoglutethimide had any effect on expression of ethanol CPP (Gabriel et al., 2004; Chester & Cunningham, 1998). Similarly, topiramate (anti-convulsant agent) had no effect on expression of ethanol CPP (Gremel et al., 2006). Taken together, data from systemic studies suggest that the opioid system, and perhaps glutamate receptors influence expression of cue-induced ethanol-seeking behaviors.

Neural areas underlying expression of ethanol CPP. Prior to this thesis (Gremel & Cunningham, 2008), no studies had examined the neural areas involved in acquisition of ethanol CPP. However, initial work investigating the specific neural areas underlying expression of ethanol CPP had begun (Hill, Ryabinin, & Cunningham, 2007; Bechtholt & Cunningham, 2005). Using a modified CPP procedure, mice received pairings of a distinctive tactile cue and ethanol. Instead of performing the normal expression test post-conditioning, mice were instead exposed to the CS+ in the absence or presence of ethanol and cFos activation within the limbic cortical ventral striatopallidal circuit was examined (Hill et al., 2007). Mice given repeated ethanol prior to CS+ exposure displayed greater locomotor activity when presented with the CS+ alone than did mice given saline on CS+ trials, suggesting that the cue was able to elicit a conditioned response. cFos activation

after CS+ alone exposure was significantly higher in the hippocampus (CA1 and CA3), BST, and VTA than that observed in mice given saline on CS+ trials (unpaired group), or in mice treated with ethanol prior to testing. Although cFos activation was measured after CS+ exposure in this study and did not capture changes in cFos that may occur during an expression test in CPP, the findings do provide information that structures within the limbic cortical ventral striatopallidal circuit are correlated with a conditioned response elicited by an ethanol-paired cue association.

Based upon data suggesting that ethanol's conditioned (Hill et al., 2007) and reinforcing effects (e.g., Gatto et al., 1994; Rodd et al., 2004) are in part linked by mechanisms within the VTA, and opioid antagonists administered directly into the VTA (June et al., 2004) and Acb (Froehlich et al., 2003; Heyser, Roberts, Schulteis, & Koob, 1999; June et al., 2004) decrease responding for ethanol, the opioid antagonist methylnaloxonium was administered either into the VTA or Acb immediately before expression testing. While there was no effect of opioid receptor blockade within the Acb, intra-VTA methylnaloxonium dose-dependently reduced expression of ethanol CPP (Bechtholt & Cunningham, 2005). The authors hypothesized that the effect of methylnaloxonium in the VTA was to block opioid inhibition of GABA interneurons, hence allowing them to exert inhibitory actions on dopamine projection neurons. Based on this hypothesis, it was predicted that other manipulations within the VTA that would serve to decrease dopamine neuron activity would also decrease expression of ethanol CPP. Confirming this prediction, administration of baclofen, thought to exert inhibitory actions through $GABA_B$ receptors localized on dopamine projection neurons (Wirtshafter & Sheppard, 2001), blocked expression of ethanol CPP (Bechtholt & Cunningham,

2005). These findings suggest that opioid and $GABA_B$ receptors within the VTA influence expression of cue-elicited ethanol-seeking behavior.

These findings raise the possibility that expression depends upon intra-VTA dopamine neuron activation leading to downstream dopamine release and receptor activation. However, as discussed earlier, data from systemic studies do not suggest a role for dopamine receptors in this behavior (e.g., Dickinson et al., 2003). This contradiction may be explained by the idea of requisite synergistic interactions of D1 and D2 receptors (for review see Marshall, Rustein, &La Hoste, 1997) that may be required for full expression of ethanol CPP. Previous work has suggested that such an effect in the Acb enhances glutamate-induced activation in the striatum (Hu & White, 1997). However, an alternative explanation is that both D1 and D2 receptor subtypes mediate expression, and blockade of one receptor subtype is insufficient to produce effects on the behavior. Alternative explanations of dopamine's effects aside, findings suggesting VTA involvement in expression of ethanol CPP raise questions about the downstream neural areas and mechanisms recruited during ethanol CPP.

Possible neural mechanisms downstream from the VTA mediating expression of ethanol CPP.

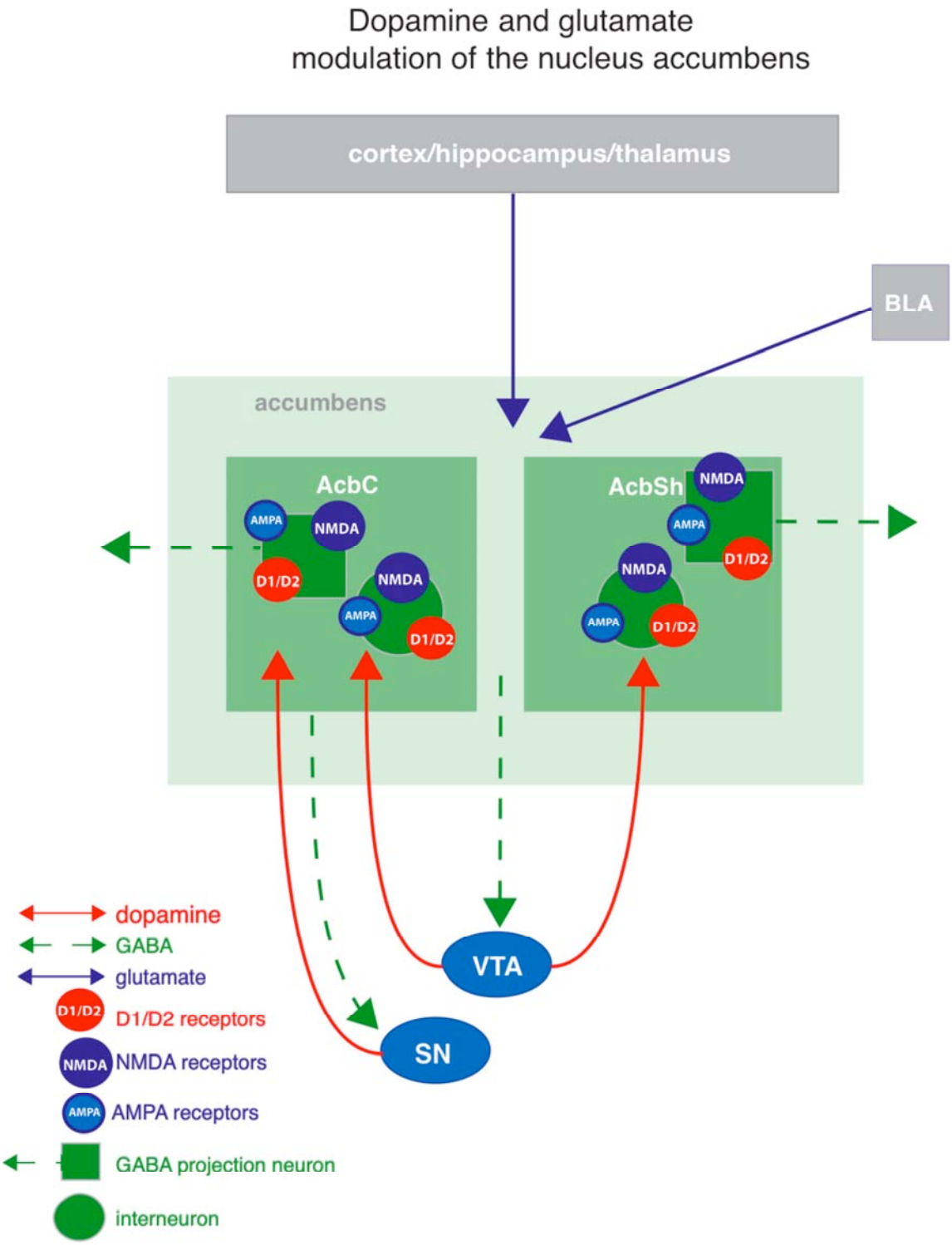
As illustrated in figure 1 and discussed earlier, the VTA sends projections, including dopamine, to several areas within the limbic cortical ventral striatopallidal circuit including the Acb, Amy, hippocampus, and various cortical areas (Ford et al., 2007; Gonzales et al., 2004; Swanson, 1982). The VTA also sends a large number of GABA projections to the PFC (Carr & Sesack, 2000) and a small number of GABA projections to the Acb (Van Bockstaele & Pickel, 1995). While the focus of this thesis is

on the contributions of the Acb and Amy to ethanol CPP, by no means should possible involvement of cortical areas or hippocampus be dismissed. This point should be heavily emphasized in light of data suggesting a role for the hippocampus in particular learning processes that may underlie CPP (e.g., White, Chai, & Hamdani, 2005) and contributions of specific cortical areas such as the anterior cingulate cortex and entorhinal cortex in appetitive conditioned behaviors (for review see Everitt & Robbins, 2005). The possible involvement of these areas will be considered further in the discussion. However, given the direction of this thesis, the following sections will concentrate on involvement and possible mechanisms within the Acb and Amy that could be mediating downstream effects of VTA dopamine projections. In general, the anatomy and physiology studies summarized below have been performed in rats unless otherwise noted. Given possible species differences, it is difficult to say whether the following pertains to mice. However, it will be noted when mice were used for a particular finding.

Nucleus accumbens: structure, dopamine, and glutamate modulation. The Acb can be subdivided into two morphologically distinct regions, the AcbSh and AcbC (see Figure 2) (e.g., Alheid & Heimer, 1998; Groenewegen et al., 1990). While the Acb receives dopaminergic input from the VTA and substanita nigra (e.g., Brog, Salyapongse, Deutch, & Zahm, 1993), distribution of dopamine inputs differs between the AcbC and AcbSh. While it appears that the AcbSh receives dopamine input from the VTA, dopamine input within the AcbC arises from both the VTA and substantia nigra (Brog et al., 1993). Both AcbC and AcbSh receive the majority of glutamatergic innervation from the cortex (e.g., Beckstead et al., 1979).

Figure 2 Diagram of dopamine and glutamate modulation of the nucleus accumbens. Connections between the ventral tegmantal area (VTA), substantia nigra (SN), cortical/hippocampus/thalamus areas, nucleus accumbens shell (AcbSh), nucleus accumbens core (AcbC), and basolateral nucleus of the amygdala (BLA) are represented via projection arrows. Dopamine projections are in solid red lines, while dashed green lines represent GABA projections, and glutamate projections are diagrammed in solid blue lines. GABA projection neurons (green squares) and interneurons (green circles) are shown, as well as the localization of D1/D2 dopamine receptors (red circles), NMDA receptors (dark blue circles), and AMPA receptors (light blue circles).

Figure 2


Dopamine and glutamate
modulation of the nucleus accumbens
cortex/hippocampus/thalamus
BLA
accumbens
AcbC
AcbSh
AMPA
NMDA
D1/D2
VTA
SN
dopamine
GABA
glutamate
D1/D2 receptors
NMDA receptors
AMPA receptors
GABA projection neuron
interneuron

However, the AcbSh and AcbC also receive glutamate projections from the Amy (e.g., Kelley, Domesick, & Nauta, 1982), thalamus (Kawaguchi, Wilson, Augood, & Emson, 1995) and hippocampus (e.g., Groenewegen, Wright, & Beijer, 1996). Acb efferents (mainly GABAergic in nature) also differ between the Acb subregions, with the AcbSh projecting to subcortical limbic areas including the lateral hypothalamus, VTA, ventro-medial ventral pallidum, and brainstem autonomic centers while the AcbC projects to classic basal ganglia output structures such as the ventral pallidum, subthalamic nucleus, and substantia nigra (for review see Kelley, 2004).

Acb afferents synapse onto medium spiny neurons (MSN), which are the principle cell type (90%) in the Acb and use GABA as their primary neurotransmitter (e.g., Meredith, Farrell, Kelleghan, Tan, Zahm, & Totterdell, 1999). The Acb also contains local circuit neurons that produce acetylcholine (Hussain, Johnson, & Totterdell, 1996), although both cell types also contain various peptides including the opioid peptides (e.g., Gerfen & Young, 1988). What is particularly interesting is that within the Acb, these different inputs may converge onto the same MSN, possibly allowing for integration of information from different neural areas (e.g., French & Totterdell, 2003). Previous findings investigating inputs on MSNs suggest that within the AcbC, prefrontal or Amy inputs converge on dopaminergic terminals, while in the AcbSh, hippocampal or Amy inputs converge with dopamine signals onto the same cell (Johnson, Aylward, Hussain, & Totterdell, 1994; Totterdell & Smith, 1989). Both NMDA and D1 and D2 receptors are seemingly distributed uniformly throughout the AcbC and AcbSh (for review see Meredith, 1999) and appear to be colocalized (Lu, Monteggia, & Wolf, 1999). Additionally, it appears that AMPA receptors are present on most neurons within the

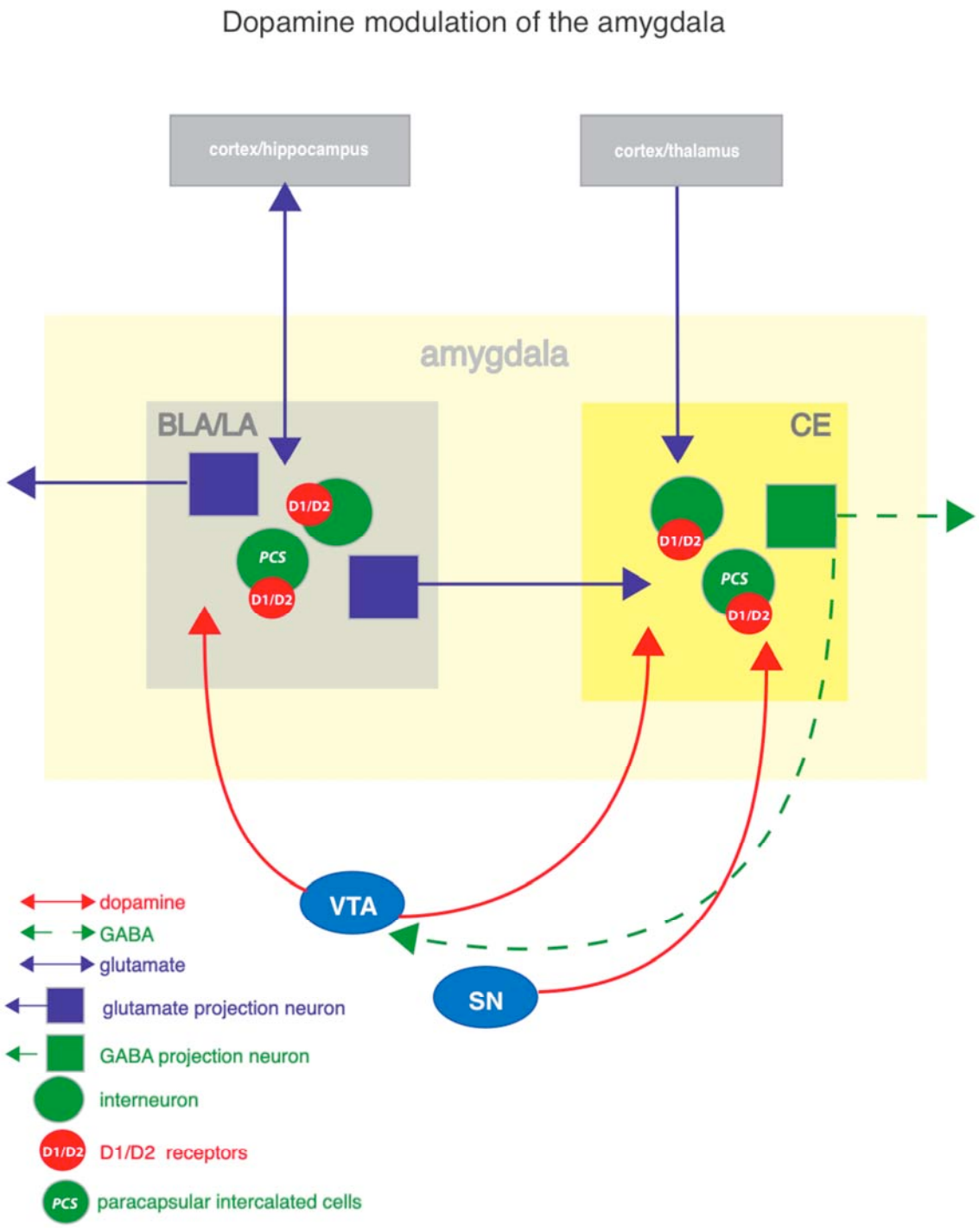
Acb, and overlap with the distribution of D1 and D2 receptors (Lu et al., 1999). While the role and distribution of NMDA receptors has been widely examined, little research has been done to examine the distribution of any other ionotropic glutamate receptor in the Acb. For example, it is not known whether AMPA receptors are differentially distributed between the AcbSh and AcbC.

Amygdala: structure and dopamine modulation. Swanson and Petrovich (1998), classified Amy areas by their structural and functional differences. Their grouping distributed Amy areas into four groups: autonomic, frontotemporal, main olfactory, and accessory olfactory systems. This viewpoint differs slightly from the traditional grouping (for review see: Sah, Faber, Armentia, Power, 2003), but does not include the proposed "extended amygdala" put forth by Alheid and Heminer (1988). Description of the Amy presented below was based upon a review of main Amy circuitry in the rat brain by Swanson and Petrovich (1998), but also similarly classified by Sah et al. (2003).

The four groupings within the Amy may be further classified on whether they contain GABAergic or glutamatergic projection neurons, with the idea that regions that contain the former are striatal and the latter cortical in nature (see Figure 3).

Figure 3 Diagram of dopamine modulation of the amgydala. Connections between the ventral tegmantal area (VTA), substantia nigra (SN), cortical/hippocampus/thalamus areas, central nucleus of the amgydala (CE), and basolateral nucleus of the amygdala (BLA) are represented via projection arrows. Dopamine projections are in solid red lines, while dashed green lines represent GABA projections, and glutamate projections are diagrammed in solid blue lines. Glutamate projection neurons (blue squares), GABA projection neurons (green squares), paracapsular intercalated cells (pcs) (green circles) and interneurons (green circles) are shown, as well as the localization of D1/D2 dopamine receptors (red circles).

Figure 3


Dopamine modulation of the amygdala
cortex/hippocampus
cortex/thalamus
amygdala
BLA/LA
CE
D1/D2
PCS
D1/D2
D1/D2
PCS
D1/D2
VTA
SN
dopamine
GABA
glutamate
glutamate projection neuron
GABA projection neuron
interneuron
D1/D2 D1/D2 receptors
PCS paracapsular intercalated cells

Beginning with the GABAergic projection areas, the autonomic group contains the CE and medial nucleus of the Amy, categorizing these areas as being more striatal in nature. However, the other areas, frontal temporal system and olfactory systems contain glutamatergic projection neurons. The BLA and lateral nucleus of the Amy generally comprise the frontal temporal system, while the BM, portions of the BLA, posterior nuclei of the Amy, piriform areas, and olfactory nucleus comprise the olfactory systems (main and accessory). While manipulations presented in chapter 1 of this thesis (Amy electrolytic lesions) may have affected all components of the Amy, the following sections focus more on the frontotemporal (BLA) and autonomic (CE) Amy systems given findings in this thesis and their hypothesized involvement in appetitive learning processes (Holland & Gallagher, 2004).

Cortical areas provide heavy glutamatergic input to the frontotemporal (BLA) system resulting in inhibition (e.g., Quirk, Likhit, Pelletier, & Paré, 2003), with both the BLA and lateral nucleus sharing bidirectional connections with the prefrontal and insular regions (McDonald, Mascagni, Guo, 1996), while the VTA provides dopaminergic innervation of the BLA (in DBA/2J mice, e.g., Ford et al., 2006). However, the lateral nucleus has connections with the temporal cortex and hippocampal regions (Swanson & Petrovich, 1998), while the BLA has connections with the somatosensory-motor areas in frontal and parietal lobes (Sripanidkulchai, Sripanidkulchai, & Wyss, 1984). Both the lateral and BLA also innervate the dorsal striatum and the Acb (e.g., Groenewegen eg al., 1996; Mascagni, McDonald, Coleman, 1993).

The BLA and lateral nucleus are generally comprised of two main groups of neurons, the glutamatergic projection neurons and the GABAergic interneurons (for

review see, Sah et al., 2003). While the BLA is under inhibitory control via cortical activation of the interneurons (in cat) (e.g., Lang & Paré, 1998), dopamine activation of the BLA lifts this inhibition (e.g., Rosenkranz & Grace, 1999). Of particular relevance to this dopamine-induced disinhibition, a separate population of interneurons was recently identified (e.g., Marowsky, Yanagawa, Obata, & Vogt, 2005). Known as paracapsular intercalated cells (pcs), they form a border around the BLA (between the lateral nuclei and BLA, and between the CE and BLA), and receive dopamine afferents from the VTA (e.g., Asan, 1998). These pcs are hypothesized to provide the gate that allows the dopamine-induced disinhibition of the Amy by suppressing the excitability of GABA interneurons via dopamine excitation of inhibitory pcs that synapse onto GABAergic interneurons (Marowsky et al., 2005).

The major cell type within the CE (autonomic system), which is very similar to the MSN found within the Acb (for review see Sah et al., 2003), sends projections down into the brainstem, as well as to the VTA, hypothalamus and periaquaductal gray (in cat) (Hopkins & Holstege, 1978). While the CE receives dopamine from the VTA and substantia nigra, it also receives a wide range of sensory information from cortical, thalamic, and brainstem inputs (for review see Swanson & Petrovich, 1998). In addition, the CE is modulated by inputs from the lateral nucleus and BLA (Pitkanen, Savanderm, & LeDoux, 1997) through pcs (Royer, Martina, & Paré, 1999; 2000).

While the above sections have focused on the BLA and CE, and dopamine input and modulation of each area, several other neurotransmitters and peptides are found in the BLA and CE. As reviewed by Sah et al. (2003), NMDA, AMPA, and kainate receptors, as well as metabotropic glutamate receptors are localized to projection neurons

within the BLA, while 5-HT and GABA $_{A,B}$ receptors appear to be localized to interneurons. Glutamatergic input to interneurons within the BLA is suggested to be moduled by AMPA, not NMDA receptors. Within the CE, peptides such as enkephaline, neurotensin, and corticotropin releasing hormone are found within interneurons and CE afferents. Further, opioid, AMPA, NMDA, and metabotropic glutamate receptors are observed within the CE, as well as members of the GABA receptor family (for review see, Sah et al., 2003).

