LOCUS COERULEUS INVOLVEMENT
IN THE DEVELOPMENT OF CLASSICALLY
CONDITIONED BRADYCARDIA

by

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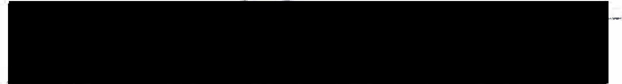

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List of Abbreviations

General

ACE	Amygdala Central Nucleus
ANOVA	Analysis of Variance
CSF	Cerebrospinal Fluid
DA	Dopamine
DNAB	Dorsal Noradrenergic Ascending Bundle
HR	Heart Rate
ICV	Intracerebroventricular
LAN	Lateral Amygdala Nucleus
LC	Locus Coeruleus
NE	Norepinephrine
NM	Nictitating Membrane
6-OHDA	Six-Hydroxydopamine
OR	Orienting Response
PBN	Parabrachial Nucleus
VNAB	Ventral Noradrenergic Ascending Bundle

Conditioning Terms

CR	Conditioned Response
CS	Conditioned Stimulus
UR	Unconditioned Response
US	Unconditioned Stimulus

Pharmacological Agents

Alpha-Helical CRF (9-41) (a-HEL) Antagonist	Corticotropin Releasing Factor
Clonidine (CLON)	Alpha-2 Noradrenergic Agonist
DAGO (D-Ala2,N-Me-Phe4,Gly5ol) enkephalin	Mu Opioid Agonist
DALA D-Ala2-Met-enkephalinamide	Mu Opioid Agonist
Idazoxan	Alpha-2 Noradrenergic Antagonist
Isoproterenol	Beta-Adrenergic Agonist
Muscarine	M2 Cholinergic Agonist
Phentolamine	Alpha-2 Noradrenergic Antagonist
Piperoxane	Alpha-2 Noradrenergic Antagonist
Propranolol	Beta-Adrenergic Antagonist
UK 14,304 (5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline	Alpha-2 Noradrenergic Agonist
Yohimbine	Alpha-2 Noradrenergic Antagonist

ABSTRACT

Previous research has shown that opioid receptor activity within the rostral fourth ventricle of the rat brain can decrement the development of a classically conditioned bradycardia response (HR CR). The purpose of the current series of experiments was to further characterize the role specific structures within the ventricle might play in mediating the opioid-induced abolition of the bradycardia response. The noradrenergic nucleus, the locus coeruleus, was a prime candidate because of its location within the ventricle, its high concentration of opioid receptors, which when activated inhibit neuronal activity, and because of previous research showing blockade of noradrenergic activity within specific areas of the brain can decrement the learning of the bradycardia CR.

Experiment I, looked at the effects of the alpha-2 agonists, Clonidine (3 ug, CLON), and UK 14,304 (5 ug, UK), a mu opioid agonist, DALA (10 ug), and the CRF antagonist, Alpha-Helical CRF (9-41) (25 ug, a-HEL) on the development of a conditioned bradycardia response. Both noradrenergic alpha-2 agonists and mu opioid agonists are known to have similar effects in suppressing the neuronal activity of LC cells whereas, alpha-helical CRF blocks stress-induced increases in LC activity. Five groups of animals were stereotaxically implanted with a chronic indwelling cannula in the rostral fourth ventricle of the brain. The drugs were dissolved in saline and infused in a 1 ul volume into the rostral fourth ventricle 5 min prior to training. A control group received an equivalent volume of saline (SAL).

Conditioning consisted of a discrimination paradigm in which one 6-s tone conditioned stimulus (CS+) was paired with a mild electric shock unconditioned stimulus (US), while a second tone (CS-) was presented alone. Training occurred on two consecutive days and was comprised of random presentations of 6 CS+ and 6 CS- trials on each day. At the end of conditioning on Day 2 the DALA, UK, and CLON groups received infusions of their respective receptor antagonists (i.e., naltrexone in the DALA group and idazoxan in the alpha-2 groups). The SAL and a-HEL groups received saline infusions. Following antagonist administration, all 5 groups received random presentations of CS+ and CS- (4 CS+ no US, 4 CS-) to test for evidence of a HR CR. Forty-eight hours later all groups were again retested with CS-alone presentations (6 CS+ no US, 6 CS-) in a non-drug state. Immediately following this test all groups received reconditioning trials (10 CS+, 10 CS-). After the reconditioning phase, the drugs used during the original training were readministered prior to CS-alone test trials (4 CS+ no US, 4 CS-).

At the end of the drug test, each group experienced the presentation of a loud noise, and the amount of movement elicited by this noise was measured (i.e., startle response). The effects of the drugs on startle responding in the presence of CS+ and CS- were evaluated in addition to effects on reflex baseline startle responding.

The administration of DALA, UK, and clonidine prior to conditioning severely decremented the development of a HR CR and significantly depressed baseline HR. The administration of the antagonists reversed the drug

effects on baseline HR but did not reverse the decrement in the bradycardia CR. The loss of the HR CR in these groups was evident even when they were tested 48 hours later in a non-drug state. Alpha-helical CRF, on the other hand, enhanced the magnitude of the bradycardia CR and this enhancement was found to cary over into the non-drug test phase suggesting perhaps that the drug enhanced the learning of the response. When the DALA, UK, and CLON groups were subsequently retrained in a non-drug state they showed the development of a normal HR CR indicating that these groups were capable of learning. The readministration of DALA, UK, and clonidine severely decremented this newly-learned CR.

During the startle test, the presence of CS+ resulted in a pronounced suppression of the reflex startle response in the SAL and a-HEL groups. Presumably this effect was due to the fact that these groups were freezing in response to CS+ and thus startle amplitudes were decreased. The administration of DALA, UK, and clonidine were found to decrement both baseline startle and the CS-induced inhibiton of startle responding. This suggests that the adverse effect of these drugs on both the learning and performance of the HR CR and fear-induced startle suppression may have been due to their ability to inhibit LC activity.

Experiment II sought to further evaluate the possible involvement of the LC and other fourth ventricle structures in the decremental effects of fourth ventricle opioid administration on HR CR formation. Animals were prepared with chronic bilateral cannulae into either the LC or the adjacent parabrachial nucleus (PBN). The procedures used in Experiment II were very similar to those used in Experiment I. Six groups of rats received LC or PBN microinjections (0.5 ul) of either the mu opioid agonist DAGO (1.6 uM), the M2 cholinergic agonist muscarine (100 uM), or an artificial CSF solution. Although, DAGO is known to have inhibitory effects in both the LC and PBN, muscarine has excitatory effects in the LC and inhibitory effects in the PBN. It was thought that because the agonist muscarine has differential effects in the LC and PBN, a better evaluation of the relative contributions of these two nuclei on the development of a HR CR could be made by using muscarine injections.

The drugs were administered prior to training on two consecutive days and a CS alone test was given at the end of Day 2. All groups were tested for a HR CR 48 hours after training in a non-drug state and then received reconditioning trials. Following reconditioning the drugs were readministered the effects of the drugs on established responses were evaluated.

The administration of DAGO in the LC produced a significant impairment in the learning of the HR CR without affecting baseline HR. No evidence of a HR CR was seen in this group on either the CS test at the end of conditioning on Day 2 or on the non-drug test that occurred 48 hours later. This group did develop a HR CR when trained with no drugs present but subsequent drug administration decremented this recently learned CR. The administration of DAGO into the PBN, in contrast to the LC, significantly augmented the magnitude of the bradycardia CR, while significantly reducing baseline HR. The ability of PBN DAGO to enhance the HR CR was only seen

during the initial training and had no clear augmenting effects on the performance of the HR CR when the animals were later tested in a non-drug state.

Like DAGO, muscarine in the PBN also reduced baseline HR but had no effects on the learning or performance of the HR CR. Muscarine administration into the LC, in contrast to the PBN, significantly elevated baseline HR but also had no effects on the learning or performance of the HR CR.

In Experiment III, it was shown that pretreating animals with the opiate antagonist naltrexone (3 mg/kg ip) blocked the decremental effects of LC DAGO administration on the development of a HR CR. In addition, the administration of DAGO at the end of reconditioning in the absence of naltrexone did not decrement the performance of a well-established HR CR. This finding suggests that LC DAGO administration is only effective in blocking recently learned HR CRs.

In Experiment IV, it was found that DAGO administration into the periaqueductal gray region above the LC or into the fourth ventricle caudal to the LC in the same volume and concentration as was given in the LC, had no effects on the learning or performance of the HR CR. The results of this experiment strongly suggest that the ability of DAGO administration to impair HR CR formation is due to its action at the LC and not due to the spread of drug to other brain sites.

It is concluded that central noradrenergic activity as mediated by the LC is critically involved in the learning and retention of a conditioned cardiovascular response.

A number of studies over the last thirty years have established that classically conditioned changes in the cardiovascular system can occur (Bykov, 1957; Cunningham, Fitzgerald, & Francisco, 1977; Fitzgerald & Hoffman, 1976; Fitzgerald, Martin, & O'Brien, 1973; Fitzgerald & Teyler, 1970; Schneiderman, Smith, Smith, & Gormezano, 1966). In the classical conditioning paradigm, it is generally accepted that an animal learns that one stimulus, the conditioned stimulus (CS) provides information about and, therefore, predicts the imminent occurrence of a second stimulus, the unconditioned stimulus (US) (Rescorla & Wagner, 1972). This associative information is stored such that upon the subsequent presentation of the CS, an appropriate, adaptive conditioned response (CR) will be emitted to the CS. In the typical heart rate (HR) conditioning paradigm, a tone CS is paired with an electric shock US and comes to elicit a CR that is a deceleration of HR or a bradycardia response. A conditioned bradycardia response can be firmly learned in 8-10 CS-US pairings. The HR CR has been used by many theorists as a measure of conditioned fear (Kapp, Gallagher, Applegate, & Frysinger, 1982; Schneiderman et al., 1966).

In recent years, classical conditioning of HR has been used as a model system for investigating the neurobiology of learning and memory and for increasing our understanding of central regulation of cardiovascular responding (Kapp & Pascoe, 1986; Schneiderman, McCabe, Haselton, Ellenberger, Jarrell, & Gentile, 1987). Previously, it was shown that rostral fourth ventricle administration of a mu opioid analog appeared to decrement the learning of the conditioned bradycardia response (Harris & Fitzgerald, 1989). It was hypothesized that the noradrenergic nucleus, the locus coeruleus (LC), could be a possible site of action for

the opioid-induced loss of the bradycardia response. The current series of studies were designed to investigate a possible role for the LC noradrenergic system in mediating the development of a classically conditioned bradycardia response.

During the past 20 years, evidence has been accumulating in support of the view that several neurotransmitter systems can modulate the ability of an organism to receive, retain, and retrieve information (Dunn, 1980). The catecholamines in particular have received a considerable amount of attention. Stein (1964) was one of the first to propose that the reinforcing properties of stimuli were subserved by their ability to activate brain noradrenergic systems. Kety (1970) proposed a general noradrenergic theory of learning in which widespread norepinephrine (NE) release after reinforcement would facilitate, at the neuronal level, the formation of a learned association. Based on the occurrence of intracranial self-stimulation in the area of the nucleus locus coeruleus (LC), Crow (1973) hypothesized that the coerulocortico NE pathway, which extensively innervates the forebrain, was the physiological substrate for NE reinforcement. In support of this theory, it has been found that amphetamine, and other drugs which facilitate NE transmission, enhance both the rate and magnitude of a learned response, whereas depletion of brain NE levels or interference with NE transmission leads to learning deficits (Dunn, 1980; Gallagher & Kapp, 1981; Gold & Zornetzer, 1983; McGaugh, 1973; Sara, 1985; Squire & Davis, 1981; Stein, Belluzzi, & Wise, 1975).

The LC contains the largest and most compact number of NE-containing cell groups and comprises more than 40% of all NE neurons in the rat brain (Swanson & Hartman, 1975). The remaining NE neurons are distributed among

several smaller groups of cells within the brain stem, collectively referred to as the lateral tegmental group (Moore & Bloom, 1979). Histochemical catecholamine fluorescence techniques have shown the LC to be a tightly packed area of cells with a few scattered subadjacent cells located bilaterally on the floor of the rostral fourth ventricle on either side of the pontine central gray region. There are between 1400-1600 cells in the rat LC (Descarries & Sauciert, 1972; Swanson, 1976), practically all belonging to the catecholamine type (Dahlstrom & Fuxe, 1964). The projections from the LC travel in the dorsal noradrenergic ascending bundle (DNAB) to provide the primary source of NE innervation to targets in the limbic system, cerebral, and cerebellar cortices and a quantitatively smaller source of NE in hypothalamic and brainstem nuclei (Korf, Bunny, & Aghajanian, 1974; Moore & Bloom, 1979; Grant & Redmond, 1981). A single LC neuron has been estimated to supply about 19 x 10(3) NE containing terminals to the rat cerebral cortex (Descarries & Lapierre, 1973). The LC has been implicated in modulating such diverse functions as respiration, micturition, sleep, control of cerebral blood flow, vigilance, affective disorders, emotions, motivation, and learning (Aston-Jones, 1985; Aston-Jones et al., 1986; Mason, 1984).

Theories of LC Function During Learning

Recently, two specific theories of LC function have been used to explain and predict the purported role of NE in learning. These include the arousal-attention hypotheses (Aston-Jones, 1985; Aston-Jones & Bloom, 1981a; 1981b; Mason & Iverson, 1979; Mason & Lin, 1980) and the anxiety hypothesis (Gray, 1982; Redmond, 1979). The assumption that NE systems mediate

learning has been extensively studied primarily with the use of either electrolytic lesions of the LC or by microinjections of the neurotoxin 6-hydroxydopamine (6-OHDA) into the LC area. Evidence has been found both supporting and refuting the NE hypotheses, making the exact role of NE in learning a controversial one (Crow, 1972; Amaral & Sinnamon, 1977).

Arousal-Attention Hypotheses

The primary evidence for these theories comes from both the electrophysiological characteristics of LC neurons in awake behaving animals and from the postsynaptic effects of NE. Recordings made in the LC of both the rat and monkey (Aston-Jones & Bloom, 1981a; 1981b; Foote, Aston-Jones, & Bloom, 1980) have shown that the spontaneous activity of LC cells reaches a maximum in the awake state, declines sharply with the onset of slow-wave sleep, and is nearly absent preceding and throughout REM sleep. The discharge of LC cells has also been shown to be decreased during grooming and consumption of sweet-water solutions (Aston-Jones & Bloom, 1981a). Thus, activities in which an animal is less likely to be attending to its environment (i.e., sleeping, eating, and grooming), appear to be highly correlated with a decrease in LC discharge rates. These findings are consistent with the view that the LC system participates in controlling cortical and behavioral arousal.

In awake behaviorally active animals, LC neurons have been found to respond vigorously to mild nonnoxious auditory, visual, and somatosensory stimuli (Foote, Aston-Jones, & Bloom, 1980). In these experiments, the excitatory response magnitude of LC cells to trains of stimuli were found to be associated with the level of vigilance, so that the largest responses

occurred for stimuli that awakened rats and the smallest were elicited by identical stimuli during uninterrupted sleep. The sensory response magnitudes of LC cells were also found to be depressed at times when behavioral orienting responses were suppressed (i.e., during grooming or consumption of sugar water). However, stimuli that successfully interrupted either of these behaviors elicited robust responses in LC neurons. Thus, sensory-evoked activity, like spontaneous activity, was found to be suppressed during sleep, grooming, and consumption, but was phasically enhanced when such ongoing behavior was disrupted, corresponding to a change in behavioral state. The version of the arousal-attention hypothesis suggested by Aston-Jones (1985) predicts that LC lesioned animals should be less attentive to external stimuli and have a narrowed scope of sensory perception.

In order to more fully comprehend the significance of changes in LC activity, it is important to know what the postsynaptic effects of NE are and how NE release might modulate cortical arousal and subsequently behavioral attention. Intracellular studies done in the intact brain have shown that NE suppresses the spontaneous activity of cells by hyperpolarizing neurons by 5 - 10 mV. In an extensive study with rat cerebellar purkinje cells (Hoffer et al., 1973), it was shown that a similar hyperpolarization could be evoked by electrical stimulation of the LC. Although NE can have a variety of actions on target cell activity by acting on different adrenergic receptor types located on both the pre- and post-synaptic elements, in general it has been reported that iontophoresis of NE onto target cells or electrical stimulation of the LC can produce a beta-receptor mediated suppression of spontaneous activity while increasing

the responsiveness of target neurons to strong or preferred stimuli (Foote, Bloom, Aston-Jones, 1983). Madison and Nicoll (1982) found that in the presence of NE, hippocampal neurons were able to respond more briskly (i.e., NE increased the number of spikes per stimulus) to a strong suprathreshold stimulus but inhibited responding to a weak stimulus that was at threshold. The mechanism by which NE exerts this effect on target cell activity appears to be more complex that would be predicted from simple membrane hyperpolarization and probably involves changes in intracellular cAMP which subsequently alters the activity of ionic channels within the membrane (Madison & Nicoll, 1982; Sessler, Cheng, & Waterhouse, 1988).

The role of the LC seems to be one of enhancing the "signal-to-noise" ratio in target cell activity. For example, in the presence of iontophoretically applied NE, excitatory responses to acetylcholine and inhibitory responses to GABA were found to be enhanced (Woodward, Moises, Waterhouse, Hoffer, Friedman, 1979). In addition, Segal and Bloom (1976a; 1976b) have demonstrated that both electrical stimulation of the LC and microiontophoresis of NE onto pyramidal cells of the hippocampus enhanced the inhibitory effects of an auditory tone on hippocampal firing or alternately enhanced the excitatory effect of the same stimulus when it predicted food. Some researchers (Aston-Jones, 1985) have suggested that NE released in terminal areas sets the stage so that only the most salient or intense stimuli will preferentially influence overall brain activity, and thereby control attention and behavior. The LC, then, is thought to serve a gating function that determines vigilance or attention, leading to the prediction that the LC should be important for learning the significance of certain sensory events.

Effects of LC Lesions on Learning: Support for the Arousal-Attention Hypothesis

The most commonly used technique for studying the functional specificity of coeruleal noradrenergic projections in many different types of learning tasks has involved injections of 6-OHDA into the area of the LC or the DNAB. The neurotoxin 6-OHDA is taken up by specific transmitter reuptake mechanisms in catecholamine-containing nerve terminals and subsequently causes degeneration of the terminals through an oxidative process (Cooper, Bloom, & Roth, 1986). Injections of 6-OHDA directly into the DNAB typically results in a profound and selective depletion of 80-90% of cortical and hippocampal NE content with minimal effects on a variety of other transmitters such as serotonin, dopamine, and GABA (Mason & Fibiger, 1979). These lesions have also been found to result in a variable and substantial depletion of hypothalamic NE content of between 30-60% which is greater than the 20% of hypothalamic NE content that actually comes from LC projections (Sawchenko & Swanson, 1982). It is thought that this loss of hypothalamic NE may be due to damage occurring in NE projections arising from NE cell bodies in the medulla, which project via the ventral noradrenergic ascending bundle (VNAB), and to variable damage to A1 catecholamine axons that leave the VNAB in the caudal midbrain and join the DNAB (Sawchenko & Swanson, 1982).

Although these types of lesions have been found to have no effects on the acquisition of appetitively motivated operant responding, it has been shown that DNAB lesioned animals show more resistance to extinction, so that it appears that their ability to suppress responses in the absence of an

appetitive reward is impeded (Mason & Iversen, 1977; 1979; Roberts, Price, & Fibiger, 1976). Two possible explanations have been made for this increased resistance to extinction in DNAB lesioned rats. Mason and Iversen (1977) proposed that the DNAB pathway innervating the hippocampus was associated with the encoding of non-reinforcement and that the lesions prevented the animals from distinguishing rewarded from non-rewarded trials. They based this hypothesis on the fact that previous research has shown an association between a specific hippocampal theta rhythm (7.7 Hz) and the coding of non-reward. Bilateral lesions of the DNAB, they reported, were found to selectively raise the threshold for this hippocampal theta rhythm.

The second explanation has centered on the concept of a selective attention deficit (Mason & Iverson, 1979). Mason and Iverson (1979), in contrast to the Aston-Jones (1985) attention-arousal hypothesis, predicted that LC lesioned animals would be more distractable and attend to more environmental stimuli. In other words, they believed that an animals ability to selectively attend to one environmental stimulus while ignoring all others would be disrupted by LC lesions. They interpreted the finding of increased resistance to extinction according to the Sutherland and Mackintosh (1971) model of selective attention. They suggested that rats with DNAB lesions sampled more stimuli in the learning situation and had formed more stimulus-reinforcer associations than nonlesioned control animals, which meant that the response took longer to extinguish.

The most convincing demonstrations supporting this attention hypothesis were found in a paper published by Mason and Lin (1980) using several paradigms including the nonreversal shift paradigm. In this paradigm, the extent to which animals have attended to previously irrelevant stimuli

(i.e., those unpredictive of reward) is measured by subsequently making them relevant (i.e., predictive of reward). According to the authors, a rat unable to ignore formerly irrelevant stimuli, should show faster acquisition of a response when these stimuli became relevant. Mason and Lin (1980), in accordance with their prediction, found faster acquisition on a nonreversal shift from a visual to a spatial dimension in a conditioned appetitive discrimination task. Pisa and Fibiger (1983), however, using DNAB-lesioned rats failed to replicate the finding of enhanced shifting from visual to position cues. Robbins et al., (1985) also found no change in nonreversal shift following DNAB lesions whether the shift was from light to tone or vice-versa. In addition, Robbins reported that DNAB lesioned rats were impaired in learning the initial discrimination. The testing of nonreversal shift learning in both the Pisa and Fibiger and the Robbins study did not occur until 7-8 weeks after the lesion, in contrast to Mason and Lin who tested their animals within two weeks after the lesion. These differences in testing time could have contributed to the conflicting findings.

Mason and Lin (1980) also looked at the effects of DNAB lesions using the latent inhibition paradigm. Latent inhibition has been widely used to assess the capacity of animals to ignore irrelevant stimuli. In this case, the stimulus is made irrelevant by preexposing the animal to the stimulus in the absence of reinforcement, thereby leading to a retarded rate of acquisition during conditioning when the stimulus is subsequently made relevant (Mackintosh, 1983). If DNAB lesioned animals have difficulty in ignoring such irrelevant stimuli, they should actually be at an advantage when the stimuli become associated with reinforcement. Mason and Lin reported an attenuation of the latent inhibition effect following DNAB

lesions, as have Mason & Fibiger (1979).

Additional evidence in support of the selective attention hypothesis has come from the finding that DNAB lesions also attenuate blocking (Lorden, Rickert, Dawson, & Pelleymounter, 1980) and overshadowing (Tsaltas, Preston, & Gray, 1983), two phenomenon that may depend on an animals ability to selectively attend to one environmental stimulus while ignoring others (Mackintosh, 1983). This explanation of blocking and overshadowing says that normal animals ignore redundant (contrasted to irrelevant as in the case of latent inhibition) information.

In classical conditioning studies, lesions of the LC in rabbits have also increased the resistance to extinction of a conditioned nictitating membrane (NM) response (McCormick & Thompson, 1982). In this study, rabbits received electrolytic lesions of the LC seven days prior to classical conditioning training of the NM response using a tone conditioned stimulus (CS) and a corneal airpuff unconditioned stimulus (US). Following training, four days of extinction were given in which the CS was presented alone. At the end of the experiment, the location of the lesions were verified and several brain areas were assayed for NE and DA content. The lesioned animals were then divided into two groups. The first group contained all the animals showing a significant depletion of NE levels relative to non-lesioned controls in all of the brain areas measured except the hypothalamus and mid thalamus, while the second group comprised all the animals showing no statistically significant depletions in any region measured. Despite the differences in NE content in the lesioned animals, all animals had electrolytic lesions in the vicinity of and including the LC.

No significant differences were found between the two lesioned groups and the non-lesioned control group in terms of the acquisition of the NM response. However, during days 3 and 4 of extinction, the group with the largest depletion of cortical/hippocampal NE showed significantly larger NM responses than either of the other groups. This extinction deficit appeared mainly in the unconditioned stimulus period and was highly correlated with the depletion of NE in both the cortex and hippocampus but not with the depletion of NE in the hypothalamus, thalamus, or cerebellum. Because animals with hippocampal lesions also show extinction deficits using this paradigm, it was suggested that the loss of hippocampal NE was the crucial factor in producing the extinction deficits seen in this study.

Lesions of the mesencephalic region of the brain that included the DNAB have been found to disrupt conditioned inhibiton using the NM response in rabbits (Berthier & Moore, 1980). The conditioned inhibition procedure consisted of random presentations of a light CS+ signaling the occurrence of a 2 mA paraorbital shock US and the same light in compound with a tone, CS-, that was presented without the US. Following acquisition of the NM response to the CS+, the rabbits received radio-frequency lesions of the mesencephalic region. Lesions which included the pretectum area of the posterior commissure, periaqueductal gray, and the DNAB produced the greatest disruption in performance primarily by reducing the capacity of the animals to suppress conditioned responses to the CS-. The findings in both of these studies (Berthier & Moore, 1980; McCormick & Thompson, 1982) are consistent with findings of instrumental extinction deficits in DNAB lesioned animals. Lesions of the DNAB under some circumstances appear to interrupt the ability of animals to suppress responses in the absence of

reinforcement and may, therefore, interfere with the development of inhibitory processes.

In summary, there are data implying that while DNAB lesioned animals are able to acquire simple appetitive responses, they are slow to extinguish responses once they are learned and are impaired in the learning of more difficult tasks that require selective attention to environmental stimuli as in the case of discrimination learning, latent inhibition, blocking, and overshadowing. It should also be mentioned that several studies have failed to confirm the above findings for latent inhibition (Pisa & Fibiger, 1983; Robbins et al., 1982) or have rejected the interpretation of the behavioral deficits in terms of failure to screen out irrelevant stimuli. In fact, suggestions have been made that NE-depleted rats integrate less information concerning context, indicating a narrowed scope of stimulus selection, rather than a broadened one (Archer, Mohammed, & Jarbe, 1983; Robbins & Everitt, 1985).

Anxiety Hypothesis

The main rival to the attentional hypothesis of LC effects on learning is the theory that the coeruleocortical system is part of a mechanism mediating behavioral suppression to anxiogenic cues, which are hypothesized to include signals of punishment, nonreward, and novelty (Gray, 1982). Because the LC is known to receive inputs from primary pain afferents, it has been proposed to play a critical role in initiating feelings of alarm, panic, fear, and anxiety associated with aversive events (Redmond & Krystal, 1984).

