CENTRAL OPIOID INFLUENCES

ON CONDITIONED ETHANOL REWARD AND AVERSION

By

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CERTIFICATE OF APPROVAL

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TABLE OF CONTENTS

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LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

5-HT - serotonin
AA – alko, alcohol
aca - and anterior commissure
BD1047
CPP – conditioned place preference
CPA – conditioned place aversion
CPu - caudate putamen
CS – conditioned stimulus
D – dopamine
DARP – dopamine and adenosine 3',5'-monophosphate-regulated phosphoprotein, 32 kDa
GABA – gamma-aminobutyric acid
GIRK – G-protein-coupled inwardly rectifying potassium channel
HAD – high alcohol drinking
i.c.v. - intracerebroventricular
KO – knockout
MGluR5 – type 5 metabotropic glutamate receptor
MPEP – 2-methyl-6-(phenylethnyl)-pyridine
ml - medial lemniscus
msP- Marchigian Sardinian alcohol-preferring rats
NAc – nucleus accumbens
NNOS – neuronal nitric oxide synthase
P – alcohol preferring
PAC - periaqueductal gray
pfc – prefrontal cortex
PRE-084 – 2-(4-morpholino)ethyl 1-phenylcyclohexane-1-carboxylate
Ro 64-6198 – (1*S*,3a*S*)-8-(2,3,3a,4,5,6-hexahydro-1*H*-phenalen-1-yl)-1-phenyl-1,3,8,triaza-spiro[4.5]dcan-4-one hydrochloride
Ro 65-6570 – 8-acenaphthen-1-yl-1-phenl-1,3,8,-triaza-spiro[4,5]decan-4-one
SNR - substantia nigra reticulata
SSRI – selective serotonin reuptake inhibitor
TAN-67 – 2-methyl-4aα-(3-hydroxyphenyl)-1,2,3,4,4a,5,12,12, aα-octahydroquinolino [2,3,3-g] isoquinoline
U50,488H – *trans*-3,4-dichloro-*N*-(2-(1-pyrrolidinyl)cyclohexyl)benzenacetamide methanesulfonate
US – unconditioned stimulus
VDB - nucleus of the vertical limb of the diagonal band
VP – ventral pallidum
VTA – ventral tegmental area

ABSTRACT

The goal of the present experiments was to identify brain areas and neurochemical substrates that mediate the expression of the conditioned rewarding properties of ethanol. Numerous previous findings suggest that opioid receptor antagonists alter the direct and conditioned rewarding effects of ethanol. These effects may occur through opioid receptors located in the ventral tegmental area (VTA) and the nucleus accumbens (NAc), two key components of the "reward circuit." This has been demonstrated for some aspects of the direct effects of ethanol, however, little is known about the mechanisms through which opioid receptor antagonists alter the conditioned rewarding properties of ethanol.

Here I examine the VTA and the NAc to determine their part in the ability of opioid receptor antagonists to decrease the expression of the conditioned rewarding properties of ethanol. Also, because VTA opioid receptor blockade altered the conditioned rewarding effects of ethanol, I tested the hypothesis that similar effects would be obtained following intra-VTA gamma-aminobutyric acid $(GABA)_B$ receptor agonist treatment. This hypothesis was generated based on previous reports that intra-VTA opioid receptor antagonist effects may occur through effects on GABA neurons and that intra-VTA $GABA_B$ receptor agonists alter behaviors associated with putative ethanol reward related pathways.

Male DBA/2J mice were implanted with chronic indwelling bilateral guide cannulae positioned just above the VTA or NAc. After a period of recovery, animals underwent a Pavlovian conditioning procedure in which they were given repeated pairings of a distinct tactile stimulus with an injection of ethanol (2g/kg). A second

stimulus was paired with saline injection. During subsequent test sessions, animals were allowed to choose between the two distinct stimuli. Just prior to testing, animals were injected either into the VTA or NAc with artificial cerebrospinal fluid (acsf) or a dose of methylnaloxonium (375 or 750 ng total infusion), a non-selective opioid receptor antagonist. Intra-VTA methylnaloxonium injection produced a dose dependent decrease in the expression of ethanol-induced conditioned place preference (CPP), while this treatment given intra-NAc had no significant effect. In a separate experiment, animals were implanted with bilateral cannulae positioned just about the VTA and underwent place-conditioning training as previously described. Just prior to testing, animals were injected into the VTA with acsf or a dose of baclofen (25 or 50 ng total infusion), a $GABA_B$ receptor agonist. Intra-VTA baclofen decreased the expression of ethanol CPP.

A second set of experiments was designed to examine the brain areas and neurochemical substrates that mediate the conditioned aversive effect of ethanol. In addition, these experiments were intended to serve as controls for the induction of a generalized aversive state and for disruption of memory retrieval in the CPP experiments. In these experiments, conditioning and testing were conducted as previously described except that ethanol was given just after the animals were taken out of the conditioning chamber to induce conditioned place aversion (CPA). Intra-VTA methylnaloxonium was without effect on the expression of ethanol-induced CPA, however the magnitude of CPA in this study was relatively weak in all groups. Further, attempts to examine the effect of intra-NAc methylnaloxonium on the expression of CPA were not successful in inducing CPA. Additional experiments demonstrated that the handling required for microinjections in mice may have interfered with the expression of ethanol-induced CPA, but did not

alter ethanol CPP. This disruption precluded continued investigations of the mediators of ethanol induced CPA. Taken together, these findings suggest that VTA opioids may not be involved in either the expression of CPA or the effect of handling on this behavior.

These findings suggest that the conditioned rewarding effect of ethanol is expressed through a VTA-dependent and NAc-independent mechanism. The effect of non-selective opioid blockade on the conditioned rewarding effect of ethanol may result from blockade of μ-opioid receptors located on GABA neurons in the VTA. Blockade of these receptors would result in a decrease in dopamine neuron activity a possibly decreased extracellular dopamine and glutamate in the NAc. In contrast, the conditioned aversive effect of ethanol may not depend on these same mechanisms. However, conclusions are tentative due to potential handling induced disruption of CPA. Taken together, these findings indicate that the conditioned response to ethanol-associated cues involves the release of endogenous opioids in the VTA that inhibit GABA neurons, preventing additional binding of GABA at the $GABA_B$ receptor.

GENERAL INTRODUCTION

Conditioned associations are thought to be important contributors to relapse to drug taking in humans. In particular, humans report that drug-associated cues induce craving, which may contribute to relapse. Non-selective opioid receptor antagonists have been shown to alter the primary and conditioned rewarding properties of ethanol, however, the mechanisms underlying these effects are largely unknown. Reviewed here is the literature involving models of ethanol reward and opioid effects, neurocircuitry of reward, and the role of a portion of the reward circuit in ethanol reward. The goal of this dissertation was to identify brain areas and neurochemical mechanisms through which the conditioned effects of ethanol are expressed. In particular, the effects of intracranial infusions of a non-selective opioid receptor antagonist on ethanol-induced conditioned place preference (CPP) and conditioned place aversion (CPA) were assessed.

Animal models of ethanol reward

Several techniques have been developed in an attempt to study the reinforcing or rewarding properties of ethanol in animals. Each technique is designed to study a particular aspect of alcoholism and has both advantages and disadvantages. Some models examine variables that alter the consumption of ethanol in an attempt to identify human determinates of ethanol intake (e.g., drinking and self-administration). Other techniques try to model the seeking of ethanol and are thought to model craving in humans (e.g., place conditioning and self-administration). Some of these models and their respective advantages and disadvantages are discussed below. However, it should be noted that general conclusions about the mediators of ethanol reward are seldom made on the basis

of a single model.

The terms reinforcing and rewarding are a topic of occasional debate in the literature. Bardo and Bevins have recently suggested that place conditioning is best described as a model of drug reward. This assertion was made to distinguish place conditioning findings from operant self-administration findings and because the particular behaviors being reinforced in place conditioning models are unclear (Bardo & Bevins, 2000). Because there is no debate about the ability of stimuli to acquire conditioned reinforcing properties (Domjan, 1998) nor whether animals seek drug-associated cues (Bardo & Bevins, 2000), perhaps a more accurate description of the problem is that the nature of the reinforcer is unclear. That is, typically the unconditioned stimulus in place conditioning studies is a pharmacological agent where the unconditioned response, and likely the conditioned response, is an internal physiological state (Cunningham, 1993). For this reason, although the instrumental behavior of approaching the drug paired floor may be "reinforced" by the induction of a rewarding internal physiological state, until this response can be pinpointed and manipulated the controversy of interpretation remains. Therefore, for simplicity, the more general term "reward" will be used in the remainder of this document to refer to the assumed appetitive nature of stimuli, rather than the ability of stimuli to maintain responding.

Drinking

Ethanol is commonly described as rewarding based on the willingness of animals to consume it over water, given a choice. Ethanol drinking models often employ the use

of a two bottle choice procedure in which animals are given access to one drinking tube containing an ethanol solution and another containing water (Richter & Campbell, 1940). Consumption of the two solutions is measured and preference is determined. If animals drink more ethanol than water, then the ethanol preference ratio is greater that 0.50, indicating a preference for ethanol because the amount consumed is greater than what would have been consumed by chance. Experimental manipulations that alter ethanol intake, without altering overall fluid intake, suggest that the manipulation strictly altered ethanol's rewarding value rather than fluid intake needs. A major advantage of ethanol drinking procedures is that it has a great deal of face validity. Like in humans, ethanol is voluntarily consumed in this paradigm. However, also like humans, many animals do not readily consume large volumes of ethanol. For this reason, techniques are employed to encourage consumption, such as, slow increases in the ethanol concentration, the addition of a sweetener to the solution and food and/or fluid deprivation (Meisch, 1977). These techniques can sometimes cloud interpretation because the underlying motivation of the behavior is not known. For example, animals might be motivated to consume ethanol solutions for the added sweetener or to fulfill a fluid or caloric need, rather than for the subjective pharmacological effects of ethanol. The increased use of ethanol preferring strains such as C57BL mice (C57BL/Crgl, McClearn & Rodgers, 1959) and selectively bred alcohol preferring rats (Li, Lumeng, McBride, & Waller, 1979) and the adoption of sucrose fading procedures (Samson, Pfeffer, & Tolliver, 1988) have largely alleviated the need to use these potentially confounding techniques. However, it has not been definitively demonstrated that ethanol is consumed for its pharmacological effects in all of these models. Also, even in the absence of these potentially confounding techniques,

interpreting differences in ethanol intake is not obvious. That is, changes in intake could have different motivational bases. For example, an increase in consumption could indicate that an animal finds the solution more rewarding and therefore consumes more. Alternatively, the animal may find the solution less rewarding and need to consume more to get the desired effect. Similarly, a decrease in ethanol intake could indicate that an animal does not find a solution rewarding and therefore consumes less or that the animal does not need to consume as much solution to achieve the desired result.

In summary, ethanol drinking provides a model that has a great deal of face validity. However, manipulations that are sometimes required to induce drinking can introduce interpretive problems and changes in intakes do not point to an obvious motivational interpretation.

Operant self-administration

Operant self-administration is a technique that is similar to ethanol drinking procedures except that animals are required to make a response, such as pressing a bar, in order to receive a small volume of ethanol. For this reason, it also has the advantage of similarity to the human condition, in that ethanol is consumed and the added advantage that animals are required to work for access to ethanol. This work requirement allows the assessment of motivation to obtain ethanol. However, without very difficult and time consuming dose-response studies, differences in responding have the same interpretational problem as seen in ethanol drinking. That is, a change in responding does not have an obvious interpretation. In addition, the activating and sedating effects of ethanol can interfere with operant responding (Cunningham, Fidler, & Hill, 2000).

Samson and Hodge have suggested that ethanol consumption can be divided into two phases, appetitive and consummatory. The appetitive phase involves the seeking of ethanol and associated behaviors, such as bar pressing. The consummatory phase involves the ingestion of ethanol (Samson & Hodge, 1996). A new method has been developed from these principles that allows the examination of the appetitive properties of ethanol in the absence of the consummatory properties. In a more traditional operant self-administration paradigm animals are required to make a fixed number of responses in order to receive a small quantity of ethanol. However, in this newer method, animals are required to make a number of responses in order to gain several minutes of access to an ethanol drinking tube. In an alteration of this model, animals are required to make an increasing number of responses across sessions in order to receive a period of access to the ethanol drinking tube. In this way, a "break point" or limit in the number of responses the animals is willing to make to obtain ethanol can be assessed (Samson, Slawecki, Sharpe, & Chappell, 1998). The procedure offers a major advantage in that, the animal's willingness to work for ethanol can be assessed without the potentially confounding locomotor effects of ethanol itself. Manipulations in this paradigm seek to alter the animal's work effort without altering the response rate, indicating that effects observed are not due to generalized locomotor effects. For example, a drug pretreatment might decrease the number of responses for ethanol (break point) that could be interpreted as a decrease in the appetitive effects of ethanol. However, if the rate of responding is also decreased, the interpretation must be qualified because decreased responding might result from an impairment in the animal's ability to respond.

One can also assess an animal's motivation to obtain ethanol by examining the

persistence of responding when ethanol is not available. Specifically, after an animal is trained to reliably respond for ethanol, the contingency is removed and responses no longer result in the delivery of ethanol. This method allows a measure of ethanol reward by determining how long an animal will respond without reinforcement. After responding has been extinguished, additional manipulations can be introduced. For example, the presentations of a cue that was previously paired with ethanol can reinstate responding (e.g., Ciccocioppo, Martin-Fardon, & Weiss, 2002). Methods such as this, allow one to investigate the conditioned effects of ethanol using operant procedures.

In conclusion, operant ethanol self-administration techniques provide good face validity and the ability to assess motivational aspects of ethanol reward. Depending on the parameters used, one can also examine persistence of responding and the ability of ethanol-paired cues to reinstate extinguished responding. Unfortunately, this procedure is extremely time consuming and as with ethanol drinking, there can be difficulties with getting animals to respond for ethanol and subsequent problems with interpretation.

Place conditioning

Place conditioning is a Pavlovian conditioning procedure that has been used to study the rewarding properties of many drugs of abuse including ethanol (for review see Tzschentke, 1998). In this procedure the animal is injected with a drug and placed in a distinct environment. On alternate trials, the animal is injected with vehicle and placed in a different environment. For example, the animal might be placed into a chamber that has black walls after an ethanol injection and one with white walls after a saline injection. This two-part pairing is carried out several times, after which an ethanol-free choice test

is done. During the choice test the animal is given access to both the compartment previously paired with ethanol and the compartment previously paired with saline and is therefore allowed to choose where to spend time. More time spent on the ethanol-paired side is termed a conditioned place preference (CPP). It is assumed that the animal experiences a conditioned rewarding response to the ethanol-paired environment that causes it to approach the area where ethanol was experienced. Alternatively, more time spent in the saline-paired side is termed conditioned place aversion (CPA) and is assumed to indicate the association of a negative experience with the ethanol-paired environment that is to be avoided (Cunningham, 1993).

The conditioning apparatus example described above (black vs. white) highlights an important methodological concern associated with this technique, bias. A recent review of the place conditioning literature suggested that a general preference for an individual compartment (bias apparatus) and the method of determining which compartment will be paired with ethanol (stimulus assignment) can be confounding to the outcome of place conditioning studies (Cunningham, Ferree, & Howard, 2003). Specifically, using a biased apparatus and stimulus assignment based on initial bias can mask the expression of CPP. These authors demonstrated that when ethanol was paired with the preferred compartment, no place conditioning was observed. However, when ethanol was paired with the less preferred compartment, ethanol elicited CPP. Conversely, when unbiased apparatus and stimulus assignment procedures where used, ethanol CPP was observed. These findings suggest that if the direction of a drug's conditioned effect is unknown at the time of stimulus assignment, the use of a biased apparatus could prevent the observation of conditioning. Finally, the use of unbiased

stimulus assignment procedures allows the detection of the presence of CPP within treatment groups without the need for comparisons between pre- and post-conditioning preference testing or comparison to animals treated with saline in both compartments (Cunningham, Ferree et al., 2003).

Like the modified operant task previously discussed, because this model lacks a consummatory phase, it allows the examination of appetitive effects alone. Models that employ ethanol consumption often examine both the conditioned and the primary rewarding effects of ethanol in combination. That is, the conditioned effects of ethanol are inseparable from the direct effects of ethanol when ethanol is present. CPP allows the study of the conditioned effects separately from the direct effects (expression studies) or the two in combination (acquisition studies). Specifically, manipulations on the choice-test day are thought to alter the conditioned response to ethanol alone, since ethanol is not present during the test. This phase can be likened to the appetitive phase of operant self-administration, in that animals are thought to be ethanol-seeking (Cunningham, Fidler et al., 2000). In contrast, manipulations during the conditioning phase of ethanol CPP may alter both the drug's direct effects and the development of the conditioned rewarding properties of ethanol.

One potential disadvantage of this model is that ethanol is given by injection. This may be a disadvantage because the animal does not take the drug voluntarily and because it is delivered via injection, a manner in which humans do not take ethanol. However, this disadvantage can be viewed as an advantage, in so far as the dose of ethanol is controlled by the experimenter and thus can be held constant across animals. Findings from CPP studies are often correlated with those using self-administration,

however, some evidence suggests that CPP and self-administration are dissociable (Bardo & Bevins, 2000).

To summarize, CPP is relatively quickly assessed and allows the examination of both the conditioned rewarding properties of ethanol either alone or in combination with the direct effects of ethanol. However, the forced nature of ethanol administration decreases its face validity.

CPP vs. CPA

Many compounds have been shown to have either rewarding or aversive effects in the place preference paradigm. However, it has recently been reported that drugs of abuse, including ethanol, paradoxically possess both rewarding and aversive effects the expression of which, depends upon the temporal relationship between drug injection and exposure to the conditioned stimulus (CS+). For example, if ethanol is injected just prior to exposure to the CS+, CPP is typically observed. Conversely, if ethanol is injected just after exposure to the CS+, CPA is typically observed (e.g. Cunningham, Smith, & McMullin, 2003). These findings suggest that a single drug injection can have both rewarding and aversive effects. It seems that the existence of these two states is revealed in the place conditioning paradigm because of their time courses and subsequent proximity to CS+ exposure (Cunningham, Okorn, & Howard, 1997). It has been proposed that the aversive component of the ethanol experience has a very short time-course that may be related to the rapid transition from the sober to the intoxicated state. In contrast, the rewarding component has been proposed to occur over a much longer time course (Cunningham, Smith et al., 2003). Taken together with principles of

Pavlovian conditioning, these findings suggest that in the post-CS+ exposure condition, the rapid aversive component is most closely associated in time with CS+ exposure. While in the pre-CS+ injection procedure, the animals experience the rapid aversive component followed by a more prolonged rewarding component, which becomes associated with CS+ exposure. Several findings suggest that these two components represent independent effects of ethanol (Cunningham & Ignatoff, 2000; Cunningham, Tull, Rindal, & Meyer, 2002; Hill et al., 2002; Le et al., 2001).

Ethanol CPP neurochemical mechanisms

Place preference for ethanol is routinely observed in mice (Cunningham, Okorn, & Howard, 1996). Conversely, place aversion is more commonly observed in rats (e.g., Cunningham, 1979) except under specific experimental conditions such as repeated ethanol pre-exposure (e.g., Reid, Hunter, Beaman, & Hubbell, 1985) the induction of fear stress (e.g., Matsuzawa, Suzuki, & Misawa, 1998) or the use of a biased apparatus and/or stimulus assignment (e.g., Biala & Langwinski, 1996). The following summary focuses on place conditioning models using mice.

The literature attempting to identify the neurochemical mechanisms underlying ethanol CPP in mice can be divided into three major experimental approaches; acquisition (Table 1), expression (Table 2), and null mutation experiments (Table 3). Acquisition experiments generally involve the use of drug treatments given in combination with ethanol during the conditioning phase. As previously mentioned, these experiments cannot differentiate between effects on the primary and conditioned rewarding effects of ethanol. That is, these two effects are inseparable during CPP

acquisition. In experiments examining drug effects on the expression of ethanol CPP, conditioning is carried out identically without drug pre-treatment in all groups. However, a drug treatment is given prior to the preference test. Because ethanol itself is not present during the test, changes in behavior are assumed to result from changes in the conditioned rewarding effect of ethanol. Knock-out (KO) experiments compare ethanol-induced CPP in wildtype mice to that of mice lacking a gene of interest. The KO models used thus far include gene deletions that are present at conception. Therefore, effects on the acquisition and expression of ethanol CPP are indistinguishable. Findings involving each of these three approaches are described in the subsequent sections.

As shown in the summary tables, opioid systems are implicated by all three of these approaches. Because opioid systems are the focus of this thesis, these data will be discussed later in the context of opioid effects on ethanol reward.

GABA

The $GABA_A$ receptor antagonists picrotoxin and bicuculline have both been shown to enhance the acquisition of ethanol CPP (Chester & Cunningham, 1999b). However, the inverse agonist at the benzodiazepine binding site of the $GABA_A$ receptor, Ro 15-4513, was without effect (Risinger, Malott, Riley, & Cunningham, 1992). Moreover, the $GABA_B$ receptor agonist, baclofen did not alter the acquisition of ethanol CPP (Chester & Cunningham, 1999a). These findings suggests that $GABA_A$, but not $GABA_B$ receptors are involved in the acquisition of ethanol CPP. Further, recent KO studies suggest that specific subunits of the $GABA_A$ receptor might be of particular importance since the deletion of the β2 subunit gene prevented ethanol CPP, while

deletion of the α1 subunit gene was without effect (Blednov et al., 2003). These findings suggest that in particular, the β2 subunit of the $GABA_A$ receptor might be important in ethanol CPP, but it is not clear whether these effects were on acquisition or expression since the deletion was present during both. In summary, $GABA_A$ receptors can influence the acquisition of ethanol CPP. However, beyond the potential implications of KO studies, the role of GABA receptors in the expression of ethanol CPP is unknown.

Dopamine

Dopamine systems have been broadly implicated in the rewarding properties of ethanol in various self-administration models (Weiss & Porrino, 2002). However, few studies involving place conditioning support this idea. To illustrate, haloperidol, the D2-like (D2, D3, D4) receptor antagonist and clozapine, the D4 receptor antagonist, had no significant effect on the acquisition of ethanol CPP (Risinger, Dickinson, & Cunningham, 1992; Thrasher, Freeman, & Risinger, 1999). On the other hand, both D2 (Cunningham, Howard et al., 2000) and DARP-32 (dopamine and adenosine 3',5'-monophosphate-regulated phosphoprotein, 32 kDa) KOs display decreased ethanol CPP (Risinger, Freeman, Greengard, & Fienberg, 2001). In contrast, the D3 receptor antagonist, U-99194A has been shown to enhance the acquisition of ethanol CPP in two separate experiments (Boyce & Risinger, 2000, 2002), while other findings demonstrate that the deletion of the D3 receptor gene was without effect (Boyce-Rustay & Risinger, 2003).

Results from expression experiments do not help to explain differences between acquisition and KO experiments because no dopamine receptor antagonist tested has yielded effects on ethanol CPP expression. In particular, haloperidol (D2, D3, D4

antagonist), SCH23390 (D1 antagonist), raclopride (D2 antagonist), and U99194A (D3 antagonist) did not alter the expression of ethanol CPP (Cunningham, Malott, Dickinson, & Risinger, 1992; Dickinson, Lee, Rindal, & Cunningham, 2003). To summarize, the role of dopamine receptors in the conditioned rewarding effects of ethanol is not entirely clear. In particular, only D3 receptor antagonists or D2 and DARP32 receptor mutaion alter the conditioned effects of ethanol and there is not agreement between results obtained from pharmacological manipulations and KO models suggesting possible developmental compensation effects in KOs.

Serotonin (5-HT)

The 5-HT2 receptor antagonist mianserin and the 5-HT1A receptor antagonist pindobind-5HT1A were both shown to enhance the acquisition of ethanol CPP (Risinger & Boyce, 2002; Risinger & Oakes, 1996). In contrast, deletion of the 5-HT1B receptor decreased ethanol CPP (Risinger, Bormann, & Oakes, 1996). Finally, fluoxetine, the serotonin selective reuptake inhibitor (SSRI) had no effect on the acquisition of ethanol CPP (Risinger, 1997). While serotonin may play an important role in the acquisition of ethanol CPP, these data suggest that specific serotonin receptor subtypes might be important, rather than serotonin more generally.

Glutamate

Various glutamate receptor antagonists including the mGluR5 receptor antagonist MPEP (McGeehan & Olive, 2003b), two NR2B receptor antagonists (ifenprodil and CP-101-606) and the NMDA channel blockers MK-801 and ketamine reportedly have no

effect on the acquisition of ethanol CPP (Boyce-Rustay & Cunningham, 2004). In addition, the glycine$_B$ receptor partial agonist, (+)-HA-966 was also without effect on the acquisition of ethanol CPP (Boyce-Rustay & Cunningham, 2004). In contrast, acamprosate, which may act through glutamate receptors decreased the acquisition of ethanol CPP (McGeehan & Olive, 2003a). As well, CGP-37849, a competitive NMDA receptor antagonist, has been shown to decrease both CPP and CPA. However, based on these and additional experiments, these authors concluded that the observed effects were likely due to learning impairments induced by CGP-37849 (Boyce-Rustay & Cunningham, 2004). In summary, some evidence implicates the glutamate systems in the acquisition of ethanol CPP, although it is unclear whether these effects are on the rewarding effect of ethanol or the learning of ethanol CPP. Finally, no experiments have been conducted to examine the role of this neurotransmitter system on the expression of ethanol CPP.