Rationale

Given the paucity of information on the functional neural mechanisms controlling cue-induced ethanol-seeking behavior, the following studies used the CPP procedure to gain a further understanding of the processes involved in acquisition and expression of this behavior. The CPP procedure provides a way to directly investigate the neural mechanisms through which an ethanol-paired cue can gain control of ethanol seeking behaviors, since the behavior observed during expression is controlled by the retrieved memory of the ethanol-cue association. This is especially important since drug-predictive cues may be involved in maintaining seeking behaviors and triggering relapse after periods of abstinence or extinction (e.g., Volkow, Wang, Telang, Fowler, Logan, Childress, Jayne, Ma, & Wong, 2006, Weiss, 2005; Heinz, Siessmeier, Wrase, Hermann, Klein, Grüsser, Grüsser-Sinopoli, Flor, Braus, Buchholt, Gruiner, Schredchenberger, Smolka, Rösch, Mann, & Bartenstein, 2004; Grüsser et al., 2004), and understanding the neural mechanisms involved in expression of this control may offer new insights into developing effective pharmacotherapies and treatment strategies. Further, since very little is known about the neural areas involved in the acquisition of any ethanol-conditioned

behavior, the CPP procedure may be useful for examining areas that influence the acquisition of the cue-ethanol association.

The goal of this thesis was to identify specific nuclei and neural mechanisms involved in the acquisition and expression of ethanol CPP. Since initial findings suggested that expression of ethanol CPP involves activation of dopamine neurons within the VTA (Bechtholt & Cunningham, 2005), I hypothesized that ethanol CPP was dependent upon dopamine activation of the Amy and Acb. As discussed in the preceding sections, the Acb and Amy have been widely implicated in both reinforcing effects of ethanol and cue-induced seeking behaviors (see Everitt & Robbins, 2005; Day & Carelli, 2007). By combining site-specific manipulations of stereotaxically guided electrolytic lesions with the CPP procedure, I first examined the role of the Acb and Amy in the acquisition and expression of ethanol CPP (Chapter 2). Based on findings in Chapter 2 and reported neural mechanisms of cocaine conditioned reinforcement (Di Ciano & Everitt, 2001), I further hypothesized that expression of ethanol CPP was dependent upon dopamine modulation of the Amy and glutamate modulation of the Acb (Chapter 3). Chapter 3 investigates the role of dopamine and NMDA receptors in the Acb and dopamine receptors in the Amy via intra-cranial drug infusions given immediately before expression of ethanol CPP. Further, I investigated whether dopamine-induced activation of the Amy leading to NMDA receptor activation within the Acb was necessary for expression of ethanol CPP using a serial neuropharmacological disconnection procedure.

Chapter 2

Roles of the nucleus accumbens and amygdala in the acquisition and expression of ethanol-conditioned behavior in mice[1]

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1

Abstract

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Although progress has been made identifying the neural areas underlying the primary reinforcing effects of ethanol, few studies have examined the neural areas mediating ethanol-induced conditioned effects. Previous work using the CPP procedure implicates the VTA (Bechtholt & Cunningham, 2005), but the downstream neural areas modulating ethanol's conditioned rewarding effects have not been identified. While the Acb and Amy, which both receive dopamine innervation from the VTA, have been implicated in ethanol's primary reinforcing effects, the roles these areas play in ethanol-conditioned behaviors are unknown. In the present set of experiments, we use the CPP procedure along with selective electrolytic lesions to examine the neural areas underlying the acquisition and expression of ethanol conditioned behavior. In the acquisition experiment, male DBA/2J mice received bilateral lesions of the Acb or Amy before CPP training. In the expression experiments, mice received bilateral lesions of the Acb, AcbSh, AcbC, and Amy, or unilateral lesions of the Amy (Uni) after training but before testing. Lesions of the Acb and Amy before training disrupted acquisition and expression of ethanol CPP. However, when given after training, only lesions of the Amy disrupted expression, while lesions of the AcbC facilitated loss of responding, of ethanol CPP. For the first time, these results demonstrate the role of the Acb and Amy in the acquisition and expression of ethanol-induced conditioned reward.

Introduction

Ethanol-seeking behavior is strongly influenced by learning about the Pavlovian relationship between environmental cues and ethanol's rewarding or aversive effects. Such learning is important because it can alter physiological/behavioral responses, motivational states (e.g., craving) or expectancies that affect the probability, vigor or cue-directed nature of ethanol-seeking behavior (e.g., Corbit & Janak, 2007; Cunningham, 1994, 1998; Krank, 1989, 2003). Pavlovian learning may be especially involved in triggering relapse to ethanol seeking after periods of abstinence or extinction (e.g., Ciccocioppo et al., 2001, 2002, 2003; Dayas et al., 2007; Katner & Weiss, 1999; Krank & Wall, 1990; Nie & Janak, 2003; Zironi, Burattine, Aicardi, & Janak, 2006). Despite the prevalence of alcohol abuse and dependence, surprisingly little is known about the neural areas that mediate the acquisition and expression of ethanol-seeking behaviors.

Substantial work has been done to identify and characterize neural mechanisms within brain regions involved in the primary rewarding effects of ethanol and other abused drugs (Koob & LeMoal, 2006). Moreover, significant effort has been devoted to identifying brain areas that regulate conditioned effects of cues associated with cocaine (Everitt & Robbins, 2005; Di Ciano & Everitt, 2005; Weiss, 2005). In contrast, very few studies have addressed the neurocircuitry underlying conditioned effects of cues paired with ethanol. Two recent studies reported increased FOS expression in brain areas activated by exposure to cues previously associated with ethanol either as a discriminative stimulus (S+) in an operant self-administration task (Dayas et al., 2007) or as a conditioned stimulus (CS+) in a Pavlovian procedure (Hill et al., 2007). Only one previous study, however, has addressed the functional role of any brain area in the

expression of behavior elicited by an ethanol-paired stimulus. That study, which used a CPP procedure, showed that microinfusion of a nonselective opioid antagonist or $GABA_B$ agonist into VTA reduced CPP expression (Bechtholt & Cunningham, 2005), suggesting that VTA dopamine neurons might play as important a role in the expression of ethanol-induced conditioned effects as they have previously been shown to play in the direct effects of ethanol (e.g., Brodie et al., 1990; 1999; Gatto et al., 1994; Gessa et al., 1985; Rodd et al., 2004; Samson & Hodge, 1996).

The downstream targets of ethanol-induced conditioned changes in VTA dopamine neurons are unknown, although previous studies strongly suggest that both the Acb and CE might be involved. For example, microdialysis studies have shown that ethanol increases dopamine release in Acb (Di Chiara & Imperato, 1988) and in CE (Yoshimoto et al., 2000). Moreover, microinfusion of opioid or GABA receptor antagonists into Acb and CE reduces ethanol self-administration (e.g., Hyytia & Koob, 1995). Although both areas have been implicated in ethanol's primary reinforcing effects, their roles in the conditioned rewarding effects of ethanol remain unknown. In the present studies, we use selective electrolytic lesions in a well-established model of ethanol-conditioned behavior in mice, CPP (e.g., Cunningham et al., 2006[a]; 2006[b]), to examine whether the Acb and Amy influence the acquisition and expression of this behavior.

Materials and Methods

Subjects

Male DBA/2J (n = 289) mice were obtained from the Jackson Laboratory (Bar Harbor, ME or Davis, CA) at 6-7 weeks of age. Mice were selected for these studies

instead of rats because of the substantial difficulty in establishing a reliable ethanol CPP in rats (e.g., Fidler et al., 2004; Tzschentke, 2007), which may reflect a reduced sensitivity to ethanol's rewarding effect as indexed by this procedure (Cunningham et al., 1993). In contrast, many previous studies have shown that DBA/2J mice develop a strong preference for ethanol-paired cues at a dose of 2 g/kg (e.g., Cunningham, Ferree, & Howard, 2003; 2006[a]). Animals were housed in groups of four on a Thoren rack (Thoren caging systems Inc., Hazleton, PA) in polycarbonate cages. Animals were kept at an ambient temperature of 21±1°C on a 12-h light-dark cycle (lights on at 0700 hours). Experiments were carried out during the light portion of the cycle beginning at 1300 h. "Labdiet" rodent chow (Richmond, IN) and bottled water were continuously available in the home cage. The National Institutes of Health (NIH) "Principles of Laboratory Animal Care" were followed in conducting these studies and the protocol was approved by the Oregon Health & Science University IACUC.

Surgery

Electrolytic lesions were administered to examine the role of the Amy and Acb in the acquisition and expression of ethanol CPP. We chose electrolytic over excitotoxic lesions in light of previous studies showing differences across mouse genotypes in the induction of lesions with excitotoxic agents in the striatum and hippocampus (Schauwecker & Steward, 1997; Schauwecker, 2005) and the paucity of excitotoxic lesion studies in DBA/2J mice. Moreover, we chose not to use temporary chemical inactivation of these nuclei in order to avoid potential problems associated with multiple injections into mouse brain and to control for the number and type of intracranial manipulations between acquisition and expression experiments. Mice were fully

anesthetized with a cocktail (0.1 ml/25 g) containing ketamine (30.0 mg/ml) and xylazine (3.0 mg/ml). Electrodes (Rhodes Medical Instruments, Woodland Hills, CA) were lowered through small burr holes in the skull to a specified depth under stereotaxic guidance (model no. 1900, Kopf Instruments, Tujunga, CA). For bilateral and unilateral (Uni) lesions of the Amy, electrodes were positioned according to the mouse brain atlas (Paxinos & Franklin, 2001) (from Bregma: A -1.46, L ± 2.75, V -5.0) and 0.5 mA of current (model no. 3500, Ugo Basile, Schwenksville, PA) was passed for 10 s, while for lesions of the Acb (from Bregma: A +1.2, L ± 1.0, V -4.5), 0.5 mA of current was passed for 15 s. Specific lesions of the Acb Core (AcbC) and Acb Shell (AcbSh) were induced by electrodes positioned at A +1.42, L ± 1.0, V -4.25 and A +1.54, L ± 1.0, V -4.75 respectively, and 0.5 mA of current was passed for 5 s. For sham lesioned mice, electrodes were lowered into the Amy, Acb, AcbC, AcbSh or Fimbria Fornix[2], although no current was passed. Order of administered lesions (i.e., left hemisphere first, right hemisphere first) and side of lesion for the unilateral lesions (left vs. right) were counterbalanced across subjects. Mice were allowed 4 – 12 days of recovery prior to the start of conditioning trials or testing. Additionally, to control for possible effects of recovery time, we counterbalanced the number of recovery days between lesion groups.

[2] Initially, a group of subjects (not included in total *n*) received lesions of the fimbria fornix consisting of a midline lesion (from Bregma: A -0.5, L 0.0, V -2.2) of 1.0 mA of current passed for 20 or 30 sec. All subjects with fimbria fornix lesions were excluded since the lesions either extended into the surrounding cortices, or verification was impossible due to the difficulty involved in maintaining the integrity of the slice. However, Sham subjects with electrodes lowered into the fimbria fornix were included since analyses did not reveal any differences among sham sites.

Apparatus

A detailed description and picture of the apparatus has been published (Cunningham et al., 2006[a]). Briefly, the apparatus consisted of 12 identical acrylic and aluminum boxes (30 x 15 x 15 cm) enclosed in individual ventilated, light- and sound-attenuating chambers (Coulbourn Instruments Model E10-20). Six sets of infrared light sources and photodetectors mounted 2.2 cm above the floor at 5 cm intervals along the long wall of the box detected general activity, location in the box, and time spent on each side of the chamber (10 ms resolution).

CSs consisted of two interchangeable distinctive floor halves placed beneath each box. The hole floor was made from perforated stainless steel sheet metal (16 gauge) containing 6.4 mm round holes on 9.5 mm staggered centers. The grid floor was constructed from 2.3 mm stainless steel rods mounted 6.4 mm apart in acrylic rails. This floor texture combination was selected on the basis of many previous studies demonstrating that drug-naïve control DBA/2J mice spend about half their time on each floor type during choice tests (e.g., Cunningham et al., 2003). The inside and floors of the box were wiped with a damp sponge and the litter paper underneath the flooring was changed between animals.

Drugs

Ethanol (95%) was diluted in 0.9% saline (20% v/v) and administered at a dose of 2 g/kg (12.5 ml/kg). In previous experiments, this ethanol dose has reliably induced a strong CPP in DBA/2J mice (e.g., Cunningham et al., 2003) without detrimental behavioral effects of repeated injections at this dose or concentration (Cunningham et al., 1997). Saline was administered in a volume of 12.5 ml/kg.

Experimental Design

In experiment 1 (n = 72), mice were allowed to acclimate to their surroundings for 2 days before undergoing surgery. Mice were then given bilateral lesions of the Amy or Acb, or sham lesions. Surgeries were conducted across 8 days, with an additional 4 days of recovery before subsequent ethanol place conditioning and testing (see Table 3). In experiment 2, which was performed in two replications (total n = 169), mice were lesioned after ethanol place conditioning, but before testing. Mice in the first replication received Sham, Acb, or Amy lesions. Based on the results from the first replicate, we included a unilateral Amy lesion (Uni) Group along with Sham and Amy Groups in a second replicate to determine whether one intact Amy was sufficient for expression of a CPP. In experiment 3 (n = 48), to examine the role of Acb subregions in expression of CPP, mice received specific AcbC, AcbSh, or sham lesions after ethanol place conditioning. For experiments 2 and 3, ethanol place conditioning began 2 weeks after arrival. Surgical procedures began 24 h after the last conditioning trial and spanned a course of 8 days. An additional 4 days of recovery were allowed, with testing beginning a total time of 13 days after the last conditioning trial.

Table 3 Experimental Design

	Lesion Group	Order of Events
Experiment 1		
Acquisition	Sham Acb Amy	1. Surgery & Recovery 2. Pretest 3. Ethanol Place Conditioning 4. Testing
Experiment 2		
Expression	Sham Acb Uni Amy	1. Pretest (Rep 1) or Habituation (Rep 2) 2. Ethanol Place Conditioning 3. Surgery & Recovery 4. Testing
Experiment 3		
Expression	Sham AcbSh AcbC	1. Pretest 2. Ethanol Place Conditioning 3. Surgery & Recovery 4. Testing

CPP Procedure

Each experiment involved three phases: pretest or habituation (1 session), conditioning (8 sessions), and one test session. Each animal was given an intraperitoneal (i.p.) injection immediately before being placed in the center of the apparatus for each session.

Pretest. A 30 min pretest was conducted in experiment 1 to determine whether the lesions or surgical procedure affected initial bias for the tactile cues. Subjects were weighed and given an i.p. injection of saline just before placement in the apparatus on a test floor containing both floor types (grid and hole). A pretest was also conducted for the first replication of experiment 2 and for experiment 3. Subjects in the second replication of experiment 2 underwent a 5-min habituation trial where they were given an injection of saline and exposed to the apparatus on a smooth paper floor to reduce the novelty and stress associated with handling, injection and exposure to the apparatus.

Conditioning. Mice were randomly assigned to groups that received a specific lesion type: Amy, Uni (unilateral Amy), Acb, AcbC, AcbSh, or Sham lesions (see Table 3). Within each lesion group, mice were randomly assigned to one of two conditioning subgroups (Grid + or Grid -) using an unbiased, one-compartment procedure (Cunningham et al., 2003; 2006[a]). Both subgroups were exposed to a differential Pavlovian conditioning procedure in which they received four CS+ and four CS- trials. Mice in the Grid+ condition received ethanol paired with the grid floor (CS+) and saline paired with the hole floor (CS-). Mice in the Grid- condition received ethanol paired with the hole floor (CS+) and saline paired with the grid floor (CS-). Each animal received

four 5-min conditioning trials of each type on alternating days over a period of 8 days, with the presentation order of CS+ and CS- trials counterbalanced within each group.

Place Preference Test. The test began 24 h after the last conditioning trial for experiment 1 or 13 days (surgery and recovery) after the last conditioning trial for experiment 2. The test duration was 30 min for experiments 1 and 2. Test duration was lengthened to 60 min for experiment 3 based upon unpublished pilot data suggesting that deficits in Acb modulation of ethanol CPP become apparent during a longer test session. Mice in all experiments were given a saline injection before the test session. Immediately after the injection, subjects were placed in the center of the apparatus with both test floors (half grid/half hole). Position (i.e., left vs. right) of each floor type was counterbalanced within subgroups.

Histology

Immediately after testing, animals were given an overdose of sodium pentobarbital (150 mg/kg), mice were decapitated and heads were postfixed in 4% (w/v) paraformaldehyde in isotonic sodium phosphate buffered saline (PBS). After 24 h, brains were dissected from the skull and placed into a solution of 2 % paraformaldehyde for an additional 24 h. After fixation, brains were cryoprotected using a sucrose saturation procedure consisting of 24 h incubations in 20% and then 30% sucrose in PBS and 0.1% NaN_3. Frozen 40 μm sections were collected through, as well as caudal and rostral to, the lesion site. Slices were directly mounted onto slides and thionen stained. Inclusion criteria for the Amy and Uni Groups included lesions of at least two of the following areas in each hemisphere lesioned: CE, BLA, basomedial nucleus, basomedial amygdala posterior, medial amygdala posterior dorsal and medial, amygdalohippocampal

area, and bed nucleus stria terminalis intraamygdaloid nucleus. In the Acb Group, lesions of the AcbC and AcbSh were included. Additionally, Acb and AcbC lesions that extended into the anterior commissure (anterior) were included given that the anterior commissure is surrounded by the AcbC. Subjects in the AcbSh Group were included if most (≈90%, blind determination) of the lesion was located in the shell region. For the AcbC Group, mice were included if ≈ 90% (blind determination) of the lesion encompassed the core. The possibility of some lesion spread extending into either the core or the shell for the AcbSh or AcbC Groups, respectively, could not be avoided.

Data Analysis

The primary dependent variable was the amount of time spent on the grid floor during the test session. In this unbiased design, the magnitude of the difference in time spent on the grid floor between the Grid+ and Grid- conditioning subgroups is indicative of CPP. See Cunningham et al. (2003) for a more complete discussion of dependent variables used in place conditioning studies. Data from each experiment were evaluated separately by analysis of variance (ANOVA) with the alpha level set at 0.05. To control overall alpha level within each experiment, p-values were Bonferroni corrected for the number of post-hoc comparisons between group means. Paired t-tests were performed where appropriate. Lesion Group, Conditioning Subgroup (Grid+ vs. Grid-), Hemisphere (left vs. right), and Sham Site were treated as between-group factors, whereas Trial Type (CS+ vs. CS-) was treated as a within-subject factor. Replication was included as an additional between-group factor in experiment 2 analyses involving only the Amy and Sham groups.

Results

Subject removal

Reasons for exclusion and the number of subjects removed are detailed in Table 4. The final n's for each group are indicated in the figure captions and tables. A z-test conducted on the two proportions revealed that significantly more mice were lost following surgical procedures in the Amy Group than either Acb or Sham Groups in experiment 1 ($z's > 3.45$, $p's < 0.05$), as well as more Amy lesioned subjects than either Acb or Sham subjects in experiment 2 ($z's > 3.9$, $p's < 0.05$).

Histological verification of lesions

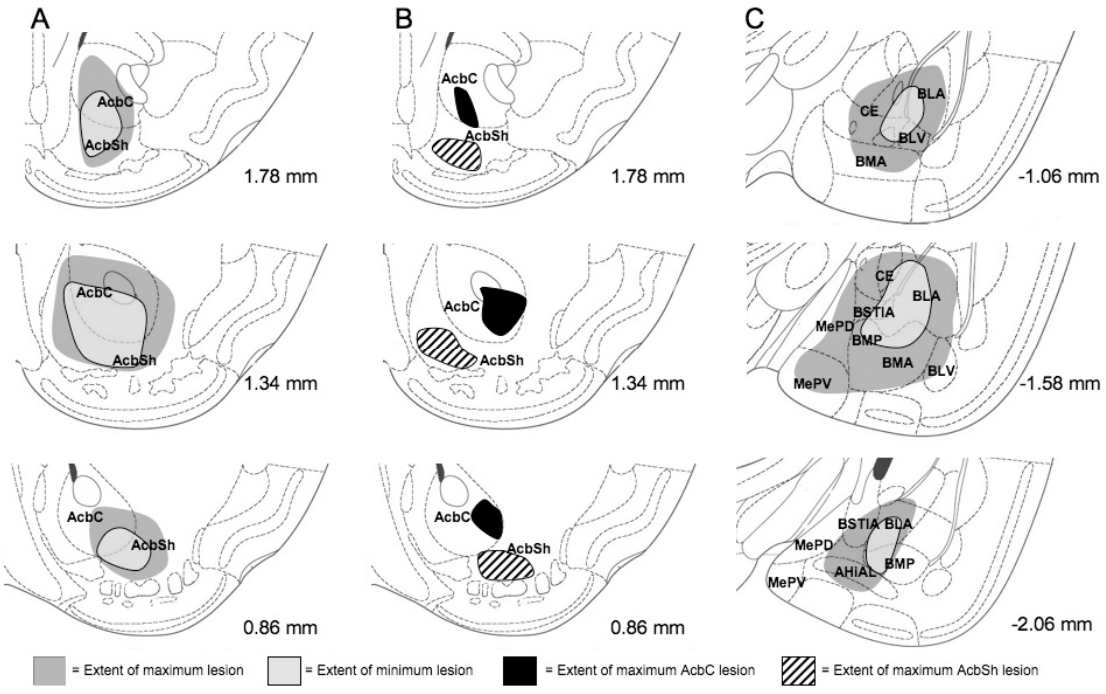
Histological verification was performed on the remaining subjects. All histological analyses were performed blind to conditioning subgroup assignment and final test outcome. A total of 41 subjects were removed from the final data analyses (see Table 4). Although of potential interest in relation to neural modulation of conditioned reinforcement and Pavlovian approach behaviors (for review see Everitt & Robbins, 2005), we were unable to separate contributions of the BLA and CE on the acquisition or expression of ethanol CPP because most subjects had lesions encompassing portions of both regions (Exp 1: 11 out of 14 subjects; Exp. 2: 25 out of 32 subjects). Schematic diagrams are shown in Figure 4.