This hypothesis accounts for the reports of retarded extinction of

appetitive behaviors following DNAB lesions as being due to a reduction in the aversive effects of "frustrative" nonreward (i.e., the lesioned animals continue to respond because the loss of reinforcement is not aversive). Robbins and Everitt (1982) have reported that NE turnover was elevated in the coeruleocortical projection areas in response to a variety of stressors or stressful conditions. They believe that the LC may be activated under conditions of stress or in response to stimuli of significance.

Rasmuss and Jacobs (1986) did a study which assessed the activity of LC neurons during the acquisition of classically conditioned responses involving both aversive and appetitive reinforcers in cats. This study provided an opportunity to examine whether LC unit activity changed in response to the acquisition of new information or whether it was specific for the learning of aversive or appetitive tasks. They found that LC neurons dramatically and specifically increased their activity in response to a tone that predicted the occurrence of a noxious event, but not to a tone that predicted the occurrence of an appetitive event. The authors felt these data provided support for the hypothesis that LC neurons play a role in anxiety.

Effects of LC Lesions on Learning: Support for the Anxiety Hypothesis

In aversive learning paradigms, lesions of the DNAB have been found to have conflicting results. Some studies show no deficit in the acquisition of either one or two-way active avoidance (Mason & Fibiger, 1979; Ogren & Fuxe, 1974; 1977), while other studies show impaired shock avoidance (Cooper, Breese, Grant, & Howard, 1973) and loss of retention in a passive avoidance task (Crow & Wenlandt, 1976). When combined with adrenalectomy,

DNAB lesions have been found to seriously impair the performance and relearning of shock avoidance (Ogren & Fuxe, 1974). Two studies have found that lesions of the DNAB made with the neurotoxin DSP4 (N-2-chloroethyl-N-ethyl-2-bromobenzylamine) consistently impaired the acquisition of two-way active avoidance while one-way active avoidance was only marginally effected (Archer 1982; Ogren, Archer, & Ross, 1980). The neurotoxin DSP4 appears to have more specificity for the NE system than does 6-OHDA. It causes a NE depletion pattern that differs in extent from 6-OHDA lesions or electrolytic lesions in the sense that there is a more marked degeneration of the LC system.

Conflicting results have also been found in terms of lesion effects on conditioned emotional behavior as measured in a conditioned suppression paradigm. In the conditioned suppression paradigm, a stimulus which previously signaled the occurrence of an electric shock is presented while an animal is bar pressing for a food reward. The suppression of on going food responding is considered a measure of the fear evoked by that stimulus. Lesions of the DNAB have been found to have little effect upon conditioned suppression in some studies (Lorden, Rickert, Dawson, & Pelleymounter, 1980; Lorden, Rickert, & Berry, 1983), while another study reported marked blocking of response suppression (Tsaltas, Gray, and Fillenz, 1984). The discrepancy between these studies may be explained by differences in shock intensity used, with the former using a 1 mA shock and the latter a 0.2 mA shock. If the lesion decreases the ability of a given shock intensity to elicit an emotional response then increasing shock intensity may compensate for this depression. Cole and Robbins (1987) using an intermediate shock level (0.5 mA) found impaired acquisition of conditioned suppression in

lesioned animals but found no effect of the lesion on suppression of responses if animals were trained before the lesions were made.

In summary, it seems that the DNAB lesion deficit becomes apparent only under difficult or stressful conditions, particularly during acquisition of learning. Thus, these lesions impair the acquisition of both conditioned appetitive discriminations and conditioned emotional responses. In contrast, once established, none of these forms of conditioning appears to be affected by DNAB lesions. It has been suggested (Robbins et al., 1985) that acquisition involves more complex processing by cortical and limbic mechanisms than does maintaining established performance. Conceivably, the coeruleocortical NE projections are critically involved in the acquisition process of some forms of learned behavior but not in the maintenance of that behavior.

Problems of Interpretation Using Electrolytic or Chemical Lesions

The variability in the behavioral effects of LC lesions may result from differences in the types of paradigms used, the amount of time that elapses between the lesioning and testing, and differences in the amount and extent of the lesions. There is considerable evidence now available that a significant amount of recovery of function can occur within the damaged projection areas in addition to functional compensatory mechanisms that occur one to two weeks after the lesion (Acheson, Zigmond, & Stricker, 1980; Harik, Duckrow, La Manna, Rosenthal, Sharma, & Bannerjee, 1981; U'Prichard, Reisine, Mason, Fibiger, & Yamamura, 1980). After depletion of cortical NE by lesions to the LC, there is a marked change in oxidative metabolism (La Manna, Harik, Light, & Rosenthal, 1981) and in cAMP production. These

processes gradually return to normal 2-8 weeks after the lesion, even while forebrain NE remains depleted (Harik et al., 1981). The exact mechanisms of recovery are not known, but the effects of DNAB lesions could be counteracted by the development of supersensitivity in the denervated receptors to the action of NE released from residual nerve terminals (Segal & Bloom, 1974; Sporn, Harden, Wolfe, & Molinoff, 1976). Furthermore, the amount of NE available for release is apparently increased by a rise in the activity of tyrosine hydroxylase in terminals spared following 6-OHDA lesions (Acheson et al., 1980) and by desensitization of the alpha-2 autoreceptor, which would lead to increased firing in LC neurons (Chiodo, Acheson, Zigmond, & Stricker, 1983).

In fact, LC efferents have been found to have such remarkable properties of plasticity in response to injury that they are excellent models for the study of regeneration and sprouting (e.g., Pickel, Krebs, & Bloom, 1973; Pickel, Segal, & Bloom, 1974). Locus coeruleus neurons have been found to sprout new axons and to reinnervate terminal areas after the induction of chemical lesions in both adult and neonatal animals (Levitt & Moore, 1980; Bjorklund & Lindvall, 1979). It has even been suggested that non-LC systems may change to partially replace the functions of the LC and thereby obscure LC lesion effects (Sara, 1985). It is possible that dopamine or serotonin may take over the function of NE at least in some brain regions. In fact, many neurons in the rat frontoparietal cortex have been found to be inhibited by all three types of monoamines (Reader, 1983). Some support for this notion comes from a study by Harik (1984), who found cortical dopamine activity was enhanced after depletion of NE. Because recovery of function can occur following such lesions, studies involving

lesions may not accurately reflect the effect of a complete loss of NE innervation but instead may reflect the effect of a damaged NE system and its subsequent compensation. This recovery then could account for the lack of dramatic effects of NE lesions on behavior. The functional recovery of the system has been shown to begin as early as two weeks after the lesion, and this is well within the recovery and testing period of the behavioral effects of the lesions. It is important perhaps to emphasize that this recovery occurs even when NE levels in target brain areas remain depleted. Most investigators control for the effectiveness and durability of lesions by measuring forebrain NE after the behavioral experiment which could lead to unwarranted conclusions.

An alternative approach to electrolytic and chemical lesions that is being employed in the current studies is the use of selective pharmacological agents that cause an immediate but reversible "lesion" of the NE system by inhibiting LC neuronal activity. An advantage of this approach is that animals can be tested immediately following pharmacological blockade before the NE system has a chance to recover or compensate. In addition, tests for learning can occur later after the drug-induced blockade has been eliminated.

Pharmacology of LC Inhibition

The activation of presynaptic autoreceptors (Young & Kuhar, 1979) on the cell bodies of LC neurons by locally applied adrenergic agonists has been found to inhibit spontaneous LC neuronal activity (Cedarbaum & Aghajanian, 1976; Williams, Henderson, & North, 1985). The alpha-adrenergic antagonists, piperoxane and yohimbine, on the other hand, have been found to

cause an increase in LC spontaneous activity (Cedarbaum & Aghajanian, 1976; Aghajanian, Cedarbaum, & Wang, 1977). Together, these data suggest that under normal circumstances LC neurons are under tonic feedback inhibition by autoreceptors on the cell body. The activity of an LC cell can be dampened by either inhibitory afferents from brain stem projections (possibly adrenergic in nature), by collaterals from LC axons themselves (Bunney, Walters, Kuhar, Roth, & Aghajanian, 1975), or perhaps under some circumstances by the contralateral LC (Buda, Roussel, Renaud, & Puiol, 1975; Sakai, Touret, Salvert, Leger, & Jouvet, 1977). Clonidine, an antihypertensive drug that acts as an alpha-adrenergic agonist, has been reported to be the most potent agent in completely inhibiting LC neurons at extremely low systemic doses (6 - 10 ug/kg) (Svensson, Bunney, Aghajanian, 1975). Biochemical studies have verified that clonidine decreases while the antagonist piperoxane increases the outflow of 3-methoxy-4hydroxy-phenethyleneglycol the principal metabolite of NE (Maas, Hattox, Landis, & Roth, 1977). The responses of the LC to clonidine are thought to be relatively specific for NE neurons because the dopaminergic neurons of the substantia nigra are insensitive to clonidine (Nyback, Walters, Aghajanian, & Roth, 1975; Svensson et al., 1975).

The LC has one of the highest densities of opioid receptors in the rat brain (Pert, Kuhar, & Snyder, 1975; Simon, 1975). Systemic morphine has been found to depress spontaneous LC neuronal activity (Korf, Bunney, & Aghajanian, 1974) as well as LC firing induced by noxious sensory stimulation (Sasa, Munekiyo, & Takaori, 1975). Local administration of various opioids in the LC has also been found to inhibit both basal and neuronal activity evoked by noxious stimuli (Bird & Kuhar, 1977; Duggan &

North, 1983; Scott-Young, Bird, & Kuhar, 1977). The depression of LC neuronal activity by various opioid agonists is receptor mediated and can be reversed by the opioid antagonist naloxone (Aghajanian, 1978; Guyenet & Aghajanian, 1977; Williams & North, 1984). Neurons in the LC are only inhibited by opioids with an affinity for the mu receptor (North & Williams, 1983; North, Williams, Surprenant, & Christie, 1987; Werling, Brown, & Cox, 1987), suggesting that LC neurons possess only mu opioid receptors. Stimulation of a beta-endorphin pathway that originates in the vicinity of the arcuate nucleus of the hypothalamus causes the inhibition of LC firing (Strahlendorf, Strahlendorf, & Barnes, 1980), and may provide a major source of endogenous opioid innervation to the LC.

While alpha-2 adrenergic and mu opioid agonists act to inhibit LC activity through different receptor types (Aghajanian, 1978; Aghajanian & Wang, 1987; North & Williams, 1985), both cause a hyperpolarization of neurons in the LC through a common mechanism which leads to an increased potassium conductance (Aghajanian & Wang, 1987; North & Williams, 1985; Williams, Egan, & North, 1982). This hyperpolarization is thought to lead to both the inhibition of spontaneous LC activity and to a reduction in the effectiveness of excitatory synaptic inputs to activate LC neurons (North & Williams, 1985). Therefore, both types of agonists provide a powerful technique to turn off LC activity in order to test the hypothesis that such activity is relevant to a given behavior.

Considerable evidence for the anxiety hypothesis of LC function has been found with the use of pharmacological agents that alter LC activity (Crow, 1968; Fuxe et al., 1975; Gray, 1982; Lidbrink, Corrodi, Fuxe, & Olsen, 1972; Redmond, Huang, Snyder, & Maas, 1976; Redmond & Huang, 1979;

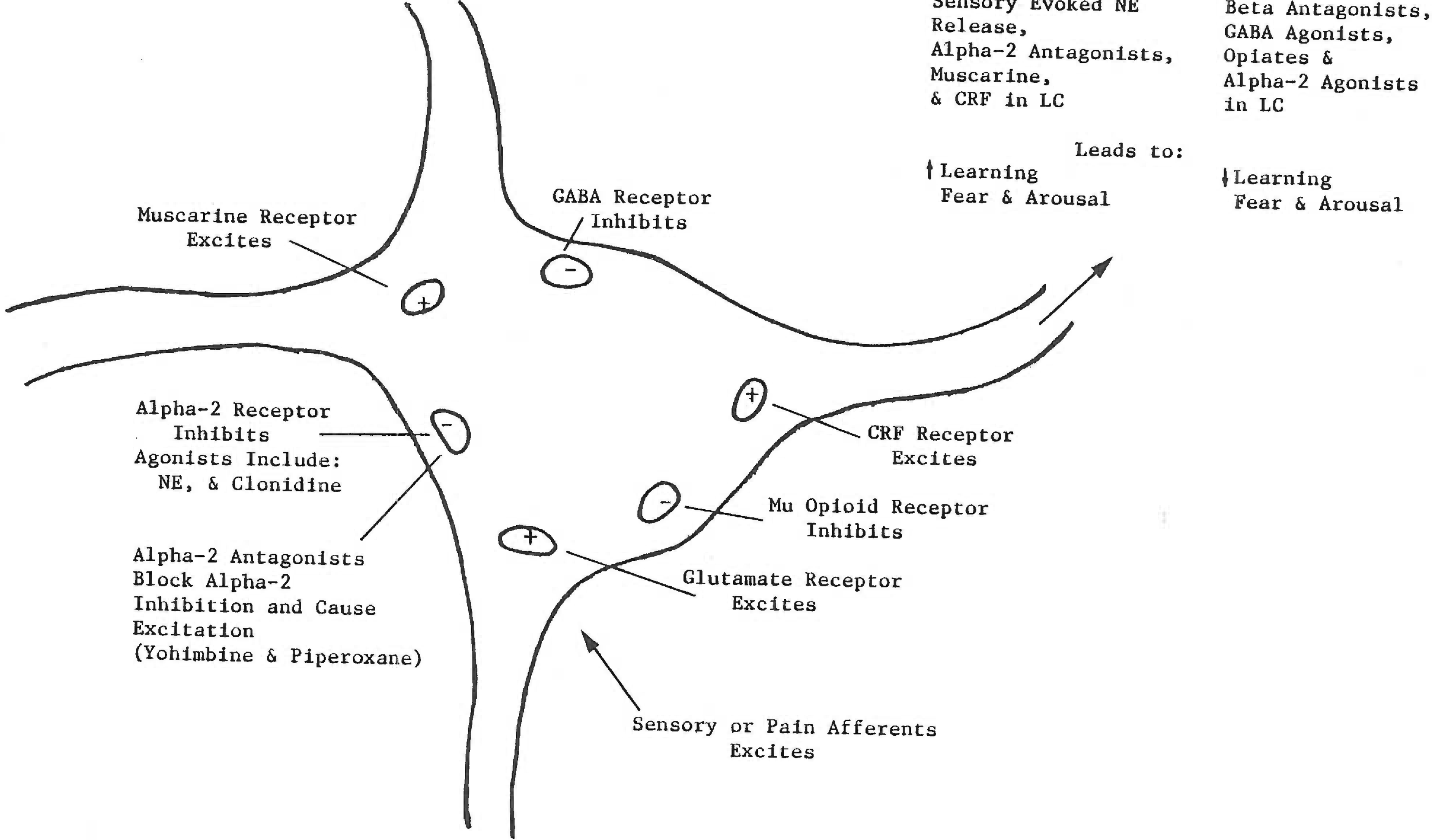
Weiss et al., 1982). Increases in NE activity, caused by either electrical stimulation of the LC or by administration of alpha-2 antagonists such as piperoxane, produce fear reactions in animals indistinquishable from naturally occurring fear responses to environmental stressors (Redmond et al., 1976; Redmond & Huang, 1979). The agonist clonidine, on the other hand, exerts an anti-anxiety effect and has been shown to reduce behaviors associated with environmental threats and to block the effects of piperoxane administration and LC stimulation. Much of the work done in animals on the role of the LC in anxiety has been replicated in human studies. For example, the administration of the alpha-2 antagonists piperoxane and yohimbine have been reported to cause anxiety (Charney, Heninger, & Redmond, 1983; Goldenberg, Snyder, & Aranow, 1947; Soffer, 1954; Holmberg & Gershon, 1961), electrical stimulation the LC was reported to produce feelings of fear and imminent death (Nashold, Wilson, & Slaughter, 1974), and clonidine has been shown to reduce anxiety similar to benzodiazepines (Charney, Heninger, & Redmond, 1983; Hoehn-Saric, Merchant, Keyser, & Smith, 1981). Clonidine has also been found to be effective in alleviating the symptoms and anxiety associated with opiate and alcohol withdrawal (Gold et al., 1978; Redmond & Krystal, 1984; Svensson et al., 1978). The effects of various neurotransmitter systems on LC activity and how these systems might affect NE release in target areas are diagramed in Figure 1.

<u>Effects of Drugs That Alter LC Activity: Appetitively Motivated Learning</u>

Sara (1985) has shown that drugs that facilitate NE transmission (i.e., amphetamine and the alpha-2 antagonist, yohimbine can facilitate memory retrieval in an appetitive maze learning task. This task involved the

Figure 1. Diagram of a single LC neuron and the possible excitatory (+) and inhibitory (-) influences on cell body activity. This figure also describes how these factors may alter beta-adrenergic receptor activity in taget areas and subsequently affect behavior.

Figure 1.



learning of spatial discriminations, so that successful completion of the maze was highly dependent on the presence of very subtle intramaze cues in the form of complex configurations of shadows on the floor and walls of the maze. In this paradigm, rats were trained to a criterion performance and then were given a retention test 3-4 weeks later. Normal rats show a considerable amount of forgetting during the retention test as measured by run time and errors. Both amphetamine, which releases NE and dopamine, and yohimbine, which increases the firing rate of LC cells, were found to significantly facilitate retrieval when injected before the retention test but not if injected during acquisition.

Systemic doses of clonidine have been found to suppress operant responding for food reward with many different schedules of reinforcement in the dose range of 0.01 - 0.8 mg/kg (Dwoskin & Sparber, 1983; Kleven & Sparber, 1987; McClearly & Leander, 1981). The efficacy of clonidine to depress operant responding appears to be due to its ability to stimulate alpha-2 receptors because the depression can be completely reversed by administration of the alpha-2 antagonists, yohimbine (Dwoskin & Sparber, 1983; Obersztyn & Kostowski, 1983; Smith, 1987) or piperoxane (Debarre & Schmitt, 1974), but not by alpha-1 adrenergic antagonists (Dwoskin & Sparber, 1983). Systemic clonidine administration has certain effects suggesting that central NE activity is suppressed. These effects could be responsible for the depression of operant responding. For instance, clonidine causes sedation (Debarre & Schmitt, 1971), is known to reduce anxiety in both humans and animals, depresses the activity of the sympathetic nervous system (Charney, Heninger, & Redmond, 1983; Hoehn-Saric, Merchant, Keyser, & Smith, 1981), and has anti-nociceptive properties

(Fielding, Wilker, Hynes, Szewczak, Novick, & Lal, 1978; Paalzow, 1974; Paalzow & Paalzow, 1972; Schmitt, Le Douarec, & Petillot, 1974).

In support of the hypothesis that clonidine has anxiolytic properties (Redmond & Krystal, 1984), clonidine has been reported to block the partial reinforcement extinction effect (Halevy, Feldon, & Weiner, 1986). The partial reinforcement extinction effect refers to the increased resistance to extinction shown by animals trained in an appetitive running task on a partial reinforcement schedule relative to animals trained on a continuous reinforcement schedule. This increased resistance to extinction may be considered to reflect the fact that partially reinforced animals learn to respond in the presence of stimuli associated with non-reward, which arouses frustration (Amsel, 1962; Capaldi, 1967; Gray, 1975; Mackintosh, 1974). Drugs with anti-anxiety properties have been found to block the partial reinforcement extinction effect when given during acquisition (i.e., learning to respond in the presence of frustration does not occur) and to increase the partial reinforcement extinction effect when given during extinction (i.e., the drug blocks the frustration of non-reward that would normally extinguish responding). It is thought that anxiolytic drugs including clonidine antagonize the emotional effects of non-reward (Feldon, Guillamon, Gray, DeWit, & McNaughton, 1979; Feldon & Gray, 1981; McNaughton, 1984). Lesions of the NE system with 6-OHDA have also been found to abolish the partial reinforcement extinction effect suggesting a possible role for NE systems in mediating this phenomonon (Owen, Boarder, Gray, & Fillenz 1982).

In contrast to the above findings, clonidine has been found to facilitate learning in animals whose NE function has been impaired through

either age or lesions. For example, clonidine has been found to improve memory on a delayed-response task in aged primates and in young primates with 6-OHDA lesions (Arnsten & Goldman-Rakic, 1985). Memory improvements have also been found following clonidine administration in patients with Korsakoff's psychosis (McEntee & Mair, 1980). It is thought that clonidine improves memory by facilitating NE transmission at postsynaptic alpha-2 receptors in the frontal cortex. These receptors proliferate in the cortex following damage to the NE system that occurs through either age, disease, or lesions (Arsten & Goldman-Rakic, 1985; McEntee & Mair, 1980; U'Prichard, Bechtel, Rouot, & Snyder, 1979).

Effects of Drugs That Alter LC Activity: Fear-Motivated Learning

In aversive learning situations, clonidine (0.1 mg/kg ip) has been found to be more potent than morphine (10 mg/kg) in decreasing responding on a Sidman avoidance task and in increasing the frequency of shocks received (Smith, 1985). No tolerance was found to this effect of clonidine in up to 30 days of testing. Clonidine (0.015 - 0.05 mg/kg) has also been found to depress other types of conditioned avoidance responses (Robson, Antonaicio, Saelens, & Liebman, 1978) but has not been shown to affect escape responding (Delbarre & Schmitt, 1974; Grilly, Johnson, Minardo, Jacoby, & LaRiccia, 1984). In a two-way active avoidance task, clonidine (0.025 - 0.1 mg/kg) depressed, while yohimbine facilitated, responding (Obersztyn & Kostowski, 1983). A 0.2 mg/kg dose of clonidine completely blocked acquisition of the response (Kostowski, Plaznik, & Pucilowski, 1980). These effects of clonidine may be due to the reported ability of this compound to inhibit LC activity and thereby reduce anxiety (Redmond & Krystal, 1984).

The amygdala central nucleus (ACE) receives a major input from the LC (Moore & Bloom, 1979; Fallon, Koziell, & Moore, 1978), has a well established role in the learning of fear-motivated behaviors (Hitchcock & Davis, 1986), and could be an important structure in mediating the effects that are seen when LC output changes in aversive learning situations. Interference with NE activity in the ACE has been shown to disrupt long term memory formation in passive avoidance conditioning (Gallagher & Kapp, 1981). In this study, bilateral administration of the beta-adrenergic antagonist propranolol (34 nmole) into the ACE was found to produce retrograde amnesia of the passive avoidance response, that was both time and dose dependent. Administration of the alpha-2 adrenergic antagonist, phentolamine (8.5 nmole) into the ACE, on the other hand, was found to significantly increase the retention of a passive avoidance response if given immediately following training, but not if given 6 hours after training. A group that received concurrent administration of both phentolamine and propranolol showed normal retention, with latencies similar to the saline-injected controls. The authors felt that the differential effects of phentolamine and propranolol were due to the fact that phentolamine increases NE transmission through blockade of inhibitory presynaptic alpha-2 receptors, while propranolol decreases NE transmission by blocking postsynaptic beta-receptors.

Clonidine has been shown to attenuate the potentiated startle response in rats, which is a widely accepted measure of conditioned fear (Davis, 1979; Davis, Cedarbaum, Aghajanian, & Gendelman, 1977; Davis, Redmond, & Baraban, 1979). The amplitude of the startle reflex to a loud auditory stimulus can be augmented by presenting the eliciting stimulus in the presence of a cue that has previously been paired with a shock (Brown,

Kalish, & Farber, 1951). This phenomenon has been termed the fear-potentiated startle effect and can be blocked by drugs which reduce fear and anxiety (Chi, 1965; Davis 1979; Davis, Redmond, & Baraban, 1979). Systemic administration of clonidine (10 - 40 ug/kg) was found to produce a dose-dependent reduction of fear as measured in the potentiated startle paradigm (Davis, Redmond, Baraban, 1979). The reduction seen in the potentiated startle response could not be accounted for entirely by a general depressant effect of clonidine on baseline startle nor by an acceleration of fear extinction. Piperoxane and yohimbine both caused an increase in potentiated startle. These results were interpreted to support the hypothesis that NE transmission is important in the expression of fear and anxiety.

Evidence linking alpha-2 receptors and NE neurons with the modulation of cardiovascular and other autonomic responses to behavioral stressors has also been found. For example, it has been demonstrated that areas of the brain that influence cardiovascular responses to stress, the amygdala, hypothalamus, pons, and nucleus of the solitary tract in the medulla, contain high densities of alpha-2 receptors and are richly innervated by NE neurons (Moore & Bloom, 1979; Unnerstall, Kopajtic, & Kuhar, 1984; Van der Kooy, Koda, McGinty, Gergen, & Bloom, 1984). In addition, the LC projects to hypothalamic, medullary, and spinal sympathetic areas that mediate the tachycardia, piloerection, gastrointestinal hypermotility, urination, and diarrhea often observed during anxiety. Several studies have shown that NE neurons and alpha-2 receptors exert a dominant influence on the cardiovascular system through alterations in the activity of the sympathetic and parasympathetic nervous systems and neuroendocrine function (Bolme,

Forsyth, Ishizaki, and Melmon, 1975; Goldberg & Robertson, 1983; Scriabine, Sweet, Ludden, Stavorski, Wenger & Bohidar, 1977).

In a study done by Hubbard, Cox, Sanders, & Lawler (1986), intracerebroventricular (icv) injections of clonidine (8 ug) were found to attenuate classically conditioned pressor responses in rats. In this study, both borderline hypertensive rats (BHR) and normotensive rats were used. The freely-moving rats received paired presentations of a tone CS with a tail shock US. Following training, both groups received third ventricle infusions of either clonidine or saline.

Clonidine produced a significant fall in baseline blood pressure (BP) and a pronounced bradycardia in both groups. Clonidine also was found to lower plasma epinephrine (EPI) levels without altering plasma NE levels. After conditioning, the onset of the CS produced a biphasic pressor response, and tachycardia in both normotensive and BHR saline rats. Clondine treated BHR rats continued to show the biphasic pressor response but it was reduced in magnitude. Normotensive animals given clonidine, on the other hand, showed a significant attenuation of the pressor response to the onset of the CS. Clonidine had no effects on the heart rate (HR) response to the CS in either group despite the reduction in baseline HR. Conditioning caused a significant increase in plasma NE and EPI levels in saline treated groups, while the groups given clonidine failed to show significant increases in plasma NE during conditioning and EPI levels were also significantly decreased. The authors felt that the ability of clonidine to block conditioned increases in BP and concentrations of plasma EPI and NE in normotensive animals might have been due to its reported anxiolytic properties.

