CHARACTERIZATION OF NALOXONE'S EFFECTS ON THE EXPRESSION OF ETHANOL-INDUCED CONDITIONED PLACE PREFERENCE IN MICE

by

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TABLE OF CONTENTS

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LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Recent studies have suggested a role of the endogenous opioid system in the reinforcing properties of ethanol. Many of these studies have shown that nonselective opioid antagonists decrease consumption of ethanol in rats, mice and monkeys. In addition, relapse rates in human alcoholics have been found to decrease when patients are administered naltrexone, an opioid antagonist. These findings have also been extended to another model of ethanol reward, the conditioned place preference paradigm. The nonselective opioid antagonist naloxone (1.5-10 mg/kg) has been shown to decrease maintenance of expression of ethanol-induced conditioned place preference in mice. However, at the doses used in these studies naloxone can occupy μ, δ or κ receptors.

The experiments described here were designed to assess the role of the individual opioid receptor types in the expression of ethanol place preference. In addition, the current set of studies further characterized the effect of naloxone by extending the dose-response curve and determining stereospecificity. Finally, these studies examined the importance of certain procedural variables thought to influence the magnitude of naloxone's effect.

Experiments 1-3 used selective antagonists to block the three receptor types during a preference test where mice typically spend more time on a floor paired with ethanol than a floor paired with saline. If naloxone's effects

on the expression of this preference are mediated through either the μ, δ or κ receptors, results of one or more of these studies should mimic those seen with naloxone. However, blockade of individual receptor types in Experiments 1-3 had no effect on expression of ethanol place preference.

Experiments 4A and 4B examined the stereospecificity of naloxone's effects. (+)-Naloxone, the inactive stereoisomer of naloxone, was administered prior to preference testing. No effects of (+)-naloxone were seen, suggesting that naloxone does attenuate place preference by acting on opioid receptors. Unexpectedly, naloxone did not have a significant effect on the first preference test.

In Experiments 5A and 5B (reported here as one experiment), the effects of high doses (20 and 30 mg/kg) of naloxone on expression were examined. Only the highest dose decreased maintenance of expression, suggesting that the dose-response curve for naloxone had been shifted to the right.

Experiment 6 assessed whether mice trained with a two-day break during conditioning would show a different response to naloxone than mice conditioned on consecutive days. It was suggested that conditioned preference would be stronger in mice not given a break, and that naloxone would, therefore, be less effective. Naloxone significantly attenuated maintenance of expression in both break and no-break groups, indicating that conditioning on consecutive days does not alter sensitivity to naloxone.

Experiment 7 indicated that the temporal proximity of cage changing to the test day significantly affected the magnitude of naloxone's effect. It was suggested that cage changing may be a stressor which raises levels of endogenous opioids, thereby shifting the dose-response curve for naloxone to the right.

The findings of Experiments 1-3, that selective blockade of opioid receptors did not have the same effect as administration of naloxone, led to the alternative hypothesis that preference may be maintained by activation of redundant opioid systems. Therefore, more than one receptor may need to be blocked simultaneously to mimic the effects of naloxone.

The present studies indicate the extreme importance of procedural variables in behavioral research and suggest alternative explanations for reduced or absent effects of naloxone.

INTRODUCTION

In 1988 approximately 15.3 million Americans met DSM-III-R criteria for alcohol abuse, dependence, or both (U.S. Alcohol and Health Report, 1993). Winnick (1992) states that alcohol is the most widely used drug in America, with about 70% of the population using alcohol in a given recent year.

According to Bozarth (1987), "...compulsive drug-taking behavior is the defining characteristic of an addiction, and drug reinforcement is a primary determinant in drug-taking behavior. Thus, the study of drug reinforcement is, in essence, the study of drug addiction." Positive reinforcement in humans is often associated with the subjective sensation of pleasure (especially in drug use), and it is natural to assume that animals experience similar sensations (Liebman, 1989). To this end, several animal models of drug reward have been developed.

Assessment of Ethanol's Rewarding Effects

In the last 30 years, several experimental techniques have been developed to assess the rewarding or motivational effects of abused drugs (see Bozarth, 1987 and Goudie, 1991 for reviews). These techniques include 'direct' measures of reinforcement such as self-administration as well as 'indirect' measures such as place conditioning. Each method has advantages and disadvantages that vary with the characteristics of the drug being tested. The

present discussion focuses on the use of various techniques to measure the rewarding effects of ethanol.

Drug discrimination

The subjective effects produced by drugs of abuse, including ethanol, are believed to play an important role in abuse liability (Colpaert, 1987). However, since subjective effects are sensory and are generally measured by self-report in humans, they can be difficult to assess in animals. The drug discrimination procedure is used to establish drugs as discriminative stimuli that guide behavioral responses. Typically, the drug discrimination paradigm involves training animals to make one response (e.g., press lever A) when treated with one drug (the training drug) and another response (e.g., press lever B) when treated with vehicle (or another drug). In this example, responses on lever A would be reinforced only after drug treatment and would have no consequences after vehicle treatment. Thus, the animal must discriminate its internal state and make the appropriate response to obtain a reward (often a food pellet).

Conger (1951) first reported that rats could be trained to run down an alley after administration of ethanol and to withhold the response when not drugged (or vice versa). Recent data showing that partial generalization occurs between centrally and peripherally administered ethanol suggest that the discriminative stimulus effects of ethanol are centrally mediated (Hodge, 1994).

An advantage of the drug discrimination procedure is that most centrally active drugs are discriminable from a non-drug state. The subjective effects of different drugs can be compared by testing with a drug different from the training drug. Drugs that produce drug-appropriate responses are said to substitute for the training drug and may have subjective effects similar to the training drug. This paradigm can, therefore, be used to categorize various drugs according to their subjective effects and can be useful in predicting abuse liability. A recent review compared drug discrimination findings in animals with 28 published studies of drug discrimination in humans, demonstrating that results were qualitatively similar between humans and animals (Kamien, Bickel, Hughes, Higgins & Smith, 1993). With regard to ethanol data, Kamien et al. (1993) suggested that drug discrimination might be used as a diagnostic tool for predicting alcoholism.

A major disadvantage of the drug discrimination paradigm is the labor and time intensive nature of these experiments (Overton, 1987). Months of training and testing are often involved which can result in tolerance or sensitization to some effects of the training drug, thereby altering its subjective effects. In addition, the requirement of extensive training precludes examination of mechanisms underlying acquisition of the behavior.

Operant Self-administration

According to the principles of operant conditioning, a positive reinforcer is defined as a stimulus (e.g., a drug) which, when its presentation is made contingent on a specific response (e.g., lever pressing), will increase the likelihood of that response (Skinner, 1953). As pointed out by Goudie (1991), in an operant sense, drugs are defined as reinforcers in terms of their effects on behaviors, not in terms of their hedonic or subjective rewarding properties. Therefore, the fact that a drug is self-administered does not necessarily mean that it has positive hedonic or pleasurable subjective qualities. However, as pointed out by Brady, Griffiths, Heinz, Ator, Lukas & Lamb (1987), there is a correspondence in the drugs self-administered by animals and those abused by humans. In addition, IV self-administration in rhesus monkeys has been proposed to be a "nearly pure, uncontaminated measure of the intrinsic, pharmacological property of abuse potential" of the drug being administered (Falk, 1993).

One advantage of the self-administration paradigm is that it appears to have high predictive validity. However, this relationship breaks down when i.v. self-administration of ethanol is considered. While monkeys have been reported to self-administer ethanol intravenously (e.g., Altshuler, Phillips, & Feinhandler, 1980), i.v. self-administration of ethanol in rats has not consistently been demonstrated (e.g., compare Collins et al. 1984 and DeNoble, Mele, & Porter, 1985 with Smith & Davis, 1974 and Lyness & Smith, 1992).

Recent studies have indicated that some inbred mice will nose-poke for i.v. administration of ethanol when ethanol delivery is signalled by a cue light (Grahame & Cunningham, 1996). However, without the cue light, others have not demonstrated ethanol-reinforced responding (T. J. Phillips, personal communication), raising the possibility that behavior is not being controlled by ethanol alone. Overall, ethanol has not proved to be a strong reinforcer in the i.v. self-administration paradigm.

While i.v. studies have shown that ethanol can act as a reinforcer, i.v. self-administration may not be an appropriate model of human alcohol use since humans do not typically inject ethanol. In response to this criticism, as well as to other disadvantages of the i.v. paradigm (e.g., short life-span of catheters, invasive surgery), oral self-administration procedures have been developed. A review by Samson and colleagues describes a variety of procedures that can initiate oral ethanol self-administration in non-deprived rats (Samson, Pfeffer, & Tolliver, 1988). Of these procedures, one of the most successful is sucrose-fading (Samson, 1986). In this procedure, rats are initially trained to bar-press for a reinforcer of 20% sucrose. Once stable responding has been acquired, increasing concentrations of ethanol are added to the sucrose as the concentration of sucrose is lowered. Eventually, rats respond reliably for a 10% ethanol solution in the absence of sucrose.

One important advantage of the sucrose-fading procedure is that rats need not be food or fluid-deprived during acquisition of self-administration.

In general, oral self-administration of ethanol has high face validity as it is the route of administration commonly used by humans.

Drinking

While many humans readily drink alcoholic beverages and some drink to excess, most animals will not, although there are exceptions (e.g., C57BL/6J inbred mice, Syrian golden hamsters, selectively bred alcohol-preferring P rats). Technically, drinking can be viewed as a form of operant behavior with licking as the operant response. However, drinking studies (especially two-bottle choice procedures) are conceptually different from what are typically termed operant conditioning experiments and belong to a different research tradition (Meisch & Lemaire, 1993).

In 1940, Richter and Campbell demonstrated that rats prefer ethanol in concentrations of 1.8 and 6% (w/v) to water in a two-bottle choice procedure. While preference for low concentrations of ethanol has been replicated, it has been suggested that this preference can be attributed to taste rather than pharmacological effect (Cicero, 1980). Attempts to establish a preference with higher ethanol concentrations have largely been unsuccessful, with the exception of some inbred mouse strains and selectively bred rat lines.

Although oral self-administration procedures have high face validity and make intuitive sense with respect to models of human ethanol consumption (see Amit, Smith, & Sutherland, 1987 for a review), they are not without disadvantages. Because of the low potency and aversive taste of

ethanol, it can be difficult to experimentally induce consumption of sufficient ethanol to produce pharmacological effects. To overcome the palatability issue, tastants such as sucrose or saccharin are often added to the ethanol solution. However, this manipulation can make interpretation of results more difficult since intake may be controlled by the tastant rather than the pharmacological effects of ethanol. In addition, with oral drug consumption there may be a considerable delay between drinking behavior and post-absorptive drug effects.

Place Conditioning

Although self-administration procedures provide 'direct' assessment of the reinforcing properties of drugs, there are advantages to using 'indirect' procedures such as place conditioning. For example, drug effects may be examined in drug-naive animals because extensive training with the drug is not necessary as in many self-administration procedures. In addition, place conditioning paradigms allow for assessment of both positive and negative hedonic drug effects.

The central tenet of place conditioning is that rewarding stimuli will elicit approach and contact behaviors whereas aversive stimuli will elicit withdrawal responses (Carr, Fibiger, & Phillips, 1989). In a typical place conditioning procedure, administration of a drug and the resulting pharmacological effects are paired with one distinct environment while drug vehicle administration is paired with another distinct environment (e.g.,

Cunningham, Dickinson, & Okorn, 1995). With repeated pairings, an association is made between the internal effects of the drug and distinct external cues. Via Pavlovian conditioning processes, these external cues assume the hedonic properties of the drug effect. After repeated exposures, the animal is given a choice between the two environments in a drug-free test. If the animal chooses to spend more time in the drug-paired environment, a conditioned place preference is demonstrated, and it is inferred that the drug effects were rewarding. Alternatively, a place aversion is demonstrated if the animal spends more time in the vehicle-paired environment, and it is concluded that the drug effects are aversive. Many variations of the place conditioning procedure have been developed with visual, tactile and/or olfactory cues serving as the distinct environmental stimuli (for recent reviews see Carr et al., 1989 and Swerdlow, Gilbert & Koob, 1989).

One advantage to using the place conditioning paradigm with ethanol is that palatability is not an issue since the drug is typically injected. In addition, treatments aimed at affecting ethanol reward may be used during either the acquisition or testing phase, allowing examination of the processes underlying the initial reward as well as expression of a conditioned preference. Finally, since testing may be done drug-free, possible motor effects of ethanol or other agents need not impact the expression of the conditioned preference.

The place preference paradigm was first reported by Olds and Milner (1954) who observed that rats preferred the area of the experimental apparatus that was associated with brain stimulation. Conditioned place preferences have since been shown to occur with many drugs abused by humans, including amphetamine (Reicher & Holman, 1977), cocaine (Spyraki, Fibiger, & Phillips, 1982), morphine (Mucha & Iversen, 1984) and heroin (Bozarth & Wise, 1981). Place conditioning in rats with ethanol, however, has generally resulted in a conditioned place aversion rather than preference (Cunningham, 1979, 1981; Stewart & Grupp, 1986; van der Kooy, O'Shaughnessy, Mucha, & Kalant, 1983), although place preference has occasionally been reported (Black, Albiniak, Davis, & Schumpert, 1973; Bozarth, 1990; Reid, Hunter, Beaman, & Hubbell, 1985; Suzuki, Shiozaki, Morizumi, & Misawa, 1992).

In contrast to the place aversion typically seen in rats, several inbred mouse strains, selectively bred lines of mice, and genetically heterogeneous mice have recently been shown to exhibit a robust conditioned place preference for tactile cues paired with ethanol (e.g., Cunningham, Hallett, Niehus, Hunter, Nouth, & Risinger, 1991; Cunningham et al., 1995; Cunningham, Niehus, Malott, & Prather, 1992; Cunningham, Niehus, & Noble, 1993; Risinger, Dickinson, & Cunningham, 1992). Not all mice develop a conditioned place preference (e.g., C57BL/6 mice do not show evidence of conditioning), but as yet no strain has shown a place aversion

with a conditioning procedure that pairs injection of ethanol with immediate placement on the CS. Why some mice show a conditioned place preference for cues paired with ethanol while rats typically show an aversion is not known. A study by Cunningham et al. (1993) demonstrating preference in mice and aversion in rats with identical place conditioning procedures indicates that procedural differences are not the source of the discrepancy. While the underpinnings of the species difference in ethanol place conditioning are unknown, it appears that the positive hedonic effects of ethanol in mice may be assessed with this paradigm.

Mechanisms of Ethanol Reward

While it is assumed that the widespread use of alcohol and other drugs of abuse is due in part to the subjective rewarding effects of these drugs, delineation of the biological basis of these effects has proved difficult, especially in the case of alcohol. Psychomotor stimulants such as amphetamine and cocaine are known to exert many of their effects via the dopamine system (e.g., Weiss, Hurd, Ungerstedt, Markou, Plotsky, & Koob, 1992; Wise & Bozarth, 1987), while morphine binds to specific membrane-bound opioid receptors (e.g., Pasternak, 1993).

Membrane fluidization

It is known that ethanol can fluidize the lipid bilayer of cell membranes (Chin & Goldstein, 1977) and thus perhaps interfere with the

function of membrane-bound receptors. However, it has also been pointed out that high, physiologically attainable concentrations (100 mM) of ethanol fluidize membranes to the same extent as a 0.5° C increase in body temperature, an event that happens regularly in humans (Tabakoff, Hoffman, & McLaughlin, 1988). Therefore, while the membrane-fluidizing properties of ethanol are undoubtedly important for some effects, it is unlikely that this can account for all of its effects.

Neurotransmitters

It is generally accepted that ethanol does not act on specific neuronal receptors but instead may exert its effects through "receptive elements" which may include receptor proteins or intracellular signalling proteins (Tabakoff & Hoffman, 1992). At one time or another, nearly every neurotransmitter system has been implicated in ethanol's rewarding effects (Koob, 1992; Koob, Rassnick, Heinrichs, & Weiss, 1994). A recent review by Koob et al. (1994) presents evidence that the γ-aminobutyric acid (GABA), dopamine, serotonin, glutamate, and opioid peptide systems are involved in ethanol reinforcement.

At various times, different agents have been proposed to reduce, reverse or prevent various effects of ethanol (see Tabakoff & Hoffman, 1992). Many investigators have found that drugs that act at the GABA receptor have these capabilities (e.g., reviews by Lister & Nutt, 1987; Ticku, 1990; Ticku &

Kulkarni, 1988), suggesting that some of ethanol's effects may be mediated through the GABA system.

The mesolimbic DA system has been implicated in the rewarding properties of abused drugs such as cocaine, amphetamine, the opiates, and ethanol, as well as natural rewards such as food, water and sex (see review by Wise, 1991; Wise & Rompre, 1989). Various investigators have found evidence that ethanol activates the mesocorticolimbic DA system (e.g., Brodie, Shefner, Dunwiddie, 1990; Di Chiara & Imperato, 1986; Imperato & Di Chiara, 1988) and that DA antagonists decrease ethanol self-administration (Pfeffer & Samson, 1988). However, not all studies have reported effects of DA antagonists on indices of ethanol reward (e.g., Brown, Gill, Abitbol, & Amit, 1982; Cunningham, Malott, Dickinson, & Risinger, 1992; Risinger, Dickinson, & Cunningham, 1992).

Parenteral administration of ethanol has been shown to increase release of 5-HT (as well as DA) in rat nucleus accumbens (Yoshimoto, McBride, Lumeng, & Li, 1991; 1992). Interestingly, it appears that treatments that enhance 5-HT function decrease ethanol intake. The 5-HT reuptake inhibitors zimeldine (Gill, Amit, & Ogren, 1985), fluoxetine (Haraguchi, Samson, & Tolliver, 1990; Lyness, & Smith, 1992) and sertraline (Gill, Amit, & Koe, 1988) have been found to reduce ethanol consumption.

Recent evidence indicates that ethanol may antagonize NMDA type glutamate receptors (Lovinger, White, & Weight, 1989). In addition,

acamprosate, a compound thought to have anti-glutamate effects via actions on calcium channels, decreases ethanol self-administration in rats (Rassnick, D'Amico, Pulvirenti, Zieglgansberger, & Koob, 1992) and reduces relapse rates in human alcoholics (Lhuintre, Moore, Tran, Steru, Lancrenon, Daoust, Parot, Ladure, Zarnitsky, Boismare, & Hillemand, 1990).

Endogenous Opioids

In recent years, several lines of research have provided evidence that the endogenous opioid system may be involved in the subjective rewarding effects of ethanol and, hence, may play a role in maintaining ethanol drinking behavior (e.g., Froehlich, 1993; Gianoulakis, 1993; Reid & Hunter, 1984). The remainder of this thesis focuses on the opioid system and its role in the rewarding effects of ethanol.

Opioid System

Opioid Receptors

Since the discovery of specific opiate binding sites in brain tissue in 1973 (Pert & Snyder; Simon, Hiller, & Edelman; Terenius), the opioid receptors have been the focus of intense research. Initially these receptors were thought to be a homogeneous group, but this idea was dispelled by studies by Martin and colleagues demonstrating that morphine and some of its analogs had different pharmacological profiles (Gilbert & Martin, 1976; Martin, Eades, Thompson, Huppler, & Gilbert, 1976). These findings led

Martin et al. to postulate the existence of three types of opioid receptors, μ, κ and σ, each named for the drugs used in the studies. After the discovery of the enkephalins, it was found that these peptides were capable of binding to another type of opioid receptor, the δ receptor (Lord, Waterfield, Hughes, & Kosterlitz, 1977). Other types of receptors have since been postulated, but the μ, δ and κ receptors are the most studied and best characterized. Subsequent studies on the σ receptor have indicated that it is a binding site for phencyclidine and its analogs (Vincent, Kartolovski, Geneste, Kamemka, & Lazdunski, 1979; Zukin and Zukin, 1979) and is not a true opioid receptor (i.e., its effects are not reversed by opiate antagonists).

In addition to the μ, δ and κ type opioid receptors, subtypes of each of these have been postulated, such as μ_1 and μ_2 (Wolozin & Pasternak, 1981), δ_1 and δ_2 (Jiang, Takemori, Sultana, Portoghese, Bowen, Mosberg, & Porreca, 1991; Sofuoglu, Portoghese, & Takemori, 1991), and κ_1, κ_2, and κ_3 (e.g., Attali, Gouarderes, Mazarguil, Audigier, & Cros, 1982; Zukin, Eghbali, Olive, Unterwald, & Tempel, 1988). With the advent of more highly selective antagonists, the existence and functional importance of receptor subtypes will likely be confirmed. However, for the present discussion, only the three major receptor types are considered.

Recent cloning of the opioid receptors indicates that they are members of a family of G-protein-coupled receptors with seven transmembrane regions (Childers, 1993). The μ, δ and κ receptors are homologous at the nucleic acid and amino acid levels, appear to be negatively coupled to adenylate cyclase via Gi, and increase K+ and decrease Ca++ conductances (Uhl, Childers, & Pasternak, 1994). The three receptor types have differential distribution patterns in the brain (see Mansour, Fox, Akil, & Watson, 1995, for a recent review). For example, regions of rat caudate-putamen and thalamic nuclei exhibit a high density of μ binding sites while δ sites are densely distributed throughout the nucleus accumbens, caudate-putamen, and olfactory tubercle, with the thalamus showing only low receptor binding density.

Endogeneous Opioid Peptides

Opioid peptides are synthesized as part of large molecular weight precursor molecules and are then post-translationally cleaved into their active forms by specific peptidases. The opioid peptides are derived from one of three precursors: proopiomelanocortin, proenkephalin, and prodynorphin. Proopiomelanocortin (POMC) is the precursor for several peptide hormones and transmitters, including the opioid peptide β-endorphin (β-END) as well as the nonopioid peptides adrenocorticotropin (ACTH) and α-melanocyte stimulating hormone (α-MSH). The enkephalins [Met^5] and [Leu^5]enkephalin (met-ENK & leu-ENK) are derived from proenkephalin, as

are two other biologically active opioids, [Met^5]enkephalin-Arg^6-Gly^6-Leu^8 (the octapeptide) and [Met^5]enkephalin-Arg^6-Phe^7 (the heptapeptide). The dynorphins are synthesized from the prodynorphin molecule and include dynorphin-A, dynorphin-B, and α-neo-endorphin.

Proopioimelanocortin

β-END, the only opioid product of POMC, was first isolated from the pituitary gland (Li & Chung, 1976), the primary location of POMC synthesis. In the brain, the major site of POMC synthesis is the arcuate nucleus of the hypothalamus, with the nucleus tractus solitarius as an additional site (see Khachaturian, Lewis, Schaefer & Watson, 1985, for a review of this system). Arcuate POMC neurons have extensive projections to other brain regions, with the septum, amygdala and other nuclei of the hypothalamus comprising some of the main targets. Other targets include the medial preoptic area and the thalamus. Arcuate neurons also innervate autonomic and nociceptive regions including the periaquaductal gray and raphe nuclei.

The most biologically active form of β-END is β-END 1-31, which can be enzymatically cleaved to the shorter peptides β-END 1-26 and β-END 1-27 with reduced opiate activity and to the N-acetylated forms of these compounds, which have no opiate activity (Akil, Young, Watson, & Coy, 1981; Deakin, Dostovsky, & Smyth, 1980; Zakarian & Smyth, 1982). The various forms of β-END-like peptides show different brain distribution

patterns, with lower levels of conversion of β-END 1-31 to the shorter, less active forms in the arcuate nucleus and periaquaductal gray and higher levels of β-END 1-27 in the amygdala, hippocampus and nucleus accumbens (Young et al., 1993). Interestingly, some effects of β-END 1-31 are antagonized by β-END 1-27 (Bals-Kubik, Herz, & Shippenberg, 1988; Suh, Tseng & Li, 1987), suggesting that it may be an endogenous antagonist of opioid activity (Young, Bronstein & Akil, 1993). Thus, various cells may use post-translational processing to control opioid agonistic/antagonistic concentrations at the synapse, and, therefore, the physiological effects of neural stimulation. In addition, relative proportions of the various forms of β-END can be altered by morphine or repeated exposure to stressors (Akil, Shiomi, & Matthews, 1985; Bronstein, Kelsey, & Akil, 1991), or treatment with haloperidol (Ham & Smyth, 1985) or ethanol (Gianoulakis & Gupta, 1986; Seizinger, Bovermann, Maysinger, Höllt, & Herz, 1983).

Proenkephalin

The first endogenous opioid compounds to be discovered were the enkephalins. In 1975 met-ENK and leu-ENK were first isolated from the brain and shown to be active (Hughes, Smith, Kosterlitz, Fothergill, Morgan & Morris, 1975). Enkephalin biosynthesis takes place in the adrenal medulla and the brain, with each proenkephalin gene coding for four copies of met-ENK and one copy each of leu-ENK, the octapeptide, and the heptapeptide.

Proenkephalin mRNA has also been found in mammalian reproductive tissue (Kilpatrick, Howells, Noe, Bailey, & Udenfriend, 1985), glial cells (Vilijn, Vaysse, Sukin, & Kessler, 1988; Yoshikawa & Sabol) and stimulated T-helper cells (Zurawski, Benedik, Kamb, Abrams, Zurawski, & Lee, 1986).

Neurons synthesizing proenkephalin are widely distributed in the brain in a number of species, including rat, monkey and man (Khachaturian, Schaefer, & Lewis, 1993). This extensive list of brain regions includes layers of the cerebral cortex, striatum, globus pallidus, amygdala, septum, hippocampus, most hypothalamic nuclei, periventricular thalamus, periaquaductal gray, superior and inferior colliculi and the substantia nigra. Proenkephalin-containing cells are also found in many nuclei of the pons and medulla as well as nociceptive regions of the spinal cord. Levels of met-ENK in brain tissue are at least one order of magnitude higher than POMC and prodynorphin-derived peptides (Giraud, Castanas, Patey, Oliver, & Rossier, 1983), indicating the importance of this peptide.

Processing of proenkephalin is apparently slightly different in the adrenal than in brain tissue, leading to the accumulation of peptides I, E, F and B in the adrenal gland but not in the brain (Liston, Patey Rossier, Verbanck, & Vanderhaeghen, 1984). It has also been suggested that alternate processing of proenkephalin could yield a variety of peptides, some without the amino-terminal sequence Tyr-Gly-Gly-Phe that confers opioid activity, which could be physiologically important (Rossier, 1993).

Prodynorphin

Dynorphin (known as dynorphin A 1-17), a potent prodynorphin peptide, was first obtained from pituitary extracts and characterized by Goldstein and colleagues (Goldstein, Tachibana, Lowney, Hunkapiller, & Hood, 1979). Like several other prodynorphin-derived peptides, including α-neo-endorphin, dynorphin A 1-8, β-neo-endorphin, and dynorphin B, dynorphin A 1-17 contains the amino acid sequence for L-ENK at its amino-terminus. As with POMC-derived peptides, the various opioid peptide products of prodynorphin result from posttranslational enzymatic processing of the prohormone and, thus, can vary from cell to cell depending on which enzymes are present. Although leu-ENK is a potential end product of this process, the cleavage sites that would result in formation of leu-ENK appear to be rarely used (Day, Trujillo, & Akil, 1993).

Like the enkephalins, prodynorphin peptides are found in many brain areas, including cerebral cortex, striatum, substantia nigra, hippocampus, hypothalamic nuclei, amygdala, periaquaductal gray, globus pallidus and raphe nuclei. prodynorphin products are also contained in cell bodies of the medullary spinal trigeminal nuclei and the nucleus tractus solitarius, as well as in the spinal cord dorsal gray laminae (Khachaturian et al., 1993). Synthesis of prodynorphin also occurs in the anterior lobe of the pituitary gland (Seizinger, Grimm, Höllt, & Herz, 1983). Low levels of prodynorphin have

also been detected in the adrenal gland (Day, Schäfer, Collard, Watson, & Akil, 1991) and the testis (Collard, Day, Akil, Uhler, & Douglass, 1990).

Opioid Peptide-Receptor Relationships

The opioid peptides are considered to be endogenous ligands for the opioid receptors. However, while each known opioid peptide will exhibit some selectivity for one of the opioid receptor types, no peptide will show specificity for a particular receptor (Day et al., 1993). For example, while β-END is considered a μ receptor ligand, it has a similar affinity for the δ receptor. Similarly, although met-ENK and leu-ENK exhibit high affinities for the δ receptor, they also bind with reduced affinity to μ receptors, and the affinity of α-neo-endorphin for the κ receptor is only about one order of magnitude higher than for the δ and μ receptor. Finally, a fundamental concept regarding opioid peptides and receptors is that there is no consistent, simple anatomical relationship between the two (Mansour & Watson, 1993). Dynorphin fibers are not consistently co-localized with κ receptors, nor are enkephalin neurons consistently found with δ receptors.

Ethanol and the Opioid System

Given that ethanol does not bind directly to receptors, ethanol may affect the endogenous opioid system in three main ways. First, it could affect the receptors themselves and alter the binding capacity for endogenous

ligand. Second, its metabolites could bind to opioid receptors and activate effector systems. Third, it could affect levels of endogenous opioids by altering synthesis, release and/or degradation of the peptides.

Ethanol and Opioid Receptors

At physiologically attainable concentrations (25-100 mM), acute exposure of tissues to ethanol can slightly enhance μ receptor binding by increasing receptor B_{max} (Hiller, Angel & Simon, 1981; Levine, Hess, & Morley, 1983; Tabakoff & Hoffman, 1983). Chronic exposure to low concentrations has occasionally been shown to decrease affinity and/or density of μ receptors, (Gianoulakis, 1983; Khatami, Hoffman, Shibuya, & Salefsky, 1987), and to increase density or affinity of δ receptors (Gianoulakis, 1983; Pfeiffer, Seizinger, & Herz, 1981; Przewlocka & Lason, 1990). In contrast, higher concentrations (>200 mM) of ethanol acutely decrease δ receptor binding by decreasing receptor affinity (Charness, Gordon, & Diamond, 1983; Hiller et al., 1981; Khatami et al., 1987). Clinically relevant concentrations of ethanol (25-100 mM) have been reported to increase δ receptor gene expression in neuronal cell lines (Charness, Hu, Edwards, & Querimit, 1993). In general, results of binding studies are dependent on ligand and ethanol concentration, assay conditions, tissue/cell type and, in the case of chronic studies, species and route of administration.

Ethanol Metabolites and the Opioid System

The metabolism of ethanol takes place primarily in the liver where it is converted to acetaldehyde by alcohol dehydrogenase and nicotinamide adenine dinucleotide (NAD) as a coenzyme. In turn, acetaldehyde can react with other compounds, forming potentially pharmacologically active substances including tetrahydroisoquinolines (TIQs) (Goldstein, 1983). One such substance is tetrahydropapaveroline (THP), a condensation product of dopamine and aldehyde. Administration of ethanol increases the amount of THP in brain homogenates (Cohen & Collins, 1970; Davis & Walsh, 1970). However, an increase in TIQ levels in humans after ethanol consumption has not been consistently seen, although this may be because of insensitive techniques (Haber, Putscher, Georgi, & Melzig, 1995).

THP has been shown to produce analgesia and to bind to α_2-adrenergic receptors and opioid receptors (Fertel, Greenwald, Schwarz, Wong, & Bianchine, 1980). THP infused i.c.v. has been shown to increase ethanol consumption in rats (Duncan & Fernando, 1991; Myers & Oblinger, 1977; Tuomisto, Airaksinen, Peura, & Eriksson, 1982) and monkeys (Myers & Melchior, 1977; Myers, McCaleb, & Ruwe, 1982). Interestingly, THP-induced drinking can be suppressed by naloxone, a nonselective opioid receptor antagonist, suggesting a role of the opioid receptor in this action (Myers & Critcher, 1982).

While a role of metabolites in the mediation of ethanol's effects has not been conclusively determined, research in this area has dwindled somewhat in recent years. However, a recent review by Myers (1990), proposes a "two channel, brain metabolite" theory of alcoholism whereby metabolites of alcohol permanently alter receptor and neurotransmitter activity in specific limbic structures. This theory is based largely on the ability of TIQs to increase ethanol consumption when infused into the brain but as yet is not strongly supported by data showing that TIQs are produced in vivo after ethanol consumption.

Ethanol and Opioid Peptides

Acute or chronic administration of ethanol in vivo has been found to alter levels of endogenous peptides in several studies. In rats, plasma β-END was increased 30 - 45 min after systemic injection of 1-3.5 g/kg ethanol (Gianoulakis & Barcomb, 1987; Guillaume & Gianoulakis, 1992; Ho & Allen, 1981; Patel & Pohorecky, 1989; Rivier & Vale, 1988). In addition, hypothalamic, but not pituitary, immunoreactive-β-END (ir-β-END) was found to be increased at 20 and 60 min after 2.5 g/kg ethanol in one study (Schulz, Wuster, Duka, & Herz, 1980) but was unchanged in either region 60 min after the same dose of ethanol in another study (Seizinger, Bovermann, Maysinger, Höllt, & Herz, 1983). However, under the same experimental conditions, Seizinger et al. (1983) found increased levels of met-ENK immunoreactivity in the hypothalamus, striatum and midbrain. Acute

ethanol administration did not change levels of the prodynorphin-derived peptides ir-dynorphin or ir-α-neoendorphin (Seizinger et al., 1983).

In contrast to their finding with acute ethanol, Schulz et al. (1980) found no effect of chronic ethanol (5% or 20% in drinking water) on hypothalamic β-END levels, although β-END content of the pituitary was decreased. Likewise, Seizinger et al. (1983) found reduced levels of opiate active β-END in the pituitary after chronic ethanol (ethanol liquid diet, 7% ethanol v/v). Consumption of ethanol liquid diet also decreased levels of ir-dynorphin and ir-α-neoendorphin in the hypothalamus and hippocampus (Seizinger et al., 1983).