Table 4 Subject removal

	Lesion Group	Initial n	Final n	Surgery & Recovery	Procedural error	Miss
Experiment 1						
	Sham	24	24	-	-	-
	Acb	24	18	1	-	5
Acquisition	Amy	24	14	7	-	3
Experiment 2			*replication* *1* *2*			
	Sham	58	25 32	1	-	-
	Acb	24	19 -	-	-	5
Expression	Uni	32	- 27	-	-	5
	Amy	55	11 21	9	2	12
Experiment 3						
	Sham	16	16	-	-	-
	AcbSh	16	12	-	-	4
Expression	AcbC	16	9	-	-	7

Figure 4. Representative diagram of Acb (nucleus accumbens) and Amy (amygdala) lesions from Bregma. (**A**) The extent of maximum (dark grey) and minimum (light grey) Acb lesions in experiment 1 and 2. (**B**) Maximum AcbSh (nucleus accumbens shell) (striped) and AcbC (nucleus accumbens core) (black) lesions in experiment 3. (**C**) The extent of maximum (dark grey) and minimum (light grey) Amy and Uni (unilateral amygdala) lesions in experiments 1 and 2.

Figure 4


A
B
C
AcbC
AcbSh
1.78 mm
1.78 mm
-1.06 mm
CE
BLA
BLV
BMA
1.34 mm
1.34 mm
-1.58 mm
BSTIA
MePD
BMP
MePV
0.86 mm
0.86 mm
-2.06 mm
AHiAL
= Extent of maximum lesion
= Extent of minimum lesion
= Extent of maximum AcbC lesion
= Extent of maximum AcbSh lesion

Pretest

A pretest was conducted before conditioning for experiments 1 and 3, and for one replication of experiment 2 to examine initial biases for the conditioned stimuli (Figure 5). Surgical procedures or lesions of the Acb or Amy in experiment 1 did not affect initial biases for the grid or hole floors as measured during a pretest (Figure 5A). These findings were supported by a two-way ANOVA (Conditioning Subgroup x Lesion Site), which yielded no significant main effects or interaction. Further, the site of the sham lesion did not affect initial bias as demonstrated by a lack of significant interaction or main effect of lesion site on pretest results. Lesion and sham subjects had similar levels of activity during the pretest (see Table 5). Additionally, basal activity levels were not different between the sham groups.

Table 5 Locomotor Activity

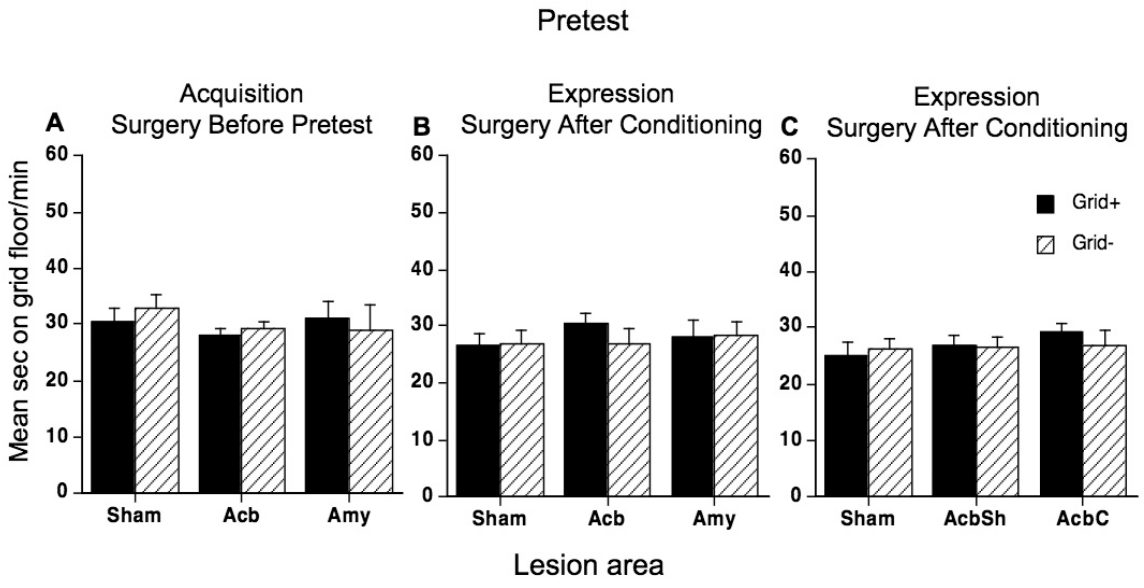
	Lesion Group	n	Mean pretest activity counts/min ± SEM	n	CS+ conditioning trials (EtOH)	CS- conditioning trials (Sal)	Mean test activity counts/min ± SEM	
Experiment 1								
	Sham	24	46.65 ± 3.7	24	172.8 0 ± 5.6	64.10 ± 3.6	36.28 ± 1.5	
Acquisition	Acb	18	54.36 ± 3.9	18	151.47 ± 7.5	64.99 ± 3.1	48.92 ± 1.3[a]	
	Amy	14	43.60 ± 4.0	14	141.77 ± 6.4[d]	58.37 ± 1.8	47.55 ± 2.2 [a]	
		Lesion Group: F(2,53) = 1.8 Sham Site: F(2,21) = .3		Lesion Group: F(2,53) = 5.5** Trial Type: F(1,53) = 518.9*** Interaction: F(2,53) = 4.3*			Lesion Group: F(2,53) = 19.2*** Sham Site: F(2,21) = 0.5	
Experiment 2								
	Sham	25	47.73 ± 2.3	57	191.56 ± 4.3	61.72 ± 1.0	39.60 ± 1.3	
	Acb	19	45.73 ± 1.7	19	204.30 ± 5.0	69.18 ± 4.4	62.11 ± 4.1 [a]	
Expression	Uni	-	-	27	176.93 ± 4.6[f]	51.03 ± 2.3[f]	47.63 ± 2.1 [b, c]	
	Amy	11	45.86 ± 1.7	32	194.30 ± 4.7	59.40 ± 1.9	54.85 ± 2.2 [a]	
		Lesion Group: F(2,52) = .7 Sham Site: F(2,22) = .7		Lesion Group: F(3,131) = 6.3** Trial Type: F(1,131) = 2851.5*** Interaction: F(3,131) = 0.84 Side (Uni): F(1,25) = .3			Lesion Group: F(3,131) = 21.6*** Sham Site: F(2,54) = 2.9	
Experiment 3							First 30 min	Last 30 min
	Sham	16	42.56 ±1.4	16	172.74 ± 4.9	50.56 ± 2.0	38.24 ± 2.2	34.95 ± 1.4 [e]
Expression	AcbSh	12	44.21 ± 1.6	12	167.03 ± 5.7	48.05 ± 3.1	47.35 ± 4.3	42.79 ±3.6
	AcbC	9	42.36 ± 2.2	9	161.72 ± 6.3	52.98 ± 2.7	45.99 ± 2.3	49.78 ± 3.4 [a]
		Lesion Group: F(2,34) = .4 Sham Site: F(1,14) = .01		Lesion Group: F(2,34) = 0.5 Trial Type: F(1,34) = 1669.9*** Interaction: F(2,34) = 1.86			Lesion Group: F(2,34) = 2.88 Sham Site: F(1,14) = 1.3	Lesion Group: F(2,34) = 7.5** Sham Site: F(1,14) = 5.0 *

Uni = Unilateral amygdala lesion
* $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$ (p-values for all group comparisons are Bonferroni-corrected).

[a] = difference from Sham, $p < .001$, [b] = difference from Acb $p = .01$, [c] = difference from Sham $p < .05$, [d] = difference from Sham $p < .01$, [e] = Sham Site AcbC and Sham Site AcbSh group means significantly different $p < .05$, and [f] = Group Uni mean activity different from Sham, Acb, and Amy $p < .05$.

Figure 5. Lesions of the Acb (nucleus accumbens) or Amy (amygdala) do not affect initial stimulus bias. Mean sec per min (+SEM) spent on the grid floor during the 30-min pretest session. Subjects in the Grid+ conditioning subgroups (solid bars) were assigned to receive ethanol paired with the grid floor on CS+ trials, and saline paired with the hole floor. These contingencies were reversed in the Grid- conditioning subgroup subjects (hatched bars). **(A)** Subjects in the acquisition experiment received either sham lesions or lesions of the Acb or Amy before the pretest. N's for Grid+ and Grid- conditioning subgroups are: Sham n = 12 and 12; Acb n = 9 and 9; and Amy n = 6 and 8. **(B)** Pretest data for one replication of the expression experiment, in which subjects did not receive lesions until after conditioning. Grid+ and Grid- conditioning subgroup n's are: Sham n = 13 and 12; Acb n = 10 and 9; and Amy n = 5 and 6. **(C)** Subjects in experiment 3 were given lesions after ethanol conditioning. N's for each conditioning subgroup (Grid+ and Grid-) are: Sham n = 8 and 8; nucleus accumbens shell (AcbSh) n = 6 and 6; nucleus accumbens core (AcbC) n = 4 and 5.

Figure 5


Pretest
A
Acquisition
Surgery Before Pretest
B
Expression
Surgery After Conditioning
C
Expression
Surgery After Conditioning
Mean sec on grid floor/min
60
50
40
30
20
10
0
Grid+
Grid-
Sham
Acb
Amy
Sham
Acb
Amy
Sham
AcbSh
AcbC
Lesion area

Pretest results in experiments 2 (replicate 1) and 3 replicated previous findings (e.g., Cunningham et al., 2003) in that non-lesioned subjects did not display an unconditioned bias for one floor type, nor did lesion group assignment affect this lack of bias (see Figures 5B and 5C). This conclusion was supported by two-way ANOVAs (Conditioning Subgroup x Lesion Site) that showed no significant main effects or interaction. Due to a procedural error, subjects in the second replicate of experiment 2 were exposed to a habituation session instead of a pretest. To determine whether development of CPP differed between the experiment 2 replicates that did (replicate 1) and did not (replicate 2) receive a pretest, a two-way ANOVA (Replication x Conditioning Subgroup) was performed on preference test results in the Sham Group and in the Amy Group. The Sham replicates in Experiment 2 did not differ, suggesting that the pretest performed in the first replication did not affect later expression of preference. Moreover, the Amy replicates in Experiment 2 did not differ, suggesting that post-conditioning lesions did not induce an effect of the pretest on later preference expression. Thus, the two replicates were combined for the preference test analyses described in the next section. Additionally, as expected, there were no activity differences between assigned lesion groups in experiment 2 or in experiment 3 (see Table 5). Overall, since no differences in initial preference or activity were observed during the pretest, differences observed during the final test can be attributed to the effects of conditioning and pre- or post-conditioning lesions on CPP.

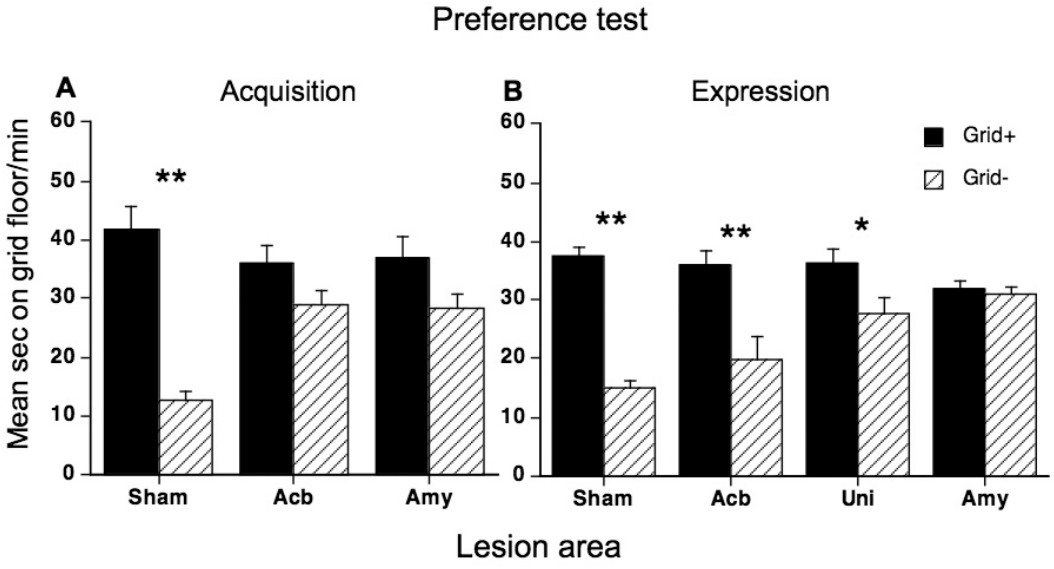
Preference testing

Lesion effects on acquisition and expression of ethanol CPP. In experiment 1, the roles of the Acb and Amy in the acquisition and expression of ethanol CPP were examined. After ethanol place conditioning, sham subjects expressed a strong place preference (see Figure 6A). However, Acb and Amy Groups did not express a place preference, i.e., there was no difference between Grid+ and Grid- conditioning subgroups after ethanol place conditioning. Moreover, both Lesion Groups differed significantly from the sham control group. Thus, lesions of the Acb or Amy prevented the acquisition and/or expression of ethanol-induced CPP.

A two-way (Lesion Group x Conditioning Subgroup) ANOVA performed on test session data revealed a main effect of Conditioning Subgroup (Grid+ vs. Grid-) [$F(1,50) = 35.8$, $p < 0.001$] as well as a significant interaction [$F(2,50) = 9.0$, $p < 0.001$]. The main effect of lesion was not significant. Post-hoc analyses of the interaction revealed a significant difference between conditioning subgroups in the Sham Group (Bonferroni corrected $p < 0.001$) that was not observed in either the Acb or the Amy Group ($ps > 0.05$). Further, two-way (Lesion Group x Conditioning Subgroup) ANOVAs between pairs of specific lesion groups suggested that the Acb Group differed from the Sham Group, and that the Amy Group differed from the Sham Group ($Fs > 9.5$, $ps < 0.01$), although the Acb and the Amy Groups did not differ from each other. A two-way ANOVA of Sham Site x Conditioning Subgroup showed no effect of sham lesion site on the magnitude of preference expressed in the Sham Group.

Figure 6. Lesions of the nucleus accumbens (Acb) and amygdala (Amy) disrupt acquisition, but only lesions of the Amy block expression of ethanol CPP. Mean sec per min (+SEM) spent on the grid floor during the 30-min test session. **(A)** In the acquisition experiment, subjects received sham lesions, or lesions of the Acb or Amy before ethanol place conditioning. Grid+ and Grid- conditioning subgroup n's are: Sham n = 12 and 12; Acb n = 9 and 9; and Amy n = 6 and 8. **(B)** However, subjects in the expression experiment received sham lesions or lesions of the Acb, unilateral Amy, or Amy after conditioning but before preference testing. Grid+ and Grid- Conditioning subgroup n's are: Sham n = 29 and 28; Acb n = 10 and 9; Uni (unilateral amygdala) n = 13 and 14; and Amy n = 17 and 15. Difference between conditioning subgroups Grid+ and Grid-: ** = Bonferroni corrected ps < 0.001; * = Bonferroni corrected p < 0.05

Figure 6


Preference test
A
Acquisition
B
Expression
Grid+
Grid-
Mean sec on grid floor/min
60
50
40
30
20
10
0
**
**
**
*
Sham
Acb
Amy
Sham
Acb
Uni
Amy
Lesion area

Lesion effects on expression of ethanol CPP. Contrary to findings in experiment 1 which suggested a role for both Amy and Acb, the findings in experiment 2 suggested that expression of ethanol CPP was solely dependent upon a fully intact Amy (see Figure 6B). After ethanol conditioning, surgery, and recovery, strong place preference was observed in the Sham and Acb Groups. However, unilateral Amy lesions (Uni Group) partially reduced expression of ethanol place CPP and bilateral Amy lesions completely disrupted expression of CPP.

These findings were supported by a two-way ANOVA (Lesion Group x Conditioning Subgroup) that revealed a main effect of Lesion Group [$F(3,127) = 5.0$, $p < 0.01$], Conditioning Subgroup [$F(1,127) = 68.4$, $p < 0.001$], and a significant interaction [$F(3,127) = 13.0$, $p < 0.001$]. Post hoc analysis of the interaction showed a significant effect of Conditioning Subgroup in the Sham, Acb, and Uni Groups (Bonferroni corrected $ps < 0.05$), but not in the Amy Group ($p > 0.05$). Comparisons between pairs of lesion groups (Lesion Group x Conditioning Subgroup ANOVAs) found that Groups Sham and Acb did not differ ($p > 0.1$). However, the Sham Group did differ from the Uni Group [$F(1,80) = 12.4$, $p < 0.01$] and from the Amy Group [$F(1,85) = 45.3$, $p < 0.001$]. Further, the Amy Group was significantly different from the Acb Group [$F1,47) = 11.4$, $p < 0.01$], and the Amy Group showed a trend towards a difference compared to the Uni Group [$F(1,55) = 3.4$, $p = 0.072$]. Groups Acb and Uni did not differ ($p > 0.2$).

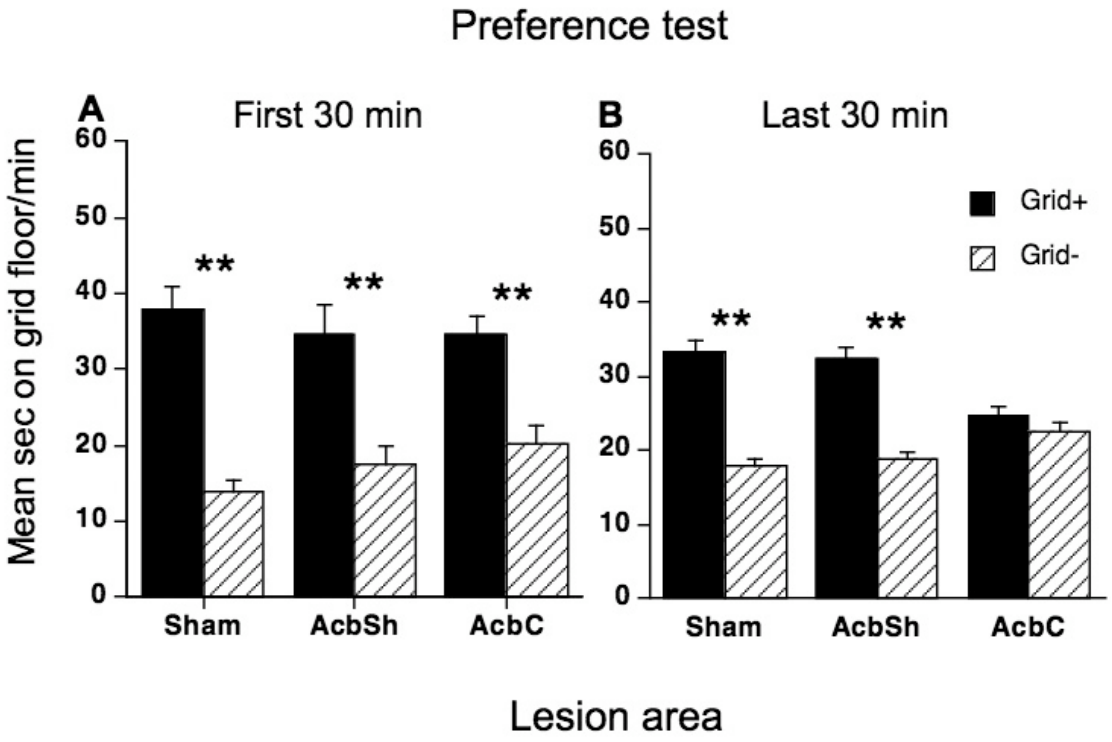
A series of analyses were performed to examine extraneous factors that might have contributed to these findings. Site of the sham lesions had no effect as confirmed by a two-way (Sham Site x Conditioning Subgroup) ANOVA on combined replicates. Additionally, since the effects of Amy and Sham lesions on expression of ethanol CPP

were performed in two replicates, separate two-way (Replication x Conditioning Subgroup) ANOVAs were performed for each brain area. These analyses showed that the effects of Amy or Sham lesions did not change across replicates (p's > 0.1), suggesting similar effects in both replicates. Further, a two-way (Hemisphere x Conditioning Subgroup) ANOVA of the Uni Group found no evidence that hemisphere lesioned (left vs. right) contributed to effects on preference observed. Overall, these analyses indicate that preference differences between lesion groups were due to the brain area lesioned and not to procedural or surgical differences.

Specific accumbens core vs. shell lesion effects on expression of ethanol CPP. Examination of the specific contributions of the Acb core and shell revealed differences in their impact on expression of ethanol CPP (see Figure 7). All lesion groups expressed similar levels of preference during the first half of the test session. However, lesions of the AcbC disrupted expression during the last 30 min of the test session, while mice with AcbSh lesions expressed a preference similar to that of the Sham Group.

Figure 7. Lesions of the nucleus accumbens core (AcbC) disrupt expression of ethanol CPP during the last half of the test session. Mean sec per min (+SEM) spent on the grid floor during the first and last 30 min of the test session. **(A)** First 30 min of the test session. The Sham, AcbSh (nucleus accumbens shell), and AcbC Groups that were lesioned after ethanol place conditioning all express strong ethanol CPP. **(B)** However, during that last 30 min of the test session, subjects in the AcbC Group no longer expressed ethanol CPP while preference in the AcbSh and Sham Groups was still significant. Grid+ and Grid- conditioning subgroups N's are respectively: Sham n = 8 and 8; AcbSh n = 6 and 6; AcbC n = 4 and 5. Difference between conditioning subgroups Grid+ and Grid-: ** = Bonferroni corrected ps < 0.01.