The ACE has been found to be a critical structure in the development of conditioned bradycardia with the use of both lesions (Kapp, Frysinger, Gallagher, & Haselton, 1979) and pharmacological blockade studies (Gallagher, Kapp, McNall, & Pascoe, 1981; Gallagher, Kapp, & Pascoe, 1982). Blockade of NE input to the ACE can attenuate the development of a classically conditioned bradycardia response in restrained rabbits (Gallagher et al., 1980). In this study (Gallagher et al., 1980), microinjections of either saline or the beta-adrenergic antagonist dl-propranolol (40 nmol) were made bilaterally into the ACE prior to training. Administration of dl-propranolol had no effects on baseline HR or the HR orienting responses to the CS when it was presented alone. However, the drug did significantly impair the development of the conditioned bradycardia response relative to saline treated animals. The unconditioned HR response to the US following dl-propranolol treatment significantly decreased over the course of conditioning. A group that received a beta-adrenergic agonist, l-isoproterenol, in combination with dl-propranolol developed conditioned responses similar in magnitude to saline treated controls. These results suggest that NE release within the ACE contributes to the acquisition of a conditioned bradycardia response.

Rostral fourth ventricle infusions of mu opioid agonists in the vicinity of the LC and the periaqueductal gray (PAG) have been reported to block the development of a conditioned bradycardia response in rats (Harris & Fitzgerald, 1989) and to prevent the performance of a recently established bradycardia response in rabbits (Lavond, Mauk, Madden, Barchas, & Thompson, 1983). In the study by Harris & Fitzgerald, animals received infusions of either saline, D-ala-methionine-enkephalinamide (DALA) (10 ug), or a

combination of DALA and naltrexone (5 ug) prior to classical conditioning training. A fourth group, which served as a location control, received DALA in the brain stem area directly below the fourth ventricle. The conditioning procedure, which was carried out over two days, consisted of a discrimination paradigm in which one tone, CS+, was paired with a chest shock US and a different tone, CS-, was presented alone. Both the brain-stem-injected group and the agonist/antagonist group showed the development of a normal bradycardia conditioned response (CR), comparable to that seen in the saline treated group. The DALA ventricle group, however, failed to learn a HR CR during conditioning despite the presence of normal URs to the shock. The absence of a CR in this group was evident even when testing occurred 48 hours after training in a non-drugged state, providing further evidence that learning was blocked. These results suggest that the decremental effects of DALA on the HR CR were due to the activation of opioid receptors located in the rostral fourth ventricle, possibly in the LC or PAG. The authors felt that the activation of opioid receptors in this area of the brain might have prevented the development of a learned association by decreasing emotional awareness of stimuli.

Summary

Although the exact role that LC activity and NE release might play during learning is not yet known, two hypotheses have emerged that are consistent with the majority of findings. First, it appears that the LC modulates attentional processes, and second, that the LC modulates emotional and autonomic responses during aversive learning. Central NE innervation provided by the LC appears to play a role in allowing animals to ignore

irrelevant environmental stimuli and to alter their responses to stimuli when environmental conditions change. This was evident in the ability of DNAB lesions to retard extinction (Mason & Iverson, 1977; McCormick & Thompson, 1982), to block the development of latent inhibition (Mason & Fibiger, 1979), blocking (Lorden et al., 1980), and of overshadowing (Tsaltas, Preston, & Gray, 1983), and to abolish conditioned inhibition (Berthier & Moore, 1980). There were also some indications that LC neurons were more responsive during the learning of an aversively motivated conditioned response as opposed to an appetitively motivated response (Rasmuss & Jacobs, 1986). This fact may relate to the LC's reported role in regulating fear and anxiety responses to environmental stressors (Redmond & Haung, 1979). And finally, there was indirect evidence that LC activity may be important in the development of classically conditioned autonomic responses (Gallagher & Kapp, 1981; Gallagher, et al., 1980; Harris & Fitzgerald, 1989) and in the performance of established autonomic CRs (Hubbard et al., 1986).

Experiment I

Rationale

The findings of Harris and Fitzgerald (1989) have suggested that stimulation of opioid receptors in the rostral fourth ventricle can block the learning of a HR CR. The aim of the present study was to isolate the site within the fourth ventricle where this blockade occurred. Because the LC appears to be involved in the behavioral expression of fear and anxiety and in autonomic responses such as HR that accompany fear (Redmond & Haung, 1979), it is conceivable that the LC was involved in the HR CR loss. Inhibition of LC activity by the administration of opioids in the ventricle could potentially affect several forebrain structures important to the learning of this response.

The present experiment examined the HR conditioning effects of alpha-2 noradrenergic agonists following administration into the rostral fourth ventricle. Tests of the effects of the corticotropin releasing factor (CRF) antagonist, alpha-helical CRF (9-41), which can block increases in LC activity due to the release of endogenous CRF that might occur during conditioning, were also made. In the initial study, intraventricular infusions were chosen over direct intracranial microinjections into the LC to see if the opioid-blockade effect seen earlier (Harris & Fitzgerald, 1989) would occur using a non-opioid agent to inhibit LC output. Given such an outcome, the more technically difficult and time consuming LC microinjection technique could be utilized to isolate LC involvement.

It was thought pharmacological manipulation appeared to have several advantages over the more traditional approaches of electrolytic or neurotoxic lesions. First, with the pharmacological approach, LC activity

would only be inhibited during the conditioning session so that the animals behavior would be relatively unaffected between conditioning sessions. Second, because this type of lesion is reversible, LC effects on the performance of the CR can be separated from effects on the learning of the response by testing animals for evidence of conditioning after the drug effects have been removed. Third, a time limited pharmacological lesion would not be expected to result in the significant recovery of function in LC target areas that is seen following electrolytic and neurotoxic lesions.

This study looked at the effects of rostral fourth ventricle administration of two different alpha-2 agonists, a mu opioid agonist, and a CRF antagonist on the development of a HR CR. A saline-injected group served as a procedural control group to insure that it was the drug administration and not the surgical procedures that altered HR learning. In this experiment, the drugs were administered prior to classical conditioning training and later tests were given to assess the evidence of a HR CR in a non-drug state.

Previous research (Harris & Fitzgerald, 1989) has shown that this design may allow distinctions to be made between drug effects on the HR CR that are due to actions on associative mechanisms from those that are due to actions on performance mechanisms. For example, animals can be tested for evidence of a CR at a time when the drug is no longer present. In this way, any attributes of the drugs which might effect the performance of the response can be eliminated. Also, by giving the drugs prior to conditioning, any possible effects of the drugs on the HR UR can be assessed throughout the conditioning session. This paradigm does not, however, rule out any state-dependent decrements in learned responses due to the change in

drug state that occurs between training and testing.

The two alpha-2 agonists used in this study were clonidine and UK 14,304. Although clonidine has been the most widely used alpha-2 adrenergic agonist in behavioral studies and has been shown to be potent in inhibiting LC neurons, its selectivity for alpha-2 receptors is reported to be marginal and it is not considered to be a full agonist (Grant & Scrutton, 1980). The alpha-2 agonist UK 14,304 (5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline), on the other hand, is considered to be a full agonist at alpha-2 receptors (Grant & Scrutton, 1980), is more potent than clonidine at alpha-2 receptors, and shows little specificity for alpha-1 receptors even at high concentrations (Cambridge, 1981). Any differences seen in the ability of these two compounds to block the learning of a HR CR might be attributed to the greater selectivity of UK 14,304 for alpha-2 receptors. The failure to find any difference could mean that all the effects of clonidine were mediated through the alpha-2 adrenergic receptor.

The opioid compound chosen was a highly potent analog of methionine-enkephalin, D-ala2-met-enkephalinamide (DALA). The compound DALA (Pert, Pert, Chang, & Fong, 1976) was selected because of its specificity for the mu receptor, its conformational protection from enzymatic attack (Pert et al., 1976), and because it was previously shown to be effective in blocking the development of a HR CR (Harris & Fitzgerald, 1989). The use of DALA allowed a direct comparison to be made between opioid and alpha-2 effects on HR CR development. If both mu and alpha-2 compounds had similar effects in blocking HR CR development then the LC would be a highly likely site for this effect to occur. The LC is the primary structure in the rostral fourth ventricle closest to the injection site with the highest

concentrations of both opioid and alpha-2 receptors (Unnerstall, Palacios, & Kuhar, 1981). The mesencephalic PAG which borders the rostral fourth ventricle also has mu and alpha-2 receptors, but they are much lower in density. The activation of mu opioid receptors in this PAG region has well-known analgesic actions (Yakash & Rudy, 1978), whereas blockade of these same receptors with naloxone results in increased shock sensitivity and fearful behaviors (Hammer & Kapp, 1986). The effect of activating alpha-2 receptors in the PAG (located primarily lateral to the cerebral aqueduct, Unnerstall, et al., 1984), on the processing of nociceptive information, is not presently known.

The fourth compound used was the CRF antagonist, alpha-helical CRF (9-41) (Rivier, Rivier, & Vale, 1984). Endogenous CRF is known to be released in response to electric shock and subsequently activates central NE systems (Berridge & Dunn, 1986). Exogenous CRF has been shown to increase the spontaneous activity of LC neurons by 100% (Valentino, Foote, & Aston-Jones, 1983; Valentino, Martin, & Suzuki, 1986). Intraventriclular administration of the antagonist alpha-helical CRF has been shown to be effective in blocking increases in LC activity due to hemodynamic stress (Valentino & Wehby, 1988), and to block behavioral responses of stress and fear (Cole, Britton, & Koob, 1987; Sherman, Barksdale, Takahaski, & Kalin, 1987). For instance, icv alpha-helical CRF (25 ug/ul) significantly attenuated shock-elicited freezing but had no effect on hot-plate tests of pain sensitivity (Sherman et al., 1987). In addition, it has been reported that alpha-helical CRF can significantly attenuate the ability of a CS signaling shock to suppress bar pressing for a food reward (Cole et al., 1987). It is thought that alpha-helical CRF may have anxiolytic effects

similar to benzodiazepines and that endogenous CRF may be involved in conditioned fear (Cole et al., 1987). It is possible that CRF could be released during HR conditioning and be involved in the behavioral expression of fear and anxiety through the activation of the LC noradrenergic system. This activation of the LC by endogenous CRF could alter the rate and degree of HR conditioning. Blockade of endogenous CRF binding in the LC could decrement the level of HR conditioning.

At the end of classical conditioning training, antagonists were administered to the alpha-2 and mu agonists groups, while saline was given to the alpha-helical CRF and saline groups. The alpha-2 agonist groups were given the highly selective alpha-2 antagonist, idazoxan (RX 781094) (Fredman & Aghajanian, 1984), while the DALA group was given the opiate antagonist, naltrexone. Following this infusion, CS alone test trials were given to assess the status of HR responding. Both mu opioid and alpha-2 noradrenergic agonists are known to have potent effects on the cardiovascular system leading to a suppression of sympathetic outflow and an increase in parasympathetic outflow (Holaday, 1983; Laubie, Schmitt, & Drouillat, 1977; Ward & Gunn, 1976). It is believed that both mu and alpha-2 agonists can affect cardiovascular activity by stimulating receptors located on brain stem cardiovascular regulatory nuclei that contain both types of receptors (Unnerstall, Kopajtic, & Kuhar, 1984). Based on previous findings (Gurtu, Sharma, Sinha, & Bhargava, 1983; Harris & Fitzgerald, 1989; Laubie, Schmitt, & Drouillat 1977), both the mu and alpha-2 drugs were expected to cause a fall in baseline HR. Therefore, the antagonist test was necessary in the mu and alpha-2 groups to show that these drugs do not block HR conditioning simply by blocking the performance of the CR. Presumably,

the antagonists should block all of the cardiovascular performance effects of these drugs.

In order to control for any residual drug effects not blocked by the antagonists that could have affected HR CR performance, a non-drug CS-alone test was given 48 hours after the antagonist test to determine whether a HR CR occurred in the different drug groups. Following the non-drug test, reconditioning trials were given to all groups in the absence of drugs to show that learning could occur in all groups in the absence of the drugs. Following the reconditioning phase, the drugs were readministered and CS-alone test trials were given to determine whether the drugs interfered with the established CRs.

The final phase of the experiment took place after the drug test and consisted of a startle test that used a loud sound as the startle eliciting stimulus presented during CS+ or CS-. The startle paradigm has been widely used to measure the presence of conditioned fear by presenting a startle stimulus in the presence of a CS that has previously been paired with an aversive US (Brown, Kalish, & Farber, 1951; Davis, 1979). It has generally been found that in this situation, the reflex startle reaction is augmented or potentiated by the CS. Presumably, this is because of a conditioned fear state elicited by the CS. The intent of the current study was to use the startle procedure to provide a measure of conditioned fear and to compare the effects of the drugs on fear potentiated startle with those that occurred for the HR CR.

Methods

Subjects

The subjects were 40 male Sprague-Dawley albino rats (Simonsen), weighing between 350 and 400 g. The rats were housed in individual cages with food and water available ad libitum. The housing of the animals complied with the NIH guidelines set forth in the *Guide for the Care and Use of Laboratory Animals.* The animals were on a normal 12 hr. light and dark cycle. Because alpha-receptor binding in the rat is reported to be the highest in the afternoon (Kafka, Wirz-Justice, Naber, & Lewy, 1981), all animals were tested during this part of the light cycle.

Surgical Procedures

Four days prior to the start of the experiment each rat was implanted with two electrodes for recording the electrocardiogram (ECG) and with one brain cannula for infusing compounds into the fourth ventricle.

ECG Electrodes . The ECG electrodes were made of 34 ga. stainless-steel wire and non-insulated metal butt connectors. The wire was threaded through the butt connector and three loops were made subcutaneously through the skin on either side of the rat's thoracic cavity. The bundle of wires with the butt connector was then crimped.

Brain Cannula . The cannula was composed of two parts. The external guide cannula was made from a 1-cm. long 22-ga. stainless-steel hypodermic needle. The hub of the needle was removed to make the cannula less cumbersome for the animal. A stylet made from 30-ga. stainless-steel tubing was placed inside the cannula in order to isolate the ventricular space from the external environment. The length of the stylet was adjusted so that it extended 1-mm beyond the end of the guide cannula.

Anesthesia

A 0.65-ml dose of a solution of Ketamine (100 mg/ml) and Rompun (20 mg/ml) mixed in the ratio of 2:1 was given ip to anesthetize the animals.

Surgeries . A 1.5-cm2 area on both sides of the thoracic cavity was shaved. The top of the animal's head, from the eyes to the base of the skull, was also shaved. All shaved areas were wiped with a Betadine antiseptic solution to cleanse the skin. The ECG electrodes were then put into place.

The rat was placed in a Kopf stereotaxic apparatus. Kopf rat ear plugs were used to prevent damage to the ear drums. The skull was then exposed. A burr hole was made using a high speed dental drill to the right of the midsagittal suture line and one stainless-steel screw was implanted in the skull. A second burr hole was made for entry of the cannula according to the stereotaxic coordinates: AP: -1.5 mm (posterior to lamda), L: 0.0 mm (lateral to the midline), V: -5.5 mm (verticle to the dura) (Pellegrino, Pellegrino, and Crushman, 1979). The incisor bar was set so that the head was in a horizontal position. Once the cannula was lowered into place, it was cemented to the skull and skull screw with dental acrylic. After the cement dried, the skin was sutured closed around the cannula with stainless-steel wound clips. All animals were allowed to recover for 4 days.

Infusion Procedure

The infusion needle was made from 30-ga stainless-steel tubing. The length of the tubing was measured carefully to make certain that it would extend 1-mm beyond the guide cannula. The length of the infusion needle was important because the scar tissue that forms around the ventricular end of

the guide cannula might hinder the flow of solution from the infusion needle. The infusion needle was connected to a 10-ul Hamilton microsyringe by a 1-cm length of PE-10 tubing. The infusion was given by opening the experimental chamber and placing the infusion needle into the guide cannula while the rat rested undisturbed in the experimental chamber. The infusion needle was left in place for 1 minute following the infusion to allow the drug to diffuse away from the infusion site.

Apparatus

The rats were restrained in an inverted U-shaped plastic holder produced by Narco Biosystems. Adjustable inserts were located at each end of the holder to provide restraint. A 3-cm diameter hole at the top of the restrainer provided access to the guide cannula.

The rats were placed in an Industrial Acoustics sound-isolated chamber during conditioning. The rats were located 10 cm away from two 8-cm speakers, through which the CSs were presented. A third speaker provided white noise to mask extraneous sounds. The chamber was ventilated by a 7.5-cm fan.

Conditioned Stimuli. The CS+, which was paired with the shock, consisted of a 6-s electronically generated click that occurred at a frequency of 3 Hz. The interval between the onset of the CS+ and the onset of the US was 6 s. The CS-, which was not paired with shock, was a continuous 6-s 1-kHz tone. The UCS was a 0.5-s train of 0.5-ms, 250-Vdc pulses of shock (1-2 pulses per 0.5-s), delivered through the ECG electrodes.

An intermittent tone was chosen to be used in this experiment as the CS+ because this type of stimulus, as was used previously (Harris &

Fitzgerald, 1989), appears to be more salient and results in faster acquisition during classical conditioning than do continuous tones (Papsdorf, Fishbein, & Gormezano, 1964). It was reasoned that using a stimulus for the CS+ that optimizes conditioning should maximize any chances of seeing conditioning in the drug pre-exposed groups. The failure of conditioning to occur in the drug pre-exposed groups, could not then be explained by the fact that the CS+ used in this experiment was not conspicuous enough. Previously, we have shown that this CS+ yields robust HR conditioned responses in saline pre-treated animals (Harris & Fitzgerald, 1989).

The discrimination conditioning paradigm allows an assessment to be made of the reported ability of animals to suppress responses in the presence of non-reinforced stimuli (i.e., CS-). Previous reports (Berthier & Moore, 1980; McCormick & Thompson, 1982), have indicated that lesions of the LC system prevent animals from suppressing responding to a CS-. Therefore, in the current experiment it might be expected that inhibition of LC activity might increase HR responding to CS-.

Recording of HR. An Apple II Plus microcomputer controlled data acquisition and the experimental procedure. Experimental events were timed to a resolution of 10 ms by a clock card set to provide interruptions at 100-Hz. A disk drive attached to the computer provided permanent storage of data on magnetic disks.

The ECG was sensed through a Massey Dickinson logic circuit. This circuit converted each heart beat to a +5-volt square wave pulse that was then transferred into the Apple II microcomputer by way of a California Computer Systems parallel interface card (Model 7720). An assembly language

program, developed by Cunningham (1982), measured each interbeat interval (IBI) to the nearest 0.01 s. In order to reduce the influences of movement artifacts, IBIs that were less than 90 ms or greater than 300 ms were automatically scored as errors as were IBIs that differed from the previous interval by more than 30 ms. These errors were not included in the data analyses. Mean IBIs were calculated for selected measurement intervals on each trial and the numbers were retained in the Apple II's memory until the end of each trial when this information was stored on a floppy disk. Interbeat interval data were converted to beats per minute (BPM) for both CS+ and CS- trials by means of an off-line BASIC program.

Each trial was 18-s long and consisted of a 6-s pre-stimulus baseline period, a 6-s CS period (divided into three 2-s measurement intervals), and a 6-s post-US period (divided into three 2-s measurement intervals). Heart rate was recorded in the same manner on each trial regardless of whether stimuli were presented on that trial or not. On each trial, an average HR in BPM was generated from the 6-s pre-CS baseline period and this measure represented baseline HR for that trial. Difference scores were calculated by the off line BASIC program for each trial by subtracting the pre-CS BPM rate from the BPM rate during each of the three 2-s measurement intervals for the CS period and during each of the three 2-s measurement intervals following the US.

Recording of Startle. The startle testing apparatus, a modified version of one described previously (Cunningham, Crowell, Eaton, & Brown, 1973), consisted of a platform attached to a springboard-like suspension system located 5 cm from the CS speakers. The restrained animals rested on the platform. Two Astatic phonocartridges (No. 24) with 1.5 in metal rods

in place of the usual needle, positioned underneath to the platform, recorded movement. Small platform movements resulted in a voltage output from each phonocartridge. These outputs were connected to a voltage summing circuit and then amplified by a Grass 5P1 preamplifier. The signal was then fed into a voltage-to-frequency converter which sent the signals into the computer. Recording of the movement data was similar to that previously described for HR. Each trial was 18-s long and consisted of a 6-s pre-stimulus period, a 6-s CS period, and a 6-s post-startle stimulus period. The startle-eliciting stimulus was presented 3-s after CS onset, a time when conditioned fear to CS+ should be present. In addition to amplifying the signal, the Grass preamp also provided a visual representation of the startle reponse on the polygraph. Calibration was accomplished by dropping a 19-g clay ball from a height of 3 cm onto the platform. The startle eliciting stimulus was provided by a flush horn activated for 100 ms at a 100 dB noise level. The horn was located 10 cm behind the rat in the experimental chamber.

Experimental Procedure

Before the start of the experiment, all of the animals were randomly assigned to one of five treatment groups (n = 8 for each group). One group (DALA) received a 10-ug dose of DALA. A second group (CLON) received a 3-ug dose of clonidine. The third group (UK) received a 5-ug dose of UK 14,304. The fourth group (a-HEL) received 25-ug dose of alpha-helical CRF (9-41). And the fifth group (SAL) received an equivalent volume of 0.9% saline.

Drug dosages were chosen on the basis of effective dosages previously reported in the literature (alpha-helical CRF, Cole, Britton, & Koob 1987; clonidine, Svensson et al., 1975; DALA, Harris & Fitzgerald, 1989) and on

the basis of pilot data (UK 14,304) showing that this dosage was able to block the development of the HR CR.

All drugs except alpha-helical CRF were dissolved in 0.9% saline. Alpha-helical CRF (9-41) was first dissolved in distilled water with the pH adjusted to 6.5 to control for the acidity of this compound. It was then mixed with saline. The volume of all infusions was restricted to 1 ul. The experimental procedure covered a span of four consecutive days. Table 1 summarizes the procedure on each day.

Day 1 . On Day 1 of the experiment, the rats were placed in the restrainers and allowed to habituate to the experimental chamber for 30 min. Following this period, a 15-min habituation period began in which four 6-s samples of baseline HR were recorded to provide information on pre-injection baseline HR. At the end of this period, each group was infused with the appropriate compound. Four 6-s HR samples were then recorded at 30-s intervals beginning 1 min following the infusion. This time of approximately 5 min was allowed for drug diffusion to receptor sites. All groups were then given two presentations of each CS to assess the HR orienting responses (ORs) to the novel CSs.

The conditioning phase began after the CS-alone phase. In the conditioning phase only 6 CS+ and 6 CS- trials were presented to ensure that the drug effects did not dissipate while the conditioning trials were being given. The trials were presented in a quasi-random order with the stipulation that no more than two trials of a given type could occur consecutively. The intertrial interval varied randomly among 1.0 min, 1.5 min, and 2.0 min intervals (mean 1.5 min). At the end of this phase, all groups were returned to their home cages. The total time of testing

Table 1. Experimental Procedure

Day	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7
Day 1.	Habituation (30 min)	Pre-Drug Baseline HR (15 min)	Drug Infusion (1 min)	Drug Distribution (5 min)	CS Alone (2CS+ no US, 2CS-) (10 min)	Conditioning (6CS+, 6CS-) (25 min)	
Day 2.	Pre-Drug Baseline HR (15 min)	Drug Infusion (1 min)	Drug Distribution (5 min)	Conditioning (6CS+, 6CS-) (25 min)	Drug Infusion (1 min)	Drug Distribution (5 min)	Antagonist Test (4CS+ no US, 4CS- (20 min)
Day 3.	All groups left in home cage						
Day 4.	Pre-Test Baseline HR (15 min)	Non-Drug Test (6CS+ no US, 6CS-) (20 min)	Reconditioning (10CS+, 10 CS-) (40 min)	Drug Infusion (1 min)	Drug Distribution (5 min)	Drug Test (4CS+ no US, 4CS-) (15 min) — Startle Test (3CS+, 3CS-) (20 min)	

following drug infusion was approximately 45 min.

Day 2 . The procedure on Day 2 was similar to that used on Day 1. Baseline HR was recorded prior to and following drug infusion. Again only 6 CS+ and 6 CS- conditioning trials were presented immediately following the post-injection period. The timing of the conditioning trials was identical to the quasi-random schedule described for Day 1. At the end of conditioning on Day 2, animals in the DALA group were given a 5-ug dose of naltrexone, animals in the CLON and UK groups were given a 10-ug dose of idazoxan, and animals in the SAL and a-HEL groups were given an equivalent volume of saline. The dosage of naltrexone was chosen on the basis of effective dosages previously reported (Harris & Fitzgerald, 1989) and in the case of idazoxan on pilot data which showed this dose to be effective in reversing the changes in baseline HR produced by the agonists. The drug administration procedure was identical to that described previously and baseline HR was recorded for 5 min post-infusion. After this period, each group received random presentations of 4 CS+ (no US) and 4 CS- test trials to determine the status of the CR with the drug effects blocked.

Day 3 . The groups were left in their home cage for 24 hr and received no treatments. This one day period allowed for the dissipation of any residual drug effects.

Day 4 . The groups were brought back to the experimental chamber, allowed to acclimate for 15 min in the restrainers and then given nonreinforced test trials while in a non-drug state. The test trials included the random presentations of six CS+ (no US) and six CS- trials. It was hoped that the trials given on this day would provide information on the presence or absence of a HR CR in the different drug groups. If the animals

in the drug groups had learned the CR earlier during conditioning, but were unable to perform it due to the presence of the drugs, then the CR should appear when the drugs were no longer present.

At the end of the non-drug test phase, each group received further conditioning consisting of random presentations of 10 CS+ and 10 CS- conditioning trials. Following this re-conditioning phase, all groups were infused with the same drugs that they experienced during the initial conditioning on Days 1 and 2. After a 5-min post-drug infusion period, each group was given random presentations of 4 CS+ (no US) and 4 CS- test trials. This drug-test phase was used to assess the effects of the various drug compounds on established HR CRs.

The startle test occurred immediately following the drug test. The animal, while still in the restrainer, was placed on top of the movement detecting platform. During the first two trials, the startle eliciting stimulus was presented alone to measure baseline startle. Following these trials, random presentations of 3 CS+ and 3 CS- trials occurred with the startle stimulus occurring in the middle of the CSs.