In normal human volunteers, plasma β-END was increased after consumption of 1 ml/kg of 80% rum (0.64 g/kg) (Naber, Soble, & Pickar, 1981) and in self-described "voluntary" drinkers who were seen at an emergency center with blood alcohol levels greater than 2 mg/ml (Aguirre, del Arbol, Rio, Raya, & Ruiz-Requena, 1995). However, in this study, administration of 1 g/kg ethanol to non-drinking volunteers tended to decrease β-END levels (Aguirre et al., 1995).

In vitro release experiments have demonstrated a biphasic effect of ethanol on release of β-END-like peptides from rat hypothalamus. Gianoulakis and colleagues have shown that low concentrations of ethanol

increase release of β-END-like peptides with maximal release occurring at 20 mM. However, higher concentrations decrease release of these peptides (DeWaele & Gianoulakis, 1993; DeWaele, Papachristou, & Gianoulakis, 1992; Gianoulakis, 1990). In addition, exposure of dispersed mouse pituitary cells to 17 mM ethanol increased release of β–END (Keith, Crabbe, Robertson, & Kendall, 1986). Repeated in vivo administration of ethanol increased in vitro release of α-neoendorphin from rat hypothalamus but decreased release of the octapeptide, indicating that chronic ethanol seems to have opposite effects on the sensitivity of hypothalamic opioid neurons (Przewlocka & Lason, 1991).

Genetic Differences in Ethanol/Opioid Interactions

Several studies have reported different levels of endogenous opioids in rodent lines known to differ in ethanol consumption, suggesting that the opioid system may play a role in this behavior (see Gianoulakis and DeWaele, 1994 for a recent review). In early studies comparing the alcohol-preferring C57BL/6J (B6) mouse with the alcohol-avoiding DBA/2J (D2) mouse, B6 mice were found to have higher basal levels of pituitary β-END (Crabbe, Keith, Kosobud, & Stack, 1983; Gianoulakis & Gupta, 1986). In vitro perfusion studies show a larger and more pronounced release of β-END from B6 hypothalami than from D2 hypothalami under both basal and ethanol-stimulated conditions (DeWaele et al., 1992). DeWaele et al. (1992) also found

higher POMC mRNA levels in B6 hypothalami, although hypothalamic tissue content of β-END-like peptides did not differ between B6 and D2 mice.

Recently, the alcohol-preferring AA rat was found to have higher hypothalamic and pituitary levels of POMC mRNA and higher anterior pituitary β-END levels than the alcohol-avoiding ANA rat (Gianoulakis, DeWaele, & Kiianmaa, 1992). Similar findings have been reported with the alcohol-preferring P and alcohol-nonpreferring NP rat lines. Intragastric administration of 2.5 g/kg ethanol induced an increase in POMC mRNA levels in the pituitary of P rats but not NP rats (Froehlich, Wand, Ochs, & Li, 1991).

Though not as frequently studied, there also appear to be genetically determined differences in the enkephalin system of rodents. Blum and colleagues have demonstrated a negative correlation between brain met-ENK levels and ethanol consumption in both the B6 and D2 strains (Blum, Elston, DeLallo, Briggs, & Wallace, 1983). While tissue levels of met-ENK in various brain regions did not differ between B6 and D2 mice, B6 mice exhibited higher levels of striatal enkephalinase activity relative to D2 mice (George, Roldan, Lui, & Naranjo, 1991). Additionally, pre-proenkepahlin mRNA levels in the posterior striatum of P, but not NP, rats were increased by intragastric administration of 2.5 g/kg ethanol (Li, Li, & Froehlich, 1992).

Finally, it has also been suggested that there are opioid system differences in human populations at high vs low risk for alcoholism.

Gianoulakis and colleagues found that basal levels of ir-β-END-like peptides were lower in individuals with a three generation history of alcoholism (defined as high risk) than in individuals without a family history of alcoholism (defined as low risk) (Gianoulakis, Béliveau, Angelogianni, Meaney, Thavundayil, Tawar, & Dumas, 1989). In the same subjects, ethanol (0.5 g/kg) increased plasma β-END-like peptides in high risk but not low risk individuals.

In general, it is agreed that acute or chronic ethanol can alter the activity of the endogenous opioid system (for reviews see Froehlich & Li, 1993; Gianoulakis, 1989; Gianoulakis, 1993). The direction and magnitude of ethanol's effects are, however, still controversial. Inconsistencies among various studies exist, perhaps, because of the dose or route of administration of ethanol used, the duration of ethanol exposure and/or genetic factors (Gianoulakis & DeWaele, 1994).

The Opioid Deficit, Surfeit and Response Hypotheses

The assumption that ethanol can stimulate endogenous opioid activity is common to three general theories of alcoholism. The first theory, the "opioid deficit hypothesis," contends that a deficiency in endogenous opioid activity predisposes individuals to drink alcohol in order to stimulate the system and increase endogenous opioid levels (e.g., Blum, Briggs, Elston, Hirst, Hamilton, & Vereby, 1980; Erickson, 1990; Trachtenberg & Blum, 1987).

This theory suggests that alcohol is consumed to compensate for low basal levels of endogenous opioids.

The second general theory is known as the "opioid surfeit hypothesis" which suggests that an excess or surfeit in endogenous opioid activity leads to alcohol craving and increased alcohol drinking (Beaman, Hunter, Dunn, & Reid, 1984; Hubbell & Reid, 1990; Hunter, Beaman, Dunn, & Reid, 1984; Reid & Hunter, 1984). Drinking is then further reinforced by alcohol-induced increases in opioidergic activity. The opioid surfeit hypothesis was developed by Reid and colleagues in response to data showing that small doses of morphine increase, rather than decrease, alcohol consumption (Beaman et al., 1984; Hubbell, Czirr, Hunter, Beaman, LeCann, & Reid, 1986; Hubbell & Reid, 1990; Reid & Hunter, 1984). In a recent paper, these authors present data in support of this theory (Reid, Delconte, Nichols, Bilsky, & Hubbell, 1991).

A third hypothesis, the "opioid response hypothesis," has been proposed by Gianoulakis (1993). According to this hypothesis, basal levels of opioidergic activity are not critical for excessive consumption of ethanol. Rather, it is a significant increase in opioid activity produced by ingestion of ethanol that may mediate ethanol's rewarding effects, thereby reinforcing drinking behavior and increasing alcohol consumption

Although these hypotheses differ in regard to the importance of basal opioid activity, the ability of ethanol to stimulate release of endogenous opioids is essential to each. Assuming that endogenous opioids then bind to

brain opioid receptors, blockade of these receptors with opioid receptor antagonists would prevent binding and the resulting neurochemical cascade which presumably contributes to ethanol's rewarding effects. Thus, all three theories suggest that opioid antagonists should be effective in reducing ethanol reward and therefore ethanol consumption.

Opioid Antagonists and Ethanol

Physiology and Behavior

Many studies have indicated an interaction between opioid antagonists and ethanol. Naloxone has been shown to prevent ethanol-induced depletion of Ca^{++} in mice (Ross, Medina, & Cardenas, 1974). Recently, Benjamin, Grant, and Pohorecky (1993) demonstrated that naltrexone (a nonselective opioid antagonist very similar to naloxone but with a longer half-life) dose-dependently reversed ethanol-induced dopamine release in rat nucleus accumbens.

Several studies have found that opioid antagonists attenuate withdrawal symptoms in animals made physically dependent on ethanol. Naloxone or naltrexone (2 mg/kg) given 10 min prior to twice daily intragastric administration of 40% ethanol (5-10 g/kg) decreased the percentage of rats exhibiting audiogenic seizures after seven days of ethanol treatment (Kotlinska & Langwinski, 1987). Similar findings were reported by Blum and colleagues who found that naloxone, given before and during

ethanol intoxication, reduced ethanol withdrawal convulsions in mice (Blum, Futterman, Wallace, & Schwertner, 1977).

Opiate antagonists have also been shown to reverse or reduce several of ethanol's behavioral effects in mice and rats. In mice, ethanol-induced sleep time (4 g/kg ethanol) was reduced by naltrexone (8 mg/kg, Tamborska, Kotlinska, & Langwinski, 1984; 10 mg/kg, Harris & Erickson, 1979). Another study found genetic differences in the ability of naltrexone to decrease sleep time in inbred mouse strains (Kiianmaa, Hoffman, & Tabakoff, 1983). Loss of righting reflex induced by 3.5 g/kg ethanol was reversed by 1 mg/kg naltrexone in BALB/c mice, while 8 mg/kg naltrexone was required to reverse this effect in B6 mice. In contrast, sleep time in D2 mice was not affected by 8 mg/kg naltrexone, the highest dose tested. In both BALB/c and D2 mice, ethanol caused an increase in locomotor activity that was decreased by 0.1 mg/kg naltrexone. However, in Swiss-Webster mice, 10 mg/kg naltrexone or naloxone did not affect ethanol-stimulated activity (2 g/kg) unless mice were fasted for 16 to 18 h (Harris & Erickson, 1979).

In one study, hypothermia induced by 3.5 or 4 g/kg ethanol was reduced by 8 mg/kg of naltrexone or naloxone, respectively (Tamborska et al., 1984). In contrast, Harris and Erickson (1979) reported no effect of 1 or 10 mg/kg naltrexone on ethanol-induced hypothermia (2.5 or 4 g/kg). Both studies used Swiss-Webster mice, however, ambient temperature in the Tamborska et al. study was lower than that reported by Harris and Erickson

(20° vs 25°C, respectively). In addition, Tamborska et al. calculated the loss of body temperature using the mean of six measurements (every 30 min starting 30 min after ethanol) minus initial temperature, while Harris and Erickson assessed body temperature only at 5 min prior to and 30 min after ethanol administration. Neither naloxone nor naltrexone antagonized ethanol-induced hypothermia in rats, although the nonselective opiate antagonist diprenorphine (8 μg/kg) was effective against hypothermia (Kotlinska & Langwinski, 1990).

Ethanol generally decreases locomotor activity in rats (e.g., Cunningham, Niehus, & Noble, 1993), although there is at least one report of ethanol inducing an increase in activity (Prunell, Boada, Feria, & Benitez, 1987). Prunell et al. (1987) used doses of 2 and 4 g/kg ethanol (p.o.) to induce stimulation and depression, respectively, in Sprague-Dawley rats. Naloxone (0.5 and 2 mg/kg) blocked the locomotor activating effects of ethanol, while having little effect on the locomotor depressant effects.

It has been suggested that opiates may have a critical modulatory effect on the actions of ethanol (e.g., Castellano & Pavone, 1984). In a passive-avoidance paradigm, posttrial administration of 1 or 2 g/kg ethanol to mice dose-dependently decreased step-through latencies when tested the following day (Castellano & Pavone, 1984). Posttrial treatment with morphine or 60-min immobilization stress also decreased step-through latencies in this paradigm. All of these effects were blocked by 1 mg/kg naloxone. Prunell et

al. (1987) found that 2 g/kg ethanol reduced avoidance latency in a one-way shuttle-box test and that this reduction was prevented by pretreatment with 0.5 or 2 mg/kg naloxone.

Early studies in humans suggested that naloxone could reverse the psychomotor effects of low doses of ethanol (Jeffcoate, Herbert, Cullen, Hastings, & Walder, 1979) and ethanol-induced coma (Jeffreys, Flanagan, & Volans, 1980).

The endogenous opioid system has also been suggested to play a role in ethanol's stimulus properties. For example, naloxone (1 and 10 mg/kg) has been shown to interfere with the discriminative stimulus produced by the initial excitatory effects of 1 g/kg of ethanol in rats, although it had no effect on the later sedative phase (Shippenberg & Altshuler, 1985). Additionally, opiate antagonists have been shown to modulate ethanol-induced conditioned taste aversion (Broadbent, Linder, & Cunningham, in press; Miceli, Marfaing-Jallat, & LeMagnen, 1979; Ng Cheong Ton & Amit, 1984), a paradigm considered to assess the negative motivational properties of a drug.

Consumption

Further evidence for a role of the opioid system in ethanol's hedonic effects comes from studies in rodents and monkeys where administration of opiate antagonists has been shown to decrease ethanol drinking and operant self-administration of ethanol (e.g., Froehlich, Harts, Lumeng, & Li, 1987;

Samson & Doyle, 1985). However, opiate anatagonists have been shown to decrease ingestive behavior in general, a point that will be considered later.

Early studies on ethanol self-administration with the opiate antagonists naloxone and naltrexone resulted in mixed findings. For example, Altshuler, Phillips and Feinhandler (1980) found that 1, 3 or 5 mg/kg naltrexone initially increased (days 1-5) then decreased (days 6-15) i.v. self-administration of ethanol in rhesus monkeys. In contrast, Blum, Wallace, Eubanks, and Schwertner (1975) found no effect of naloxone. However, as pointed out by Hubbell and Reid (1990), since most early studies measured intake over periods longer than the pharmacologial effects of the antagonists, inconsistencies are not surprising. Recent studies have generally found that the nonselective opiate antagonists decrease ethanol intake.

Operant self-administration

Several studies have been conducted to determine the effects of opioid antagonists on operant responding for ethanol. In food-deprived rats, Samson and Doyle (1985) found that 20 mg/kg naloxone (but not 5 or 10 mg/kg) injected 30 min prior to a 30-min session decreased responding for 5% ethanol without systematically affecting water responding in a concurrent access procedure. In contrast, a recent concurrent access study using 10% ethanol vs. 10% ethanol/10% sucrose as reinforcers, also in food-deprived rats, found that only low doses of naloxone (0.125, 0.25 and 1.0 mg/kg) administered 10 min prior to a 45-min session selectively attenuated

responding for the ethanol mixture (Petry, 1995). All other doses decreased both sucrose and ethanol/sucrose responding.

Alcohol-preferring AA rats were trained to lever-press for 10% ethanol during daily 60 min access periods (indicated by a stimulus light) with food and water available at all times (Hyytiä & Sinclair, 1993). Naloxone (0.05 - 2.5 mg/kg) had no effect on initial response rates, but dose-dependently decreased responding later in the session. Effects of naloxone on water responding could not be assessed in this procedure. In another study with non-deprived rats, naloxone (0.1, 0.3 and 1.0 mg/kg) reduced responding for 10% ethanol and sweetened ethanol (10% ethanol/2% sucrose) solutions in a dose-dependent manner (Schwarz-Stevens, Files, & Samson, 1992). However, when water was available concurrently with 10% ethanol or 5% sucrose, naloxone did not affect water responding although it decreased responding for both ethanol and sucrose.

Drinking

In studies with ethanol-naive rats, Pulvirenti and Kastin (1988) found that naloxone (1, 2 and 4 mg/kg) decreased intake of a 3% ethanol solution when it was presented concurrently with water for 2 h each day. The effects of naloxone were most evident in "high-preferring" rats (those where ethanol consumption was greater than 60% of total fluid intake). Naloxone had no effect on water intake when all rats were considered together, but did decrease water intake in rats determined to be "low-preferring" (ethanol was less than

30% of total fluid intake). Similar effects of naloxone on ethanol consumption were seen in rats selectively bred for oral ethanol preference (High Alcohol Drinking or HAD line). Naloxone (0.05-18 mg/kg), given 30 min prior to a two-bottle choice 2-h access period, dose-dependently decreased intake of 10% ethanol without affecting water intake. However, when water was presented as the only fluid source, naloxone also attenuated water consumption (Froehlich, Harts, Lumeng, & Li, 1990).

In another study, rats were made dependent on ethanol by daily intragastric administration (10 g/kg/day) and then given 8 h access to 10% ethanol or water on alternating days for 6 days (Marfaing-Jallat, Miceli, & LeMagnen, 1983). During the next 2 days, rats were injected with 1 mg/kg naloxone 30 min prior to fluid access and again 4 h into the 8 h access period. Naloxone significantly reduced ethanol consumption without affecting water intake, suggesting that in these dependent rats the opioid system was involved in maintaining high ethanol consumption. Similar findings were reported by Kornet, Goosen and Van Ree (1991) who demonstrated that ethanol intake in chronically drinking monkeys was decreased by naltrexone (0.5 - 1.5 mg/kg). During the first two h after injection of naltrexone, both ethanol and water intake were suppressed; however, ethanol consumption remained suppressed over the next 15 h while water intake was increased.

In addition to the studies described above, naloxone (2.5, 4 or 10 mg/kg) has been shown to decrease consumption of a sweetened ethanol solution

(Hubbell, Czirr, Hunter, Beaman, LeCann, & Reid, 1986; Reid et al., 1991; Reid & Hunter, 1984). Finally, 5 mg/kg naloxone injected prior to forced exposure to a weak ethanol solution selectively attenuated ethanol consumption in two-bottle choice tests on three subsequent days (Sandi, Borrell, & Guaza, 1988a).

Human studies

Recent clinical studies based on these and other findings indicate that daily oral administration of 50 mg naltrexone to human alcoholics decreases craving for alcohol as well as the likelihood of relapse (O'Malley, Jaffe, Chang, Schottenfeld, Meyer, & Rounsaville, 1992; Volpicelli, Alterman, Hayashida, & O'Brien, 1992; Volpicelli, Clay, Watson, & O'Brien, 1995). In addition, a recent clinical study demonstrated that naltrexone attenuated the positive reinforcing effects of ethanol, altered some of its subjective effects, and augmented its sedative effects (Swift, Whelihan, Kuznetsov, Buongiorno, & Hsuing, 1994).

Opioid Antagonists and Ingestive Behavior

The preceding sections presented data suggesting that opioid antagonists such as naloxone and naltrexone are capable of decreasing ethanol intake in several species. However, in many studies, the effects of naloxone have not always been specific for ethanol consumption, decreasing intake of water and/or sweetened fluids as well (e.g., Schwarz-Stevens et al., 1992;

Weiss, Mitchiner, Bloom, & Koob, 1990). In fact, the endogenous opioid system is well known to play a large role in ingestive behavior, such that opioid antagonists tend to decrease consumption (both feeding and drinking) while agonists and the opioid peptides generally increase these behaviors (Cooper & Kirkham, 1990; Levine, Morley, Gosnell, Billington, & Bartness, 1985).

Most studies have assessed ethanol's rewarding effects using procedures involving ingestive behaviors. Given the detrimental effects of opioid antagonists on drinking in general, reduction of ethanol consumption by agents such as naloxone may reflect a nonspecific decrease in consummatory behavior rather than a specific attenuation of ethanol's rewarding properties. One way to circumvent this problem is to use a procedure to measure ethanol reward that does not involve drinking, such as the place conditioning procedure.

Naloxone Effects on Ethanol-Induced Place Preference

A recent series of studies in this laboratory was conducted to examine the effect of naloxone administration on both the acquisition and expression of ethanol-induced conditioned place preference (Cunningham et al., 1995). In the acquisition studies, D2 mice underwent a differential Pavlovian conditioning procedure whereby drug administration was paired four times with placement on one distinct floor texture and saline administration was

paired four times with another distinct texture. Two injections, 15 min apart, were given on all trials; the first injection consisted of naloxone (1.5 or 10 mg/kg) or saline and the second injection consisted of ethanol (2 g/kg) or saline. After eight trials, mice were given a saline injection and presented with a choice between the ethanol and saline paired floors. In all ethanol-treated groups, mice showed a preference for the floor cue that had been paired with ethanol. Pretreatment with naloxone had no effect on the magnitude of the ethanol preference, although naloxone without ethanol did produce a conditioned place aversion. These findings indicate that blockade of opiate receptors during conditioning does not prevent acquisition of ethanol-induced conditioned place preference and suggest that the initial rewarding effects of ethanol are not mediated via the opioid system.

The lack of effect of naloxone pretreatment on the acquisition of ethanol place preference is somewhat surprising in light of previous drinking and operant studies which show a significant effect of opiate antagonists on ethanol reward (e.g., Froehlich et al., 1990; Froehlich et al., 1991; Hyttiä & Sinclair, 1993). However, as pointed out by Cunningham et al. (1995), data from clinical trials suggest that opiate antagonists may act on the craving for ethanol in the absence of the drug itself. As stated earlier, one advantage of the place conditioning paradigm is that it can be used to assess the effects of an antagonist treatment not only on the direct effects of ethanol (i.e., during conditioning trials), but also on the craving for or anticipation of ethanol

during the preference test when ethanol is absent. In fact, Hand, Stinus, and LeMoal (1989) suggested that the mechanisms responsible for acquisition vs expression of place preference are different. Accordingly, Cunningham et al. (1995) examined the effect of opiate receptor blockade during the expression of ethanol preference.

D2 mice underwent a place conditioning procedure that paired one distinctive floor type with ethanol and another type with saline. No antagonist treatments were given during conditioning. However, 15 min prior to a saline injection and placement in the conditioning apparatus for the 60 min choice test, mice were injected with saline or naloxone (0.15, 1.5, 3 or 10 mg/kg, i.p.). At all doses except 0.15 mg/kg, naloxone attenuated the expression of place preference in a dose- and time-dependent manner. Specifically, mice receiving saline or 0.15 mg/kg naloxone showed a place preference that developed in the first few minutes and was fairly constant throughout the rest of the 60 min session (data from saline-treated animals are plotted in Figure 1). However, mice pretreated with 1.5, 3 or 10 mg/kg showed place preference during the first 10-20 min of the session, but spent equal amounts of time on the two floor types for the remainder of the session, i.e., showed no preference for the ethanol-paired cue (Figures 1 and 2). To facilitate dose-response comparisons, the 60-min test session was divided into three successive time intervals; Minutes 1-10, Minutes 11-30 and Minutes 31-60 (Figure 2).

Figure 1. Mean (±SEM) seconds per minute spent on the grid floor during each minute of the preference test for animals pretreated with saline (upper graph) and 1.5 mg/kg naloxone (lower graph) 15 min prior to testing. Grid+ and Grid- refer to conditioning subgroups within each treatment group. Grid+ mice had the grid floor paired with ethanol and the hole floor paired with saline during conditioning. Grid- animals received the opposite treatment (grid floor paired with saline and hole floor with ethanol). Data from Cunningham, Dickinson, and Okorn, 1995.

Figure 1

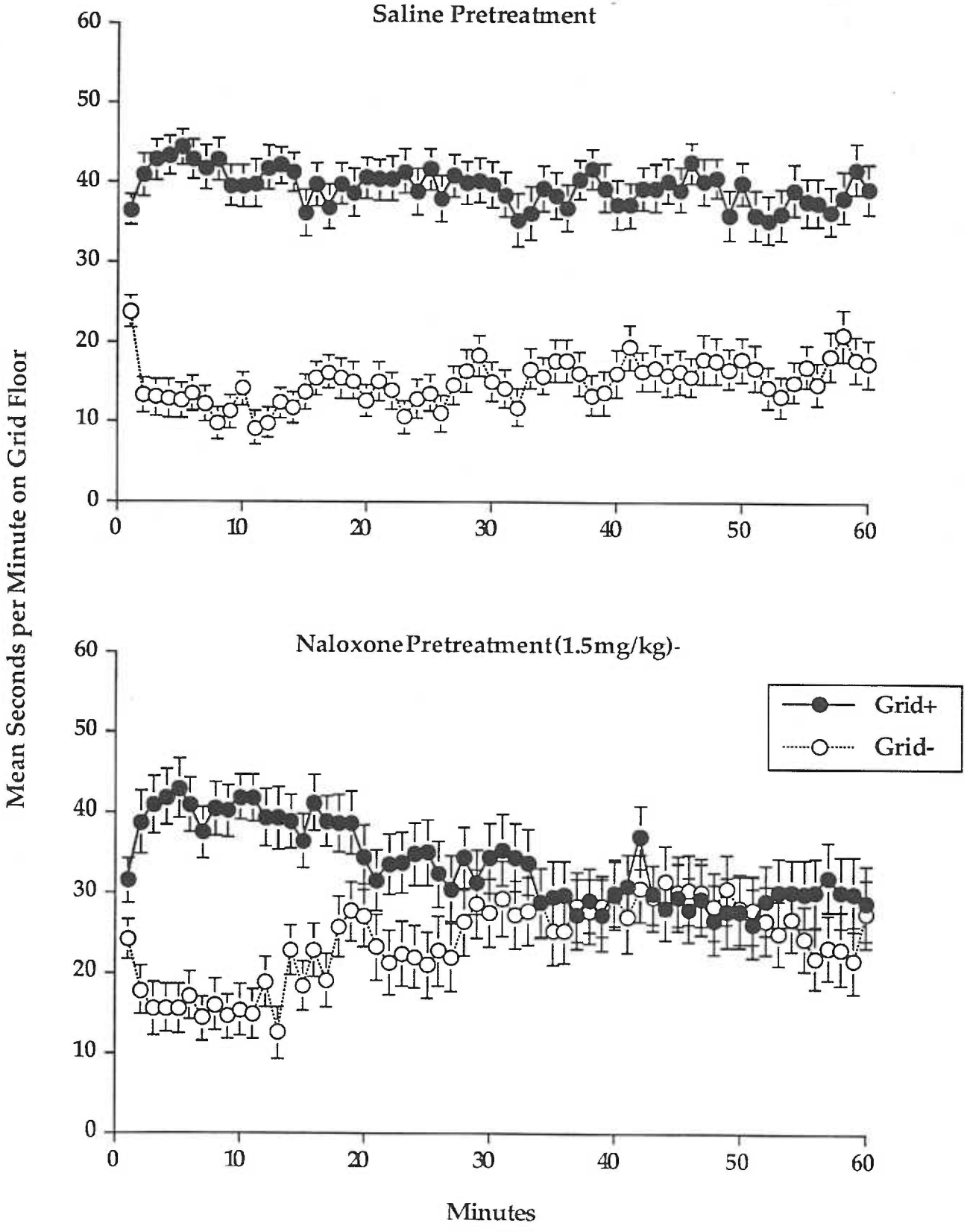
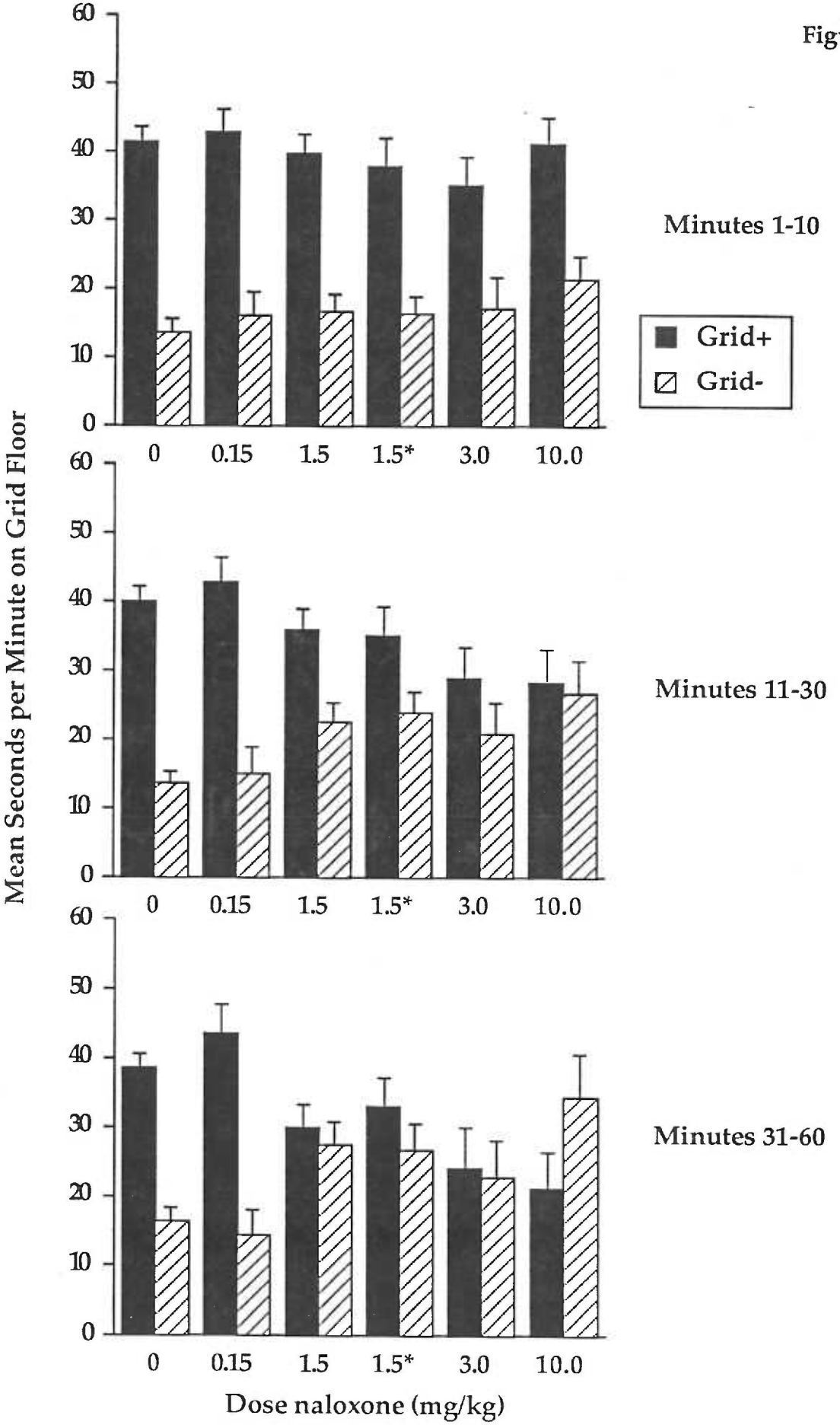

Saline Pretreatment
Naloxone Pretreatment (1.5mg/kg)-
Grid+
Grid-
Mean Seconds per Minute on Grid Floor
Minutes
0
10
20
30
40
50
60

Figure 2. Mean (+SEM) seconds per minute spent on the grid floor during Minutes 1-10, 11-30 and 31-60 of the preference test for the naloxone dose groups. All groups received naloxone 15 min prior to testing, except for Group 1.5*, which received naloxone 45 min before the test session. Grid+ and Grid- conditioning groups are as in Figure 1. Data from Cunningham, Dickinson, and Okorn, 1995.

Figure 2


Mean Seconds per Minute on Grid Floor
60
50
40
30
20
10
0
0
0.15
1.5
1.5*
3.0
10.0
Minutes 1-10
Grid+
Grid-
Minutes 11-30
Minutes 31-60
Dose naloxone (mg/kg)

To determine whether naloxone's effect on preference was permanent, mice previously receiving 0, 3 or 10 mg/kg naloxone were injected with saline prior to a second preference test. Strong place preference was seen in the saline control group while both naloxone groups showed much weaker preference even though no naloxone was present. Overall, these data indicate that maintained expression of ethanol-induced conditioned place preference requires intact opiate pathways. Cunningham et al. (1995) suggested that naloxone served to facilitate the extinction of the conditioned place preference.

The temporal pattern of ethanol preference with naloxone pretreatment, i.e., early preference that disappears after 10-20 min, suggested that pharmacokinetics of naloxone may be important in the effect. To examine this possibility, another experiment was conducted in which 1.5 mg/kg naloxone was administered 45 min prior to the preference test (Group 1.5* in Figure 2). Interestingly, naloxone attenuated the expression of ethanol place preference with the same temporal characteristics as when administered 15 min prior to testing, indicating that the temporal pattern seen in earlier work was not due to pharmacokinetic factors.

Since naloxone is capable of supporting a conditioned place aversion (Cunningham et al., 1995; Mucha & Walker, 1984), it is possible that some aversive properties of naloxone were responsible for attenuating expression of ethanol preference. To examine this possibility, a control experiment was

conducted in which mice underwent a standard ethanol conditioning procedure but were injected with lithium chloride (0, 1.5 or 3 mEq/kg) 15 min prior to the preference test. Lithium chloride produces general malaise and supports a conditioned place aversion (Shippenberg, Millan, Mucha, & Herz, 1988). All groups showed a significant preference for the floor type paired with ethanol, regardless of lithium treatment, indicating that simply feeling unwell during the test did not disrupt expression of preference.

In sum, the experiments by Cunningham and colleagues demonstrated that naloxone attenuates the expression of ethanol-induced conditioned place preference without affecting its acquisition. In addition, it was determined that this effect was not a consequence of pharmacokinetics or a nonspecific aversive property of naloxone. Overall, the findings from this series of experiments were interpreted as evidence of facilitation of extinction of ethanol-induced conditioned place preference by naloxone (Cunningham et al., 1995). This conclusion is in general agreement with the idea that opioid antagonists serve to extinguish alcohol-reinforced drinking behavior (Sinclair, 1990). However, the specific opioid receptor system mediating this effect remains to be elucidated.

RATIONALE

As a non-selective opioid antagonist, naloxone can bind to the μ, δ, or κ opioid receptor (Corbett, Paterson, & Kosterlitz, 1993; Froehlich, Zweifel,

Harts, Lumeng, & Li, 1991). Therefore, antagonists selective for these opioid receptor types must be used to determine which type (if any) mediates the naloxone effect seen in earlier place conditioning studies.

The current studies were intended to assess the role of the individual opioid receptor types in the expression of ethanol place preference. In addition, the current set of studies further characterized the effect of naloxone by extending the dose-response curve and determining stereospecificity. Finally, these studies examined the importance of certain procedural variables thought to influence the magnitude of naloxone's effect.

HYPOTHESIS

For the following reasons, it was hypothesized that the observed effects of naloxone would be mimicked by selective blockade of the δ receptor. First, as reported in Cunningham et al. (1995), the doses of naloxone that effectively attenuated maintenance of expression (1.5 - 10 mg/kg) bind both μ and δ receptors. Second, Hiller et al. (1981) showed that ethanol affected binding of agonists to δ receptors, but not μ receptors. Finally, Froehlich and colleagues reported that a selective δ receptor antagonist was as effective as naloxone in suppressing ethanol consumption in selectively-bred high alcohol drinking rats (Froehlich, Zweifel, Harts, Lumeng, & Li, 1991). These authors also

demonstrated that potentiation of the action of endogenous enkephalins with an enkephalinase inhibitor increased alcohol intake.