Steroids

One recent study showed that corticosterone can either diminish or enhance the acquisition of ethanol CPP depending on the timing of the conditioning trials (Brooks, Hennebry, Croft, Thomas, & Little, 2004). That is, if CS+ and CS- trials are given on alternate days, corticosterone before CS+ exposure decreased ethanol CPP. However, when CS- trials were given immediately before CS+ trials, corticosterone just before CS+ trials enhanced ethanol CPP. Nevertheless, a different study showed that aminoglutethimide, the steroid synthesis inhibitor had no effect on either acquisition or expression of ethanol CPP, suggesting that significantly reducing corticosterone levels

Table 1. Summary of drug effects on the acquisition of ethanol CPP.

System	Pharmacological Mechanism	Agent	Outcome[1]	Citation
Opioid	Non-selective Antagonist	Naloxone	No Effect	(Cunningham, Dickinson, & Okorn, 1995)
	Non-selective Antagonist	Naloxone	Decrease[2]	(Biala & Langwinski, 1996)
	Non-selective Antagonist	Naloxone	CPA; Enhance[2]	(Bormann & Cunningham, 1997)
	Non-selective Antagonist	Naloxone	Decrease[2,3]	(Matsuzawa, Suzuki, Misawa, & Nagase, 1998)
	μ Antagonist	β-funalrexamine	Decrease[2,3]	"
	δ Antagonist	Naltridole	Decrease[2,3]	"
	κ Antagonist	Nor-binaltor-phimine	No Effect[2,3]	"
	μ Agonist	Morphine	Enhance[2,3]	"
	δ Agonist	TAN-67	Enhance[2,3]	"
	κ Agonist	U50,488H	No Effect[2,3]	"
	μ Agonist	Morphine	Enhance[2,3]	(Matsuzawa, Suzuki, Misawa, & Nagase, 1999)
	δ Agonist	TAN-67	Enhance[2,3]	
	ORL1 (NOR) Agonist	Nociceptin/ OrphaninFQ	Decrease	(Kuzmin, Sandin, Terenius, & Ogren, 2003)
	ORL1 (NOR) Agonist	Ro 64-6198	Decrease	"
	Non-selective Antagonist	Naloxone	No Effect	"
	ORL1 (NOR) Agonist	Nociceptin/ OrphaninFQ	Decrease[2,4]	(Ciccocioppo, Panocka, Polidori, Regoli, & Massi, 1999)
GABA	$GABA_A$ Benzodiazepine Inverse Agonist	Ro 15-4513	No Effect	(Risinger, Malott et al., 1992)
	$GABA_A$ Antagonist	Picrotoxin	Enhance	(Chester & Cunningham, 1999b)
	$GABA_A$ Antagonist	Bicuculline	Enhance	"
	$GABA_B$ Agonist	Baclofen	No Effect	(Chester & Cunningham, 1999a)
Dopamine	D2, D3, D4 Antagonist	Haloperidol	No Effect	(Risinger, Dickinson et al., 1992)

Table 1: continued

System	Pharmacological Mechanism	Agent	Outcome[1]	Citation
	D4 Antagonist	Clozapine	No Effect	(Thrasher et al., 1999)
	D3 Antagonist	U-99194A	Enhance	(Boyce & Risinger, 2000)
	D3 Antagonist	U-99194A	Enhance	(Boyce & Risinger, 2002)
Serotonin	5-HT2 Antagonist	Mianserin	Enhance	(Risinger & Oakes, 1996)
	SSRI	Fluoxetine	No Effect	(Risinger, 1997)
	5-HT1A Antagonist	Pindobind-5HT1A	Enhance	(Risinger & Boyce, 2002)
Glutamate	MGluR5 Antagonist	MPEP	No Effect	(McGeehan & Olive, 2003b)
	NMDA competitive antagonist	CPG-37849	Decrease	(Boyce-Rustay & Cunningham, 2004)
	NMDA competitive antagonist	CPG-37849	CPA; Decrease[5]	"
	NMDA channel blocker	MK-801	No Effect	"
	NMDA channel blocker	Ketamine	No Effect	"
	NR2B Antagonist	Ifenprodil	No Effect	"
	NR2B Antagonist	CP-101,606	No Effect	"
	$Glycine_B$ partial agonist	(+)-HA-966	No Effect	"
	Unknown	Acamprosate	Decrease	(McGeehan & Olive, 2003b)
Steroid	Synthesis Inhibitor	Amino-glutethimide	No Effect	(Chester & Cunningham, 1998)
	Agonist	Corticosterone	Decrease	(Brooks et al., 2004)
	Agonist	Corticosterone	Enhance	
Other	nNOS Inhibitor	7-nitroindazole	Decrease	(Itzhak & Martin, 2000)
	σ_1 Agonist	PRE-084	Enhance	(Maurice, Casalino, Lacroix, & Romieu, 2003)
	σ_1 Antagonist	DB1047	Decrease	

[1] The term decrease is used to describe experiments where CPP was either decreased or eliminated.
[2] Studies carried out in rats
[3] Ethanol place preference was induced by preexposure to a shock associated stimulus
[4] Studies carried out in msP rats.
[5] Ethanol injections given after CS+ exposure

Table 2. Summary of drug effects on the expression of ethanol CPP.

System	Pharmacological Mechanism	Agent	Outcome[1]	Citation
Opioid	Non-selective Antagonist	Naloxone	Decrease	(Cunningham et al., 1995)
	Non-selective Antagonist	Naloxone	CPA; No Effect[2]	(Bormann & Cunningham, 1997)
	Non-selective Antagonist	Naloxone	Decrease	(Cunningham, Henderson, & Bormann, 1998)
	Non-selective Antagonist	Naloxone	CPA; Enhance[3]	"
	Non-selective Antagonist	Naltrexone	Decrease	(Middaugh & Bandy, 2000)
	ORL1 (NOR) Agonist	Nociceptin/ OrphaninFQ	Decrease	(Kuzmin et al., 2003)
	ORL1 (NOR) Agonist	Ro 64-6198	Decrease	"
	Non-selective Antagonist	Naloxone	Decrease	"
Dopamine	D2, D3, D4 Antagonist	Haloperidol	No Effect	(Cunningham et al., 1992)
	D1 Antagonist	SCH23390	No Effect	(Dickinson et al., 2003)
	D2 Antagonist	Raclopride	No Effect	"
	D3 Antagonist	U99194A	No Effect	"
Steroid	synthesis inhibitor	Amino-glutethimde	No Effect	(Chester & Cunningham, 1998)

[1] The term decrease is used to describe experiments where CPP was either decreased or eliminated.
[2] Studies carried out in rats
[3] Ethanol injections given after CS+ exposure

Table 3. Summary of null mutation effects ethanol CPP.

System	Deletion	Outcome[1]	Citation
Opioid	μ Receptor	Decrease	(Hall, Sora, & Uhl, 2001)
	Preproenkephalin	No Effect	(Koenig & Olive, 2002)
	Nociceptin/ OrphaninFQ	No Effect	(Kuzmin et al., 2003)
GABA	α1 Receptor	No Effect	(Blednov et al., 2003)
	β2 Receptor	Decrease	
Dopamine	D2 Receptor	Decrease	(Cunningham, Howard et al., 2000)
	DARP-32	Decrease	(Risinger et al., 2001)
	D3 Receptor	No Effect	(Boyce-Rustay & Risinger, 2003)
Serotonin	5-HT_{1B} Receptor	Decrease	(Risinger et al., 1996)
Other	GIRK2	Decrease	(Hill, Alva, Blednov, & Cunningham, 2003)
	ApoE	Enhance	(Bechtholt, Smith, Raber, & Cunningham, 2004)

[1] The term decrease is used to describe experiments where CPP was either decreased or eliminated.

does not alter ethanol CPP (Chester & Cunningham, 1998). While some data suggest that steroids might play a role in the acquisition of ethanol CPP the data are few and the results are somewhat tentative.

Other

Finally, several single reports implicate other systems and proteins including neuronal nitric oxide synthase (nNOS), σ_1 receptors and G-protein-coupled inwardly rectifying potassium channel 2 (GIRK2) in ethanol CPP. The acquisition of ethanol CPP is decreased by 7-nitroindazole, the nNOS inhibitor (Itzhak & Martin, 2000). A σ_1 receptor agonist enhanced while an antagonist decreased the acquisition of ethanol CPP (Maurice et al., 2003). Finally, deletion of the GIRK2 gene decreased ethanol CPP (Hill et al., 2003).

In short, alterations of many systems including, GABA, dopamine, serotonin, steroids and glutamate have changed the acquisition of ethanol-induced CPP. In contrast, few systems have been examined for their role in the expression of the conditioned effects of ethanol. Several experiments have suggested that the opioid, but not the dopamine system might be involved in the expression of ethanol-induced CPP.

Ethanol and opioid peptides

The effects of non-selective opioid receptor antagonists on ethanol related behaviors have been extensively studied. As a result, a large body of literature suggests that the rewarding properties of ethanol might be mediated by the endogenous opioid system (for review see Herz, 1997). This relationship has been demonstrated in many

species and using several behavioral paradigms. Reviewed here are effects of opioid system manipulations observed in the treatment of alcoholism and in animal models of ethanol drinking, self-administration and place conditioning.

Effects in humans

Numerous single center and multicenter placebo-controlled clinical trials have demonstrated that a non-selective opioid receptor antagonist improved treatment outcomes for alcoholics. In particular it has been reported that naltrexone decreased relapse (Anton et al., 1999; Guardia et al., 2002; Heinala et al., 2001; Latt, Jurd, Houseman, & Wutzke, 2002; O'Malley et al., 1992; Oslin, Liberto, O'Brien, Krois, & Norbeck, 1997; Volpicelli, Alterman, Hayashida, & O'Brien, 1992), craving (Chick et al., 2000; Heinala et al., 2001; Volpicelli et al., 1992), days of drinking (Monti et al., 2001; O'Malley et al., 1992; Volpicelli et al., 1992) and number of drinks if the patient drank (Anton et al., 1999; Chick et al., 2000; Monti et al., 2001). These findings were further supported by a meta-analysis that demonstrated that naltrexone treatment was superior to placebo treatment in that it significantly decreased the relapse episodes and increased abstinence (Streeton & Whelan, 2001).

In contrast, some studies indicated that naltrexone was without effect (Gastpar et al., 2002; Kranzler, Modesto-Lowe, & Van Kirk, 2000; Krystal, Cramer, Krol, Kirk, & Rosenheck, 2001). In response to one of these negative findings, Fuller and Gordis (2001) suggested that it is important to consider the patient population and total treatment program when attempting to assess the effectiveness of naltrexone. In particular, they suggested that it may be important to identify what types of patients are most likely to

benefit from naltrexone treatment and that naltrexone should be given in conjunction with counseling. In support of this suggestion, some findings indicate that naltrexone might be most effective in highly motivated patients since naltrexone is more effective with a high rate of compliance (Chick et al., 2000; Volpicelli et al., 1997). Similarly, some studies suggest that the particular type of treatment program might influence the effects of naltrexone since certain approaches seem to be more effective in conjunction with naltrexone. For example, it has been reported that naltrexone is most effective in patients who drank during the treatment (Oslin et al., 1997; Volpicelli et al., 1992). In contrast, Gastpar and colleagues reported no effect of naltrexone treatment in their study where complete abstinence was advocated (Gastpar et al., 2002).

To summarize, non-selective opioid receptor antagonists are somewhat effective in the treatment of alcoholism. However, naltrexone appears to be most effective in certain patient populations and in conjunction with particular types of therapy. Further, the mechanism through which naltrexone is effective remains unknown. Therefore, further investigations into the mechanisms underlying the effects of non-selective opioid receptor antagonists on the effects of ethanol are required.

Effects on drinking in animals

A role for the endogenous opioid system in ethanol drinking was suggested by many studies beginning in the early 1980s. In particular, several early studies demonstrated that given a choice between water and ethanol, animals drank more ethanol if pre-treated with morphine (Hubbell et al., 1986; Reid & Hunter, 1984; Wild & Reid, 1990), while naloxone decreased ethanol consumption (Hubbell et al., 1986; Reid &

Hunter, 1984). However, the specificity of this effect on ethanol consumption was unclear because 1) in order to induce drinking, rats were typically fluid deprived and then allowed access to a sweetened ethanol solution or water and 2) these studies demonstrated that consumption of both the ethanol solution and water were decreased by naloxone.

Problems in interpretation based on the procedures used to induce consumption have been largely avoided by the use of animals that have a propensity to consume ethanol, such as animals selectively bred for preference for ethanol over water. Because these animals preferred to drink an ethanol solution with a 2:1 ratio over water, deprivation or added sweeteners were not necessary to induce ethanol consumption (Froehlich, Harts, Lumeng, & Li, 1987, 1990). Using this or similar models of ethanol drinking, several groups have reported effects of non-selective opioid receptor antagonists on ethanol consumption in the absence of changes in water consumption (Froehlich et al., 1987, 1990; Myers & Lankford, 1996; Parkes & Sinclair, 2000; Phillips, Wenger, & Dorow, 1997). Specifically, High Alcohol Drinking (HAD) (Froehlich et al., 1987, 1990; Myers & Lankford, 1996), and Alko, Alcohol (AA) (Parkes & Sinclair, 2000) selectively bred rat lines and the ethanol preferring mouse strain, C57BL/6J (Phillips et al., 1997), all showed decreased ethanol consumption following non-selective opioid receptor antagonist treatment without altered water consumption. A decrease in ethanol consumption was also observed in separate studies using C57BL/6J mice (Middaugh & Bandy, 2000) and Alcohol-preferring (P) rats (Overstreet et al., 1999), however, water consumption results were not reported.

Several studies suggest that the specific effects of non-selective opioid receptor

antagonists on ethanol consumption are derived from their effects at μ and or δ_2-opioid receptors, but not κ-receptors or the opioid-like orphan receptor. ORL1 (also known as NOR). In particular it has been reported that μ- and δ_2-receptor antagonists can decrease ethanol consumption without altering water consumption (Hyytia, 1993; Krishnan-Sarin, Portoghese, Li, & Froehlich, 1995; Krishnan-Sarin, Wand, Li, Portoghese, & Froehlich, 1998). On the contrary, κ-receptor *agonists* are required to obtain similar effects (Lindholm, Werme, Brene, & Franck, 2001). Similarly, repeated orphaninFQ/nociceptin, the endogenous ligand to the ORL1 receptor decreases ethanol consumption in Marchigian Sardinian alcohol-preferring rats (msP) (Ciccocioppo et al., 1999).

A much earlier study provides insight for the specificity of non-selective opioid receptor antagonist effects on ethanol consumption in preferring animals (Pulvirenti & Kastin, 1988). That is, when animals were categorized based on their initial preference for ethanol, naloxone decreased both ethanol and water consumption in low preference animals, but only ethanol consumption in high preference animals (Pulvirenti & Kastin, 1988). Likewise, a recent study conducted a meta-analysis of rat studies investigating the effects of naloxone on ethanol consumption. While all of the studies included in the analysis reported that control solution consumption was not altered by naloxone treatment, control solutions were significantly decreased overall. However, control solution data were extremely variable and subsequent experiments demonstrated that the degree of naloxone-induced reduction in fluid intake was correlated with the amount of the solution initially consumed (Cichelli & Lewis, 2002). Specifically, the more an animal drank of a given solution, the greater the percentage of suppression elicited by naloxone. These findings may explain why such variation is observed in the literature

with some studies reporting that the consumption of many solutions is decreased by naloxone (e.g., Goodwin, Campisi, Babinska, & Amit, 2001) while others report no effect on control solution consumption. These effects might explain why decreases in control solution consumption have not been reported in animals selectively bred to prefer ethanol (e.g., Froehlich et al., 1990). Specifically, because such animals drink more ethanol than water, it is more likely that non-selective opioid receptor antagonists would decrease ethanol intake.

The effects of non-selective opioid receptor antagonists on ethanol drinking have also been examined under repeated treatment protocols. These results have been relatively inconsistent across experiments. In particular, repeated (every other day) (Davidson & Amit, 1997; Parkes & Sinclair, 2000) and periodic (5 days, every other 5 days) (Reid, Gardell, Chattopadhyay, & Hubbell, 1996) naltrexone treatments were reported to continually decrease ethanol consumption. However, some of these experiments used forced ethanol consumption (Davidson & Amit, 1997) or fluid deprivation (Reid et al., 1996) to induce ethanol consumption. Conversely, others report a progressive tolerance to the effects of non-selective opioid receptor antagonists effects on ethanol consumption as evidenced by decreased suppression with repeated treatment in P rats (Overstreet et al., 1999), fawn-hooded rats (Cowen, Rezvani, Jarrott, & Lawrence, 1999) and C57BL/6J mice (Phillips et al., 1997). In addition, chronic treatment, via a subcutaneous pellet, actually increased ethanol consumption (Phillips et al., 1997). Although, some of these studies report opposite effects of repeated non-selective opioid receptor antagonists on drinking, there is a consensus that non-selective opioid receptor antagonists result in the upregulation of opioid receptors (Cowen et al., 1999; Overstreet

et al., 1999; Parkes & Sinclair, 2000).

In summary, it has been repeatedly demonstrated that non-selective opioid receptor antagonists decrease the consumption of ethanol. However, some findings suggest that these effects may only be selective for ethanol in animals that prefer ethanol. Further, some findings suggest that the effectiveness of non-selective opioid receptor antagonists might be short lived.

Effects on operant self-administration in animals

As in the drinking literature, results regarding the specificity and time-course of effects of opioid receptor antagonists on operant self-administration are sometimes conflicting. While some studies reporting decreases have not examined effects on control solution consumption (Boyle et al., 1998; Gonzales & Weiss, 1998; Le et al., 1999), most do. Several studies using a variety of procedures and species report that ethanol and not control solution responding is decreased by non-selective opioid antagonism (e.g., Heyser, Roberts, Schulteis, & Koob, 1999; Samson & Doyle, 1985; Sinden, Marfaing-Jallat, & Le Magnen, 1983; Williams, Kane, & Woods, 2001). Rats that were rationed food in order to encourage ethanol self-administration demonstrated a naloxone induced decrease in ethanol responding without altered sucrose responding (Samson & Doyle, 1985). Similarly, methylnaloxonium, a modified form of naloxone that does not cross the blood-brain-barrier, administered intracerebroventricular (i.c.v.) or intra-nucleus accumbens (NAc) decreased responding for ethanol in non-deprived rats without altering concurrent water responding (Heyser et al., 1999). And, rats that underwent continuous passive ethanol administration and were later allowed to self-administer ethanol

intragastrically demonstrated decreased responding for ethanol after naloxone treatment that did not alter food or water consumption (Sinden et al., 1983). Finally, rhesus monkeys trained to respond for ethanol and water demonstrated decreased responding only for ethanol after both morphine and naltrexone treatment (Williams et al., 2001).

Like the effects observed for ethanol drinking, the selective effects of opioid receptor antagonists on ethanol self-administration may be mediated by δ_2-opioid receptors since δ_2-receptor antagonists decreased ethanol, but not sucrose responding (June et al., 1999). Interestingly, additional studies using msP rats suggest a selective role for the ORL1 receptor in ethanol reward since orphaninFQ/nociceptin decreased responding for ethanol without altering sucrose responding (Ciccocioppo et al., 2004). In contrast, many findings, including some from the same laboratories, suggest that non-selective opioid receptor antagonists are not selective for ethanol in that control solution responding is also reduced (Gallate & McGregor, 1999; Schwarz-Stevens, Files, & Samson, 1992; Sharpe & Samson, 2001; Shelton & Grant, 2001; Williams & Woods, 1999). For example, naltrexone decreased responding for ethanol and Tang (Shelton & Grant, 2001) or sucrose (Williams, Winger, Pakarinen, & Woods, 1998) in monkeys and responding for both beer and near beer in rats (Gallate & McGregor, 1999).

A more recent study examined the effects of naloxone on the appetitive (i.e., drug seeking) and consummatory (i.e., drug taking) aspects of ethanol self-administration separately. Animals were required to make several responses on a lever (appetitive) in order to gain 20 min access to ethanol or sucrose (consummatory). These authors reported that naloxone decreased the amount of ethanol or sucrose consumed, but did not significantly alter ethanol or sucrose seeking (Sharpe & Samson, 2001). However, the

authors did observe relatively profound effects of naloxone on appetitive behavior in some animals at some doses. Therefore, they concluded that naloxone may affect appetitive behavior to some degree in some animals (Sharpe & Samson, 2001). Although the authors did not address this question, it is possible that between animal variation is derived from variation in preference for ethanol between animals.

As suggested in ethanol drinking, the specificity of non-selective opioid receptor antagonists on operant responding for ethanol may be influenced by ethanol preference. This was suggested by a study using rhesus monkeys trained to respond for ethanol and water concurrently. The authors varied the concentration of ethanol, which was effective in altering the intake of ethanol and thus, the preference for ethanol. At lower concentrations ethanol was preferred and naltrexone decreased responding only for ethanol. At intermediate ethanol concentrations, ethanol and water were equally preferred and responding for both were decreased by naltrexone. In contrast, at higher concentrations, water was preferred over ethanol and only water responding was decreased by naltrexone (Williams & Woods, 1999).

The vast majority of studies report that naltrexone or naloxone decreased responding for ethanol. In contrast to drinking studies, acute administration of non-selective opioid receptor antagonists in some cases did not induce a decrease in operant responding for ethanol. Instead, repeated antagonist administration was required to decrease ethanol responding (Altshuler, Phillips, & Feinhandler, 1980; Bienkowski, Kostowski, & Koros, 1999; Hyytia & Sinclair, 1993). In his seminal paper, Altshuler and colleagues reported that naltrexone treatment actually increased intravenous ethanol self-administration in rhesus monkeys for the first 5 days of treatment, which was followed by

a decrease in the following 10 days (Altshuler et al., 1980). Similarly, both Wistar and AA rats did not show acute effects of non-selective opioid antagonism until several doses had been administered (Bienkowski et al., 1999; Hyytia & Sinclair, 1993).

To summarize, the findings regarding the effects of non-selective opioid receptor antagonists on the operant self-administration of ethanol are very similar to those for ethanol drinking. In particular, non-selective opioid receptor antagonists have been reported to decrease responding for ethanol and may only be selective for ethanol if it is the preferred solution. In contrast to drinking findings, although acute effects have been reported, some findings suggest that repeated treatment is required to decrease responding for ethanol.

Place conditioning in animals

It seems clear that the opioid system is involved in ethanol drinking and operant self-administration of ethanol, although the effects may only be selective for ethanol under certain circumstances. Similar findings have been reported with respect to the acquisition of ethanol CPP in rats. Non-selective (Biala & Langwinski, 1996; Matsuzawa, Suzuki, Misawa et al., 1998) as well as μ and δ-opioid receptor selective antagonists have been reported to decrease ethanol CPP in rats, while κ-opioid receptor antagonists are without effect (Matsuzawa, Suzuki, Misawa et al., 1998). In the same way, μ (Matsuzawa, Suzuki, Misawa et al., 1998; Matsuzawa et al., 1999) and δ-opioid receptor agonists enhance ethanol CPP in rats, while κ-receptor agonists were without effect (Matsuzawa, Suzuki, Misawa et al., 1998; Matsuzawa et al., 1999). In contrast, in experiments where ethanol CPA was observed in rats, naloxone given during

conditioning (acquisition) enhanced CPA, but was without effect when administered just before the preference test (expression) (Bormann & Cunningham, 1997).

As previously noted, place aversion for a CS paired with ethanol is more commonly observed in rats (e.g., Cunningham, 1979) except under specific experimental conditions. All of the findings just described where CPP was observed, also involved induction via exposure to a cue previously paired with foot shock (Matsuzawa, Suzuki, Misawa et al., 1998; Matsuzawa et al., 1999) or the use of biased stimulus assignment (Biala & Langwinski, 1996; Matsuzawa, Suzuki, Misawa et al., 1998; Matsuzawa et al., 1999), which might explain inconsistencies between these and other data (e.g., Bormann & Cunningham, 1997). Animals that were not exposed to a fear arousing stimulus did not express ethanol CPP, suggesting that opioid effects were not directly on ethanol's rewarding effects but were due instead to an effect on the response to stress or to some interactive effects of ethanol and stress (Matsuzawa, Suzuki, Misawa et al., 1998; Matsuzawa et al., 1999). Further, it has been reported that the use of biased stimulus assignment can mask some effects (Cunningham, Ferree et al., 2003). In these studies by Matsuzawa and colleagues, the use of biased apparatus and stimulus assignment might have created a floor effect that prevented the observation of CPA in animals that were not shocked (Matsuzawa, Suzuki, & Misawa, 1998; Matsuzawa, Suzuki, Misawa et al., 1998). Specifically, ethanol was paired with the animals least preferred compartment. Since this initial aversion to the ethanol-paired compartment was extreme, it is unlikely that a further decrease in the time spent in the ethanol-paired compartment could be detected. Finally, in the case where preference was observed without the use of shock (Biala & Langwinski, 1996), the use of biased stimulus assignment might reflect an

"antiaversive" effect of ethanol (Fidler, Bakner, & Cunningham, 2004). Thus, it is not clear whether opioid treatments in these experiments decreased the reinforcing properties of ethanol or made the initially aversive compartment more aversive.