Histology

At the end of the experiment, the animals were deeply anesthetized with Nembutal (40 mg/kg ip) and perfused through the heart with a 10.0% neutralized buffered formalin solution. Evans blue dye was infused into the cannula to locate the position of the cannula. The brains of those animals with dye in the ventricle were examined. The brains were blocked so that the section of brain containing the cannula track was separated from the rest of the brain. The cannula tract was visually inspected to ensure that the cannula extended to the roof of the fourth ventricle but did not

penetrate beyond the ventricle into the brain stem.

Data Analyses

The HR responses were analyzed by a 4-way analysis of variance (ANOVA). There was one between groups factor and three within groups factors. The factors compared were groups (5) x type of CS (2) x trial blocks (3) x measurement intervals (3). Each day of the experiment was analyzed separately as was each of the phases during conditioning (i.e. CS alone, and conditioning). Follow-up analyses of significant interactions were performed using the Newman-Keuls' test to determine the nature of the significant differences.

Results

Histology

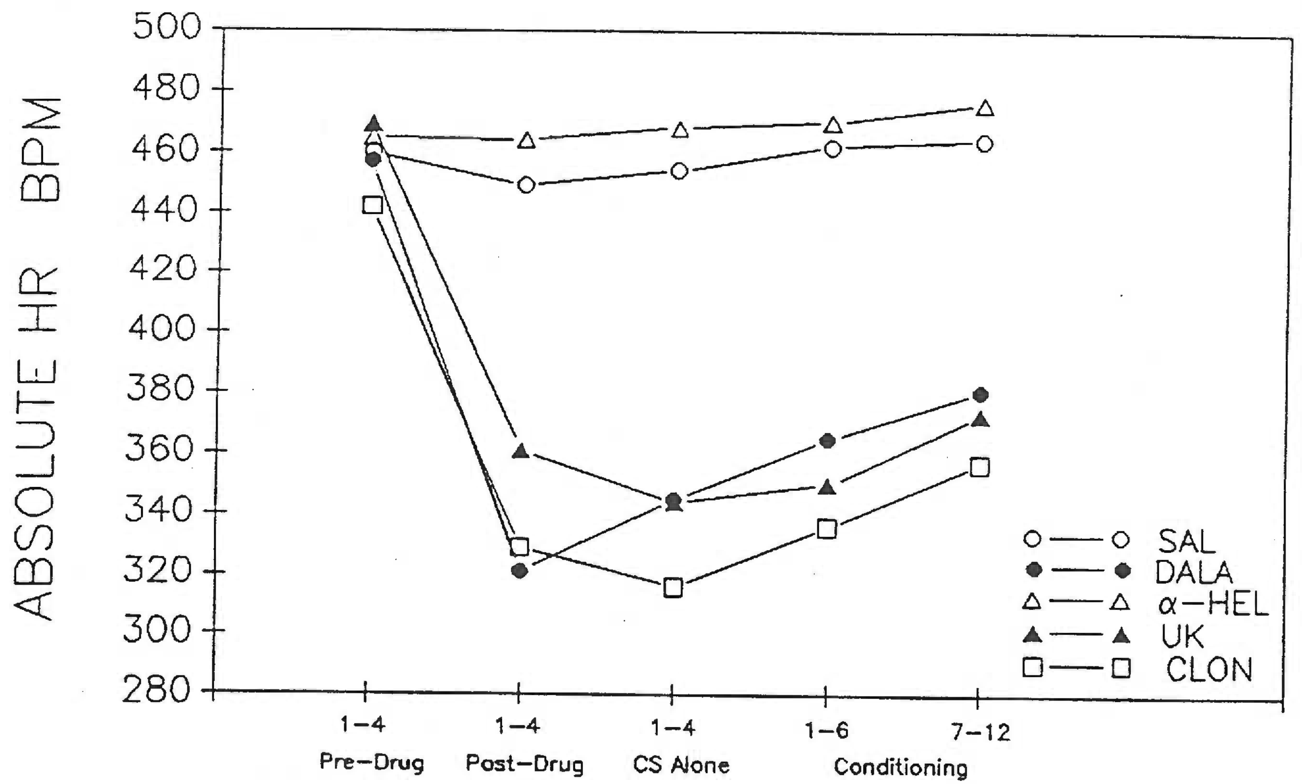
In each of the five groups, only those animals with cannulas correctly located in the roof of the rostral portion of the fourth ventricle, based on dye staining in the ventricle, were included in the data analyses. The data from animals who did not show dye staining in the ventricle, were discarded. This elimination process resulted in 8 subjects in the saline (SAL), DALA, and clonidine (CLON) groups and 9 subjects in the UK 14,304 (UK) and alpha-helical CRF (a-HEL) groups who met the above criteria. Only 1 - 2 animals per group were discarded due to a failure of cannula placement.

Baseline Heart Rate

Figures 2 and 3, repectively, illustrate baseline heart rate (HR) of all groups during successive phases on Day 1 and on Day 2. It may be seen from these figures that on each day resting HR in the DALA, UK, and CLON groups fell immediately (within 2 min) following drug administration. Baseline HR of these three groups generally reached a low point just prior to conditioning and remained depressed throughout the conditioning sessions on both days. On Day 2, the administration of the antagonists, naltrexone in the DALA group and idazoxan in the UK and CLON groups, reversed the depression of baseline HR and returned it to levels seen prior to drug administration. No systematic change in baseline HR was seen in the SAL or a-HEL groups on either day.

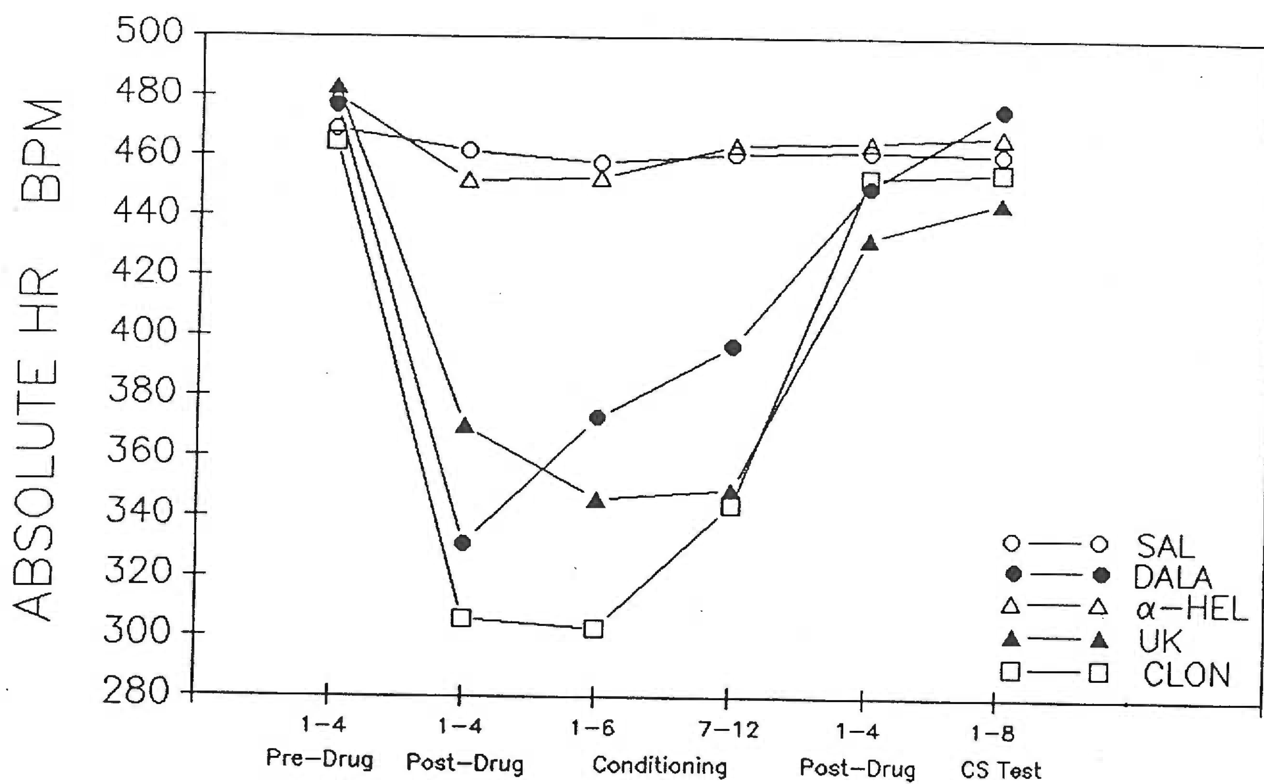
On Day 1, a 5 x 6 (Groups x Trial Blocks) ANOVA provided a significant groups effect, $F\ (4,37) = 48.22$, $p < .01$, and a significant groups x trial blocks interaction, $F\ (16,148) = 15.91$, $p < .01$. Follow-up tests at each

Figure 2. Baseline HR for each group during Day 1 of conditioning. Scores were calculated from mean HR during the 6-s pre-CS period of each trial and were averaged over one four-trial block during the pre-drug phase, post-drug phase, CS-alone phase, and two six-trial blocks during conditioning (SAL = saline, a-HEL = alpha helical CRF, UK = UK 14,304, CLON = clonidine).



ABSOLUTE HR BPM
500
480
460
440
420
400
380
360
340
320
300
280
1-4
Pre-Drug
1-4
Post-Drug
1-4
CS Alone
1-6
7-12
Conditioning
SAL
DALA
α-HEL
UK
CLON

Figure 3. Baseline HR for each group during Day 2 of conditioning. Scores were calculated from mean HR during the 6-s pre-CS period of each trial and are averaged over one four-trial block during the pre-drug phase and both post-drug phases, two six-trial blocks during conditioning, and one-eight trial block during CS-test phase.



ABSOLUTE HR BPM
500
480
460
440
420
400
380
360
340
320
300
280
1-4
Pre-Drug
1-4
Post-Drug
1-6
7-12
Conditioning
1-4
Post-Drug
1-8
CS Test
SAL
DALA
α-HEL
UK
CLON

trial block showed that while the groups were not significantly different from each other prior to drug administration, the DALA, UK, and CLON groups were significantly different from the SAL group at each trial block following drug infusion, $p < .05$.

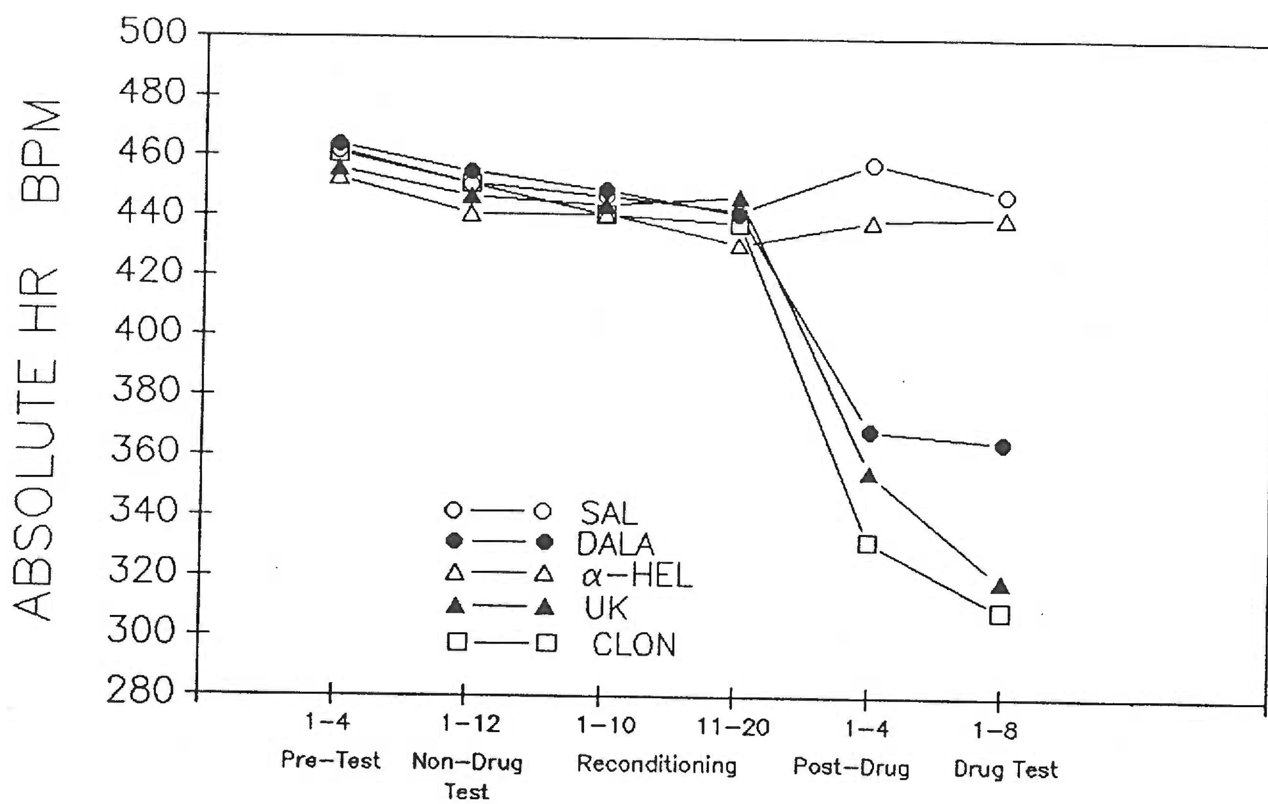
On Day 2, a 5 x 6 (Groups x Trial Blocks) ANOVA produced a significant groups effect, $F(4,37) = 28.90$, $p < .01$ and a significant groups x trial blocks interaction, $F(20,185) = 23.90$, $p < .01$. A follow-up test indicated that the DALA, UK, and CLON groups had suppressed baseline HRs relative to the SAL group immediately following drug infusion and during conditioning, $p < .05$, but were not significantly different from the SAL group prior to drug infusion or following the administration of the antagonists.

Baseline HR during Day 4 of the experiment is shown in Figure 4. All groups exhibited similar baseline HRs on this day until the drugs were re-administered at the end of the reconditioning phase, at which point HR immediately fell in the DALA, UK, and CLON groups. A 5 x 6 (Groups x Trial Blocks) ANOVA revealed a significant groups effect $F(4,37) = 11.55$, $p < .01$, and a groups x trial blocks interaction, $F(20,185) = 30.21$, $p < .01$. A follow-up test affirmed that while the groups were not significantly different from each other during the non-drug phase, the subsequent administration of drug in the DALA, UK, and CLON groups again resulted in a significant depression of baseline HR, $p < .05$.

Orienting Responses

Figure 5 depicts HR orienting responses (ORs) to CS+ and CS- for each group during three 2-s CS measurement intervals averaged over two-trial blocks. This figure shows that the SAL, DALA, and a-HEL groups all

Figure 4. Baseline HR during Day 4. Scores were calculated from mean HR during the 6-s pre-CS period for each trial and were averaged over one four-trial block during the pre-test phase and post-drug phase, one twelve-trial block during the non-drug test, two ten-trial blocks during reconditioning, and one eight-trial block during the drug test phase.


ABSOLUTE HR BPM
500
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340
320
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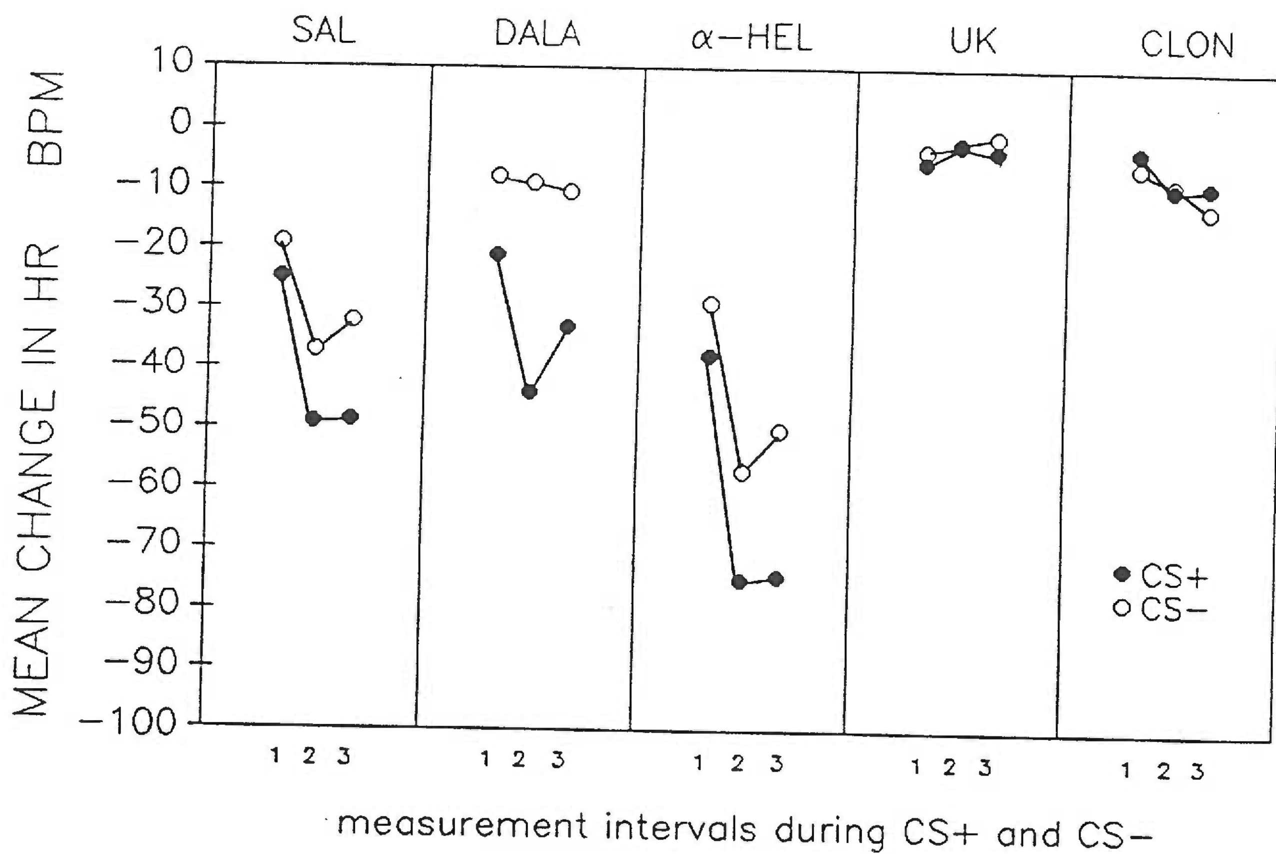
SAL
DALA
α–HEL
UK
CLON
1–4
Pre–Test
1–12
Non–Drug
Test
1–10
11–20
Reconditioning
1–4
Post–Drug
1–8
Drug Test

Figure 5. Heart rate orienting responses during three 2-s CS measurement intervals for each group to CS+ and to CS- averaged over a two-trial block during the CS-alone phase that occurred prior to conditioning. Responses are plotted as CS minus pre-CS heart rate difference scores.


SAL
DALA
α−HEL
UK
CLON
MEAN CHANGE IN HR BPM
10
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
1 2 3
CS+
CS−
measurement intervals during CS+ and CS−

exhibited a bradycardia OR to CS+ and CS-, while the UK and CLON groups showed very little response to either CS. A 5 x 2 x 3 (Groups x CS Type x Measurement Intervals) ANOVA demonstrated a significant groups effect, F (4,37) = 18.64, $p < .01$, a significant CS type effect, F (1,37) = 21.11, $p < .01$, a significant groups x CS type interaction, F (4,37) = 4.34, $p < .01$, and a significant groups x CS type x measurement intervals interaction, F (8,74) = 2.24, $p < .05$.

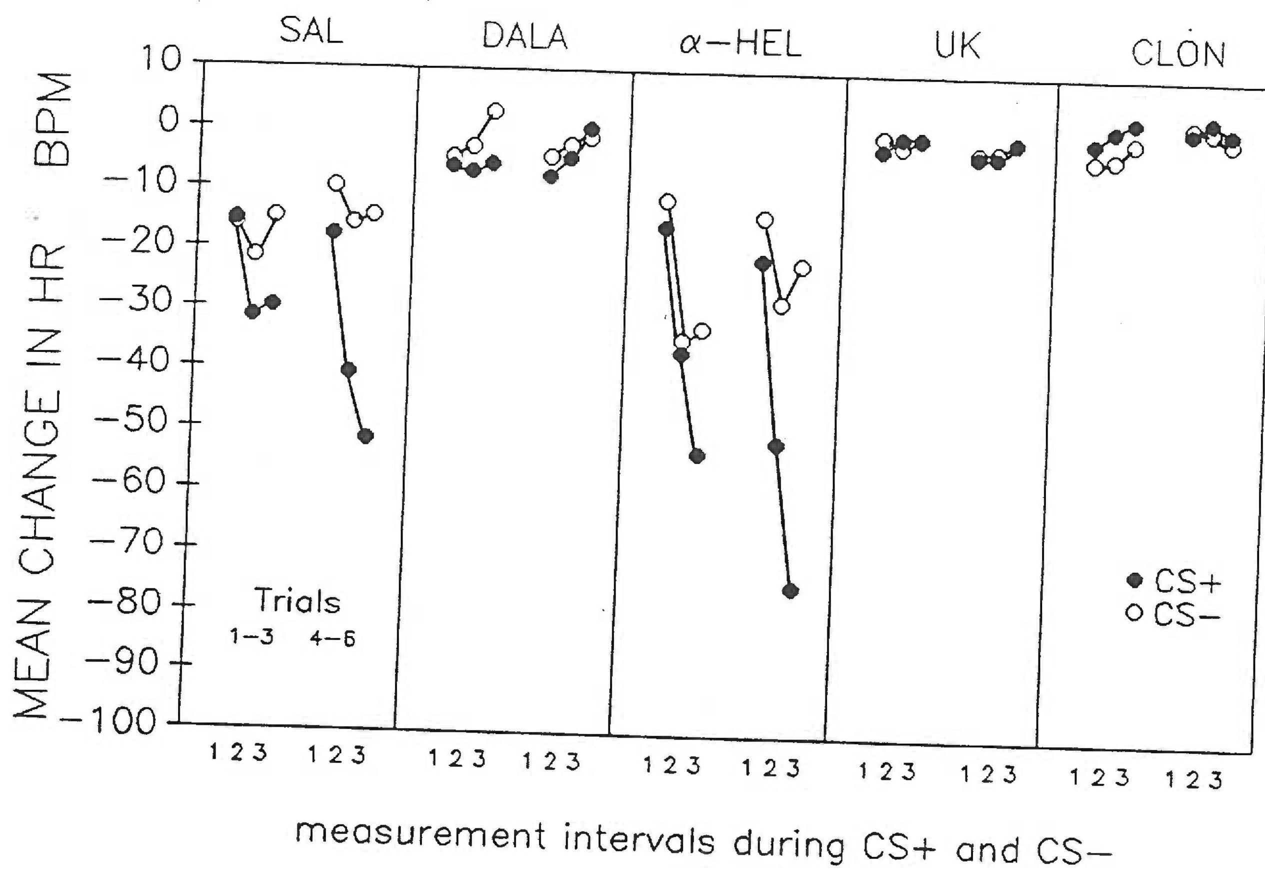
A follow-up test denoted that the CLON and UK groups were significantly impaired relative to the SAL group in their responses to both CS+ and CS- during the second and third measurement intervals, $p < .05$. The a-HEL group, on the other hand, showed a significant enhancement relative to the SAL group in their responses to CS+ during the second and third measurement intervals, $p < .05$.

Conditioned Responses

Day 1 Conditioning. Figure 6 illustrates the mean HR responses of each group during three 2-s CS measurement intervals to CS+ and CS- averaged over successive blocks of three trials on Day 1. It may be seen that the DALA, UK, and CLON groups exhibited little or no response to CS+ or to CS-. By contrast, the a-HEL group showed the development over trials of a major decelerative HR CR to CS+ that was somewhat larger than that displayed by the SAL group.

The reliability of the conditioning outcomes was tested by means of a 5 x 2 x 2 x 3 (Groups x CS Type x Trial Blocks x Measurement Intervals) ANOVA. This test established a significant groups effect, F (4,37) = 46.16, $p < .01$, a significant groups x CS-type interaction, F (4,37) = 20.70, $p < .01$, and a significant groups x trial blocks interaction, F (4,37) = 1.67,

Figure 6. Heart rate responses on Day 1 of conditioning during three 2-s CS measurement intervals for each group to CS+ and to CS- averaged over two blocks of three trials. Responses are plotted as CS minus pre-CS heart rate difference scores.

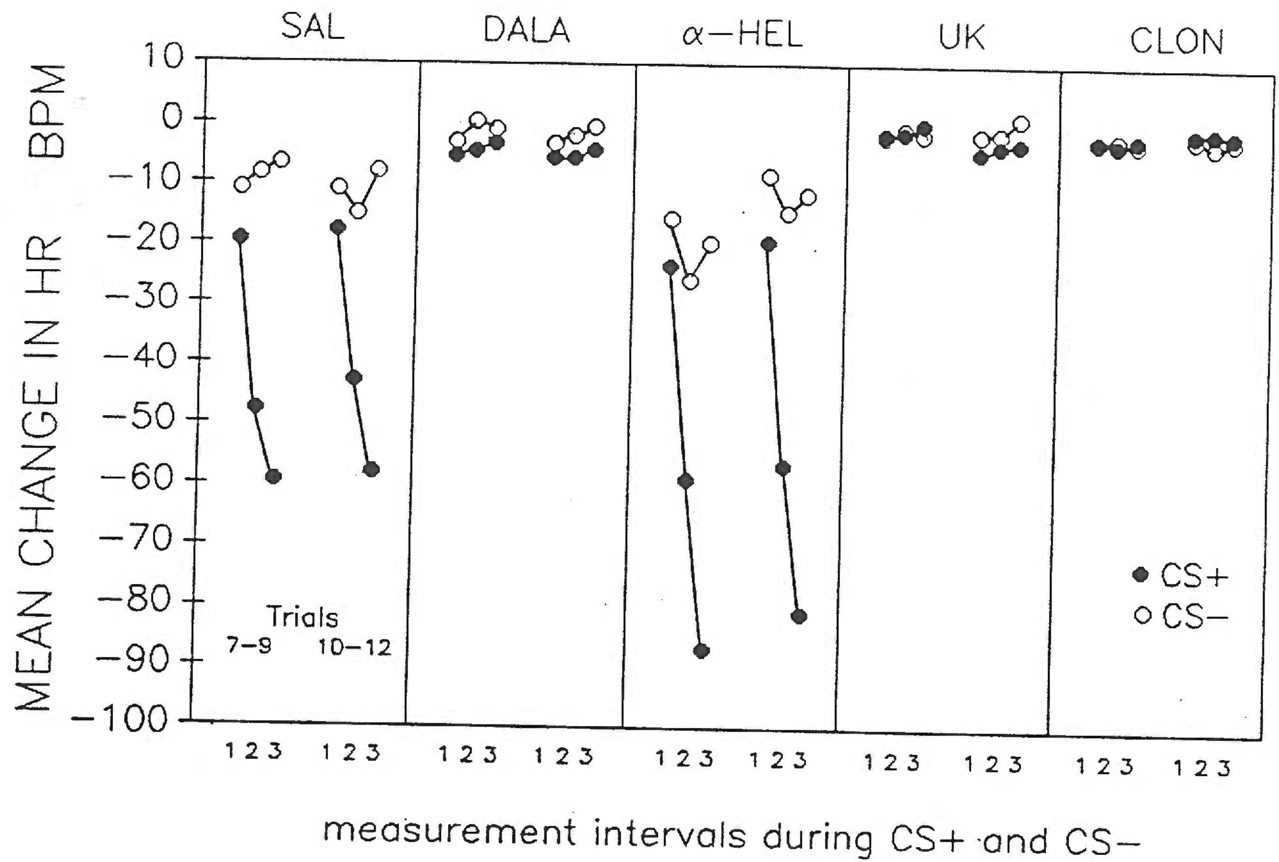

SAL
DALA
α−HEL
UK
CLON
MEAN CHANGE IN HR BPM
10
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
Trials
1−3 4−6
CS+
CS−
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
measurement intervals during CS+ and CS−

$p < .05$. In addition, there was a significant CS type x trial blocks interaction, $F(1,4) = 16.76$, $p < .01$, indicating that conditioning occurred over trials, and a significant groups x CS type x trial blocks x measurement intervals interaction, $F(8,74) = 5.96$, $p < .01$, demonstrating that the level of conditioning was not the same in all groups.