EXPERIMENTS 1-3: SELECTIVE OPIOID ANTAGONISTS AND EXPRESSION OF ETHANOL PLACE PREFERENCE

While many studies have examined the effects of nonselective opioid antagonists such as naloxone and naltrexone on the physiological effects and consumption of ethanol, there are relatively few published studies on the selective blockade of μ, δ or κ receptors and ethanol's effects. Experiments 1-3 were intended to determine if one particular opioid receptor type mediates the maintenance of expression of ethanol-induced conditioned place preference.

Experiment 1: Effects of Blockade of δ Receptors

Introduction & Rationale

As stated earlier, naloxone is a nonselective antagonist whose affinity for the three receptor types varies by less than 10-fold. Naloxone's affinity for the δ receptor is only 2-3 fold lower than for the μ receptor (Goldstein & Naidu, 1989). Thus, the δ opioid receptor may mediate the effects of naloxone on the expression of ethanol place preference.

Binding levels and mRNA expression of the δ receptor are high in the frontal cortex, caudate putamen, nucleus accumbens, and amygdala (Mansour et al., 1995). Like μ receptor agonists, δ agonists are generally reinforcing. Conditioned place preferences are produced by i.c.v. administration of the δ agonists [D-Ala^2-D-Leu^5]-ENK (DADLE) (Katz & Gormezano, 1979) and [D-Pen^2, D-Pen^5]-ENK (DPDPE) (Bals-Kubik et al., 1990). Bals-Kubik et al. (1990) also reported that the selective δ receptor antagonist ICI 174864 attenuated the conditioned rewarding effects of DPDPE while the μ antagonist CTOP did not, indicating that δ receptors were indeed responsible for DPDPE reinforcement. Interestingly, Bals-Kubik et al. also found that ICI 174864 also blocked β-END-induced conditioned place preference (as did CTOP), demonstrating that both μ and δ receptors mediate the reinforcing effects of β-END. In contrast to the μ antagonists, antagonism of δ receptors with ICI 174864 does not result in a place aversion (Bals-Kubik, Herz & Shippenberg, 1989).

The development of highly selective agonists and antagonists for the δ receptor was instrumental in demonstrating a role of the δ receptors in various behaviors. Though controversial for some time, it is now generally accepted that δ receptors are involved in supraspinal antinociception (Heyman, Vaught, Raffa, & Porreca, 1988). For example, in mice treated with

the selective μ receptor antagonist β-funaltrexamine (β-FNA), but not in vehicle-treated control mice, morphine analgesia was antagonized by ICI 174864, suggesting that when μ receptors were blocked, δ receptors mediated the antinociceptive response (Takemori & Portoghese, 1987).

Three selective δ antagonists are currently available: ICI 154129, ICI 174864, and naltrindole (NTI). The peptide ICI 154129 is highly selective for δ receptors but its affinity for these receptors is low (Shaw, Miller, Turnbull, Gormley, & Morley, 1982). ICI 174864 is a stable peptide analog of ICI 154129 with high δ-selectivity and affinity (Cotton, Giles, Miller, Shaw, & Timms, 1984). However, because these compounds are peptides, their use in vivo is limited to i.c.v. or i.t. administration. In addition, ICI 174864 has been found to exhibit agonist activity at δ receptors (Cohen, Shuman, Osborne, & Gesellchen, 1986).

NTI is a highly selective and potent nonpeptide δ receptor antagonist synthesized by Portoghese and colleagues (Portoghese, Sultana, & Takemori, 1988). Unlike the peptide antagonists, NTI readily crosses the blood-brain barrier and is, therefore, centrally active after peripheral administration (Portoghese et al., 1988). Antinociception produced by i.c.v. DPDPE, but not morphine, is dose-dependently attenuated by s.c. administration of NTI (Ayres, Davis, & Burks, 1990).

Recent studies suggest that δ receptors may play a role in the effects of drugs of abuse, including cocaine, morphine and ethanol. Pretreatment with NTI has been shown to block cocaine-induced conditioned place preference (Menkens, Bilsky, Wild, Portoghese, Reid, & Porreca, 1992; Suzuki, Mori, Tsuji, Misawa, & Nagase, 1994; but see de Vries, Babovic-Vuksanovic, Elmer, & Shippenberg, 1995). Morphine-induced hyperlocomotion and release of mesolimbic DA, thought to mediate reward (Wise & Rompre, 1989), are attenuated by NTI.

That the δ receptor is important in the effects of ethanol is supported by the findings of Hiller et al. (1981) showing that ethanol inhibited binding to δ receptors. ICI 174864 (10 μM) decreases ethanol-induced DA release from striatal slice preparations (Widdowson & Holman, 1992). In freely moving rats treated with ethanol (1 g/kg), DA release in the nucleus accumbens is prevented by infusion of 1.0 μM NTI (Acquas, Meloni, & Di Chiara, 1993). To the extent that release of mesolimbic DA is important for ethanol reward, these findings suggest that blockade of δ receptors may attenuate the reinforcing effects of ethanol.

Froehlich and colleagues have investigated the role of δ receptors in ethanol consumption. In rats of the HAD line given 30 min of limited access to 10% ethanol and water, ICI 174864 (1 and 3 mg/kg, i.p.) 15 min prior to fluid access decreased ethanol intake without affecting water consumption

(Froehlich, Zweifel, Harts, Lumeng, & Li, 1991). In a separate experiment with a 60-min access period, injection of the enkephalinase inhibitor thiorphan (30 mg/kg) 5 min prior to fluid access increased ethanol intake during the first 30 min, suggesting that increased duration of action of enkephalins increases ethanol intake (Froehlich et al., 1991). Similar effects of ICI 174864 were seen in non-deprived P rats given one h access to 10% ethanol and water every four h (Krishnan-Sarin, Jing, Kurtz, Zweifel, Portoghese, Li, & Froehlich, 1995). The ICI compound (3, 5 and 8 mg/kg given i.p. 15 min before the first access period) did not affect water consumption but decreased ethanol intake during the first access period of the day. Using the same procedures, Krishnan-Sarin et al. (1995) also found that i.p. administration of 10, 15 or 20 mg/kg NTI (but not 5 mg/kg) suppressed ethanol intake during the first two access periods but did not decrease water intake. Finally, these investigators demonstrated that NTI (15 mg/kg) equally suppressed consumption of both a saccharin/ethanol solution and a saccharin solution, suggesting that the effects of NTI on intake are not selective for ethanol. This conclusion is supported by the finding that i.c.v. NTI (1-20 μg) dose-dependently decreased saccharin intake over a 1-h access period, but, interestingly, did not affect consumption of a maltose-dextrin solution (Beczkowska, Koch, Bostock, Leibowitz, & Bodnar, 1993). Since intake of neither water nor complex carbohydrate solution was decreased, NTI does not appear to affect consummatory behavior in general, but instead these findings

suggest that both ethanol and sweets have reinforcing effects mediated by an opioid pathway.

As mentioned earlier, the place preference procedure can be used to assess the rewarding effects of drugs without the possible confound of drug effects on ingestive behaviors. Experiment 1 was conducted to assess the role of δ opioid receptors in the expression of ethanol-induced conditioned place preference. NTI was chosen as the antagonist because it is highly selective for the δ receptor and, in contrast to the ICI compounds, it crosses the blood-brain barrier readily as it is not a peptide. If expression of conditioned ethanol reward is mediated by δ receptors, blockade of these receptors should attenuate preference. Furthermore, if naloxone's effects are mediated by the δ receptor, the results with NTI should mimic those seen with naloxone.

Methods

As discussed earlier, procedures in our laboratory have conclusively established that ethanol can produce a conditioned place preference in some inbred strains and selectively bred lines of mice (Cunningham et al., 1991; Cunningham et al., 1995; Cunningham et al., 1992; Cunningham et al., 1993; Risinger et al., 1992). To date, the D2 inbred strain has been used most successfully and was the strain used in previous naloxone studies. Therefore, this strain was also chosen for the current studies.

In pilot studies, the grid and hole floors described in detail below were found to be, on average, equally preferred in baseline, non-drug tests (reported in Cunningham et al., 1992). In other words, animals spent about 50% of their time on each floor type. Use of two equally preferred stimuli makes our conditioning procedure an 'unbiased' procedure. Some investigators use a 'biased' place conditioning procedure whereby the putatively rewarding drug is paired with the initially unpreferred cue. An increase from baseline in time spent on the unpreferred side is then interpreted as a conditioned place preference. However, as pointed out in a review by Swerdlow, Gilbert, and Koob (1989), this outcome is technically a decrease in aversion, which may not be the same as a true preference. Another advantage to using an unbiased procedure is that both preferences and aversions may be assessed, i.e., it is less subject to floor or ceiling effects. This is especially important when the direction of the conditioned effect is unknown prior to experimentation or when parametric aspects of the procedure are being examined.

In a dose-response study with D2 mice (Cunningham et al., 1992), 3 and 4 g/kg ethanol produced a significant conditioned place preference, a nonsignificant trend toward preference was found with 2 g/kg and no conditioning was seen with 1 g/kg. In this study, duration of the conditioning trial was 30 min. Cunningham and Prather (1992) examined the role of trial duration in this paradigm, using trials of 5, 15 or 30 min and a

dose of 2 g/kg. Their findings indicated that conditioned place preference was inversely related to trial duration, i.e., the 5-min group spent more time (83%) on the ethanol-paired floor than did the 30-min group (66%). Thus, the combination of 2 g/kg ethanol and a 5 min conditioning trial results in a robust conditioned preference for ethanol-paired cues.

For the reasons just discussed and to facilitate comparison with previous studies by Cunningham et al. (1995), the same mouse strain (DBA/2J), ethanol dose (2 g/kg) and basic place conditioning paradigm (grid vs hole floor, 5 min conditioning trial) used previously were used in the current studies.

Subjects

In all experiments, male D2 mice (n=95 in Experiment 1) were obtained from The Jackson Laboratory (Bar Harbor, ME) at 6 weeks of age and allowed to acclimate for 10-14 days before experimentation. Mice were approximately 8 weeks old at the beginning of each experiment and were tested at approximately 10 weeks of age. Mice were housed in groups of four in standard polycarbonate cages (27.9 x 9.5 x 12.7 cm) with corn cob bedding in a Thoren rack. The colony rooms were maintained at 22 $\pm$ 1° C and all experiments occurred during the light phase of a 12-h light-dark cycle (lights on at 0700). Food and water were available ad lib in the home cage.

In Experiments 1-4A mice were transported from the colony room to the experimental room approximately one h prior to experimentation and

remained in the room for at least 30 min after experimentation. In all other experiments (4B, 5, 6 and 7), the mice were housed in the experimental room and transport was not required.

Apparatus

The place conditioning apparatus consisted of 12 identical acrylic and aluminum boxes (30 x 15 x 15 cm) with six sets of infrared light sources and photodetectors mounted opposite each other at 5-cm intervals on the sides of each box, 2.2 cm above the floor. Occlusion of the infrared beams was used to measure locomotor activity and to determine the animal's position (left vs right) in the box. Total activity counts and time spent on each side of the box were recorded each minute by an Apple II computer (10 msec resolution). The floor of each box was composed of interchangeable halves of one of two distinct textures, 'grid' and 'hole'. Grid floors were made of 3.2-mm (Experiments 1-4A) or 2.3-mm (Experiments 4B-7) stainless-steel rods mounted 6.4-mm apart in acrylic rails, and hole floors were perforated 16-gauge stainless steel with 6.4-mm round holes on 9.5-mm staggered centers mounted on acrylic rails. The floors and the inside of the boxes were wiped with a damp sponge and the litter paper beneath the floors changed after each animal.

Each conditioning chamber was enclosed in a sound and light attenuating chamber equipped with a ventilating fan (Coulbourn Model E10-20).

Drugs

NTI, a selective δ opioid receptor antagonist, was purchased from Research Biochemicals Inc., Natick, MA. NTI was dissolved in physiological saline and administered i.p. in a volume of 10 ml/kg. Doses of NTI used (1.3, 3, 10 and 20 mg/kg) fall in a dose range shown to be effective in blocking DPDPE-induced analgesia (Ayres et al., 1992) and attenuating consumption of ethanol (Krishnan-Sarin et al., 1995). A 20-min pretreatment time was chosen based on findings that DPDPE analgesia is blocked 15-30 min after NTI administration (Ayres et al., 1992; Drower, Stapelfeld, Rafferty, De Costa, Rice, & Hammond, 1991). In addition, a pretreatment time of 20 min was suggested by Jackson, Ripley, and Nutt (1989) to be optimal in assessing attenuation of swim stress-induced antinociception, a model for endogenous δ receptor activation.

In all experiments, commercial grade ethanol (95%) was obtained from university stores, mixed in physiological saline (20% v/v) and injected i.p. in an injection volume of 12.5 ml/kg.

Procedure

All experiments included three phases: one habituation session, eight conditioning sessions, and at least one test session. During the habituation session, animals received a saline injection before being placed in the conditioning box on a smooth, paper covered floor. The habituation session

was intended to reduce the stress and novelty of the handling and injection procedure.

In all experiments, mice were randomly assigned to one of several treatment groups. Within each of these treatment groups (three in Experiment 1), mice were randomly assigned to one of two conditioning subgroups (Grid+ or Grid-) and exposed to a Pavlovian differential conditioning procedure. On all conditioning trials, both halves of the floor were homogenous (either grid or hole), and mice had access to both sides of the apparatus. During preference tests, the floor was half grid and half hole with left/right position of each texture counterbalanced within groups When second preference tests were conducted, the left/right floor positions were the same as in the first test.

As seen in Cunningham et al. (1995), a second preference test can be conducted after a drug effect has been observed on the first test. In this case, a second test under saline conditions can be used to assess whether effects of drug administration on the first test resulted in permanent alteration of the preference response. For example, mice that received naloxone prior to the first test showed much weaker conditioned preference on a second test (conducted three days later) than mice that did not receive naloxone, even though all mice received saline prior to the second test (Cunningham et al., 1995). A second preference test can also be useful if no effects of drug administration on the first test are observed. As long as a saline-only group is

retained, a second test with higher doses or a different drug can be conducted. The saline-only group is important since a preference test constitutes an extinction trial, therefore, a decrease in the magnitude of preference seen on a second test may be a result of extinction processes and not of drug administration. Regardless of the inclusion of control groups, conclusions drawn from second test data are generally more tentative than those drawn after the initial preference test. Finally, performance on second tests may be more variable from experiment to experiment, making direct comparison between experiments difficult.

On alternating days throughout conditioning, mice in the Grid+ subgroup received a 2 g/kg i.p. injection of ethanol prior to placement on the grid floor (CS+ days) and saline injection before placement on the hole floor (CS- days). Conversely, mice in the Grid- subgroup received saline paired with the grid floor and ethanol prior to placement on the hole floor. Only one injection was given on each conditioning trial. The conditioning subgroups within each treatment group were matched for overall exposure to floor type and drug. The order of exposure to ethanol and saline was counterbalanced within groups and all subjects received four complete conditioning trials, each comprised of one CS+ day and one CS- day. Conditioning trials took place on consecutive days with the exception of Experiments 6 and 7. In Experiment 6, half the animals received a two-day break between Trials 2 and 3 (i.e., between days five and six). In Experiment 7,

all mice received the two-day break. A 60-min preference test was conducted 24 h after the final conditioning trial.

For Experiment 1, three groups of D2 mice underwent ethanol place conditioning as described above. For the first preference test, mice received injections of saline (Group 0), 3 mg/kg NTI (Group 3), or 10 mg/kg NTI (Group 10) 20 min prior to saline injection and placement on a mixed floor for the 60-min test. A second preference test was conducted 48 h later. Mice receiving saline on the first test also received saline on the second test to serve as a control for extinction effects. Mice from Groups 3 and 10 were randomly reassigned to two new dose groups, Groups 1.3 (1.3 mg/kg NTI) and 20 (20 mg/kg NTI) and injected with the appropriate dose of NTI 20 min prior to the test. Reassignment was done to control for possible effects of treatment condition on the first test that could carry-over to the second test. All mice received a saline injection just before placement in the apparatus for the test.

Data Analysis

Because all groups were treated identically during conditioning trials (with the exception of Experiments 6 and 7), the conditioning trial data are not of central importance to this dissertation. Thus, all analyses involving conditioning trial activity data are reported separately in Appendix A.

The primary dependent variable for preference tests was time spent on the grid floor. As in Cunningham et al. (1995), the average time spent on the grid floor was determined for three successive time intervals of the 60-min

test session: Minutes 1-10, Minutes 11-30 and Minutes 31-60. Preference data from each interval were analyzed by analysis of variance (ANOVA). Activity data on the preference test were also divided into the three time intervals and analyzed by ANOVA. When significant interactions or main effects were found, follow-up comparisons were conducted using ANOVAs or Tukey's test as appropriate. See Results of each experiment for specifics.

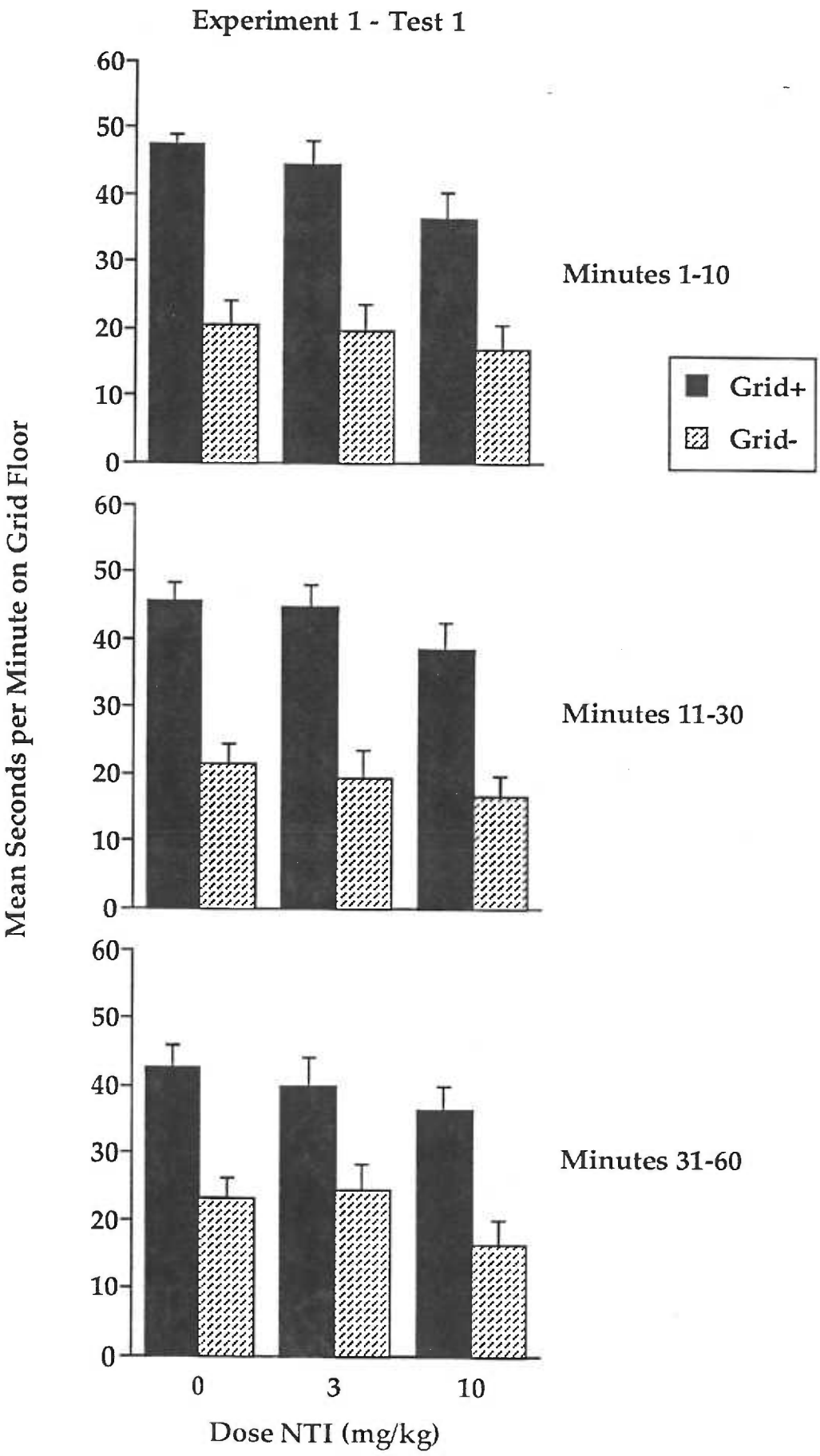
Results

One mouse from Group 10 was found caught in the wire cage top and was excluded from the study. A mouse from Group 3 received a misplaced injection during the conditioning phase, and data from this mouse were also excluded. Because of equipment problems, data from one Group 3 mouse were lost on the first test and data from two mice (one Group 0, one Group 10) were lost on the second test.

Place preference data from the three antagonist dose groups on the first test are shown in Figure 3. Data are presented as mean (+SEM) seconds per minute spent on the grid floor by the Grid+/Grid- conditioning subgroups in each dose group during Minutes 1-10, Minutes 11-30 and Minutes 31-60 of the 60-min test session. Ethanol produced a conditioned place preference in all three dose groups and in all three time intervals, with NTI pretreatment having no effect. Two-way ANOVAs (Dose x Conditioning Group) supported these observations and revealed significant effects of conditioning group in each time interval, $F(1,86) = 80.0$, Minutes 1-10; $F(1,86) = 96.6$ for Minutes

Figure 3 - Experiment 1: Test 1 Preference. Mean (+SEM) seconds per minute spent on the grid floor during Minutes 1-10, 11-30 and 31-60 of the first preference test for mice in the three NTI dose groups. All groups received the appropriate dose of NTI 20 min before the test session. Conditioning groups (n=14-16) are as in Figure 1.

Figure 3


Experiment 1 - Test 1
Mean Seconds per Minute on Grid Floor
60
50
40
30
20
10
0
Minutes 1-10
Grid+
Grid-
Minutes 11-30
Minutes 31-60
0
3
10
Dose NTI (mg/kg)

11-30; $F(1,86) = 44.2$, Minutes 31-60, all $ps<.001$. No effects involving dose were seen (all $Fs<2.6$).

Activity was highest during the first part of the test and declined over time (Figure 4). NTI pretreatment tended to decrease activity but this effect was only significant in Minutes 11-30. One-way ANOVA showed a significant main effect of antagonist dose during this interval, $F(2,89) = 3.1$, $p=.05$ while the effect failed to reach significance in Minutes 31-60, $F(2,89) = 2.9$, $p=.06$. Follow-up analyses (Tukey's) indicated no significant differences in activity in the antagonist dose groups ($ps>.05$).

Figure 5 depicts preference data from the second preference test for the reassigned antagonist dose groups. As in the first test, no effects of NTI on the expression of preference were seen. A two-way ANOVA (Antagonist Dose x Conditioning Group) yielded only significant main effects of conditioning group in each time interval, $F(1,85) = 97.0$, Minutes 1-10; $F(1,85) = 58.7$, Minutes 11-30; $F(1,85) = 26.1$, Minutes 31-60, all $ps<.001$.

Significant effects of NTI were seen on activity during the second preference test, indicating that the highest dose was behaviorally active (Figure 6). A one-way ANOVA applied to the activity data from test two yielded a significant main effect of antagonist dose in Minutes 11-30, $F(2,88) = 4.7$, $p<.05$, and in Minutes 31-60, $F(2,88) = 6.5$, $p<.01$. Follow-up analyses showed that during Minutes 11-30 and Minutes 31-60 activity was lower in

Figure 4 - Experiment 1: Test 1 Activity. Mean (+SEM) activity counts per minute for mice in the three NTI dose groups (n=31/group) during Minutes 1-10, 11-30 and 31-60 of the first preference test.

Figure 4

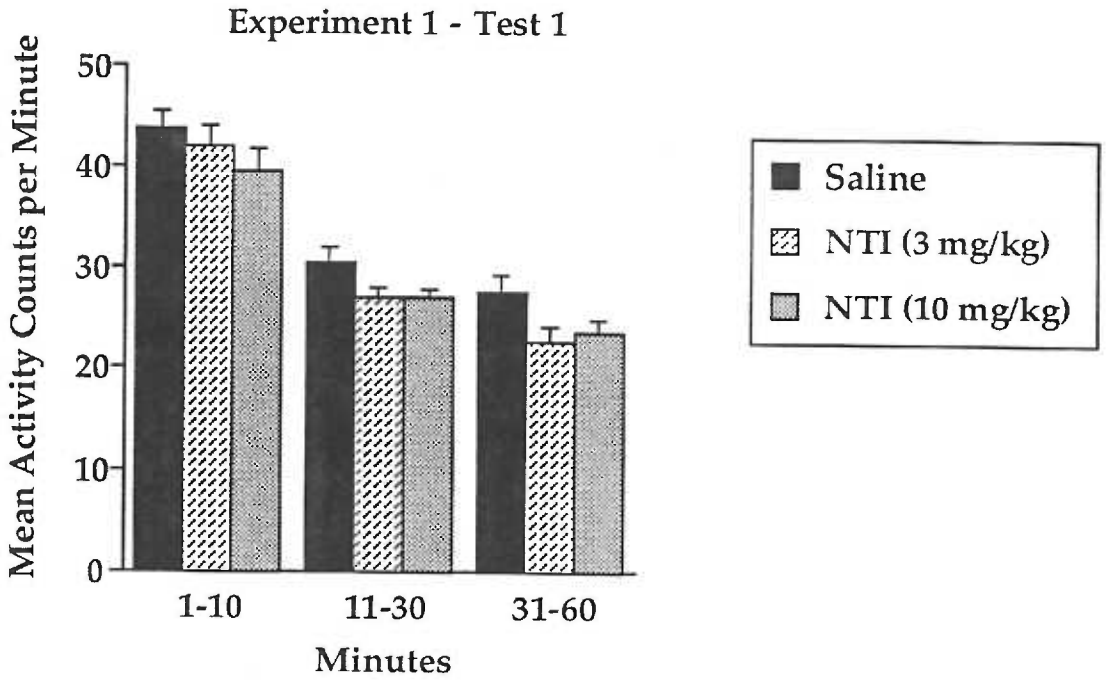

Experiment 1 - Test 1
Mean Activity Counts per Minute
50
40
30
20
10
0
1-10
11-30
31-60
Minutes
Saline
NTI (3 mg/kg)
NTI (10 mg/kg)

Figure 5 - Experiment 1: Test 2 Preference. Mean (+SEM) seconds per minute spent on the grid floor during Minutes 1-10, 11-30 and 31-60 of Test 2 for mice in the three new NTI dose groups. Mice receiving 3 or 10 mg/kg NTI on Test 1 received either 1.3 or 20 mg/kg NTI 20 min prior Test 2 (see text for explanation). Conditioning groups (n=14-16) are as in Figure 1.

Figure 5

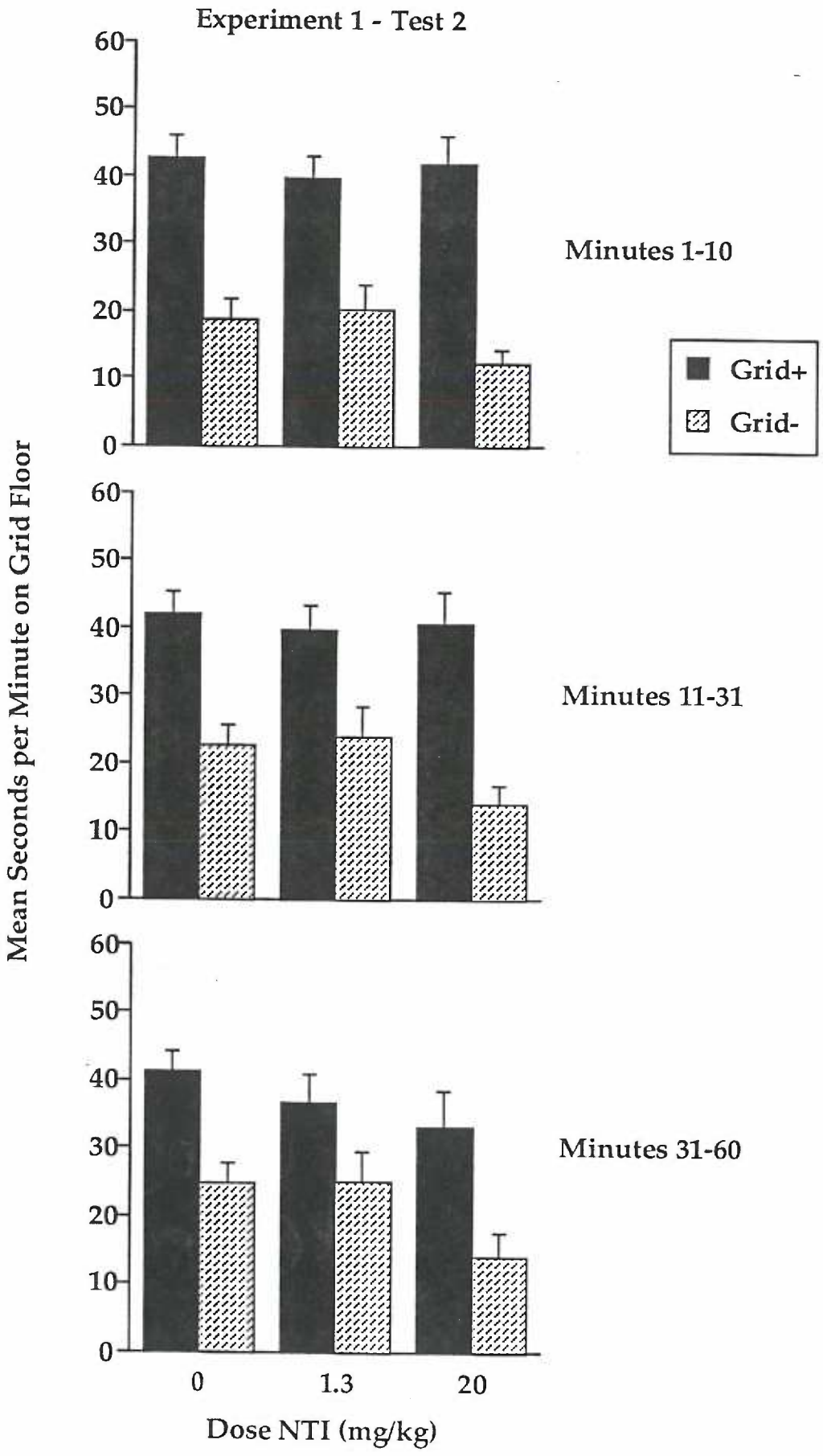
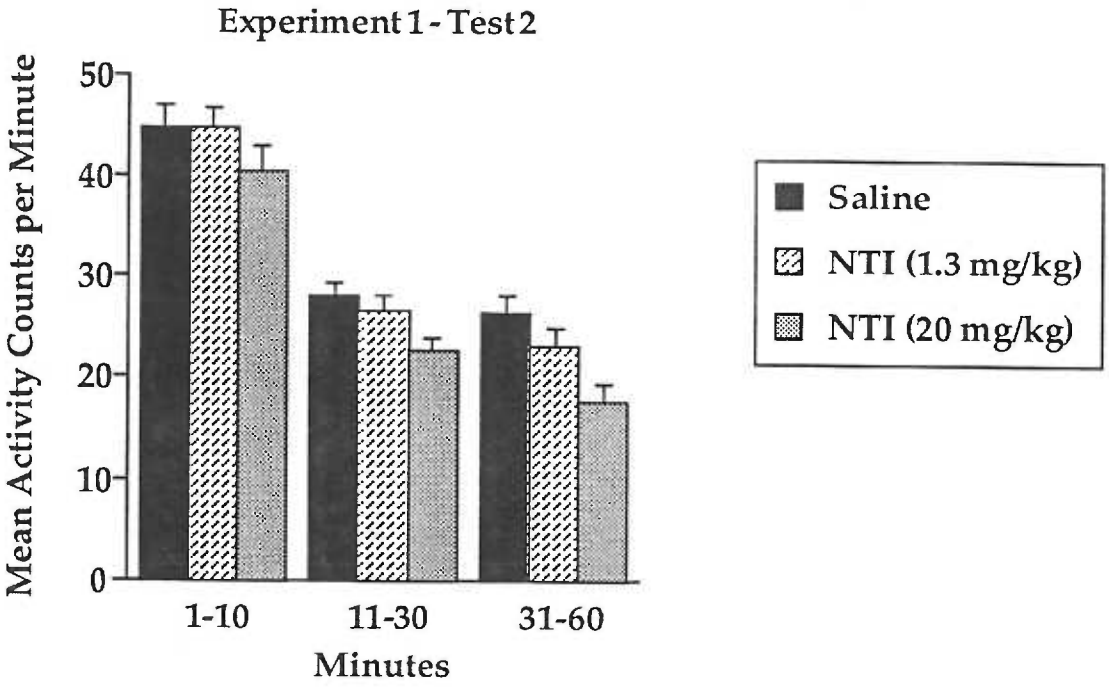

Experiment 1 - Test 2
Mean Seconds per Minute on Grid Floor
60
50
40
30
20
10
0
Minutes 1-10
Grid+
Grid-
Minutes 11-31
Minutes 31-60
0
1.3
20
Dose NTI (mg/kg)

Figure 6 - Experiment 1: Test 2 Activity. Mean (+SEM) activity counts per minute for mice in the three NTI dose groups (n=30-31) during Minutes 1-10, 11-30 and 31-60 of the second preference test.

Figure 6


Experiment 1 - Test 2
Mean Activity Counts per Minute
50
40
30
20
10
0
1-10
11-30
31-60
Minutes
Saline
NTI (1.3 mg/kg)
NTI (20 mg/kg)

the 20.0 mg/kg dose group than in either of the other dose groups (Tukey's, ps<.05).