In contrast to the rat literature, relatively consistent opioid effects on ethanol CPP have been observed in mice. Specifically, pre-treatment with naloxone on conditioning days (acquisition) was without effect on ethanol CPP regardless of whether ethanol was administered intra-gastric (Kuzmin et al., 2003) or intra-peritoneal (Cunningham et al., 1995). In contrast, non-selective opioid receptor antagonists have been reported to decrease the expression of ethanol CPP (Cunningham et al., 1995; Cunningham et al., 1998; Kuzmin et al., 2003; Middaugh & Bandy, 2000). In addition, mice lacking μ-opioid receptors exhibit decreased ethanol CPP (Hall et al., 2001). Because opioid receptor antagonists decrease expression, but not acquisition of CPP, these findings suggest that the effects observed in the μ-receptor KO are derived from effects on expression, rather than acquisition of ethanol CPP. Importantly, naloxone has also been reported to enhance the expression of ethanol CPA in mice (Cunningham et al., 1998). These findings complement the self-administration literature by suggesting that opioid receptor antagonists may decrease ethanol-seeking behavior by decreasing the conditioned rewarding and increasing the conditioned aversive properties of ethanol. Also, the commonality between opioid receptor antagonist effects on ethanol self-administration and place conditioning in mice may result from effects on the expression of the conditioned effects of ethanol. Specifically, because the conditioned effects of ethanol presumably influence behavior in self-administration paradigms, the expression of conditioned effects of ethanol may be the common element that is altered by opioid

receptor antagonists.

In addition, deletion of the preproenkephalin gene did not alter ethanol CPP, suggesting that POMC, rather than preproenkephalin derived peptides may mediate the conditioned rewarding properties of ethanol (Koenig & Olive, 2002). Finally, the opioid-like receptor, ORL1, can influence both the acquisition (Ciccocioppo et al., 1999; Kuzmin et al., 2003) and the expression (Kuzmin et al., 2003) of ethanol CPP . More specifically, orphaninFQ/nociceptin (Ciccocioppo et al., 1999; Kuzmin et al., 2003), the endogenous ligand for the ORL1 receptor, and a synthetic ORL1 receptor agonist (Kuzmin et al., 2003) have been shown to decrease the acquisition (Ciccocioppo et al., 1999; Kuzmin et al., 2003) and expression of ethanol CPP (Kuzmin et al., 2003). However, deletion of the nociceptin/orphaninFQ gene did not alter ethanol CPP (Kuzmin et al., 2003). This dissociation between pharmacological and genetic manipulations may suggest developmental compensation in nociceptin/orphaninFQ KO mice.

Summary; Ethanol and opioid peptides

Nonselective opioid receptor antagonists can improve treatment outcomes for alcoholics (O'Malley et al., 1992; Volpicelli et al., 1992). The practice of including opioid receptor antagonists in treatment regimens for alcoholism was derived primarily from animal studies demonstrating that opioid receptor antagonists such as naltrexone and naloxone decrease ethanol consumption in several species including monkeys, rats and mice (e.g., Altshuler et al., 1980; Froehlich et al., 1987; Phillips et al., 1997). These drugs have also been shown to decrease operant ethanol self-administration (e.g., Samson & Doyle, 1985). Unfortunately, it is unclear whether the effects of opioid receptor

antagonists are specific to the ingestion of ethanol or reflect a more general suppression of ingestive behavior. This uncertainty arises because opioid receptor antagonists also decrease the consumption and operant self-administration of other reinforcers such as water or sucrose under some circumstances (e.g., Hubbell et al., 1986; Schwarz-Stevens et al., 1992). Place conditioning studies are informative in this regard because they typically lack a consumption element. While there are profound behavioral and procedural differences between species in ethanol place conditioning, the evidence generally indicates that the conditioned rewarding effect of ethanol (CPP) is decreased by naloxone, while the conditioned aversive effect (CPA) may be increased (e.g., Cunningham et al., 1998). The exact neurochemical mechanisms and brain areas through which these effects occur remain unknown.

Reward circuit connectivity

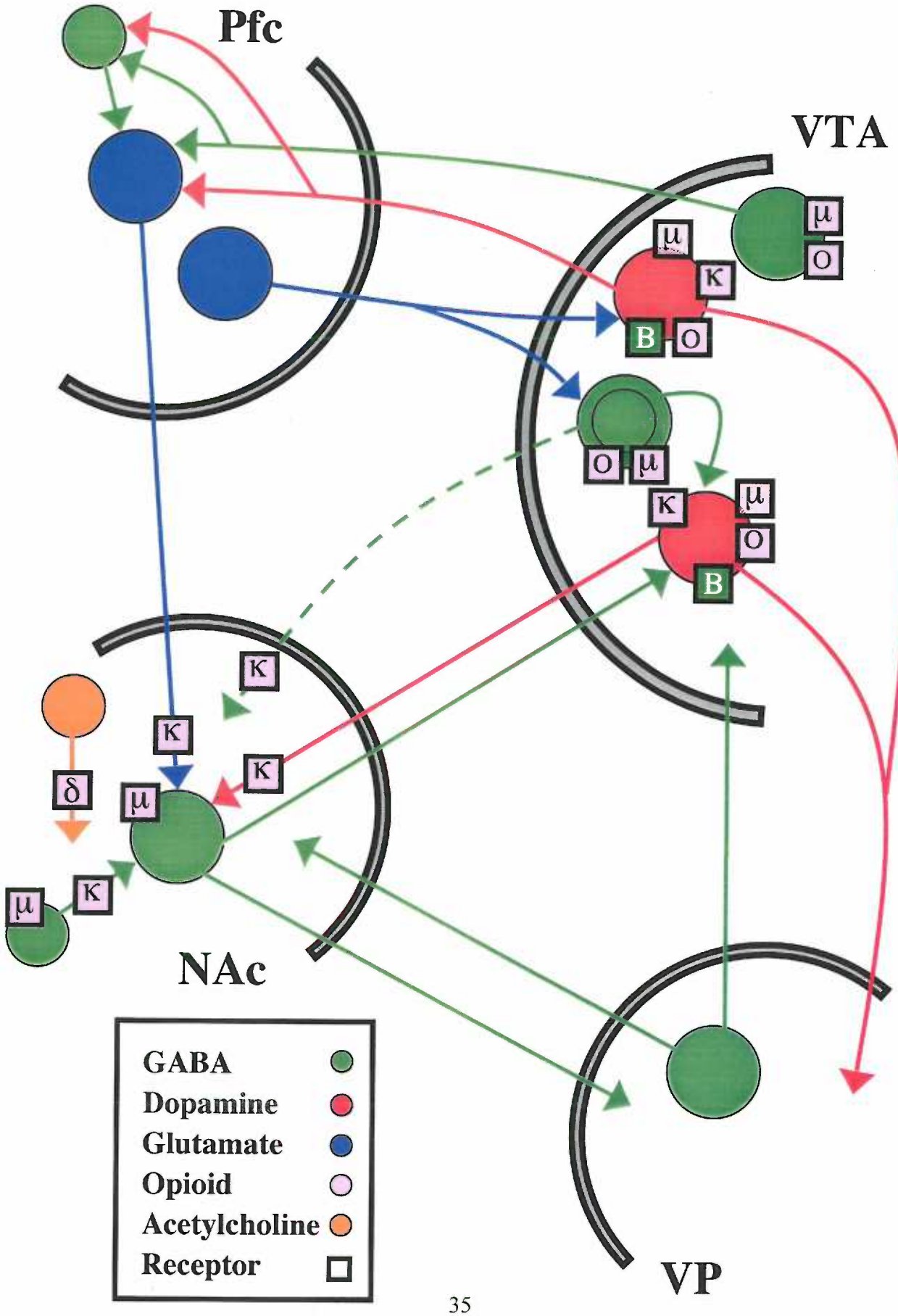
The ventral tegmental area (VTA), NAc and the prefrontal cortex are the primary components of the reward circuit, also called the mesocorticolimbic dopamine system (for review see Tzschentke, 2001). This system includes multiple feedback mechanisms between these three brain areas and several others. Mesencephalic neurons in the VTA project to several brain areas including the NAc, the prefrontal cortex (pfc) and the ventral pallidum (Figure 1, modified from Sesack, Carr, Omelchenko, & Pinto, 2003). Dopamine neurons projecting from the VTA to the pfc form synapses with inhibitory local GABA neurons (Carr & Sesack, 2000a) and excitatory glutamatergic neurons projecting to the GABA-output neurons in the NAc (Carr, O'Donnell, Card, & Sesack, 1999). In addition to dopaminergic projections, the VTA sends GABAergic projections to

the pfc that, like dopamine neurons, form synapses with both local GABA neurons and glutamatergic neurons projecting to the NAc (Carr & Sesack, 2000a). A separate group of dopaminergic and GABAergic neurons project from the VTA to the NAc. The dopaminergic component is known to make contact with medium spiny GABA neurons that in turn, project back to the VTA. In contrast, the GABA component is relatively small (Swanson, 1982) and the nature of the synapses these neurons form remains unknown. In addition, while somewhat controversial, some evidence suggests that local GABA neurons might also be present in the VTA exerting tonic inhibition on dopamine neurons (e.g., Johnson & North, 1992). The pfc sends excitatory glutamatergic projections to the VTA that contact dopamine neurons projecting back to the pfc and the small population of the GABA neurons that project to the NAc (Carr & Sesack, 2000b). Also, both the VTA (dopamine, Klitenick, Deutch, Churchill, & Kalivas, 1992) and the NAc (GABA, Phillipson & Griffiths, 1985) send projections to the ventral pallidum which sends reciprocal inhibitory GABA projections (Kalivas, Churchill, & Klitenick, 1993). Finally, a non-dopaminergic projection from the VTA to the tegmental pedunculopontine nucleus has been recently proposed, although no direct anatomical studies have been done (Laviolette, Gallegos, Henriksen, & Van Der Kooy, 2004). Likewise, a projection from the prefrontal cortex to the pedunculopontine nucleus and back to the VTA has also been suggested without exact neuroanatomical confirmation (Tzschentke & Schmidt, 2000).

Opioid receptor localization and function

Opioid receptors are present throughout the reward circuit where they inhibit

Figure 1. Schematic representation of reward circuit projections including the ventral tegmental area (VTA), nucleus accumbens (NAc), prefrontal cortex (pfc) and ventral pallidum (VP). *Large circles* indicate projection neurons. *Small circles* represent local neurons. GABA neurons and projections are represented in *green*. The GABA neuron in the VTA shown as a *circle within a circle* indicates that these neurons may be local or projection neurons. Dopamine neurons and projections are represented in *red*. Glutamate neurons and projections are represented in *blue*. Acetylcholine neurons represented in *orange*. Boxes indicated receptors. *Purple boxes* represent opioid receptors with the interior symbol indicating the receptor subtype μ, δ, κ. The opioid-like receptor ORL1 (O) is also indicated. *Semi-transparent purple boxes* indicate that only 10% of those neurons express this receptor. *Green boxes* indicate $GABA_B$ receptors. In cases where projections actually make contact with other schematic neurons, known contacts are depicted. Projections shown into an area without contacting a schematic cell indicates that it is not clear which neurons form synapses. In addition, smaller neurons represent inter-neurons, while larger ones represent projection neurons. Finally, the *dashed* projection indicates that very few neurons make up this projection.


Pfc
VTA
NAc
VP
μ
κ
δ
O
B
GABA
Dopamine
Glutamate
Opioid
Acetylcholine
Receptor

various cell types. Of particular interest are the μ and κ opioid receptors, which may exibit tonically active opposing actions (Spanagel, Herz, & Shippenberg, 1992). Discussed below are findings suggesting the locations of μ-, κ-, and δ-opioid receptors as well as opioid-like ORL1 receptors within the VTA and NAc (figure 1). In some cases, dual labeling studies indicate the exact cell types that express opioid receptors, however, in some cases conclusions are based on functional pharmacological findings.

Opioid receptors in the VTA

μ-opioid receptors are expression in the VTA at moderate levels, based on binding studies, (Mansour, Burke, Pavlic, Akil, & Watson, 1996; Mansour, Khachaturian, Lewis, Akil, & Watson, 1987) and are primarily expressed in GABA neurons (Svingos, Garzon, Colago, & Pickel, 2001). Further, those neurons that project to the pfc express these receptors (Svingos, Garzon et al., 2001). However, μ-opioid receptors are also thought to be expressed by intrinsic VTA GABA neurons (Svingos, Garzon et al., 2001) that exert tonic inhibition on dopamine neurons projecting to the NAc (Johnson & North, 1992). In this way, activation of opioid receptors in the VTA is thought to functionally disinhibit dopamine neurons. Additional findings suggest that μ receptors are indeed predominately located on non-dopaminergic cells in the VTA, with only 10% of tyrosine hydroxylase positive neurons expressing them (Garzon & Pickel, 2001). Functionally, μ receptor agonists in the VTA have been shown to enhance dopamine release in the NAc, while μ antagonists decrease dopamine release in the NAc (Devine, Leone, Pocock, & Wise, 1993; Spanagel et al., 1992). In contrast, κ-receptor expression in the VTA is reportedly low (Mansour et al., 1996; Mansour et al., 1987) and activation of VTA κ-

receptors does not affect dopamine release in the NAc (Devine, Leone, Pocock et al., 1993; Spanagel et al., 1992). However, more recent findings suggest that κ-receptor activation directly inhibits tyrosine hydroxylase positive neurons in the VTA (Margolis, Hjelmstad, Bonci, & Fields, 2003). Although δ-opioid receptor binding in undetectable in the VTA (Mansour et al., 1996; Mansour et al., 1987), pharmacological experiments suggest that δ-activation in the VTA increases (Devine, Leone, Carlezon, & Wise, 1993; Devine, Leone, Pocock et al., 1993), while blockade decreases, NAc dopamine release (Devine, Leone, Pocock et al., 1993). Finally, in situ hybridization studies indicate that the opioid-like receptor, ORL1, is expressed in the VTA (Darland, Heinricher, & Grandy, 1998; Neal et al., 1999). Additional findings suggest that these receptors are primarily expressed in dopamine neurons in this region (Maidment, Chen, Tan, Murphy, & Leslie, 2002; Norton, Neal, Kumar, Akil, & Watson, 2002), however, electrophysiological findings indicate that activation of ORL1 receptors in the VTA inhibits both dopaminergic and non-dopaminergic neurons (Zheng, Grandy, & Johnson, 2002). Microdialysis studies suggest that one functional outcome of this inhibition in the VTA is a decrease in dopamine release in the NAc (Maidment et al., 2002). Functionally, the ORL1 differs from μ-, κ-, and δ-opioid receptors, in that, it is regarded as "naloxone-insensitive" (Darland et al., 1998) and therefore may only be affected by non-selective opioid antagonism at very high doses (Calo et al., 2000).

Opioid receptors in the NAc

Very dense binding of μ-opioid receptors is reported in the NAc (Mansour et al., 1996; Mansour et al., 1987). These receptors are primarily expressed in GABAergic

neurons (Svingos, Moriwaki, Wang, Uhl, & Pickel, 1997) and activation or blockade of these receptors does not alter dopamine release (Spanagel et al., 1992). In contrast, the localization of κ-receptors is more ubiquitous. That is, κ-receptors appear to be located on the terminals of dopamine (Svingos, Chavkin, Colago, & Pickel, 2001), glutamate (Meshul & McGinty, 2000) and GABA (Svingos, Colago, & Pickel, 1999) neurons in the NAc. Further, activation of κ-receptors has been shown to inhibit the release of dopamine (Spanagel et al., 1992), glutamate (Hjelmstad & Fields, 2001) and GABA (Hjelmstad & Fields, 2003) in the NAc. In contrast, δ-receptors in the NAc are primarily located on terminals that oppose dopamine transporter containing terminals (Svingos, Clarke, & Pickel, 1999),which are likely cholinergic (Svingos, Clarke, & Pickel, 1998). Finally, ORL1receptors have thus far not been detected in this region (Darland et al., 1998; Neal et al., 1999).

Reward circuit; conclusions

It has been firmly established that many drugs of abuse, including ethanol, share a common effect in their ability to increase dopamine (DA) levels in the NAc (for review see Di Chiara, 1995) and that this increase in extracellular dopamine in the NAc is correlated with reward. However, four major problems make this reductionist view of the reward circuit overly simplistic. First, as described in the previous section, dopamine release occurs within a neural circuit that provides multiple feedback mechanisms that can actually counteract the effects of dopamine. However, some evidence suggests that repeated drug use might strengthen some of these connections while simultaneously weakening others (Pierce & Kalivas, 1997). Second, multiple cell types exist in each

region and in some cases share receptor types, making it difficult to determine the effects of a pharmacological manipulation on the output of the nucleus. Third, while many of the major projections within the circuit are known, few retrograde and double labeling studies have been conducted to determine the exact connectivity of different cell types both within and between regions. While these data are increasing, predictions are difficult since it is not always clear what specific cells within nuclei will be altered by a pharmacological manipulation. Fourth, the majority of these finding have been established in the rat, making generalizations to other species tentative.

Reward circuit and ethanol reward

The reward circuit has been repeatedly implicated in the actions of ethanol. Ethanol increases the firing rate of VTA dopamine neurons with bath application in vitro (Brodie, Shefner, & Dunwiddie, 1990) and intravenous administration in vivo (Gessa, Muntoni, Collu, Vargiu, & Mereu, 1985). Also, ethanol is self-administered directly into the VTA (Gatto, McBride, Murphy, Lumeng, & Li, 1994; Rodd-Henricks, McKinzie, Crile, Murphy, & McBride, 2000), which has been shown to be dependent on dopamine neurons in the posterior VTA (Rodd et al., 2004). Further, systemic ethanol administration (Di Chiara & Imperato, 1988) and self-administration increase extrasynaptic DA in the NAc (Gonzales & Weiss, 1998). Dopamine receptor agonists injected into the NAc increase operant ethanol self-administration (Hodge, Samson, & Haraguchi, 1992; Samson, Tolliver, Haraguchi, & Hodge, 1992), while dopamine receptor antagonists given intra NAc have the opposite effect (Samson et al., 1992). While DA may play a significant role in ethanol reward, multiple neurotransmitter and

peptide systems are likely involved. For example, ethanol administration increases serotonin levels in the NAc (Yan, 1999) and serotonin receptor antagonists decrease ethanol self-administration (e.g., 5HT3: Hodge, Samson, Lewis, & Erickson, 1993). Likewise, ethanol administration decreases extracellular glutamate levels in the NAc and glutamate receptor antagonism in the NAc has also been shown to decrease ethanol self-administration (Rassnick, Pulvirenti, & Koob, 1992). Finally, as previously described, opioid peptides may also have an important role in ethanol reward (for review see Herz, 1997).

Effects of non-selective opioids are thought to be due to blockade of μ-opioid receptors on local GABA neurons in the VTA that would result in less inhibition of GABA neurons and consequently more inhibition of dopamine neurons (Johnson & North, 1992). Indeed, several studies suggest that the effects of opioid receptor antagonists on the self-administration of ethanol may be mediated by the reward circuit. Responding for ethanol in an operant paradigm increases dopamine levels in the NAc. Systemic opioid receptor antagonists decrease this responding for ethanol and prevent ethanol-induced increases in NAc dopamine (Gonzales & Weiss, 1998). Further, the administration of opioid receptor antagonists directly into the VTA (June et al., 2004) or NAc (Froehlich, O'Malley, Hyytia, Davidson, & Farren, 2003; Heyser et al., 1999; June et al., 2004) are sufficient to decrease responding for ethanol in an operant paradigm.

Dopamine release in the NAc may be a critical response that maintains approach behavior and responding in the presence of ethanol-associated cues. Others have reported that NAc dopamine levels are increased when ethanol-associated environmental cues are presented (Gonzales & Weiss, 1998) and generally that dopamine cells are activated in

response to conditioned stimuli (for review see Schultz, 1998). Two key findings, however, suggest that dopamine is not involved in the expression of the conditioned effects of ethanol. Haloperidol, the non-selective D_2, D_3 and D_4 dopamine receptor antagonist had no effect on the expression of ethanol-induced CPP (Cunningham et al., 1992). Likewise, selective D_1, D_2 and D_3 receptor antagonists did not alter ethanol CPP expression (Dickinson et al., 2003). Further, locomotor effects were observed in these experiments, suggesting that behaviorally effective doses were used. These findings are in direct contradiction to the suggestion that the activation of dopamine receptors is critical to the expression of the conditioned effects of ethanol. Therefore, these data diminish the likelihood that opioid receptor antagonists alter conditioned ethanol reward via downstream alterations in dopamine release. Opioid receptors located on GABA neurons in the VTA that project to the prefrontal cortex (Svingos, Garzon et al., 2001) could be responsible for opioid effects on the conditioned effects of ethanol in a manner that is independent of dopamine.

Recent findings suggest that, gamma-aminobutyric acid $(GABA)_B$ receptor agonists may decrease ethanol consumption and craving in alcoholics (Addolorato et al., 2000; Addolorato et al., 2002). A double blind study of male alcoholics demonstrated that ethanol intakes decreased over a 4-week treatment period in subjects treated with baclofen, a $GABA_B$ receptor agonist, compared to placebo treated controls. While the literature is somewhat inconsistent, this finding is supported by preclinical data demonstrating that $GABA_B$ receptor agonists alter ethanol related behaviors, including withdrawal (Colombo et al., 2000; File, Zharkovsky, & Gulati, 1991), locomotion (Boehm, Piercy, Bergstrom, & Phillips, 2002; Cott, Carlsson, Engel, & Lindqvist, 1976),

and drinking (Colombo et al., 2000; Daoust et al., 1987). Systemic or i.c.v. pretreatment with baclofen attenuates ethanol-induced locomotor stimulation (Boehm et al., 2002; Cott et al., 1976). Additional studies demonstrated that these effects are mediated by the VTA (Boehm et al., 2002). The finding that baclofen, especially in the VTA, decreases ethanol stimulated locomotion is of particular interest since drug-induced locomotion and reward are often correlated (Wise & Bozarth, 1987). This finding was also demonstrated following systemic baclofen treatment in an ethanol CPP paradigm. However, while baclofen decreased ethanol-stimulated activity, no effect was observed on the acquisition of CPP (Chester & Cunningham, 1999a). The effect of baclofen on the expression of ethanol CPP has not been examined. Animals treated with baclofen demonstrated decreased ethanol intake without decreasing water consumption in a choice paradigm (Colombo et al., 2000; Daoust et al., 1987). $GABA_B$ receptor agonists may act similarly, but down-stream to non-selective opioid receptor antagonists in the VTA, to alter dopamine neuron activity. This relationship has been previously reported in that activation of $GABA_B$ receptors in the VTA inhibits the ability of μ-opioid stimulation to elicit dopamine release in the NAc (Kalivas, Duffy, & Eberhardt, 1990).

Rationale

In summary, a large body of literature suggests that the reward circuit, and specifically the activation of dopamine neurons in the VTA, may be involved in the rewarding effects of ethanol. Some data suggest that systems that mediate self-administration may also influence the conditioned effects of ethanol. In contrast, other findings suggest that the conditioned effects of ethanol are expressed through a dopamine

independent mechanism.

The purpose of the following experiments was to identify the brain areas and neurochemical mechanisms through which the conditioned effects of ethanol are expressed. The basic premise was first that the conditioned effects of ethanol might be expressed through neural circuits that are similar to those that mediate ethanol self-administration and second that the effects of systemic opioid receptor antagonists could be localized in the brain. Further, a GABA receptor agonist was used to probe the possibility that intra-VTA opioid receptor antagonist effects on the conditioned effects of ethanol occur through a GABA mechanism.

Chapter 1 describes a series of experiments in which methylnaloxonium, a non-selective opioid receptor antagonist, was injected into either the VTA or the NAc before the expression of ethanol CPP. A non-selective opioid receptor antagonist was chosen because such drugs were shown to decrease the expression of CPP and to enhance the expression of CPA when given systemically. Methylnaloxonium was specifically chosen because it diffuses away from the injection site more slowly than other non-selective opioid receptor antagonists. Intra-VTA methylnaloxonium was effective in decreasing the expression of ethanol CPP, an effect that may result from disinhibition of local GABA neurons in the VTA. In order to further support this suggestion, the VTA experiment was replicated using baclofen, a $GABA_B$ receptor agonist.

Chapter 2 describes similar experiments to examine the role of the VTA and NAc in the expression of the conditioned aversive properties of ethanol because they are also altered by non-selective opioid receptor antagonists. However, interpretations of these data were tentative because in some cases CPA was not expressed in the vehicle treated

groups. Additional experiments demonstrate that the handling required for intracranial injections can be disruptive to the expression of CPA but did not appreciably alter CPP.