The four-way interaction was tested with separate 5 x 2 x 3 (Groups x Trial Blocks x Measurement Intervals) ANOVAs on the CS+ and the CS- data. For the CS+ data, the ANOVA revealed a significant groups effect, $F(4,37) = 54.01$, $p < .01$, a significant groups x trial blocks interaction, $F(4,37) = 6.06$, $p < .05$, and a groups x trial blocks x measurement intervals interaction, $F(8,74) = 5.08$, $p < .01$. The decelerative HR responses to CS+ in the DALA, UK, and CLON groups were found to be significantly smaller than those of the SAL group at each trial block during the second and third measurement intervals, $p < .05$. The a-HEL group exhibited a significant enhancement in its response to CS+ relative to the SAL group at each trial block during the third measurement interval. The CS- analysis showed a significant groups effect, $F(4,37) = 21.59$, $p < .01$, and a significant group x trial block x measurement interval interaction, $F(8,74) = 2.59$, $p < .05$. A follow-up test indicated that the DALA, UK, and CLON groups showed a significantly smaller response to CS- at each trial block during the second and third measurement intervals with respect to the SAL group, $p < .05$.

Day 2 Conditioning. The HR responses of each group to CS+ and CS- averaged over successive blocks of three trials during three 2-s CS measurement intervals on Day 2 of conditioning are represented in Figure 7. It may be noted in this figure that the CRs acquired on Day 1 by the a-HEL

Figure 7. Heart rate responses on Day 2 of conditioning during three 2-s CS measurement intervals for each group to CS+ and to CS- averaged over two blocks of 3 trials. Responses are plotted as CS minus pre-CS heart rate difference scores.

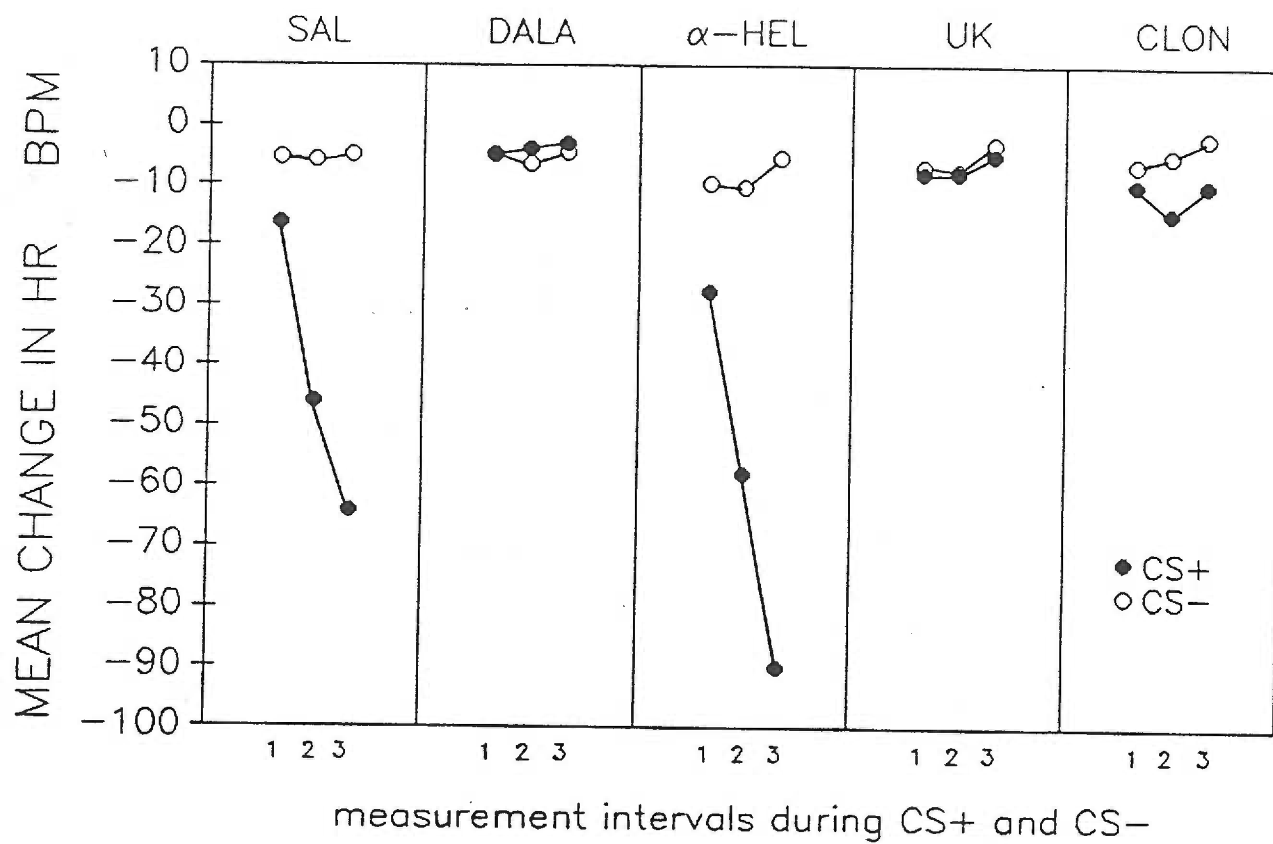

SAL
DALA
α−HEL
UK
CLON
MEAN CHANGE IN HR BPM
10
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
Trials
7−9
10−12
● CS+
○ CS−
1 2 3
measurement intervals during CS+ and CS−

and SAL groups persisted on Day 2, whereas the DALA, UK, and CLON groups continued to display no evidence of a HR CR.

A 5 x 2 x 2 x 3 (Groups x CS Type x Trial Blocks x Measurement Intervals) ANOVA affirmed a significant groups effect, F (3,37) = 103.11, $p < .01$, a significant groups x CS type interaction, F (4,37) = 47.43, $p < .01$, and a significant groups x CS type x measurement intervals interaction, F (8,74) = 36.83, $p < .01$. A follow-up 5 x 3 (Groups x Measurement Intervals) ANOVA on just the CS+ data provided a significant groups effect, F (4,37) = 118.60, $p < .01$ and a groups x measurement intervals interaction F (8,74) = 72.07, $p < .01$. A subsequent test showed that the DALA, UK, and CLON groups were significantly supressed in their responding to CS+ relative to the SAL group during measurement intervals 2 and 3, $p < .05$. The a-HEL group was found to be significantly augmented in its response to CS+ during the third measurement interval, $p < .05$. A 5 x 3 (Groups x Measurement Intervals) ANOVA on CS- data produced a significant group effect F (4,37) = 13.72, $p < .01$, and a significant group x measurement intervals interaction F (8,74) = 3.71, $p < .05$. However, no significant differences among groups were found at any measurement interval.

Day 2 Antagonist Test. Figure 8 shows the HR responses of each group to CS+ and CS- during three 2-s CS measurement intervals averaged across the four CS-alone test trials. Despite the antagonist reversal of the drug-induced depression of baseline HR seen in the DALA, UK, and CLON groups (see Figure 2) no significant changes occurred in the magnitude their CS+ responding. The a-HEL group continued to show an enhanced bradycardia to CS+. A 5 x 2 x 3 (Groups x CS Type x Measurement Intervals) ANOVA denoted a significant groups effect, F (4,37) = 43.86, $p < .01$, a significant groups

Figure 8. Heart rate responses for the antagonist CS test during three 2-s CS measurement intervals for each group to CS+ and to CS- averaged over four trials. Responses are plotted as CS minus pre-CS heart rate difference scores.

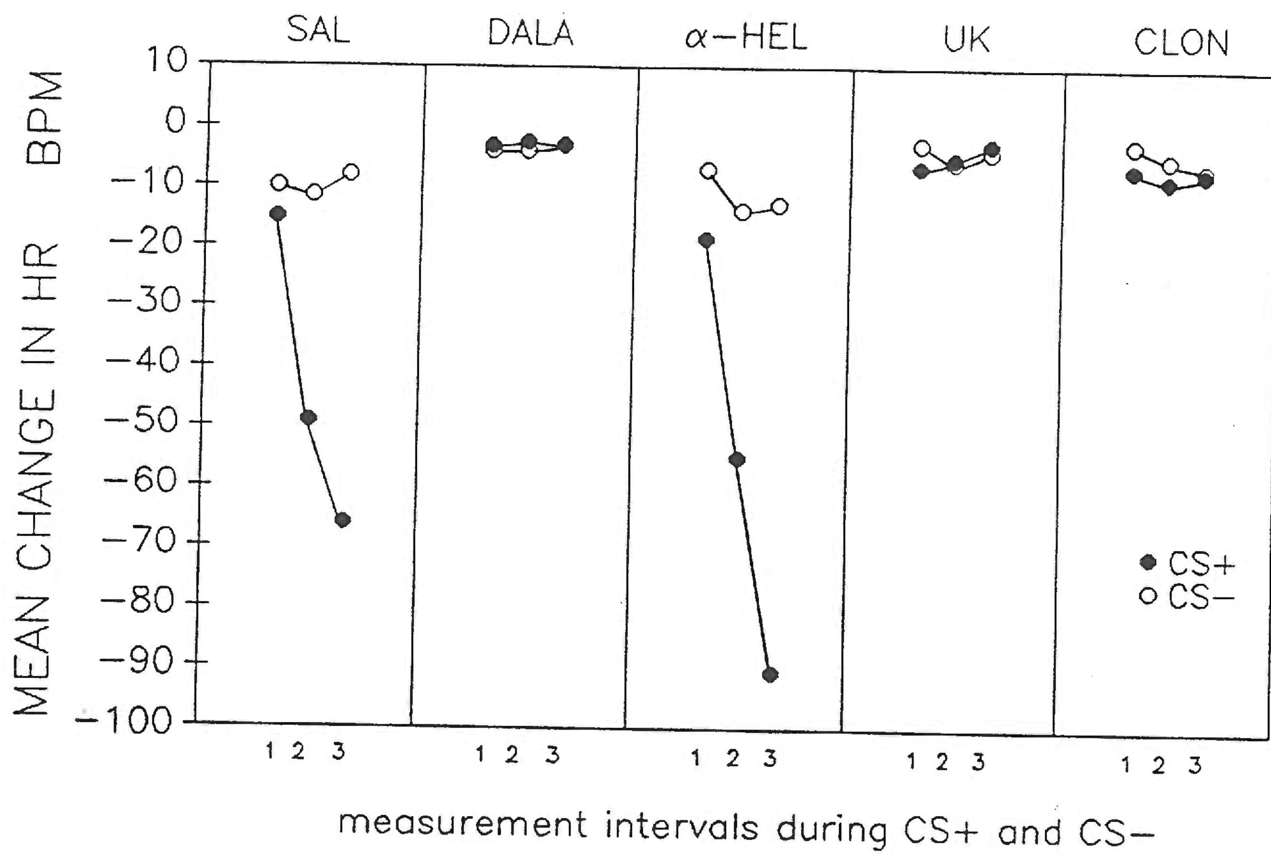

SAL
DALA
α−HEL
UK
CLON
MEAN CHANGE IN HR BPM
10
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
CS+
CS−
1 2 3
measurement intervals during CS+ and CS−

x CS type interaction, $F(4,37) = 42.36$, $p < .01$, and a significant groups x CS type x measurement intervals interaction, $F(4,37) = 34.56$, $p < .01$. A follow-up 5 x 3 (Groups x Measurement Intervals) ANOVA on the CS+ data produced a significant groups effect, $F(4,37) = 58.78$, $p < .01$, and a significant groups x measurement intervals interaction, $F(8,74) = 49.83$, $p < .01$. A subsequent test pointed out that the DALA, UK, and CLON groups were significantly depressed in their responses to CS+ relative to the SAL group during the second and third measurement intervals, while the response of the a-HEL group was significantly augmented during the third measurement interval, $p < .05$. A similar test on the CS- data provided a significant groups effect, $F(4,37) = 3.70$, $p < .05$ and a groups x measurement interval interaction, $F(8,74) = 2.23$, $p < .05$, but no significant group differences were found in a subsequent test.

Non-Drug Test Phase. Heart rate responding to CS+ and to CS- during three 2-s CS measurement intervals collapsed across the six non-drug test trials given one day after the second day of conditioning is shown in Figure 9. The HR responses of the DALA, UK, and CLON groups to CS+ were again small in magnitude, while those of the a-HEL group were very large.

A 5 x 2 x 3 (Groups x CS Type x Measurement Intervals) ANOVA revealed a significant groups effect, $F(4,37) = 61.69$, $p < .01$, a significant groups x CS type interaction, $F(4,37) = 31.97$, $p < .01$, and a significant groups x CS type x measurement intervals interaction, $F(8,74) = 28.26$, $p < .01$. A follow-up 5 x 3 (Groups x Measurement Intervals) ANOVA on the CS+ data demonstrated a significant groups effect, $F(4,37) = 63.77$, $p < .01$, and a significant groups x measurement intervals interaction, $F(8,74) = 44.07$, $p < .01$. The responses of the DALA, UK, and CLON groups were found to be

Figure 9. Heart rate responses for the non-drug test during three 2-s CS measurement intervals for each group to CS+ and to CS- averaged over six trials. Responses are plotted as CS minus pre-CS heart rate differences scores.

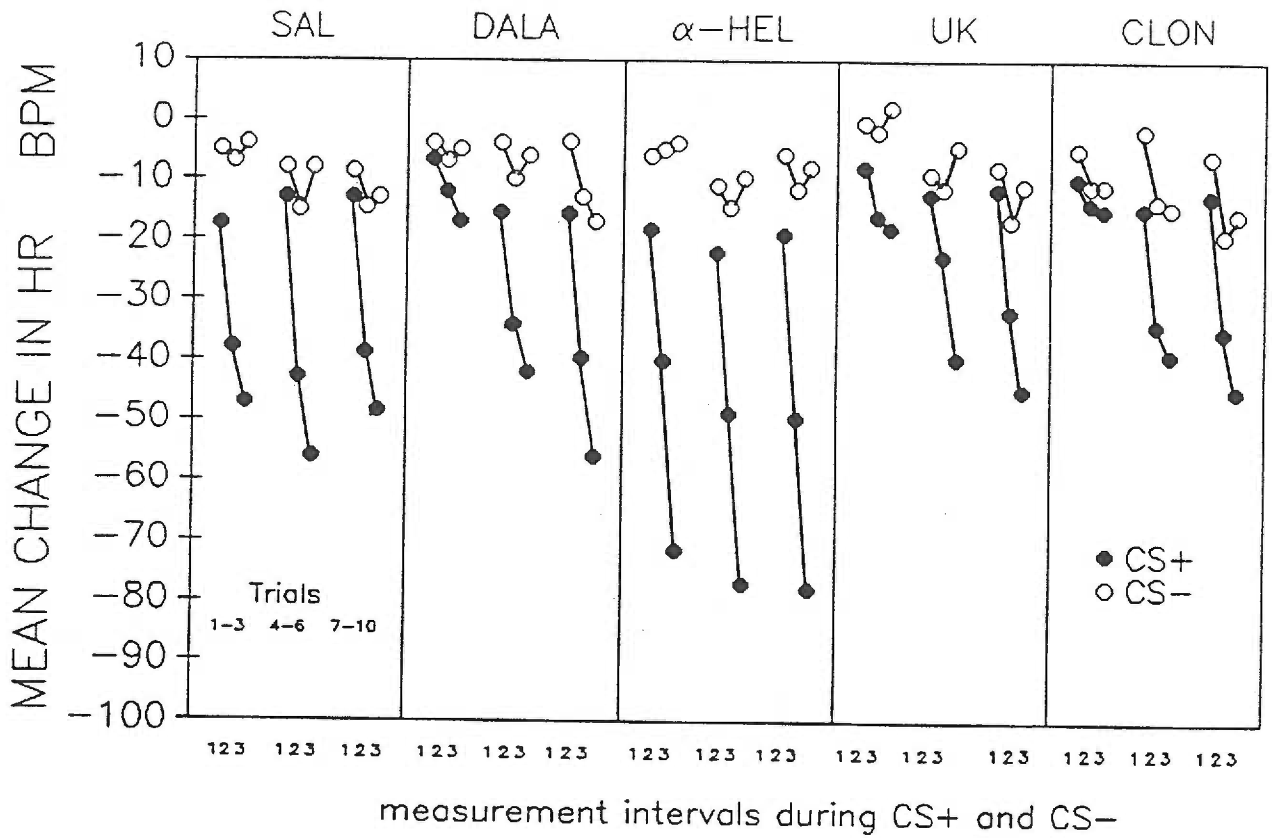

SAL
DALA
α–HEL
UK
CLON
MEAN CHANGE IN HR BPM
10
0
–10
–20
–30
–40
–50
–60
–70
–80
–90
–100
1 2 3
• CS+
○ CS–
measurement intervals during CS+ and CS–

significantly attenuated when compared to the SAL group on the second and third measurement intervals, $p < .05$. The a-HEL group showed a significant facilitation, with respect to the SAL group, in the magnitude of the CR to CS+ during the third measurement interval, $p < .05$. An analysis of the CS- data showed that the groups were not significantly different in their responses to CS-.

Non-Drug Reconditioning Phase. Figure 10 depicts the HR CRs to CS+ for each group during three 2-s CS measurement intervals averaged over trial blocks 1-3, 4-6, and 7-10 during reconditioning. As can be seen from this figure the DALA, UK, and CLON groups all expressed the development over trials of a CR to the CS+ and the a-HEL group continued to show a large HR CR. A 5 x 2 x 3 x 3 (Groups x CS Type x Trial Blocks x Measurement Intervals) ANOVA established a significant groups effect, $F(4,37) = 10.23$, $p < .01$, a groups x CS type interaction, $F(4,37) = 17.27$, $p < .01$, and a groups x CS type x measurement intervals interaction, $F(8,74) = 5.46$, $p < .01$.

A follow-up 5 x 3 x 3 (Groups x Trial Blocks x Measurement Intervals) ANOVA on CS+ data revealed a significant groups effect, $F(4,37) = 27.61$, $p < .01$, and a significant groups x trial blocks x measurement intervals interaction, $F(16,222) = 1.96$, $p < .05$. A follow-up test verified that the DALA, UK, and CLON groups showed significantly smaller responses when compared to the SAL group during measurement intervals 2 and 3 during the first trial block only (trials 1-3), $p < .05$. No significant differences were seen on the remaining trial blocks. The a-HEL group showed a significant facilitation of CS+ responding relative to the SAL group during the third measurement interval during trial blocks 4-6 and 7-10, $p < .05$.

Figure 10. Heart rate responses for reconditioning during three 2-s CS measurement intervals for each group to CS+ and to CS- averaged over trials 1-3, 4-6, and 7-10. Responses are plotted as CS minus pre-CS difference scores.


SAL
DALA
α−HEL
UK
CLON
MEAN CHANGE IN HR BPM
10
0
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−50
−60
−70
−80
−90
−100
Trials
1−3 4−6 7−10
CS+
CS−
123 123 123 123 123 123 123 123 123 123 123 123 123 123 123
measurement intervals during CS+ and CS−

A similar analysis of CS- data demonstrated no significant group differences.

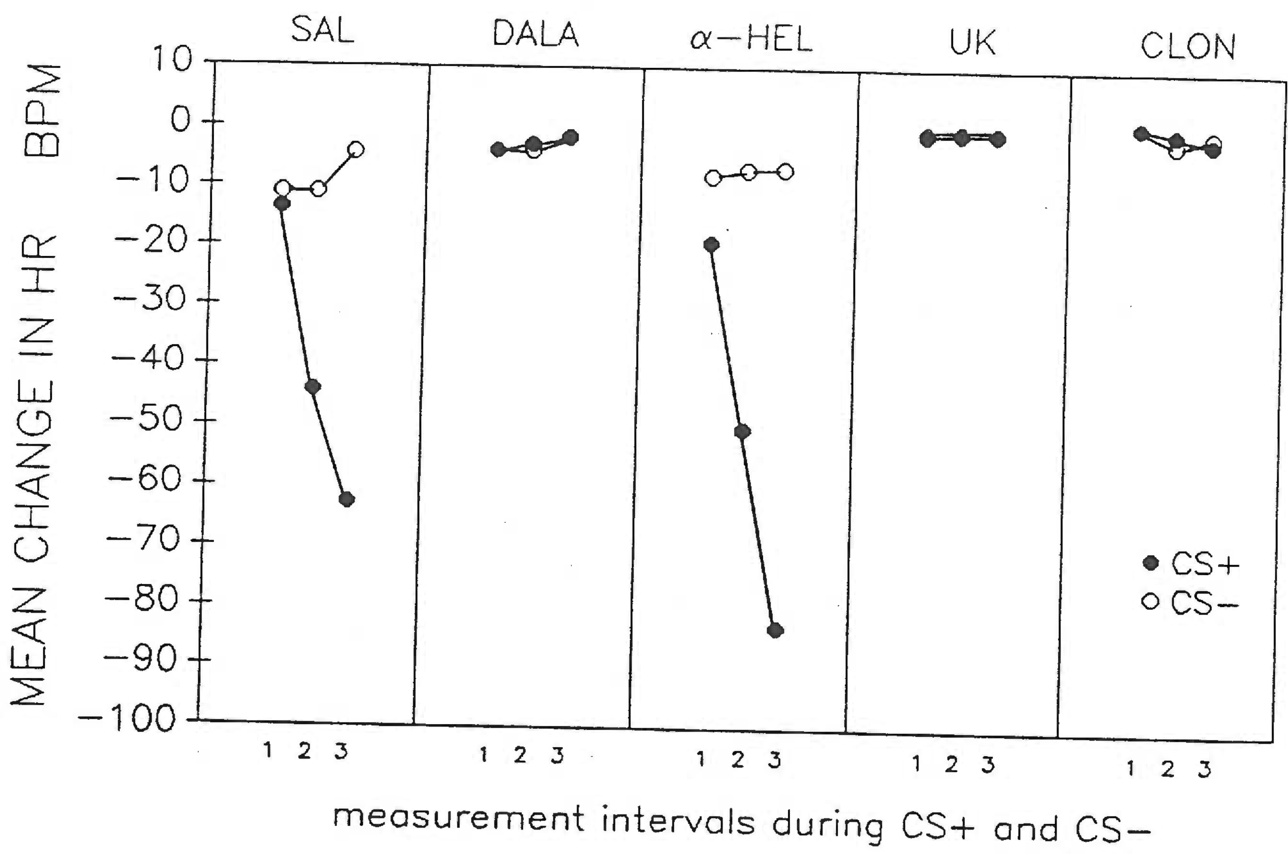
Examination of the individual ANOVAs for each group separately showed a significant conditioning effect (i.e., CS type x trial blocks interaction, $p < .01$) only in the DALA, UK, and CLON groups illustrating that the development of a conditioned response was evident in these groups only. The a-HEL and SAL groups showed no increase from previously established CR levels, confirming that conditioning was at a maximum in these groups.

Drug Test Phase. The responses of each group to CS+ and CS- during three 2-s CS measurement intervals collapsed across four CS-alone trials on the drug test can be seen in Figure 11. This figure illustrates that the CRs previously established in the DALA, UK, and CLON groups (see Figure 10) were abolished when the drugs used during the initial training procedure were re-administered. The CR in the a-HEL group was relatively unchanged. A 5 x 2 x 3 (Groups x CS Type x Measurement Intervals) ANOVA on drug test data indicated a significant groups effect, $F\ (4,37) = 74.02$, $p < .01$, a groups x CS type interaction, $F\ (4,37) = 30.58$, $p < .01$, and a significant groups x measurement intervals interaction, $F\ (8,74) = 21.52$, $p < .01$. The DALA, UK, and CLON groups were found to be significantly suppressed in their responses to CS+ thoughout the drug test relative to the SAL group during measurement intervals 2 and 3, while the a-HEL group was found to be significantly facilitated in its response to CS+ in the third measurement interval, $p < .05$.

Unconditioned Responses

The HR URs of each group to the shock US averaged over trials for Days 1 and 2 of conditioning and during reconditioning are shown in Figure 12.

Figure 11. Heart rate responses for the drug test phase during three 2-s CS measurement intervals for each group to CS+ and to CS- averaged over four trials. Responses are plotted as CS minus pre-CS difference scores.