Discussion

Ethanol produced a reliable conditioned place preference in all groups. Blockade of δ opioid receptors with NTI on the test day had no effect on the expression of preference. Increasing the dose range by administering a second preference test with different doses of the antagonist also had no effect on expression of preference, although NTI decreased activity. This finding suggests that attenuation of preference by naloxone is not mediated via the δ receptor.

The highest dose of NTI (20 mg/kg) significantly reduced activity and the effects of 10 mg/kg approached, but did not reach, statistical significance. These findings are consistent with previous studies demonstrating a reduction in locomotor activity in rats with NTI (de Vries et al., 1995).

Levels of activity during preference testing may affect expression of place preference, raising the possibility that the activity decreasing effects of NTI may have masked its effects on expression. This interpretation is supported by previous findings that low levels of activity tend to enhance, rather than decrease, preference (Cunningham, 1995; Neisewander, Pierce, & Bardo, 1990; Vezina & Stewart, 1987). Thus, it is possible that NTI does attenuate the expression of preference but that this attenuation is offset by an enhancement of preference resulting from a drug-induced decrease in

activity. However, the previous findings with naloxone indicate that this is not necessarily the case. Naloxone decreased activity and, in the same mice, attenuated maintenance of preference (Cunningham et al., 1995), an effect opposite to the above suggestion. It could be argued that since both NTI and naloxone decrease activity, this effect of naloxone may be mediated by δ receptors while its effects on expression of preference occur via another receptor type that is not affected by NTI. The current study does not exclude this possibility; however, these data do suggest that naloxone's effects on ethanol preference are not mediated by the δ opioid receptor.

The current findings are somewhat surprising in light of reports by Froehlich and colleagues (Froehlich et al., 1991; Krishnan-Sarin et al., 1995) indicating that the δ receptor may play an important role in mediating ethanol drinking. However, two primary differences exist between the present study and those reported by Krishnan-Sarin et al.. First, they used a limited access two-bottle choice procedure that resulted in extensive ethanol exposure prior to antagonist treatment whereas in the current study, animals received ethanol only four times before testing. Second, Krishnan-Sarin et al. used rats, whereas mice were used here. This raises the possibility that species differences may be responsible for the discrepancy. It is also possible that drinking is not a good index of ethanol reward inasmuch as factors such as

taste and nutritive properties may control behavior rather than the pharmacological properties of ethanol.

Not all studies in rats have seen alterations in ethanol's effects after administration of δ antagonists. Jørgensen and Hole (1986) found that ICI 154129, given either before or after ethanol administration, did not affect ethanol-induced hypothermia, ataxia or loss of righting reflex. The absence of an effect of δ receptor blockade on extinction is consistent with a report by Benton, Dalrymple-Alford, McAllister, Brain, & Brain (1984). These investigators found that while administration of naloxone facilitated extinction of food-rewarded alley running in food-deprived mice, the δ antagonist ICI 154129 had no effect.

A potentially serious problem with the studies using either of the ICI compounds is that these agents are peptides and cross the blood-brain barrier with great difficulty (Portoghese, 1993). Therefore, the observed effects of ICI 174864 on drinking could result from blockade of peripheral, not central, δ receptors. Similarly, the negative findings reported with ICI 154129 (Jørgensen & Hole, 1986; McAllister, Brain, & Brain, 1984) may be a result of peripheral administration of the peptide, not truly evidence for non-involvement of the δ receptor in these effects.

Experiment 2: Effects of Blockade of μ Receptors

Introduction & Rationale

For some time, naloxone was considered the prototypical μ receptor antagonist, though it is now known that this antagonist exhibits selectivity for the μ receptor only at doses well below those used effectively in Cunningham et al. (1995). In light of the negative findings of Experiment 1 suggesting that the δ receptor does not mediate the previously observed effects of naloxone, the μ receptor should also be examined.

As mentioned earlier, the opioid receptor types have different, overlapping, brain distribution patterns. High levels of μ receptor mRNA expression and binding are found in the amygdala, many thalamic nuclei, the substantia nigra and in clusters and patches in the nucleus accumbens and caudate-putamen with moderate binding density in the ventral tegmental area and the periaquaductal gray (Mansour et al., 1995).

Striatal clusters and patches of μ receptors are thought to be important in mediating the rewarding (Shippenberg, 1993) and locomotor activating (Kalivas, Widerlöv, Stanley, Breese, & Prange, 1993) effects of μ receptor agonists via the mesolimbic DA system. Agonists at these receptors increase striatal release of dopamine (Di Chiara & Imperato, 1988; Spanagel, Herz, &

Shippenberg, 1992), probably via GABA interneurons (Johnson & North, 1992).

Agonists at μ receptors, including morphine and [D-Ala^2,MePhe^4,Gly-ol^5]-ENK (DAMGO), have positive motivational effects and can function as reinforcers. In fact, the capacity of opioids to be rewarding or reinforcing appears to be characteristic of agents that function as μ receptor agonists (Shippenberg, 1993). Conditioned place preferences are produced by morphine (Mucha & Herz, 1985), DAMGO and β-END (Bals-Kubik, Herz, & Shippenberg, 1988). In contrast, antagonism of μ receptors with naloxone (Cunningham et al., 1995; Mucha & Iversen, 1984) or selective antagonists (Bals-Kubik, Herz, & Shippenberg, 1989) results in conditioned place aversions.

The development of μ-selective antagonists has been crucial in determining the role of μ receptors in various behaviors. One such antagonist, β-FNA, is a naltrexone derivative synthesized by Portoghese, Larson, Sayre, Fries, and Takemori (1980) that has been shown to have short-acting (one to four h) reversible κ agonist activity (Ward, Portoghese, & Takemori, 1982). However, its primary usefulness lies in its ability to alkylate μ receptors, resulting in irreversible, selective μ receptor antagonism generally reported to remain for up to four days (e.g., Negus, Pasternak, Koob,

& Weinger, 1993; Ward et al., 1982). β-FNA has been reported to show remarkable selectivity against antinociception produced by μ agonists, but not δ or κ agonists (Ward & Takemori, 1983).

Recent studies have examined the ability of β-FNA to affect the motivational properties of opiates. For example, Negus, Henriksen, Mattox, Pasternak, Portoghese, Takemori, Weinger and Koob (1993) and Martin, Dworkin, and Smith (1995) demonstrated that β-FNA pre-treatment (5 - 20 mg/kg SC and 40 nmol i.c.v., respectively) increased responding for heroin in rats. This effect was attributed to a decrease in effect of the infused dose of heroin. In another study, Suzuki, Funada, Narita, Misawa, & Nagase (1993) showed that 10 mg/kg β-FNA attenuated acquisition of morphine-induced conditioned place preference in mice. In addition, β-FNA (5 μg, i.c.v.) was found to decrease food and water intake (Ukai & Holtzman, 1988), behaviors known to be reduced by opioid antagonists (e.g., Lynch, 1986).

In addition to β-FNA, another selective μ receptor antagonist is the cyclic somatostatin derivative CTOP (D-Pen-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2) developed by Hruby and colleagues (Pelton, Gulya, Hruby, Duckles, & Yamamura, 1985; Pelton, Kazmierski, Gulya, Yamamura, & Hruby, 1986). CTOP has been shown to antagonize morphine analgesia and to precipitate withdrawal in morphine-dependent mice (Gulya, Krivan, Nyolczas, Sarnyai,

& Kovacs, 1988). In addition, administration of CTOP prevents DAMGO-induced antinociception (Fanselow, Calcagnetti, & Helmstetter, 1989) and conditioned place preference (Bals-Kubik, Shippenberg, & Herz, 1990).

Few studies have examined the effects of selective μ receptor antagonists on ethanol's effects. In one recent study, Lê, Poulos, Quan, and Chow (1993) used β-FNA to assess the role of the μ receptor in ethanol consumption in ethanol-preferring B6 mice. After 11 days of daily access to 12% ethanol and water for one h in a two-bottle restricted access paradigm, mice were injected with β-FNA (20 mg/kg) 14 h prior to the access period on day 12. β-FNA had no effect on the amount of ethanol consumed, although naltrexone reduced ethanol consumption by 50%, suggesting that naltrexone's effects are not mediated by the μ receptor. In contrast, Hyttiä (1993) demonstrated that blockade of μ receptors suppresses ethanol intake in the alcohol-preferring AA rat. Rats with extensive experience with limited access to 10% ethanol received i.c.v. administration of CTOP (1 μg) 15 min prior to the 30-min access period. The antagonist was given on three successive days and significantly decreased ethanol intake after the second day. Consumption remained suppressed the day after termination of CTOP treatment. Interestingly, the AA rat presents a higher density of μ receptor binding in

limbic regions than the alcohol-avoiding ANA rat (DeWaele, Kiianmaa, & Gianoulakis, 1995).

To assess the role of μ receptors in the expression of ethanol place preference, a selective μ antagonist is necessary. Of the selective μ receptor antagonists currently available, β-FNA, CTOP, and cyprodime, β-FNA was chosen for the current study. CTOP, a fairly widely used and selective μ antagonist, does not cross the blood-brain barrier well, making it unsuitable for systemic use. While i.c.v. administration is certainly possible in mice, it requires anesthesia, which in itself could alter expression of place preference. In addition, given the labor-intensive nature of this route of administration and the large number (n=96) of mice used in our standard place conditioning procedure, CTOP was not used. Cyprodime can be administered peripherally in mice but it has low potency and large doses must be used (Portoghese, 1993). In addition, cyprodime is insoluble in saline but highly soluble in ethanol, an unacceptable solvent in this study.

In Experiment 2, mice were tested for ethanol-induced conditioned place preference after administration of β-FNA. If naloxone's effect on place preference is mediated by the μ receptor, selective blockade of this receptor should affect the expression of place preference and the results should mimic those seen with naloxone.

Methods

Subjects

Subjects were 96 male D2 mice housed as in Experiment 1. Mice were 6 weeks old upon arrival and approximately 10 weeks old at the time of testing.

Apparati

Place conditioning

The 12-box place conditioning apparatus was as described in Experiment 1.

Analgesia

The hot-plate apparatus consisted of a 15-cm high square Plexiglas enclosure (10 cm x 10 cm) resting on the aluminum plate of a Thermolyne Dri-Bath. A stopwatch was used to determine the time (0.1 sec resolution) until the behavioral response occurred.

Drugs

β-FNA was obtained from the National Institute of Drug Abuse Drug Supply System (Baltimore, MD) and morphine sulfate was purchased from Mallinckrodt. Both drugs were dissolved in physiological saline and administered s.c. in injection volumes of 10 ml/kg. β-FNA doses of 5 and 10 mg/kg (i.p.) were chosen based on previous studies showing them to be effective in preventing morphine analgesia (Pick, Paul, & Pasternak, 1991; Ward et al., 1982) and morphine-induced conditioned place preference (Suzuki et al., 1993) in mice. A pretreatment time of 18-20 h was chosen based

on reports that agonist activity at the κ receptor is absent by this time and only μ antagonist activity is present (e.g., Paronis, Waddell, & Holtzman, 1993; Suzuki et al., 1993).

Morphine sulfate was used to elicit analgesia for the hot-plate test. The dose of 5 mg/kg (s.c.) has previously been shown to produce tail-flick analgesia in mice (Pick et al., 1991) and is in the range of doses used to elicit analgesia in rats using a hot-plate (Paronis et al., 1993).

Procedure

Place conditioning

Three groups of D2 mice underwent ethanol place conditioning as described in Experiment 1. Mice received injections of vehicle (Group 0), 5 mg/kg β-FNA (Group 5) or 10 mg/kg β-FNA (Group 10) 2-3 h after the final conditioning trial. Eighteen to 20 h later, subjects received a saline injection immediately prior to placement in the conditioning apparatus on a mixed floor for a 60-min choice test. The saline injection was given so that cues normally present during conditioning (including handling and injection cues) would also be present during testing.

Analgesia testing

Twenty-four h after the preference test a morphine analgesia test was conducted to determine whether μ receptors were blocked, i.e., whether β-FNA was effective. Half of the mice in each antagonist dose group received

an injection of 5 mg/kg morphine (10 ml/kg, s.c.) and the other half received a saline injection (10 ml/kg, s.c.). Thirty min later, mice were placed on a hot-plate (56° C) where a Plexiglas enclosure limited locomotion to an area 10 cm x 10 cm. Latency to respond to the heat stimulus was measured to the nearest 0.1 sec with a stopwatch by an experienced observer (Jeff Mogil) blind to drug treatment. Response was defined as a paw lick or paw shake/flutter of a hindpaw. If no response was seen in 60 sec, the mouse was removed to avoid tissue damage.

Ten days after analgesia testing, mice were reassigned to one of two groups (n=26-27/group) for a second preference test. Equal numbers of mice from each β-FNA dose group and conditioning subgroup received an injection of either saline or 1.5 mg/kg naloxone (n=22-24/subgroup) 15 min prior to a saline injection and placement in the test apparatus on a mixed floor.

Data analysis

Place conditioning data were analyzed as in Experiment 1. The dependent variable in the analgesia test was latency to paw shake. These data were analyzed by two-way ANOVA (Antagonist Dose x Drug). Significant main effects were further analyzed by Tukey's test.

Results

Data from three mice were excluded from analysis. Two mice (one each from Groups 0 and 10) were injured by injections during conditioning.

An additional mouse from Group 10 sustained a foot injury during the course of the experiment which caused the foot to be deformed. Because the CS was a tactile cue, data from this mouse were not included.

Place preference data from the three antagonist dose groups on the first test are shown in Figure 7. Data are presented as mean (+SEM) seconds per minute spent on the grid floor by the Grid+/Grid- conditioning subgroups during Minutes 1-10, Minutes 11-30 and Minutes 31-60 of the 60-min test session. Ethanol produced a conditioned place preference in all three groups and in all three time intervals, with β-FNA pretreatment having no effect on the magnitude of the preference. Two-way ANOVAs (Dose x Conditioning Group) supported these observations and revealed significant effects of conditioning group in each time interval, $Fs(1,87) = 98.4$ for Minutes 1-10, 181.2 for Minutes 11-30 and 118.6 for Minutes 31-60, all $ps<.001$. No effects involving dose were seen (all $Fs<2$).

Activity during the first preference test was highest during the first 5 min and declined over the remainder of the test (Figure 8). β-FNA had no effect on activity. A one-way ANOVA of each time interval yielded no significant effects of dose (all $Fs<2.4$).

Results from the hot plate test indicated that β-FNA pretreatment dose-dependently attenuated paw-shake latencies. These data are presented in Figure 9. A two-way ANOVA (Antagonist Dose x Drug) supported this

Figure 7 - Experiment 2: Test 1 Preference. Mean (+SEM) seconds per minute spent on the grid floor during Minutes 1-10, Minutes 11-30 and Minutes 31-60 of the first 60 min preference test for the three β-FNA dose groups. Mice received β-FNA 18-20 h prior to the test. Grid+ animals had ethanol paired with the grid floor during conditioning and Grid- animals had ethanol paired with the hole floor. The magnitude of conditioned preference is indicated by the difference between Grid+ and Grid- conditioning groups in each dose group. n=14-16/conditioning group.

Figure 7

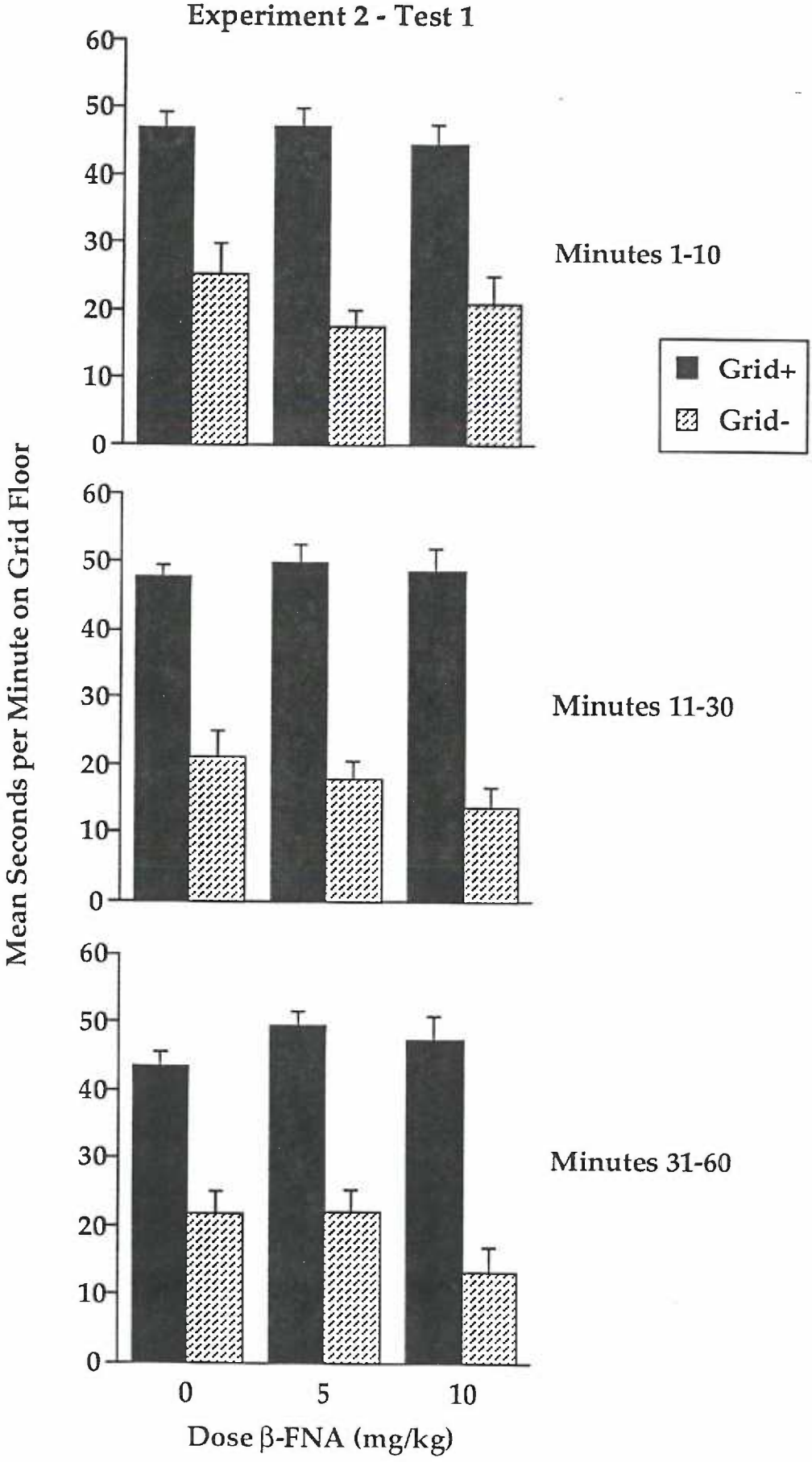

Experiment 2 - Test 1
Mean Seconds per Minute on Grid Floor
60
50
40
30
20
10
0
Minutes 1-10
Grid+
Grid-
Minutes 11-30
Minutes 31-60
0
5
10
Dose β-FNA (mg/kg)

Figure 8 - Experiment 2: Test 1 Activity. Mean (+SEM) activity counts per minute during Minutes 1-10, 11-30 and 31-60 of the first preference test for each β-FNA dose group (n=30-32) in Experiment 2.

Figure 8

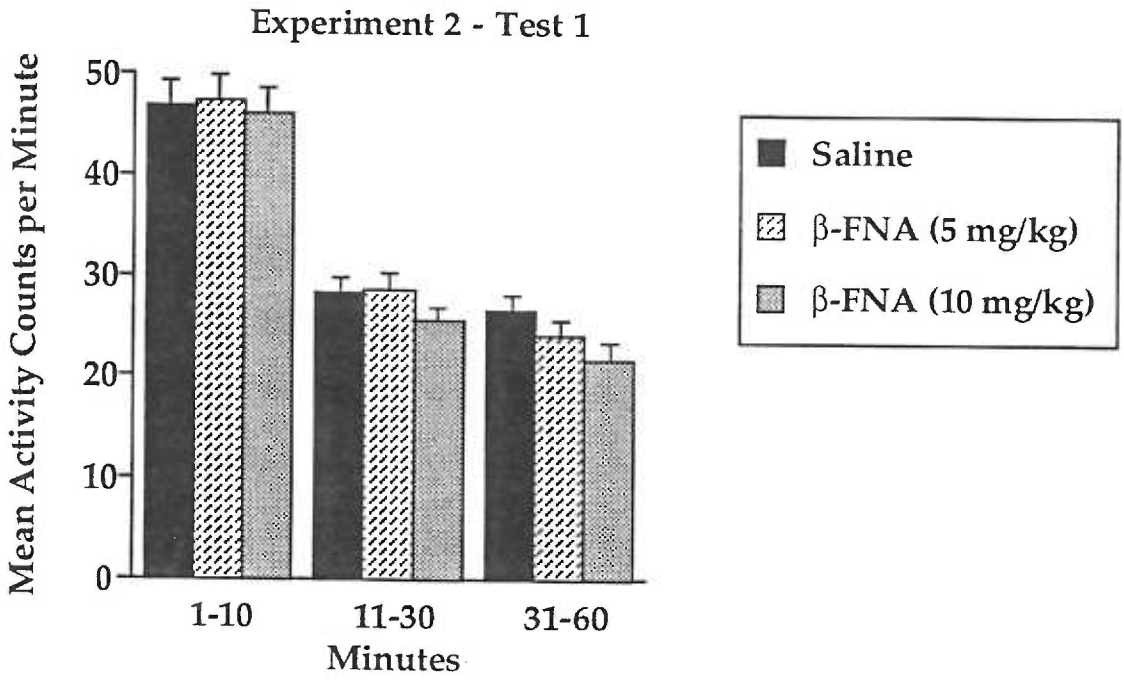
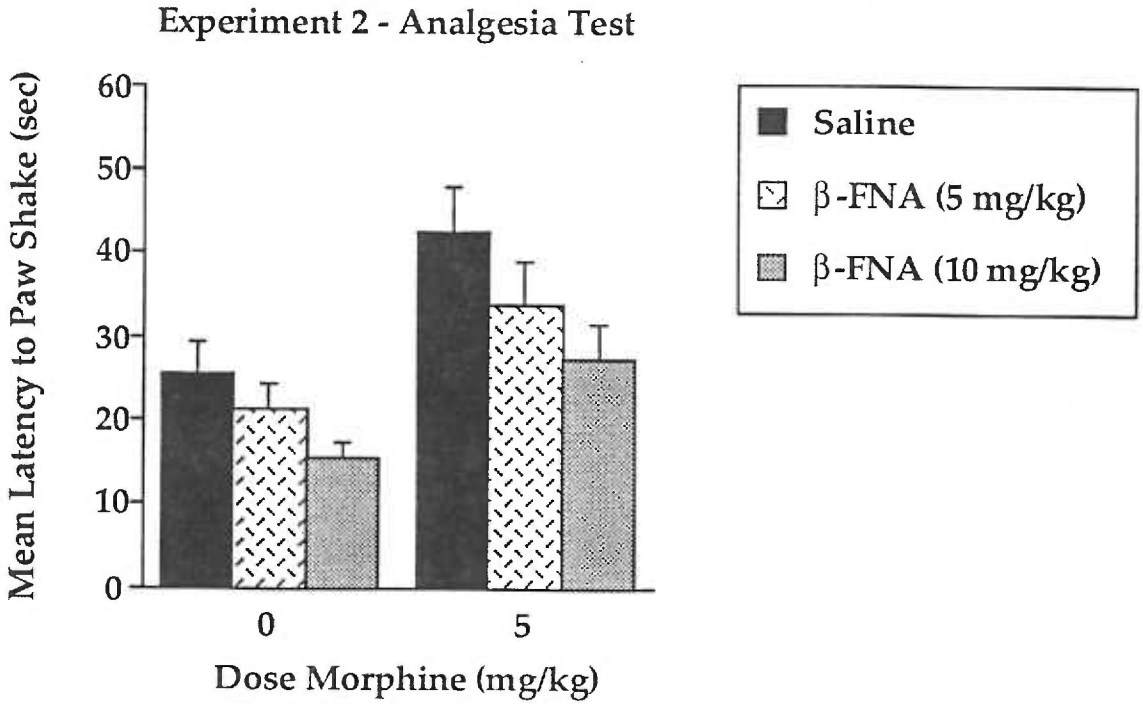

Experiment 2 - Test 1
Mean Activity Counts per Minute
50
40
30
20
10
0
1-10
11-30
31-60
Minutes
Saline
β-FNA (5 mg/kg)
β-FNA (10 mg/kg)

Figure 9 - Experiment 2: Analgesia. Mean (+SEM) latency to paw shake 30 min after morphine (5 mg/kg) or saline in mice treated with 0, 5 or 10 mg/kg β-FNA approximately 44 h earlier.

Figure 9

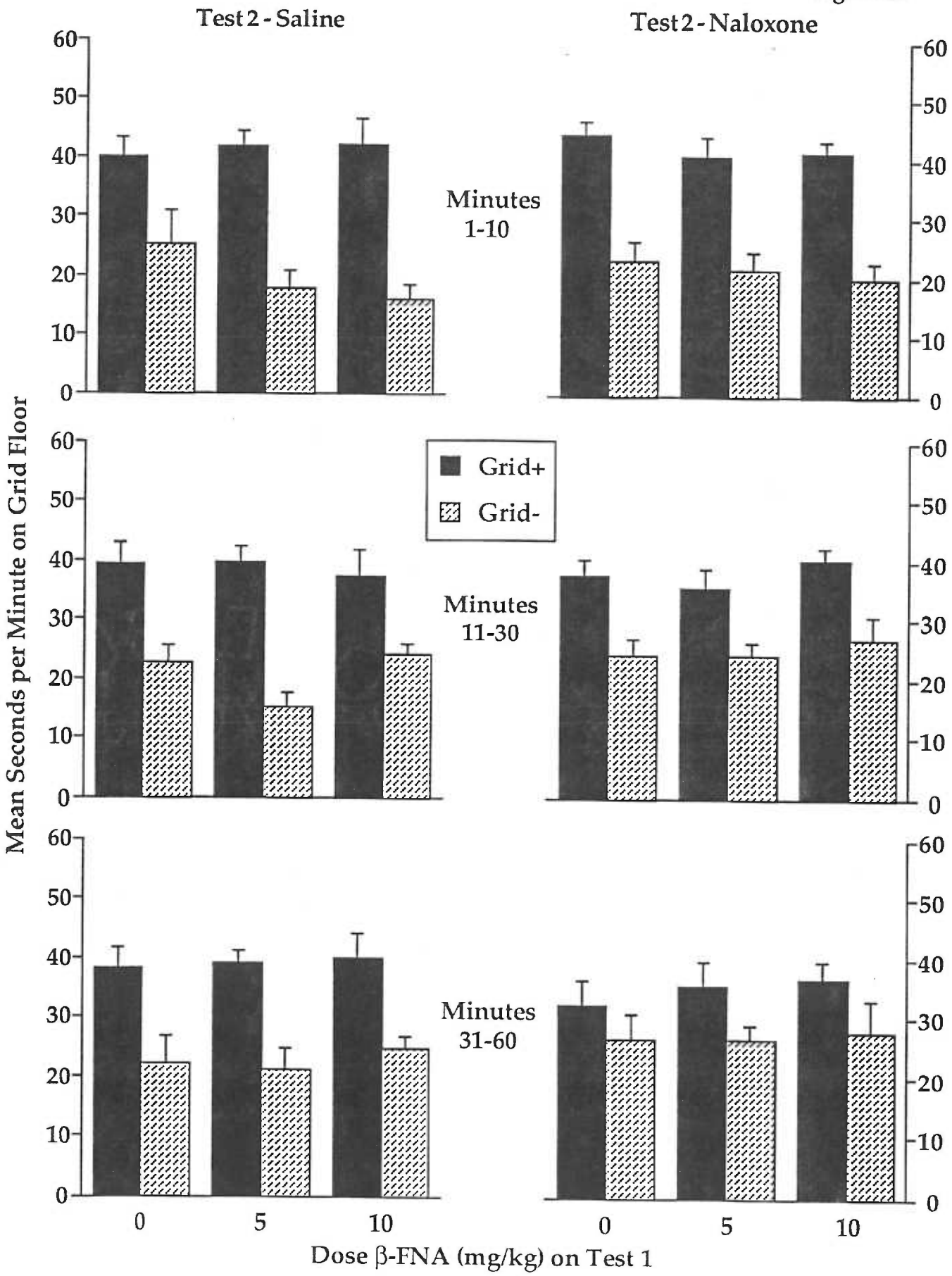

Experiment 2 - Analgesia Test
Mean Latency to Paw Shake (sec)
60
50
40
30
20
10
0
0
5
Dose Morphine (mg/kg)
Saline
β-FNA (5 mg/kg)
β-FNA (10 mg/kg)

observation with significant main effects of antagonist dose, $F(2,87) = 4.6$, $p<.05$, and drug (morphine vs saline), $F(1,87) = 17.1$, $p<.001$. Follow-up analyses (Tukey's) indicated that latency to paw shake was significantly increased in morphine treated animals and was decreased by 10 mg/kg β-FNA relative to saline or 5 mg/kg β-FNA (ps<.05). No interaction of β-FNA and morphine treatment was present ($F<1$), indicating that β-FNA decreased latency to paw shake in both saline and morphine treated animals.

Since β-FNA is an irreversible antagonist, preference data from the second test (naloxone vs saline) were analyzed with respect to β-FNA treatment on the first test (see Figure 10). A significant place preference was seen in the saline group, and, as in the first test, this preference was not affected by earlier β-FNA pretreatment. Naloxone had no effect on the expression of preference during the first two time intervals, but decreased preference in the last 30 min. Three-way ANOVAs (Antagonist Dose Test 1 x Drug Test 2 x Conditioning Group) supported these observations, revealing significant effects of conditioning group in all intervals, Minutes 1-10, $F(1,81) = 117.5$; Minutes 11-30, $F(1,87) = 77.4$; Minutes 31-60, $F(1,87) = 31.9$ (all ps<.001). In Minutes 31-60 the drug test 2 x conditioning group interaction was also significant, $F(1,81) = 4.1$, $p<.05$. Follow-up analyses (Tukey's) showed that the two conditioning groups were different only in the saline group ($p<.001$), not in the naloxone group ($p>.05$). No effects involving β-FNA dose were seen.

Figure 10 - Experiment 2: Test 2 Preference. Mean (+SEM) seconds per minute spent on the grid floor during Minutes 1-10, 11-30 and 31-60 of the second preference test. Conditioning groups are as in Figure 1. The left column depicts preference data from mice receiving saline and the right column depicts data from mice receiving 10 mg/kg naloxone 15 min prior to Test 2. The x-axis indicates the dose of β-FNA given before Test 1. Each bar depicts data from 7-8 mice.

Figure 10


Test 2 - Saline
Test 2 - Naloxone
Minutes 1-10
Minutes 11-30
Minutes 31-60
Grid+
Grid-
Mean Seconds per Minute on Grid Floor
Dose β-FNA (mg/kg) on Test 1
0
5
10
60
50
40
30
20
10
0

No significant effects of either naloxone or prior β-FNA treatment were seen on activity during the second test, all ps>.05. Mean activity counts are presented in Table 1.

Discussion

Ethanol produced a conditioned place preference that was not affected by administration of β-FNA. However, paw shake latencies measured 24 h after preference testing were significantly attenuated by administration of β-FNA, indicating that the doses used were active and that μ receptors were inactivated. β-FNA dose dependently decreased latency to paw shake in both morphine- and saline-treated animals. This suggests that a basal level of opioid activity contributes to baseline latencies in the paw flick test. Alternatively, it may be that the handling and injection procedure normally results in a release of endogenous opioids that is prevented by β-FNA pretreatment.

These place preference data suggest that maintenance of expression of ethanol-induced conditioned place preference is not dependent on μ opioid receptors. Alternatively, given that β-FNA was necessarily administered 18-20 h prior to testing (and, therefore, that μ receptors were non-functional for an extended period), it is possible that some compensatory change took place during this time that allowed another system to mediate expression of

Table 1. Mean Activity Counts per Minute (±SEM) in Minutes 1-10, 11-30 and 31-60 on Test 2 of Experiment 2.

Group[a]	Minutes 1-10	Minutes 11-30	Minutes 31-60
β-FNA 0/saline	59.9 ± 4.89	40.3 ± 2.56	34.2 ± 3.27
β-FNA 0/nal 10	52.8 ± 3.90	35.4 ± 1.84	29.7 ± 1.58
β-FNA 5/saline	53.7 ± 3.60	33.3 ± 2.05	30.4 ± 2.03
β-FNA 5/nal 10	49.5 ± 3.29	34.7 ± 2.11	33.6 ± 2.00
β-FNA 10/saline	52.0 ± 2.50	34.6 ± 2.54	28.9 ± 1.69
β-FNA 10/nal 10	57.2 ± 3.81	40.8 ± 2.41	33.9 ± 1.85

[a] Dose of β-FNA on Test 1/Drug Treatment on Test 2
nal 10 = 10 mg/kg naloxone

preference.

Although general consensus exists regarding the ability of β-FNA to antagonize behaviors mediated by μ receptors, results of binding studies have been less consistent. Recent studies have demonstrated that while β-FNA can completely abolish effects of opiates, it binds irreversibly only to a subset of μ receptors, resulting in changes in affinity or density of μ binding sites much smaller than expected (Liu-Chen, Li, Wheeler-Aceto, & Cowan, 1991; Martin et al., 1995; Recht & Pasternak, 1987). The reason for this dichotomy between behavioral and binding studies is not known, although it has been suggested that perhaps only one μ receptor subtype or conformation is sensitive to β-FNA (Recht & Pasternak, 1987; Rothman, Long, Bykov, Jacobson, Rice, and Holaday, 1988). Additionally, it is possible that the μ binding sites not affected by β-FNA are not fully functional receptors (Martin et al., 1995). Therefore, it may be that maintenance of expression of preference is mediated by a subset of μ receptors not susceptible to alkylation by β-FNA.