Figure 7


Preference test
A
First 30 min
B
Last 30 min
Mean sec on grid floor/min
60
50
40
30
20
10
0
**
Grid+
Grid-
Sham
AcbSh
AcbC
Lesion area

A two-way ANOVA (Conditioning Subgroup x Lesion Group) conducted on the first 30 min of the test session revealed a main effect of Conditioning Subgroup [$F(1,31) = 65.5$, $p < 0.001$], but no main effect of lesion group or interaction. However, the same analysis conducted on the last 30 min of the test revealed a main effect of Conditioning Subgroup [$F(1,31) = 22.7$, $p < 0.001$] and a significant interaction [$F(2,31) = 3.3$, $p = 0.05$]. There was no main effect of lesion during the last half of the test. Post hoc analyses of the interaction demonstrated a significant preference in both the Sham and AcbSh Groups (Bonferroni corrected p's < 0.001], but not in the AcbC Group. Comparisons between pairs of Lesion Groups found that the Sham and AcbSh Groups did not differ in level of preference expressed during the last 30 min ($p > 0.7$). However, the magnitude of preference expressed was significantly different between the Sham and AcbC Groups [$F(1,21) = 7.8$, $p < 0.02$] and there was a trend towards a significant difference between the AcbC and AcbSh Groups [$F(1,17) = 3.8$, $p = 0.067$]. The Sham lesion area did not contribute to these differences, as there were no effects of Sham site on preference expressed during the first or last 30 min of the test session.

Conditioning Activity

To investigate whether lesions of the Acb or Amy created before conditioning trials would affect development of sensitization to the locomotor stimulating effects of ethanol previously reported in CPP (e.g., Cunningham, Tull, Rindal, & Meyer, 2002), initial analyses were performed on activity data across conditioning trials. A three-way ANOVA (Lesion Group x Trial Type x Trial) revealed a significant interaction [$F(6, 159) = 2.2$, $p = 0.05$], that was driven by a difference in activity levels between lesioned

subjects during CS- conditioning trials [$F(6,159) = 2.4$, $p < 0.05$]. More specifically, a difference between lesioned subjects was observed during the first CS- conditioning trial [$F(2,53) = 4.0$, $p < 0.05$], in that Acb lesioned subjects had significantly lower activity levels than Amy lesioned mice (Bonferroni corrected $p < 0.05$). All subjects showed similar levels of sensitization to ethanol, as revealed by a two-way ANOVA performed on CS+ conditioning trials [$F(2,53) = 6.0$, $p < 0.01$] and a follow-up paired t-test performed between the first and last ethanol conditioning trial ($t = 2.4$, $p < 0.05$). Since no other differences between lesions groups developed across conditioning trials, and for ease of presentation, conditioning activity data were averaged across trials to create single means for the CS+ and CS- trials. As in previous studies in DBA/2J mice (e.g., Gremel & Cunningham, 2007; Cunningham et al., 2006[b]), ethanol given immediately before the conditioning trial induced increases in locomotor activity (see Table 5 for group means and statistical comparisons). In experiment 1, lesions of the Amy reduced this ethanol-induced activation.

In experiments 2 and 3 where lesions were administered after conditioning trials, there was no effect of lesion group assignment on the sensitization to ethanol's locomotor stimulating effects that developed across trials [Trial x Trial Type: $Fs' > 12.5$, $ps' < 0.001$, paired t-test performed on first CS+ versus last CS+ conditioning trial: $ts' > 1.9$, $ps' \leq 0.05$]. For this reason, conditioning activity data were averaged across trials to create single means for the CS+ and CS- trials. Ethanol also induced large increases in locomotor activity when administered before conditioning trials in experiments 2 and 3 (see Table 5). However, in experiment 2, mean activity in the Uni Group was slightly lower than other lesion groups, reflecting sampling differences among groups before

lesion administration. There was no effect of replication in a separate analysis of the Amy and Sham groups. In experiment 3, groups did not differ in their response to ethanol before the lesion was induced (see Table 5).

Test Activity

A history of ethanol treatment or conditioning procedures differentially affected test activity levels between lesion groups (see Table 5 for group means and statistical comparisons). Amy and Acb lesioned subjects had significantly higher test activity levels than the Sham Group in experiments 1 and 2. Moreover, unilateral Amy lesions before testing increased activity levels relative to Sham lesions in experiment 2. In experiment 2, there was also a significant Lesion x Replication interaction in a separate analysis of the Amy and Sham Groups [$F(1,85) = 12.1$, $p = .001$]. This interaction was due to slightly higher test activity levels in the Amy Group during the second replication (increase of about 10.6 counts/min). In experiment 3, test activity levels did not differ between lesion groups during the first 30 min of the test session. However, during the last portion of the test, subjects with lesions of the AcbC had significantly higher levels of activity than Sham subjects.

Discussion

These experiments are the first to reveal the functional importance of the Acb and Amy in the learning and expression of behavior controlled by a cue previously paired with ethanol. Lesions of Acb and Amy disrupted acquisition, while lesions of the Amy disrupted expression of ethanol CPP. CPP allows for investigation of ethanol-conditioned behaviors, without the confounding effects of ethanol on reinforced responding observed in self-administration models. Further, CPP has the unique advantage over self-administration procedures in the measurement of conditioned behavior, since the response controlled by the conditioned cue has never produced the primary reinforcer (i.e., ethanol). The response measured during expression testing is approach and maintenance of contact with the previously drug-paired cue, without administration of the conditioning drug. Although theoretically, Pavlovian approach behavior, conditioned reinforcement, and conditioned incentive may all be operating in CPP, it is likely that only the latter two processes are involved in our procedure because animals are responding to a tactile cue in the dark (Cunningham et al., 2006[b]).

Lesion-induced deficits in the acquisition of CPP could be attributed to a disruption in the primary rewarding effects of ethanol or to a decrement in learning the association between the cue and ethanol (e.g., Cunningham et al. 2006[a]; Bardo & Bevins, 2001). However, lesions made before conditioning also affect CPP expression, hence not allowing for separation between the neural areas involved in the learning versus the manifestation of the behavior. For this reason, in experiments 2 and 3, lesions were administered after conditioning. Lesion effects on expression of CPP may reflect a decrease in the conditioned value of the cue, an inability to retrieve the memory of the

association between the drug and cue, or a decrement in the learning or performance of the approach response. Although the present studies do not distinguish among these possibilities, they strongly suggest that different neural areas mediate the acquisition and expression of behavior controlled by ethanol-paired stimuli.

Acquisition of ethanol CPP is dependent upon Acb and Amy. In experiment 1, lesions of the Acb and Amy before conditioning interfered with the expression of ethanol CPP (see Figure 4). Unlike other drugs of abuse such as cocaine and amphetamine, there has been little direct evidence for involvement of the Acb or the Amy in the acquisition of ethanol's conditioned effects. Previous work found that intra-Acb dopamine depletion by 6-hydroxydopmaine (6-OHDA) lesions did not alter the acquisition of ethanol self-administration (Lyness & Smith, 1992; Rassnick, Stinus, & Koob, 1993; Myers & Quarfordt, 1991), suggesting that the Acb may not be necessary for ethanol's primary reinforcing effects. However, the current findings are more in line with previous studies examining the effect of Acb and Amy lesions on the acquisition of CPP induced by other abused drugs. For example, excitotoxic lesions of the Amy prevented the acquisition of cocaine CPP (e.g., Brown & Fibiger, 1993) and excitotoxic lesions of the lateral Amy attenuated amphetamine CPP (Hiroi & White, 1991). Further, lesions of the Acb disrupted amphetamine CPP (e.g., Olmstead & Franklin 1996), but had no effect on cocaine CPP (Spyraki, Fibiger, & Phillips, 1982). There have been mixed results as to the role of the Acb and Amy in morphine CPP. For example, there have been reports of 6-OHDA and electrolytic lesions of the Acb disrupting acquisition of morphine CPP (e.g., Shippenberg, Bals-Kubik, & Herz, 1993; Kelsey, Carlezon, & Falls, 1989; White et al.,

2005), while Olmstead and Franklin (1997) found excitotoxic lesions of the Acb or Amy failed to disrupt acquisition of morphine CPP.

Ethanol CPP is expressed through an Amy-dependent mechanism. In contrast to findings from the acquisition study (Exp. 1), only lesions of the Amy had an effect on expression of ethanol CPP (Exp. 2). Bilateral lesions performed after conditioning completely disrupted CPP, while unilateral lesions attenuated expression of ethanol conditioned behavior (see Figure 6). Of particular interest, the findings of Amy involvement in ethanol CPP are in parallel to studies examining ethanol reinforcement in self-administration procedures (e.g., Schroeder, Olive, Koenig, & Hodge, 2003; Heyser, Roberts, Schulteis, & Koob, 1999; Roberts et al., 1996).

In contrast to the Amy findings, lesions of the Acb did not affect initial expression of CPP. This finding is not only at odds with studies demonstrating the importance of the Acb in the primary reinforcing effects of ethanol (for review see McBride & Li, 1998), but is also divergent from findings examining contributions of the Acb in ethanol-seeking using a self-administration procedure that suggested a role for the Acb (particularly the core region) in stimulus processing of alcohol-related cues (e.g., Samson & Chappell, 2003; 2004; Czachowski et al., 2001; Czachowski, 2005). It may be that control over ethanol seeking behavior by a conditioned cue only involves some of the same areas, mainly the Amy, as those recruited in an ethanol self-administration model.

However, one issue in comparing our studies is the difference in time elapsed between conditioning and testing in experiment 1 (1 day) versus Experiments 2 and 3 (13 days). It is possible, for example, that time-dependent compensatory mechanisms may have masked an effect of Acb lesions on expression of ethanol CPP in Experiments 2 and

3. Alternatively, Acb, in comparison to Amy, lesioned mice may be more sensitive to post-conditioning changes in the strength of CPP over time. However, a cross-experiment comparison of the Sham groups (Experiment x Conditioning Subgroup ANOVA) showed no significant differences across experiments, suggesting that the memory of the Pavlovian association controlling ethanol CPP is not affected by a delay of 13 days before testing. Although we cannot completely dismiss a possible role of post-conditioning delay, the similarity among Sham groups argues against this difference as an important determinant of the difference in the outcomes of the acquisition and expression experiments.

Lesions of the AcbC facilitate loss of responding. In experiments 1 and 2, mice in the Acb Group had lesions that extended into both the AcbSh and AcbC, not allowing for possible behavioral separation based on lesion location. Thus, experiment 3 was performed to ask whether subjects with lesions focused in the AcbC would differ from AcbSh and Sham lesioned subjects. We found that AcbSh and Sham Groups displayed a similar magnitude preference throughout the 60-min expression test. Although lesions of the AcbC had no effect on initial expression of ethanol CPP, these mice showed a more rapid loss of responding (see Figure 7B), possibly implicating the AcbC in the inhibitory learning believed to occur when the context is no longer paired with drug during CPP extinction.

Overlap with areas activated by ethanol-paired cue exposure. As expected, there is some overlap between the neural areas mediating the acquisition and expression of ethanol CPP and those activated by exposure to a cue previously paired with ethanol. For example in an ethanol reinstatement model using a S^+ task, increased FOS expression

was observed in the Acb (Dayas et al., 2007), in addition to the Acb, BLA, and CE in a separate study (Zhao et al., 2006). Further, recent findings from an ethanol cue-induced reinstatement study found increases in BLA and CE activation in response to a discrete cue (Radwanska et al., 2007). In a study that used a Pavlovian conditioning procedure similar to ours, the CS+ elicited increased FOS expression in the extended Amy and anterior VTA, but no conditioned increase was observed in the Acb (Hill et al., 2007). Although the procedural differences responsible for the above discrepancies are unknown, these findings generally suggest that the Amy and Acb play major roles in the processing of ethanol-predictive environmental stimuli and mediation of ethanol seeking behavior as indexed by CPP and ethanol reinstatement models.

The role of the Acb and Amy in locomotor activity. Present findings also shed light on the neural areas governing ethanol-induced activation and test activity in mice that are hypothesized to overlap with those involved in its reinforcing effects (Phillips & Shen, 1996; Boehm, Piercy, Bergstrom, & Phillips, 2002). In experiment 1, only lesions of the Amy attenuated ethanol-induced stimulation during conditioning trials (see Table 5). The lack of effect of Acb lesions on ethanol-induced increases in activity is suprising given that VTA-induced activation and release of dopamine in the Acb has been suggested to influence the stimulant response to ethanol (e.g., Boehm, Piercy, Bergstrom, & Phillips, 2002). Since we were unable to quantify the extent of Acb lesions, it may be the size of lesion created was insufficient to interfere with Acb modulation of ethanol-induced locomotor activation. Although the differences in activity were relatively minor, subjects with Amy and Acb lesions showed higher levels of activity during the drug-free test (see Table 5). Although level of activation can affect preference (Gremel &

Cunningham, 2007), we do not believe that the differences in activity observed in the present studies were sufficient to significantly alter the observed CPP since Acb lesioned subjects expressed a preference similar to Sham subjects (see Figure 6).

In summary, our data suggest that the Amy and Acb influence the acquisition of ethanol CPP, whereas the Amy modulates CPP expression. Overall, these findings suggest that the neural mechanisms underlying ethanol CPP mirror some mechanisms recruited during ethanol self-administration, while involving seeking mechanisms also engaged by other drugs of abuse. Although the use of electrolytic lesions in the present study prevents the interpretation of direct effects on nuclei, the results strongly implicate these areas in the mediation of ethanol CPP. Future work will aim to delineate the specific neurotransmitters in each area contributing to the acquisition, expression, and extinction of this behavior.

Chapter 3

Involvement of amygdala dopamine- and nucleus accumbens NMDA- receptors in ethanol-seeking behavior in mice[3]

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Abstract

Although progress has been made identifying neural mechanisms underlying ethanol's primary reinforcing effects, few studies have examined the mechanisms mediating ethanol-induced conditioned effects. A recent lesion study suggests that expression of ethanol-conditioned behaviors depends upon an intact Amy and AcbC. However, specific mechanisms within these nuclei are unknown. In the present experiments, we used site-specific microinfusions of dopamine D1/D2 and NMDA receptor antagonists to examine the roles of Acb and Amy in the expression of ethanol CPP in mice. In experiments 1 and 2, a D1/D2 receptor antagonist (flupenthixol) was infused into Acb or Amy before testing, while experiment 3 used pretest infusions of an NMDA antagonist (AP-5) to examine the role of intra-Acb NMDA receptors. Dopamine antagonism of Acb was without effect, but intra-Amy infusions of flupenthixol blocked CPP expression. Moreover, this effect was dependent upon dopamine antagonism within the BLA but not the CE. Antagonism of NMDA receptors in Acb also blocked CPP expression. Experiment 4 used a neuropharmacological disconnection procedure to investigate whether activation of intra-Acb NMDA receptors resulted from dopamine receptor activation in Amy. However, because both ipsi- and contra-lateral manipulations disrupted ethanol CPP, we were not able to support that hypothesis, leaving open the possibility of modulation by glutamate projections from cortex or hippocampus. These are the first studies in any species to show a role for Amy dopamine receptors and the first studies in mice to implicate Acb NMDA receptors in ethanol-induced conditioned effects.

Introduction

Although alcohol abuse and dependence are widespread, knowledge about the neurobiological mechanisms regulating ethanol-seeking behaviors is limited. Conditioned responses to environmental stimuli predictive of ethanol's effects are thought to be critical for instigating ethanol-seeking behaviors and maintaining ethanol consumption. Understanding the neuromechanisms of cue-induced ethanol seeking may offer insight into reducing the physiological/behavioral responses, motivational states, or expectancies that lead to craving and relapse (e.g., Corbit & Janak, 2007; Cunningham, 1994, 1998; Krank, 1989, 2003).

Most studies of the neural mechanisms involved in ethanol seeking have used rats trained in operant self-administration procedures. Such studies have suggested roles for dopamine D2 (Samson et al., 1993; Hodge et al., 1997) and NMDA (Rassnick et al., 1992) receptors within the Acb and for $GABA_A$ (Hyytiä & Koob, 1995) and opioid (Heyser et al., 1999) receptors within the CE. Although some studies have suggested that dopamine receptors within Acb play a greater role in mediating ethanol conditioned appetitive responses than in mediating ethanol consumption (Czachowski et al., 2001, 2002; Samson & Chappell, 2004), most self-administration studies have failed to distinguish between the mechanisms underlying ethanol's primary reinforcing effects and those underlying ethanol-induced conditioned reinforcing or conditioned motivational effects.

In contrast to self-administration, the CPP procedure is well suited for studying conditioned motivational and/or conditioned reinforcing effects of abused drugs (Tzschentke, 2007), especially because one can examine pretreatment drug effects on

CPP expression in the absence of the training drug (Cunningham et al., 2006[a]). Although two recent rat studies have suggested roles for dopamine receptors in AcbSh (Walker & Ettenberg, 2007) and for NMDA receptors in CE (Zhu, Bie, & Pan, 2007) on ethanol CPP expression, interpretation of these studies is complicated because most rat studies have reported no conditioning or conditioned place aversion with ethanol (Tzschentke, 1998, 2007; Fidler et al., 2004). In contrast, ethanol CPP is reliably observed in mice (e.g., Cunningham et al., 2006[a]; Tzschentke, 1998, 2007). Using this model, a previous study showed that opioid and $GABA_B$ receptors within the VTA mediated expression of ethanol CPP whereas blocking opioid receptors in Acb had no effect (Bechtholt & Cunningham, 2005). Moreover, a recent lesion study in mice identified functional roles for both Acb and Amy in ethanol CPP (Gremel & Cunningham, 2008).

To determine whether specific receptors within Acb or Amy modulate the conditioned motivational/conditioned reinforcing effects of ethanol, we used site-specific bilateral infusions of a D1/D2-receptor antagonist (into Acb or Amy) or an NMDA-receptor antagonist (into Acb) to assess the influence of these receptors on expression of ethanol CPP in mice. Further, we examined whether these nuclei interact serially to control ethanol-seeking behavior by using a neuropharmacological disconnection procedure (Parkinson et al., 2000). These are the first studies in any species to evaluate the role of Amy dopamine receptors and the first studies in mice to assess the roles of Acb dopamine and NMDA receptors in ethanol's conditioned motivational/conditioned reinforcing effects.

Materials and Methods

Subjects

Male DBA/2J (n =642) mice were obtained from the Jackson Laboratory (Bar Harbor, ME or Davis, CA) at 6-7 weeks of age. Previous findings have demonstrated that DBA/2J mice develop a strong preference for ethanol-paired tactile cues at a dose of 2 g/kg (e.g., Cunningham et al., 2003; 2006[a]). Animals were initially housed in groups of four on a Thoren rack (Thoren caging systems Inc., Hazleton, PA) in polycarbonate cages. After surgical procedures, animals were housed two per cage for the duration of the experiments. Animals were kept at an ambient temperature of 21±1°C on a 12-h light-dark cycle (lights on at 0700 hours). Experiments were carried out during the light portion of the cycle beginning at 1300 h. "Labdiet" rodent chow (Richmond, IN) and bottled water were continuously available in the home cage. The National Institutes of Health (NIH) "Principles of Laboratory Animal Care" were followed in conducting these studies and the protocol was approved by the Oregon Health & Science University IACUC.

Surgery

Mice were fully anesthetized with a cocktail (0.1 ml/25 g) containing ketamine (30.0 mg/ml) and xylazine (3.0 mg/ml). Bilateral indwelling cannulae were implanted under stereotaxic guidance (model no. 1900, Kopf Instruments, Tujunga, CA) aimed at the AcbC (from Bregma: anterior (AP) + 1.40, lateral (ML) ± 1.26, ventral (DV) -4.2) or BLA/CE (from Bregma: AP – 1.22, ML ± 2.85, DV – 4.5; Paxinos & Franklin, 2001). Small burr holes were drilled and stainless steel cannulae (10 mm, 25 gauge) were positioned 2 mm above the target area (Experiments 1, 2, and 3). In Experiment 4, since

dorsal/ventral placement was limited to the same depth for both cannulae, they were positioned 2.0 mm above the Acb and 2.3 mm above the BLA/CE. Cannulae were secured with stainless steel screws and carboxylate cement (Durelon™, 3M, St. Paul, MN). Thirty-two gauge stainless steel stylets were inserted into the length of each guide cannula to maintain patency. Mice were allowed 4 – 9 days of recovery prior to the start of conditioning trials. In Experiment 4, cannulae placement were counterbalanced for hemisphere (left vs. right) and disconnection group (ipsi- vs. contra-lateral). To control for possible effects of recovery time, the number of recovery days was counterbalanced across infusion groups.

Apparatus

A detailed description and picture of the apparatus has been published (Cunningham et al., 2006[a]). Briefly, the apparatus consisted of 12 identical acrylic and aluminum boxes (30 x 15 x 15 cm) enclosed in individual ventilated, light- and sound-attenuating chambers (Coulbourn Instruments Model E10-20). Six sets of infrared light sources and photodetectors mounted 2.2 cm above the floor at 5 cm intervals along the long wall of the box detected general activity, location in the box, and time spent on each side of the chamber (10 ms resolution).

CSs consisted of two interchangeable distinctive floor halves placed beneath each box. The hole floor was made from perforated stainless steel sheet metal (16 gauge) containing 6.4 mm round holes on 9.5 mm staggered centers. The grid floor was constructed from 2.3 mm stainless steel rods mounted 6.4 mm apart in acrylic rails. This floor texture combination was selected on the basis of many previous studies demonstrating that drug-naïve control DBA/2J mice spend about half their time on each

floor type during choice tests (e.g., Cunningham et al., 2003; Gremel & Cunningham, 2008). The inside and floors of the box were wiped with a damp sponge and the litter paper underneath the flooring was changed between animals.

Conditioning drugs

Ethanol (95%) was diluted in 0.9% saline (20% v/v) and administered at a dose of 2 g/kg (12.5 ml/kg). In previous experiments, this ethanol dose and concentration has reliably induced a strong CPP in DBA/2J mice (e.g., Cunningham et al., 2003) without detrimental behavioral effects of repeated injections (Cunningham et al., 1997). Saline was administered in a volume of 12.5 ml/kg.

General Procedure

Each experiment involved three phases: habituation (1 session), conditioning (8 sessions), and testing. Each animal was given an intraperitoneal (i.p.) injection immediately before being placed in the center of the apparatus for each session.

Habituation. Subjects in all experiments underwent a 5-min habituation trial where they were given an injection of saline and exposed to the apparatus on a smooth paper floor to reduce the novelty and stress associated with handling, injection and exposure to the apparatus.

Conditioning. Mice were randomly assigned to an infusion group described separately for each experiment in a later section (Intracranial Microinfusions). Within each infusion group, mice were also randomly assigned to one of two conditioning subgroups (Grid + or Grid -) using an unbiased, one-compartment procedure (Cunningham et al., 2003; 2006[a]). Both subgroups were exposed to a differential Pavlovian conditioning procedure in which they received four CS+ and four CS- trials.