SAL
DALA
α−HEL
UK
CLON
MEAN CHANGE IN HR BPM
10
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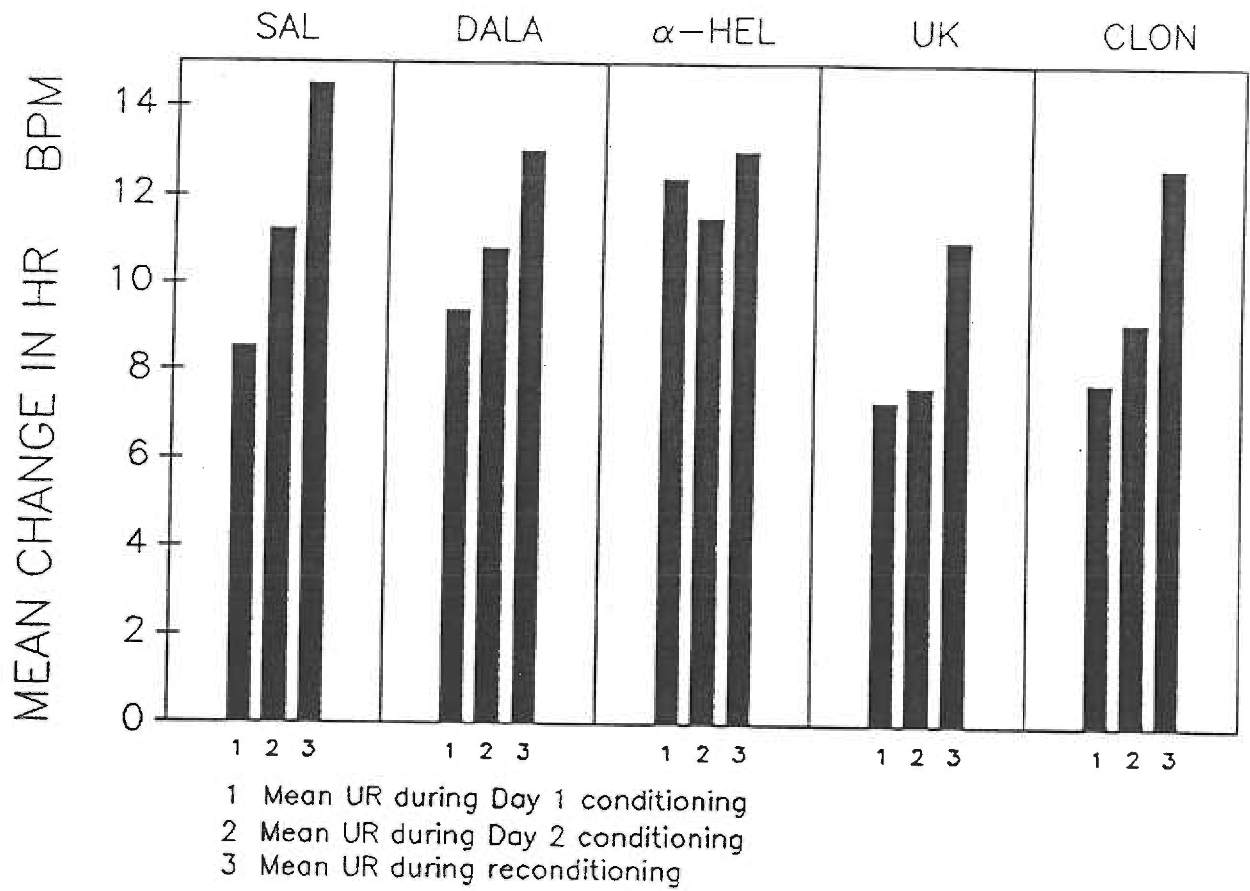
−40
−50
−60
−70
−80
−90
−100
● CS+
○ CS−
1 2 3
measurement intervals during CS+ and CS−

Figure 12. Heart rate responses on Days 1 and 2 of conditioning and reconditioning for each group averaged over the 6-s post-US period, a six trial block, and a ten trial block. Responses are graphed as post-US minus pre-CS difference scores.


SAL
DALA
α−HEL
UK
CLON
MEAN CHANGE IN HR BPM
14
12
10
8
6
4
2
0
1 2 3
1 Mean UR during Day 1 conditioning
2 Mean UR during Day 2 conditioning
3 Mean UR during reconditioning

The three 2-s post-US intervals are averaged together. It can be seen from this graph that all groups exhibted tachycardia URs to the US. A 5 x 2 (Groups x Trial Blocks) ANOVA revealed no significant group differences during any phase of the experiment.

Startle Responses

The movement responses (i.e., startle responses) of each group averaged over the two baseline startle trials (startle stimulus alone) and over the three CS+/startle stimulus and three CS-/startle stimulus trials are shown in Table 2. One can observe from this table that the DALA, UK, and CLON groups all showed a suppression of baseline startle relative to the SAL group, while the a-HEL group showed an enhancement. It can also be noted that the a-HEL and SAL groups both showed a profound suppression of startle responses when the startle eliciting stimulus was presented in the presence of the CS+. A small suppression of startle relative to baseline was also seen in the presence of CS-, but the magnitude of the suppression was not as great. The DALA, UK, and CLON groups did not show any major changes in their startle responses during the CS+ and CS- presentations. A one-way ANOVA on baseline startle responses produced a significant groups effect, F $(3,37) = 188.6$, $p < .01$. The DALA, UK, and CLON groups were found to be significantly suppressed in their responses in relation to the SAL group, while the response of the a-HEL group was significantly larger, $p < .05$.

A 5 x 2 (Groups x CS Type) ANOVA on the startle response data in the presence of the different CSs disclosed a significant groups effect, F $(4,37) = 34.12$, $p < .01$, a significant CS type effect F $(1,37) = 379.12$, $p < .01$, and a significant groups x CS type interaction, F $(4,37) = 180.07$, $p < .01$. A follow-up test substantiated the fact that the DALA, UK, and

Table 2. Mean movement to the startle eliciting stimulus recorded during baseline startle (startle stimulus alone) trails and in the presence of CS+ and CS-.

Table 2. Mean movement of each group on startle test trials.

	Baseline	CS+	CS-
SAL	2363.3	26.6	1420.6
DALA	508.9	504.0	447.6
a-HEL	3080.6	84.7	2352.9
UK	323.0	472.9	387.5
CLON	296.7	408.2	386.5

CLON groups showed a significantly elevated startle response in the presence of CS+ and a significantly suppressed startle response to CS- relative to the SAL group, $p < .05$. The a-HEL group showed a significantly greater startle response than the SAL group in the presence of CS-, $p < .05$.

Examination of individual one-way ANOVAs for each group comparing baseline, with CS+, and CS- startle responses indicated significant main effects ($p < .05$) in the SAL, a-HEL, UK, and CLON groups. Follow-up tests demonstrated that in both the SAL and a-HEL groups, the presentation of both CSs resulted in a significant depression of startle responses from baseline, $p < .05$; however, suppression during the CS+ was more severe. In the UK and CLON groups, there was a tendency for startle responses to be increased in the presence of each CS but this effect was not significant.

Discussion

Confirming what was observed in a previous study (Harris & Fitzgerald, 1989), Experiment I showed that the administration of the mu opioid agonist, DALA, into the rostral portion of the fourth ventricle prior to conditioning resulted in the failure of a HR CR to develop during conditioning. This experiment also showed that the alpha-2 noradrenergic agonists, UK 14,304 and clonidine were as effective as DALA in blocking CR development. The inability to develop a CR in these treatment groups was evidenced both during the antagonist test that occurred at the end of conditioning on Day 2 and during the non-drug test that occurred 48 hours after the last drug infusion. During the antagonist test, the drug-induced changes in baseline HR were reversed by the respective mu and alpha-2 antagonists, but the animals still did not exhibit a CR. This indicates that the drug-induced changes in baseline HR were not responsible for the HR CR losses in these groups. Alternatively, it could be argued that the antagonist treatment did not completely block all the effects of the drugs and that these effects prevented the appearance of a HR CR. However, on the non-drug test day, a time when no drug effects should have been present, these groups continued to show no evidence of a HR CR, suggesting that prior conditioning trials failed to instill a HR CR.

On the other hand, it might be argued that a CR was in fact learned but that drug-induced cardiovascular effects prevented the performance of the CR. Then with the change in drug state that occurred between the conditioning phase with the drugs and the test phase without the drugs there was a state dependent loss (Overton, 1978) of the CR on the test trials.

State dependency refers to the notion that a change in state from being trained in the presence of a drug to being tested without the drug can produce memory or retrieval deficits (Overton, 1978) leading to a loss in the performance of a response. While this view cannot be completely ruled out, some evidence against this position will be presented below.

When subsequently trained with no drugs present during reconditioning, the DALA, UK, and CLON groups showed the development, over trials, of a major decelerative HR response to the CS+. These data indicate that these animals were capable of learning and that the failure of a CR to develop during the initial conditioning phase was not due to brain damage following the cannula implants. During the reconditioning phase, the acquisition of the HR CR in these groups looked very much like that seen in the saline group on the first day of conditioning (i.e., the animals appeared to be like those experiencing the conditioning paradigm for the first time). This finding provides evidence against the view that conditioning occurred in these groups on Days 1 and 2 of conditioning. If this were true then all three groups should have showed an apparent savings of this learning when subsequently retrained.

The administration of DALA, UK 14,304 and Clonidine following reconditioning was found to decrement the recently learned response. This finding supports a previous report that fourth ventricle opioid administration given immediately after classical conditioning training decremented a recently learned bradycardia response in rabbits (Lavond et al., 1983). In that study, as well as in the current experiment, the decremental effect of the mu opioid agonist on the recently learned HR CR could be due to a drug-induced alteration in central cardiovascular

regulation (Harris & Fitzgerald, 1989), to a loss of fear motivation (Lavond et al., 1983), or to some type of memory or retrieval deficit (Kapp & Gallagher, 1979). At present it is not possible to select which explanation is most likely.

The administration of DALA, UK 14,304, and clonidine had similar effects in suppressing baseline HR. On both Day 1 and Day 2, this change was seen as an immediate bradycardia occurring after drug infusion with HR remaining suppressed throughout the conditioning session. The reductions in baseline HR are in agreement with the results of several other studies that also have found that opioid and alpha-2 agonist administration in the region of the fourth ventricle produced bradycardia (Bolme, Fuxe, Agnati, Bradley, & Smythies, 1978; Gurtu, Sinha, & Bhargava, 1982; Schoener & Pitts, 1985). The effect of the alpha-2 drugs and DALA on baseline HR suggests that the drugs might have diffused from the rostral to the caudal area of the ventricle and affected the mu opioid and alpha-2 receptor rich cardiovascular control nuclei, the nucleus tractus solitarius, nucleus ambiguus, and the dorsal motor nucleus of the vagus located there (Unnerstall, Kopajtic, & Kuhar, 1984). Previously, it has been suggested that the similarity in the cardiovascular effects of alpha-2 and mu opioid agonists may be due to the co-localization of alpha-2 and mu receptors within the same cardiovascular control nuclei (Unnerstall et al., 1984). Here, as in other studies (Bolme et al., 1978; Gurtu, Sinha, & Bhargava, 1982), the respective mu and alpha-2 antagonists, naltrexone and idazoxan, were effective in reversing the effects of the drugs on baseline HR, suggesting that this was a receptor mediated phenomenon.

It is believed that the loss of the CR induced by DALA and the alpha-2

agonists occurred independently of the change in baseline HR. This was evidenced by the failure of a CR to appear when baseline HR was returned to normal during the antagonist test and by the absence of a CR on the non-drug test day when baseline HR was normal. Previously, we have seen that major changes in baseline HR had no effect on the ability of animals to perform a HR CR (Fitzgerald, Hatton, & Foutz, 1984). The possibility that blocking the performance of the HR CR during conditioning, through direct cardiac effects, prevented conditioning of a CR is also not likely because it has been shown that such a blockade has no effect on conditioning (Fitzgerald, Martin, & O'Brien, 1973).

The administration of the alpha-2 agonists significantly reduced the magnitude of the preconditioning reflex bradycardia (i.e., the OR) to the CS+, but DALA did not. While the distributions of alpha-2 and mu receptors tend to be similar in some fourth ventricle structures, there are nuclei in which these receptor types are localized on different cell types or coexist in different ratios (Unnerstall, Palacios, & Kuhar, 1981). The alpha-2 drugs may have had a more pronounced effect on the bradycardia OR, either because they were more effective in changing activity in cardiac control nuclei or in nuclei involved in CS sensory processing. Baseline HR was lower, although not statistically so, in the alpha-2 groups than in the DALA group, which might help account for the inability of the alpha-2 groups to show a normal bradycardia OR. Regardless of the mechanism, suppression of the OR response to the CS was probably unrelated to the decremented HR CR in the alpha-2 groups because the DALA group also showed a HR CR decrement with no change in the preconditioning CS reflex response.

Relative to the SAL group, the group that received alpha-helical CRF

showed an augmented HR CR during conditioning and during the non-drug test phase. It is conceivable that the facilitation of the CR, which is known to be vagally mediated (Fitzgerald, Martin, & O'Brien, 1973), may have been due to the ability of alpha-helical CRF to enhance parasympathetic outflow (Brown et al., 1982). However, the stable baseline HR level seen in the a-HEL group suggests that autonomic tone was not changed appreciably. Also a shift in autonomic balance would not explain why the a-HEL group continued to show a significantly enhanced CR even when the drug was no longer present on the non-drug test day. An alternative possibility is that conditioning training in the presence of alpha-helical CRF in some way enhanced learning of the CR and that this was reflected on the non-drug test day as continued facilitation of the CR. This point will be addressed in more detail below. In terms of state dependent notions, the failure of the a-HEL group to show a decrement in CR performance between conditioning and the non-drug test phase suggests that drug state was not a critical part of the stimulus complex supporting the CR. This outcome also might argue against the view that a change in drug state was responsible for the absence of a CR in the alpha-2 and DALA groups on the non-drug test day.

Originally it was thought that alpha-helical CRF might decrement HR conditioning by blocking endogenous CRF activity in the LC that could be important in establishing conditioned fear. This view was based on findings showing that blockade of central CRF with the antagonist alpha-helical CRF (9-41) decremented learned fear responses in a number of other paradigms (Cole, Britton, & Koob, 1987; Sherman, Barksdale, Takahaski, & Kalin, 1987). However, there is some reason to believe that the effects of CRF blockade could vary with the location of the blockade. In all the previous studies

showing that CRF antagonism decremented conditioned fear (Cole, Britton, & Koob, 1987; Sherman et al., 1987), the drug was administered into the lateral ventricles and thereby, presumably, affected CRF receptors in limbic system structures involved in fear conditioning, most notably the amygdala central nucleus (ACE). By contrast, fourth ventricle administration of alpha-helical CRF would not be expected to affect the ACE or other limbic structures that may be critically involved in conditioned fear, and that may be why the HR CR was not decreased.

Alternatively, in keeping with the notion that increasing levels of arousal can impair learning by leading to decremental processes like hyperactivity and distractability (Kesner, 1973), it could be suggested that alpha-helical CRF lowered fear and arousal in the a-HEL group to a level more optimal for conditioning. In support of this idea, it has been found that increasing US shock intensity, which may increase arousal and anxiety, can decrement the magnitude of the bradycardia CR (Fitzgerald & Teyler, 1970). Therefore, under certain circumstances, decreasing arousal and anxiety may augment the magnitude of the HR CR.

Endogenous CRF, which has been shown to be released in the LC following electric shock and other stressful events, increases tonic or resting LC activity (Berridge & Dunn, 1986; Cassens et al., 1973; Korf, Aghajanian, & Roth, 1973). While the administration of CRF in the LC, also increases tonic activity in LC neurons, sensory evoked LC activity is disrupted (Valentino & Foote, 1987; 1988). Decrementing sensory evoked activity in the LC may be one way in which increased levels of arousal attenuate learning. Given this relationship, blockade of CRF activity in the LC, as may have occurred in the a-HEL group, might have enhanced sensory evoked

responding to the CS and US. Such an enhancement may have augmented the learning of the HR CR by facilitating NE release in target areas in conjunction with CS/US presentations. The fact that alpha-helical CRF also enhanced the startle reflex response in this experiment is consistent with the view of enhanced LC evoked activity. Also, other drugs that are known to enhance postsynaptic NE activity (i.e., cocaine and amphetamine) have been found to augment baseline startle reactivity (Davis, 1985).

The CRF antagonist, alpha-helical CRF, was found to augment the reflex bradycardia OR. This finding may be explained by either a performance related effect (i.e., enhancement of vagal outflow) or by an increase in CS sensory evoked responding in the LC after blockade of endogenous CRF levels (Valentino & Foote, 1988). Whether there was a common mechanism responsible for the facilitated OR, HR CR, and baseline startle reflex in the a-HEL group cannot be determined on the basis of current findings, but a mechanism involving sensory processing of the CS seems attractive.

The administration of DALA, UK 14,304, clonidine, and alpha-helical CRF had no effects on the performance of the tachycardia UR. This finding suggests that US sensory processing was probably not affected by the administration of these drugs. Both opioid agents and alpha-2 drugs have been shown to possess analgesic and anxiolytic effects (Redmond, 1979). It has been reported that the analgesic effects of opiate compounds may be more related to their ability to suppress emotional reactions to pain than to their ability to cause specific blunting of pain sensations (Jaffee & Martin, 1985; Kelly, 1985). Although the exact role of emotions, such as fear, in the development of HR CRs is not known, it seems plausible to suggest that in this study, DALA, UK, and Clon may not have blocked the

specific pain sensations of the shock US as evidenced by the presence of the UR. However, they could have blocked the emotional after effects of the US and as a consequence perhaps prevented the development of a HR CR.

In this study, the presentation of the CS+ in conjunction with the startle eliciting stimulus resulted in the inhibition of the reflex startle response in the SAL and a-HEL groups. This outcome is in opposition to the potentiation of the startle response that is commonly seen in freely-moving animals following fear conditioning (Brown et al., 1951; Davis, 1979). Differences in the direction of the HR CR have also been found between freely-moving and restrained animals. For example, in unrestrained freely moving animals, given a footshock US, the CR is a cardioacceleration (LeDoux, Sakaguchi, & Reis, 1984; Martin & Fitzgerald, 1980). It has been suggested (Fitzgerald & Teyler, 1970) that the potential to escape from the footshock by jumping in the air encourages the conditioning of an anticipatory locomotor state that has cardioacceleration as one of its components. Restrained rats, on the other hand, are unable to escape from the shock and may develop a state of conditioned immobility accompanied by cardiodeceleration. The bradycardia HR CR in restrained rats has been found to be related to decreases in movement and increases in electromyographic activity (Martin & Fitzgerald, 1980), suggesting that the animals are freezing and tensing their muscles in anticipation of the shock. Motor inhibition or "freezing" to a stimulus signaling inescapable shock is a naturally occurring defense behavior in rats (Blanchard & Blanchard, 1972). The CS+ induced inhibition of the startle response seen in this study, using restrained rats, may have been due to the fact that freezing behavior competed with and blocked the normal reflex startle reaction to the startle

stimulus. Less freezing was presumably elicited by the CS- and, therefore, less inhibition of startle occurred when this tone was presented. Although freely-moving animals may also freeze in response to the CS, they are also preparing to jump in the air with shock onset. Therefore, the opportunity to escape shock in the freely-moving situation may be what leads to a CS-induced enhancement of startle responding.

The administration of DALA, UK, and Clon also significantly impaired the startle reflex response to the startle stimulus alone. This finding is similar to previous findings of alpha-2 agonist inhibition of baseline startle reflexes (Davis et al., 1977), but is not consistent with previous findings of no changes in baseline startle following systemic morphine administration (Davis, 1979). In the present study, DALA, and the alpha-2 agonists drugs also suppressed the CS+ induced inhibition of the startle response that was seen in the saline and alpha-helical CRF groups. The former groups responded to the startle eliciting stimulus the same regardless of whether it was presented alone or in conjunction with CS+ or CS-.

The failure of the DALA, UK, and Clon groups to show any significant changes in startle responding in the presence of the CS+ cannot be explained solely by the depression of baseline startle found in these groups (i.e., a floor effect) because startle amplitudes dropped much lower in the SAL and a-HEL groups when CS+ was given. Instead, the failure of the CS+ to inhibit startle in the DALA and alpha-2 groups may have been due to a drug-produced loss of conditioned fear and associated freezing behavior to the CS+. This could also explain the loss of the HR CR that was caused by the drug during the drug-test phase. The drugs may have reduced the anxiety associated with

the CS and thereby reduced both the HR CR and the conditioned suppression of startle responding.

If the mu and alpha-2 agents decremented the HR CRs and conditioned suppression of the startle response because of their reported anxiolytic properties (Redmond, 1979), the most likely site of action for these drugs within the fourth ventricle is the LC. The LC possess the highest concentration of both receptor types (Unnerstall et al., 1981), is inhibited by both types of drugs, and is reported to mediate anxiety and arousal responses to environmental stimuli (Redmond & Krystal, 1984). It is plausible to suggest, based on this evidence, that decrements in LC evoked activity, which would decrease the amount of NE released in target areas such as the ACE, could decrement the learning of a HR CR as well as the performance of an already established HR CR. However, other fourth ventricle structures that may be affected by the drugs (i.e., cardiovascular control nuclei and the PAG) could also have contributed to these outcomes.

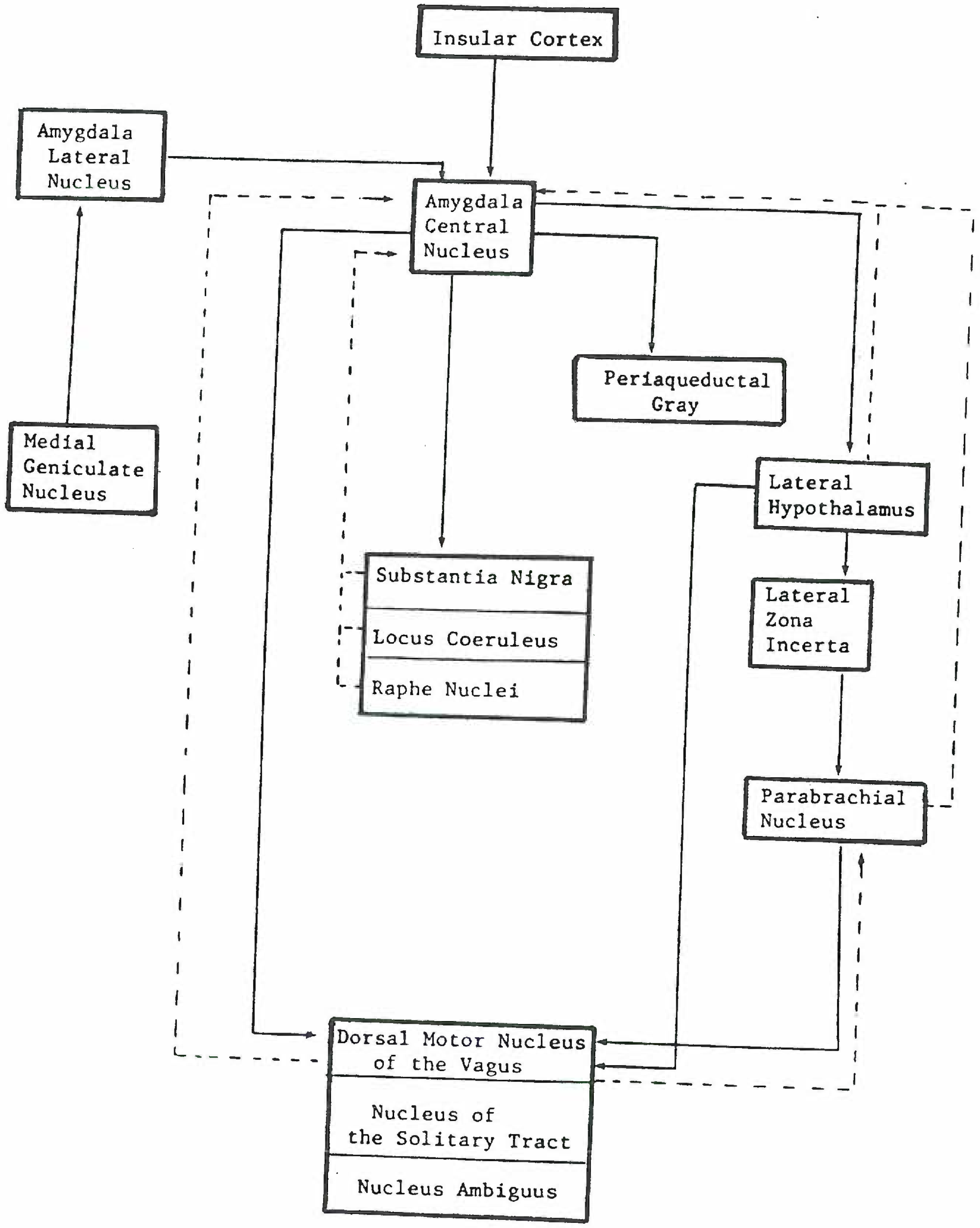
Experiment II

Rationale

The findings of Experiment I indicated that when administered into the rostral fourth ventricle, DALA, UK 14,304, and clonidine, were equally effective in decrementing both the development of a HR CR and performance of a CR established in a drug-free state. As indicated previously, there is a strong possibility given the similar actions of DALA and the alpha-2 agonists that the adverse effect of these drugs on the HR CR may have been due to their inhibitory action on LC activity.

The purpose of Experiment 2 was to test directly the possible role that LC activity might have on the HR CR by microinjecting opioid agonists into the LC. Because of the possible diffusion of LC injected drugs to the nearby opioid sensitive parabrachial nucleus (PBN), it was also important to assess the relative contributions of this nucleus to any observed decrements in conditioned bradycardia. The PBN is located lateral to the LC, and previously has been implicated in the regulation of autonomic activity (Wang & Ranson, 1930), respiration (Bertrand & Hugelin, 1971), adrenocorticotropin release (Ward, Grizzle, & Grann, 1976), and defense reactions (Coote, Hilton, & Zbrozyna, 1973). The PBN is currently thought to be a part of the conditioned bradycardia pathway in rabbits that courses from the amygdala central nucleus (ACE) through the lateral hypothalamus, lateral zona incerta, and to the PBN before reaching the vagal cells of origin located in the dorsal vagal nucleus (DVN) and the nucleus ambiguus (NA) (Schneiderman, McCabe, Haselton, Ellenberger, Jarrell, & Gentile, 1987). Figure 13 diagrams a proposed conditioned bradycardia circuitry (Kapp & Pascoe, 1986; Schneiderman, 1987). In addition, the PBN has extensive projections to

Figure 13. This diagram shows some of the major afferent and efferent projections between brain structures thought to be involved in conditioned bradycardia (Kapp & Pascoe, 1986; Schneiderman et al., 1987). This information is based on findings in both rats and rabbits. For clarity several other structures and projection systems have been omitted. Dotted lines represent major ascending projections, either direct or indirect to the central nucleus.