Ethanol-induced conditioned place preference is expressed through a ventral tegmental area dependent mechanism

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Running head: Ethanol Place Conditioning and VTA

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Abstract

The brain areas and neurochemical mechanisms that underlie the expression of the conditioned rewarding effect of ethanol remain unknown. Here we examine the role of opioid receptors in the ventral tegmental area (VTA) and nucleus accumbens (NAc), two key components of the reward circuit, in the expression of ethanol-induced conditioned place preference. Because intra-VTA opioid receptor antagonists altered the conditioned effect of ethanol, we also tested the hypothesis that a similar effect would be obtained following intra-VTA gamma-aminobutyric acid $(GABA)_B$ receptor agonist treatment. Male DBA/2J mice were implanted with bilateral cannulae positioned above the VTA or NAc. After recovery, animals underwent an unbiased Pavlovian conditioning procedure for ethanol-induced conditioned place preference. Just prior to preference testing, animals were injected either intra-VTA (Experiments 1 and 3) or intra-NAc (Experiment 2) with the non-selective opioid receptor antagonist methylnaloxonium (0, 375, or 750 ng total infusion; Experiments 1 and 2) or the $GABA_B$ receptor agonist baclofen (0, 25 or 50 ng total infusion; Experiment 3). Intra-VTA methylnaloxonium and baclofen produced dose dependent decreases in the expression of ethanol-induced conditioned place preference, while intra-NAc methylnaloxonium had no significant effect. Taken together, these findings indicate that the conditioned rewarding effect of ethanol may be expressed through a VTA dependent mechanism that involves both opioid and $GABA_B$ receptors.

Introduction

A growing body of literature has identified several potential neural targets for altering the primary rewarding effect of ethanol. However, little is known about the brain areas and neurochemical substrates that mediate the expression of the conditioned rewarding properties of ethanol. This information is of particular interest because understanding the mechanisms underlying the conditioned effects of ethanol may provide insight for preventing relapse in alcoholics.

Many previous studies suggest that opioid receptor antagonists alter the primary and conditioned rewarding effects of ethanol (for review see Herz, 1997). Specifically, opioid receptor antagonists decrease ethanol intake in a variety of species (e.g., Altshuler et al., 1980; Froehlich et al., 1987; Phillips et al., 1997), including humans, who report decreased craving (e.g., O'Malley et al., 1992; Volpicelli et al., 1992). Similarly, opioid receptor antagonists decrease the expression of the conditioned rewarding effect of ethanol, as measured by ethanol-induced conditioned place preference (CPP, Cunningham et al., 1995; Cunningham et al., 1998; Kuzmin et al., 2003; Middaugh & Bandy, 2000) and cue-induced ethanol-seeking behavior in an operant paradigm (Ciccocioppo et al., 2002).

Data suggest that the rewarding properties of ethanol may be mediated through the ventral tegmental area (VTA) and the nucleus accumbens (NAc), two key components of the reward circuit. For example, opioid receptor antagonists decrease responding for ethanol, which occurs in combination with decreased extracellular dopamine in the NAc (Gonzales & Weiss, 1998) and opioid receptor antagonists administered directly into the VTA (June et al., 2004) or NAc (Froehlich et al., 2003;

Heyser et al., 1999; June et al., 2004) are sufficient to decrease responding for ethanol. Moreover, ethanol is self administered into the VTA (Gatto et al., 1994; Rodd et al., 2004; Rodd-Henricks et al., 2000) and the firing rate of VTA dopamine neurons is increased when ethanol is administered (Brodie et al., 1990; Gessa et al., 1985). In addition to their involvement in ethanol reward, these two brain areas also mediate the aversive properties of opioid receptor antagonists (Shippenberg & Bals-Kubik, 1995).

$GABA_B$ receptor agonists may act similarly, but down-stream to non-selective opioid receptor antagonists in the VTA, to alter dopamine release. This relationship has been supported by data showing that activation of $GABA_B$ receptors in the VTA inhibits the ability of opioid stimulation to elicit dopamine release in the NAc (Kalivas et al., 1990). While the literature is somewhat inconsistent, gamma-aminobutyric acid $(GABA)_B$ receptor agonists are reported to alter ethanol related behaviors, including drinking (Daoust et al., 1987), withdrawal (File et al., 1991) and locomotion (Cott et al., 1976). In contrast, the $GABA_B$ receptor agonist, baclofen reportedly did not affect the acquisition of ethanol CPP (Chester & Cunningham, 1999a). However, the effect of this drug on the expression of ethanol CPP has not been investigated. Few data are available regarding central effects of these agents. However, intracerebroventricular and intra-VTA baclofen have been shown to alter ethanol-induced locomotion (Boehm et al., 2002). Recent findings suggest that baclofen may decrease ethanol craving and consumption in alcoholics (Addolorato et al., 2002).

The nature of the conditioned response to ethanol-associated cues that results in CPP is unknown. Here, we suggest for the first time that this response is mediated by the VTA. We demonstrate that intra-VTA methylnaloxonium decreases the expression of

ethanol-induced CPP, while intra-NAc infusions are ineffective. Next, we show that intra-VTA baclofen, a $GABA_B$ receptor agonist, eliminates the expression of ethanol-induced CPP. Taken together, these findings suggest that the conditioned effects of ethanol may be expressed through a VTA dependent mechanism that involves both opioid and $GABA_B$ receptors.

Method

Animals

Male DBA/2J mice (n = 283) were obtained from the Jackson Laboratory (Bar Harbor, ME) at 6 weeks of age. This strain was chosen because it shows robust ethanol place conditioning under the current parameters (e.g., Cunningham et al., 1998). Animals were housed four to a cage with continuous access to food and water and allowed to acclimate to the colony for 4-8 days. Experiments were carried out during the light phase of a 12-hr light-dark cycle (initiated at 7:00 a.m.). After surgery, animals were housed two per cage. The National Research Council's "Guide for the Care and use of Laboratory Animals" (1996) was followed in conducting these studies and the protocol was approved by the OHSU IACUC .

Surgery

Animals were implanted with chronic indwelling bilateral guide cannulae aimed at the posterior VTA (from bregma; A –3.62, L± 0.6, V 4.15) or the NAc shell (from bregma 20° lateral; A +1.10, L± 0.6, V 4.75) (Paxinos & Franklin, 2001). Specifically, animals were fully anesthetized using a cocktail (0.1 ml/25 g) containing ketamine (30.0

mg/ml) and xylazine (3.0 mg/ml). Small burr holes were made in the skull, through which guide cannulae were lowered to the specified depth under stereotaxic guidance (Cartesian Research, Sandy, OR). Stainless steel guide cannulae (10 mm, 25 ga.) were positioned 2 mm above the posterior VTA or NAc shell and fastened to the skull using stainless steel screws and Durelon® carboxylate cement (ESPE America, Inc., Norristown, PA). Guide cannulae were kept patent using 32 ga. stainless steel stylets. Mice were allowed 4-8 days of recovery prior to the start of the place conditioning procedures. One animal died during surgery. Six animals were removed from the experiments due to defective head-mounts or cannulae. Forty additional animals were removed from the experiments because of complications associated with infection including tissue damage, sickness or death. The latter problems occurred between the test day and the time of sacrifice, but never during conditioning. Details regarding errors in cannula placement are described below (*Histological Verification*).

Intracranial Microinjection

Microinjectors made of 32 ga. stainless steel tubing encased by 25 ga. stainless steel tubing were used. The distal ends of the injectors were attached to 10μL Hamilton syringes via polyethylene tubing (PE20) and infusions were delivered via a syringe pump (Model: A-74900-10, Cole Parmer, Vernon Hills, IL). Immediately before preference testing, stylets were removed and injectors were inserted into the guide cannulae such that they extended 2 mm beyond the guide cannulae into the posterior VTA or NAc shell. Because of the small size of these nuclei in mice, injection volumes were minimized to decrease spread of the injection to neighboring brain areas. In addition, infusion duration was lengthened to minimize seeping of the drug up the injection tract. Infusions of 100

nL/side were given simultaneously over 1 min. In order to ensure complete diffusion, injectors were left in place for an additional 30 sec, after which stylets were replaced.

Drugs

A 95% ethanol stock solution (Aaper Alcohol & Chemical Company, Shelbyville, KY) was diluted to 20% v/v with saline. A dose of 2 g/kg was administered intraperitoneally in an injection volume of 12.5 ml/kg. This dose was chosen because it has been shown to induce robust CPP (Cunningham et al., 1996) and was used in systemic naloxone experiments conducted by Cunningham et al. (Cunningham et al., 1995; Cunningham et al., 1998). Methylnaloxonium iodide (Sigma, St. Louis, MO) or baclofen (Sigma, St. Louis, MO) was dissolved in artificial cerebrospinal fluid (acsf). Methylnaloxonium is an analog of naloxone and was chosen because it diffuses away from the site of injection more slowly than naloxone and does not cross the blood brain barrier (Schroeder, Weinger, Vakassian, & Koob, 1991). The Methylnaloxonium doses used were based on pilot studies and doses reported in the literature to decrease ethanol self-administration in rats (Heyser et al., 1999). Similarly, the baclofen doses were chosen based on pilot experiments and doses reported in the literature to alter ethanol induced locomotion in mice (Boehm et al., 2002).

Apparatus

The place conditioning chambers consisted of 12 identical acrylic and aluminum boxes (30 x 15 x 15 cm) enclosed in separate light and sound attenuating chambers. Locomotor activity and the location of the mouse within the box were determined by six

infrared beams located 2.2 cm above the floor of the chamber at 5 cm intervals and recorded with a 10 ms resolution by a computer. The floor consisted of interchangeable halves, which were made of two distinct textures. Specifically, hole floors were made from perforated 16 gauge stainless steel with 6.4 mm round holes on 9.5 mm staggered centers. Grid floors were made of 2.3 mm stainless steel rods mounted 6.4 mm apart on acrylic rails. These floor textures were chosen based on previous findings demonstrating that drug naive mice spend equal time on both floors (Cunningham, 1995), which allows the use of an unbiased method of assessing CPP.

Place Conditioning Procedure

Each experiment consisted of three phases: habituation (one session), conditioning (eight sessions), and preference testing (one session). The habituation session was intended to reduce the novelty of the experimental apparatus and injection procedure. On the first day of the experiment, animals were injected intraperitoneally (IP) with saline just prior to being placed in the conditioning chamber on a smooth paper floor for 5 min. During the conditioning phase, animals were injected with saline or ethanol (2 g/kg) on alternating days just before being placed in the conditioning chamber for 5 min where both sides of the floor were the same. Animals in the Grid+ condition were placed on the grid floor on ethanol injection days and the hole floor on saline injection days. Conversely, animals in the Grid- condition were placed on the hole floor on ethanol injection days and the grid floor on saline injection days. The order of ethanol and saline exposure was counterbalanced within groups. After four conditioning trials (4 CS+, 4 CS-; days 2-9), a 30 min preference test was conducted (day 10). Immediately before the preference test session, animals were given an intra-cranial injection and placed into the

conditioning chambers. The floor was half hole and half grid and the left/right position of the floors was counterbalanced within groups. In Experiments 1 and 2, animals were injected intra-VTA and intra-NAc, respectively, with either artificial cerebrospinal fluid (acsf) or methylnaloxonium (375 or 750 ng total infusion). In Experiment 3, animals were injected intra-VTA with either acsf or baclofen (25 or 50 ng total infusion). Conditioning sessions occurred 5 days per week.

Histological Verification

Within 3-8 days of the preference test, brains were removed and post-fixed in 2% paraformaldehyde in isotonic sodium phosphate buffered saline (PBS) for 24 h. Brains were cryoprotected with overnight incubations in 20% and then 30% sucrose in PBS with 0.1% NaN_3. Frozen 40 μm coronal sections were cut through the site of the microinjectors using a cryostat and collected in PBS with 0.1% NaN_3. Sections were then mounted onto slides and thionin stained.

The dorsal boundaries of the VTA are not clearly delineated by the surrounding structures. Therefore, a neuroanatomical template was constructed in an attempt to objectively determine the accuracy of cannula placement (Figure 2). This strategy took advantage of the high dopamine content of the VTA, in comparison to the surrounding dorsal structures. In particular, a subset of unilaterally implanted animals ($n = 4$) was sacrificed and the brain tissue fixed as described above. Tyrosine hydroxylase (TH), a marker for dopamine, was identified using the immunohistochemical protocol previously described (Bachtell, Tsivkovskaia, & Ryabinin, 2002). Briefly, endogenous peroxidase activity was inhibited by pretreatment with 0.3% hydrogen peroxide. Blocking was

performed with 6% goat serum. Rabbit polyclonal antibodies against tyrosine hydroxylase (Chemicon International, Temecula, CA) were used in a dilution of 1:1,000. The immunoreaction was detected with Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Enzymatic development was performed with the Metal Enhanced DAB kit (Pierce, Rockford, IL). The template was constructed by highlighting the dense TH positive staining (Figure 2; top left) within the VTA, in combination with neuroanatomical features including the interpeduncular nucleus, medial lemniscus and the substantia nigra (Figure 2; top right). In order to determine cannula placement, the anterior-posterior (A/P) location of the section was determined (Figure 2; bottom left) and the corresponding A/P template was applied according to the orientation of the VTA with respect to interpeduncular nucleus, medial lemniscus and substantia nigra (Figure 2; bottom right). Cannulae were considered within the posterior VTA if the end of both of the injector tracts was between the A/P coordinates A –3.28 and –3.88, and came into contact with the pink area defined as the VTA (as shown in Figure 2). In contrast, the boundaries of the NAc shell are easily distinguished based on neuroanatomical boundaries. Therefore cannulae were considered within the NAc shell if both of the injector tracts ended beyond the edge of the NAc core and before the nucleus of the vertical limb of the diagonal band.

Animals given microinjections outside of the posterior VTA or NAc shell were removed from all analyses (bilateral hits $n = 140$; misses $n = 81$). Example photomicrographs of VTA (top panel) and NAc (bottom panel) bilateral injection sites are shown in Figure 3. Data from seven additional animals were removed from all

Figure 2. Photomicrographs demonstrating application of the template system used to determine VTA cannula placement. Representative sections were collected along the anterior-posterior axis and stained for tyrosine hydroxylase (top left). The area of dense staining representing the VTA was highlighted (top right). The anterior-posterior coordinates of the thionin stained sections from experimental animals (bottom left) was determined and the corresponding template was applied (bottom right).

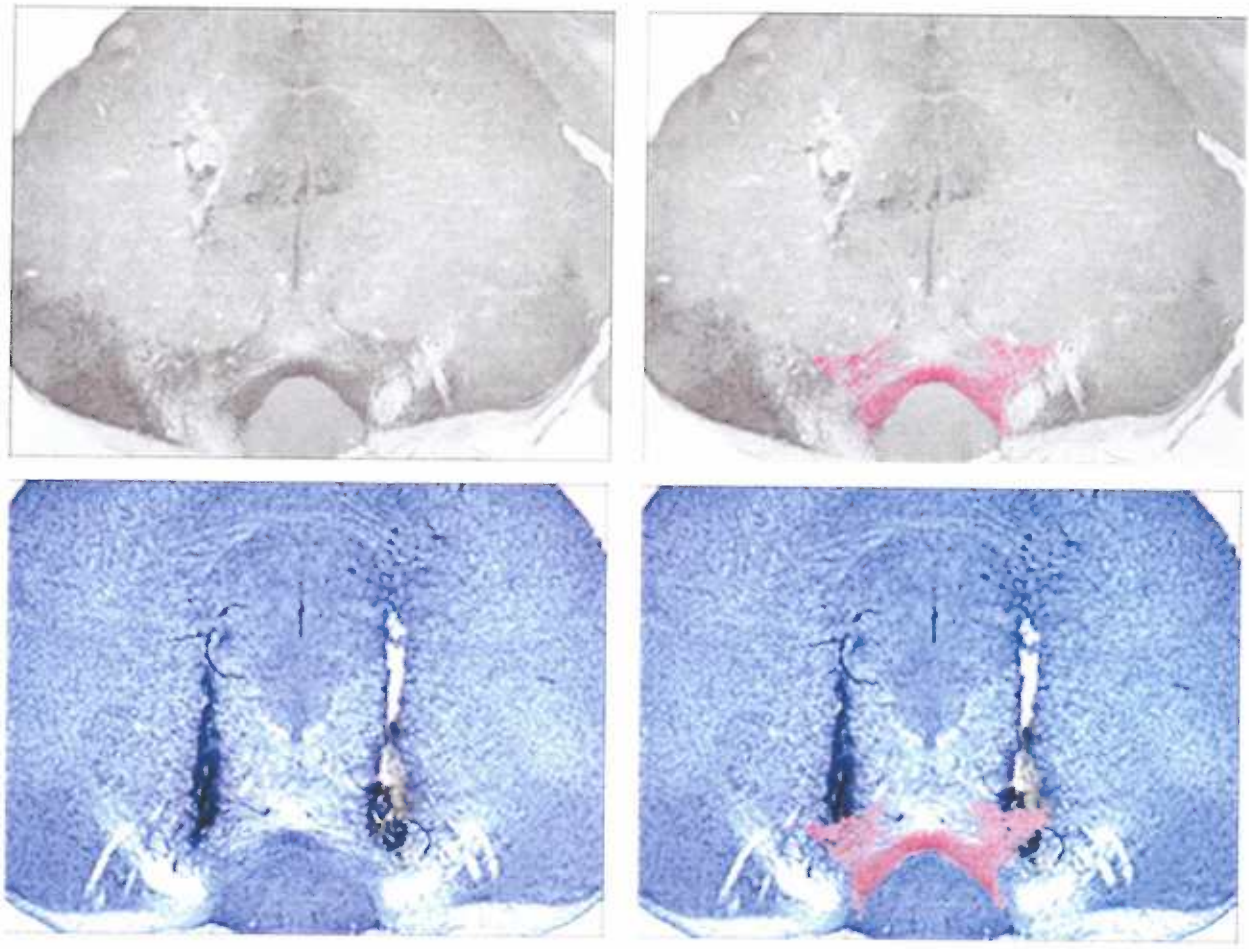
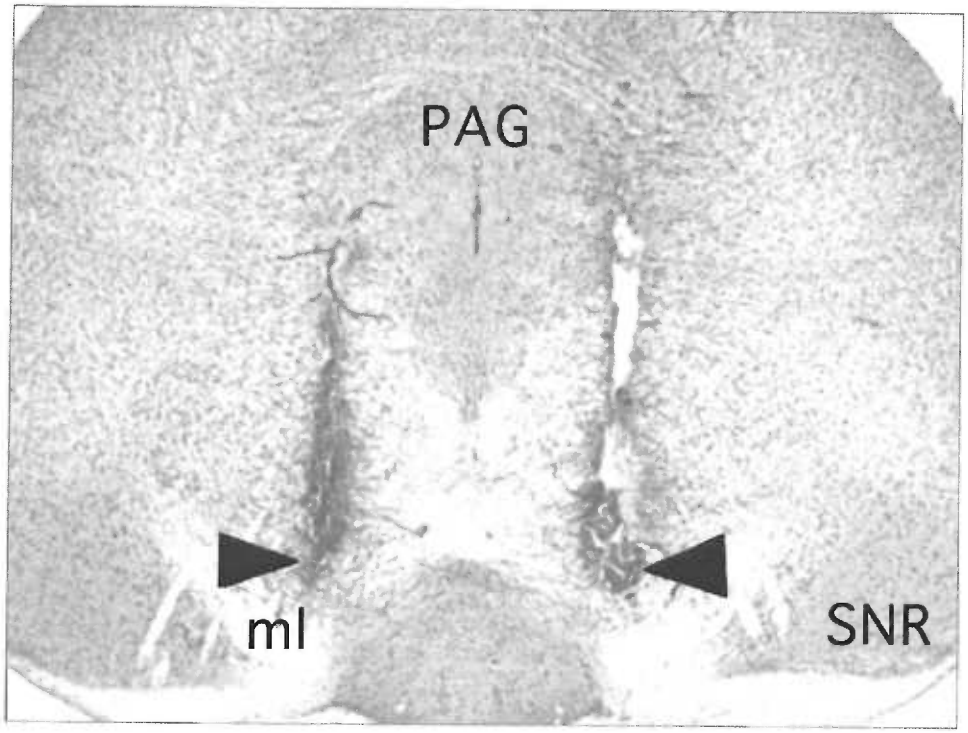
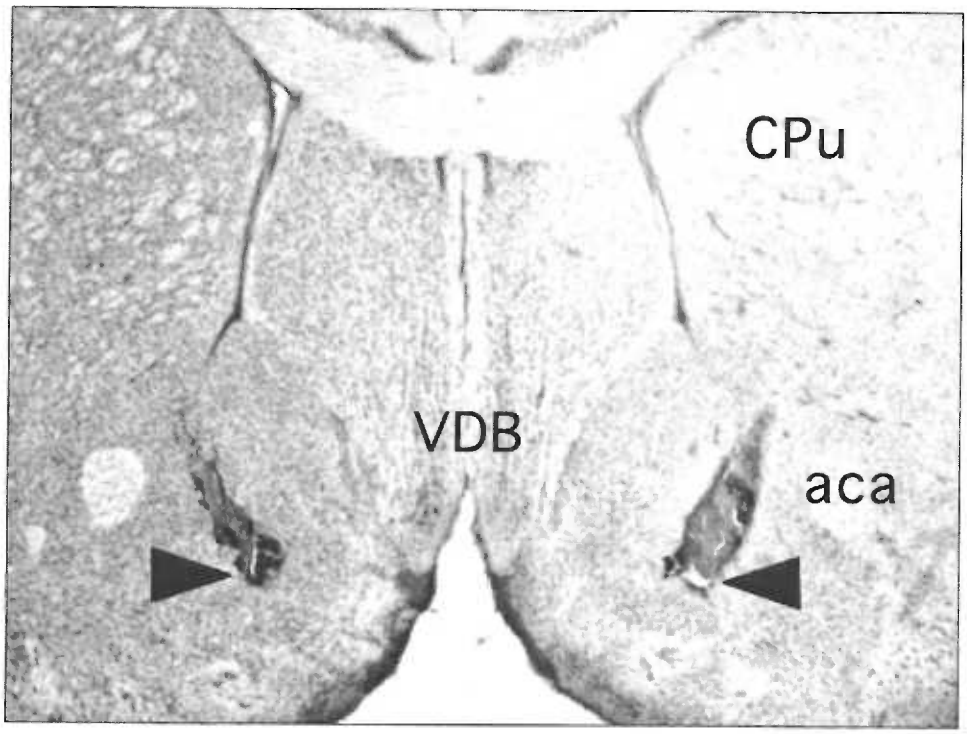


Figure 3. Photomicrographs showing thionin stained coronal sections of bilateral intra-VTA (top panel) and intra-NAc (bottom panel) injection sites. Arrows point to the site of injection within the VTA or NAc. For reference the location of the periaqueductal gray (PAG), medial lemniscus (ml), substantia nigra reticulata (SNR), caudate putamen (CPu), nucleus of the vertical limb of the diagonal band (VDB) and anterior commissure (aca) are indicated by their abbreviations.


PAG
ml
SNR


CPu
VDB
aca

analyses because of lost or damaged tissue during brain collection, slicing, staining, or mounting.

Data Analysis

Conditioning activity data were analyzed using two-way ANOVAs with the factors Test Drug Dose (acsf, dose A, dose B) and Trial Type (Ethanol or Saline). Significant effects were followed by Bonferroni corrected pair-wise comparisons between Test Drug doses. Place preference test data were analyzed with two-way ANOVAs using the factors Test Drug Dose and Conditioning Group (Grid+ or Grid-). Significant interactions were followed by two-way ANOVAs (Test Drug Dose x Conditioning Group) for each pair of test drug doses (i.e., acsf vs. dose A, acsf vs. dose B, dose A vs. dose B). In addition, Bonferroni corrected pair-wise comparisons of the two conditioning subgroups were carried out at each Test Drug Dose. Place conditioning test activity data were analyzed with one-way ANOVAs using the factor Test Drug Dose. Significant effects were followed by Bonferroni corrected pair-wise comparisons of each possible combination of Test Drug Doses. In Experiments 1 and 2, the Test Drug Dose factor was Methylnaloxonium Dose (acsf, 375 ng methylnaloxonium, 750 ng methylnaloxonium). The Test Drug Dose factor in Experiment 3 was Baclofen Dose (acsf, 25 ng baclofen, 50 ng baclofen).

Results

Experiment 1: Intra-VTA methylnaloxonium

Data were collected in 3 replications (n = 35-36 per replication). Each of the first two replications yielded accurate cannula placement in 0 to 5 animals per conditioning

sub-group in each methylnaloxonium dose group. The final replication included only animals treated with acsf or 750 ng methylnaloxonium yielding accurate cannula placement in 6 to 7 animals per conditioning sub-group in each methylnaloxonium dose group.

Conditioning Activity. Table 4 shows mean activity rates (counts/min) during CS+ (ethanol) and CS- (saline) conditioning trials. Animals were more active on ethanol treatment days than saline treatment days, demonstrating typical activation to ethanol in this apparatus in DBA/2J mice (e.g., Cunningham et al., 1995). This observation was confirmed by a two-way ANOVA (Methylnaloxonium Dose x Trial Type) on activity during conditioning trials, which revealed a significant main effect of Trial Type [$F(1,55) = 862.1; p < 0.001$]. No other significant effects were observed.