In the second preference test 12 days after β-FNA administration, naloxone (1.5 mg/kg) significantly attenuated the maintenance of expression of preference. Prior treatment with β-FNA had no effect on naloxone's ability to affect preference expression. The results with naloxone replicate earlier

findings by Cunningham et al. (1995), but do not provide new information about the mechanism of the effect.

It is interesting that β-FNA was ineffective in attenuating ethanol place preference in mice in the present study and in decreasing ethanol consumption in mice (Lê et al., 1993) while CTOP was able to decrease ethanol intake in rats (Hyttiä, 1993). It could be that ethanol reward in mice is not mediated by the μ receptor, while in rats the μ receptor does play a role in these effects. Alternatively, perhaps central administration of the μ antagonist is required to block ethanol's rewarding effects. However, this is unlikely since peripheral administration of β-FNA antagonizes analgesia produced by central administration of morphine (Ward et al., 1982) and DAMGO (Pick et al., 1991), indicating that β-FNA does cross the blood-brain barrier. Regardless, given the potential problems with β-FNA outlined above, new selective μ antagonists should be tested as they are developed.

Taken in conjunction with the findings from Experiment 1 with the selective δ antagonist NTI, these data suggest that blockade of μ or δ opioid receptors is not responsible for the observed effects of naloxone on expression of preference.

Experiment 3: Effects of Blockade of Kappa Receptors

Introduction & Rationale

It has been suggested that doses of naloxone below 1.0 mg/kg selectively bind μ receptors, doses above 1.0 mg/kg bind both μ and δ receptors while 20-30 times more naloxone is needed to antagonize κ receptors than μ receptors (Froehlich et al., 1991b; Leander, 1983). According to this schema, the fact that 1.5 mg/kg naloxone was effective in attenuating expression of preference suggests that κ receptors are not involved in this effect. However, in a recent review of opioid ligand selectivity, Corbett et al. (1993) give K_i values of 1.8, 23.0 and 4.8 nM for the inhibitory effects of naloxone at μ, δ, and κ receptors, respectively. The relative affinities of naloxone for the opioid receptors were 0.69 for the μ receptor, 0.05 for δ, and 0.26 for κ. Although these data indicate that naloxone has the highest affinity for μ receptors, they do not necessarily suggest that much higher doses of naloxone are needed to bind κ receptors. Given the difficulty in relating drug concentrations present in binding assays to drug concentrations present at critical brain regions after systemic injection, and the relatively small differences in selectivity of different opioid receptor subtypes for naloxone, it may be that the doses of naloxone used in Cunningham et al. (1995) are affecting κ receptors.

κ receptor binding sites are widely distributed throughout the forebrain, midbrain and brainstem in all species examined thus far (Mansour & Watson, 1993). High levels of κ receptor binding are present in the nucleus accumbens, amygdala, hypothalamic nuclei, periaquaductal gray, spinal trigeminal nucleus, nucleus tractus solitarius, the preoptic area, and deep layers of cerebral cortex (Mansour et al., 1995).

In contrast to μ and δ agonists, agonists at κ receptors, including U50,488H, bremazocine, and the dynorphin derivative E-2078, produce conditioned place aversions (Bals-Kubik et al., 1989; Mucha & Herz, 1985) and do not support self-administration (Woods et al., 1982; Woods & Winger, 1987). In addition, κ agonists decrease locomotor activity (von Voigtlander, Lahti, & Ludens, 1983; Jackson & Cooper, 1988). Given that κ receptor agonists also decrease DA release in the nucleus accumbens (Devine, Leone, Pocock, & Wise, 1993; Di Chiara & Imperato, 1988; Spanagel et al., 1992), it has been suggested that both the aversive and activity suppressing properties of these agonists may be mediated via the mesolimbic DA system (Di Chiara & Imperato, 1988).

As κ agonists appear to have little or no abuse liability, yet are effective in producing analgesia against chemical, pressure and probably heat stimuli, these agents are being heavily investigated as novel pharmacotherapeutic analgesics. As with μ and δ receptor systems, the development of selective

agonists and antagonists has been crucial in determining the role of the κ receptor in analgesia and other behaviors.

Portoghese and colleagues have reported on two selective κ receptor antagonists, binaltorphimine (BNI) and nor-binaltorphimine (nor-BNI) (Portoghese, Lipkowski, & Takemori, 1987; Takemori, Ho, Naeseth, & Portoghese, 1988). In pharmacologic studies, both BNI and nor-BNI attenuated κ agonist-induced antinociception but not that elicited by μ or δ agonists, indicating selectivity for the κ receptor (Portoghese et al., 1987; Takemori et al., 1988). However, in receptor binding studies, BNI exhibited little selectivity while nor-BNI was highly selective (Takemori et al., 1988). Endoh, Matsuura, Tanaka, and Nagase (1992) demonstrated that nor-BNI (5 and 20 mg/kg) partially antagonized analgesia elicited by morphine, but not by U50,488H, in the first 30 min after administration. However, between two and four h after nor-BNI administration, morphine analgesia was not altered while U50,488H analgesia was dose-dependently decreased. The κ antagonist properties were long-lasting, with 20 mg/kg nor-BNI still effective at 8 days. Nor-BNI (10 mg/kg) also suppressed diuresis induced by several κ agonists without affecting morphine-induced antidiuresis (Takemori, Schwartz, & Portoghese, 1988).

Few studies have looked at interactions of κ antagonists and ethanol. Those that have been reported were conducted with MR-2266, now known to

exhibit little selectivity for the κ receptor (Portoghese & Takemori, 1985). Sandi, Borrell, and Guaza (1988b) used a one-day forced exposure to ethanol (2.5% w/v) to induce ethanol drinking in water-deprived rats. After the forced exposure, where both bottles contained ethanol, ethanol and water were presented concurrently for 15 min on three subsequent days. Administration of the κ agonist dynorphin 1-17 on the forced ethanol drinking day had no effect on ethanol preference during retention days. However, when administered prior to the first retention test, dynorphin 1-17 reduced the ethanol preference score on all three retention days. This effect was reversed by coadministration of MR-2266, a μ/κ antagonist. Injection of MR-2266 alone during forced exposure to water or ethanol decreased intake of both. Using the same procedure, these investigators found that MR-2266 administered prior to fluid access on the forced exposure day had no effect on subsequent retention choice tests, but decreased ethanol preference scores on all three days when given after forced access (Sandi, Borrell, & Guaza, 1990). Although these findings may be interpreted as supporting the involvement of the κ receptor in ethanol consumption, and possibly reward, the possibility that this effect could be caused by actions at the μ receptor cannot be discounted.

At present, nor-BNI is the most selective and potent κ antagonist available. Therefore, this drug was chosen to assess the role of the κ receptor

in the expression of ethanol place preference. If the observed effect of naloxone on preference expression results from activity at κ receptors, nor-BNI should attenuate maintenance of expression of ethanol preference and the results should mimic those obtained with naloxone.

Methods

Subjects

Subjects were 96 male D2 mice housed as in Experiment 1. Mice arrived at 6 weeks of age and were approximately 10 weeks old at the time of testing.

Apparatus

The 12-box place conditioning apparatus was the same as in Experiment 1.

Drugs

Nor-BNI was obtained from the NIDA Drug Supply System and dissolved in saline. The doses of nor-BNI chosen (5 and 20 mg/kg) have been shown to selectively antagonize κ agonist-induced analgesia for up to eight days (Endoh et al., 1992). A pretreatment time of 18-20 h (s.c.) was chosen because nor-BNI has demonstrated antagonist activity at the μ receptor that declines after two h and is gone by four h (Endoh et al. 1992).

U50,488H, a widely used, selective κ receptor agonist, was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in saline. For the

analgesia test, a dose of 20 mg/kg U50,488H (s.c.) was initially chosen based on data showing a significant antinociceptive effect of this dose with the hot-plate test (Suzuki, Narita, Takahashi, Misawa, & Nagase, 1992). This dose was increased to 33.3 mg/kg for an additional test after the lower dose did not produce reliable analgesia.

Procedure

Three groups of D2 mice underwent ethanol place conditioning as in Experiments 1 and 2. Mice received s.c. injections of saline (Group 0), 5 mg/kg nor-BNI (Group 5) or 20 mg/kg nor-BNI (Group 20) 2-3 h after the final conditioning trial. Eighteen to 20 h later, subjects received a saline injection immediately prior to placement in the conditioning apparatus on a mixed floor for the 60-min choice test.

Twenty-four h after the preference test, an analgesia test with the κ agonist U50,488H was conducted to determine whether κ receptors were blocked, i.e., whether nor-BNI was effective. Half of the mice in each antagonist dose group received an injection of 20 mg/kg U50,488H (10 ml/kg, s.c.), and the other half received a saline injection (10 ml/kg, s.c.). Thirty min later, mice were placed on a hot-plate (56° C) and latency to paw shake was measured. If no response was seen in 60 sec, the mouse was removed to avoid tissue damage.

During the first analgesia test, the dose of U50,488H failed to produce reliable analgesia. Accordingly, a second analgesia test was conducted four

days later; the dose of U50,488H was increased to 33.3 mg/kg and the temperature of the hot plate was decreased to 52.5° C. Because of a limited amount of U50,488H, 12 mice were not injected or tested for analgesia. Analgesia data from an additional mouse (Group 20/U50,488H) were excluded from analysis because of urine on the hot plate.

Results

As in Experiments 1 and 2, ethanol produced a conditioned place preference that was not affected by pretreatment with nor-BNI. Place preference data from the three antagonist dose groups on the test are shown in Figure 11. Data are presented as mean (+SEM) seconds per minute spent on the grid floor by the Grid+/Grid-conditioning subgroups during Minutes 1-10, Minutes 11-30 and Minutes 31-60 of the 60-min test session. Two-way ANOVAs (Dose x Conditioning Group) yielded significant effects of conditioning group in each time interval, Fs(1,89) = 148.7 for Minutes 1-10, 199.3 for Minutes 11-30, and 107.4 for Minutes 31-60, all ps<.001. No effects involving dose were seen (all Fs<2).

Activity data from the preference test are shown in Figure 12. Activity during the test session was highest during the first time interval and declined over time. In contrast to Experiments 1 and 2, the highest dose of antagonist tended to increase rather than decrease activity, although this effect was seen only in the first 30 minutes of the test. ANOVAs supported this observations, indicating a significant main effect of dose in both Minutes 1-10, F(2,92) = 5.2,

Figure 11 - Experiment 3: Preference Data. Mean (+SEM) seconds per minute spent on the grid floor during Minutes 1-10, 11-30 and 31-60 of the preference test for mice in the three nor-BNI dose groups. Mice were injected with nor-BNI 18-20 h prior to the test. Conditioning groups (n=15-16) are as in Figure 1.

Figure 11

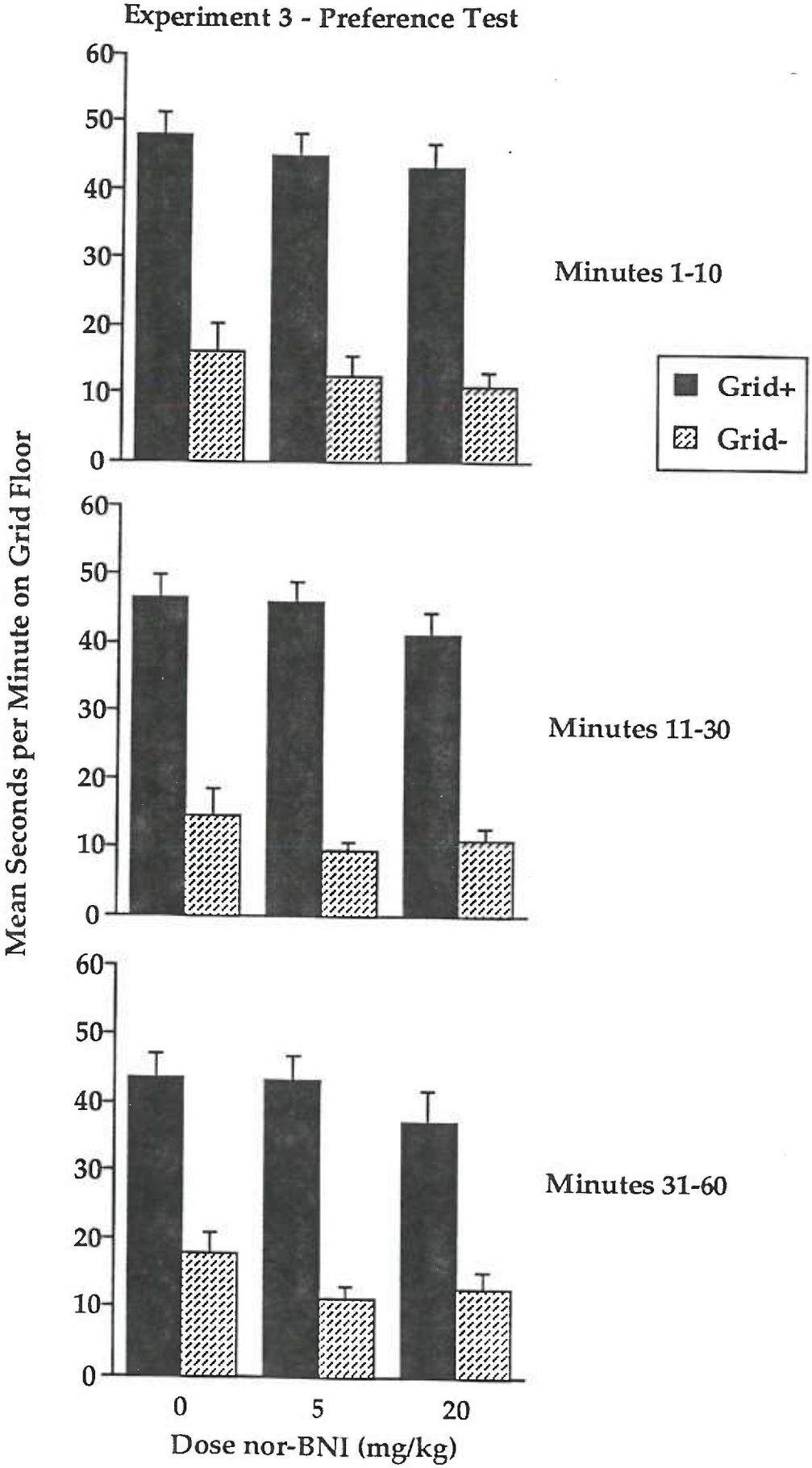
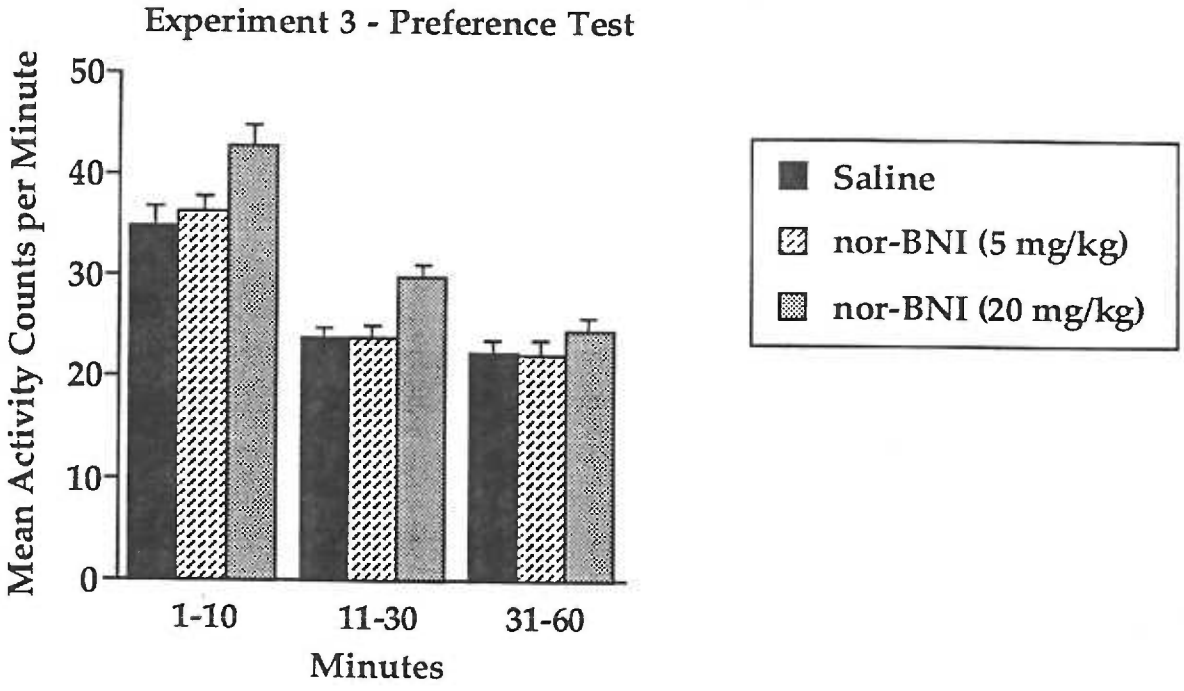

Experiment 3 - Preference Test
Mean Seconds per Minute on Grid Floor
60
50
40
30
20
10
0
Minutes 1-10
Grid+
Grid-
Minutes 11-30
Minutes 31-60
0
5
20
Dose nor-BNI (mg/kg)

Figure 12 - Experiment 3: Test Activity. Mean (+SEM) activity counts per minute during Minutes 1-10, 11-30 and 31-60 of the preference test for each nor-BNI dose group (n=31-32/dose group).

Figure 12


Experiment 3 - Preference Test
Mean Activity Counts per Minute
50
40
30
20
10
0
1-10
11-30
31-60
Minutes
Saline
nor-BNI (5 mg/kg)
nor-BNI (20 mg/kg)

$p<.01$, and in Minutes 11-30, $F(2,92) = 8.2$, $p<.01$. Follow-up analyses showed that 20 mg/kg nor-BNI significantly increased activity in both of these time intervals (Tukey's, $ps<.05$).

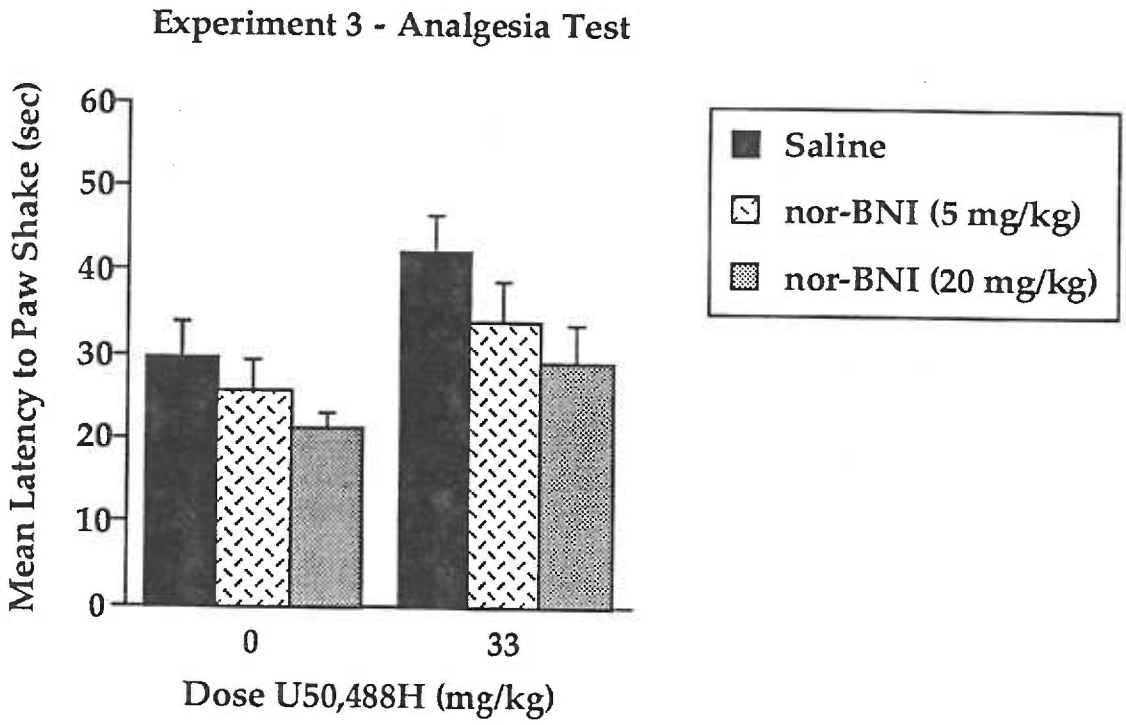
Data from the first analgesia test showed no effects of agonist treatment and are not presented. On the second analgesia test, animals treated with the κ agonist U50,488H showed an increase in latency to paw shake. Pretreatment with nor-BNI attenuated this increase and also decreased paw shake latencies in saline-treated animals (Figure 13). Two-way ANOVA (Antagonist Dose x Drug) revealed significant main effects of antagonist dose, $F(2,76) = 3.7$, $p<.05$, and drug (U50,488H vs saline), $F(1,76) = 8.8$, $p<.01$. Follow-up analyses (Tukey's) indicated that latency to paw shake was significantly increased in U50,488H treated animals and was decreased by 20 mg/kg nor-BNI relative to saline or 5 mg/kg nor-BNI ($ps<.05$). No interaction was seen ($F<1$), indicating that nor-BNI decreased analgesia in both saline and U50,488H treated animals.

Discussion

As in Experiments 1 and 2, pretreatment with a selective opioid antagonist had no effect on the expression of ethanol-induced place preference. Interestingly, 20 mg/kg nor-BNI increased locomotor activity. Analgesia produced by the κ receptor agonist U50,488H was blocked by nor-BNI pretreatment. The failure of U50,488H (20 mg/kg) to produce analgesia on the first analgesia test, was overcome by increasing the dose to 33 mg/kg

Figure 13 - Experiment 3: Analgesia. Mean (+SEM) latency to paw shake 30 min after U50,488H (33.3 mg/kg) or saline in mice treated with 0, 5, or 20 mg/kg nor-BNI approximately 140 h earlier. n=12-15/dose combination.

Figure 13


Experiment 3 - Analgesia Test
Mean Latency to Paw Shake (sec)
60
50
40
30
20
10
0
0
33
Dose U50,488H (mg/kg)
Saline
nor-BNI (5 mg/kg)
nor-BNI (20 mg/kg)

and by decreasing the temperature of the hot-plate. The results of the analgesia test, taken with the finding that nor-BNI increased locomotor activity during the preference test, indicate that doses were behaviorally active and that κ receptors were blocked. Thus, these data strongly suggest that the κ receptor is not involved in expression of ethanol-inconditioned place preference and that naloxone's effects are not mediated via this receptor system.

As the second analgesia test took place six days after nor-BNI administration, these data confirm the extremely long-lasting effects of this antagonist previously reported (Endoh et al., 1992; Horan, Taylor, Yamamura, & Porreca, 1992; Jones & Holtzman, 1992). As with β-FNA, nor-BNI decreased paw shake latencies in saline-treated animals, i.e., reduced baseline analgesia levels. In contrast, a previous study reporting effects of nor-BNI alone on baseline analgesia showed that nor-BNI elicited antinociception in rhesus monkeys for less than four h after administration (Butelman, Negus, de Costa, & Woods, 1993). Both findings suggest that a basal level of κ opioid activity contributes to baseline latencies in the paw flick test, although the direction of the effect is variable. Finally, a study in mice found no effects of nor-BNI on antinociception in saline-treated controls (Broadbear, Negus, Butelman, de Costa, & Woods, 1994). Given that different analgesia tests were used in each study (hot-plate, warm-water tail withdrawal, acetic acid-induced

writhing), the disparate effects of nor-BNI may reflect differential involvement of the κ opioid system in these various measures of analgesia.

The ability of nor-BNI to increase locomotor activity should not be surprising in light of the suppressive effect that κ agonists have on this measure (von Voigtlander et al., 1983). However, few, if any, reports of this effect exist in the literature. It has been speculated that κ agonists decrease activity by decreasing mesolimbic DA release (Di Chiara & Imperato, 1988). Arguably, antagonism of κ receptors could, therefore, increase DA release. Spanagel et al. (1990) reported exactly this result: infusion of nor-BNI into the nucleus accumbens dose-dependently increased DA release. Therefore, the increase in activity seen in the current study could result from increased dopaminergic activity in the NAC.

As discussed earlier, activity levels during preference testing can affect the expression of conditioned responses (see Discussion, Experiment 1). However, increases in activity generally are thought to reduce expression of conditioned preferences, a result not seen in the present study.

Summary and Conclusions: Experiments 1-3

Ethanol-induced conditioned place preference was consistently demonstrated. Unexpectedly, antagonists selective for the three opioid receptor types had no effect on the expression of this preference. These

findings are probably not the result of inappropriate doses since each experiment showed some behavioral effect of the antagonist (antagonism of analgesia and/or alteration of activity). In spite of this, however, it is possible that antagonist dose-response curves for these effects and that of attenuation of expression may differ. With the limited number of doses used in the current studies, this possibility cannot be completely ruled out.

EXPERIMENTS 4-5: EXTENSION OF NALOXONE'S EFFECTS

Antagonism by naloxone is generally considered to implicate opioid mechanisms in a particular drug effect, and it is assumed that one of the opioid receptor types is involved. Accordingly, it is somewhat surprising that selective blockade of the various receptors had no effect on the expression of ethanol place preference, a behavior consistently attenuated by naloxone (Cunningham et al., 1995). However, naloxone has been shown to have effects not mediated through opioid receptors (Sawynok, Pinsky, & LaBella, 1979), raising the possibility that the effect of naloxone observed by Cunningham et al. was not an opioid effect.

Using other opioid antagonists (as in Experiments 1-3) in addition to naloxone is one method of determining involvement of endogenous opioids in a given drug effect or behavior (Sawynok et al., 1979). Another method is to preclude nonopioid effects of naloxone by testing (+)-naloxone, the opioid inactive stereoisomer of naloxone (Corbett et al., 1993; Sawynok et al., 1979).

Experiments 4A and 4B: Stereospecifity of Naloxone's Effects

Introduction & Rationale

Opioid recognition sites are stereochemically selective (Portoghese, 1993), consequently, the unnatural isomer of naloxone, (+)-naloxone, is inactive as an opioid antagonist (Gayton, Lambert, & Bradley, 1978; Iijima, Minamikawa, Jacobson, Brossi, Rice, & Klee, 1978). Therefore, replication of a naloxone effect with (+)-naloxone leads to the conclusion that a nonopioid mechanism is involved. The absence of an effect with (+)-naloxone does not, however, ensure that naloxone is working through opioid receptors, although it is generally inferred.

Stereospecific actions of naloxone have been observed in many experimental situations. Antinociception elicited by morphine (Stein, Millan, Yassouridis, & Herz, 1988) or nitrous oxide (Moody, Mattson, Newman, Rice, & Skolnik, 1989) is attenuated by naloxone but not (+)-naloxone (up to 40 mg/kg). Naloxone-induced decreases in sham feeding were not mimicked by 1.25, 2.5 or 10.0 mg/kg (+)-naloxone (Kirkham & Cooper, 1995), confirming the involvement of opioids in ingestive behaviors. Finally, the aversive motivational effects of naloxone administration are not seen with (+)-naloxone. Mucha and Walker (1987) found that 1.0 mg/kg naloxone produced a conditioned taste aversion while the same dose of (+)-naloxone did not. Similarly, i.v. administration of 0.5 mg/kg (+)-naloxone

did not result in a conditioned place aversion, although the same dose of naloxone did (Mucha, van der Kooy, O'Shaughnessy, & Bucenieks, 1982).

To determine if some non-opioid receptor mediated effect of naloxone could be mediating its effects on expression of place preference, (+)-naloxone was administered prior to preference testing in Experiments 4A and 4B. In Experiment 4A, two doses of (+)-naloxone were tested while Experiment 4B used one dose each of naloxone and (+)-naloxone. Results for the two experiments are reported separately.

Methods

Subjects

Subjects were 96 (Experiment 4A) and 92 (Experiment 4B) male D2 mice (tested at approximately 10 weeks of age) housed as in Experiment 1.

Apparatus

For Experiment 4A the 12-box place conditioning apparatus was the same as in Experiments 1-3. The apparatus for Experiment 4B was identical with the exception of the grid floors which were made of 2.3-mm stainless steel rods rather than the 3.2-mm rods used in Experiments 1-3 and 4A.

Drugs

(+)-Naloxone hydrochloride was obtained from the NIDA Drug Supply System and naloxone hydrochloride was purchased from Sigma. Both drugs were dissolved in physiological saline and injected i.p. in a volume of 10 ml/kg. In Experiment 4A, doses of 1.5 and 10.0 mg/kg (+)-naloxone were

used. These were the lowest and highest effective doses of naloxone used by Cunningham et al. (1995). If the effects seen in those studies were a result of nonopioid activity, the same doses of (+)-naloxone should have the same effect. In Experiment 4B, naloxone and (+)-naloxone were both administered at a dose of 10 mg/kg. In both experiments a pretreatment time of 15 min was used based on findings of Cunningham et al. (1995).

Procedure

In Experiment 4A, mice were transported to the experimental room as in Experiments 1-3. In Experiment 4B, mice were housed in the experimental room. For each experiment, subjects were randomly assigned to one of three treatment groups and exposed to a differential place conditioning procedure. For the 60-min preference test, mice in Experiment 4A received an injection of saline (Group 0), 1.5 mg/kg (+)-naloxone (Group 1.5+), or 10 mg/kg (+)-naloxone (Group 10+) 15 min prior to a saline injection and placement in the apparatus for testing.

For both 60-min preference tests in Experiment 4B, mice received an injection of saline (Group 0), 10 mg/kg (+)-naloxone (Group 10+) or 10 mg/kg naloxone (Group 10) 15 min prior to a saline injection and testing. Test 2 took place three days after Test 1.

Results: Experiment 4A

Poor injection placement resulted in the exclusion of three mice (one Group 0, two Group 10+) from analyses.

Ethanol induced a conditioned place preference that was not affected by administration of (+)-naloxone. Preference data from the three time intervals for each dose group are depicted in Figure 14. Two-way ANOVA (Dose x Conditioning Group) showed a significant main effect of conditioning group in each interval; Minutes 1-10, $F(1,87) = 108.5$; Minutes 11-30, $F(1,87) = 150.3$; Minutes 31-60, $F(1,87) = 84.8$, $ps<.001$. No main effects or interactions involving dose were evident ($Fs<1$).

Activity during the preference test decreased over time (Figure 15). (+)-Naloxone had no effect on activity in any time interval ($Fs<1$).

Results: Experiment 4B

One Group 10 mouse suffered leg and foot injury after getting caught in the metal cage top and data from this mouse were excluded. Data from one Group 10+ mouse were excluded from Test 2 analyses after a poorly placed injection.

Ethanol induced a place preference that was not significantly affected by treatment with (+)-naloxone or naloxone. While naloxone appeared to attenuate expression in the last half of the test session, the interaction was not significant. Data from the first preference test are shown in Figure 16. Two-way ANOVA (Drug x Conditioning Group) revealed a significant main effect of conditioning group in each time interval; Minutes 1-10, $F(1,85) = 168$, Minutes 11-30, $F(1,85) = 98.2$, Minutes 31-60, $F(1,85) = 18.9$, all $ps<.001$. There were no significant effects involving drug treatment.

Figure 14 - Experiment 4A: Preference Data. Mean (+SEM) seconds per minute spent on the grid floor during Minutes 1-10, 11-30 and 31-60 of the preference test for mice injected with saline or (+)-naloxone 15 min prior to testing. Conditioning groups (n=14-16) are as in Figure 1.

Experiment 4A - Preference Test

Figure 14

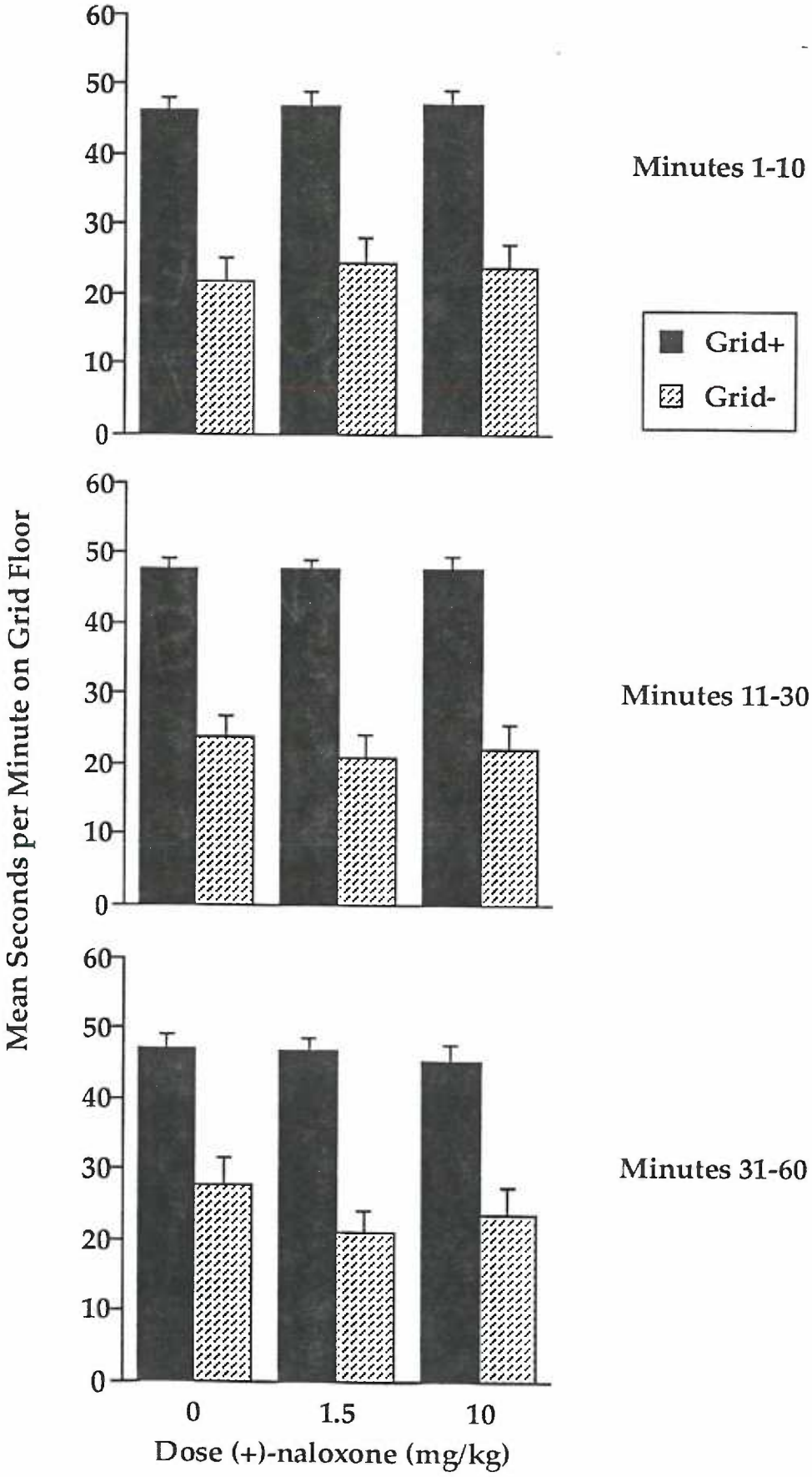


Figure 15 - Experiment 4A: Test Activity. Mean (+SEM) activity counts per minute during Minutes 1-10, 11-30 and 31-60 of the preference test for each (+)-naloxone dose group (n=30-32/dose group).