Insular Cortex
Amygdala Lateral Nucleus
Amygdala Central Nucleus
Medial Geniculate Nucleus
Periaqueductal Gray
Lateral Hypothalamus
Substantia Nigra
Locus Coeruleus
Raphe Nuclei
Lateral Zona Incerta
Parabrachial Nucleus
Dorsal Motor Nucleus of the Vagus
Nucleus of the Solitary Tract
Nucleus Ambiguus

other limic structures known to be involved in the production of the conditioned bradycardia response. These nuclei include the ACE, lateral preoptic region, medial forebrain bundle, bed nucleus of stria terminalis, anterior, and lateral hypothalamus, and lateral zona incerta (Schneiderman et al., 1987). It would appear, based on this evidence, that PBN might provide a neural interface between the limbic structures that may be responsible for the plasticity involved in the learning of the bradycardia CR and the autonomic structures involved in the performance of the CR.

The administration of opioids in the PBN produces hyperpolarization of the neurons through activation of the mu receptor subtype in a manner similar to that seen in the LC (Christie & North, 1988; Williams & North, 1984). However, LC and PBN neurons show opposite effects to the administration of other agonists such as muscarine. For instance, muscarine, acting at the same receptor subtype (M2), excites LC neurons (Egan & North, 1985), but hyperpolarizes PBN neurons (Christie & North, 1988; Egan & North, 1986). The differential responses of LC and PBN neurons to the agonist muscarine provides a possible technique for separating out the relative contributions of these two nuclei in the opioid-induced decrements in the learning and performance of conditioned bradycardia. In Experiment 2 microinjections of either the selective mu opioid agonist (D-Ala2,N-Me-Phe4,Gly5-ol)-enkephalin (DAGO), muscarine, or of a vehicle solution were made into the LC or into the PBN.

It was expected that in the current study DAGO and muscarine administration should have opposite conditioning effects in the LC with DAGO blocking LC output and impeding the learning of the HR CR and with muscarine enhancing LC output and perhaps facilitating the CR. In the PBN, it was

expected that DAGO and muscarine administration should have similar conditioning effects and perhaps alter the performance of the HR CR by inhibiting PBN output. If muscarine placed in the LC impeded the CR, it should raise the possibility that this effect occurred because muscarine diffused to the PBN. Such an effect could mean that any impairment in the HR CR following LC DAGO administration could also be due to diffusion into the PBN.

Methods

Subjects

The subjects were 32 male Sprague-Dawley albino rats (Simonsen), the same age and weight as those used in Experiment I.

Surgical Procedure

All animals had either bilateral cannulae implanted into the LC or in the PBN. The coordinates for the LC were AP: +1.1 mm (anterior to lamda), L: +- 1.1 mm (lateral to the midline), V: -4.2 mm (ventral to the dura). The coordinates for the PBN were AP: +1.1, L: +- 1.6, V: -4.5 mm. All other surgical procedures were identical to those used in Experiment I.

Apparatus

The same apparatus was used in Experiment II as was used in Experiment I.

Experiment Procedure

The cannulae used in this experiment were constructed from 1-cm long 24-ga stainless steel tubing with a 1.1-cm long 32-ga stainless steel internal stylet. The infusion needles were made from 1.5-cm long 32-ga stainless steel tubing. The infusions were performed by hand using two mounted gastight and liquidtight 5 ul SGE syringes. The syringes were attached to the infusion needles with PE-20 tubing. The volume and time course of infusions was 0.5 ul over 5 min. The infusions were done slowly by gently twisting the syringe plunger. This allowed for an approximate rate of .025 ul every 15 s. Drugs were dissolved in an artificial cerebrospinal fluid (CSF), and vehicle injections were also made with artificial CSF. The dose of DAGO was 1.6 uM, while the dose of muscarine

was 100 uM. The dose of DAGO that was choosen is consistent with previous reports of behaviorally effective brain concentrations (Locke & Holtzman, 1986), while the dose of muscarine was chosen to be similar to concentrations found to be effective in vitro (Christie & North, 1988).

All animals were randomly assigned to one of six treatment conditions prior to the surgery (see Table 3). The conditioning procedures and design were similar to those used in Experiment I (see Table 4). Briefly, this included two days of conditioning in the presence of the drugs, a CS test phase at end of the second day of conditioning, test trials given in the absence of the drugs 48 hours after Day 2 conditioning, reconditioning in the absence of drugs, and finally a second set of test trials following drug administration to evaluate the effects of the drugs on established CRs.

Histology

Following removal of the brain, tissue blocks containing the cannulae tract were frozen and sectioned (30 microns) on a cryostat. The sections were then mounted on glass slides and stained with thionin. The stereotaxic atlas of Paxinos and Watson (1982) was consulted to determine the precise location of cannulae on microscopically inspected slides.

Data Analyses

The data in Experiment II were analyzed using an analysis of variance in which brain location and drug type were the main between group variables, while trial blocks, CS type, and measurement intervals were the major within group variables. First, each brain location site was analyzed separately, using drug treatment as the between groups factor, and then an overall ANOVA comparing brain location and drug treatment was performed. Each day of the experiment was analyzed separately, and so was each of the phases within

Table 3.

	DAGO	Muscarine	Vehicle
LC	N=6	N=6	N=4
PBN	N=6	N=6	N=4

Table 4. Experimental Procedure

Day						
Day 1.	Habituation (30 min)	Pre-Drug Baseline HR (15 min)	Drug Infusion (1 min)	Drug Distribution (5 min)	CS Alone (2CS+ no US, 2CS-) (10 min)	Conditioning (6CS+, 6CS-) (25 min)
Day 2.	Pre-Drug Baseline HR (15 min)	Drug Infusion (1 min)	Drug Distribution (5 min)	Conditioning (6CS+, 6CS-) (25 min)	CS-Test (4CS+ no US, 4CS-) (20 min)	
Day 3.	All groups left in home cage					
Day 4.	Pre-Test Baseline HR (15 min)	Non-Drug Test (6CS+ no US, 6CS-) (20 min)	Reconditioning (10CS+, 10 CS-) (40 min)	Drug Infusion (1 min)	Drug Distribution (5 min)	Drug Test (4CS+ no US, 4CS-) (15 min)

each day (i.e., CS-alone, and conditioning). All follow-up tests to compare group means after the identification of significant group interactions were done with the use of a Newman-Keuls test.

Results

Histology

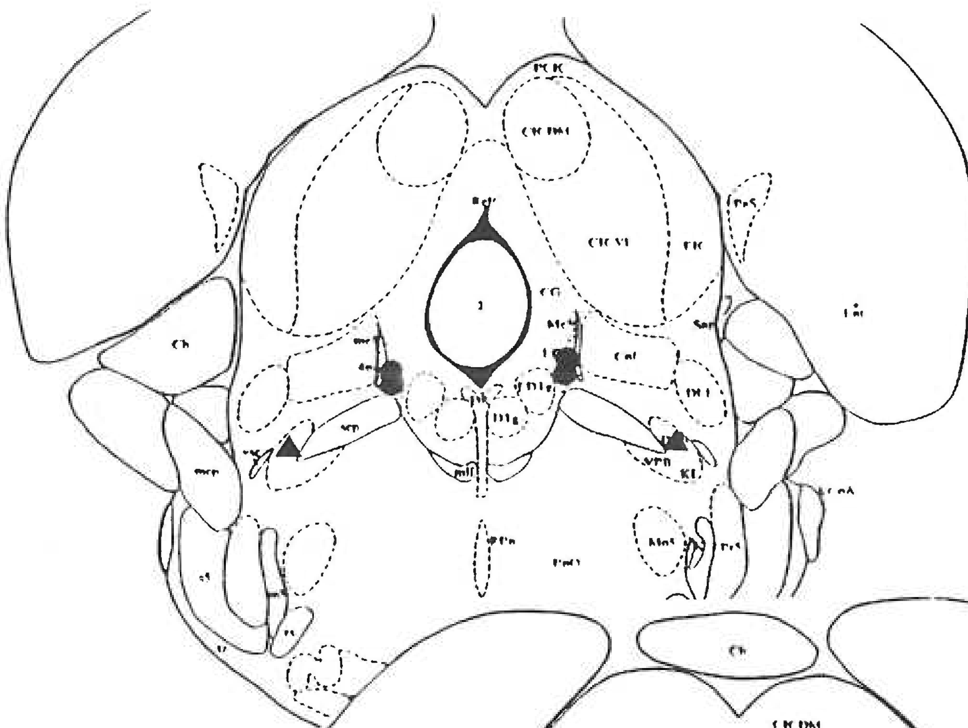
In each of the six groups, only those animals with cannulae located in the LC or PBN were included in the data analyses. Microscopic examination of slides containing the injection sites for each animal resulted in the selection of six animals in each of the drug injected groups and four animals in each of the Vehicle injected groups (only 2 - 3 animals per group were discarded due to missed placements). Figure 14 shows the injection sites of each individual animal in the LC-DAGO and the PBN-DAGO groups. While there was some variation in placement on the anterior-posterior plane, behaviorally the animals were indistigushible, and, therefore, placements in either the rostral or caudal portions of the nuclei were accepted as correct placements. The majority of animals in each group did, however, fall into the more centralized locations. Figures 15 and 16, respectively, show the location of injection sites for individual animals in the Muscarine and Vehicle injected groups. Microscopic examination of the slides revealed tissue damage and necrosis around the injection site. However, this damage did not extend for more than 60-90 microns (in the rostral-caudal plane) from the injection site, thus leaving the majority of each nucleus intact.

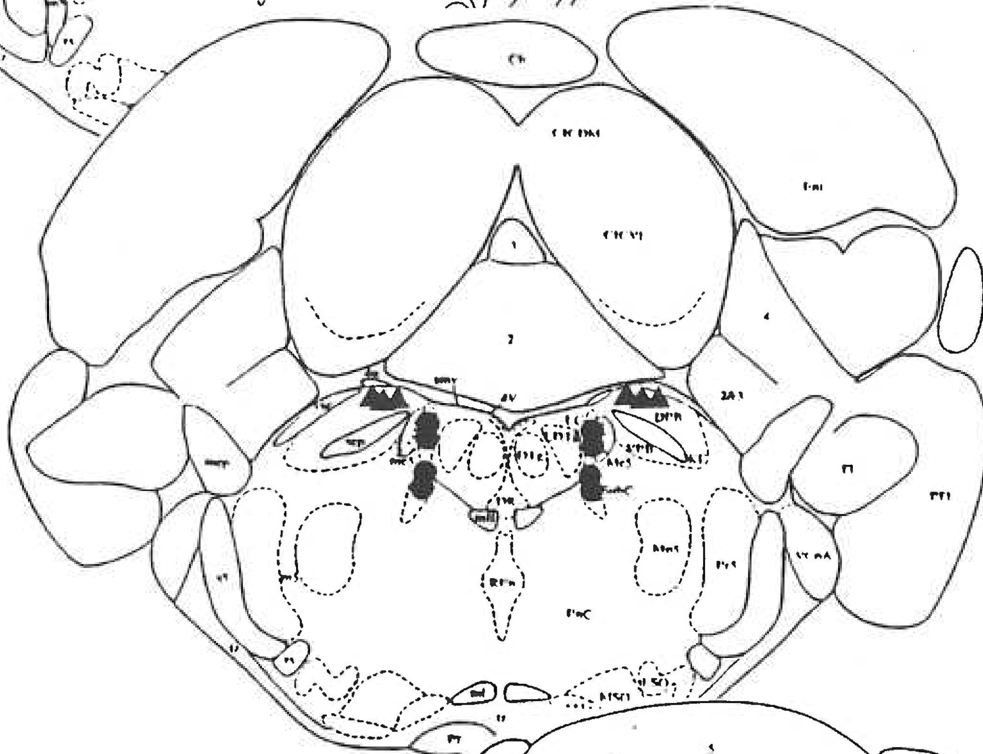
Baseline Heart Rate for LC Injected Groups

Figures 17 and 18, respectively, illustrate baseline HR of LC injected groups during successive phases on Day 1 and Day 2. It may be noted from these figures that on each day, the DAGO and Vehicle injected groups maintained a steady fixed level of baseline HR, whereas the Muscarine injected group showed some elevation of HR immediately following drug administration. Baseline HR in this group remained slightly elevated

Figure 14. The circles in this figure (taken from the Paxinos & Watson, 1982 stereotaxic atlas) depict the location of the infusion sites for individual animals in the LC-DAGO group, while the triangles represent the individual placements for animals in the PBN-DAGO group.

● LC
▲ PBN





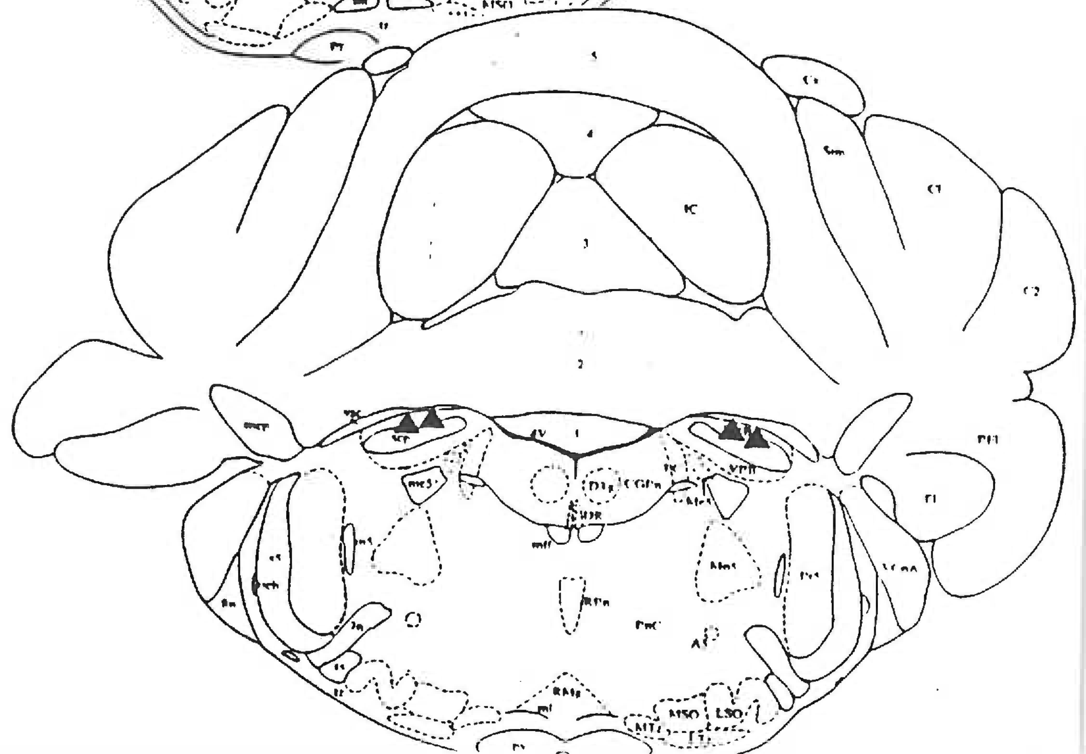
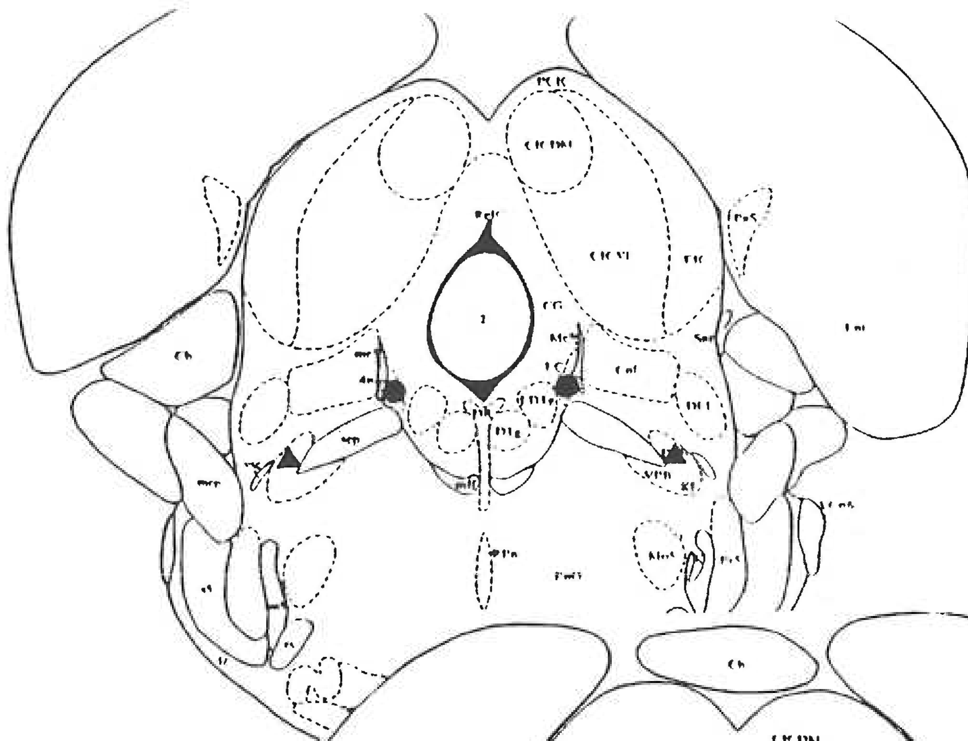
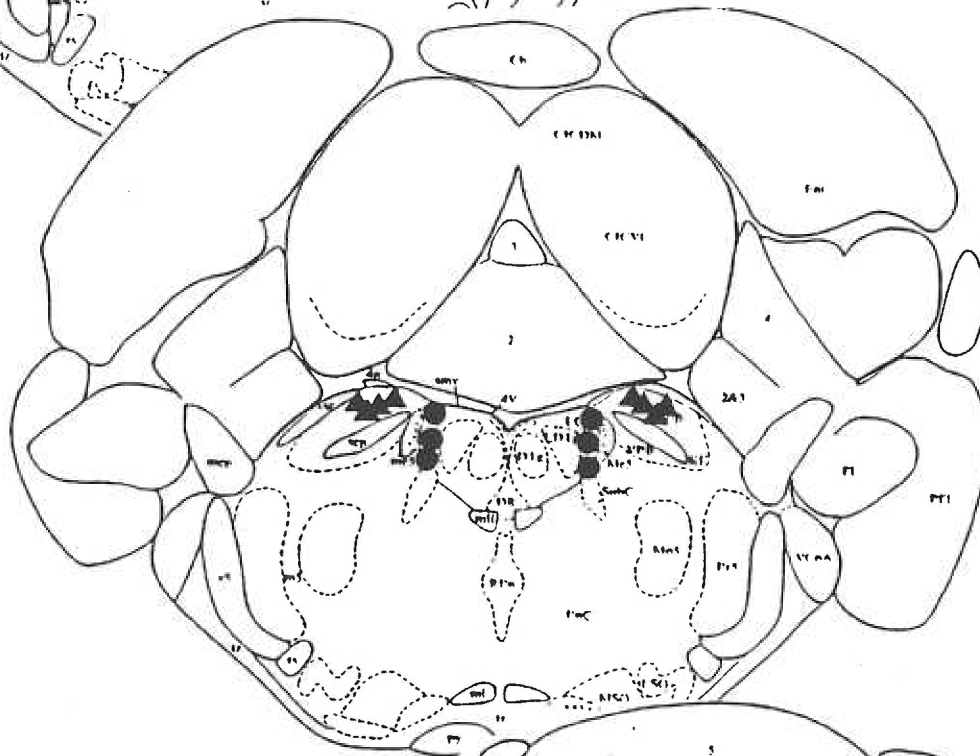
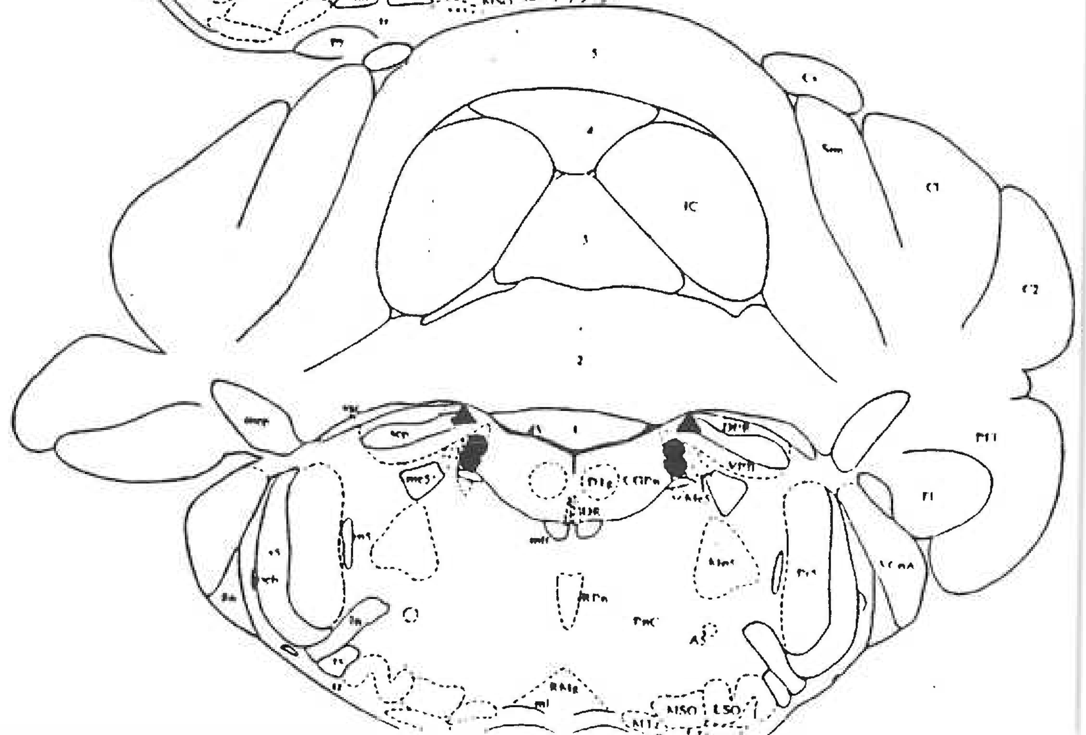


Figure 15. The circles in this figure depict the location of the infusion sites for individual animals in the LC-Muscarine group, while the triangles represent the individual placements for animals in the PBN-Muscarine group.







● LC
▲ PBN

Figure 16. The circles in this figure depict the location of the infusion sites for individual animals in the LC-Vehicle group, while the triangles represent the individual placements for animals in the PBN-Vehicle group.

● LC
▲ PBN

Figure 17. Baseline HR for each of the LC injected groups during Day 1 of conditioning. Scores were calculated from mean HR during the 6-s pre-CS period of each trial and were averaged over one four-trial block during the pre-drug phase, post-drug phase, CS-alone phase, and two six-trial blocks during conditioning.

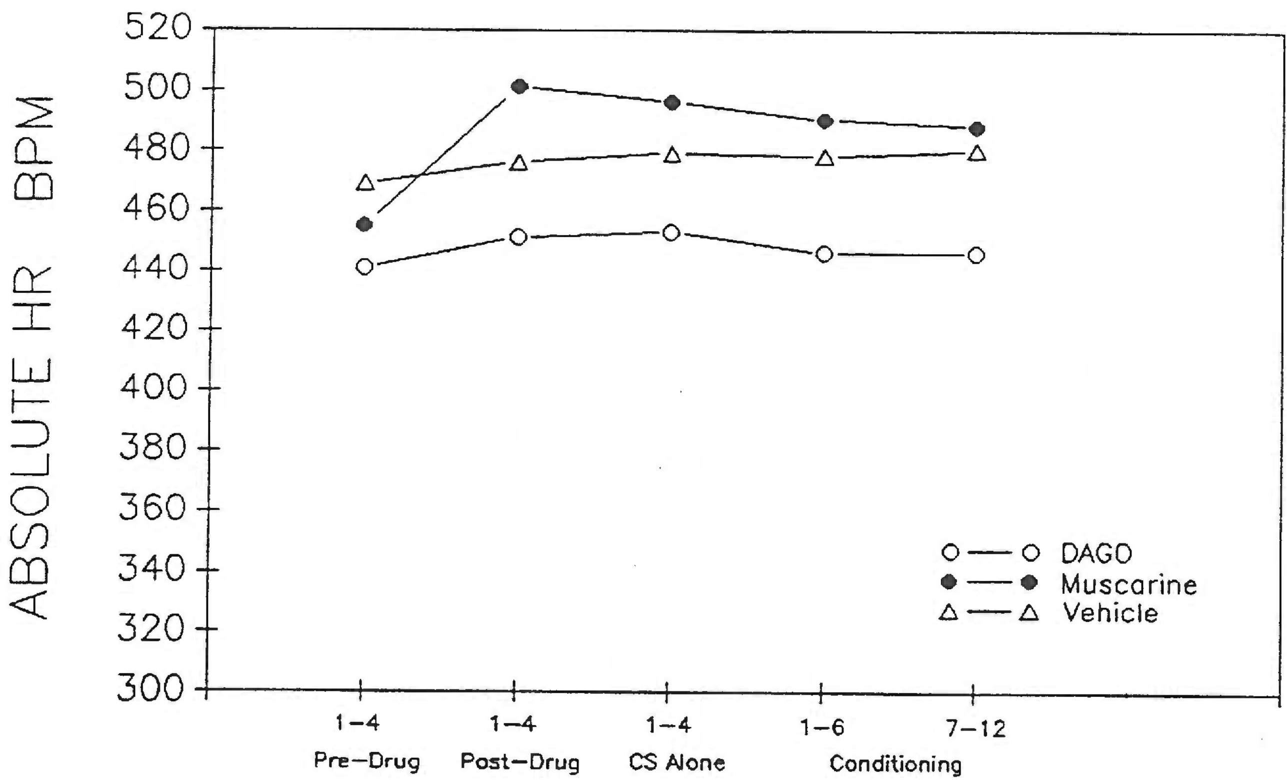
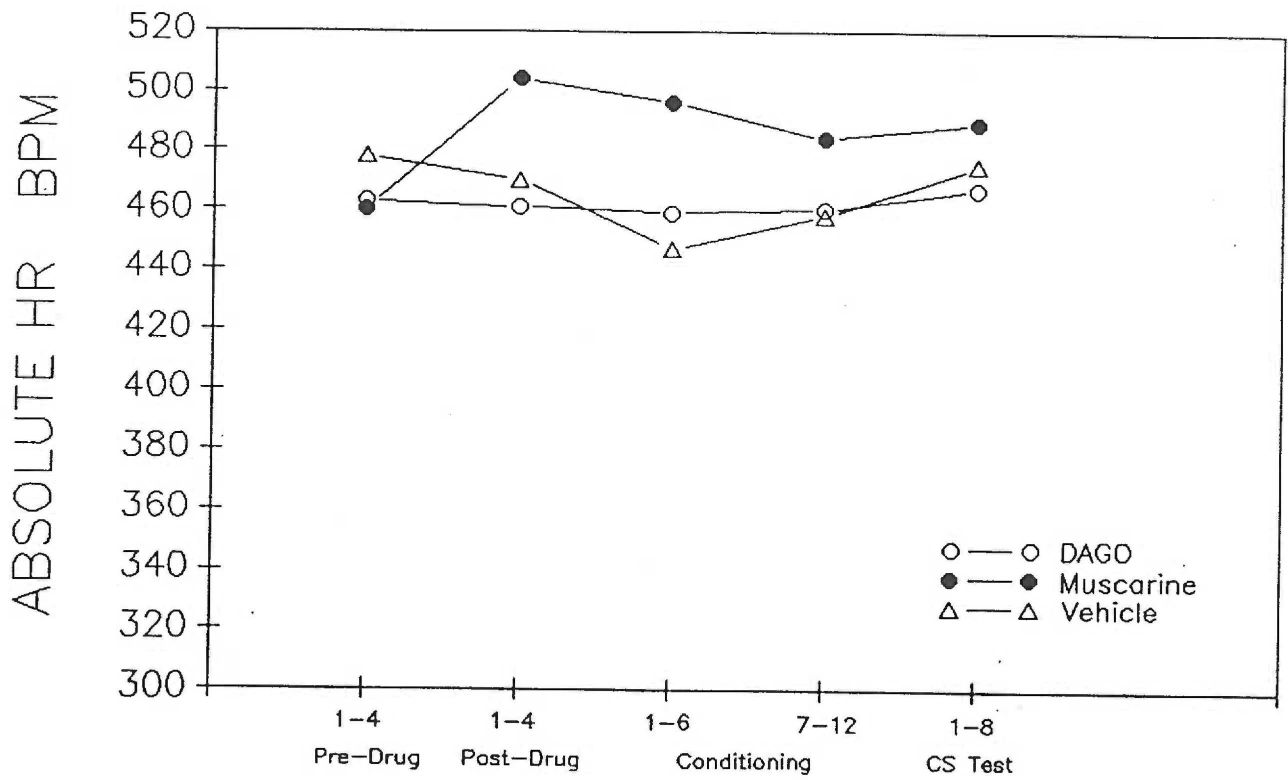

ABSOLUTE HR BPM
520
500
480
460
440
420
400
380
360
340
320
300
1–4
Pre–Drug
1–4
Post–Drug
1–4
CS Alone
1–6
7–12
Conditioning
DAGO
Muscarine
Vehicle

Figure 18. Baseline HR for each of the LC injected groups during Day 2 of conditioning. Scores were calculated from mean HR during the 6-s pre-CS period of each trial and were averaged over one four-trial block during the pre-drug phase and post-drug phase, two six-trial blocks during conditioning, and one eight-trial block during the CS-test.