Preference Test. Figure 4 shows the mean (+SEM) time spent on the grid floor for the two conditioning groups (Grid+ and Grid-) at each methylnaloxonium dose. Animals in the Grid+ condition generally spent more time on the grid floor than animals in the Grid- condition, indicating development of ethanol-induced CPP. However, methylnaloxonium dose-dependently decreased the expression of ethanol-induced CPP (Figure 4). This observation was supported by a two-way ANOVA (Methylnaloxonium Dose x Conditioning Group) that revealed a significant main effect of Conditioning Group [$F(1,52) = 60.0; p < .001$] and a Methylnaloxonium Dose x Conditioning Group interaction [$F(2,52) = 4.3; p < .02$] for time spent on the grid floor during the test. Follow-up two-way ANOVAs (Methylnaloxonium Dose x Conditioning Group) for each pair of methylnaloxonium doses revealed that the Methylnaloxonium Dose x Conditioning Group interaction was only significant when comparing the acsf and 750 ng

Table 4. Group Mean ± SEM Activity Counts During Conditioning Trials.

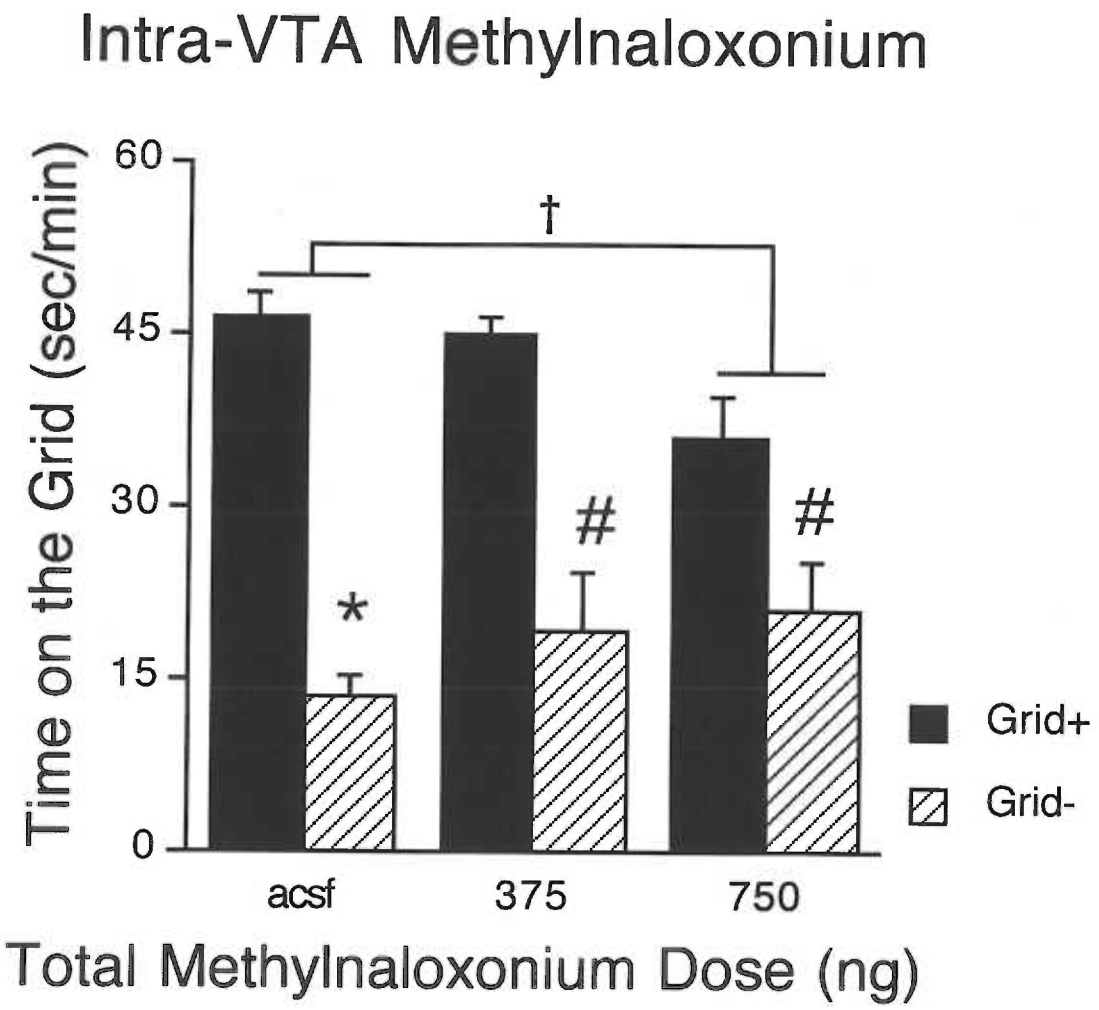
	Conditioning Trial Activity (counts/min)		
	Saline Trials (CS-)	Ethanol Trials (CS+)	Average
Experiment 1: Intra-VTA Methylnaloxonium			
acsf[c]	61.8±2.7	190.7±6.3[b]	126.3±3.9
375 ng	67.0±4.7	183.0±8.9	125.0±6.0
750 ng	67.5±3.0	192.5±6.8	130.0±4.2
Experiment 2: Intra-NAc Methylnaloxonium			
acsf	70.2±3.4	201.0±6.6[b]	135.6±4.7
375 ng	77.5±3.9	200.3±10.2	138.9±6.4
750 ng	71.4±4.1	200.8±8.8	136.1±5.3
Experiment 3: Intra-VTA Baclofen			
acsf	63.8±3.8	192.2±10.6[b]	128.0±6.7
25 ng	82.5±34.0	219.8±6.5	151.1±4.2[a]
50 ng	73.0±3.8	212.4±7.5	142.7±5.1

[a] different from acsf ($p<.01$)

[b] main effect of trial type ($p<.001$)

[c] Methylnaloxonium dose group designations are not meaningful until after conditioning since treatment is given prior to the test.

Figure 4. Intra-VTA methylnaloxonium decreases ethanol CPP. Mean time in sec/min (+SEM) spent on the grid floor during a 30-min test after intra-VTA methylnaloxonium (left panel). Animals in the Grid+ condition were given ethanol paired with the grid floor during conditioning trials (n = 11, acsf; n = 4, 375 ng methylnaloxonium; n = 14, 750 ng methylnaloxonium). Animals in the Grid- condition were given ethanol paired with the hole floor during conditioning trials (n = 13, acsf; n = 5, 375 ng methylnaloxonium; n = 11, 750 ng methylnaloxonium). Bracket indicates a significantly greater magnitude of CPP in the acsf treated group compared to the 750 ng methylnaloxonium dose group. (significant Methylnaloxonium Dose x Conditioning Group interaction). †, $p < .01$; #, $p < .005$; *, $p < .001$.


Intra-VTA Methylnaloxonium
Time on the Grid (sec/min)
60
45
30
15
0
†
*
#
#
Grid+
Grid-
acsf
375
750
Total Methylnaloxonium Dose (ng)

methylnaloxonium dose groups [$F(1,45) = 8.2; p < .006$], indicating a greater magnitude CPP in the acsf group compared to the 750 ng methylnaloxonium group. In addition, Bonferroni corrected pair-wise comparisons between conditioning groups (Grid+ vs. Grid-) at each methylnaloxonium dose demonstrated that while diminished in the 750 ng methylnaloxonium treated group, significant CPP was expressed in all dose groups ($.001 < p < .003$).

Test activity. Mean (SEM) activity rates during the test for the acsf, 375 ng and 750 ng methylnaloxonium dose groups, were 34.6 (2.4), 40.6 (3.8) and 34.1 (2.5) counts per min, respectively. In general, all groups showed similar activity levels. A one-way ANOVA (Methylnaloxonium Dose) supported this observation, yielding no significant effect. Thus, interpretation of floor preference data was not confounded by differences in test activity.

Experiment 2: Intra-NAc methylnaloxonium

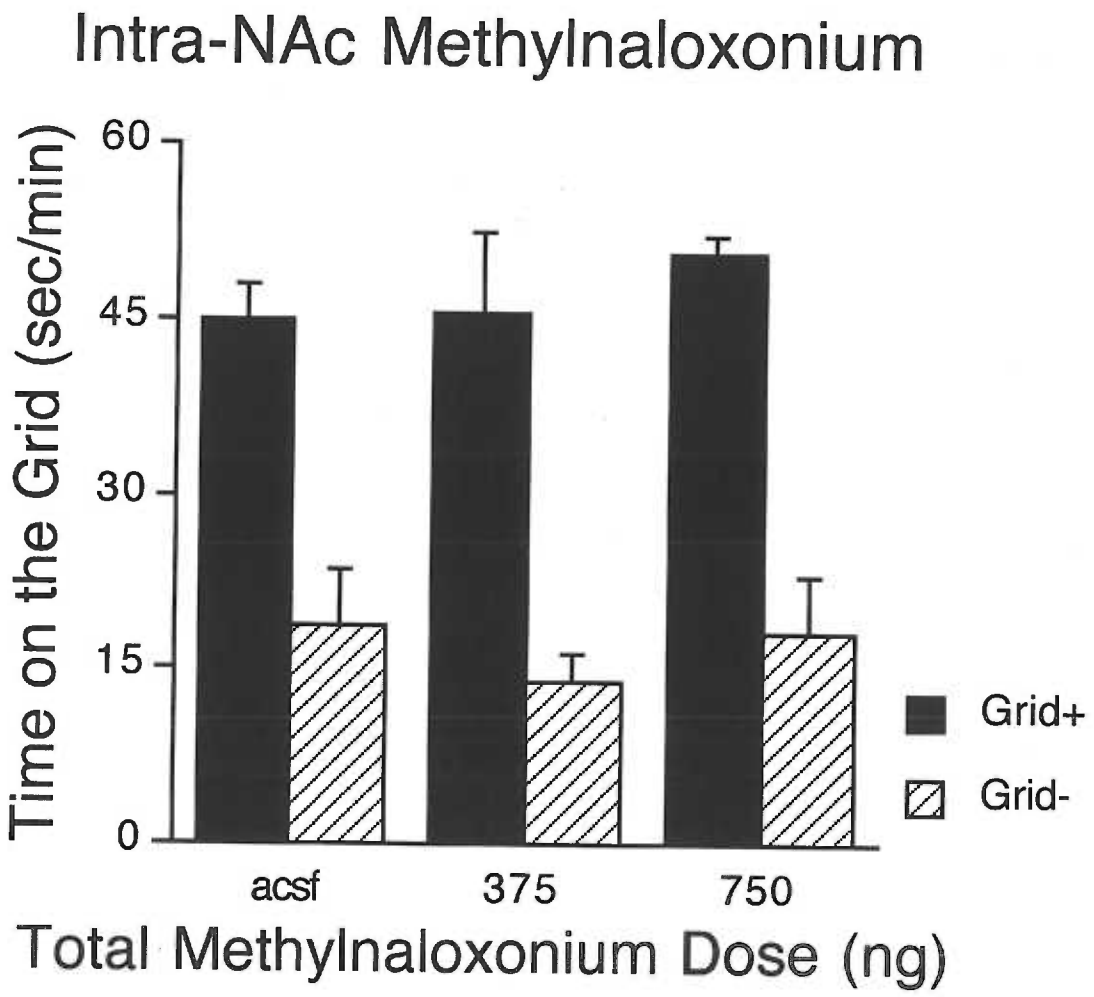
One animal, in addition to those previously mentioned, was removed from these analyses because of a procedural error. Data were collected in 2 replications ($n = 36$ per replication). Each replication yielded accurate cannula placement in 1 to 5 animals per conditioning sub-group in each methylnaloxonium dose group.

Conditioning Activity. All groups showed stimulation to ethanol compared to saline (Table 4). This conclusion was confirmed by a two-way ANOVA (Methylnaloxonium Dose x Trial Type) on activity during conditioning trials that revealed a significant main effect of Trial Type [$F(1,35) = 906.7; p < 0.001$]. No other effects were significant.

Preference Test. Figure 5 shows the mean (+SEM) time spent on the grid floor for the two conditioning groups (Grid+ and Grid-) at each methylnaloxonium dose. Animals in the Grid+ condition spent more time on the grid floor than animals in the Grid- condition, indicating development of ethanol-induced CPP. Ethanol CPP was not altered by intra-NAc methylnaloxonium treatment (Figure 5). This observation was supported by a two-way ANOVA (Methylnaloxonium Dose x Conditioning Group) that revealed a significant main effect of Conditioning Group [$F(1,32) = 94.6$; $p < .001$] for time spent on the grid floor during the test, but no Methylnaloxonium Dose x Conditioning Group interaction.

Test activity. Mean (SEM) activity rates during the test for the acsf, 375 ng and 750 ng methylnaloxonium dose groups were 39.8 (2.2), 45.7 (2.8) and 52.8 (2.2) counts per min, respectively. Methylnaloxonium treatment increased locomotor activity during the test. A one-way ANOVA (Methylnaloxonium Dose) supported this observation, yielding a significant main effect [$F(2,35) = 7.8$; $p < .001$]. Pair-wise Bonferroni corrected comparisons between the methylnaloxonium dose groups demonstrated that mice treated with 750 ng methylnaloxonium were more active than acsf treated mice ($p < .001$). However, mice treated with 375 ng methylnaloxonium were not significantly different from the other two groups. Previous findings have suggested that differences in locomotor activity during the test can influence the expression of CPP (e.g., Cunningham, 1995; Vezina & Stewart, 1987). However, the observed increase in locomotion during the test does not readily explain the lack of effect of methylnaloxonium on ethanol CPP, since increased locomotor activity would be expected to reduce the expression of CPP. That is, no change in the expression of CPP was observed despite increased locomotion.

Figure 5. Intra-NAc methylnaloxonium does not alter ethanol CPP. Mean time in sec/min (+SEM) spent on the grid floor during a 30-min test after intra-NAc methylnaloxonium. Animals in the Grid+ condition were given ethanol paired with the grid floor during conditioning trials (n = 7, acsf; n = 3, 375 ng methylnaloxonium; n = 7, 750 ng methylnaloxonium). Animals in the Grid- condition were given ethanol paired with the hole floor during conditioning trials (n = 7, acsf; n = 8, 375 ng methylnaloxonium; n = 6, 750 ng methylnaloxonium). Significant main effect of Conditioning Group (Grid+ vs. Grid-) [$F(1,32) = 94.6$; $p < .001$]), but no significant interaction.


Intra-NAc Methylnaloxonium
Time on the Grid (sec/min)
60
45
30
15
0
acsf
375
750
Total Methylnaloxonium Dose (ng)
Grid+
Grid-

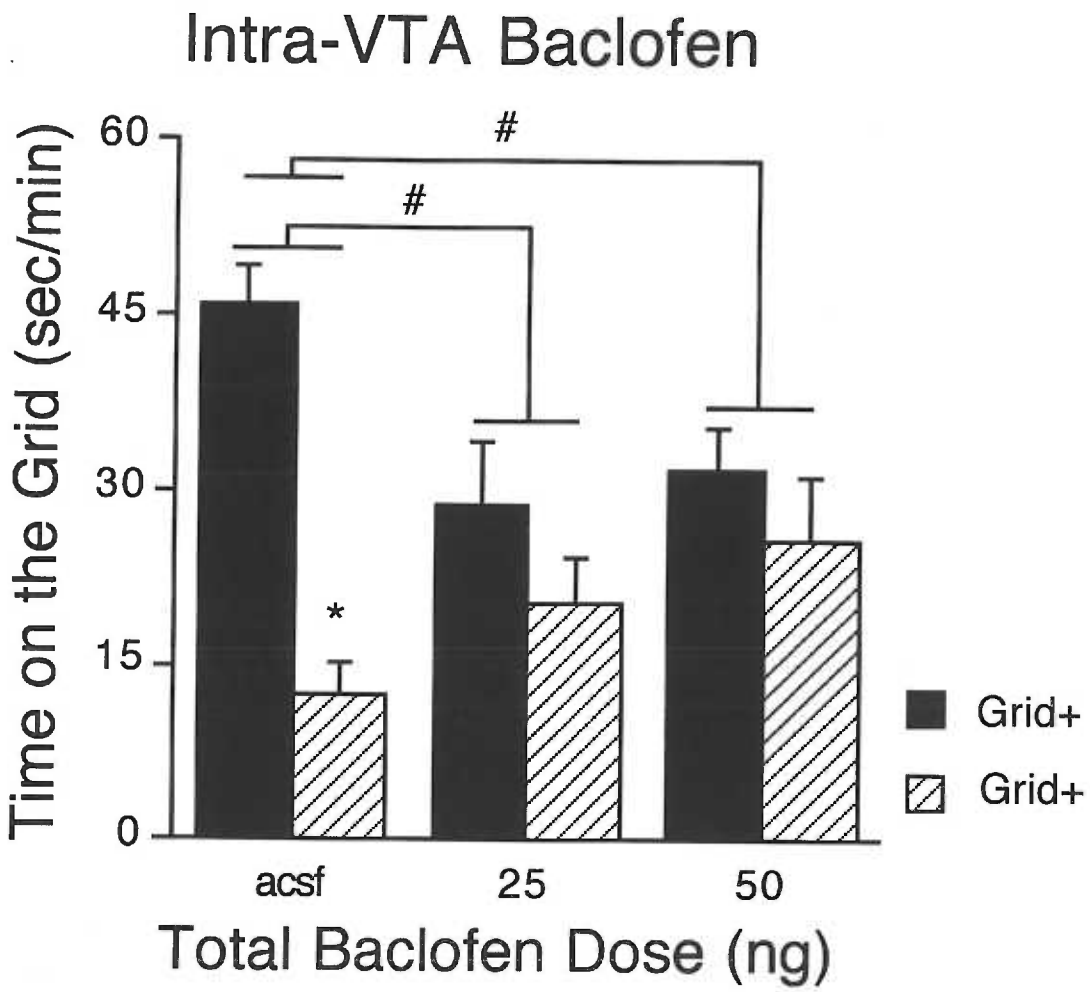
Experiment 3: Intra-VTA baclofen

Data were collected in 3 replications (n = 36 per replication). Each replication yielded accurate cannula placement in 1 to 5 animals per conditioning sub-group in each baclofen dose group.

Conditioning Activity. Compared to saline, all groups showed stimulation to ethanol (Table 4). This conclusion was supported by a two-way ANOVA (Baclofen Dose x Trial Type) on activity during conditioning trials, which revealed a significant main effect of Trial Type [$F(1,54) = 1075.5$; $p < 0.001$]. A significant main effect of Baclofen Dose was also observed [$F(2,54) = 4.4$; $p < 0.02$]. Follow-up Bonferroni corrected comparisons of this effect demonstrated that animals later treated with 25 ng baclofen on the test day were more active during conditioning trials than animals later treated with acsf. This effect is presumably due to sampling error since baclofen treatment did not occur until after conditioning.

Preference Test. The expression of ethanol-induced CPP was decreased by baclofen treatment (Figure 6). This observation was supported by a two-way ANOVA (Baclofen Dose x Conditioning Group) that revealed a significant main effect of Conditioning Group [$F(1,51) = 18.934$; $p < .001$] and a Baclofen Dose x Conditioning Group interaction [$F(2,51) = 5.4$; $p < .008$] for time spent on the grid floor during the test. Follow up two-way ANOVAs (Baclofen Dose x Conditioning Group) demonstrated that this interaction was significant when comparing the acsf group to either the 25 ng [$F(1,34) = 9.5$; $p < .004$] or 50 ng [$F(1,31) = 9.5$; $p < .004$] baclofen doses, but not when comparing the two baclofen doses. Further, Bonferroni-corrected comparisons of Conditioning Group at each baclofen dose demonstrated that acsf treated mice

Figure 6. Intra-VTA baclofen decreases ethanol CPP. Mean time in sec/min (+SEM) spent on the grid floor during a 30-min test after intra-VTA baclofen (left panel). Animals in the Grid+ condition were given ethanol paired with the grid floor during conditioning trials (n = 9, acsf; n = 7, 25 ng baclofen; n = 10, 50 ng baclofen). Animals in the Grid- condition were given ethanol paired with the hole floor during conditioning trials (n = 7, acsf; n = 12, 25 ng baclofen; n = 12, 50 ng baclofen). Brackets indicate a significantly weaker magnitude of CPP in each baclofen dose group compared to the acsf group (significant Baclofen Dose x Conditioning Group interaction). *, p< .001; #, p< .005.


Intra-VTA Baclofen
Time on the Grid (sec/min)
60
45
30
15
0
#
#
*
acsf
25
50
Total Baclofen Dose (ng)
Grid+
Grid+

demonstrated significant CPP ($p < .001$), while this was not true of either baclofen treated group.

Test activity. Mean (SEM) activity rates during the test for the acsf, 25 ng and 50 ng baclofen dose groups were 34.2 (3.2), 25.9 (4.2) and 26.0 (4.2), respectively. In contrast to the findings during the conditioning phase, all groups showed similar activity levels during the test. A one-way ANOVA (Baclofen Dose) supported this observation, yielding no significant effects. Thus, interpretation of the baclofen effect on place preference was not confounded by differences in test activity.

Discussion

While several genetic and pharmacological manipulations have been shown to block the acquisition of the conditioned effects of ethanol, few have been effective in blocking the expression of these effects. Manipulations that alter the expression of these effects are of particular importance since they occur after the conditioned-association has been established. Changes in the expression of the conditioned effects of ethanol may have important implications for alcoholics because ethanol-related-associations are already established at the time of treatment. Here we show for the first time that the expression of the conditioned rewarding effect of ethanol is disrupted by either blockade of opioid receptors or activation of $GABA_B$ receptors in the VTA. Specifically, the expression of ethanol-induced CPP was dose-dependently decreased by intra-VTA methylnaloxonium and baclofen at doses that did not significantly alter locomotor activity.

The current findings extend and are in agreement with the literature

demonstrating that the VTA is central in ethanol reinforcement (Gatto et al., 1994; Rodd-Henricks et al., 2000). These effects may occur through disinhibition of GABA neurons in the VTA, resulting in a decreased dopamine neuron activity. The present findings lend support to the hypothesis that the expression of the conditioned effects of ethanol depends upon stimulation of opioid receptors in the VTA that are up-stream of effects at $GABA_B$ receptors. This relationship between opioid and GABA receptors in the VTA has been proposed in a number of electrophysiological and neurochemical experiments (for review see Xi & Stein, 2002). For example, activation of $GABA_B$ receptors in the VTA inhibits the ability of VTA μ-opioid receptor stimulation to elicit dopamine release in the NAc and prefrontal cortex (Kalivas et al., 1990).

Dopamine release might be a critical response that maintains approach behavior and responding in the presence of ethanol-associated cues. Others have reported that NAc dopamine levels are increased when ethanol-associated environmental cues are presented (Gonzales & Weiss, 1998) and generally that dopamine cells are activated in response to conditioned stimuli (for review see Schultz, 1998). However, the interpretation of the present findings in terms of effects on dopamine is not supported by previous reports that systemic blockade of D1-like or D2-like dopamine receptors does not alter the expression of ethanol CPP (Cunningham et al., 1992; Dickinson et al., 2003). It is possible that the expression of ethanol CPP does not depend on dopamine and that effects of methylnaloxonium and baclofen occur through a non-dopaminergic VTA mechanism. Indeed, opioid receptor expressing GABA neurons (Svingos, Garzon et al., 2001) project from the VTA to the prefrontal cortex (Carr & Sesack, 2000a) and could mediate these effects through an indirect mechanism. In particular, projections have been proposed

from the prefrontal cortex to the pedunculopontine nucleus (Tzschentke & Schmidt, 2000) and from the VTA to the pedunculopontine nucleus (Laviolette et al., 2004) that may serve this function. However, as previously mentioned, dopamine receptor antagonists did not alter ethanol CPP when administered systemically (Cunningham et al., 1992; Dickinson et al., 2003). Systemic administration provides the potential for differential diffusion throughout the brain making direct comparison of systemic results to those of site-specific injections problematic.

In contrast to opioid receptor effects, the effects of baclofen in the VTA are not easily explained by a mechanism excluding dopamine neurons because $GABA_B$ receptors are mainly present on dopamine neurons. Double labeling studies suggest that tyrosine hydroxylase, a dopamine marker, and $GABA_B$ receptors are located on the same neurons with only very light staining detected in non-dopaminergic cells (Wirtshafter & Sheppard, 2001). Moreover, $GABA_B$ receptors were reportedly only located on cells in the VTA lacking glutamic acid decarboxylase, a GABA marker, suggesting that $GABA_B$ receptors are not located on GABA neurons (Margeta-Mitrovic, Mitrovic, Riley, Jan, & Basbaum, 1999).

The lack of dopamine blockade effects previously reported and the apparent necessity for intra-VTA baclofen to act through dopamine neurons lends support to the notion that glutamate released from dopamine neurons might be important in reward (Charles et al., 2001). In particular, dopamine neurons immunostain for glutamate and form glutamatergic synapses *in vitro* (Sulzer et al., 1998). Furthermore, VTA dopamine neurons have excitatory actions that are too fast to result from dopamine effects that have been shown to be mediated by glutamate (Chuhma et al., 2004). These authors suggest

that the salience signal proposed by Schultz and colleagues (for review see Schultz, 1998) may be initiated by glutamate released from dopamine neurons and modulated by dopamine (Chuhma et al., 2004).