Mice in the Grid+ condition received ethanol paired with the grid floor (CS+) and saline paired with the hole floor (CS-). Mice in the Grid- condition received ethanol paired with the hole floor (CS+) and saline paired with the grid floor (CS-). Each animal received four 5-min conditioning trials of each type on alternating days over a period of 8 days, with the presentation order of CS+ and CS- trials counterbalanced within each group.

Place Preference Test. Testing began 48 h after the last conditioning trial for all animals. The test duration was 30 min and in each test session mice were first given an intracranial infusion (see next section for details). Immediately after the infusion, mice were given an i.p. saline injection and placed in the center of the apparatus with both test floors (half grid/half hole). Position (i.e., left vs. right) of each floor type was counterbalanced within subgroups.

Intracranial Microinfusions

All mice received an intracranial microinfusion immediately before testing (Table 6). For microinfusions, stylets were removed and injectors made of 32-gauge stainless steel tubing encased by 25-gauge stainless steel were lowered beyond the tip of the guide cannula into the Amy or Acb. Injectors were attached via polyethylene tubing (PE20) to 10 μl Hamilton syringes, and infusions were delivered via a syringe pump (Model A-74900-10: Cole Palmer, Vernon Hills, IL). Simultaneous infusions of 100 nl/side were given over 60 sec to limit injection spread into neighboring brain areas, as well as to minimize diffusion up the injector track. Further, to ensure complete diffusion, injectors were removed 30 sec after completion of the infusion and stylets were replaced.

Table 6 Subject removal

	Initial *n*	Final *n*	Surgery & Recovery	Procedural error	Histology error	Miss	Infection
Experiment 1 *Intra-Acb flupenthixol*	216	109	6	3	8	39	51
Experiment 2 *Intra-Amy flupenthixol*	190	74	36	4	7	50	19
Experiment 3 *Intra-Acb AP-5*	94	63	-	4	2	7	18
Experiment 4 *Disconnection (AP-5/Flupenthixol)*	142	103	3	6	6	12	12

Choice of dopamine and NMDA receptor antagonists. The broad D1/D2 type receptor antagonist *cis*-(Z)-flupenthixol dihydrochloride (flupenthixol), which has similar binding affinities for D1 and D2 type receptors (e.g., Cresse & Hess, 1986), has been previously used to investigate the role of dopamine receptors in the Acb in ethanol reinforcement (e.g., Rassnick et al., 1992) and in the Amy in cocaine-induced conditioned reinforcement (e.g., Di Ciano & Everitt, 2004[a]). Additionally, we chose to use the competitive NMDA receptor antagonist D-(-)-2-Amino-5-phosphonopentanoic acid (AP-5) to investigate the role of intra-Acb NMDA receptors in expression of ethanol CPP, since it has previously been shown that Acb infusions of AP-5 reduced ethanol reinforcement (e.g., Rassnick et al., 1992) and disrupted acquisition of reinforced responding (Kelley, Smith-Roe, & Holahan, 1997).

Experiment 1: Bilateral infusions of flupenthixol into Acb. To examine the role of intra-Acb dopamine receptors in the expression of ethanol CPP, a mixed D1/D2 dopamine receptor antagonist was infused immediately prior to testing. The dopamine antagonist flupenthixol, was obtained from Sigma-Aldrich (St. Lois, MO) and dissolved in artificial cerebrospinal fluid (aCSF). Doses were chosen from previous findings demonstrating effects on cocaine seeking behaviors (Di Ciano & Everitt, 2004) as well as data from pilot studies demonstrating minor effects on locomotor activity in Acb. Mice in six replications (n = 216) were implanted with bilateral cannula aimed at AcbC. Immediately before testing, injectors were lowered 2 mm into AcbC and subjects were infused with aCSF or flupenthixol (1, 10, or 20 μg/side).

Experiment 2: Bilateral infusions of flupenthixol into Amy. To investigate whether intra-Amy D1/D2 receptor activation modulated the expression of ethanol CPP, aCSF or

flupenthixol (10 or 20 μg/side) was infused immediately before testing through bilateral cannula aimed at the BLA/CE. The lowest flupenthixol dose (1 μg/side) was not used. A total of 4 replications (n = 190) were performed.

Experiment 3: Bilateral infusions of AP-5 into Acb. To examine the role of intra-Acb glutamate activation in the expression of ethanol CPP, a NMDA antagonist was infused before testing. The NMDA receptor antagonist D-(-)-2-Amino-5-phosphonopentanoic acid (AP-5) (Ascent Scientific, Weston-Super-Mare, UK; Tocris, Ellsville, MO) was dissolved in aCSF. Initial doses were chosen in part based on findings from a previous report demonstrating deficits in response-reinforcement learning (Kelley et al., 1997). In two replications, mice (n = 94) were infused immediately before testing with aCSF or AP-5 (0.5 or 1.0 μg/side) through bilateral cannula aimed at AcbC.

Experiment 4: Disconnection of the Amy and Acb. To investigate possible dopamine-induced Amy activation and subsequent activation of Acb NMDA receptors via glutamatergic BLA afferents (e.g., Groenewegen et al., 1996) in the expression of ethanol CPP, we utilized a neuropharmacological disconnection procedure in mice. The premise behind the disconnection procedure is that unilateral manipulations of two interconnected nuclei located in opposite hemispheres should reduce behavior if the behavior is dependent upon interactions between the two areas, compared to manipulations within the same hemisphere (e.g., Di Ciano & Everitt, 2004). Initially two replications were performed (n = 94), with disconnection of Amy and Acb made by infusing flupenthixol into the Amy in one hemisphere, while simultaneously infusing AP-5 into Acb in either the same (ipsi-lateral) or opposite (contra-lateral) hemisphere. In the first replication, flupenthixol doses of 10 or 20 μg/side were infused into Amy, while AP-

5 doses of 0.5 or 1.0 μg/side were infused into Acb (see Table 7). Since all treatments reduced preference in the first replication, lower doses of AP-5 (0.05 and 0.15 μg/side) were infused, in combination with the same doses of flupenthixol (10 or 20 μg/side) in the second replication.

Because the second replication also failed to show differences between the ipsi-lateral and contra-lateral groups, a third replication (n = 48) was performed to examine the effect of a single intracranial microinfusion of drug into one area in one hemisphere (e.g., the effect of AP-5 antagonism in a single Acb nucleus on CPP expression). As in the previous replications, all mice were implanted with cannulae aimed at the Amy and AcbC, either ipsi- or contra-lateral to each other. Immediately before testing, however, mice received a unilateral intracranial microinfusion of either flupenthixol (10 or 20 μg/side) into Amy or AP-5 (0.15 or 0.5 μg/side) into Acb. To control for the number of intra-cranial infusions between experiments, mice were given a simultaneous aCSF infusion into the other brain area.

Table 7 Disconnection Groups

Final Group	*n*	Rep	Dose Groups AP-5/Flu (µg/side)	*n*
aCSF	29	1	aCSF	9
		2	aCSF	12
		3	aCSF	8
Contra	20	2	0.05/10	6
			0.15/20	5
		1	0.5/10	5
			1.0/20	4
Ipsi	24	2	0.05/10	6
			0.15/20	5
		1	0.5/10	6
			1.0/20	7
Unilateral AP-5	16	3	0.15	9
			0.5	7
Unilateral-Flu	14	3	10	5
			20	9

Histology

Animals were given an overdose of sodium pentobarbital (150 mg/kg). Heads were removed and postfixed in 4% (w/v) paraformaldehyde in isotonic sodium phosphate buffered saline (PBS). After 24 h, brains were dissected from the skull and placed into a solution of 2% paraformaldehyde for an additional 24 h. After fixation, brains were cryoprotected using a sucrose saturation procedure consisting of 24 h incubations in 20% and then 30% sucrose in PBS and 0.1% NaN_3. Frozen 40 μm sections were collected through the infusion site. Slices were directly mounted onto slides and thionen stained. Placements were subjectively assessed blind to dose, hemisphere, disconnection group, disconnection placement, and test outcome. Inclusion criteria were as follows: subjects with bilateral injector tracks within AcbC were included in analyses in Experiments 1 and 3. Although inclusion criteria specified injector tracks within AcbC, given the close proximity and possibility of drug diffusion into the AcbSh we present results as infusions in Acb and do not make AcbC/AcbSh distinction. In experiment 2, subjects with bilateral injector tracks located within BLA and/or CE were included in analyses. For experiment 4, subjects were included if they had one injector track within AcbC and one injector track located within BLA and/or CE. To identify possible contributions of specific nuclei in the Amy within experiment 2, a subset of subjects with bilateral injector tracks specifically in BLA or CE were compared with each other and to subjects with injector tracts in both areas. Although drug diffusion spread was not specifically examined in these studies, subjects who met inclusion criteria were also compared to subjects with injector placement in the basomedial nuclei (BM) (directly below the BLA and CE) of Amy as a site control.

Data Analyses

The primary dependent variable was the amount of time spent on the grid floor during the test session. In this unbiased design, the magnitude of the difference in time spent on the grid floor between the Grid+ and Grid- conditioning subgroups is indicative of CPP. See Cunningham et al. (2003) for a more complete discussion of dependent variables used in place conditioning studies. Data from each experiment were evaluated separately by ANOVA with the alpha level set at 0.05. To control overall alpha level within each experiment, p-values were Bonferroni corrected for the number of post-hoc comparisons between group means. Dose, Conditioning Subgroup (Grid+ vs. Grid-), Replication, Amy Site, Disconnection Placement (ipsi- vs. contra-lateral), Disconnection Group (Experiment 4), and Hemisphere (left vs. right) were treated as between-group factors, whereas Trial Type (CS+ vs. CS-) was treated as a within-subject factor.

Results

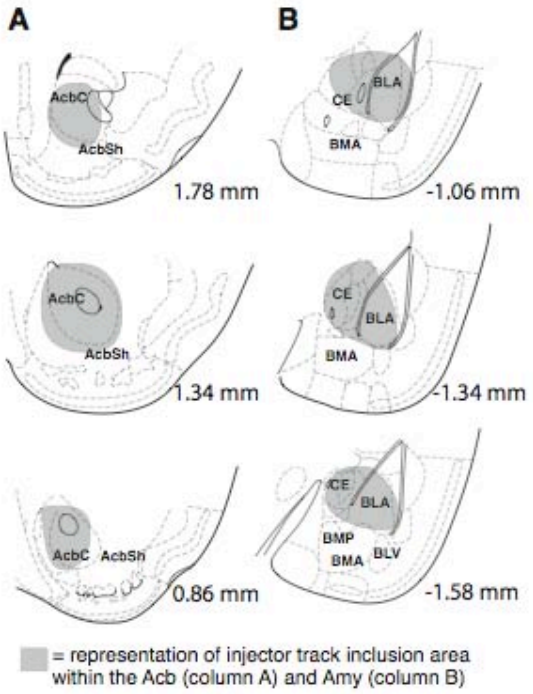
Histological verification and subject removal

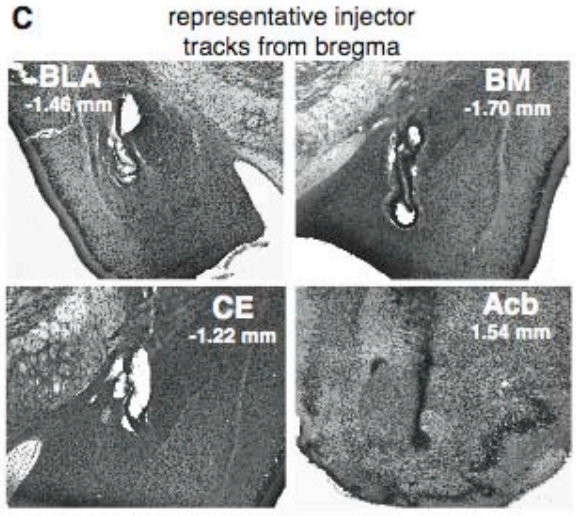
Schematic diagrams of inclusion criteria and representative photomicrographs are shown in Figure 8. A total of 291 subjects were removed from the final analyses for various reasons, including: poor health during recovery following surgical procedures (Surgery& Recovery), procedural errors during conditioning and testing (Procedural error), an inability to accurately assign injector placement due to problems with histological assessment (Histology error), incorrect injector placement (Miss), or an infection at the injector and/or cannula site (Infection) (see Table 6). In experiment 1, and to a lesser degree in experiments 2 and 3, a relatively large percentage of mice had infections located near the end of the cannula. Although the causes are unknown, this

higher rate of infection may have been due to a longer delay and additional microinfusions between the first microinfusion and brain removal. In experiment 1, brains were removed after all mice had received three microinfusion tests at 24-h intervals (only data from the first test are reported here). In experiments 2 and 3, however, about half of the brains were removed immediately after the first test while the others were removed after only one additional test. In experiment 4, which showed the lowest attrition due to infection, all brains were removed immediately after the first test.

Figure 8 Representative diagram and photomicrograhs of nucleus accumbens (Acb) and amygdala (Amy) injector placements. Representative injector inclusion area criteria are shown for Acb (column A) and Amy (Column B). In column C, photomicrographs of representative injector tracks into basolateral amygdala (BLA) (upper left panel), central nucleus of the amygdala (CE) (lower left panel), basomedial nucleus of the amygdala (BM) (upper right panel), and Acb (lower right panel). Numbers indicate the distance from bregma in millimeters of the section (Paxinos & Franklin, 2001).

Figure 8


A
B
AcbC
AcbSh
1.78 mm
CE
BLA
BMA
-1.06 mm
AcbC
AcbSh
1.34 mm
CE
BLA
BMA
-1.34 mm
AcbC
AcbSh
0.86 mm
CE
BLA
BMP
BMA
BLV
-1.58 mm
= representation of injector track inclusion area within the Acb (column A) and Amy (column B)


C
representative injector tracks from bregma
BLA
-1.46 mm
BM
-1.70 mm
CE
-1.22 mm
Acb
1.54 mm

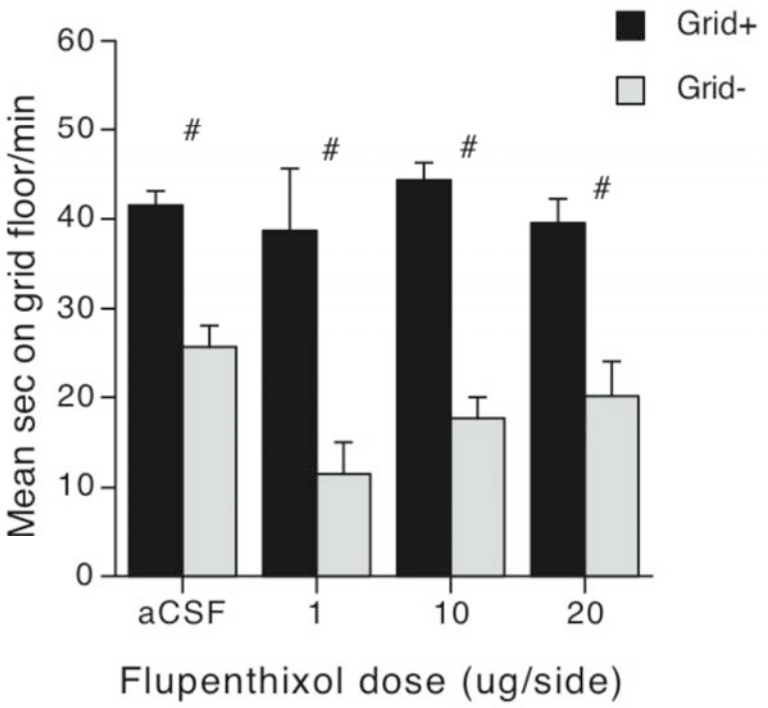
Additionally, many mice in Experiment 2 were lost during surgical recovery (Table 6). We previously reported significantly larger losses during recovery in mice with bilateral electrolytic lesions of the Amy in comparison to mice with Acb lesions (Gremel & Cunningham, 2008). In the current studies, significantly larger losses were observed during recovery in mice with Amy cannulae than in those with Acb cannulae ($z = 9.16$, $p < 0.05$). Because no infusions were made into Amy until testing, it may be that the region just above the Amy in mice is especially sensitive to bilateral cannula insertion.

Place Preference Test

Experiment 1: Effects of intra-Acb D1/D2 type receptor antagonism on CPP expression. In experiment 1, we examined the effect of an intra-Acb flupenthixol infusion (1, 10, or 20 mg/side) on expression of ethanol CPP. As can be seen in Figure 9, pretreatment with intra-Acb flupenthixol had no impact at any dose, yielding CPP similar to that seen in aCSF control mice. A two-way (Dose x Conditioning Subgroup) ANOVA revealed a significant main effect of Conditioning Subgroup (Grid+ vs. Grid-) [$F(1,101) = 90.0$, $p < 0.001$], but no effect of dose or interaction. Further analysis showed no effect of replication in aCSF control mice ($p > 0.05$) (to create reasonable subgroup *n's* for this analysis, data were collapsed across replicates 1-3, then compared to replicates 4-6). Thus, expression of ethanol CPP did not depend upon D1/D2 type receptor activation in Acb.

Figure 9 Intra-Acb (nucleus accumbens) microinfusions of flupenthixol did not affect expression of ethanol CPP. Mean sec per min (+SEM) spent on the grid floor during the 30-min test session. Subjects in the Grid+ conditioning subgroups (solid bars) received ethanol paired with the grid floor on CS+ trials, and saline paired with the hole floor. These contingencies were reversed in the Grid- conditioning subgroup subjects (grey bars). N's for Grid+ and Grid- conditioning subgroups are: aCSF n = 28 and 18; 1 μg/side n = 5 and 4; 10 μg/side n = 13 and 12, and 20 μg/side n = 15 and 14. # = Main effect of conditioning between Conditioning Subgroups, $p < 0.001$.

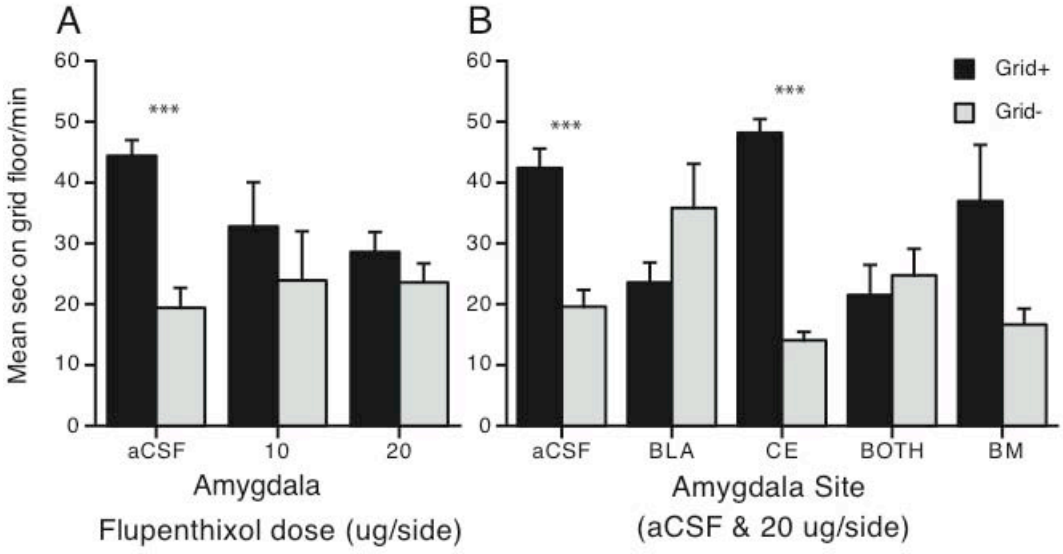
Figure 9


Grid+
Grid-
Mean sec on grid floor/min
60
50
40
30
20
10
0
#
#
#
#
aCSF
1
10
20
Flupenthixol dose (ug/side)

Experiment 2: Effects of intra-Amy D1/D2 type receptor antagonism on CPP expression. To determine whether dopamine receptor activation in Amy modulated expression of ethanol CPP, mice in experiment 2 were given intra-Amy infusions of flupenthixol immediately before testing. As in experiment 1, aCSF-treated mice displayed a strong CPP in experiment 2 (see Figure 10A). In contrast, intra-Amy flupenthixol infusion disrupted CPP expression at both doses (10 and 20 μg/side), i.e., there was no difference between Grid+ and Grid- conditioning subgroups. A two-way (Dose x Conditioning Subgroup) ANOVA revealed a significant main effect of Conditioning Subgroup (Grid+ vs. Grid-) [$F(1,68) = 11.8$, $p < 0.01$] and a significant interaction [$F(2,68) = 4.9$, $p < 0.05$]. There was no effect of dose. Post hoc analysis of the interaction showed a significant CPP in the aCSF group (Bonferroni corrected $p < 0.001$), but not in the 10 or 20 mg/side dose groups (p's > 0.05). Moreover, follow-up ANOVAs revealed that preference in the highest flupenthixol dose group (20 μg/side) was significantly lower than that in aCSF control mice (Dose x Conditioning Subgroup interaction: $F(1,62) = 9.8$, $p < 0.01$), whereas mice infused with 10 μg/side did not differ from either the aCSF or 20 μg/side groups (p's > 0.05). A separate analysis performed on data from aCSF-treated mice showed no effect of replication, indicating that preference was similar in the control group across all four replicates. Thus, D1/D2 type receptor antagonism within the Amy blocked ethanol CPP expression.

Figure 10 Flupenthixol infused into the amgydala (Amy) disrupts expression of ethanol CPP. Mean sec per min (+SEM) spent on the grid floor during the 30-min test session. (A) Effects of intra-Amy [basolateral amgydala (BLA) and central nucleus of the amgydala (CE)] infusions of flupenthixol on expression of ethanol CPP. Grid+ and Grid- conditioning subgroup N's are: aCSF n = 13 and 18; 10 μg/side n = 4 and 4; and 20 μg/side n = 18 and 17. (B) Flupenthixol infusions into the BLA, but not CE disrupt expression of ethanol CPP. Test data for aCSF and 20 μg/side dose groups grouped by injector site within the Amy, combined with subjects (aCSF and 20 μg/side) with injector placements within the basomedial nucleus of the amgydala (BM). Grid+ and Grid- Conditioning subgroup N's are: aCSF n = 15 and 22; BLA n = 10 and 4; CE n = 4 and 6; Both n = 4 and 7, and BM n = 3 and 3. Difference between conditioning subgroups Grid+ and Grid-: *** = Bonferroni corrected ps < 0.001.