Figure 15

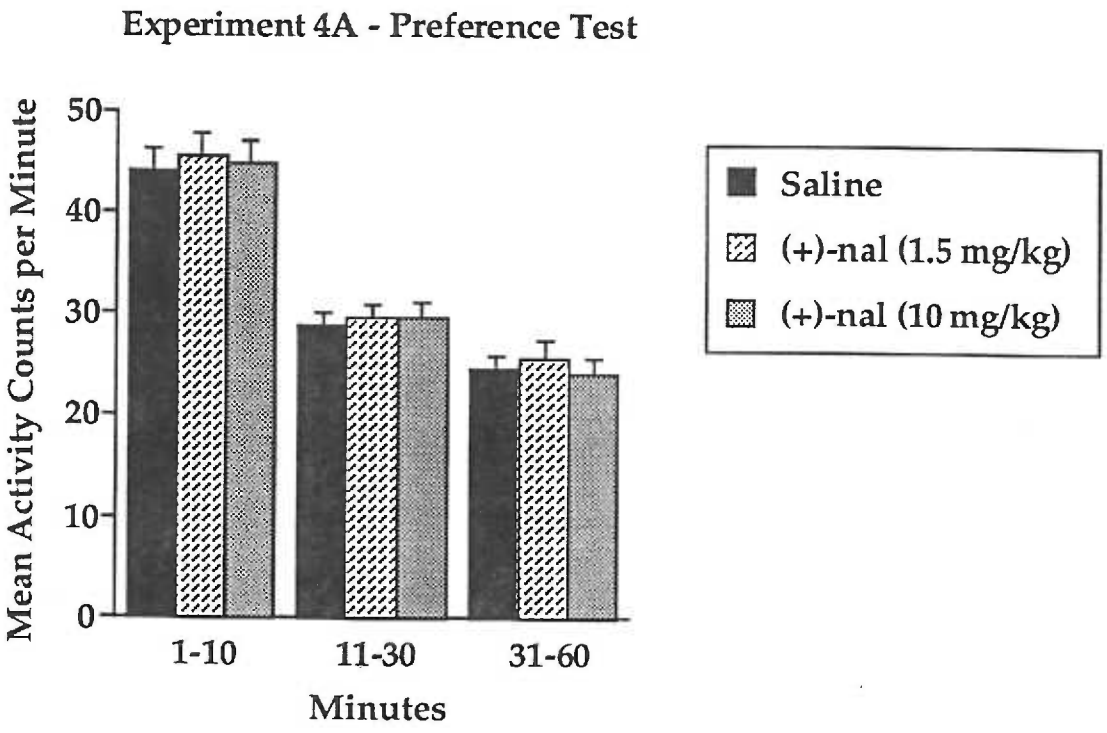
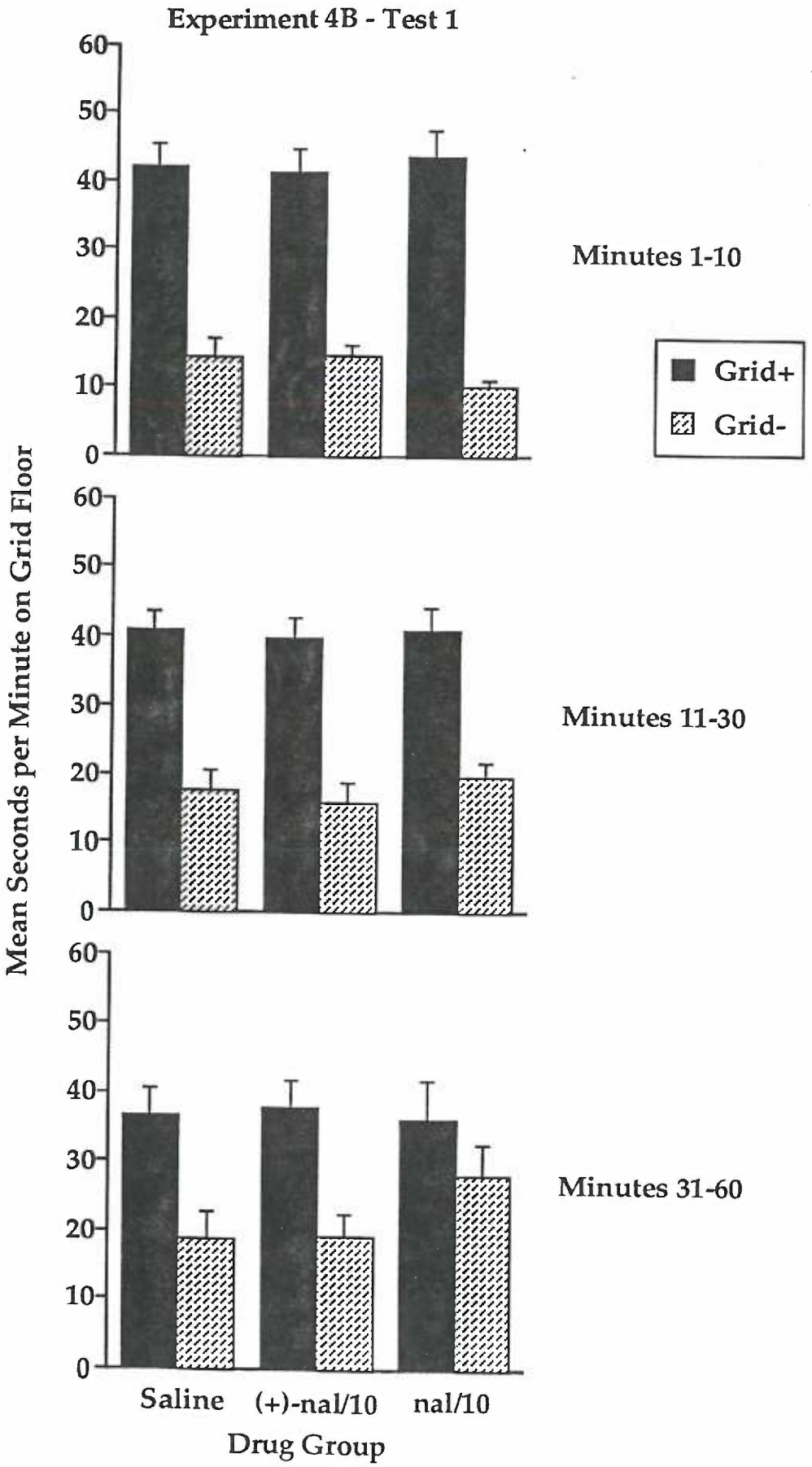

Experiment 4A - Preference Test
Mean Activity Counts per Minute
50
40
30
20
10
0
1-10
11-30
31-60
Minutes
Saline
(+)-nal (1.5 mg/kg)
(+)-nal (10 mg/kg)

Figure 16 - Experiment 4B: Test 1 Preference. Mean (+SEM) seconds per minute spent on the grid floor during Minutes 1-10, 11-30 and 31-60 of the first preference test for mice in the three treatment groups. Conditioning groups (n=14-16) are as in Figure 1. (+)-nal/10 = 10 mg/kg (+)-naloxone; nal/10 = 10 mg/kg naloxone.

Figure 16


Experiment 4B - Test 1
Mean Seconds per Minute on Grid Floor
60
50
40
30
20
10
0
Minutes 1-10
Grid+
Grid-
Minutes 11-30
Minutes 31-60
Saline
(+)-nal/10
nal/10
Drug Group

As in earlier studies, naloxone pretreatment decreased activity on the test while (+)-naloxone had no effect (Figure 17). ANOVA revealed significant main effects of drug in all three time intervals; Minutes 1-10, $F(2,88) = 7.1$, $p<.01$, Minutes 11-30, $F(2,88) = 8.4$, $p<.001$, Minutes 31-60, $F(2,88) = 5.2$, $p<.01$. Follow-up analyses (Tukey's) indicated that naloxone decreased activity relative to (+)-naloxone in Minutes 1-10 and relative to both saline and (+)-naloxone in Minutes 11-30 and 31-60.

On the second preference test, expression of preference was blocked by naloxone but not by (+)-naloxone. Data from Test 2 are presented in Figure 18. Two-way ANOVA (Drug x Conditioning Group) revealed a significant main effect of conditioning group in the first two time intervals only; Minutes 1-10, $F(1,84) = 33.0$, $p<.001$, Minutes 11-30, $F(1,84) = 8.3$, $p<.01$. Significant main effects of drug were also present in Minutes 11-30, $F(2,84) = 3.6$, $p<.05$, and in Minutes 31-60, $F(2,84) = 3.7$, $p<.05$. However, the drug x conditioning group interaction was significant in all three intervals; $Fs(2,84) = 5.9, 7.8$, and 7.2, $ps<.01$, for Minutes 1-10, 11-30, and 31-60, respectively. Follow-up analyses indicated that Grid+ and Grid- subgroups of Group 10+ were significantly different in all three intervals and in Minutes 1-10 and 11-30 for subgroups in Group 0. No preference was seen in Group 10 in any interval (all $ps<.05$).

Figure 17 - Experiment 4B: Test 1 Activity. Mean (+SEM) activity counts per minute during Minutes 1-10, 11-30 and 31-60 of the first preference test for each treatment dose group (n=28-32). (+)-nal/10 = 10 mg/kg (+)-naloxone; nal/10 = 10 mg/kg naloxone.

Figure 17

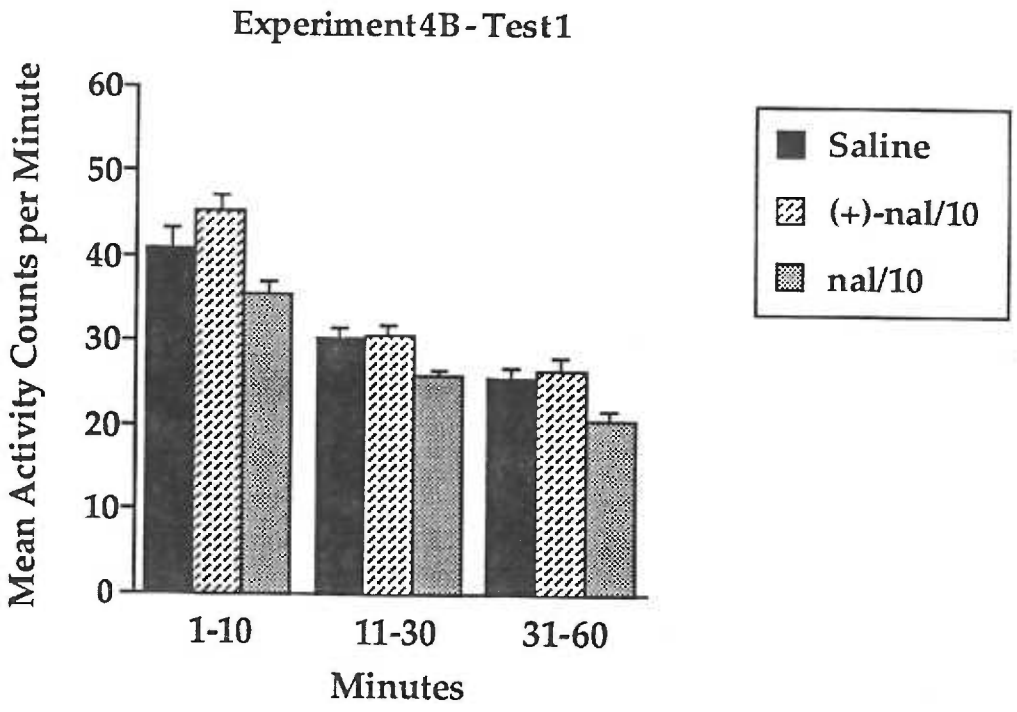
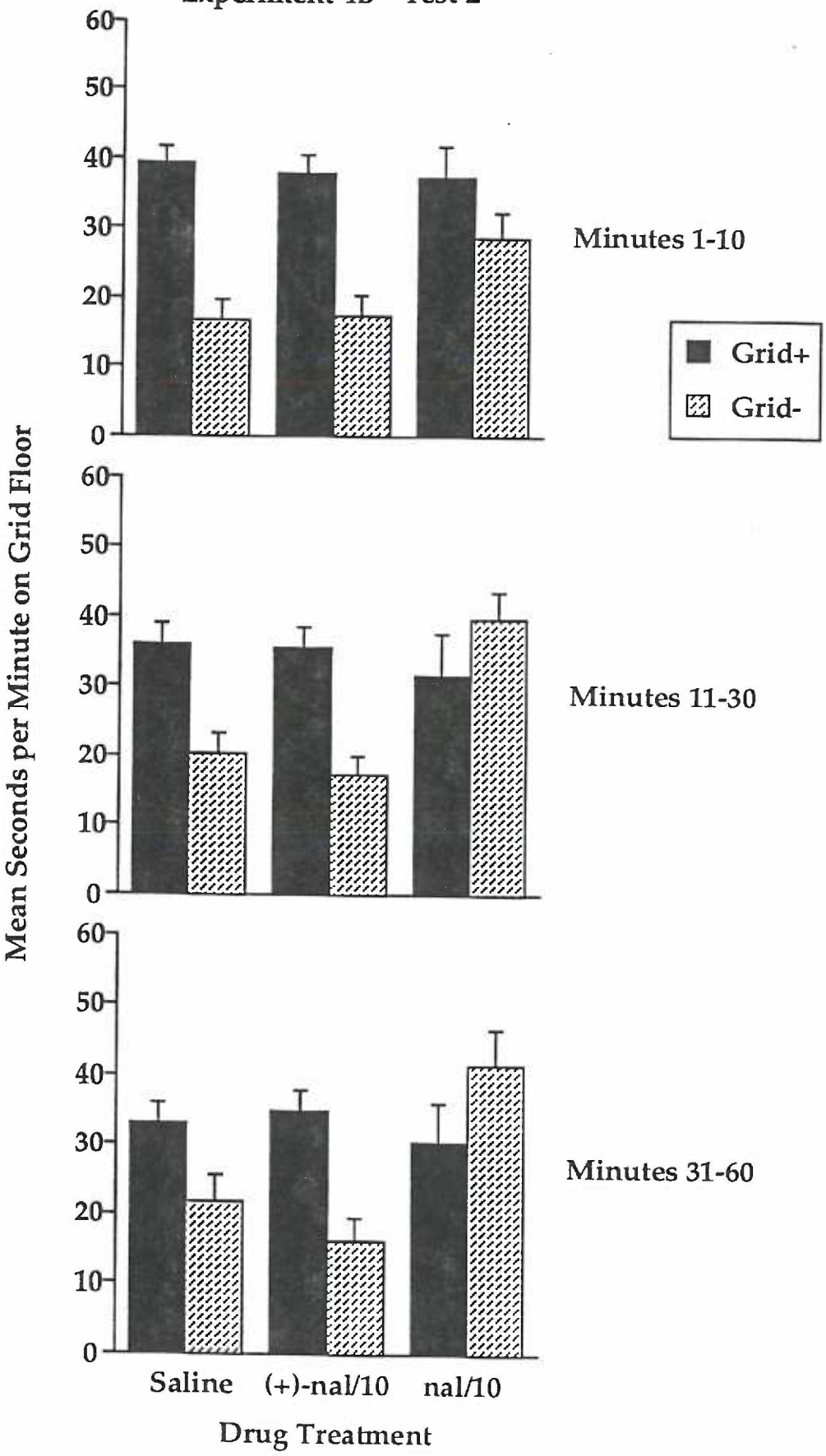

Experiment4B-Test1
Mean Activity Counts per Minute
60
50
40
30
20
10
0
1-10
11-30
31-60
Minutes
Saline
(+)-nal/10
nal/10

Figure 18 - Experiment 4B: Test 2 Preference. Mean (+SEM) seconds per minute spent on the grid floor during Minutes 1-10, 11-30 and 31-60 of Test 2 for mice in the three treatment groups. Conditioning groups (n=14-16) are as in Figure 1. Treatment groups are as in Figures 16 and 17.

Figure 18


Experiment 4B - Test 2
Mean Seconds per Minute on Grid Floor
60
50
40
30
20
10
0
Minutes 1-10
Grid+
Grid-
Minutes 11-30
Minutes 31-60
Saline
(+)-nal/10
nal/10
Drug Treatment

As on the first test, naloxone decreased activity while (+)-naloxone had no effect (Figure 19). One-way ANOVA yielded significant effects of drug in each interval; Minutes 1-10, $F(2,87) = 5.0$, $p<.01$; Minutes 11-30, $F(2,87) = 15.5$, $p<.001$;Minutes 31-60, $F(2,87)= 9.5$, $p<.001$. Post-hoc analyses (Tukey's) revealed that activity in Group 10 was significantly lower than that in Groups 0 and 10+ in all three intervals ($ps<.05$).

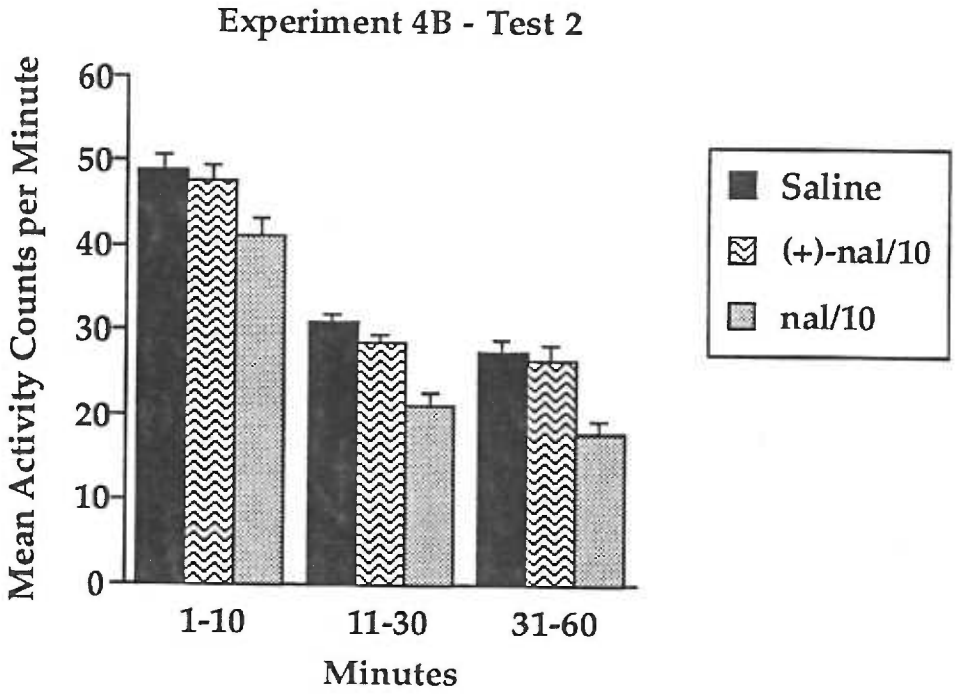
Discussion

The expression of ethanol-induced place preference was not affected by administration of (+)-naloxone, a stereoisomer of naloxone that does not bind specifically to opioid receptors but shares other non-specific effects of naloxone (Sarne, Flitstein, & Oppenheimer, 1991). These findings suggest that the ability of naloxone to attenuate expression of place preference is not caused by non-specific actions at sites other than opioid receptors.

In accord with the results of Cunningham et al. (1995), 10 mg/kg naloxone attenuated maintenance of expression of preference in Experiment 4B. Although this effect did not reach significance in the overall ANOVA on the first test, the effect was highly significant on the second test. As argued by Cunningham et al. (1995), this effect is not likely to be caused by naloxone's effects on activity since a decrease in activity is generally thought to result in an enhanced expression of preference.

Figure 19 - Experiment 4B: Test 2 Activity. Mean (+SEM) activity counts per minute during Minutes 1-10, 11-30 and 31-60 of the second preference test for each treatment group (n=28-32). Legend is as in Figure 17.

Figure 19


Experiment 4B - Test 2
Mean Activity Counts per Minute
60
50
40
30
20
10
0
1-10
11-30
31-60
Minutes
Saline
(+)-nal/10
nal/10

That administration of (+)-naloxone had no effect on locomotor activity is consistent with a report by Jacquet (1980) showing that 100 mg/kg (+)-naloxone had no effect on novelty-induced behavioral activation in mice.

Had administration of (+)-naloxone attenuated the expression of preference in Experiment 4, it could be concluded that previously observed effects of naloxone were not mediated by specific opioid receptors. In the absence of such an effect, no definitive conclusion can be reached. Finally, although maintenance of expression was diminished by naloxone as reported by Cunningham et al., (1995), this effect was significant only on Test 2.

The lack of an obvious effect of naloxone administration on Test 1 in Experiment 4B is surprising in light of the robust effect seen in previous studies reported by Cunningham et al. (1995). One possible explanation is that previous results on Test 1 were spurious. This is highly unlikely, as naloxone was shown to attenuate preference expression in four separate experiments, with several different doses and two different pretreatment times. A more plausible explanation is that, for some reason, the strength of preference conditioning was stronger in Experiment 4B than in previous studies. Thus, naloxone was not significantly effective until Test 2, after mice had undergone 60 min of extinction during Test 1.

As mentioned earlier, a preference test is an extinction trial, in that the CS+ (the floor) is present, but ethanol (the US) is not. With repeated presentation of the CS in the absence of the US, the learned association

between the two stimuli is weakened and the strength of the conditioned response may also be decremented. Accordingly, in Experiment 4B, it may be that the conditioned preference was too strong on Test 1 to be attenuated by naloxone. However, after 60 min of extinction, the conditioned response was sufficiently weakened so that naloxone could be effective. To accept this explanation, it must be argued that the conditioned response was stronger on Test 1 than in previous experiments but was of similar strength after 60 min of extinction.

Experiments 5A and 5B: Effects of High Dose Naloxone

Introduction & Rationale

If naloxone was ineffective in Experiment 4B because preference was stronger than in previous experiments, increasing the dose of naloxone may overcome the increased preference strength. To investigate this possibility, Experiments 5A and 5B were conducted to replicate previous results with naloxone and to extend the dose range studied. Two studies that were identical in procedure and differed only in the dose of naloxone administered on the preference tests are described as Experiments 5A and 5B. Data from these experiments were combined as explained below and are presented as Experiment 5.

Methods

Subjects

Subjects were 216 adult male D2 mice (Experiment 5A, n=96; Experiment 5B, n=120) housed as in Experiment 1. Mice were obtained at 6 weeks and tested at approximately 10 weeks of age.

Apparatus

The 12-box place conditioning apparatus was as in Experiment 4B.

Drugs

Naloxone was administered in doses of 1.5, 10, 20, and 30 mg/kg. These doses were chosen to replicate (1.5 and 10 mg/kg) and extend (20 and 30 mg/kg) the previous findings.

Procedure

Fifteen min prior to a saline injection and placement in the apparatus for the first 60-min preference test, mice in Experiment 5A received an injection of saline (Group 0) or naloxone (1.5 or 10 mg/kg; Groups 1.5 and 10, respectively) and mice in Experiment 5B received saline, 10, 20 or 30 mg/kg of naloxone (Groups 0, 10, 20 and 30, respectively). A second test was conducted two or three days after Test 1 in Experiments 5A and 5B, respectively. For the second preference test in Experiment 5A, mice in Group 1.5 and Group 10 received an injection of 10 or 20 mg/kg naloxone, respectively, prior to testing. Mice in Experiment 5B received the same dose of naloxone on Test 2

as on Test 1. Left/right positions of the test floors were the same for both tests.

Results

In Experiment 5A, equipment error caused Test 2 data from one Group 0 mouse to be lost. Test 2 data from a mouse in Group 10 were excluded because of an incorrect injection. In Experiment 5B, data from four mice each from Groups 0, 10 and 20 were excluded because of experimenter error. Three mice (two from Group 10 and one from Group 30) were excluded because of misplaced or incorrect injections. One Group 30 mouse died before the study began, and two additional mice (one each from Groups 10 and 30) were excluded because of deformed or crippled paws.

Initial analyses of data from comparable dose groups in Experiments 5A and 5B indicated that no there were no significant differences between experiments. Thus, Test 1 data from all mice injected with a given dose were combined, as were Test 2 data, and are presented as such. As with all previous studies, data are presented for three successive time intervals. Figure 20 depicts preference data from Test 1 for each naloxone dose group. Within each interval, ethanol induced a significant place preference that was not affected by naloxone pretreatment, although a dose effect was seen in Minutes 31-60. Two-way ANOVA (Dose x Conditioning Group) yielded significant main effects of conditioning group in each interval; Minutes 1-10, $F(1,188) = 221.9$; Minutes 11-30, $F(1,188) = 115.3$; Minutes 31-60, $F(1,188) = 51.4$,

Figure 20 - Experiment 5: Test 1 Preference. Mean (+SEM) seconds per minute spent on the grid floor during Minutes 1-10, 11-30 and 31-60 of the first preference test for mice in each naloxone dose group. Naloxone was injected 15 min prior to the test. Conditioning groups (n=13-29) are as in Figure 1.

Figure 20

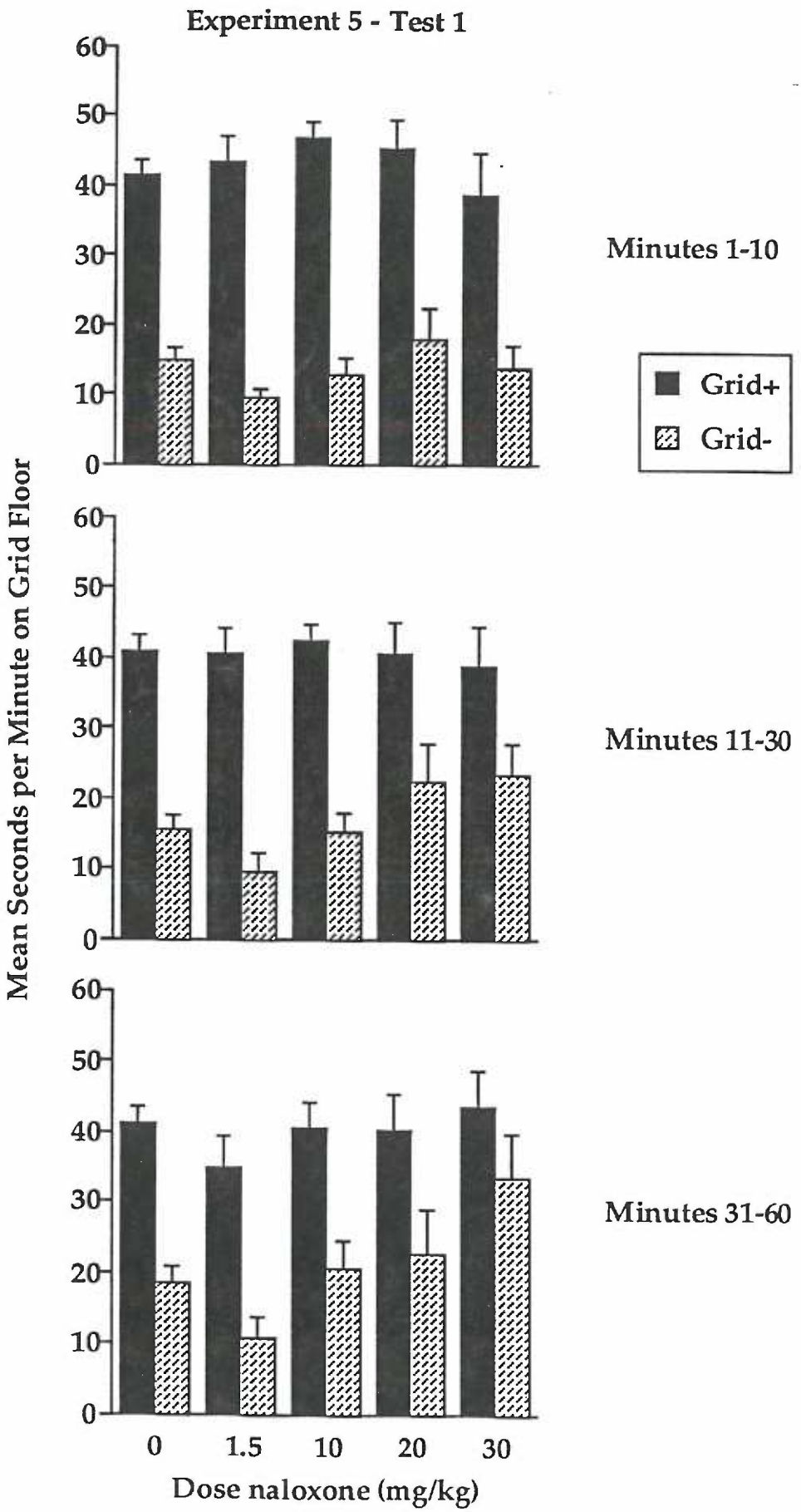

Experiment 5 - Test 1
Mean Seconds per Minute on Grid Floor
60
50
40
30
20
10
0
Minutes 1-10
Grid+
Grid-
Minutes 11-30
Minutes 31-60
0
1.5
10
20
30
Dose naloxone (mg/kg)

Figure 21 - Experiment 5: Test 1 Activity. Mean (+SEM) activity counts per minute during Minutes 1-10, 11-30 and 31-60 of the first preference test for each naloxone dose group (n=26-58).