The ineffectiveness of dopamine receptor antagonists to alter the expression of ethanol CPP might also be explained by requisite synergistic interactions of D1 and D2 dopamine receptors (for review see Marshall, Ruskin, & LaHoste, 1997). Although the mechanisms underlying these interactions are largely unknown, requisite synergism has been demonstrated and refers to situations where the concomitant activation of the two receptors mediates a given effect. This relationship has been suggested in the NAc because the application of either D1 or D2 dopamine receptor antagonists are without effect while, coadministration decreases the duration of VTA stimulation evoked periods of depolarization (Goto & O'Donnell, 2001). Interestingly, this alternative explanation may not be mutually exclusive to the formerly suggested glutamate mechanism of ethanol CPP (Chuhma et al., 2004) because synergistic interactions of D1 and D2 dopamine receptors are reported to enhance glutamate induced activation in the striatum (Hu & White, 1997). Taken together, these data suggest that either glutamate or concomitant D1/D2 dopamine antagonism might be required to disrupt the expression of ethanol CPP in a way similar to the disruption in the present VTA experiments.

The current findings are in agreement with some data regarding place preference for other rewarding stimuli suggesting that some mechanisms of place preference might be shared (Table 5). For example, non-selective opioid antagonism decreases the expression of place preference for, cocaine (Gerrits, Patkina, Zvartau, & van Ree, 1995), sucrose (Delamater, Sclafani, & Bodnar, 2000) and an estrous female (in males)

(Mehrara & Baum, 1990; Miller & Baum, 1987). Also, the expression of cocaine (Lawley & Kantak, 1990), d-methamphetamine (Li, Yin, Ren, Pan, & Zheng, 2001), nicotine (Ashby et al., 2002) and heroin (Paul, Dewey, Gardner, Brodie, & Ashby, 2001) CPP is decreased by GABA receptor agonists or GABA transaminase inhibitors, which increase GABA concentrations. However, as is highlighted by Table 5, findings are not necessarily in agreement between laboratories and vary based on the unconditioned stimulus. For example, individual dopamine receptor antagonists have been shown to decrease the expression of amphetamine CPP (Hiroi & White, 1991; Liao, Chang, & Wang, 1998) and have no effect on ethanol CPP (Dickinson et al., 2003), while coadministration of D1 and D2 dopamine receptor antagonists may be required to decrease the expression of cocaine CPP (Liao et al., 1998). In summary, the literature on the mechanisms of the expression of CPP demonstrates that some commonalities exist between unconditioned stimuli, suggesting that our findings may generalize to other rewarding stimuli. However, especially with regard to the individual dopamine receptors, the role of dopamine in the expression of CPP is tentative and may warrant further experiments using concomitant administration of D1 and D2 like dopamine receptor antagonists either systemically or into the NAc and pfc.

One possible explanation for a decrease in ethanol CPP after VTA opioid receptor blockade is the induction of an aversive hedonic state, since intra-VTA opioid antagonism alone can condition a place aversion (Shippenberg & Bals-Kubik, 1995). However, it is unclear why this aversive state would be associated specifically with the ethanol-paired conditioned stimulus (Cunningham et al., 1998). Further, not only intra-VTA, but also intra-NAc opioid antagonism produces a conditioned place aversion and it

Table 5. Results of opioid, GABA and Dopamine drug treatments on the expression of place conditioning sorted by the unconditioned stimulus.

US	System	Pharmacological Mechanism	Agent	Outcome[1]	Citation
Ethanol	*Opioid*	Non-selective antagonist	Naloxone	Decrease	(Cunningham et al., 1995)
		Non-selective antagonist	Naloxone	CPA; No Effect[2]	(Bormann & Cunningham, 1997)
		Non-selective antagonist	Naloxone	Decrease	(Cunningham et al., 1998)
		Non-selective antagonist	Naloxone	CPA; Enhance[3]	"
		Non-selective	Naltrexone	Decrease	(Middaugh & Bandy, 2000)
		ORL1 (NOR) agonist	Nociceptin/ orphaninFQ	Decrease	(Kuzmin et al., 2003)
		ORL1 (NOR) agonist	Ro 64-6198	Decrease	"
		Non-selective antagonist	Naloxone	Decrease	"
	Dopamine	D2, D3, D4 antagonist	Haloperidol	No Effect	(Cunningham et al., 1992)
		D1 Antagonist	SCH23390	No Effect	(Dickinson et al., 2003)
		D2 Antagonist	Raclopride	No Effect	"
		D3 Antagonist	U99194A	No Effect	"
Cocaine	*Opioid*	Non-selective Antagonist	Naloxone	Decrease	(Gerrits et al., 1995)
		ORL1 (NOR) agonist	Nociceptin/ orphaninFQ	Decrease	(Kotlinska, Wichmann, Legowska, Rolka, & Silberring, 2002)
		NOR (ORL1) agonist	Ro 65-6570	No Effect	"
	GABA	Benzodiazepine Agonist	Pentobarbital	Decrease	(Lawley & Kantak, 1990)
		Transaminase Inhibitor	ACC[4]	Decrease	(Ashby et al., 2002)
	Dopamine	D2, D3, D4 Antagonist	Haloperidol	No Effect	(Lawley & Kantak, 1990)
		D2, D3, D4 Antagonist	Haloperidol	Decrease	(Adams, Careri, Efferen, & Rotrosen, 2001)

Table 5: continued

US	System	Pharmacological Mechanism	Agent	Outcome[1]	Citation
		D1 + D2 Antagonist	SCH23390 + Spiperone	Decrease	(Liao et al., 1998)
		D1 Antagonist	SCH23390	No Effect	(Cervo & Samanin, 1995)
		D1 Antagonist	SCH23390	No Effect	(Liao et al., 1998)
		D1 Antagonist	SCH23390	No Effect	(Adams et al., 2001)
		D2 Antagonist	Sulpiride	No Effect	(Cervo & Samanin, 1995)
		D2 Antagonist	Spiperone	No Effect	(Liao et al., 1998)
		D2 Antagonist	Raclopride	No Effect	(Adams et al., 2001)
		D3 Partial Agonist	BP 897	Decrease	(Duarte, Lefebvre, Chaperon, Hamon, & Thiebot, 2003)
Amphetamine	*Dopamine*	Depletes Vesicular Dopamine	Reserpine	Decrease	(Hiroi & White, 1990)
		Non-selective Antagonist	∝-flupenthixol	Decrease	(Hiroi & White, 1990)
		Non-selective Antagonist	∝-flupenthixol	Decrease	(Hiroi & White, 1991)
		D1 Antagonist	SCH23390	Decrease	(Hiroi & White, 1991)
		D1 Antagonist	SCH23390	Decrease	(Liao et al., 1998)
		D2 Antagonist	Metoclopramide	Decrease[4]	(Hiroi & White, 1991)
		D2 Antagonist	Sulpiride	Decrease[4]	(Hiroi & White, 1991)
		D2 Antagonist	Spiperone	Decrease	(Liao et al., 1998)
Meth-amphetamine	*GABA*	$GABA_B$ Agonist	Baclofen	Decrease	(Li et al., 2001)
Nicotine	*GABA*	Transaminase Inhibitor	ACC[6]	Decrease	(Ashby et al., 2002)
Heroin	*Opioid*	Non-selective	Naloxone	No Effect	(Hand, Stinus, & Le Moal, 1989)
	GABA	Transaminase Inhibitor	Gamma-Vinyl GABA	Decrease	(Paul et al., 2001)
	Dopamine	D2 and 5-HT7 Antagonist	Pimozide	Decrease	(Hand et al., 1989)

Table 5: continued

US	System	Pharmacological Mechanism	Agent	Outcome[1]	Citation
		D2, D3, D4 Antagonist	Haloperidol	No Effect	(McFarland & Ettenberg, 1999)
Morphine	*Opioid*	Non-selective Antagonist	Naloxone	Enhance[5]	(Neisewander, Pierce, & Bardo, 1990)
		Non-selective Antagonist	Naloxone	Enhance	(Noble, Fournie-Zaluski, & Roques, 1993)
	Dopamine	D3 Agonist	7-OH-DPAT	Decrease	(Rodriguez De Fonseca et al., 1995)
		D3 Partial Agonist	BP 897	No Effect	(Duarte et al., 2003)
Food	*Dopamine*	D2, D3, D4 Antagonist	Haloperidol	No Effect	(McFarland & Ettenberg, 1999)
		D3 Partial Agonist	BP 897	No Effect	(Duarte et al., 2003)
Sucrose	*Opioid*	Non-selective Antagonist	Naltrexone	Decrease	(Delamater et al., 2000)
Estrous Female	*Opioid*	Non-selective Antagonist	Naloxone	Decrease	(Miller & Baum, 1987)
		Non-selective Antagonist	Naloxone	Decrease	(Mehrara & Baum, 1990)
Novelty	*Opioid*	μ Agonist	Morphine	No Effect	(Bardo, Neisewander, & Pierce, 1989)
		Non-selective Antagonist	Naltrexone	No Effect	(Bardo et al., 1989)
	Dopamine	D2, D3, D4 Antagonist	Haloperidol	Decrease	(Bardo et al., 1989)

[1] The term "decrease" is used to describe experiments where place conditioning was either decreased or eliminated.
[2] Studies in rats where conditioned place aversion (CPA) was observed
[3] Ethanol injections given after CS+ exposure results in CPA in mice
[4] The doses required to decrease expression were 2-4 times greater than that required to block acquisition. The authors suggest that these effects are not selective for the D2 receptor.
[5] After additional experiments the authors concluded that the enhancement of CPP observed was an artifact of suppressed locomotor activity.
[6] 1*R*,4*S*-4-Amino-Cyclopent-2-Ene-Carboxylic Acid

has been reported that the NAc is the primary site for mediating this effect via non-dopaminergic mechanisms (Shippenberg & Bals-Kubik, 1995). In the present experiments, intra-NAc methylnaloxonium injections had no effect on the expression of ethanol CPP. This lack of effect further supports the suggestion that the conditioned effects of ethanol are mediated by a VTA-opioid mechanism and are not due to the general aversive properties of opioid receptor antagonists. Here we show that opioid receptor activation at the level of the cell bodies located in the VTA, rather than the terminal field (NAc), is critical for mediating the conditioned effects of ethanol. It is unlikely that this lack of effect was due to an ineffective dose range since opioid antagonism produced a dose dependent increase in activity during the test, suggesting that behaviorally effective doses were used.

Finally, the finding that intra-NAc methylnaloxonium did not alter ethanol CPP is not in agreement with previous findings suggesting that NAc opioid receptor blockade decreases ethanol self-administration (Froehlich et al., 2003; Heyser et al., 1999; June et al., 2004). However, these data are in agreement with a growing body of literature suggesting that CPP and self-administration represent two dissociable phenomena (Bardo & Bevins, 2000). Therefore, the present findings suggest that previous reports of opioid receptor antagonist effects in the NAc are derived from alterations in the direct, rather than conditioned rewarding properties of ethanol.

In conclusion, we suggest for the first time that the conditioned response underlying the expression of CPP depends upon the VTA and is mediated through an opioid and $GABA_B$ receptor dependent mechanism. These effects likely occur by suppression of dopamine neurons in the VTA, however, the downstream effects of this

attenuation remain unknown. These data taken together with previous findings implicate the possible role of glutamate and/or requisite synergism of dopamine receptors in the expression of ethanol CPP. Finally, these findings provide a possible site of action and mechanism through which the both non-selective opioid receptor antagonists and $GABA_B$ receptor agonists decrease craving in alcoholics.

Intra-VTA methylnaloxonium does not affect ethanol-induced conditioned place aversion: Effects of handling on place conditioning in mice

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Running head: Handling and place conditioning

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Key Words: handling, aversion, preference, place conditioning, ethanol, methylnaloxonium, locomotor activity, DBA/2J, mice

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Abstract

Previous findings implicate endogenous opioids in the expression of the conditioned rewarding and aversive properties of ethanol. We have recently reported that the conditioned rewarding effect of ethanol is mediated by opioid receptors in the ventral tegmental area (VTA). To determine whether the VTA also mediates the expression of the conditioned aversive properties of ethanol, animals were implanted with cannulae above the VTA. After recovery, animals underwent a Pavlovian conditioning procedure for ethanol conditioned place aversion (CPA). Just before preference testing, animals were injected with vehicle or the non-selective opioid receptor antagonist, methylnaloxonium (Experiment 1). No significant effects of methylnaloxonium were observed. However, the magnitude of CPA was not consistent with our previous findings. We hypothesized that the handling required for microinjections in mice alters the expression of CPA, but not conditioned place preference (CPP). Therefore, non-operated animals underwent conditioning for either ethanol CPA (Experiment 2) or CPP (Experiment 3). Just before testing, half of the animals were held by the scuff of the neck to mimic microinjection handling. Animals conditioned for CPA did not express CPA if they were handled. Conversely, animals conditioned for CPP exhibited robust CPP, regardless of handling. Taken together, these findings suggest that the conditioned aversive effect of ethanol and the disruptive effect of handling on CPA may not be mediated by opioids in the VTA.

Key Words: handling, aversion, preference, place conditioning, ethanol, methylnaloxonium, locomotor activity, DBA/2J, mice

Introduction

A large body of literature suggests that non-selective opioid receptor antagonists decrease the rewarding effect of ethanol. This suggestion has been made based on many reports demonstrating that ethanol consumption (e.g., Froehlich et al., 1987; Phillips et al., 1997), operant responding (e.g., Altshuler et al., 1980) and conditioned place preference (CPP) (Cunningham et al., 1995; Cunningham et al., 1998; Kuzmin et al., 2003; Middaugh & Bandy, 2000) are attenuated by these drugs in animals. In addition, both consumption and craving are reduced in alcoholics given non-selective opioid receptor antagonists (e.g., O'Malley et al., 1992; Volpicelli et al., 1992). It has also been reported that non-selective opioid receptor antagonists enhance the conditioned aversive effect of ethanol. In particular, naloxone enhances the expression of ethanol-induced conditioned place aversion (CPA) in mice (Cunningham et al., 1998). However, the brain areas that mediate the conditioned rewarding and aversive effects of ethanol are largely unknown.

We recently demonstrated that the conditioned rewarding effect of ethanol is expressed through a ventral tegmental area (VTA) dependent mechanism that involves both opioid and $GABA_B$ receptors (Bechtholt & Cunningham, 2004). In particular, we showed that the non-selective opioid receptor antagonist, methylnaloxonium, or a $GABA_B$ receptor agonist, baclofen, injected into the VTA decreased the expression of ethanol induced CPP in mice while intra-nucleus accumbens (NAc) methylnaloxonium was without effect (Bechtholt & Cunningham, 2004).

CPA experiments take advantage of recent findings from our laboratory demonstrating that the same dose of ethanol that produces CPP can yield CPA, depending

on the temporal relationship between ethanol and conditioned stimulus (CS) presentations. That is, ethanol given just before CS+ exposure results in CPP, while ethanol given just after CS+ exposure results in CPA. These paradoxically opposite behaviors are suggested to reflect independently mediated rewarding and aversive properties of ethanol (Cunningham & Ignatoff, 2000; Cunningham et al., 2002; Hill et al., 2002; Le et al., 2001). The finding that both of these behaviors are altered by systemic non-selective opioid antagonism suggests that they may be mediated by similar neural substrates, although in opposite directions (Cunningham et al., 1998). Indeed, while it has been repeatedly suggested that rewarding stimuli activate midbrain dopamine neurons (Schultz, 1998), it has been recently suggested that aversive stimuli inhibit these neurons (Ungless, Magill, & Bolam, 2004). Therefore, we hypothesized that the conditioned aversive effect of ethanol might be mediated by similar neural substrates to those of the conditioned rewarding effect of ethanol, but in the opposite direction.

Here we present data collected to determine the effect of intra-VTA methylnaloxonium on the expression of the conditioned aversive effect of ethanol (Experiment 1). We hypothesized that methylnaloxonium would enhance ethanol's conditioned aversive effect. Contrary to this hypothesis, no significant effect of methylnaloxonium on the expression of ethanol CPA was observed, suggesting that VTA opioid mechanisms are not involved in the expression of the conditioned aversive effect of ethanol. However, the CPA was relatively weak compared to previous findings of our laboratory (e.g., Cunningham et al., 2002). In addition, initial attempts to assess the effects of intra-NAc methylnaloxonium on ethanol CPA (data not shown) yielded no significant CPA in acsf treated animals, which may have prevented us from testing our

hypothesis.

We speculated that the handling required for microinjections was disruptive to the expression of the conditioned aversive effect of ethanol, perhaps because mice are more sensitive to such effects than rats. It is well known that rats habituate to handling such that they can be handled by simply placing the hand under the abdomen and thorax. Further, rats can be trained to remain still for intracranial injections using minimal restraint. In contrast, mouse handling usually involves a more forceful technique in which mice are virtually immobilized by clutching the scruff of the neck. Moreover, mice do not seem to habituate to this procedure (Tabata, Kitamura, & Nagamatsu, 1998; Wilson & Mogil, 2001). These observations are commonly reported among researchers who work with both rats and mice. However, literature on this topic is scarce. A recent review notes that mice typically require more aggressive handling techniques and that repeated handling makes them "increasingly agitated" (Wilson & Mogil, 2001). These authors also point out that the small size and speed of mice necessitates such handling practices (Wilson & Mogil, 2001). Other investigators have also noted that rats and mice differ in their response to handling. Specifically, glucose levels increase when mice are handled. In contrast, rats showed little or no glucose response to handling (Tabata et al., 1998).

In the current studies, we tested the hypothesis that the nature of the handling techniques required for microinjections in mice was disruptive to the expression of the conditioned aversive effect of ethanol in a CPA procedure (Experiment 2). However, because we had successfully conducted similar experiments examining the conditioned rewarding effect of ethanol, we hypothesized that ethanol-induced CPP would not be altered by handling (Experiment 3).

Method

Animals

Two-hundred-seventy-three male DBA/2J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) at 6 weeks of age. This strain was chosen because it shows robust ethanol place conditioning under the current parameters (e.g., Cunningham et al., 1998). Animals were housed 4 to a cage with continuous access to food and water and allowed to acclimate to the colony for 4-8 days. After surgery, animals in Experiment 1 were housed 2 per cage to minimize damage to implanted cannulae. Therefore, to match housing conditions, animals in Experiments 2 and 3 were also housed 2 per cage after 4-8 days of acclimation to the colony. Experiments were conducted 5 days per week during the light phase of a 12-hr light-dark cycle (lights on at 7:00 a.m.). The NIH "Principles of laboratory animal care" (1996) were followed in conducting these studies and the protocol was approved by the OHSU IACUC.

Surgery

Animals in Experiment 1 (n = 107) were implanted with chronic bilateral guide cannulae aimed at the posterior VTA (from bregma; A –3.62, L± 0.6, V 4.15) (Paxinos & Franklin, 2001). Specifically, animals were anesthetized using a cocktail (0.1 ml/25g) containing ketamine (30.0 mg/ml) and xylazine (3.0 mg/ml). Small holes were drilled in the skull, through which guide cannulae were lowered to the specified depth under stereotaxic guidance. Stainless steel cannulae (25 ga.) were positioned 2 mm above the VTA and fixed to the skull using stainless steel screws and Durelon® carboxylate cement

(ESPE America, Inc., Norristown, PA). Cannulae were kept patent using 32 ga. stainless steel stylets. Mice were allowed 4-8 days of recovery prior to the start of the place conditioning procedures. Eighteen animals were removed from Experiment 1 because of complications associated with infection including tissue damage, sickness or death. The latter problems occurred between testing and the time of sacrifice, but not during conditioning. Animals in Experiments 2 (n = 84) and 3 (n = 82) were not exposed to any surgical procedures. Details regarding errors in cannula placement are given below (*Histological Verification*).

Intracranial Microinjection and Handling

Microinjectors made of 32 ga. stainless steel tubing encased by 25 ga. stainless steel tubing were used. The distal ends of the injectors were attached to 10μL Hamilton syringes with polyethylene tubing (PE20) and infusions were delivered via a syringe pump (Model: A-74900-10 Cole Parmer, Vernon Hills, IL). Immediately before preference testing, animals in Experiment 1 were gently, but firmly held by the scruff of the neck such that all four paws were touching a flat surface as shown in Figure 7. Stylets were then removed and injectors were inserted into the guide cannulae such that they extended 2 mm beyond the guide cannulae into the NAc or VTA. Infusions of acsf (100 nL/side) were given over 1 min. In order to ensure complete diffusion, injectors were left in place for an additional 30 sec, after which stylets were replaced. Total time for this procedure was approximately 2.25 min. During Experiments 2 and 3, animals in the Handled groups were similarly held by the scruff of the neck (Figure 7) for 2.25 min. Animals appeared quite calm during this procedure and struggled very little.

Figure 7. Photograph of handling technique used for microinjections and handling experiments.

Drugs

A 95% ethanol stock solution (Aaper Alcohol & Chemical Company, Shelbyville, KY) was diluted to 20% v/v with saline. A dose of 2 g/kg was administered intraperitoneally in an injection volume of 12.5 ml/kg. This dose was chosen because it has been shown to induce robust CPP and CPA (Cunningham et al., 1997). Methylnaloxonium iodide (Sigma, St. Louis, MO) or baclofen (Sigma, St. Louis, MO) was dissolved in artificial cerebrospinal fluid (acsf). The Methylnaloxonium doses used were chosen based on previous doses reported to decrease ethanol CPP (Bechtholt & Cunningham, 2004) and self-administration in rats (Heyser et al., 1999).

Apparatus

The place conditioning chambers consisted of 12 identical acrylic and aluminum boxes (30 x 15 x 15 cm) enclosed in separate light and sound attenuating chambers. Locomotor activity and the location of the mouse within the box were determined by six infrared beams located 2.2 cm above the floor of the chamber at 5 cm intervals and recorded with a 10 ms resolution by a computer. The floor consisted of interchangeable halves, which were made of two distinct textures. Specifically, hole floors were made from perforated 16 gauge stainless steel with 6.4 mm round holes on 9.5 mm staggered centers. Grid floors were made of 2.3 mm stainless steel rods mounted 6.4 mm apart on acrylic rails. These floor textures were chosen based on previous findings demonstrating that drug naive mice spend equal time on both floors (Cunningham, 1995), which allows the use of an unbiased method of assessing CPP (Cunningham, Ferree et al., 2003).

Place Conditioning Procedure

Each experiment consisted of three phases: habituation (one session), conditioning (eight sessions), and preference testing (one session). The habituation session was intended to reduce the novelty of the experimental apparatus and injection procedure. On the first day of the experiment, animals were injected intraperitoneally (IP) with saline (12.5 ml/kg) just after (CPA; Experiments 1 and 2) or just before (CPP; Experiment 3) being placed in the conditioning chamber on a smooth paper floor for 5 min. During the conditioning phase, animals were injected with saline or ethanol on alternating days just after (CPA; Experiments 1 and 2) or just before (CPP; Experiment 3) being placed in the conditioning chamber for 5 min where both sides of the floor were the same. Animals in the Grid+ condition were placed on the grid floor on ethanol injection days and the hole floor on saline injection days. Conversely, animals in the Grid- condition were placed on the hole floor on ethanol injection days and the grid floor on saline injection days. The order of ethanol and saline exposure was counterbalanced within groups. After four conditioning trials (4 CS+, 4 CS-; days 2-9), a 30 min preference test was conducted (day 10). In Experiment 1, immediately before the preference test session, animals were given an intra-cranial injection of acsf or methylnaloxonium (375 ng or 750 ng) and placed into the conditioning chambers where the floor was half hole and half grid and the left/right position of the floors was counterbalanced within groups. In contrast, during Experiments 2 and 3, animals were either held by the scruff (Handled) as previously described or placed into the conditioning chamber without prolonged handing (Standard).