Figure 10


A
B
Mean sec on grid floor/min
60
50
40
30
20
10
0
***
***
***
aCSF
10
20
Amygdala
Flupenthixol dose (ug/side)
aCSF
BLA
CE
BOTH
BM
Amygdala Site
(aCSF & 20 ug/side)
Grid+
Grid-

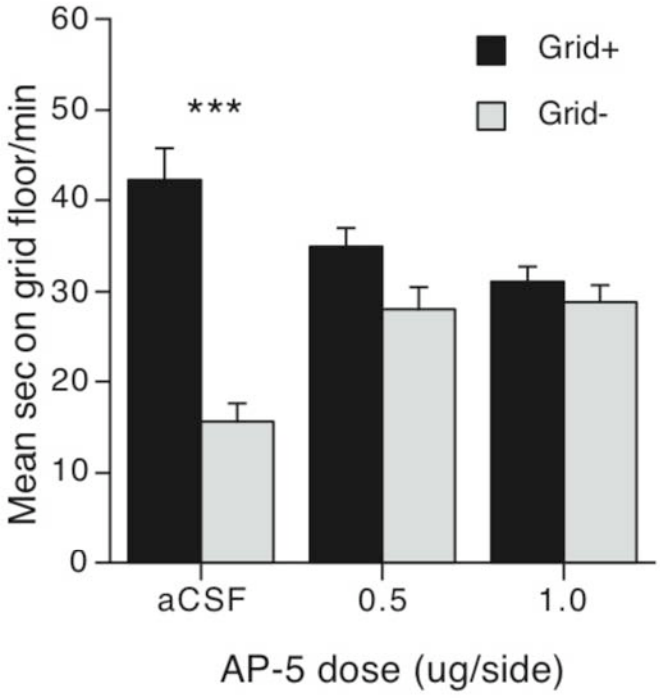
Experiment 2: Differing effects of D1/D2 receptor antagonism in the BLA or CE on CPP expression. To examine the contributions of specific nuclei within the Amy on CPP expression, comparisons were made between mice with bilateral infusions of flupenthixol (20 mg/side) into only BLA (n = 14) or only CE (n = 10) and mice with bilateral infusions into BLA and CE (Both Group, n = 11). Further, for a site control comparison, these groups were compared to mice with bilateral infusions (20 μg/side) into BM (n = 6), which was a common histological miss site.

As shown in Figure 10B, magnitude of ethanol CPP varied between flupenthixol-treated mice depending on Amy Site [Site x Conditioning Subgroup interaction: $F(3,24) = 11.3$, $p < 0.001$]. Of particular interest, mice in the BLA and Both Groups did not display preference after flupenthixol infusion (p's > 0.05), whereas subjects with flupenthixol infusions into the CE showed strong ethanol CPP (Bonferroni corrected $p < 0.001$). Although there was an arithmetic trend toward preference in the BM group, the difference fell short of significance ($p = .11$), most likely due to the low *n* ($n = 6$). Additional two-way (Amy Site x Conditioning Subgroup) ANOVAs performed between specific pairs of Amy Sites did not reveal any differences between CE and BM (p's > 0.05). However, these analyses showed that the BLA and Both Groups each differed significantly from both the CE and BM groups (F's > 4.7, p's < 0.05). Moreover, the BLA and Both groups did not differ from each other (p's > 0.05). Overall, these findings suggest that any D1/D2 type receptor antagonism within the BLA (as evidenced in BLA and Both groups), but not CE, significantly disrupts expression of ethanol CPP. Separate analysis of aCSF control mice showed that CPP did not depend upon site of the aCSF infusion [Amy Site x Conditioning Subgroup interaction: $F(3,29) = 1.8$, $p > 0.05$].

Experiment 3: Effects of intra-Acb NMDA receptor antagonism on CPP expression. Experiment 3 examined the role of the NMDA receptor in Acb on expression of ethanol CPP. Intra-Acb infusion of the NMDA receptor antagonist AP-5 significantly disrupted CPP expression (Figure 11). Two-way (Dose x Conditioning Subgroup) ANOVA revealed a significant main effect of Conditioning Subgroup (Grid+ vs. Grid-) [$F(1,57) = 36.7$, $p < 0.001$] and a significant interaction [$F(2,57) = 15.1$, $p < 0.001$]. Control (aCSF) mice showed strong ethanol CPP as indicated by the large difference between the Grid+ and Grid- conditioning subgroups (Bonferroni corrected $p < 0.001$). However, intra-Acb AP-5 infusions (0.5 or 1.0 μg/side) immediately before testing interfered with expression of ethanol CPP (Bonferroni corrected p's > 0.05). Although the two AP-5 dose groups did not differ from each other (p's > 0.05), both were significantly different from aCSF controls (Dose x Conditioning Subgroup interactions: F's > 14.7, p's < 0.001). A separate two-way (Replication x Conditioning Subgroup) ANOVA showed no effect of replication in aCSF controls. Overall, these findings demonstrate that expression of ethanol CPP is dependent upon NMDA receptor activation within the Acb.

Figure 11 Infusions of AP-5 into the nucleus accumbens core (AcbC) disrupted expression of ethanol CPP. Mean sec per min (+SEM) spent on the grid floor during the 30 min of the test session. Grid+ and Grid- conditioning subgroups N's are respectively: aCSF n = 10 and 13; 0.5 μg/side n = 9 and 12; 1.0 μg/side n = 11 and 8. Difference between conditioning subgroups Grid+ and Grid-: *** = Bonferroni corrected ps < 0.001.

Figure 11


60
50
40
30
20
10
0
Mean sec on grid floor/min
***
Grid+
Grid-
aCSF
0.5
1.0
AP-5 dose (ug/side)

Experiment 4: Disconnection of the Acb and Amy. To examine whether dopamine activation of the Amy leading to glutamatergic modulation of the Acb modulates expression of ethanol CPP, we used a neuropharmacological disconnection procedure antagonizing dopamine receptors in the Amy in one hemisphere, and intra-Acb NMDA receptors in the ipsi- or contra-lateral hemisphere. The first two replications yielded a strong CPP in aCSF controls that was reduced by ipsi- or contra-lateral drug infusions (data not shown). However, disconnection group did not differentially affect ethanol CPP in either replication (p's > 0.05 for main effect and all interactions with Disconnection Group). Because the subgroup *n's* within each replicate were low (see Table 7), we conducted an additional analysis in which the data from the disconnection groups were pooled to increase statistical power (see Figure 12). This analysis revealed a significant Disconnection Group x Conditioning Subgroup interaction [$F(2,67) = 10.2$, $p < 0.0001$], reflecting significant CPP in the aCSF control group (Bonferroni corrected $p < 0.0001$) and ipsi-lateral group (Bonferroni corrected $p = 0.02$), but not in the contra-lateral group ($p > 0.2$). However, there was no significant difference in a direct comparison between the ipsi- and contra-lateral groups [Disconnection group x Conditioning Subgroup interaction: $F(1,40) = 0.3$, $p > 0.5$], indicating no difference between the disconnection manipulations on the expression of ethanol CPP. Moreover, the aCSF group showed significantly greater CPP when compared to either the ipsi- [$F(1,49) = 11.2$, $p < 0.005$] or contra-lateral [$F(1,45) = 16.7$, $p < 0.0001$] groups. Thus, we were unable to confirm a unique role for glutamatergic modulation of Acb via dopamine activation of Amy.

Figure 12 Effects of neuropharmacological disconnection of the amygdala (Amy) and nucleus accumbens core (AcbC). Subjects were given intra-Amy aCSF or flupenthixol infusions and contra- or ipsi-lateral intra-AcbC infusions of aCSF or AP-5. Groups were given the following infusions: aCSF mice were given infusions of aCSF into both the Amy and AcbC. Mice in the Contra group were infused with flupenthixol (10 or 20 μg) into the Amy in one hemipshere, with AP-5 (0.05, 0.15, 0.5, or 1.0 μg) infused into the Acb in the opposite hemisphere. Ipsi mice were given intra-Amy infusions of flupenthixol (10 or 20 μg) and intra-Acb (0.05, 0.15, 0.5, or 1.0 μg) into the same hemisphere. Grid+ and Grid- conditioning subgroups N's are respectively: aCSF n = 14 and 15; Contra n = 10 and10; Ipsi n =12 and 12. Difference between conditioning subgroups Grid+ and Grid-: * = Bonferroni corrected ps < 0.05; *** = Bonferroni corrected ps < 0.001.

Figure 13 To examine the effects of NMDA or D1, D2 receptor antagonism of a single nucleus accumbens (Acb) or amygdala (Amy) nucleus, unilateral drug infusions were given. Mice in the AP-5 group were infused with aCSF into one Amy, and AP-5 (0.15 or 0.5 μg) into one AcbC. Mice in the Flu group were given a flupenthixol infusion (10 or 20 μg) into one Amy, and aCSF into one AcbC. Infusions were counterbalances between hemispheres with both ipsi- and contra- lateral placements. Unilateral AP-5 n = 9 and 7; and Unilateral Flu n = 7 and 7. Difference between conditioning subgroups Grid+ and Grid-: *** = Bonferroni corrected ps < 0.001.

Figure 12

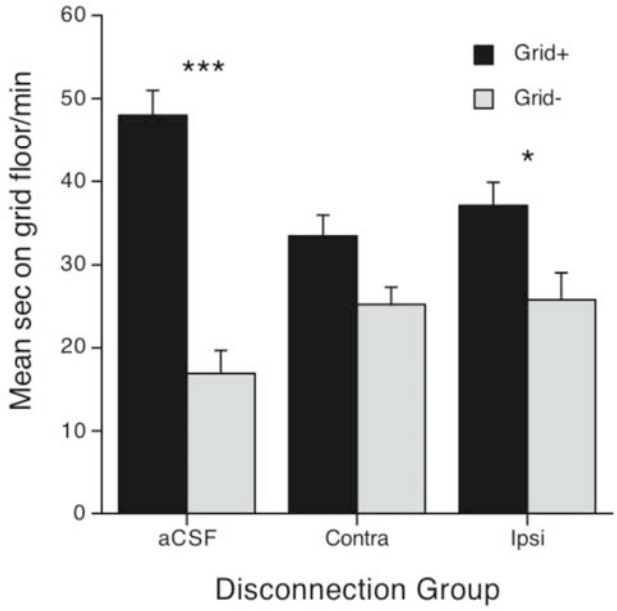
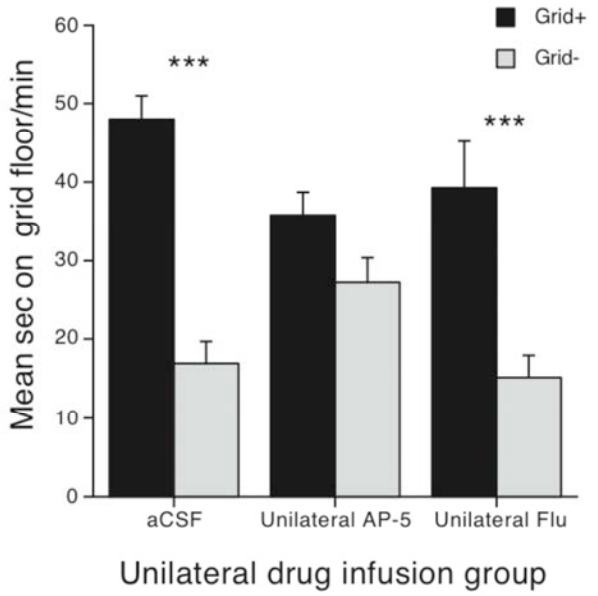


Figure 13



In an effort to better understand the reasons behind the outcome of our disconnection studies, mice in replicate 3 received unilateral infusions of AP-5 into Acb or Flu into Amy (see Table 7). As shown in Figure 13, unilateral drug infusions had a significant effect on CPP [Unilateral Dose x Conditioning Subgroup interaction: $F(2,53) = 5.7$, $p < 0.01$], reflecting significant CPP in the aCSF control group (Bonferroni corrected $p < 0.0001$) and unilateral Flu (Amy) group (Bonferroni corrected $p = 0.0001$), but not in the unilateral AP-5 (Acb) group ($p > 0.3$). Pair-wise group comparisons showed that aCSF controls differed from the unilateral AP-5 (Acb) group [$F(1,41) = 12.5$, $p = 0.001$], but not from the unilateral Flu (Amy) group [$F(1,39) = 0.9$, $p > 0.3$]. The two unilateral infusion groups were marginally different [$F(1,26) = 4.2$, $p = 0.052$]. A separate analysis showed that there was no effect of ipsi- versus contra-lateral infusion in aCSF controls. Findings from the unilateral AP-5 (Acb) group suggest that intra-Acb NMDA receptor antagonism in one hemipshere may be sufficient to disrupt preference independent of dopamine blockade in Amy.

Locomotor Activity

Group means and statistical comparisons for conditioning and test activity are shown in Table 8.

Table 8 Locomotor Activity

	Dose µg/side	*Final n*	CS+ conditioning trials	CS- Conditioning trials	Mean test activity counts/min ± SEM
Experiment 1					
Intra- AcbC Flupenthixol	aCSF	46	195.97 ± 5.4	76.23 ± 2.3	46.78 ± 1.7
	1	9	178.25 ± 5.2[e]	62.96 ± 4.0[e]	33.652 ± 3.3[c]
	10	25	210.08 ± 6.2	79.32 ± 3.2	36.28 ± 2.4[b]
	20	29	203.38 ± 6.6	79.97 ± 4.9	30.478 ± 2.2[a]
			Dose Group: F(3,105) = 3.2* Trial Type: F(1,105) = 966.9*** Interaction: F(3,105) = 0.7		Dose Group: F(3,105): 12.8***
Experiment 2					
Intra-Amy Flupenthixol	aCSF	31	177.11 ± 4.5	69.3 ± 2.5	39.38 ± 2.1
	10	8	158.55 ± 12.4	65.94 ±6.3	33.09 ± 2.5
	20	35	179.1 ± 5.6	67.7 ± 2.6	26.97 ± 1.6[a]
			Dose Group: F(2,71) = 1.4 Trial Type: F(1,71) = 527.2*** Interaction: F(2,71) = 1.2		Dose Group: F(2,71) = 11.7***
Experiment 3					
Intra-AcbC AP-5	aCSF	23	182.51 ± 7.7	69.04 ± 2.3	41.17 ± 2.3
	.5	21	189.21 ± 7.9	74.18 ± 3.5	51.46 ± 3.0
	1.0	19	190.86 ± 10.6	72.1 ± 4.2	73.77 ± 6.1[a,d]
			Dose Group: F(2,60) = 0.4 Trial Type: F(1,60) = 870.6*** Interaction: F(2,60) = 0.2		Dose Group: F(2,60) = 17.8***
Experiment 4					
Disconnection AP-5 and/or Flupenthixol	aCSF	29	189.78 ± 5.6	62.00 ± 2.1	35.62 ± 1.4
	Contra	20	189.83 ± 7.5	63.97 ± 2.9	35.36 ± 2.2
	Ipsi	24	192.37 ± 5.9	67.65 ± 2.3	37.81 ± 2.6
			Dose Group: F(2,70) = 0.3 Trial Type: F(1,70) = 1509.7*** Interaction: F(2,70) = 0.9		Disconnection Group: F(2,70) = 0.4
	Unilateral AP-5	16	187.65 ± 7.8	68.60 ± 3.7	45.19 ± 2.4[b,f]
	Unilateral Flu	14	182.79 ± 7.3	66.79 ± 4.0	30.47 ± 2.3
			Disconnection Group: F(6,96) = 0.3 Trial Type: F(1,96) = 1802.1*** Interaction: F(6,96) = 0.6		Disconnection Group: F(6,96) = 3.9***

* $p < 0.05$, *** $p < 0.001$ (p-values for all group comparisons are Bonferroni-corrected).
[a] = difference from aCSF Group, $p < 0.001$, [b] = difference from aCSF Group, $p < 0.01$, [c] = difference from aCSF Group, $p < 0.05$, [d] = 1.0 µg/side Group significantly different from 0.5 µg/side Group, $p < 0.05$, [e] = 1 µg/side Group activity means significantly different from 10 µg/side Group mean, $p < 0.05$, [f] = Unilateral AP-5 test activity means significantly different from Unilateral Flu, $p < 0.001$.

Conditioning Activity. Since a three-way ANOVA (Dose Group x Trial x Trial Type) performed on conditioning activity data from each experiment did not reveal any effects of assigned expression dose group on locomotor activity during conditioning trials [Fs' < 0.8, ps' > 0.2], and all infusion groups in each experiment displayed a similar level of locomotor sensitization to ethanol [Trial x Trial Type interaction: Fs' > 12, ps' < 0.0001; paired t-tests (first CS+ trial versus fourth CS+ trial): ts' >3.8, ps' < 0.001], conditioning activity data were collapsed across trials to create single means for the CS+ and CS-. As in previous experiments, ethanol induced large increases in locomotor activity on CS+ trials (e.g., Cunningham et al., 2003; Cunningham et al., 2006[a]; Gremel & Cunningham, 2007). In experiment 1, overall activity levels (combined CS+ and CS- trials) were slightly higher in the 10 μg/side group than in the 1 μg/side group, reflecting a small sampling difference between dose groups (i.e., there was no difference in experimental manipulations between the groups at this point). However, no group effects were seen in any of the other experiments, suggesting that all groups within each experiment had similar activity responses during conditioning.

Test Activity. D1/D2 type receptor antagonism in the Acb decreased test activity levels (experiment 1). All flupenthixol-treated groups (1, 10, or 20 μg/side) showed significantly lower levels of activity than aCSF infused controls, but there were no differences among the flupenthixol groups. Similarly, intra-Amy flupenthixol (20 μg/side) significantly lowered test activity levels in comparison to aCSF (experiment 2). However, there were no differences between the aCSF and 10 mg/side dose groups, or between the 10 and 20 μg/side dose groups in experiment 2. In contrast, NMDA receptor antagonism in the Acb generally increased test activity levels (experiment 3). Infusions of

the high AP-5 dose (1.0 μg/side) significantly increased activity levels compared to either the low AP-5 dose (0.5 μg/side) or aCSF. However, aCSF and 0.5 mg/side Dose Groups did not differ. In experiment 4, the unilateral AP-5 (Acb) group had higher activity levels than either the unilateral Flu (Amy) or aCSF groups. There were no effects of replication on test activity levels in the aCSF groups in any of these experiments.

Discussion

These are the first studies in any species to demonstrate involvement of dopamine receptor activation within BLA and the first studies in mice to implicate Acb NMDA receptors in the expression of an ethanol-conditioned behavior (CPP). Moreover, these studies show that dopamine receptors within CE and Acb are <u>not</u> involved in the expression of such behavior. Although it is not known whether these effects reflect a decrease in the conditioned value of the cue, impaired retrieval of the cue-drug association, or a decrement in the learning or performance of the approach response, these studies offer important new information about the specific receptor systems within Amy and Acb that modulate behaviors controlled by an ethanol-paired cue.

Ethanol CPP expression does not depend on dopamine activation in Acb. Surprisingly, expression of ethanol-induced CPP in mice was not dependent upon D1/D2-type receptor activation in Acb. This finding contrasts with previously reported dopamine antagonist effects on the reinforcing effect of ethanol in rats as indexed by operant SA procedures (e.g., Rassnick et al., 1992; Samson et al., 1993, Hodge et al., 1994) and by ethanol conditioned appetitive responding in an SA procedure (Czachowski et al., 2001, 2002; Samson & Chappell, 2004). A possible explanation may be that the target response in CPP (i.e., approach towards the ethanol-paired cue) has never produced ethanol,

whereas the target response in an SA procedure (e.g., barpressing) has previously produced the primary reinforcer and may therefore depend upon intra-Acb dopamine transmission. However, it is also possible that there is a more fundamental species (i.e., mouse vs. rat) difference in the role played by Acb in the expression of ethanol-conditioned behaviors. This possibility is supported by a recent study in which an intra-AcbSh dopamine antagonist was reported to reduce expression of CPP induced by an intra-cerebroventricular (icv) ethanol injection in rats (Walker & Ettenberg, 2007), a finding that is at odds with our finding of no effect on ethanol CPP in mice. Thus, although Acb dopamine receptors may be involved in the expression of ethanol conditioned behaviors in rats (e.g., Samson & Chappell, 2004; Walker & Ettenberg, 2007), the current findings suggest that the hypothesized alterations in Acb dopamine receptor activation resulting from increases in VTA dopamine neuron firing do not contribute to expression of ethanol-induced CPP in mice.

Ethanol CPP expression depends on dopamine activation in Amy. These studies provide the first experimental evidence for the role of intra-Amy dopamine receptors in the expression of any ethanol-conditioned behavior in either rats or mice. Moreover, our data suggest that nuclei within the Amy play different roles in dopamine mediation of ethanol-conditioned behavior because CPP expression was blocked in mice that received flupenthixol infusions into BLA, but not in mice that were infused only into CE. Although several other receptors within CE have been implicated in the modulation of ethanol SA (e.g., CRF: Funk et al., 2006; Funk & Koob, 2007; $GABA_A$: Hytiaä & Koob, 1995; Roberts et al., 1996; Serotonin: Dyr & Kostowski, 1995) or ethanol CPP in rats (NMDA: Zhu et al., 2007), it does not yet appear that any ethanol SA study has shown a

functional role for dopamine receptors within CE. Reports of increased FOS activation in the BLA in rats after exposure to a cue previously paired with ethanol (Radwanska et al., 2007; Zhao et al., 2006), as well as the observation of VTA dopamine projections to BLA in DBA/2J mice (Ford et al., 2006) lend support to our conclusion of intra-BLA dopamine receptor involvement in the modulation of ethanol conditioned behavior.