Figure 21

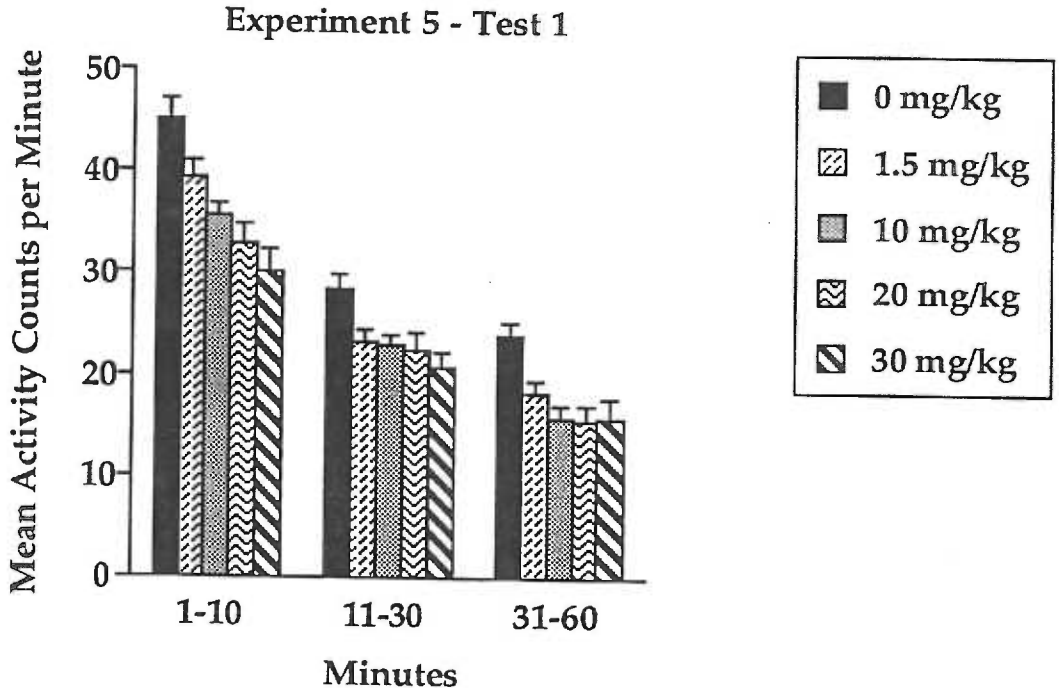
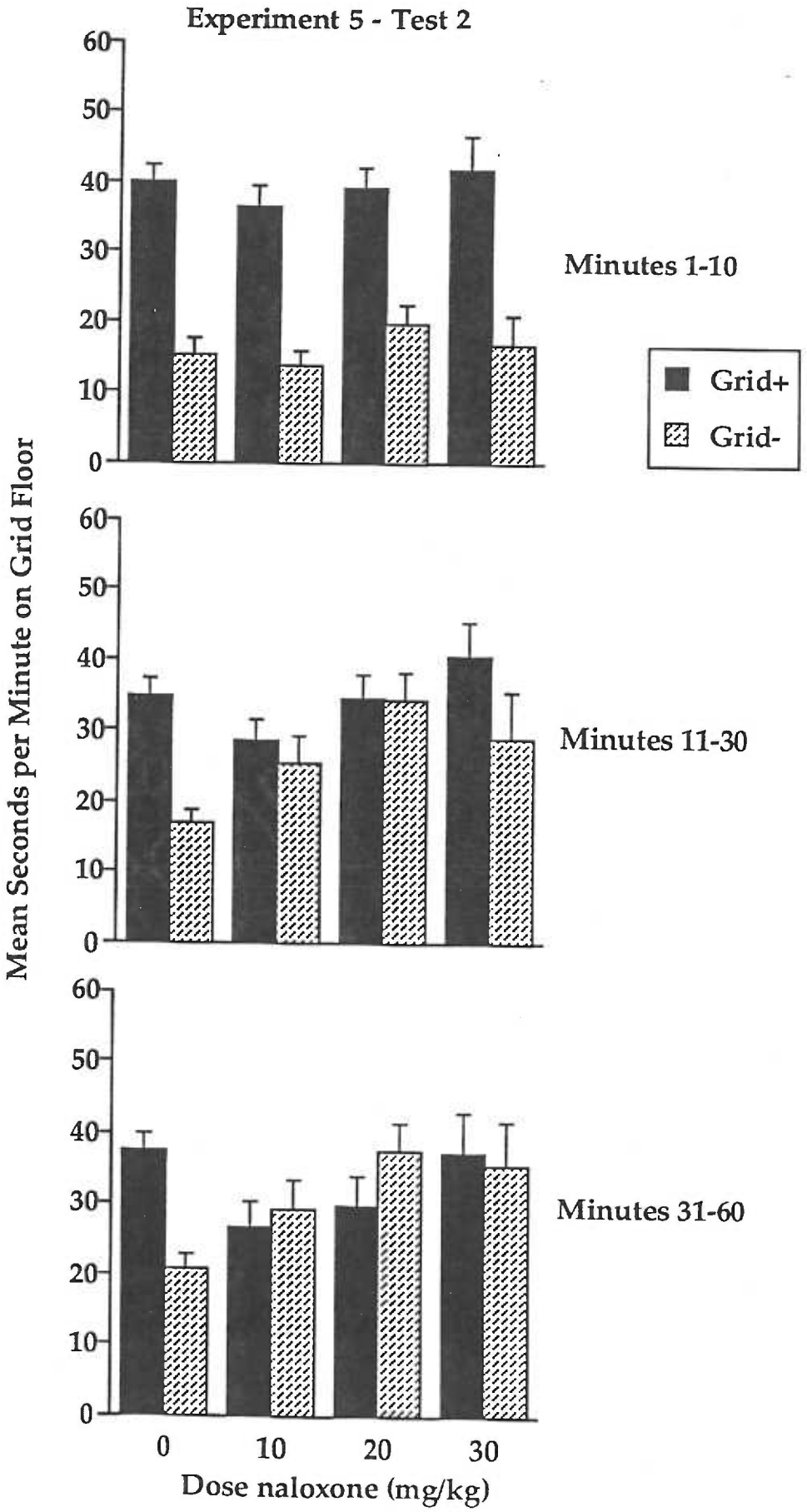

Experiment 5 - Test 1
Mean Activity Counts per Minute
50
40
30
20
10
0
1-10
11-30
31-60
Minutes
0 mg/kg
1.5 mg/kg
10 mg/kg
20 mg/kg
30 mg/kg

Figure 22 - Experiment 5: Test 2 Preference. Mean (+SEM) seconds per minute spent on the grid floor during Minutes 1-10, 11-30 and 31-60 of Test 2 for mice in each naloxone dose group. Naloxone was given 15 min prior to testing. Conditioning groups (n=13-29) are as in Figure 1.

Figure 22


Experiment 5 - Test 2
Mean Seconds per Minute on Grid Floor
60
50
40
30
20
10
0
Minutes 1-10
Grid+
Grid-
Minutes 11-30
Minutes 31-60
0
10
20
30
Dose naloxone (mg/kg)

11-30, $F(3,188) = 3.5$, $p<.05$. In both Minutes 11-30 and Minutes 31-60, however, the dose x conditioning group interaction was significant; Minutes 11-30, $F(3,188) = 3.1$, $p<.05$; Minutes 31-60, $F(3,188) = 4.3$, $p<.01$. Follow-up analyses (Tukey's) of the interactions in both intervals indicated that the Grid+ and Grid- subgroups differed only in animals treated with saline (ps<.05).

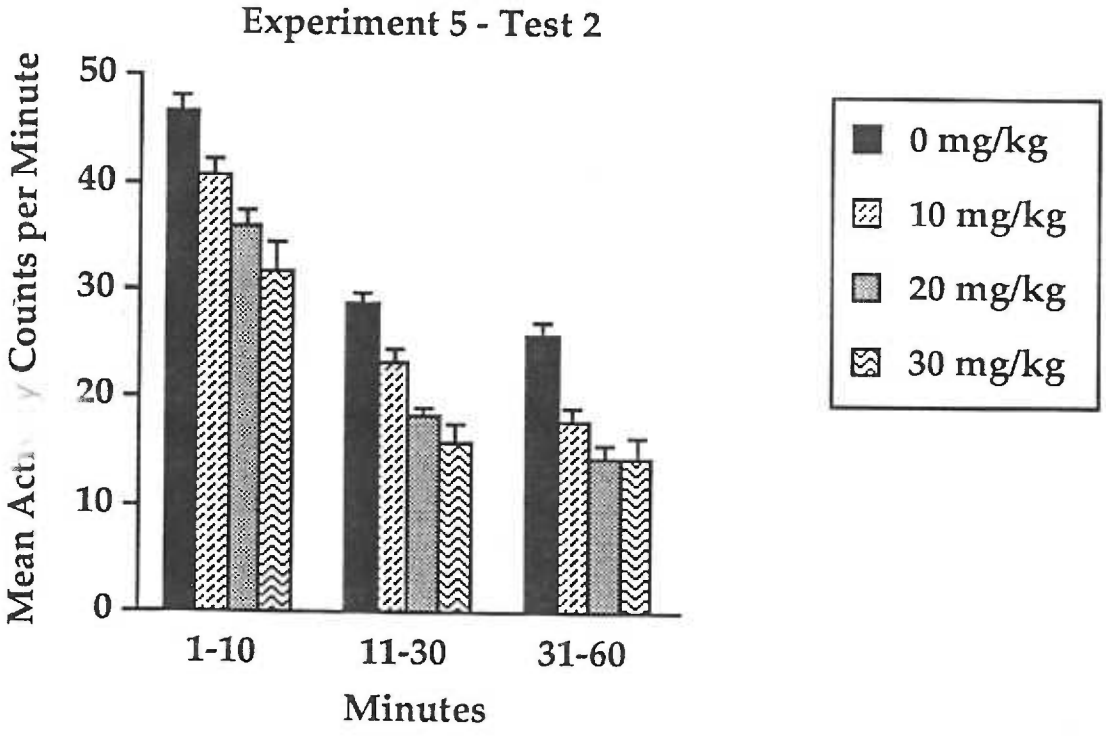
Activity data from Test 2 are depicted in Figure 23. Naloxone produced a dose-dependent decrease in activity. ANOVAs applied to data from each interval revealed a significant main effect of dose in each of the three time intervals; Minutes 1-10, $F(3,192) = 12.0$; Minutes 11-30, $F(3,192) = 22.6$; Minutes 31-60, $F(3,192) = 17.0$, all ps<.001. Post-hoc tests indicated that in all intervals, each dose of naloxone reduced activity relative to saline (Tukey's, ps<.05). In addition, activity in Group 30 was significantly lower than that in Group 10 in Minutes 1-10 and in Minutes 11-30. In Minutes 11-30, Group 10 activity was also decreased relative to Group 20 (all ps<.05).

Discussion

Ethanol-induced conditioned place preference persisted across two preference tests in saline treated animals. Effects of naloxone administration (1.5, 10, 20 or 30 mg/kg) prior to the first test on expression of preference approached, but did not reach, significance. However, naloxone (10, 20 or 30 mg/kg) attenuated expression of preference when given again before the

Figure 23 - Experiment 5: Test 2 Activity. Mean (+SEM) activity counts per minute during Minutes 1-10, 11-30 and 31-60 of the second preference test for each naloxone dose group (n=27-58).

Figure 23


Experiment 5 - Test 2
Mean Activity Counts per Minute
50
40
30
20
10
0
1-10
11-30
31-60
Minutes
0 mg/kg
10 mg/kg
20 mg/kg
30 mg/kg

second test. As expected, naloxone decreased locomotor activity throughout the test; this effect was dose-dependent in Minutes 1-10.

The finding that Group 30 mice showed a bias for the grid floor on Test 1 suggests that the lack of effect of 30 mg/kg naloxone may be the result of an unconditioned bias. Visual inspection of Figure 20 reveals that while Grid- mice in Group 30 spent half of each minute on the grid floor (indicating no preference), Grid+ mice retained a preference for the grid floor. Assuming that this subgroup of mice began the experiment with an innate preference for the grid floor, the ethanol-induced conditioned preference for this floor would be added to the basal level. Therefore, it is possible that naloxone did indeed attenuate expression of conditioned preference (as seen in Grid- animals) but had no effect on the unconditioned bias for the grid floor in Grid+ animals.

Though ineffective on Test 1, 10 mg/kg naloxone on Test 2 blocked maintenance of expression after 10 min of the test. The same pattern was seen with the higher doses of naloxone. These findings are consistent with the idea that the conditioning procedure resulted in a stronger conditioned preference than in previous studies, thereby shifting the dose-response curve for naloxone to the right. In other words, mice were resistant to naloxone-induced facilitation of extinction, making high doses necessary.

The fact that a high dose of naloxone (30 mg/kg) was necessary to produce an effect on expression of preference in Experiment 5 raises the

possibility that a non-opioid mechanism was involved. It has been suggested that high doses of naloxone can act as an antagonist at the GABA receptor (Dingledine, Iversen, & Breukker, 1978). However, these investigators used doses in excess of 200 mg/kg to demonstrate proconvulsant activity. Pretreatment of mice with diazepam increased the IC_{50} of naloxone to around 400 mg/kg, leading to the suggestion that the convulsant properties were mediated by GABA. It is doubtful that the dose of 30 mg/kg used in the current study could have this effect. Finally, given that 1.5 mg/kg naloxone was effective in studies by Cunningham et al. (1995), and that (+)-naloxone had no effect on expression of preference, it is unlikely that a non-opioid mechanism mediates naloxone's effects.

Summary & Conclusions: Experiments 4-5

In Experiments 4A and 4B, the effect of naloxone was found to be stereospecific, suggesting but not proving, that it is indeed mediated through opioid receptors. Although naloxone was effective in attenuating maintenance of expression, the effect was significant only on Test 2, leading to speculation that naloxone's dose-response curve may have been shifted to the right. Experiment 5 tested this hypothesis by increasing the dose of naloxone. While the effects of high dose naloxone did not reach statistical significance on the first test, a strong trend was apparent in the data, substantiating the idea of a shift in the dose-response curve. On the second test, all doses of

naloxone were effective in attenuating maintenance of preference. As stated earlier, this is also consistent with stronger preference in Experiments 4B and 5 and a concomitant shift to the right of naloxone's dose-response curve.

EXPERIMENTS 6 & 7: PROCEDURAL VARIABLES AND NALOXONE'S EFFECTS ON EXPRESSION OF PREFERENCE

The suggestion that the dose-reponse curve for naloxone was shifted to the right in Experiments 4B and 5 implies that, for some reason, sensitivity to naloxone in these mice was lower than in mice used in Cunningham et al. (1995). Since inbred D2 mice (which are theoretically genetically identical to one another) from Jackson Labs were used in the previous studies as well as in the current studies, the apparent differences in naloxone sensitivity are not likely to be inherent in the mice. Similarly, the naloxone used in both sets of studies was purchased from Sigma and was from the same lot number. Accordingly, Experiments 6 and 7 examined the effects of slight procedural differences on naloxone sensitivity.

Experiment 6: Effects of a Break During Conditioning

Introduction & Rationale

One possible explanation for the disparity in findings between Experiment 5 and previous studies reported in Cunningham et al. (1995) is a slight procedural difference. Experiments 1-5B were run on consecutive days

until after the first preference test. In contrast, in previous studies a two-day break occurred between conditioning trials two and three (after day five). It is possible that training on consecutive days resulted in a stronger association between the CS and ethanol's effects than when conditioning was interrupted by a two-day break. However, since magnitude of preference does not differ with the two procedures, it must be argued that strength of conditioning does not translate directly into magnitude of preference. Alternatively, it may be that a greater magnitude of preference cannot be behaviorally expressed, regardless of strength of conditioning, i.e., a ceiling exists which limits performance.

Experiment 6 was designed to determine whether a two-day break between conditioning trials two and three would affect naloxone's ability to attenuate expression of preference. If conditioning on consecutive days does indeed shift naloxone's dose-response curve to the right by making the conditioned preference stronger, the same dose of naloxone should be effective in mice given a break during conditioning but not in those run consecutively.

Methods

Subjects

Subjects were 96 adult male D2 mice (obtained at 6 weeks and tested at approximately 10 weeks old) housed as in Experiment 1.

Apparatus

The 12-box place conditioning apparatus was as in Experiments 4B-5.

Drugs

A dose of 10 mg/kg naloxone was effective against preference expression on Test 1 in Cunningham et al. (1995) and was ineffective on Test 1 in Experiments 4B and 5. Therefore this dose was chosen.

Procedure

Mice were randomly assigned to one of two dose groups (0 or 10 mg/kg) within one of two procedural groups, B or NB (break or no-break, respectively). For mice in Group NB, conditioning proceeded as in Experiments 4B and 5. For Group B mice, the only change was a two-day break after the second conditioning trial. To accommodate these differences and maintain a constant test date, habituation day for Group NB took place two days after that for Group B. The first preference test took place 24 h after the final conditioning session for both groups. For the first 60-min preference test, mice received an injection of saline (Group B/0, Group NB/0) or 10 mg/kg naloxone (Group B/10, Group NB/10) 15 min prior to a saline injection and placement in the conditioning apparatus. For the second preference test three days later, mice received one saline injection immediately before placement in the test apparatus.

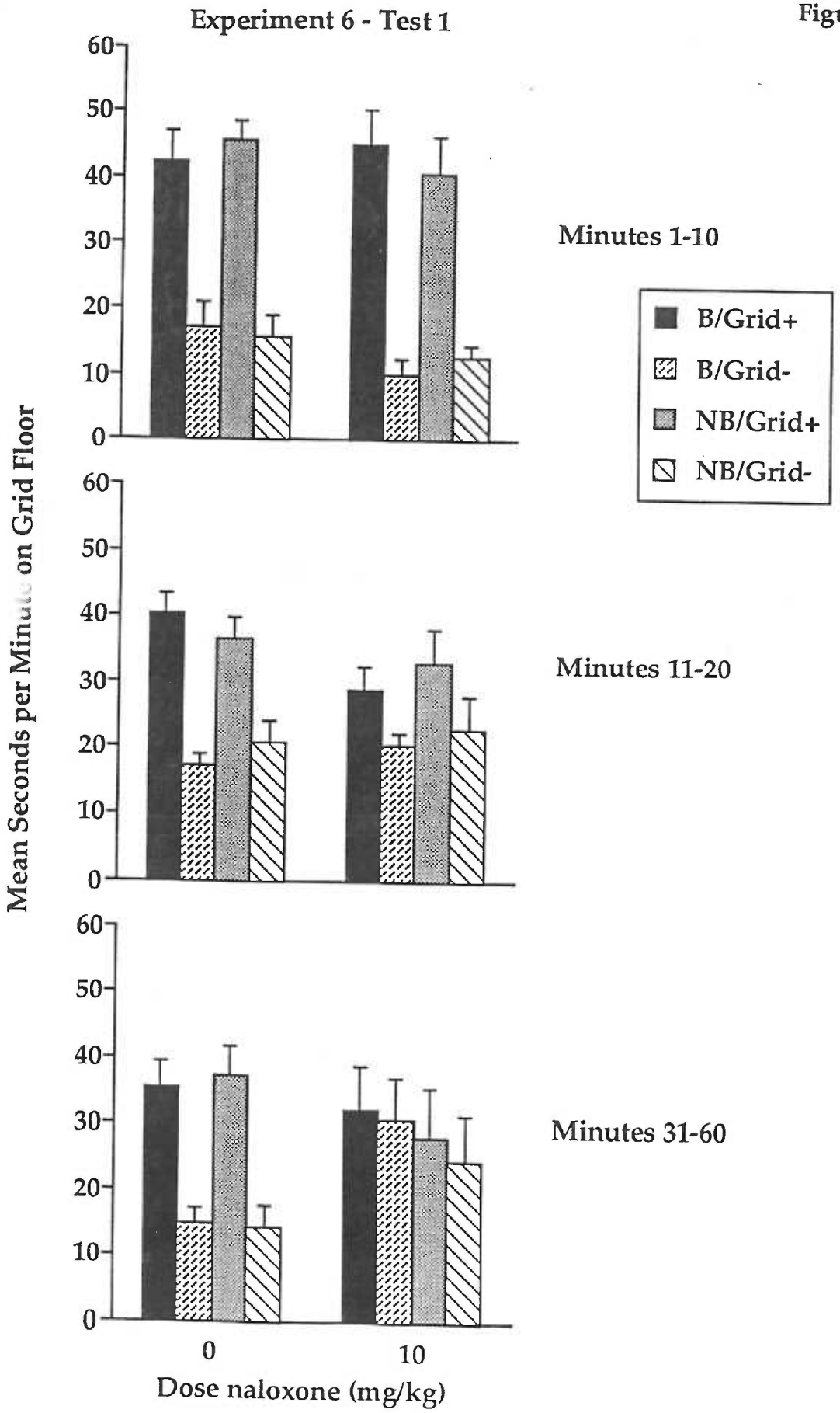
Results

Two mice from Group NB/10 were caught in the metal cage top, causing damage to their feet; data from these mice were excluded. One Group NB/0 mouse died just prior to the first test day. Because of equipment error, Test 1 data were lost from an additional 24 mice (eight Group B/0, four Group B/10, four Group NB/0, and eight Group NB/10). Data from one of these Group B/0 mice were also excluded from analyses of Test 2 after a misplaced injection.

Ethanol produced a conditioned preference for the drug-paired floor in both break and no-break animals. As expected based on previous studies, naloxone (10 mg/kg) attenuated expression of preference in a time-dependent manner in Group B/10 (where a two-day break was present). Naloxone also attenuated preference in the no-break group (Group NB/10). Data from Test 1 are presented in Figure 24. The preceding observations were supported by three-way ANOVA (Group x Dose x Conditioning Group) applied to each time interval. The main effect of conditioning group was significant in all intervals; Minutes 1-10, $F(1,61) = 100.1$; Minutes 11-30, $F(1,61) = 25.9$; Minutes 31-60, $F(1,61) = 9.6$, $ps<.01$. However, in Minutes 31-60, the dose x conditioning group interaction was also significant, $F(1,61) = 6.2$, $p<.05$. Post-hoc analysis (Tukey's) indicated that the Grid+ and Grid- subgroups were only different in saline animals, not in mice treated with naloxone. No significant effects involving break/no-break were seen ($Fs<2$).

Figure 24 - Experiment 6: Test 1 Preference. Mean (+SEM) seconds per minute spent on the grid floor during Minutes 1-10, 11-30 and 31-60 of the first preference test for mice in the four treatment groups (n=10-12). Group designations are as follows: B/Grid+ mice received a two-day break between conditioning trials 2 and 3 and had the grid floor paired with ethanol; B/Grid- mice also had a break but had the grid floor paired with saline; NB/Grid+ mice did not have a break during conditioning and received the grid floor paired with ethanol; NB/Grid- mice had no break and had the grid floor paired with saline.

Figure 24


Experiment 6 - Test 1
Mean Seconds per Minute on Grid Floor
60
50
40
30
20
10
0
Minutes 1-10
B/Grid+
B/Grid-
NB/Grid+
NB/Grid-
Minutes 11-20
Minutes 31-60
0
10
Dose naloxone (mg/kg)

As on other tests, activity was highest initially and declined over time. Naloxone decreased activity throughout the test. These data are presented in Figure 25. Two-way ANOVA (Group x Dose) revealed significant main effects of dose in each time interval; Minutes 1-10, $F(1,65) = 16.0$, $p<.001$; Minutes 11-30, $F(1,65) = 10.0$, $p<.01$; Minutes 31-60, $F(1,65) = 11.5$, $p<.01$. No effects involving break/no-break were seen ($Fs<2$).

All groups received saline before Test 2; data from this test are shown in Figure 26. Ethanol-induced place preference on the second test was only marginally affected by Test 1 naloxone administration. Three-way ANOVAs (Group x Dose x Conditioning Group) on data from each time interval yielded significant main effects of conditioning group in all three bins; Minutes 1-10, $F(1,84) = 58.6$; Minutes 11-30, $F(1,84) = 35.2$; Minutes 31-60, $F(1,84) = 22.6$, all $ps<.001$. In Minutes 1-10 and Minutes 11-30 the dose x conditioning group interaction was also signifiant; $Fs(1,84) = 5.2$ and 4.6, $ps<.05$, for Minutes 1-10 and 11-30, respectively. However, follow-up analyses (Tukey's) indicated that Grid+ and Grid- subgroups in both dose groups were significantly different in both of these time intervals ($ps<.01$). No effects involving break/no-break were evident.

Activity on the second test followed the same pattern as in other tests by declining over time. Interestingly, activity after the first 10 minutes was suppressed by Test 1 naloxone administration only in Group NB animals. These data are presented in Figure 27. Two-way ANOVA (Group x Dose)

re 25 - Experiment 6: Test 1 Activity. Mean (+SEM) activity counts per minute during Minutes 1-10, 11-30 and 31-60 of the first preference test for each treatment group (n=22-24). B and NB refer to the presence or absence of a two-day break during conditioning (see text for details). 0 and 10 mg/kg refer to the dose of naloxone given 15 min prior to the preference test.

Figure 25

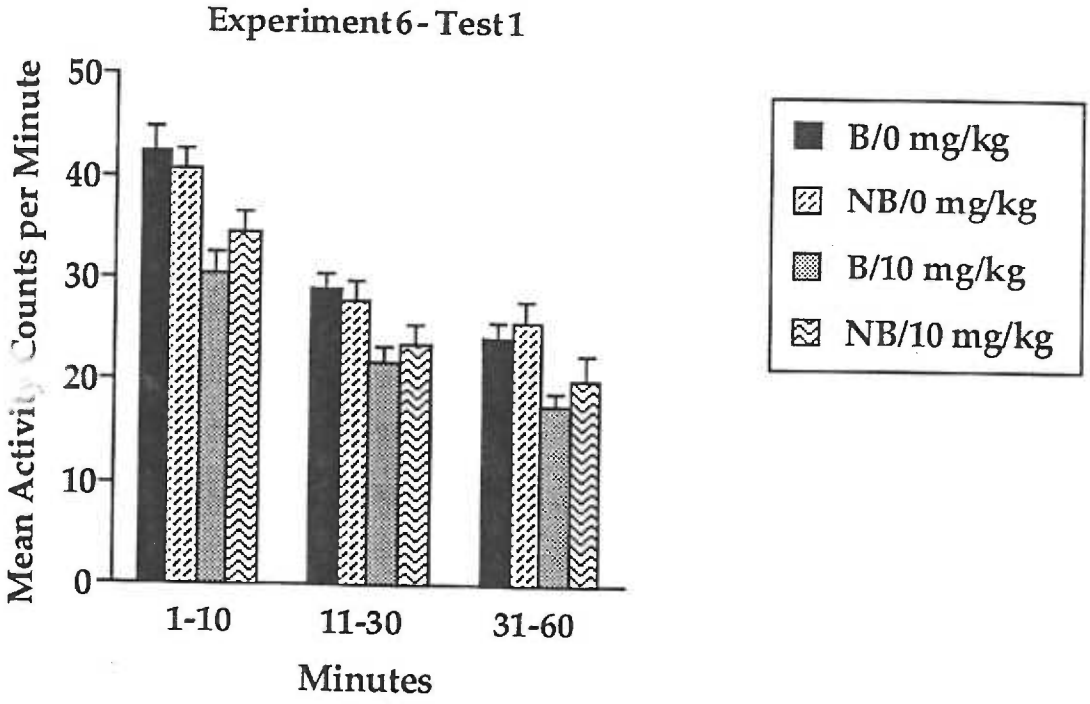

Experiment 6 - Test 1
Mean Activity Counts per Minute
50
40
30
20
10
0
1-10
11-30
31-60
Minutes
B/0 mg/kg
NB/0 mg/kg
B/10 mg/kg
NB/10 mg/kg

Figure 26 - Experiment 6: Test 2 Preference. Mean (+SEM) seconds per minute spent on the grid floor during Minutes 1-10, 11-30 and 31-60 of the second preference test for mice in the four treatment groups (n=10-12). Group designations are as in Figure 24. Dose naloxone refers to treatment on Test 1, all mice received saline prior to Test 2.

Figure 26

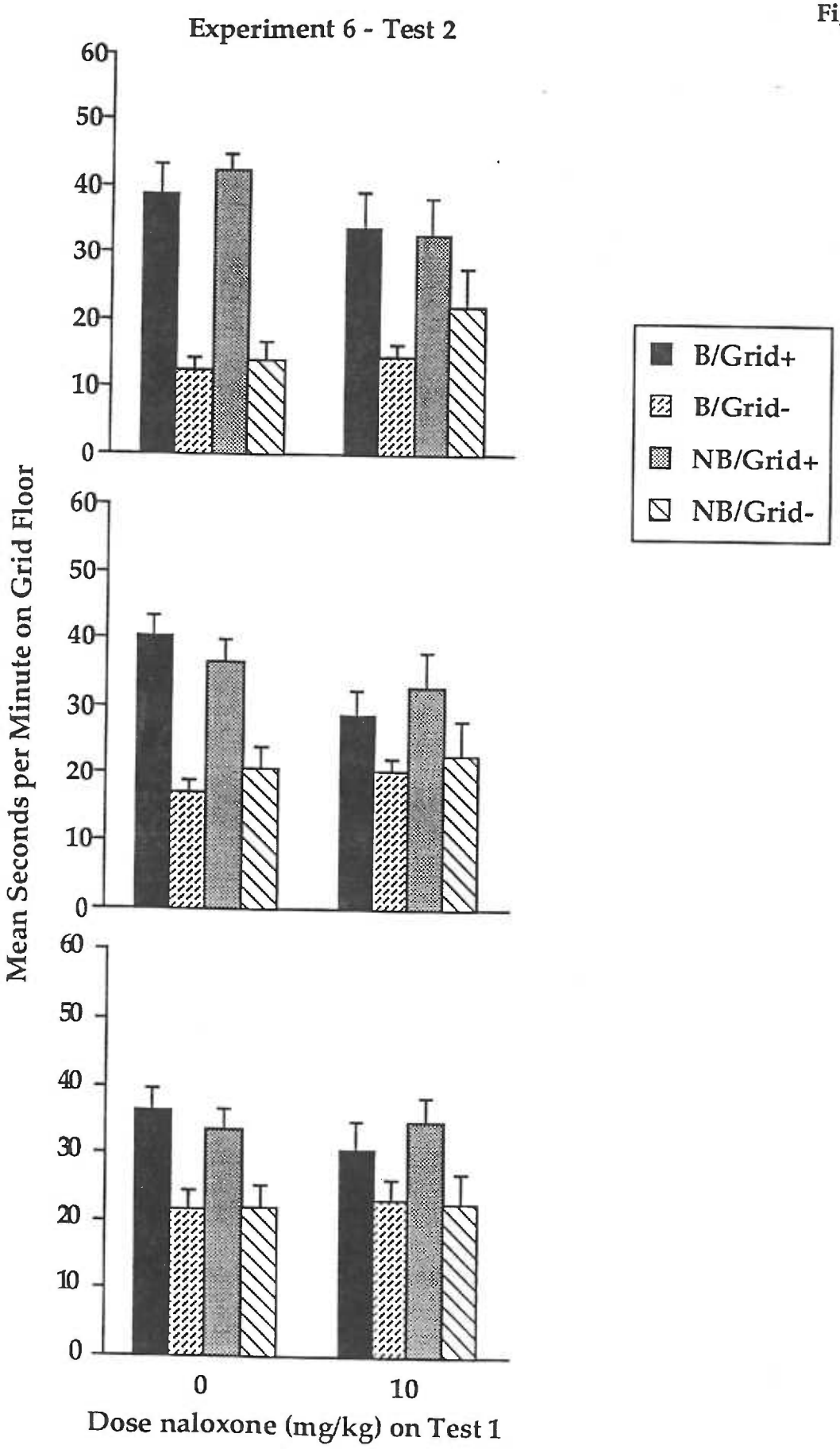
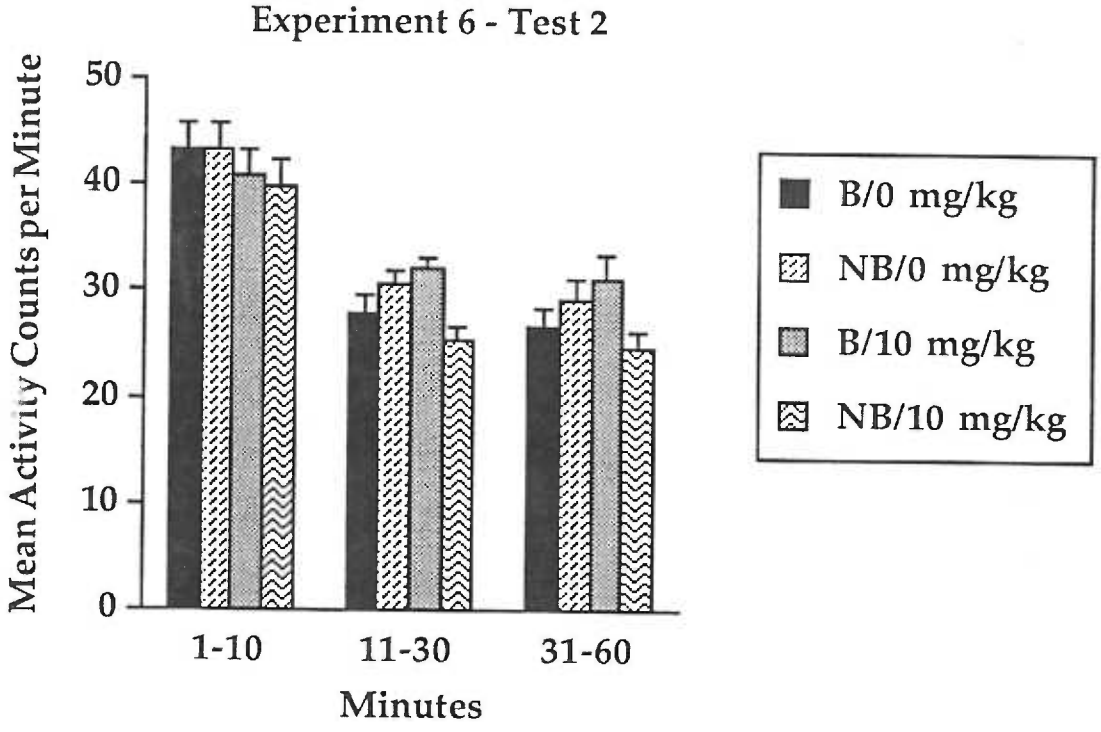

Experiment 6 - Test 2
Mean Seconds per Minute on Grid Floor
60
50
40
30
20
10
0
B/Grid+
B/Grid-
NB/Grid+
NB/Grid-
0
10
Dose naloxone (mg/kg) on Test 1

Figure 27 - Experiment 6: Test 2 Activity. Mean (+SEM) activity counts per minute during Minutes 1-10, 11-30 and 31-60 of Test 2 for each treatment dose group (n=22-24). Group designations are as in Figure 25 but the dose of naloxone refers to treatment on Test 1. All mice received saline prior to Test 2.

Figure 27


Experiment 6 - Test 2
Mean Activity Counts per Minute
50
40
30
20
10
0
1-10
11-30
31-60
Minutes
B/0 mg/kg
NB/0 mg/kg
B/10 mg/kg
NB/10 mg/kg

revealed significant group x dose interactions in Minutes 11-30, $F(1,88) = 12.2$, $p<.01$, and in Minutes 31-60, $F(1,88) = 5.3$, $p<.05$. Follow-up analysis indicated that activity in Group NB/10 was suppressed relative to Group B/10 and Group NB/0 during Minutes 11-30 (Tukey's, $p<.05$). However, in Minutes 31-60 no significant differences were found, although the difference between Groups NB/10 and B/10 suggested a trend toward significance (Tukey's, $p=.08$).

Discussion

Conditioning with ethanol produced a place preference, regardless of the presence of a break during the acquisition phase. In both break and no-break mice, naloxone prevented the maintenance of expression of preference but did not affect initial expression.

Naloxone administration on Test 1 suppressed Test 2 activity after the first 10 minutes in no-break animals relative to mice given a two-day break during conditioning. However, this effect was not robust, reaching significance only during Minutes 11-30. Inasmuch as no differences involving the break were seen in the preference data, this finding is of little importance for the current studies.

Given that 10 mg/kg naloxone was effective in attenuating preference in both experimental groups, it does not appear that the discrepancy between the findings of Cunningham et al. (1995) and Experiments 4B and 5 of the present series can be attributed to the break during conditioning.

As in Cunningham et al. (1995), naloxone significantly attenuated expression of preference when mice received a two-day break during conditioning. Unexpectedly, naloxone had identical effects on mice that did not receive a break. Given that the identical procedure was used in Experiments 4B, 5A and 5B with no significant effect of naloxone on Test 1, there is no ready explanation for the results seen in Group NB of Experiment 6.