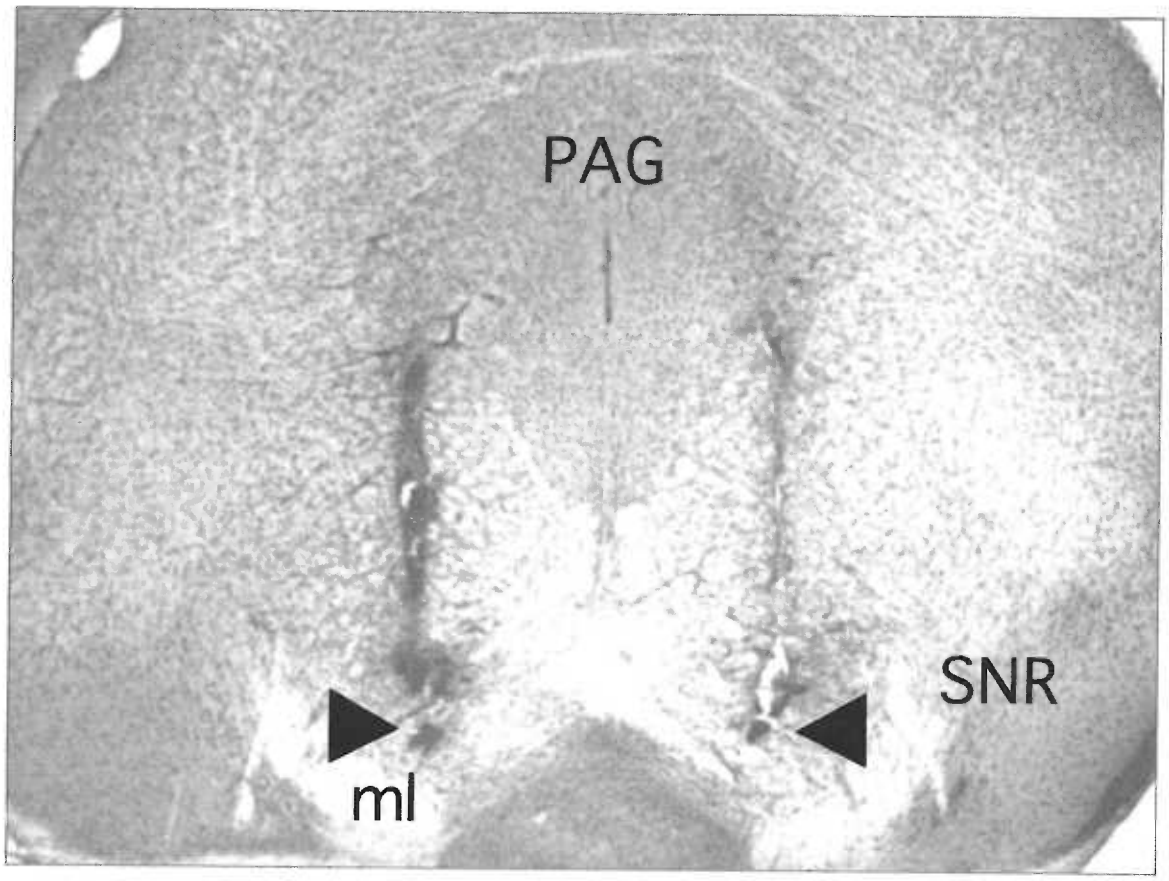
Histological Verification

Animals with cannula implants were sacrificed after completion of the preference test and brains were post-fixed in 2% paraformaldehyde in isotonic sodium phosphate buffered saline (PBS) for 24 h. Brains were cryoprotected with overnight incubations in 20% and then 30% sucrose in PBS with 0.1% NaN_3. Frozen 40μm coronal sections were cut through the site of the microinjectors using a cryostat and collected in PBS with 0.1% NaN_3. Sections were then mounted onto slides and thionin stained. Animals given microinjections outside of the VTA were eliminated from all analyses. An example photomicrograph of bilateral VTA injection sites are shown in Figure 8. The template system described in Chapter 1 (pg 53) was used to determine cannula placement (Figure 2, pg 56). Data from twenty-five additional mice were removed from all analyses because of cannula placement outside of the posterior VTA (bilateral hits; $n = 64$). The final numbers of mice in each subgroup are shown in the figure captions.

Data Analysis

Conditioning activity data were analyzed using two-way ANOVAs with the factors Trial Type (Ethanol or Saline) and Methylnaloxonium Dose (375 ng or 750 ng; Experiment 1) or Handling Group (Standard or Handled; Experiments 2 and 3). Because the general conclusions were unchanged, conditioning activity data were collapsed across the individual CS+ and CS- trials to simplify presentation. Grid time test data were analyzed with two-way ANOVAs and the factors Conditioning Group (Grid+ or Grid-) and Methylnaloxonium Dose (Experiment 1) or Handling Group (Experiments 2 and 3). Because we had a specific, directional hypothesis about the outcome of Experiment 2,

Figure 8. Example photomicrograph of bilateral cannula placement in the VTA. Arrows point to the site of injection within the VTA. For reference, the location of the periaqueductal gray (PAG), medial lemniscus (ml) and substantia nigra reticulata (SNR) are indicated by their abbreviations.


PAG
SNR
ml

pair-wise planned comparisons were conducted for the two conditioning subgroups within each handling group to determine whether place conditioning had occurred. To determine whether interpretation of preference data was complicated by group differences in activity, test activity data were analyzed with one-way ANOVAs using the factor Methylnaloxonium Dose (Experiment 1) or Handling Group (Experiments 2 and 3).

Results

Experiment 1: Intra-VTA methylnaloxonium effects on CPA

One animal, in addition to those previously mentioned, was removed from these analyses due to an experimental error. Data were collected in 3 replications (n =35-36 per replication). Each replication yielded accurate cannula placement in 1 to 6 animals per conditioning sub-group in each methylnaloxonium dose group.

Conditioning Activity. Table 6 shows mean activity rates (counts/min) during CS+ (ethanol) and CS- (saline) conditioning trials. All groups showed slightly higher activity on CS+ trials compared to CS- trials. This conclusion was confirmed by a two-way ANOVA (Trial Type x Methylnaloxonium Dose) on activity during conditioning trials that revealed a significant main effect of Trial Type [$F(1,60) = 4.4$; $p < 0.04$]. This finding has been occasionally reported in the literature (Cunningham et al., 2002). No other significant effects were observed.

Preference Test. Figure 9 shows the mean (+SEM) time spent on the grid floor for the two conditioning groups (Grid+ and Grid-) at each methylnaloxonium dose. Animals in the Grid+ condition generally spent less time on the grid floor than animals in the Grid- condition, indicating development of ethanol-induced CPA. Ethanol CPA was not

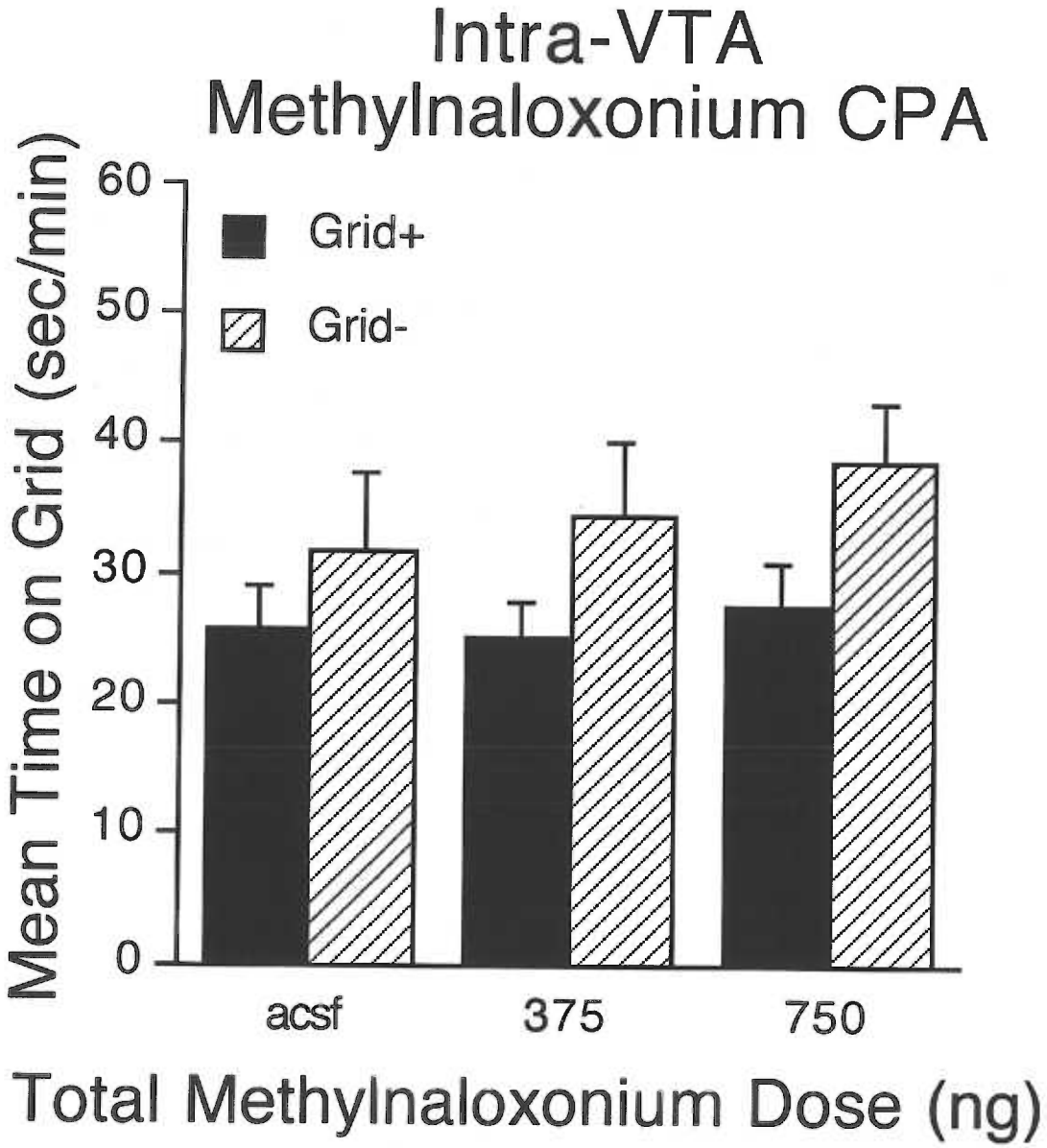
Table 6. Group Mean ± SEM Activity Counts During Conditioning Trials.

	Conditioning Trial Activity (counts/min)	
	Saline Trials (CS-)	Ethanol Trials (CS+)
Experiment 1: Intra-VTA Methylnaloxonium effects on CPA		
acsf	38.9±2.6	42.6±2.4[a]
375ng	42.8±2.8	45.5±2.6
750ng	41.9±2.9	44.2±4.6
Experiment 2: Handling Effects on CPA		
Standard	35.1±2.1	37.4±1.8
Handled	36.6±1.7	37.4±1.7
Experiment 3: Handling Effects on CPP		
Standard	61.5±2.9	179.6±5.4[a]
Handled	55.7±2.0	177.7±5.0

[a] Main effect of trial type

[c] Methylnaloxonium dose and handling group designations are not meaningful until after conditioning since treatment is given prior to the test.

Figure 9. Mean time in sec/min (+SEM) spent on the GRID floor during the 30-min post-conditioning test for each methylnaloxonium dose. Animals in the GRID+ condition were given ethanol paired with the grid floor during conditioning trials (acsf, $n = 14$; 375 ng methylnaloxonium, $n = 9$, 750 ng methylnaloxonium, $n = 10$). Animals in the GRID- condition were given ethanol paired with the hole floor during conditioning trials (acsf, $n = 11$; 375 ng methylnaloxonium, $n = 10$; 750 ng methylnaloxonium, $n = 9$). Significant main effect of Conditioning Group (Grid+ vs Grid-) ($p < .02$), but no significant interaction.


Intra-VTA
Methylnaloxonium CPA
Mean Time on Grid (sec/min)
60
50
40
30
20
10
0
Grid+
Grid-
acsf
375
750
Total Methylnaloxonium Dose (ng)

altered by intra-VTA methylnaloxonium treatment (Figure 9). This observation was supported by a two-way ANOVA (Conditioning Group x Methylnaloxonium Dose) that revealed a significant main effect of Conditioning Group [$F(1,57) = 5.7$; $p < .02$] for time spent on the grid floor during the test, but no Conditioning Group x Methylnaloxonium Dose interaction. However, visual inspection indicated that the magnitude of CPA was generally weak across all groups when compared to findings in previously published reports (e.g., Cunningham et al., 1998).

Test activity. Mean (SEM) activity rates during the test for acsf, 375 ng and 750 ng methylnaloxonium dose groups were 31.8 (2.1), 30.6 (2.1) and 37.9 (2.6) counts per min, respectively. Methylnaloxonium treatment did not significantly alter locomotor activity during the test. A one-way ANOVA (Methylnaloxonium Dose) supported this observation, yielding no significant main effect. Thus, locomotor effects did not confound the interpretation of preference findings.

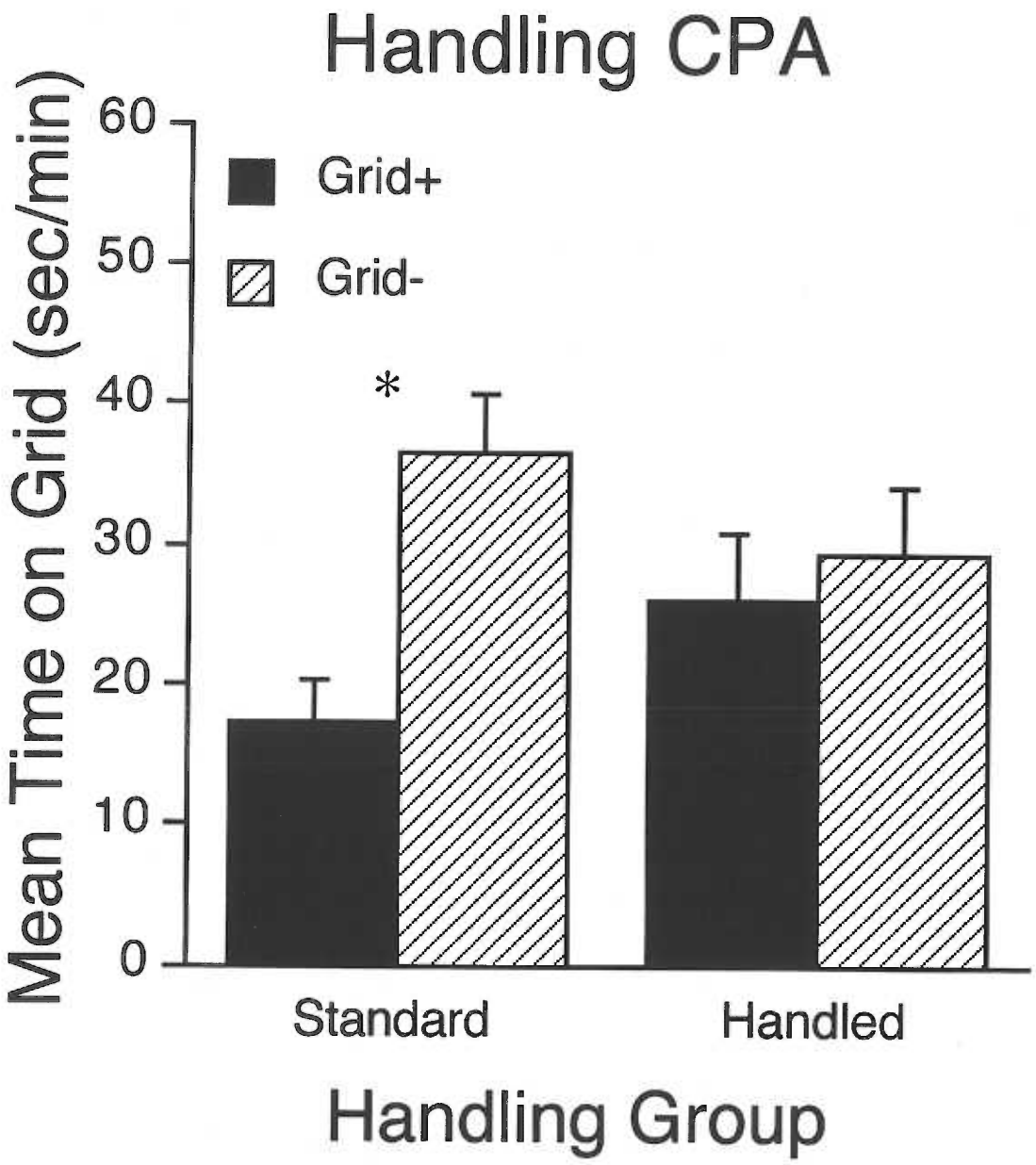
Experiment 2: Handling effects on CPA

Data were collected in 2 replications (n = 36-48 per replication). Data from 6 animals were removed from analyses due to an experimental error; one animal was removed due to an injection injury.

Conditioning Activity. Activity during conditioning trials was similar on ethanol and saline days across the handling groups (Table 6). This conclusion was confirmed by a two-way ANOVA (Trial Type x Handling Group) on activity during conditioning trials that yielded no significant effects. Thus, the small difference in activity between trial types in Experiment 1 was not replicated in Experiment 2.

Preference Test. Animals in the Grid+ condition spent less time on the grid floor

Figure 10. Mean time in sec/min (+SEM) spent on the GRID floor during the 30-min post-conditioning test for each handling group. Animals in the Handled groups were held by the scruff of the neck for 2.25 min just before being place into the chamber on the post-conditioning test day. Animals in the Standard groups were placed into the chamber without prolonged handling post-conditioning test day. Animals in the GRID+ condition were given ethanol paired with the grid floor during conditioning trials (Standard, $n = 19$; Handled, $n = 19$). Animals in the GRID- condition were given ethanol paired with the hole floor during conditioning trials (Standard, $n = 20$; Handled, $n = 19$). Significant main effect of Conditioning Group ($p < .01$). Conditioning Group x Handling Group interaction ($p = .071$). *, $p = .001$ for the planned comparison between conditioning groups (Grid+ vs. Grid-) in the standard condition.


Handling CPA
Mean Time on Grid (sec/min)
60
50
40
30
20
10
0
Grid+
Grid-
*
Standard
Handled
Handling Group

during the test than the animals in the Grid- condition, indicating an overall development of CPA (Figure 10). This conclusion was confirmed by a two-way ANOVA (Conditioning Group x Handling Group) that revealed a significant main effect of Conditioning Group [$F(1,73) = 6.8$; $p < .01$]. This analysis also revealed a trend toward a Conditioning Group x Handling Group interaction [$F(1,73) = 3.4$; $p = .071$] for time spent on the grid floor during the test. Planned comparisons between the Grid+ and Grid- conditioning groups within each handling group demonstrated that animals in the standard group exhibited significant CPA [$F(1,37) = 13.3$; $p = .001$] while animals in the handled group did not, suggesting that handling disrupted the expression of ethanol CPA.

Test activity. Mean (SEM) activity rates during the test for Standard and Handled animals were 24.2 (1.3), and 21.7 (1.5) counts per min, respectively. Handling did not significantly alter locomotor activity during the test. A one-way ANOVA (Handling Group) yielded no significant main effect, suggesting that interpretation of floor preference data was not confounded by differences in test activity.

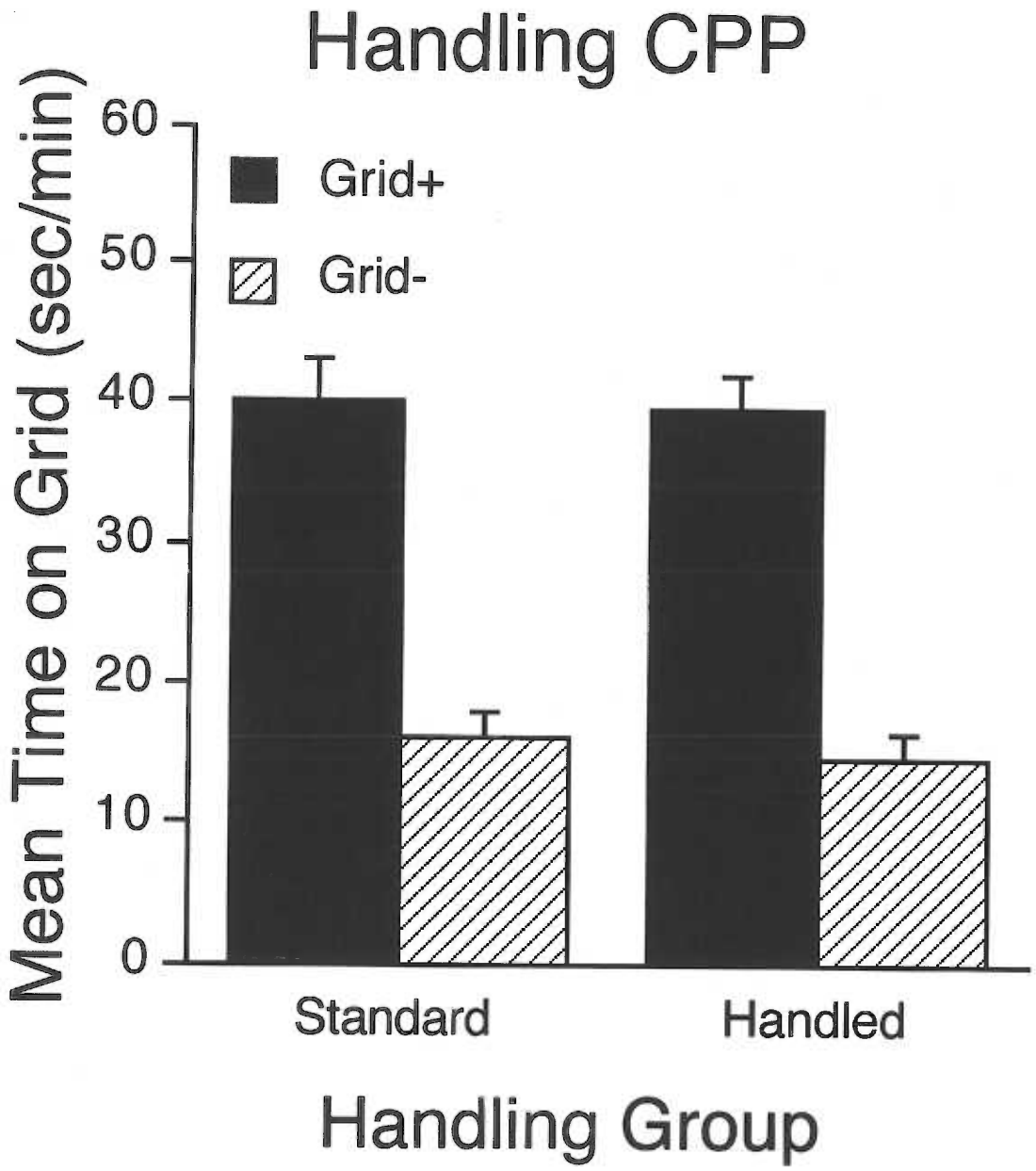
Experiment 3: Handling effects on CPP

Data were collected in 2 replications (n = 36-48 per replication).

Conditioning Activity. Animals were more active when treated with ethanol than saline as is typically seen in DBA/2J mice (Table 6) (e.g., Cunningham et al., 1995). This observation was confirmed by a two-way ANOVA (Trial Type x Handling Group) on conditioning trial activity, which yielded a significant main effect of Trial Type [$F(1,80) = 1329.8$; $p < .001$], but no other significant effects.

Preference Test. As expected, animals in the Grid+ condition spent more time on the grid floor than animals in the Grid- condition, indicating development of CPP.

Figure 11. Mean time in sec/min (+SEM) spent on the GRID floor during the 30-min post-conditioning test for each handling group. Animals in the Handled groups were held by the scruff of the neck for 2.25 min just before being place into the chamber on the post-conditioning test day. Animals in the Standard groups were placed into the chamber without prolonged handling post-conditioning test day. Animals in the GRID+ condition were given ethanol paired with the grid floor during conditioning trials (Standard, $n = 21$; Handled, $n = 20$). Animals in the GRID- condition were given ethanol paired with the hole floor during conditioning trials (Standard, $n = 20$; Handled, $n = 21$). Significant main effect of Conditioning Group ($p < .001$).


Handling CPP
Mean Time on Grid (sec/min)
60
50
40
30
20
10
0
Grid+
Grid-
Standard
Handled
Handling Group

Moreover, handling did not alter the development of CPP (Figure 11). Two-way ANOVA (Conditioning Group x Handling Group) demonstrated a significant main effect of Conditioning Group [$F(1,78) = 105.8$; $p < .001$] and no significant Conditioning Group x Handling Group interaction for time spent on the grid floor during the test. Thus, consistent with our prediction, handling did not affect expression of CPP.

Test activity. Mean (SEM) activity rates during the test for Standard and Handled animals were 38.2 (1.4), and 38.4 (1.5) counts per min, respectively. Handling did not significantly alter locomotor activity during the test and therefore interpretation of the preference data was not confounded. A one-way ANOVA (Handling Group) supported this observation, yielding no significant main effect.

Discussion

A large body of literature has suggested a role for the endogenous opioid system in the rewarding properties of ethanol. These systems have also been implicated in the conditioned aversive properties of ethanol, in that opioid antagonism enhances the expression of ethanol-induced CPA (Cunningham et al., 1998). Here, we suggest that the expression of the conditioned aversive effects of ethanol may not be mediated by opioids in the VTA (Experiment 1). Taken together with previous findings (Bechtholt & Cunningham, 2004), this suggests that the conditioned rewarding and aversive properties of ethanol are expressed through different neural mechanisms. Moreover, we show that handling, in the absence of an infusion, is sufficient to disrupt ethanol CPA (Experiment 2), but has no discernable effect on ethanol CPP (Experiment 3). Although the mechanism underlying the effect of handling on CPA is unknown, our findings suggest

that this effect is not mediated by endogenous opioid release in the VTA.

The finding that intra-VTA methylnaloxonium was without effect on ethanol-induced CPA is in contrast to a recent report that aversive stimuli inhibit VTA dopamine neurons. However, those results were obtained using an unconditioned, rather than conditioned, aversive stimulus (Ungless et al., 2004). Although plausible, it has not been established that effects of conditioned aversive stimuli are mediated by the same substrates as primary aversive stimuli. It is possible that dopamine neurons were already maximally inhibited by the conditioned aversive stimulus, thereby preventing their activity from being further suppressed by methylnaloxonium. The relatively weak magnitude of CPA observed in these experiments make a floor effect on dopamine neuron suppression seem unlikely and suggests that it should have been possible to observe an enhancement of CPA.