NMDA receptors in Acb modulate ethanol CPP expression. Although dopamine activation in Acb is not necessary, NMDA receptor activation within Acb appears to be critical for expression of ethanol CPP. Infusions of AP-5 aimed at AcbC blocked expression of ethanol CPP. Although the highest dose of AP-5 increased locomotor activation, which may complicate interpretation of CPP results (Gremel & Cunningham, 2007), the lowest dose was sufficient to block CPP expression without locomotor effects, eliminating non-specific interpretations of this outcome. Our finding is in concordance with a previous study showing that NMDA antagonist infusion into Acb disrupted ethanol SA in rats (Rassnick et al., 1992). Moreover, expression of morphine-induced CPP in rats has also been found to depend on NMDA receptor activation in Acb (Popik & Kolasiewicz, 1999). While it has previously been shown that NMDA receptors within Acb mediate ethanol's physiological effects (e.g., Maldve et al., 2002; Nie et al., 1994), these are the first data demonstrating a functional role for intra-Acb NMDA receptors in ethanol-conditioned behavior in mice.

Learning processes underlying CPP expression. In contrast to most ethanol SA procedures, the CPP procedure provides a way to investigate ethanol-conditioned behaviors in the absence of ethanol's direct effects. CPP also allows experimenters to measure an ethanol conditioned response that has never produced the primary reinforcer

(i.e., ethanol). The conditioned response is measured as approach and maintenance of contact with the previously drug-paired cue, without administration of the conditioning drug. Theoretically, Pavlovian conditioned approach behavior, conditioned reinforcement, and conditioned incentive may all be operating in CPP (e.g., Kumar, 1972; Swerdlow, Gilbert, & Koob, 1989; Uslaner et al., 2006). However, we have hypothesized that only the latter two processes are likely involved in our procedure because mice are responding to a tactile cue presented in the dark (Cunningham et al., 2006[b;] Gremel & Cunningham, 2008).

Our finding that the Amy is importantly involved in ethanol CPP is generally consistent with a broader literature implicating Amy in the learning or expression of other conditioned appetitive behaviors (see reviews by: Everitt, Cardinal, Parkinson & Robbins, 2003; Everitt & Robbins, 2005; Holland & Gallagher, 1999). For example, several studies have suggested that conditioned reinforcement is regulated by the BLA (e.g., Burns, Robbins & Everitt, 1993, Cador, Robbins, & Everitt, 1989, Whitelaw Markow, Robbins, & Everitt, 1996), whereas CE modulates Pavlovian conditioned approach behavior (e.g., Parkinson et al. 2000). Although our previous lesion study demonstrated that expression of ethanol CPP was dependent upon an intact Amy (Gremel & Cunningham, 2008), the current findings suggest that activation of dopamine receptors specifically within BLA, not CE, is necessary for the behavior. The critical role played by BLA dopamine receptors is further corroborated by data showing that these receptors modulate cocaine-induced conditioned reinforcement on a second-order schedule in rats (Di Ciano & Everitt, 2004).

While Acb is also important in the expression of other appetitive-conditioned behaviors (see review by: Everitt & Robbins, 2005), the role of NMDA receptors within AcbC is less clear. For example, antagonism of intra-AcbC NMDA receptors with AP-5 had little effect responding for a cocaine-conditioned reinforcer (Di Ciano & Everitt, 2001) and impaired only the acquisition, not expression, of Pavlovian approach behavior (Di Ciano et al., 2001). However, AcbC NMDA receptors have been implicated in response-outcome learning (e.g., Baldwin, Holahan, Sadegian, & Kelley, 2000; Kelley et al., 1997), an outcome that may be consistent with our finding showing that blockade of Acb NMDA receptors reduced ethanol CPP expression. Overall, these findings raise an interesting hypothesis about the processes underlying ethanol CPP. Perhaps during expression testing, intra-Acb NMDA receptors govern initial learning of BLA dopamine-mediated responding for the conditioned reinforcer.

Effects of neuropharmacological disconnection of the Amy and Acb. We attempted to determine whether glutamate activation of Acb NMDA receptors was dependent upon dopamine activation of the Amy. However, because both ipsi- and contra-lateral manipulations were effective at reducing CPP expression, we were not able to support that hypothesis, leaving open the possibility of modulation by glutamate projections from cortex or hippocampus. Although glutamatergic input arising from Amy might nevertheless modulate ethanol CPP, the finding of significant blockade by a unilateral AP-5 infusion precludes any conclusions about a unique role for the Amy-Acb neuroanatomical connection. While this effect might be specific to the use of mice or CPP, Baldwin et al. (2000) reported a similar effect in rats, showing that unilateral AP-5 infusion into Acb impaired acquisition of instrumental learning.

The role of Acb and Amy dopamine receptors, and Acb NMDA receptors in locomotor activity during testing. In the present findings, D1/D2 receptor antagonism in both the Acb and Amy reduced activity levels during testing (see Table 8). Previous studies that systemically blocked D1 and D2 receptors had similar effects on locomotor activity during testing (e.g., Dickinson et al., 2003; Holmes, Lachowiez, & Sibley, 2004), suggesting that dopamine receptors within the Acb and Amy modulate activity levels in mice. Additionally, similar to previous systemic findings with competitive NMDA antagonists (Boyce-Rustay & Cunningham, 2004), AP-5 infused into the Acb increased locomotor activity during testing. We have previously shown a negative correlation between test activity levels and magnitude of ethanol CPP expressed (Gremel & Cunningham, 2006). However, since the lowest effective AP-5 dose (0.5 μg/side) had no affect on activity levels, the lack of preference expressed in AP-5 infused mice was most likely not due to increased locomotor activity levels during testing.

Summary. Our data show that expression of ethanol CPP in mice is mediated by dopamine receptors in Amy and by NMDA receptors in Acb. Moreover, within Amy, dopamine receptor activation within BLA, but not CE, modulated CPP expression. Surprisingly, dopamine receptor activation within Acb had no effect. Although several of these findings are consistent with previous findings in rats on mechanisms underlying other appetitive conditioned behaviors, our data on the role of Acb dopamine receptors in mouse CPP are at odds with operant ethanol SA and ethanol CPP findings in rats, raising the possibility of a species difference in the mechanisms underlying Acb's role in the expression of ethanol conditioned behaviors. Nevertheless, the similarity between the neural mechanisms involved in mouse ethanol CPP and those underlying conditioned

reinforcement in rats encourages continued use of the CPP procedure to examine the learning and motivational processes underlying the acquisition, maintenance and extinction of ethanol-seeking behaviors.

Chapter 4: General Discussion

The findings in this thesis identify neural areas and mechanisms underlying the expression of a cue-induced ethanol-seeking behavior. Specifically, expression of ethanol CPP was found to depend on an intact Amy, while lesions of the AcbC facilitated loss of expression responding (Chapter 2). Further, it appears that expression is dependent on Amy dopamine receptor activation, specifically in the BLA and not CE, and Acb NMDA receptor activation (Chapter 3). Additionally, I demonstrated that acquisition of cue-induced ethanol-seeking behavior depends upon an intact Acb and Amy, since lesions disrupted the acquisition and subsequent expression of ethanol CPP (Chapter 2). Based on the these findings, I suggest that the Amy and Acb influence the reinforcing, learning, and/or memory processes that underlie cue-induced ethanol-seeking behavior

Many abused drugs are thought to exert their actions through common mechanisms, such as increases in Acb dopamine (e.g., Di Chiara & Imperato, 1988), through which they control drug behavior leading to dependence. The incentive sensitization theory postulates this “commonality of action”, and suggests that chronic drug states induce an upregulation of the mesolimbic dopamine system, which establishes compulsive drug-wanting and development of dependence (Berridge & Robinson, 1998; Robinson & Berridge, 1993, 2000). Others have hypothesized that drug abuse and dependence is dependent upon opponent motivational processes with continued use driven by avoidance of dyshporia and anhedonia (e.g., Solomon, 1980; Koob & Bloom, 1988). The work presented in this thesis is focused more on the argument that addiction may be understood as drug-induced control over learning and memory systems that normally guide behaviors towards natural reinforcers and instead focuses them on drug-

seeking behaviors (e.g., Everitt, Dickinson, & Robbins, 2001). In humans, drug seeking is a foraging behavior that is guided by cues in the environment where the drug is not freely available. Associative learning processes are necessary to appropriately direct and maintain behaviors aimed at accessing the drug. Since drug predictors may maintain seeking behaviors and trigger relapse after periods of abstinence (e.g., Grüsser et al., 2004; Volkow et al., 2006), identifying the neurobiological mechanisms underlying the ability of cues in the environment to control ethanol-seeking behaviors is critical to understanding the development and maintenance of alcohol abuse and dependence.

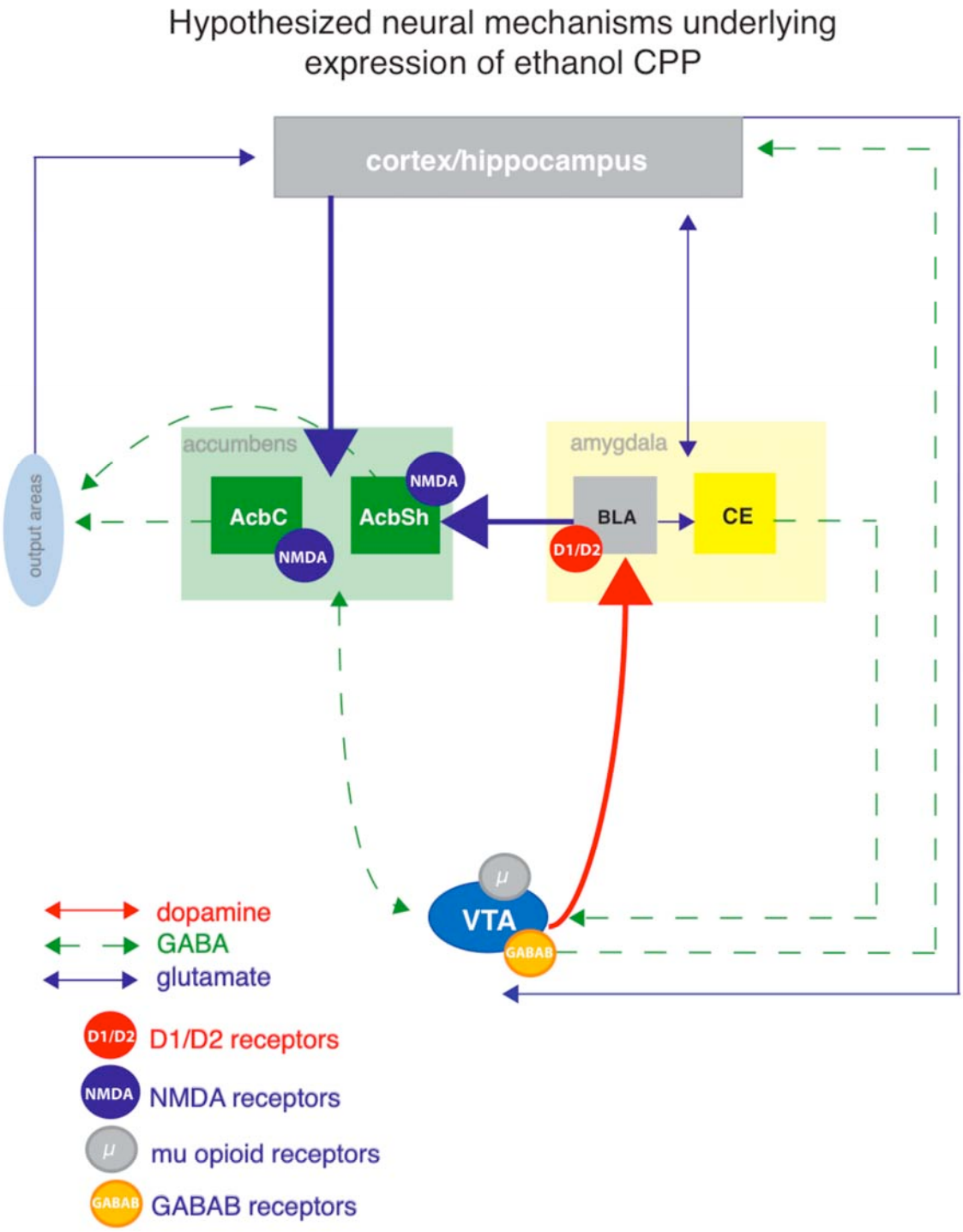
Use of CPP to examine neural areas underlying associative control over ethanol-seeking behaviors. In this thesis, I was able to examine areas and mechanisms underlying the acquisition and expression of associative control over an ethanol behavior. The use of the CPP procedure allowed me to investigate the neural areas that may be involved during acquisition in Pavlovian processes that endow an ethanol-predictive CS with motivational properties. Further, as previously discussed, Pavlovian approach behavior, conditioned reinforcement, and incentive motivation could all theoretically be influencing the behavior observed during the expression test. However, I argue that since the procedureal configuration used in this thesis involves responding to a tactile stimulus in the dark, conditioned reinforcement and potentially incentive motivation processes are most likely controlling the ethanol-seeking behavior expressed. During the test session, subjects need to be able to retrieve the memory of the ethanol-cue association, assess the motivational value of that association, learn that the approach response gains access to the conditioned reinforcer, and perform the approach response to gain reinforcement from the CS. However, in addition to learning that the approach response made during the test

session brings reinforcement, subjects are also learning that the CS no longer predicts the presence of ethanol (i.e. extinction). This second type of learning that is occurring during expression may be reflected in a decrease in responding as testing continues.

By using the CPP procedure to examine the formation and expression of associations between ethanol's rewarding effects and environmental cues, I was able to selectively assess the influence of the Acb and Amy on these behaviors (see Figure 14). Although I was unable to separate whether disruptions were due to disruptions in ethanol's rewarding effects or underlying learning processes, I demonstrated that acquisition and subsequent expression depends upon an intact Acb and Amy. Of particular interest, I observed a discrepancy between the neural areas involved in the acquisition and expression of cue-induced ethanol seeking behaviors. When I assessed the involvement of these same areas after the mice had already learned the association between ethanol's effects and the paired cue, only lesions of the Amy interfered with the initial expression of the cue-induced behavior. However, I did see a striking effect in that AcbC lesioned mice decreased their responding towards the ethanol-paired cue. This observation provides evidence that the AcbC is involved in maintaining responding for the stimulus.

Figure 14 Diagram of the hypothesized neural mechanisms underlying expression of ethanol CPP. Connections between the ventral tegmental area (VTA), cortical/hippocampal areas, basolateral amygdala (BLA), central nucleus of the amygdala (CE), and nucleus accumbens core (AcbC) and shell (AcbSh) are represented via projection arrows. Dopamine projections are shown in solid red lines, while dashed green lines represent gamma-aminobutyric acid (GABA) projections, and glutamate projections are diagrammed in solid blue lines. The hypothesized connections implicated by the current results as well as previous findings, are represented by bold projections (Bechtholt & Cunningham, 2005; Ford et al., 2006; Chapter 3), while other connections whose influence is unknown are represented by thin projection arrows. Additionally, receptors localized to specific neural areas that are implicated by the current results and previous findings are represented: D1/D2 dopamine receptors (red circles), NMDA receptors (dark blue circles), μ-opioid receptors (grey circles), and $GABA_B$ receptors (orange circles) have all been suggested to influence expression of ethanol CPP.

Figure 14


Hypothesized neural mechanisms underlying expression of ethanol CPP
cortex/hippocampus
output areas
accumbens
AcbC
AcbSh
NMDA
NMDA
amygdala
BLA
CE
D1/D2
VTA
μ
GABAB
dopamine
GABA
glutamate
D1/D2 D1/D2 receptors
NMDA NMDA receptors
μ mu opioid receptors
GABAB GABAB receptors

Focused manipulations within each of these areas demonstrated that expression of ethanol CPP is influenced by dopamine receptors in the Amy and by NMDA receptors in the Acb in mice. Moreover, within the Amy dopamine receptor activation of the BLA, but not the CE, influences CPP expression. Surprisingly, dopamine receptor activation within the Acb had no effect. These are the first studies in any species to show a role for Amy dopamine receptors and the first studies in mice to implicate Acb NMDA receptors in an ethanol-induced conditioned behavior.

Hypothesized role for Acb DA receptors. Using information from previous findings in interpretation of the current results (for review see Day & Carelli, 2007), I suggest the Acb may function in ethanol CPP as a mediator of ethanol's rewarding effects during acquisition, and as a site of converging associative information that forms the response output that is observed during expression testing. Supporting the hypothesized role of the Acb in ethanol's reinforcing effects are multiple studies demonstrating intra-Acb manipulation effects on ethanol self-administration (Hodge et al., 1992; 1997; June et al., 2004; Rassnick et al., 1992; Samson et al., 1993).

Manipulations of the Acb dopamine system, (which has been implicated in the reinforcing effects of drugs, particularly in the AcbSh) (e.g., Bassareo, & Di Chiara, 1999; Ito, Dalley, Howes, Robbins, & Everitt, 2000), generally reduce ethanol-reinforced responding. As discussed previously, intra-Acb D1 and/or D2 blockade decreased ethanol self-administration (e.g., Rassnick et al., 1992; Hodge et al., 1997; Samson et al., 1993), perhaps reflecting a decrease in the rewarding properties of ethanol. Further, activation of dopamine receptors via agonist administration increased responding, possibly suggesting an enhancement of ethanol reward (Hodge et al., 1992; Samson et al., 1993). However, it

should be noted that studies examining the function of Acb dopamine in ethanol reinforcement are not all in agreement. For example, 6-OHDA lesions did not alter the acquisition of ethanol self-administration (Myers & Quardfordt, 1992; Lyness & Smith, 1992; Rassnick et al., 1993), suggesting that Acb dopamine may not be necessary for ethanol reward. However, given the length of training involved in acquiring ethanol self-administration and the use of a sucrose/saccharine fading procedure, it is reasonable to hypothesize that compensatory mechanisms may be responsible for the acquisition of responding in such cases. Based on ethanol self-administration findings, I would predict that modulations of dopamine receptors in the Acb would affect the acquisition of ethanol CPP by altering the rewarding effects of ethanol. Recent evidence seems to offer support for this prediction. In a report of rat CPP induced by intracerebroventricular (icv) infusions of ethanol, intra-AcbSh infusions of the D1/D2 type dopamine receptor antagonist fluphenazine was reported to attenuate the acquisition of ethanol CPP (Walker & Ettenberg, 2007).

While the role of Acb dopamine receptors in the acquisition of ethanol CPP in mice is unknown, blockade before expression testing was without effect (Chapter 3). This is in direct contrast to reports of dopamine involvement in ethanol appetitive behaviors observed in ethanol self-administration procedures. Previously Samson and colleagues demonstrated that blockade of D2 receptors in the Acb reduced appetitive ethanol responding (Czachowski et al., 2001) and non-reinforced extinction responding (Samson & Chappell, 2004), thereby suggesting a role for Acb dopamine in ethanol-seeking behaviors. Further, the current findings differ from a report by Walker and Ettenberg (2007) that suggested AcbSh dopamine receptor blockade prior to expression testing

attenuated ethanol CPP that was induced in rats by icv ethanol infusions. Given the report of Acb dopamine modulation of ethanol CPP and ethanol self-administration in rats, current findings in this thesis could reflect a difference between species in the neural areas influencing ethanol-conditioned reward.

However, a note of caution should be added to the interpretation of reported Acb dopamine involvement in rat ethanol CPP. In that particular study, icv ethanol may have produced a modest preference as measured by an increase in a difference score (Time spent in drug-paired side: post test – baseline) (Walker & Ettenberg, 2007). However, the strength of CPP reported in that study was unclear, since a one sample t-test compared to zero was used as evidence of CPP, while preplanned orthogonal contrasts were used to examine differences between fluphenazine groups. Moreover, that study showed no significant CPP when experimental rats were compared to vehicle trained controls. Given this as a caveat, findings supporting a role for dopamine in acquisition of ethanol CPP may not hold under a more rigrous statistical analysis. Further the discrepancy between the lack of effect of Acb dopamine blockade observed in chapter 2 and findings from self-administration studies, may suggest that while Acb dopamine plays a role in conditioned instrumental responding, it may not influence cue-induced seeking behaviors as measured in ethanol CPP.

The Acb has also been suggested to play an important role in the acquisition and expression of associative processes that may guide food-reinforced behavior. For example, Acb appears to be involved in conditioned approach behaviors to a food-paired CS+, since lesions of the AcbC disrupted acquisition (e.g., Parkinson et al., 2000) and expression (Cardinal, Parkinson, Lachenal, Halkerston, Rudarakanchana, Hall, Morrison,

Howes, Robbins, & Everitt, 2002). Further, acquisition and expression of conditioned approach behaviors is dependent upon dopamine activation of the AcbC (e.g., Parkinson, Dalley, Cardinal, Beauford, Fehnert, Lachenal, Rudarakanachana, Halkerston, Robbins, & Everitt, 2002; Cardinal et al., 2002). Based on these findings, it could be hypothesized that antagonism of Acb dopamine receptors during acquisition or prior to expression testing would attenuate ethanol CPP. While the role of Acb DA receptors in acquisition is still unknown, blockade of Acb dopamine receptors had no effect on expression of ethanol CPP, suggesting that conditioned approach processes are not involved in the behavior.

Based on findings in chapter 3 and previous hypotheses concerning expression behavior (see Cunningham et al., 2006[b]), we suggest that conditioned reinforcement, not Pavlovian approach, controls responding during expression of ethanol CPP using a tactile procedure. With this hypothesis in mind, the lack of an effect of Acb dopamine antagonism is not surprising. Previous results have demonstrated that an intact Acb is not required for conditioned reinforcement, although increases in dopamine release or receptor activation enhance responding for a conditioned reinforcer (e.g., Cador, Taylor, & Robbins, 1991; Parkinson, Olmstead, Burns, Robbins & Everitt, 1999; Taylor & Robbins, 1984; 1986). It has been hypothesized that dopamine release in the Acb serves to increase the motivational valence of the retrieved memory of the drug-paired cue perhaps through incentive motivational processes, but does not influence the formation or performance of the underlying response (for review see Cardinal, Parkinson, Hall, & Everitt, 2002). Based on this hypothesis, manipulations that would serve to increase the release of dopamine in the Acb would enhance expression of ethanol CPP.