Experiment 7: Effects of Cage Changing

Introduction & Rationale

Experiment 6 ruled out the possibility that the presence of a break during conditioning trials could lead to changes in sensitivity to naloxone. Detailed examination of possible differences between studies reported by Cunningham et al. (1995) and the current set of studies indicated that one procedural detail varied across experiments. In the expression studies by Cunningham et al., the first preference test was always conducted three days after cages were changed. During cage changing, mice received clean cages with new corn-cob bedding, a new water bottle and a new supply of lab chow. In Experiments 1-5, the amount of time between cage changing and testing varied from one to three days. However, in Experiment 6, Groups B and NB were both tested three days after cage changing; both groups also exhibited the naloxone effect. Interestingly, the preference test for mice in Experiment 5

that were given 20 or 30 mg/kg took place the day after cages were changed, while Test 2, where naloxone was effective, occurred three days following cage changing.

These observations led to the hypothesis that cage changing somehow alters the sensitivity of mice to naloxone, shifting the dose-response curve to the right. Experiment 7 was conducted to examine the possibility that getting a clean cage the day before testing could diminish or eliminate the effects of naloxone. Since Experiment 6 demonstrated that the presence of a two-day break during conditioning had no effect on naloxone's ability to attenuate maintenance of preference, Experiment 7 was conducted with a break. By using this procedure, this experiment was identical to those reported in Cunningham et al., (1995), allowing the effects of cage changing to be compared directly with those studies.

Methods

Subjects

Subjects were 96 adult male D2 mice housed as in Experiment 1. Mice were obtained at 6 weeks of age and were approximately 10 weeks old at the time of testing.

Apparatus

The 12-box place conditioning apparatus was as in Experiments 4B-6.

Drugs

In all previous experiments that had a three-day interval between cage changing and testing, 10 mg/kg naloxone was effective in attenuating expression of preference. This dose was therefore chosen for Experiment 7.

Procedure

This experiment was conducted by Carly Henderson. Four groups of mice underwent place conditioning with ethanol as in Cunningham et al. (1995), i.e., mice received a two-day break between trials two and three. All groups had their cages changed three days prior to the preference test. Two Change groups (Ch) received an additional cage change immediately after the final conditioning trial (one day prior to testing); two No-Change groups (No-Ch) were returned to the same home cage after the last conditioning trial. On test day, half the mice in each cage treatment group received 10 mg/kg naloxone 15 min before the preference test (Groups No-Ch/10 and Ch/10), while the other half of each group received saline (Groups No-Ch/0 and Ch/0). All mice received a saline injection immediately before placement on the test floor.

Results

One mouse each from the No-Ch/0 group and the Ch/10 group died prior to the preference test, probably as the result of poor injection placement. Four mice in the Ch/0 group were excluded from analysis because their cage was flooded by a leaky water bottle and was changed two days prior to testing.

As expected, ethanol produced a conditioned place preference in both the Change and No-Change groups. However, as seen in Figure 28, naloxone was more effective in the No-Change mice at decreasing maintenance of expression, especially in Minutes 11-30. The preceding observations were supported by three-way ANOVA (Group x Dose x Conditioning Group) applied to each time interval. The main effect of conditioning group was significant in all intervals; Minutes 1-10, $F(1,82) = 83.9$; Minutes 11-30, $F(1,82) = 52.2$; Minutes 31-60, $F(1,82) = 20.9$, $ps<.001$. In Minutes 11-30 the three-way interaction was also significant, $F(1,82) = 6.4$, $p<.05$. To follow up this interaction, two-way ANOVAs (Dose x Conditioning Group) were applied separately to data from this interval in the No-Change and Change groups. These analyses revealed significant main effects of conditioning group in both ge treatment groups; No-Change, $F(1,43) = 17.9$, $p<.001$; Change, $F(1,39) = 34.1$, $p<.001$. However, the interaction was significant only in the No-Change group, $F(1,43) = 14.5$, $p<.001$, not in the Change group ($F<1$). Tukey's test indicated that in the No-Change group, Grid+ and Grid- differed only in saline treated animals ($p<.001$), not in those receiving naloxone ($p>0.9$).

As on other tests, activity was highest initially and declined over time. Naloxone decreased activity throughout the test. These data are presented in Figure 29. Two-way ANOVA (Group x Dose) revealed significant main effects of dose in each time interval; Minutes 1-10, $F(1,86) = 8.0$, $p<.01$; Minutes 11-30,

Figure 28 - Experiment 7: Test 1 Preference. Mean (+SEM) seconds per minute spent on the grid floor during Minutes 1-10, 11-30 and 31-60 of the preference test for mice in the No-Change (left column) and Change (right column) groups (n=10-12/conditioning group). No-Change refers to the groups that did not get clean cages the day prior to testing while Change mice had their cages changed within 24 h of the preference test. Saline or naloxone (10 mg/kg) was administered 15 min prior to testing. Grid+ and Grid- are as in previous figures.

Figure 28

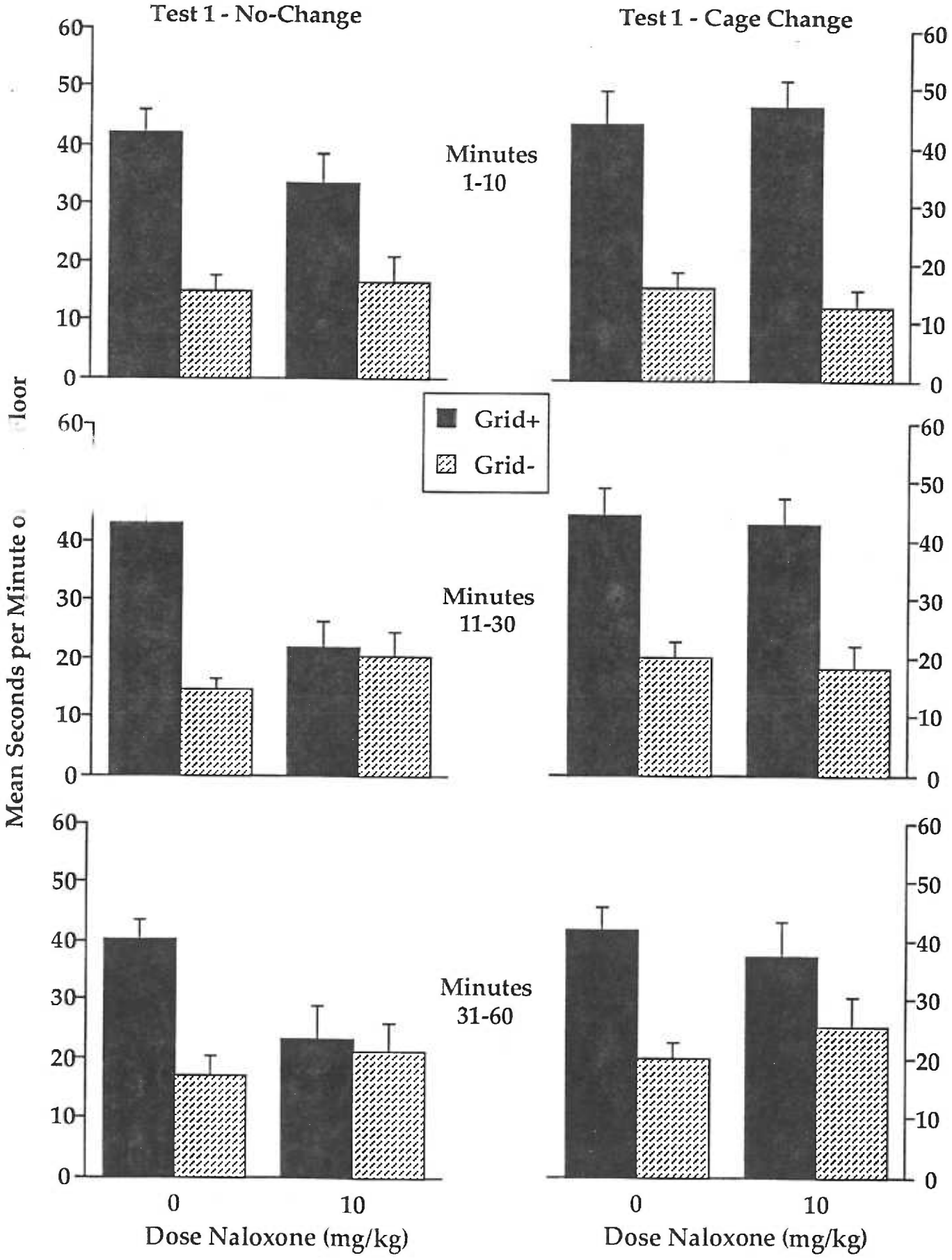
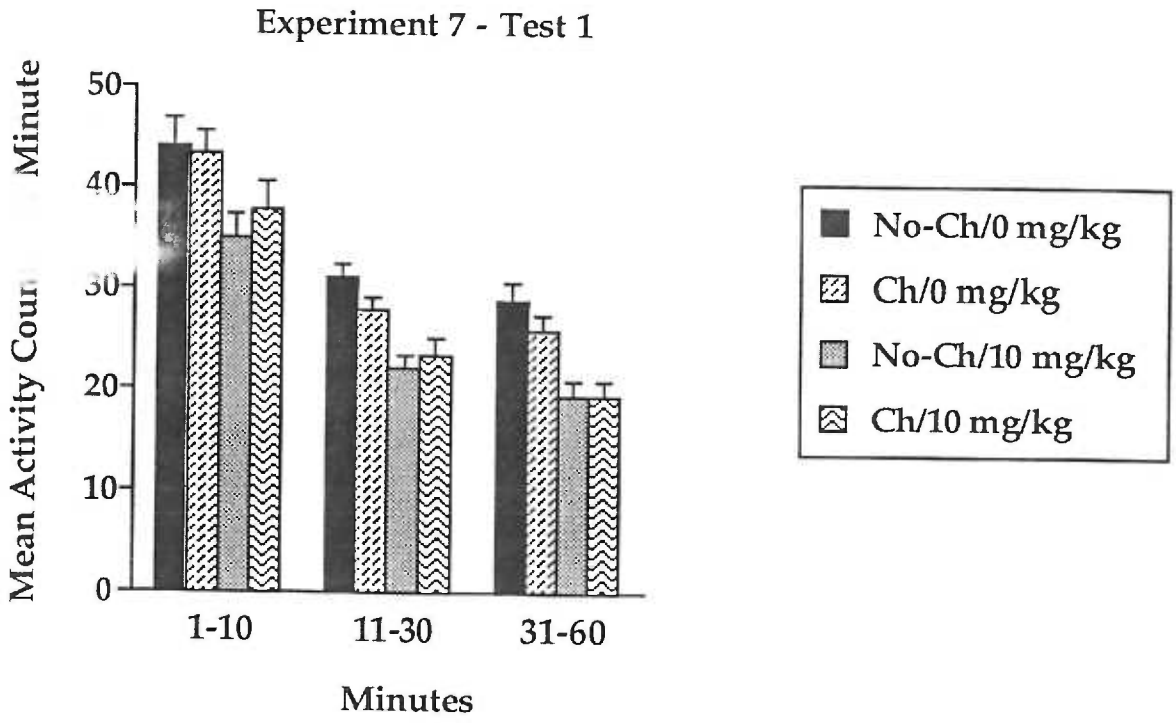

Test 1 - No-Change
Test 1 - Cage Change
Minutes 1-10
Minutes 11-30
Minutes 31-60
Grid+
Grid-
Mean Seconds per Minute o
loor
0
10
20
30
40
50
60
0
10
Dose Naloxone (mg/kg)
0
10
Dose Naloxone (mg/kg)

Figure 29 - Experiment 7: Test 1 Activity. Mean (+SEM) activity counts per minute during Minutes 1-10, 11-30 and 31-60 of the first preference test for each treatment group (n=22-24). 0 and 10 mg/kg refer to the dose of naloxone given 15 min prior to the preference test.

Figure 29


Experiment 7 - Test 1
Mean Activity Count / Minute
50
40
30
20
10
0
1-10
11-30
31-60
Minutes
No-Ch/0 mg/kg
Ch/0 mg/kg
No-Ch/10 mg/kg
Ch/10 mg/kg

$F(1,86) = 20.9$, $p<.001$; Minutes 31-60, $F(1,86) = 26.4$, $p<.001$. No effects involving cage changing were seen ($Fs<2$).

All groups received saline before Test 2; data from this test are shown in Figure 30. Mice that received naloxone prior to Test 1 exhibited a place preference only in the first 10 minutes of the second test while saline treated mice expressed preference throughout the test session. Three-way ANOVAs (Group x Dose x Conditioning Group) on data from each time interval yielded significant main effects of conditioning group in all three bins; Minutes 1-10, $F(1,82) = 45.0$; Minutes 11-30, $F(1,82) = 31.4$; Minutes 31-60, $F(1,82) = 29.7$, all $ps<.001$. In Minutes 11-30 and Minutes 31-60 the Dose x Conditioning Group interaction was also significant; $Fs(1,82) = 7.6$ and 5.3, $ps<.05$, for Minutes 1-10 and 11-30, respectively. Follow-up analyses (Tukey's) indicated that Grid+ and Grid- subgroups were significantly different only in saline-treated mice in both of these time intervals ($ps<.01$). Mice treated with naloxone on Test 1 did not show preference in either of these intervals ($ps>0.1$). No effects of cage-changing were seen on Test 2.

Activity on the second test followed the same pattern as in other tests by declining over time. No significant effects of naloxone treatment on Test 1 or cage changing were seen. Data are presented in Table 2.

Discussion

Ethanol induced a conditioned place preference that was not affected by cage changing 24 h prior to testing, i.e., equivalent preference was seen in

Figure 30 - Experiment 7: Test 2 Preference. Mean (+SEM) seconds per minute n the grid floor during Minutes 1-10, 11-30 and 31-60 of the second preference test for mice in the four treatment groups (n=10-12). Group designations are as in Figure 24. Dose naloxone refers to treatment on Test 1, all mice received saline prior to Test 2.

Figure 30

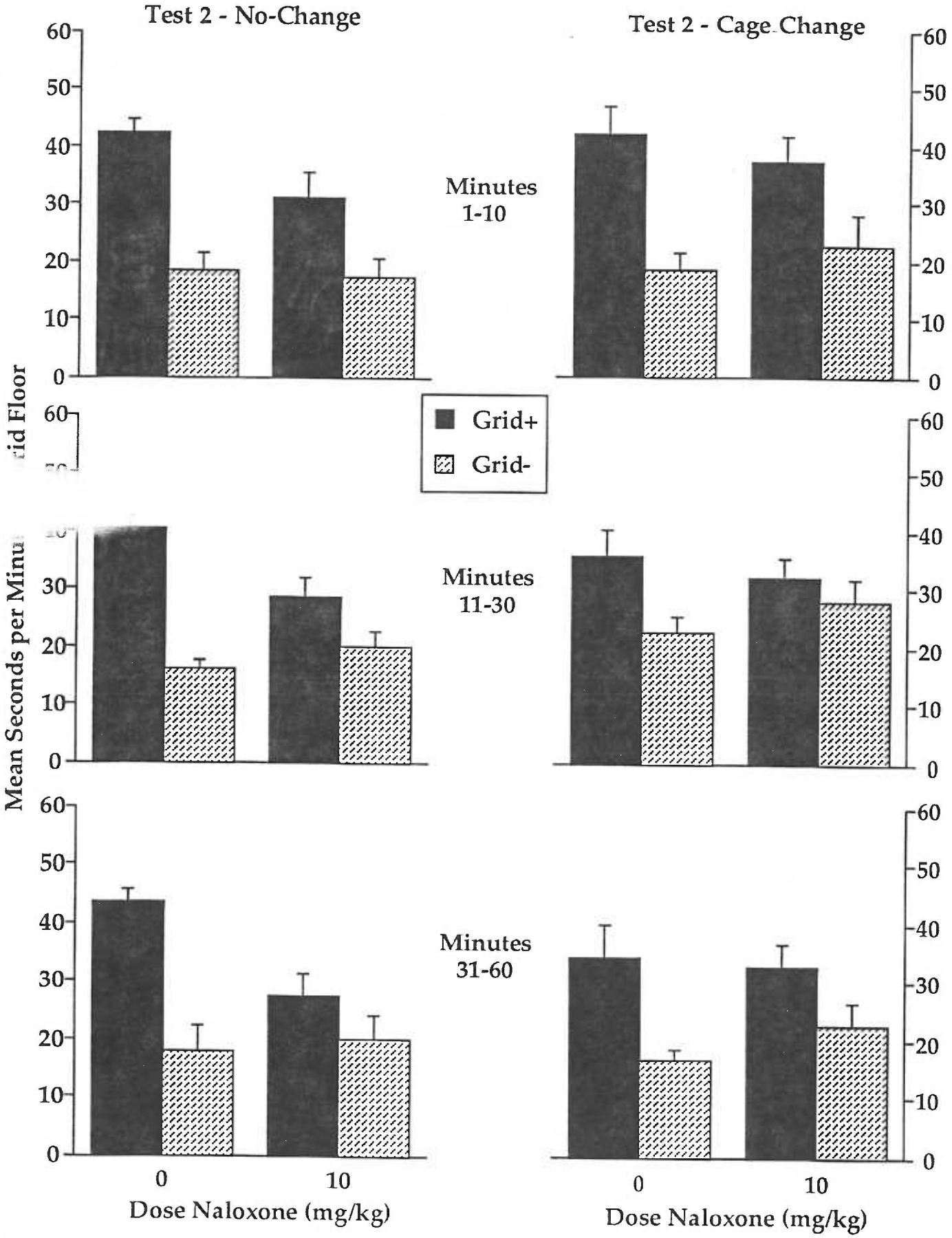

Test 2 - No-Change
Test 2 - Cage Change
Minutes 1-10
Minutes 11-30
Minutes 31-60
Grid+
Grid-
Mean Seconds per Minu
id Floor
0
10
20
30
40
50
60
Dose Naloxone (mg/kg)

Table 2. Mean Activity Counts per Minute (±SEM) in Minutes 1-10, 11-30 and 31-60 on Test 2 of Experiment 7.

Group	Minutes 1-10	Minutes 11-30	Minutes 31-60
No-Ch/saline	50.8 ± 3.01	31.6 ± 1.48	25.2 ± 1.62
No-Ch/nal 10	42.9 ± 2.43	30.5 ± 1.52	25.9 ± 1.67
Change/saline	48.5 ± 3.88	31.3 ± 1.92	24.7 ± 1.84
Change/nal 10	45.9 ± 2.78	33.9 ± 1.99	28.4 ± 1.63

No-Ch = no cage change the day before testing (3 day interval)
Change = cage change the day before testing (1 day interval)
nal 10 = 10 mg/kg naloxone

saline-treated mice in both No-Change and Change groups. However, naloxone was more effective at attenuating maintenance of preference in No-Change mice. In Minutes 11-30, both naloxone and saline-treated mice in the Change group showed a significant preference, while only saline-treated mice in the No-change group did. In this time interval, naloxone completely eliminated the conditioned preference for the ethanol paired floor in animals that did not receive a cage change the day before testing. In the last 30 min of the test, naloxone decreased expression of preference in both cage treatment groups, but the effect was more evident in the No- change animals (Minutes 31-60, Figure 28).

The conclusion indicated by this study, that timing of cage changing is important for naloxone's effects, suggests a possible explanation for the disparate results discussed earlier. Table 3 lists the experiments that used naloxone on Test 1, the number of days between cage changing and testing, and the results of planned comparisons of preference data from Minutes 31-60 in the stated dose group. Each planned comparison consisted of a one-way ANOVA with Conditioning Group (Grid+ vs Grid-) as the between group factor.

As seen in Table 3, Experiments 4B and 6 had the test separated from the cage change by three days while in Experiment 5B the interval was only one day. The results of Experiment 7 suggest that naloxone treatment would be relatively ineffective with a one-day interval but would be effective with a

Table 3. Analysis of Preference Data from Minutes 31-60.

Experiment (Interval[a])	Drug Group - Dose	F	*p*
4B (3 days)	saline	(1,26) = 5.5	<.01*
	(+)naloxone - 10	(1,29) = 16.9	<.01*
	naloxone - 10	**(1,29) = 2.2**	**.15**
5A (2 days)	saline	(1,30) = 18.7	<.01*
	naloxone - 1.5	(1,30) = 20.7	<.01*
	naloxone - 10	(1,30) = 11.3	<.01*
5B (1 day)	saline	(1,24) = 20.7	<.01*
	naloxone - 10	(1,21) = 4.7	.04*
	naloxone - 20	(1,24) = 4.5	.04*
	naloxone - 30	**(1,25) = 1.6**	**.21**
6 (3 days)	Break - saline	(1,14) = 20.1	<.01*
	No break - saline	(1,17) = 16.4	<.01*
	Break - naloxone 10	**(1,18) = 0.0**	**.89**
	No break - naloxone 10	**(1,12) = 0.1**	**.74**

All data are results of planned comparisons between Grid+ and Grid- conditioning groups (one-way ANOVA). Doses are in mg/kg.

[a] Number of days between cage change and preference test.

* Grid+ and Grid- subgroups are significantly different.

Data in **bold face** indicate that naloxone significantly attenuated expression of place preference -- i.e., Grid+ and Grid- groups do NOT differ.

three-day interval; consequences of a two-day interval were not explored. This idea is supported by the data summarized in Table 3. Specifically, in experiments with a three-day interval, namely 4B and 6, mice treated with 10 mg/kg naloxone did not show a significant place preference (i.e., the conditioning groups did not differ, $p<.05$). However, in Experiment 5B, which had a one-day interval, 10 mg/kg naloxone did not affect preference, although the 30 mg/kg dose was effective. A 10 mg/kg dose of naloxone was also ineffective in Experiment 5A, where the interval between cage change and test was two days. These findings support the general theory that cage changing one day before preference testing alters sensitivity to naloxone such that a higher dose is required to obtain an effect.

It is well known that exposure to stressors can activate the POMC system (see review by Przewlocki, 1993). Endogenous opioids appear to be mobilized by acute, mild, short-lasting stressors (Przewlocki, 1993). Exposure to clean bedding has been used experimentally as a mild stressor and has been shown to increase levels of stress hormones (Cabib, Puglisi-Allegra, & D'Amato, 1993; Marchlewska-Koj & Zacharczuk-Kaietek, 1990). It is possible that in the current and previous studies from this laboratory, exposure to a clean cage results in an increase in brain POMC activity, thereby increasing levels of endogenous opioids. Pharmacologically, this is equivalent to increasing the concentration of agonist available to receptors. With increasing agonist concentration, higher doses of a competitive antagonist are

necessary to compete for binding sites, i.e., the dose-response curve is shifted to the right. Thus, if levels of endogenous opioids are increased in a critical region by cage changing, naloxone levels would also have to be raised to compete effectively for binding sites. This may explain why the lower dose of naloxone was ineffective in Experiment 5B while the expected effect was seen with a higher dose.

Although stressors cause an immediate increase in opioid and stress hormone levels, it is not known whether cage changing could induce an increase in POMC activity that would still be present after 24 h (and possibly at 48 h). Young, Bronstein, and Akil (1993) found that rats that swam daily for 14 days had elevated plasma and pituitary β-END levels 24 h after the final swim session. This indicates that relatively long-term changes in opioid levels can result from exposure to stressors. However, exposure to a novel cage is a mild stressor compared to swim stress and would not necessarily be expected to result in a long-term increase in opioid activity (J. Weinberg, personal communication).

Alternatively, it may be that opioid levels are increased not in response to cage changing per se, but because the mice engage in territory and dominance-establishing behaviors when they are exposed to a clean cage and fresh litter. To the extent that these encounters are stressful, they would increase opioid levels. As these behaviors may continue for some time, while territories are re-established, stress responses could occur long after the

initial stress of cage changing. Thus, opioid levels may be high at the time of preference testing because of recurring stressful encounters with cage-mates. As discussed earlier, high levels of agonist (opioid peptides) necessitate increased levels of antagonist (naloxone). With a longer interval between cage changing and testing, territories and dominance would likely be established and stressful encounters would decrease in frequency, thus opioid levels would decrease, enabling a lower dose of naloxone to be effective.

In addition to casting new light on the results of Experiments 4-6, the cage-changing hypothesis may complicate interpretation of the current studies with the selective opioid antagonists (Experiments 1-3). In these , the interval between cage changing and testing was either one or two days. As naloxone (10 mg/kg) is not effective in procedures using these intervals, perhaps because of a shift in the dose-response curve, the selective antagonists may have been ineffective for the same reason. Thus, although it may be true that naloxone's effects are not mediated by the μ, δ, or κ receptor systems, antagonists at these receptors might be effective if a broader dose range (to accommodate a shift in the dose-response curve) or a three-day interval procedure were used. In Experiment 2, however, a second test was conducted with higher doses of the δ antagonist and a three-day break between cage changing and Test 2. Given that no change in the expression of preference was seen, the conclusion that δ receptors do not mediate the

expression of ethanol place preference may indeed be correct. It is unfortunate that second tests were not conducted in Experiments 1 and 3 (μ and κ antagonists, respectively) after additional antagonist treatment, as second tests with naloxone were consistently effective.

SUMMARY & CONCLUSIONS: EXPERIMENTS 1-7

Overall, the present studies replicated the finding of Cunningham et al., (1995) that administration of naloxone prior to testing attenuates the enhancement of expression of ethanol-induced conditioned place preference. Data reported here strongly suggest that naloxone's effects are not mediated solely by the δ receptor, while involvement of the μ and/or κ receptor types cannot be ruled out. However, the naloxone effect appears to be opioid receptor mediated, as (+)-naloxone did not alter expression of preference. Finally, these studies indicate that naloxone's effects can be altered by cage changing 24 h prior to testing. Table 4 contains a summary of drug effects seen in the current studies.

As seen in Table 4, NTI and nor-BNI affected locomotor activity without affecting the expression of preference. This indicates a dissociation between the two behaviors, and suggests that naloxone's ability to attenuate preference is not simply an artifact of it's activity decreasing effects. It is especially interesting that expression of preference was maintained in mice

Table 4. Summary of Drug Effects in Experiments 1-7. T1 , T2 = Test 2.

Exp. # - Drug	Preference-T1	Activity-T1	Pav	ke Lat.	Preference-T2	Activity-T2
1 - NTI	NO	NO	---		NO	↓
2 - β-FNA (T1)	NO	NO	↓		---	---
- nal (T2)	---	---	---		YES	↓
3 - nor-BNI	NO	↑	↓		---	---
4A - (+)-nal	NO	NO	---		NO	NO
4B - (+)-nal	NO	NO	---		NO	NO
- nal	NO	NO	---		YES	↓
5 - nal	NO	↓	---		YES	↓
6 - B/nal	YES	↓	---		YES	↓
- NB/nal	YES	↓	---		YES	↓
7 - Ch/nal	YES	↓	---		YES	↓
- No-Ch/nal	YES	↓	---		YES	↓

NTI = δ antagonist; β-FNA = μ antagonist; nor-BNI = κ antagonist; nal = naloxone; (+)-nal = (+)-naloxone

B = break during conditioning; NB = no break during conditioning; Ch = cage change 1 day before testing; No-Ch = cage change 3 days before testing

↑ = increase; ↓ = decrease; --- = not measured

treated with high doses of nor-BNI that increased activity; this suggests that the preference for the CS+ was strong enough to maintain the mouse's choice of spatial location in the face of the competing behavior of increased locomotion.

As stated earlier, it is highly unlikely that naloxone's effects are mediated solely by the δ receptor since a second test conducted with a high dose of NTI did not result in attenuation of preference. Definite conclusions regarding selective blockade of μ and κ receptors cannot be made, although data suggest that neither of these receptors alone mediate naloxone's effects on expression. Alternatively, it may be that concomitant blockade of more than one receptor type is necessary to reproduce naloxone's effects.

Following is a possible explanation of naloxone's effects. During conditioning, ethanol stimulates release of endogenous opioids (e.g., β-END or met-ENK) and this release becomes associated with the CS+ floor. On the preference test, contact with the CS+ results in a conditioned release of endogenous opioids, which then activate opioid receptors, resulting in a neurochemical cascade that maintains expression of preference. Given that none of the opioid peptides exhibit high selectivity or specificity for any opioid receptor type, it is possible that two separate receptor populations (e.g., μ and δ) may be activated by the released ligand. Co-activation may occur even if only <u>one</u> receptor pathway is required to maintain expression, i.e., the

pathways are redundant. As naloxone can bind to all opioid receptor types, it blocks both receptor populations, thereby preventing activation of downstream pathways subserving preference expression. Using selective antagonists only removes one receptor at a time from this scheme, leaving one system intact to maintain preference. Therefore concomitant blockade of more than one receptor type may be necessary to mimic naloxone's effects on expression.

Evidence for parallel activation of multiple opioid systems comes from a report by Watkins, Wiertelak, Grisel, Silbert, & Maier (1992). They demonstrated that stress-induced analgesia (previously termed 'non-opiate') that was attenuated by naltrexone (Watkins, Wiertelak, & Maier, 1992) was not affected by selective blockade of μ, δ, or κ receptors. However, when antagonists at these receptors were administered in combination, the analgesia was either attenuated or abolished, depending on the test used. Interestingly, blocking either μ and δ or μ and κ receptors was effective against stress-induced analgesia. These findings suggest that nonselective opiate antagonists such as naltrexone and naloxone may act by simultaneously blocking more than one receptor type. Hence, it may be that the conclusions drawn from Experiments 1-3 were correct in that selective antagonists administered alone do not mimic naloxone's effects on expression of preference but would be effective if administered in combination.

To conclusively determine the receptor type responsible for naloxone's effects on preference two series of studies should be conducted. First, place conditioning experiments using selective opioid antagonists prior to testing should be performed again, with increased dose ranges and a three-day interval between cage changing and preference testing. Second, if administration of each selective antagonist alone does not affect expression of place preference as naloxone does, antagonists should be administered in combination prior to preference testing.

The current studies point out the importance of procedural variables in behavioral studies and suggest that special attention should be paid to the potentially long-lasting effects of seemingly mild stressors (e.g., cage changing). It may be that inconsistencies in the literature regarding the effectiveness of naloxone and naltrexone (and perhaps antagonists of other systems) are a function of shifts in the dose-response curve brought about by stressors.

To determine if the observed effects of cage changing are indeed a result of long-lasting changes in the opioid system, two experiments are suggested to address the suggested shift in the naloxone dose-response curve. The first suggested experiment would be to measure plasma and/or tissue levels of the endogenous opioid peptides at various time points after cage changing. If cage changing is indeed a stressor that induces a shift in the naloxone dose-response curve by increasing opioid levels, peptide levels

should be increased immediately after cage changing and should decrease with time. The time course suggested by the cage-changing study described earlier indicates that opioid levels should be increased at around 24 h and should return to basal levels by 72 h.

The second experiment, which could also involve sampling of peptide levels, would be to conduct ethanol place conditioning in both group-housed and singly-housed mice and to determine sensitivity to naloxone's effects on expression in both sets of mice at various intervals after cage changing. If the idea that stressful encounters with cage-mates while establishing territory in a clean cage lead to increased opioid levels, the duration of the cage change-preference test interval should not alter sensitivity to naloxone.

Finally, the suggestion that sensitivity to opioid antagonists might be [illegible] responses to stressors could be of great importance in the use of [illegible] antagonists as pharmacotherapeutic agents. For example, perhaps alcoholic patients who do not respond favorably to naltrexone treatment have more pronounced physiological responses to stress than do those patients that are helped by naltrexone. The present data suggest that either increasing the dose of naloxone or decreasing the stress response may improve patient responding.

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Appendix A. Mean Activity Counts per Minute (±SEM) on Each Conditioning Trial (C1-C4).

	C1		C2		C3		C4	
Exp. # (n)	EtOH	Sal	EtOH	Sal	EtOH	Sal	EtOH	Sal
1 (n=94)	133.1 ± 3.14	53.9 ± 0.99	139.8 ± 3.32	47.6 ± 1.16	157.8 ± 4.41	47.4 ± 1.91	156.5 ± 4.26	42.6 ± 1.59
2 (n=93)	148.7 ± 3.44	56.4 ± 1.07	163.4 ± 4.24	52.2 ± 0.98	175.8 ± 3.89	51.5 ± 1.51	196.2 ± 3.99	49.1 ± 1.54
3 (n=96)	144.7 ± 2.81	56.3 ± 1.24	158.6 ± 3.01	48.3 ± 1.23	167.2 ± 3.40	44.8 ± 1.46	181.9 ± 3.64	42.2 ± 1.61
4A (n=94)	149.2 ± 3.55	58.5 ± 1.09	167.3 ± 3.80	52.5 ± 1.14	176.2 ± 4.31	50.6 ± 1.32	185.2 ± 4.60	46.7 ± 1.54
4B (n=91)	147.6 ± 3.26	57.2 ± 1.24	165.5 ± 3.89	54.8 ± 1.08	171.3 ± 3.94	51.5 ± 1.51	191.4 ± 4.33	43.1 ± 1.87
5 (n=92)	136.2 ± 3.46	65.2 ± 3.02	153.3 ± 4.15	61.6 ± 3.34	164.5 ± 4.51	61.5 ± 3.58	168.5 ± 5.04	61.4 ± 3.61
6 (n=91)	150.3 ± 3.34	58.3 ± 1.48	163.6 ± 3.60	52.9 ± 1.19	176.2 ± 2.94	50.9 ± 1.12	183.2 ± 3.94	46.6 ± 1.74
7 (n=90)	141.5 ± 3.67	58.0 ± 1.37	162.9 ± 4.02	55.3 ± 1.41	178.8 ± 4.01	48.8 ± 1.68	191.8 ± 4.36	46.4 ± 1.72

Analysis of CS+ (EtOH) data yielded a significant effect of trials in each experiment, indicating the development of sensitization (all Fs>18.5). Ethanol induced locomotor activity was higher on C4 than on C1. Analysis of CS- (saline) data revealed a significant effect of trials in all experiments except for Exp. 5 ($F<2$ for Exp. 5, other Fs>9). In all other experiments, saline-treated mice showed decreased rates of activity across trials.