Alternatively, taken together with our previous findings that ethanol CPP is reduced by this treatment (Bechtholt & Cunningham, 2004), these findings may suggest that the conditioned aversive and rewarding effects of ethanol are distinct phenomena expressed through different neural substrates. This suggestion is intriguing, given that they are elicited by the same unconditioned stimulus (US; ethanol) and are determined only on the basis of the temporal relationship between ethanol and the conditioned stimulus (CS). That is, the same dose of ethanol given before CS exposure results in CPP and after CS exposure results in CPA. In light of previous reports that a systemic non-selective opioid receptor antagonist can both enhance the conditioned aversive and decrease the conditioned rewarding effects of ethanol (Cunningham et al., 1998), these findings suggest that these two phenomena are influenced by opioid effects in different

brain areas. This disassociation of CPA and CPP is, further, supported by previous findings demonstrating that these two behaviors were not genetically correlated (Cunningham & Ignatoff, 2000; Hill et al., 2002) and that ethanol pre-exposure decreased CPA while CPP was unchanged (Cunningham et al., 2002).

Interpretations of the effects of intra-VTA methylnaloxonium on CPA, however, must be tempered by the finding that handling more generally disrupts the expression of ethanol-induced CPA. Although significant CPA was expressed when the data for the methylnaloxonium dose groups were combined, the outcome of Experiment 2 suggests that this behavior may have been suppressed by handling. This suppression of CPA may have interfered with our ability to detect methylnaloxonium's effects on CPA, thereby preventing us from testing our hypothesis. However, it is difficult to speculate about how this might occur because the mechanism underlying the handling-induced disruption of CPA remains unknown. Interestingly, some findings suggest that the endogenous opioid system might be involved.

Previous findings suggest that some handling induced behaviors are mediated by the endogenous opioid system. For example, light touch of the dorsal spine resulted in 20-32 kHz ultrasonic vocalizations in rats that decreased with repeated handling (Brudzynski & Ociepa, 1992). These vocalizations (20-32 kHz) are thought to convey an affective state (Miczek, Weerts, Vivian, & Barros, 1995) and can be mediated by the endogenous opioid system (Vivian & Miczek, 1998). Similarly handling rats, as for an intraperitoneal injection, induced analgesia and disrupted postshock freezing behavior which was blocked by the non-selective opioid receptor antagonist, naltrexone (Fanselow & Sigmundi, 1986) and decreased by repeated handling. These findings suggest that

routine experimental procedures may induce an opioid-mediated response in non-habituated animals. This may be particularly problematic in mice that do not seem to habituate to handling.

The finding that CPA is disrupted by handling while CPP is unaffected may also be explained by endogenous opioid release. As mentioned, previous findings suggest that both of these behaviors are altered by manipulations of the opioid system. Specifically, systemically administered naloxone, a non-selective opioid receptor antagonist enhances the expression of CPA and disrupts the expression of CPP (Cunningham et al., 1998). Based on these findings, one might hypothesize that opioid receptor agonists could disrupt the expression of CPA and enhance the expression of CPP. While we did not observe effects of handling on CPP, we cannot rule out the possibility that a ceiling effect prevented us from observing enhanced CPP.

Finally, important procedural differences beyond the duration of handling might explain why handling effects were observed in CPA, but not CPP. It is possible that CPP was preserved while CPA was disrupted because handling before the test was novel in CPA. During the conditioning phase in our CPP study (Experiment 3), animals were held by the scruff daily before conditioning trials, and the only procedural difference experienced by the animals on the test day was being the duration of the handling. Conversely, animals conditioned for CPA (Experiments 1-2) were handled by the scruff for injections as they were taken out of the conditioning chamber on conditioning days. They were for the first time held by the scruff of the neck for a prolonged period on the test day just before being placed into the conditioning chamber. This procedural difference between CPA and CPP might explain the observed difference in handling

effects due to the relatively greater novelty of pre-test handling for CPA mice. Alternatively, it is possible that aversive tasks are simply more sensitive to interference by extraneous stimuli. These possibilities can only be addressed by additional experiments investigating possible habituation to handling during conditioning and by experiments using different aversive stimuli.

In summary, our data suggest that opioids in the VTA are not involved in the expression of the conditioned aversive effect of ethanol as indexed by CPA. These findings suggest that different mechanisms or brain areas mediate the conditioned rewarding and aversive effects of ethanol. In addition, we demonstrate that the handling required for microinjections in mice disrupts CPA, but not CPP. Perhaps the novelty of the timing and/or duration of handling are important in this selective disruption of CPA. Previous findings suggest that endogenous opioids could be involved in these handling effects. However, our findings suggest it is unlikely that such effects occur through opioid effects in the VTA. Finally, these findings underscore the need to carefully re-evaluate the potential impact of methodologies developed in rats when they are transferred to studies involving mice.

GENERAL DISCUSSION

The neurochemical mechanisms and substrates that mediate the self-administration of ethanol are increasingly being identified. Some data suggest that similar mechanisms and substrates may underlie the expression of the conditioned effects of ethanol, however, little data on this topic exist. The present experiments suggest that a portion of the reward circuit is involved in the expression of the conditioned rewarding effect of ethanol as indexed by the CPP procedure. Here I report, that a non-selective opioid receptor antagonist injected into the VTA decreased the expression of ethanol-induced CPP, while this treatment was without effect in the NAc. In addition, the injection of a $GABA_B$ receptor agonist into the VTA was effective in decreasing the expression of ethanol CPP. Finally, I also, suggest that the expression of the conditioned aversive effect of ethanol occurs through a mechanism that is independent of opioid receptors in the VTA. However, additional studies indicated that handling may have suppressed the expression of ethanol CPA, complicating interpretation of the microinjection study. Overall, these findings suggest that the conditioned rewarding effect of ethanol is expressed through an opioid and $GABA_B$ dependent mechanism in the VTA, and that the conditioned rewarding and aversive properties of ethanol may be mediated by different neural mechanisms.

Common substrate for ethanol CPP expression and self-administration

The finding that intra-VTA methylnaloxonium decreases ethanol CPP lends further support to the notion that the VTA (e.g., Boehm et al., 2002; Brodie et al., 1990; Gatto et al., 1994; Gessa et al., 1985; June et al., 2004; Rodd et al., 2004; Rodd-Henricks

et al., 2000) and opioids (e.g., Altshuler et al., 1980; Froehlich et al., 1987; O'Malley et al., 1992; Phillips et al., 1997; Volpicelli et al., 1992) are important in the effects of ethanol. Specifically, it is in agreement with the literature demonstrating that the VTA can support the self-administration of ethanol (Gatto et al., 1994; Rodd et al., 2004; Rodd-Henricks et al., 2000) and that opioid receptor antagonists given systemically (Altshuler et al., 1980; Froehlich et al., 1987; Phillips et al., 1997) or injected into this brain area (June et al., 2004) decrease ethanol intake. Further, this finding is also in agreement with experiments demonstrating that a non-selective opioid receptor antagonist given systemically decreases the expression of ethanol CPP (Cunningham et al., 1995; Cunningham et al., 1998). Moreover, these data extend the literature to suggest that opioid systems in the VTA mediate the expression of the conditioned rewarding effect of ethanol. While some discordance has been reported between the mechanisms underlying self-administration and CPP (Bardo & Bevins, 2000), the finding that blockade of opioid receptors in the VTA decreases the expression of ethanol CPP suggests a common substrate for ethanol self-administration and CPP. However, it should be noted that the present experiments cannot rule out the possibility of diffusion of the drug to nearby brain areas. Indeed, the substantia nigra borders the VTA and projects to the NAc where it could have effects relevant to the current findings. The primary role of this nucleus involves locomotion (Cote & Crutcher, 1991). However, the lack of significant locomotor effects provides further support for the notion that the observed effects were derived from drug actions at the target site, the VTA.

Opioid receptors upstream from $GABA_B$ receptors

GABA neurons in the VTA exert a tonic inhibition on dopamine neurons that is decreased by activation of opioid receptors (Johnson & North, 1992). Blockade of opioid receptors in the VTA may alter ethanol CPP through disinhibition of local GABA neurons, resulting in a decrease in dopamine release in the NAc or pfc. Similar to the effects of methylnaloxonium, activation of $GABA_B$ receptors in the VTA decreased the expression of ethanol CPP. This apparent replication of the opioid receptor antagonist effect with a GABA receptor agonist is complementary to the idea that opioid receptor antagonists act through a GABA dependent mechanism. These findings suggest that the expression of the conditioned rewarding effect of ethanol depends upon activation of opioid receptors in the VTA that are up-stream of effects at $GABA_B$ receptors. In other words, baclofen acts on $GABA_B$ receptors located on dopamine neurons whereas nethylnaloxonium likely acts on local GABA neurons. This relationship between opioid and GABA receptors in the VTA has been proposed in a number of electrophysiological and neurochemical experiments (for review see Xi & Stein, 2002). For example, activation of $GABA_B$ receptors in the VTA inhibits the ability of VTA μ-opioid receptor activation in this region to elicit dopamine release in the NAc and pfc (Kalivas et al., 1990). Dopamine release in the NAc or pfc might be a critical response that maintains approach behavior and responding in the presence of ethanol-associated cues, given that others have reported that the presentation of ethanol-associated environmental cues increases NAc dopamine levels (Gonzales & Weiss, 1998). This is consistent with reports that reward associated stimuli activate VTA dopamine neurons (for review see Schultz,

1998).

Possible role of Dopamine neurons in the expression of ethanol CPP

In contrast, previous findings suggest that dopamine receptors are not involved in the expression of ethanol CPP (Cunningham et al., 1992; Dickinson et al., 2003). A non-selective dopamine receptor antagonist (Cunningham et al., 1992) and several selective opioid receptor antagonists (Dickinson et al., 2003) injected prior to the test in a CPP paradigm were without effect on ethanol CPP. These null outcomes were validated by the observation of dopamine receptor antagonist induced locomotor effects, indicating that behaviorally effective dose ranges were used. Therefore, these findings suggest that the effects of methylnaloxonium given into the VTA and other opioid receptor antagonists given systemically may occur through a non-dopaminergic VTA dependent mechanism. Indeed, μ-opioid receptors are located on GABA neurons in the VTA (Svingos, Garzon et al., 2001) that project to the pfc (Carr & Sesack, 2000a) and could potentially mediate these effects excluding dopamine. Such mechanisms are possible via pfc projections to the NAc and the pedunculopontine nucleus (Tzschentke & Schmidt, 2000). However, it should be noted that in the previous studies, the dopamine antagonists were administered systemically (Cunningham et al., 1992; Dickinson et al., 2003), making direct comparisons to intracranial findings somewhat difficult. In that, it is possible that diffusion following systemic administration causes an artificial targeting of particular brain areas excluding the NAc or pfc. Therefore, definitive conclusions regarding the role of dopamine receptors in the expression of ethanol CPP should be tested within specific brain regions. Further, several findings make the possibility that the conditioned

rewarding effect of ethanol is expressed via a dopamine-independent mechanism unlikely. First, systemic opioid receptor antagonists decrease dopamine release in the NAc (e.g., Gonzales & Weiss, 1998). Second, similar alternative mechanisms for the actions of baclofen in the VTA have not been identified. In particular, $GABA_B$ receptors are reportedly expressed on dopaminergic neurons (Wirtshafter & Sheppard, 2001) and not GABA containing neurons (Margeta-Mitrovic et al., 1999) in the VTA. Third, baclofen is known to decrease dopamine release in both the NAc (Westerink, Kwint, & deVries, 1996) and pfc (Westerink, Enrico, Feimann, & De Vries, 1998) through its actions in the VTA. Thus, the observed effects are likely the result of alterations in the activity of dopamine neurons in the VTA.

Candidate opioid receptor for mediating the expression of ethanol CPP

The presentation of conditioned rewarding stimuli is correlated with increased activity of dopamine neurons (Schultz, 2002). On this basis, the μ-opioid receptor is a strong candidate for mediating the effects of methylnaloxonium on ethanol CPP via its actions on GABA neurons that regulate the activity of dopamine neurons projecting to the NAc. This argument stems from the notion that μ-opioid receptor activation in the VTA increases dopamine release in the NAc (Devine, Leone, Pocock et al., 1993; Spanagel et al., 1992), while ORL1 (Maidment et al., 2002) and possibly κ receptors (Devine, Leone, Pocock et al., 1993; Spanagel et al., 1992) have the opposite effect that is correlated with decreases in reward. Further, non-selective blockade of opioid receptors in the VTA decreases NAc dopamine release, which bears the greatest similarity to the blockade of μ receptors in the VTA. Further, the relative insensitivity of

ORL1 receptors to naloxone (Calo et al., 2000) diminishes the likelihood that methylnaloxonium, a derivative of naloxone, alters ethanol CPP through actions at this receptor. Also, the replication of the methylnaloxonium results with baclofen, and the necessity for baclofen to act through activation of $GABA_B$ receptors located on dopamine neurons suggests that the conditioned rewarding effect of ethanol is not expressed through alterations in GABA projection neurons. However, as can been seen in Figure 1 (pg 35), dopamine neurons also project from the VTA to the pfc and it has been demonstrated that non-selective blockade of opioid receptors in the VTA decreases dopamine release in the pfc (Kalivas et al., 1990). However, while it can be said generally that dopamine inhibits pyramidal cells in the pfc, the effect of dopamine in the pfc is extremely complicated and dependent on many factors. In addition, the role of the pfc in reward remains unclear, making conclusions regarding the role of the pfc in conditioned ethanol reward difficult (for review see Tzschentke, 2001).

Possible role of glutamate released from dopamine neurons

Although non-selective opioid receptor blockade and $GABA_B$ receptor activation decreases dopamine levels in the NAc, some data suggest that alterations in dopamine release may not be the only result of changing the activity of VTA dopamine neurons. It has been reported that VTA dopamine neurons grown in culture form glutamatergic synapses. Stimulation of these neurons evokes fast excitatory postsynaptic potentials that are regulated by dopamine. These findings suggest that glutamate release from dopamine neurons initiates the fast actions of dopamine neurons (Sulzer et al., 1998). These findings were more definitively demonstrated recently using slice electrophysiology.

These authors suggest that this fast component of the dopamine neuron signal results from glutamate released from these neurons and might represent the salience signal (Chuhma et al., 2004) suggested by Schultz and colleagues to be conveyed by dopamine neurons (for review see Schultz, 1998). These findings suggest that the signal carried by dopamine release from these neurons may not be as prominent in drug reward as previously suspected and suggest a role for glutamate that might explain the lack of dopamine antagonist effects observed in previous experiments.

Possible role of dopamine receptor synergism

The lack of effect of dopamine receptor antagonists on the expression of CPP might be explained by findings demonstrating that D1 and D2 like receptor antagonists in the NAc do not alter membrane or local field potentials evoked from VTA stimulation (Goto & O'Donnell, 2001). However, concurrent intra-NAc D1 and D2 antagonism decreases the duration of VTA stimulation induced periods of depolarization. These findings are a demonstration of the synergistic effects of D1 and D2 receptors that has been previously reported. This synergism is specifically termed "requisite synergism" to indicate that the effects observed are more than additive (for review see Marshall et al., 1997). The possible importance of concomitant activation of dopamine receptors in the NAc in the expression of place preference may not be in opposition to the previously suggested hypothesis involving dopaminergic modulation of activation induced by glutamate release from VTA dopamine neurons. Indeed, it has been reported that concomitant D1 and D2 receptor activation is required for dopamine to enhance glutamate mediated activation in the striatum (Hu & White, 1997). It seems likely that the

current findings indeed occur through decreases in the activity of dopamine neurons in the VTA, however, the role downstream effects in the NAc play in the expression of CPP remain unknown, but may involve activation of glutamate receptors and/or concomitant activation of D1 and D2 receptors.

Similarity of mechanisms among conditioned rewarding stimuli

The current findings are in agreement with some data regarding place preference for other rewarding stimuli (Table 5). Non-selective opioid antagonism decreases the expression of place preference for cocaine (Gerrits et al., 1995), sucrose (Delamater et al., 2000) and an estrous female (in males) (Mehrara & Baum, 1990; Miller & Baum, 1987) Likewise, the expression of cocaine (Ashby et al., 2002; Lawley & Kantak, 1990), d-methamphetamine (Li et al., 2001), nicotine (Ashby et al., 2002) and heroin (Paul et al., 2001) CPP is decreased by GABA receptor agonists or GABA transaminase inhibitors, which increase GABA concentrations. As highlighted by Table 5, findings are not necessarily in agreement between laboratories and vary based on the unconditioned stimulus. For example, haloperidol, a D2-like receptor antagonist, has been reported to either decrease (Adams et al., 2001) or have no effect on the expression of cocaine CPP (Lawley & Kantak, 1990) and to have no effect on the expression of ethanol (Cunningham et al., 1992), heroin and food CPP (McFarland & Ettenberg, 1999), while place preference for novelty is decreased by haloperidol (Bardo et al., 1989). Although opioid receptors are the known mechanism of action of heroin and morphine, non-selective opioid receptor antagonists reportedly have no effect on the expression of heroin CPP (Hand et al., 1989) and may enhance morphine CPP (Neisewander et al., 1990;

Noble et al., 1993) suggesting that the mechanisms underlying primary and conditioned rewarding effects may differ. Multiple experiments suggest that D1 and D2-like receptor antagonists decrease the expression of amphetamine CPP (Hiroi & White, 1991; Liao et al., 1998), while cocaine CPP is not altered (Adams et al., 2001; Cervo & Samanin, 1995; Liao et al., 1998). However, D1 and D2-like receptor antagonists given in combination decrease cocaine CPP again suggesting synergistic effects of these two receptor types in the conditioned effects of some unconditioned stimuli (Liao et al., 1998). In summary, the available literature on the mechanisms that mediate the expression of CPP suggests that some commonalities exist between unconditioned stimuli. Especially with regard to the role of individual dopamine receptors, the role of dopamine in the expression of CPP is tentative and may warrant further experiments using concomitant administration of D1 and D2 like receptor antagonists.

Improbable induction of an aversive hedonic state

One possible explanation for a decrease in ethanol CPP by blockade of VTA opioid receptors is the induction of an aversive hedonic state, since VTA opioid antagonism alone can condition a place aversion (Shippenberg & Bals-Kubik, 1995). However, it is unclear why this aversive state would be associated specifically with the ethanol-paired conditioned stimulus. Not only intra-VTA, but also intra-NAc opioid antagonism produces CPA and it has been reported that the NAc is the primary site for mediating this effect via non-dopaminergic mechanisms (Shippenberg & Bals-Kubik, 1995). In the present experiments, intra-NAc methylnaloxonium injections had no effect on the expression of ethanol CPP. This lack of effect further supports the suggestion that

the conditioned effects of ethanol are mediated by a VTA-opioid mechanism and are not due to the general aversive properties of opioid receptor antagonists. Here we show that opioid receptors at the level of the cell bodies located in the VTA, rather than the terminal field (NAc), are critical for mediating the conditioned rewarding effect of ethanol. Further, it is unlikely that this lack of effect in the NAc was due to an ineffective dose range since opioid antagonism produced a dose dependent increase in activity during the test, suggesting that behaviorally effective doses were used.

Role of the VTA in the expression of ethanol CPA

Additional experiments were designed to more definitively determine whether a decrease in the expression of ethanol induced CPP was due to the induction of an aversive hedonic state. Because both the CS+ and CS- are presented during the test it is not entirely clear why this aversive state would be directed at the CS+ (Cunningham et al., 1998). One might suggest that because the animal is spending the majority of its time on the CS+ and therefore that the aversion is conditioned to the CS+. In essence, a decrease in the expression of CPP is actually a weakening of the reinforcing association with the CS+ resulting from the new conditioning of an aversion to the CS+. Previous findings demonstrating that a non-selective opioid receptor antagonist enhanced the expression of the CPA diminishes this possibility (Cunningham et al., 1998). In the case of CPA, animals are spending the majority of their time on the CS-. By the same argument, the aversive association would be formed with the CS-. This conditioning should result in a net decrease in CPA. In contrast, naloxone increases the expression of ethanol CPA (Cunningham et al., 1998).

Intra-VTA methylnaloxonium had no effect on ethanol-induced CPA. These findings suggest that the effects of methylnaloxonium on CPP were not due to a general aversive hedonic state because the decrease in CPA, predicted by the induction of an aversive hedonic state was not observed. In addition, these findings are in agreement with previous reports that CPP and CPA are two distinct phenomena (Le et al., 2001) that are not genetically correlated (Cunningham & Ignatoff, 2000; Hill et al., 2002). Further, although CPP and CPA are both altered by non-selective opioid receptor blockade, these findings suggest that they are mediated by opioid effects in different brain areas. These findings are not in agreement with a recent report demonstrating that aversive stimuli decrease the activity of dopamine neurons in the VTA (Ungless et al., 2004). However, it is possible that effects on CPA were not observed because VTA dopamine neurons could not be further suppressed by methylnaloxonium due to floor effects. Interestingly, the relatively weak magnitude of CPA observed in this experiment argues against the notion that a floor effect in the suppression of dopamine neurons prevented us from confirming our hypothesis and suggests that an enhancement of CPA was parametrically possible. Further, as previously mentioned, there is no clear reason to assume that unconditioned and conditioned aversive stimuli share common mechanisms. Unfortunately, these conclusions must be qualified, in that, although significant CPA was observed, the magnitude was weak and later experiments designed to examine similar treatments in the NAc, failed to yield significant CPA.

Role of VTA opioids in handling effects on expression of place conditioning

Follow-up experiments demonstrated the handling required for microinjections

might have caused the weak magnitude of CPA. Animals trained for ethanol CPP or CPA were held by the scruff of the neck for 2.25 min just prior to the preference test, in order to mimic the handling used in microinjection experiments. This handling disrupted the expression of ethanol CPA, but not CPP. Previous findings indicate that similar types of disruptions are mediated by endogenous opioid release. In the current experiments endogenous opioid release may have diminished the expression of aversive effect of ethanol, however, these effects do not appear to be mediated by the VTA since non-selective opioid receptor blockade did not rescue the expression of CPA. In addition, while it is possible that handling affects only CPA and not CPP, however, we cannot rule out the possibility that a ceiling effect masked an enhancement of CPP by handling induced endogenous opioid release.

Dissociation of the expression of ethanol CPP and self-administration

Finally, the finding that NAc opioid receptor blockade did not alter ethanol CPP is not in agreement with previous findings suggesting that NAc opioid antagonism decreases operant responding for ethanol (Froehlich et al., 2003; Heyser et al., 1999; June et al., 2004). However, these findings add further support to the notion that self-administration and CPP are dissociable (Bardo & Bevins, 2000). One major advantage to the study of the expression of ethanol CPP is that ethanol is not present during the test, allowing the exclusion of alterations to the direct pharmacological effects of ethanol. Therefore, these findings suggest that the effects observed in self-administration studies were likely due to alterations in the unconditioned effects of ethanol rather than the conditioned effects. While some aspects of the conditioned response to ethanol may be

similar to the primary effects of ethanol, as was observed in the VTA CPP experiments, these data provide support for the notion that not all of the mechanisms involved in ethanol CPP and self-administration overlap.

Role of the VTA in the conditioned response to ethanol associated cues

Drug associated cues are known to elicit conditioned responses of a Pavlovian nature. Place conditioning is a model that relies upon the principles of Pavlovian conditioning, however, unlike more typical Pavlovian models, the unconditioned and conditioned responses are not directly measured. Rather, the conditioned response is inferred to be rewarding, and possibly drug-similar, based on the animal's elective proximity to the drug-associated cue. The current findings suggest that the conditioned response to cues previously paired with ethanol is mediated by the VTA and involves both μ-opioid and $GABA_B$ receptors. Therefore, it is likely that contact with the ethanol associated cue elicits endogenous opioid release in the VTA, which binds to μ-opioid receptors, inhibiting GABA neurons, preventing additional binding of GABA at $GABA_B$ receptors. The downstream effect of this inhibition remains unknown. However, because $GABA_B$ receptors are located primarily on dopamine neurons in the VTA projecting to the NAc and pfc, dopamine neurons are a likely candidate.

Relevance to humans

Alcoholism has proven to be a challenging public health concern with only two drugs approved by the FDA for its treatment. Ethanol associated cues are thought to induce craving, which is correlated with relapse (Flannery, Volpicelli, & Pettinati, 1999)

and may be causally related to relapse to drinking. Understanding the expression of conditioned rewarding associations that might increase craving and the probability of relapse holds great promise for treatment. This approach may be particularly useful because associations between ethanol and environmental cues are already in place when alcoholics seek treatment. The present findings demonstrate that conditioned rewarding associations can be disrupted after they are acquired and provide a brain area that can mediate the effects of naltrexone and baclofen, two drugs already shown to be effective in the treatment of alcoholics. Greater understanding of the up- and down-stream mechanisms of these effects may help identify more direct or exclusive neural targets that may aid in the development of new, more effective, treatment strategies.

Summary

In summary, I suggest for the first time that the conditioned response to ethanol-associated cues depends upon the VTA and occurs through an opioid and $GABA_B$ receptor dependent mechanism that likely affects the activity of dopamine neurons. Specifically, ethanol-associated stimuli may evoke endogenous opioid release in the VTA that decreases the activity of local inhibitory GABA neurons, thereby increasing the activity of dopamine neurons. Further, these findings provide a possible site of action and mechanism through which both non-selective opioid receptor antagonists and $GABA_B$ receptor agonists decrease craving in alcoholics.

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