Hypothesized role for Acb NMDA receptors. Antagonism of NMDA receptors completely blocked expression of ethanol CPP. There is a previous report of Acb NMDA receptors influencing ethanol behavior, where blockade of NMDA receptors decreased ethanol-reinforced responding (Rassnick et al., 1992). It may be that Acb NMDA receptors mediate common mechanisms that govern the initiation of ethanol-reinforced responding, and responding for an ethanol-conditioned reinforcer.

Although there is little information regarding Acb NMDA receptor modulation of ethanol-related behaviors, not surprisingly, it has been implicated in associative learning processes. Previously it was demonstrated that AcbC NMDA receptors are necessary for the acquisition, but not later performance, of a Pavlovian conditioned approach behavior to a sucrose-paired stimulus (Di Ciano et al., 2001). Further, manipulations of the AcbC interfered with cocaine-conditioned reinforcer supported responding (Di Ciano & Everitt, 2004). However the observed effect was not due to actions at NMDA receptors, since AP-5 infused into the AcbC had no effect on cocaine-paired conditioned reinforcer responding, although blockade of AMPA receptors reduced the behavior (Di Ciano & Everitt, 2001). Considering these previous results, the effect of AP-5 blockade of Acb NMDA receptors on expression of ethanol CPP may not be due to deficits in the performance of conditioned approach or conditioned reinforcement behaviors.

Alternatively, the findings of Acb NMDA receptor involvement in expression of ethanol CPP might suggest that NMDA receptors are recruited for the learning of the response necessary to gain access to the conditioned reinforcer. It was previously shown that acquisition of an instrumental response that resulted in sucrose reinforcement was dependent upon AcbC NMDA receptors (e.g., Kelley et al., 1997). Further, only blockade

of receptors via pre trial- and not post trial-infusions prevented acquisition, suggesting that initial performance but not consolidation of the learning was dependent upon Acb NMDA receptors (Hernandez, Andrezejewski, Sadeghian, Pankseps, & Kelley, 2005). It may be that information from the Amy, cortical areas, and hippocampus relayed via glutamatergic projections, converges in the Acb and subsequently activates NMDA receptors. In turn, the NMDA receptors may influence the formation of the association between the approach behavior and the previously ethanol-paired floor as the subjects learn how to respond to gain access to the conditioned reinforcer. This proposal is in line with the more general hypothesis that the Acb serves to gate and process limbic and cortical information before it passes to motor and response output pathways (e.g., Costa, 2007).

The difference in the time course of disruption between the Acb lesion and intra-Acb AP-5 infused subjects suggests potential discrepancies in the above hypothesis. If the Acb is involved in the initial learning of the response, then lesions should have immediate effects on the level of responding. Instead, expression behavior decreased later in the test session in AcbC lesions subjects, while intra-Acb AP-5 infusions disrupted initial preference (data not shown). It may be that the effect of NMDA antagonism within the Acb is due to modulation of GABAergic projections to the VTA (e.g., Chang & Kitai, 1985) and subsequent alterations in VTA activity (see Figure 14).

While this scenario is possible, a more plausible explanation for the discrepancy in the findings may be the difference between the types of intra-cranial manipulations made. It may be that my AcbC lesions were not large enough to interfere with the initial response learning, but instead manifested later in the test session as disruptions in

responding by influencing downstream mechanisms (i.e., through potential effects on feedback mechanisms to the VTA). A potential experimental manipulation that could shed light on the contribution of Acb NMDA receptors to expression of ethanol CPP would be to administer the antagonist either before the first test, or after the response has been established (i.e., administer prior to a second test session). If NMDA receptors are necessary only for the initial formation of the choice response, antagonism after learning (i.e., prior to the second test session) should not affect the level of responding (e.g., Baldwin et al. 2000).

Hypothesized role of Amy DA receptors. Blockade of D1/D2 receptors in the Amy disrupted expression of ethanol CPP. Of particular interest, when analyses were performed on mice with infusion locations identified as located in specific regions of the Amy, expression appeared to be dependent on dopamine receptor activation in the BLA. While there have been no reports of BLA functional involvement in any ethanol-related behaviors, neural activation in response to a ethanol-paired S^+ stimulus has been observed (Radwanska et al., 2007; Zhao et al., 2006). However, numerous studies have implicated the CE in ethanol behaviors. In particular, there is a growing body of work suggesting that CRF in the CE modulates aspects of ethanol-reinforced responding in dependent rats (e.g., Funk et al., 2006; Funk & Koob, 2007), as well data implicating CE $GABA_A$ systems in ethanol reinforcement (Hyytia & Koob, 1995; Roberts et al., 1996). However, whether $GABA_A$ manipulation effects on responding are due to alterations in ethanol seeking or taking behaviors is unknown.

A recently published research report implicated CE NMDA and non-NMDA receptors in the expression of an ethanol CPP in rats (Zhu et al., 2007). In this procedure,

ethanol was “mostly” paired with the non-preferred side of apparatus in which rats spent ~ 20% of their time. Ethanol increased the percentage of time spent in the paired chamber to around 50% (only includes time spent in paired and unpaired chamber, time spent in middle “neutral” zone was not included). When NMDA or non-NMDA receptors were blocked during a 2nd test session, a reduction in the percentage time spent in the paired-chamber was observed. The authors hypothesized that this effect was due to a decrease in the conditioned rewarding properties of ethanol. However, the authors did not report locomotor activity levels during testing, which could have been a potential confound (e.g., Gremel & Cunningham, 2007). We would like to present a different interpretation of the data. Instead of interference with reward, the data suggest that NMDA and non-NMDA receptor antagonism blocked the ethanol-conditioned alleviation of an initial aversion to the paired chamber. This interpretation is more in line with previous data demonstrating that rats normally do not condition an ethanol place preference to a non-biased apparatus or at the low dose of ethanol used (0.5 g/kg) (see Fidler et al., 2004 for summary).

In contrast to the hypothesis about Acb contributions, I hypothesize that in ethanol CPP the Amy is involved in learning about the cue-drug relationship during acquisition, and then the cue depends on the Amy to retrieve the motivational value of the cue-drug association during expression testing. This is in line with current thinking about the role of the BLA and CE in emotional processing (Cardinal et al., 2002; Holland & Gallagher, 1999). In chapter 2, I demonstrate that acquisition of ethanol CPP is dependent upon an intact Amy, since lesions completely disrupted the subsequent expression of the behavior. The Amy has been widely implicated in the acquisition and expression of

conditioned reinforcement and Pavlovian approach behaviors (for review see Cardinal et al., 2002; Robbins & Everitt, 2005). Of particular interest, a double dissociation between the BLA and CE was observed in the acquisition of Pavlovian approach behavior and conditioned reinforcement (Hitchott & Phillips, 1998). In particular, they found that administration of the D3 receptor antagonist R+ 7-OH-DPAT into the BLA disrupted acquisition of conditioned reinforcement, while infusion into the CE blocked development of Pavlovian conditioned approach. While in the current set of experiments I was unable to identify specific regions or receptors in the Amy involved in acquisition, we would predict that acquisition of ethanol CPP using a tactile procedure is dependent upon intra-BLA dopamine receptor activation based upon the above findings.

It is possible that the effect of Amy lesions we observed in the acquisition experiment may have been due to effects on expression alone. In support of this, when lesions were performed after acquisition, BLA-lesioned rats did not respond for a conditioned reinforcer (e.g., Burns, Robbins, & Everitt, 1993; Cador et al., 1989), and were insensitive to changes in the value of the US (e.g., Hatfield et al., 1996; Killcross, Everitt, & Robbins, 1998), whereas these behaviors were intact in CE-lesioned rats (for review see Cardinal, et al., 2002). However, CE lesions do disrupt expression of Pavlovian conditioned approach behaviors (Hall, Parkinson, Robbins, & Everitt, 2001), and Pavlovian instrumental transfer, whereas BLA lesions have no effect (Hall, Parkinson, Connor, Dickinson, & Everitt, 2001; Killcross et al., 1998). Taken together, these studies suggest the observed effect on Amy lesions on expression of ethanol CPP may be due to disruptions in conditioned reinforcement and/or Pavlovian approach behaviors.

Since my lesions (chapter 2) were not localized to the BLA or CE, findings in chapter 3 provide more detailed information about Amy regions and mechanisms involved in expression of ethanol CPP. While previous work by Harmer and Phillips (1999) demonstrated that Amy dopamine levels rise in response to a CS, more localized roles for dopamine receptors have been demonstrated in the BLA and CE. Antagonism of D3 receptors with R(+) 7-OH-DPAT within the CE, but not BLA, disrupted expression of Pavlovian approach behavior. However, D3 receptor blockade within the BLA, not CE, disrupted acquisition of new response supported by a conditioned reinforcer (Hitchcott & Phillips, 1998). Further, BLA infusions of the D1 receptor antagonist SCH-23390 alone or in combination with raclopride (D2 antagonist) decreased cocaine-conditioned responding (See, Kruzich, & Grimm, 2001). Additionally, blockade of D1/D2 receptors in the BLA decreased cocaine-seeking behavior under a second-order schedule of reinforcement (Di Ciano & Everitt, 2004). The finding that expression of ethanol CPP was dependent upon dopamine receptor activation specifically in the BLA, and not CE, strongly suggests that conditioned reinforcement processes are contributing to the observed behavior. However, further speculation about the specific receptor subtype does not seem justifiable given that both D1 and D2 type receptor manipulations within the BLA have had effects on conditioned reinforcement.

Potential upstream-downstream mechanisms. Increased dopamine-induced activation of the BLA that influences expression behavior is most likely a result of increased activity of VTA dopamine neurons modulated by mu opioid receptors (e.g., Ford et al., 2006). This is in concordance with previous findings from this laboratory that showed effects of opioid- and $GABA_B$-receptor modulation on expression of ethanol CPP

(Bechtholt & Cunningham, 2005). Bechtholt and Cunningham (2005) hypothesized that effects were due to decreases in VTA dopamine neuron activity and downstream release in the Acb. However, given the present findings I modify this hypothesis and now implicate the VTA dopamine projection to the Amy and its downstream projection areas (see Figure 14). Since the BLA has dense connectivity with cortical areas, it may be that information regarding the cue-drug association received via dopamine input to the BLA, in turn modulates cortical processing. For example, Pre-L areas have been implicated in action-outcome contingencies, in that lesioned rats were insensitive to contingency manipulations (Balleine & Dickinson, 1998). Further, orbital frontal areas may represent aspects of reinforcer value. Similar to BLA manipulations, rats with lesions of the orbital frontal cortex did not adjust their conditioned responding following US devaluation (Hatfield et al., 1996). Additionally, although the Ac has been widely implicated in modulating Pavlovian conditioned approach behavior, in ethanol CPP it may be involved in interpreting specific cue-drug relationships (for review see Cardinal et al., 2002).

Another possibility is that activation of BLA dopamine receptors may modulate information output via the CE and its hypothalamic, brainstem and midbrain connections (see Sah et al., 2003). Even though CE dopamine receptor antagonism did not alter behavior, inputs from the BLA to the CE (Pitkanen et al., 1997) could modify output independent of CE dopamine receptors. An interesting alternative explanation of previous findings in the VTA is that since the CE sends projections to the VTA (Hopkins & Holstege, 1978), the BLA may regulate VTA activity via its glutamate projections to the CE, hence also possibly mediating Acb activity downstream from the VTA (as mentioned previously). However since effects are seen in both regions, means to dissociate

involvement of VTA dopamine neuron modulation of the BLA from BLA modulation of the VTA via CE connections in the modulation of this behavior are currently not available.

We attempted to examine another possible BLA information output pathway. Given the serial connection between the BLA and Acb (e.g. Groenwegen et al., 1996), and the findings of decreased expression of ethanol upon manipulations within each area, we hypothesized that expression of this behavior activates BLA dopamine receptors which directly leads to increases in Acb NMDA receptor activation via BLA glutamate efferents. Cocaine-seeking behavior has been shown to be dependent upon a similar mechanism of information transfer between the BLA and Acb (Di Ciano & Everitt, 2004). From the Acb, it may be hypothesized that information is passed to cortical areas via the dorsal striatum and substantia nigra, where it gains access to behavioral output mechanisms (e.g., Haber et al., 2000; Day & Carelli, 2007). However, both ipsi- and contra-lateral drug manipulations decreased expression of ethanol CPP. This finding may suggest that while BLA glutamatergic innervation of the Acb is involved in the expression behavior, heavy cortical and hippocampal glutamatergic input may also modulate the activity at NMDA receptors. However, our single nucleus manipulation results suggest that interpretations made from the use of the disconnection procedure may be limited. Mice may be more sensitive than rats to disruption from single nucleus manipulations, given the use of the disconnection procedure in rats to investigate drug-seeking behavior (e.g., Di Ciano & Everitt, 2004[a]). This finding is discouraging for the future use of mouse models to investigate serial connections between brain areas underlying motivated behaviors.

A recent paper by Cole and McNally (2007) provides an interesting hypothesis about the current pattern of results presented in this thesis. In a purely Pavlovian task (fear conditioning), they were able to demonstrate that it was possible to differentially manipulate the underlying motivation of the retrieved association from learning associated with the response output. Further, they were able to identify specific neurotransmitter mechanisms that differentially influenced these separate components of behavior. While opioid receptor manipulations altered the motivation of the learned association, NMDA receptors appeared to influence the learning of the association. Taking some liberty in transferring this hypothesis to the current work that involves both appetitive Pavlovian and instrumental learning processes, it may be possible to explain the observed behavior. Expression of ethanol CPP may depend on VTA opioid receptor modulation of the underlying motivation that is communicated through alterations in VTA dopamine neuron activity (e.g., Fields, Hjelmstad, Marolis, & Nicola, 2007), which is then assessed through dopamine activation of the BLA. The BLA in turn sends "motivational" information to the Acb, where it converges with information from cortical sources to direct the learning that involves NMDA receptors. Further, for expression of the behavior, the motivational value of the retrieved cue-drug association needs to be compared to the current perceived value, which may be communicated through cortical input to the VTA. However, this hypothesis remains purely speculative and only provides an interesting suggestion about mechanisms that might be especially relevant to decreasing ethanol-seeking behaviors.

Contributions to the understanding of ethanol-induced locomotor activation. Present findings also shed light on the neural areas governing ethanol-induced activation

and test activity in mice. The neural areas modulating ethanol-induced increases in locomotor activity are thought to overlap with those involved in its reinforcing effects (Phillips & Shen, 1996). For example, $GABA_B$ receptors in the VTA have been implicated in ethanol's activating effects (Boehm et al., 2002). In chapter 1 only lesions of the Amy attenuated ethanol-induced stimulation during conditioning trials, suggesting a role for this nucleus in the activating effects of ethanol. However, there was not a difference between lesion groups in the sensitization to the stimulating effects of ethanol that developed across ethanol conditioning trials, suggesting that perhaps the amygdala is not involved in ethanol-induced locomotor sensitization. Although the differences in activity were relatively minor, subjects with Amy and Acb lesions showed higher levels of activity during the drug-free test. However, the relative small effect of lesions on locomotor activity may have been due to the size of the lesions. Since we were unable to quantify the extent of lesions in the Acb and Amy, it may be that larger lesions would have induced a greater affect on either test or conditioning trial activity levels and ethanol-induced locomotor sensitization.

Within chapter 3, I observed similar effects of dopamine receptor manipulations on activity levels independent of area. That is, D1/D2 receptor antagonism in both the Acb and Amy decreased locomotor activity, suggesting that a general decrease in dopamine activation within the limbic cortical ventral striatal system reduces locomotor activity. This suggestion is in line with previous studies showing that systemic manipulation of dopamine receptors has effects on locomotor activity in mice (e.g., Dickinson et al., 2003; Holmes et al., 2004). Further, I saw an increase in locomotor activity when Acb NMDA receptors were blocked with AP-5, which is similar to a

previous finding of increased activity with systemic blockade of the NMDA receptor with a competitive antagonist (e.g., Boyce-Rustay & Cunningham, 2004). It is worth noting however, that in the disconnection procedure, very little alterations in locomotor activity were observed. Further, there were no differences in activity observed between ipsi and contralateral drug-manipulations, suggesting that any single nuclei manipulation (i.e. as observed in unilateral AP-5 infused subjects) may have been canceled out by a drug infusion into the contrasting area.

Previously, we demonstrated a negative correlation between magnitude of preference expressed and level of activation observed during testing (Gremel & Cunningham, 2007). However, I do not believe that the differences in locomotor activity observed in the present studies were sufficient to significantly alter the magnitude of preference observed. For example, in Chapter 1, Acb lesioned subjects were the most activated, but still expressed a preference similar to that of Sham controls.

Insight from human studies. There is overlap between findings in this thesis implicating specific neural areas and mechanisms in the limbic cortical ventral striatopallidal circuit, and neural areas activated by alcohol-associated stimuli in humans. In abstinent alcoholics, presentation of an alcohol-associated visual stimulus increased neural activation of the ventral striatum (Braus, Wrase, Grüsser, Hermann, Ruf, Flor, Mann, & Heinz, 2001) and the ventral striatum, Ac, and mPre (Grüsser, Sabine, Wrase, Klein, Hermann, Smolka, Ruf, Weber-Fahr, Flor, Mann, Braus, & Heinz, 2004) compared to a neutral stimulus. Further, in both of these studies, relapse was associated with the level of activation observed within these areas (Braus et al., 2001; Grüsser et al., 2004). In this thesis, I also found involvement of the ventral striatum in a model of cue-

induced ethanol-seeking behavior in mice, suggesting that our findings may have some level of predictive validity.

Of particular interest, both NMDA and dopamine receptors have been targets in pharmacotherapy development for the treatment of alcoholism. While antagonism of dopamine receptors with fluphenthixol seems to have no effect on craving or may even serve to increase relapse (Wiesbeck, Weijers, Lesch, Glaser, Toennes, & Boening (2001), results from clinical studies using NMDA receptor antagonists have been more positive. In addition to the use of acamprosate (a mixed NMDA, mGluR5, and GABA antagonist), which at least in Europe has shown slight effects on decreasing alcohol drinking without effects on craving (for review see Johnson, 2008), more specific NMDA antagonists have been investigated. Initially, a pilot study reported that memantine, a non-competitive NMDA receptor antagonist, attenuated alcohol craving, but not consumption of alcohol (Bisaga & Evans, 2004). In contrast, a subsequent pilot study suggested that when memantine was given to moderate drinkers (not diagnosed as dependent), the decrease in drinking was less than placebo-treated moderate drinkers (Evans, Levin, Brooks, & Garawi, 2007). However, in that study cue-induced craving was not investigated. More recently, the effect of memantine on cue-induced craving was examined (Krupitsky, Neznanova, Masalov, Burakov, Didenko, Romanoa, Tsoy, Bespalov, Slavina, Grineko, Petrakis, Pittman, Gueorguieva, Zvartau, & Krystal, 2007). After memantine administration, alcohol dependent subjects were presented with an alcohol cue and their drink of choice (although they were told to not consume the drink) and subsequent craving was assessed. Memantine dose dependently reduced craving elicited by the cue

and drink (Krupitsky et al., 2007), suggesting that cue-induced alcohol craving in humans is modulated by NMDA receptors.

Future directions. The findings presented in this thesis show that expression of an initial ethanol CPP in a tactile procedure depends upon dopamine receptor activation in the Amy and NMDA receptor activation in the Acb. Future work could aim to delineate these areas in the involvement in retrieval of the cue-drug association, assignment of motivation value to the cue-drug relationship, learning of approach response to gain access to the cue, or in the performance of the approach response remains unknown. For example, given previous findings suggesting that opioid receptors modulate the underlying motivational value of the cue-drug association (e.g., Cunningham et al., 1998), and the hypothesis that this assignment may be performed by intra-VTA opioid receptors which then passes that information to the Amy for processing, opioid receptor blockade in the VTA or dopamine receptor blockade in the Amy should alter the underlying motivation guiding expression behavior. It may be that preference examined during a later test (i.e. after receptor manipulations in the first test), would reveal a change in the direction of preference expressed, such that subjects would subsequently display a CPA towards the previously ethanol-paired cue. While the current data did not examine preference during a second test session, the preference expressed when flupenthixol was infused into the BLA (Chapter 3) was in the direction of a CPA, suggesting that indeed the BLA may be involved in the processing of motivational information. Further, cortical information regarding the current value of the association sent via glutamatergic projections to the VTA (for review see Cardinal et al., 2002) may be cricital in comparing the retrieved value and current value of the previously ethanol-

paired cue. Indeed, previous findings suggest glutamatergic input into the VTA is essential for the acquisition and expression of a cocaine-induced CPP (Harris & Aston-Jones, 2003). Based on this, blockade of glutamate receptors in the VTA would block expression of preference, but may also inhibit extinction of CPP if subjects cannot assess current value of the retrieved association.

Besides processes regulating responding during the expression test itself, the current findings offer hypothesis about the involvement of these neural areas in the acquisition and extinction of cue-control over ethanol-seeking behaviors. It may be that similar neural mechanisms within the Acb and Amy are critically involved in the learning of or processing of ethanol's rewarding effects, or the learning involved in extinction of this behavior. Additionally, there is evidence that the modality of CS used may recruit different learning mechanisms that may be subserved by other neural areas (e.g., White et al., 2005). It may be that expression behavior towards a different type of CS (such as a visual instead of tactile cue) would recruit a different associative process (e.g., sign-tracking) that would then rely upon the CE instead of the BLA (see Cardinal et al., 2002 for review). Additional investigations into the neural mechanisms of cue-induced ethanol-seeking behavior using the CPP procedure may offer much needed insight into the different associative processes that instigate seeking and relapse behavior.

Summary. In this thesis, I have identified neural areas and mechanisms that appear to influence the acquisition and expression of a cue-induced ethanol-seeking behavior. Specifically, I found that acquisition of ethanol CPP depends upon an intact Acb and Amy, while expression seems to require an intact Amy. However, the findings in Chapter 2 do support a role for the Acb in expression, since lesions facilitated loss of responding

of the expression response. Results from experiments in Chapter 3 confirmed these findings by identifying specific mechanisms within each of these areas. Dopamine receptor activation within the BLA, but not the CE or Acb, is necessary for cue-induced ethanol-seeking behavior. Further, expression of ethanol CPP was dependent upon Acb NMDA receptor activation. The findings presented in this thesis offer novel contributions to the understanding of the neural mechanisms influencing conditioned stimulus control over ethanol-seeking behaviors.

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