ABSOLUTE HR BPM
520
500
480
460
440
420
400
380
360
340
320
300
1-4
Pre-Drug
1-4
Post-Drug
1-6
7-12
Conditioning
1-8
CS Test
DAGO
Muscarine
Vehicle

throughout conditioning on both days.

On Day 1, a 3 x 5 (Groups x Trial Blocks) ANOVA revealed a significant groups effect, F (2,13) = 5.99, $p < .05$. A follow-up test showed that the baseline HR of the Muscarine group was significantly elevated relative to the DAGO group but not with respect to the Vehicle injected group, $p < .05$.

On Day 2, a 3 x 5 (Groups x Trial Blocks) ANOVA provided a significant groups effect, F (2,13) = 6.33, $p < .05$ and a groups x trial blocks interaction, F (8,52) = 6.29, $p < .01$. A follow-up test at each trial block revealed that while the groups were not significantly different from each other prior to drug administration, the Muscarine group had a significantly elevated HR following drug infusion and during conditioning but was not different from the other groups during the CS test, $p < .05$.

Baseline HR in the LC groups during Day 4 of the experiment is shown in Figure 19. All groups exhibited similar baseline HRs on this day until the drugs were re-administered at the end of the reconditioning phase, at which point the Muscarine group again showed an elevated HR. A 3 x 5 (Groups x Trial Blocks) ANOVA revealed a significant groups effect, F (2,13) = 5.21, $p < .05$, and a significant groups x trial blocks interaction, F (8,52) = 9.10, $p < .01$. A follow-up test affirmed that while the groups were not significantly different from each other during the non-drug phase, the subsequent administration of drug in the Muscarine group again resulted in a significant elevation of baseline HR, $p < .05$.

Baseline Heart Rate in PBN Injected Groups

Figures 20 and 21, respectively, show the baseline HR of the PBN-injected groups during successive phases on Day 1 and Day 2. It may be seen from these graphs that DAGO and Muscarine administration into the PBN

Figure 19. Baseline HR during Day 4 for LC injected groups. Scores were calculated from mean HR during the 6-s pre-CS period for each trial and were averaged over one four-trial block during the pre-test phase and post-drug phase, one twelve-trial block during the non-drug test, one twenty-trial block during reconditioning, and one eight-trial block during the drug test phase.

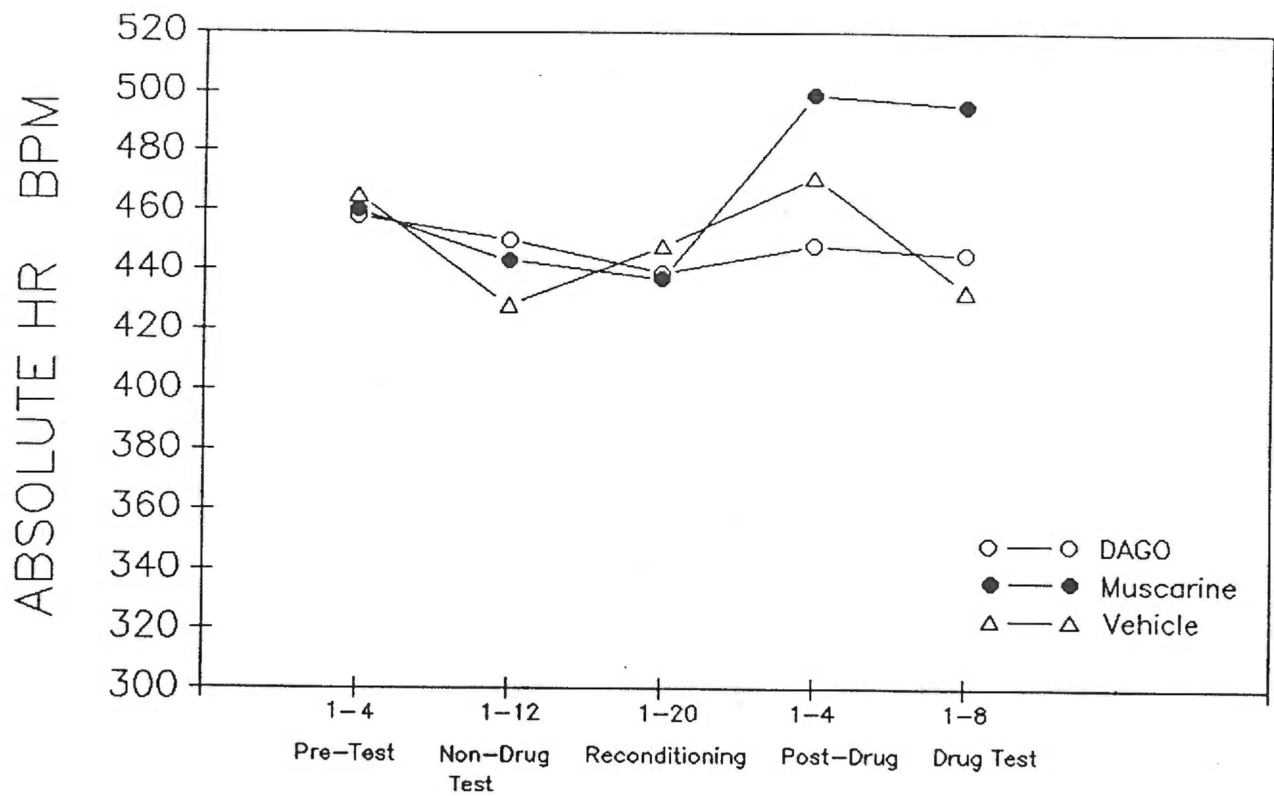
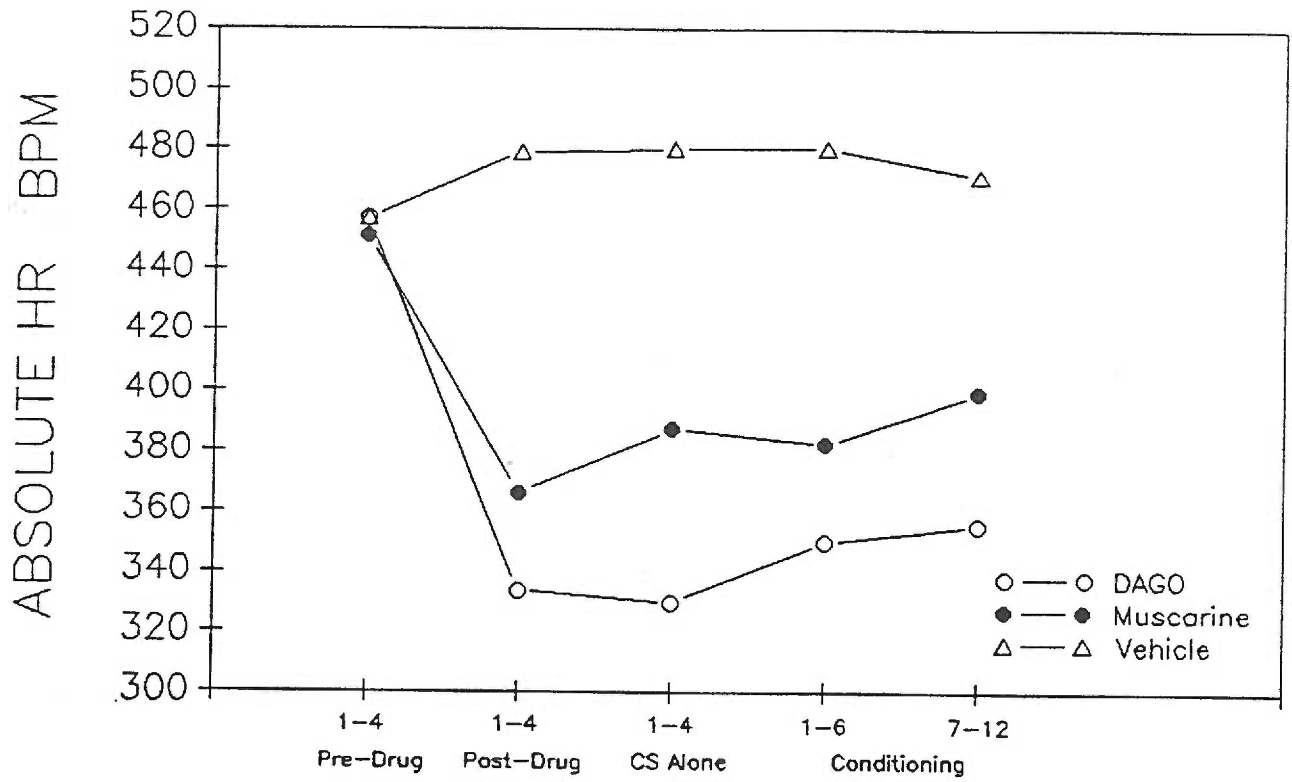
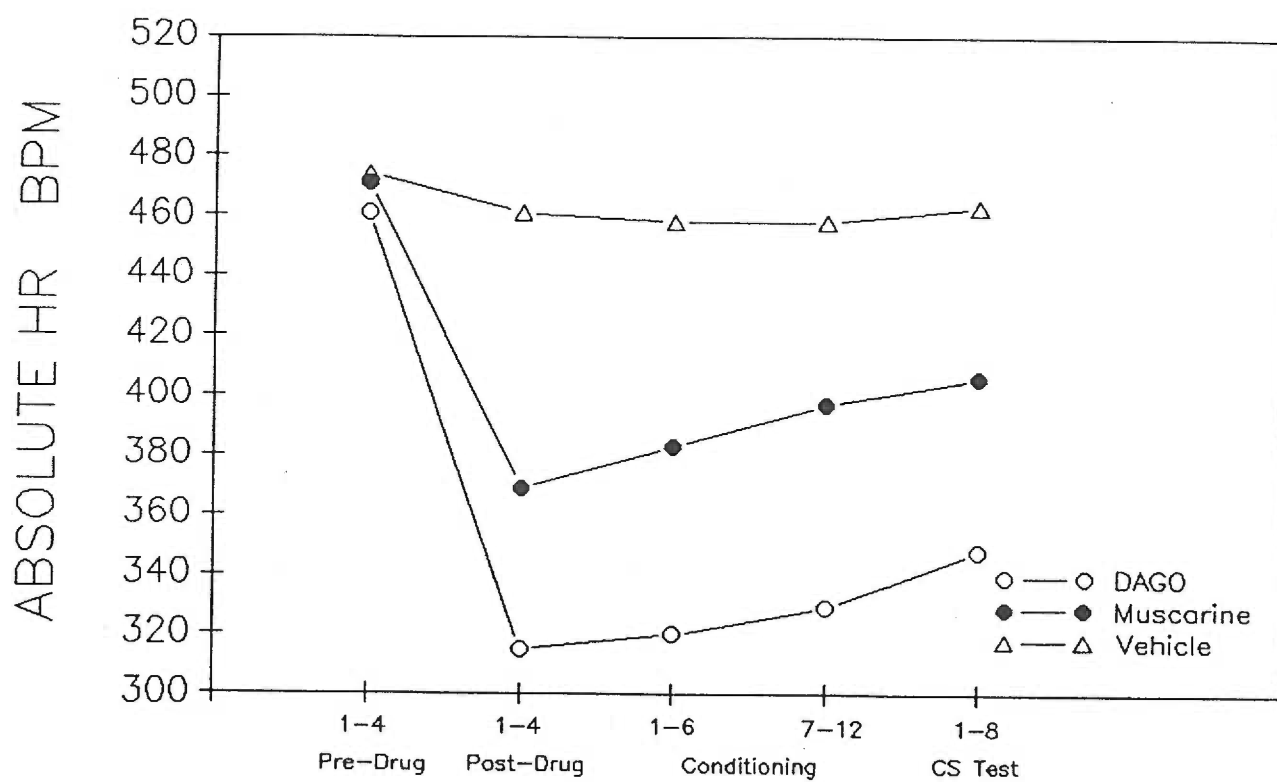

ABSOLUTE HR BPM
520
500
480
460
440
420
400
380
360
340
320
300
1–4
Pre–Test
1–12
Non–Drug Test
1–20
Reconditioning
1–4
Post–Drug
1–8
Drug Test
DAGO
Muscarine
Vehicle

Figure 20. Baseline HR for each of the PBN injected groups during Day 1 of conditioning. Scores were calculated from mean HR during the 6-s pre-CS period of each trial and were averaged over one four-trial block during the pre-drug phase, post-drug phase, CS-alone phase, and two six-trial blocks during conditioning.



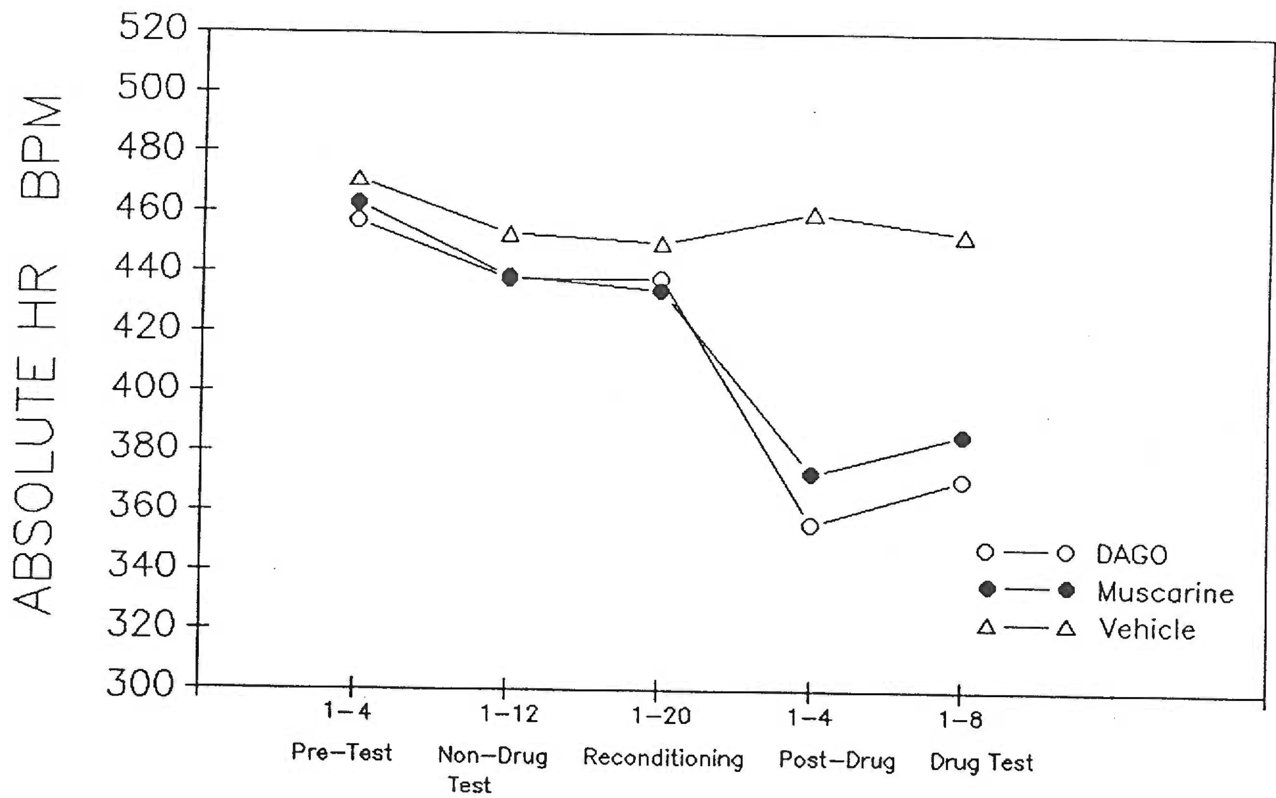
ABSOLUTE HR BPM
520
500
480
460
440
420
400
380
360
340
320
300
1-4
Pre-Drug
1-4
Post-Drug
1-4
CS Alone
1-6
7-12
Conditioning
DAGO
Muscarine
Vehicle

Figure 21. Baseline HR for each of the PBN injected groups during Day 2 of conditioning. Scores were calculated from mean HR during the 6-s pre-CS period of each trial and were averaged over one four-trial block during the pre-drug phase and post-drug phase, two six-trial blocks during conditioning, and one-eight trial block during the CS-test.



ABSOLUTE HR BPM
520
500
480
460
440
420
400
380
360
340
320
300
1–4
Pre–Drug
1–4
Post–Drug
1–6
7–12
Conditioning
1–8
CS Test
DAGO
Muscarine
Vehicle

Figure 22. Baseline HR during Day 4 for PBN injected groups. Scores were calculated from mean HR during the 6-s pre-CS period for each trial and were averaged over one four-trial block during the pre-test phase and post-drug phase, one twelve-trial block during the non-drug test, one twenty-trial block during reconditioning, and one eight-trial block during the drug test phase.


ABSOLUTE HR BPM
520
500
480
460
440
420
400
380
360
340
320
300
1-4
Pre-Test
1-12
Non-Drug Test
1-20
Reconditioning
1-4
Post-Drug
1-8
Drug Test
DAGO
Muscarine
Vehicle

resulted in an immediate fall in baseline HR (within 2 min) and HR remained depressed throughout the session on both days. No changes in baseline HR were seen in Vehicle injected groups.

On Day 1, a 3 x 5 (Groups x Trial Blocks) ANOVA revealed a significant groups effect, $F\ (2,13) = 66.67$, $p < .01$, and a significant groups x trial blocks interaction, $F\ (8,52) = 12.64$, $p < .01$. A follow-up test indicated that the DAGO and Muscarine groups had a lower baseline HR than the Vehicle group following drug administration and at each subsequent trial block throughout conditioning, $p < .05$.

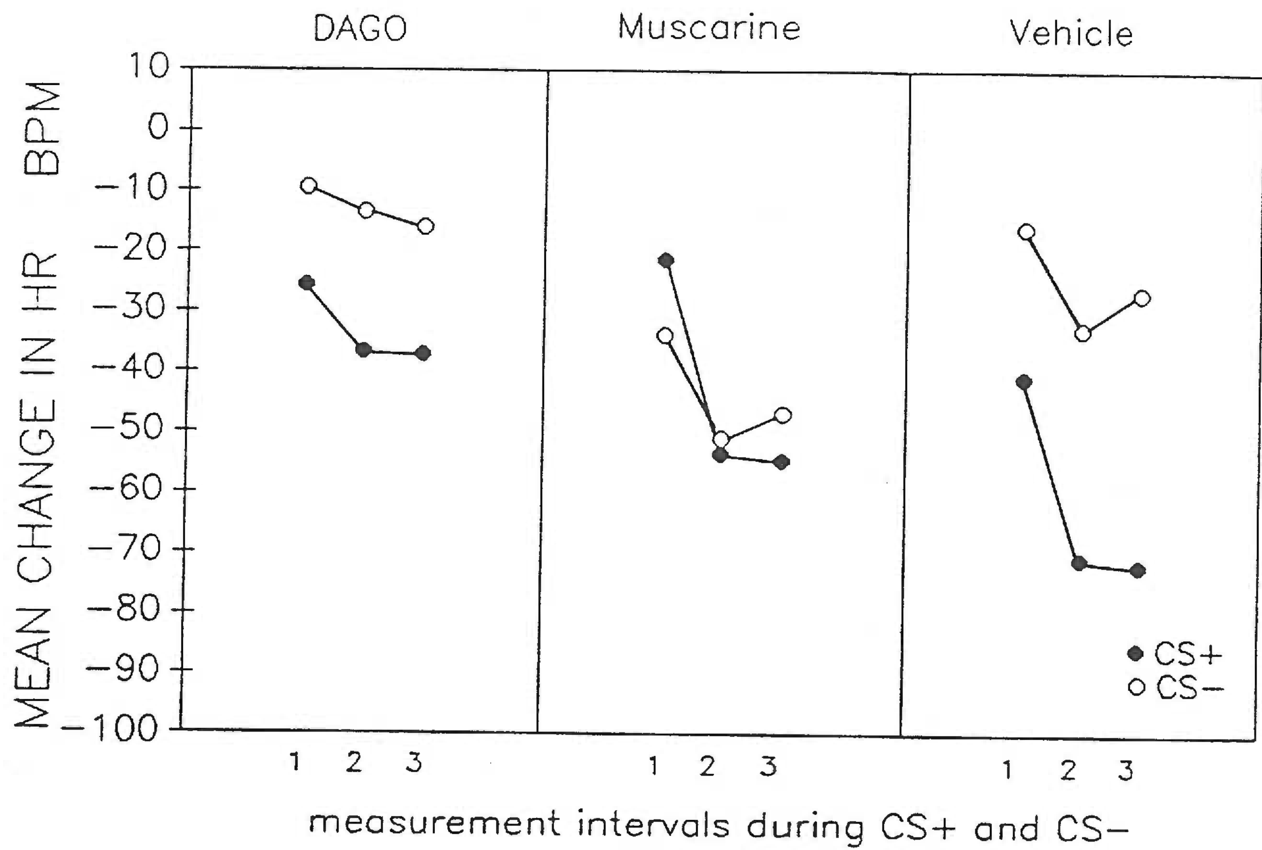
On Day 2, a 3 x 5 (Groups x Trial Blocks) ANOVA again produced a significant groups effect, $F\ (2,13) = 40.85$, $p < .01$, and a significant groups x trial blocks interaction, $F\ (8,52) = 7.69$, $p < .01$. A follow-up test again affirmed that the DAGO and Muscarine groups had suppressed baseline HR relative to the Vehicle injected group immediately following drug administration and throughout the rest of the session, $p < .05$.

Baseline HR during Day 4 for PBN-injected groups is depicted in Figure 22. All groups displayed a similar baseline HR on this day until the drugs were re-administered at the end of reconditioning, at which time the DAGO and Muscarine injected groups show a depression of baseline HR. A 3 x 5 (Groups x Trial Blocks) ANOVA produced a significant groups effect, $F\ (2,13) = 11.8$, $p < .01$, and a significant groups x trial blocks interaction, $F\ (8,52) = 11.17$, $p < .01$. A follow-up test showed that both the DAGO and Muscarine animals were significantly suppressed following drug administration and during the drug test phase, $p < .05$.

Orienting Responses

Figure 23 depicts the original preconditioning HR orienting responses

Figure 23. Heart rate orienting responses during three 2-s CS measurement intervals for each of the LC injected groups to CS+ and to CS- averaged over a two-trial block during the CS-alone phase that occurred prior to conditioning. Responses are plotted as CS minus pre-CS heart rate difference scores.


DAGO
Muscarine
Vehicle
MEAN CHANGE IN HR BPM
10
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
1 2 3
1 2 3
1 2 3
CS+
CS−
measurement intervals during CS+ and CS−

(ORs) to CS+ and to CS- for the LC injected groups during three 2-s CS measurement intervals averaged over two trial blocks. This figure shows that responding to CS+ was greater than responding to CS- in the DAGO and Vehicle groups but not in the Muscarine group. Responding to CS+ in the Vehicle group was larger than in the other groups, while responding to CS- was larger in the Muscarine group. A 3 x 2 x 3 (Groups x CS Type x Measurement Intervals) ANOVA demonstrated a significant CS type effect, $F(1,13) = 36.55$, $p < .01$, and a significant groups x CS type interaction, $F(2,13) = 12.34$, $p < .01$. A follow-up on this interaction affirmed that the DAGO group was suppressed in their responding to CS+ relative to the Vehicle group, and that the Muscarine group showed an elevated response to CS- relative to the Vehicle group.

The HR OR to CS+ and to CS- in the PBN groups is shown in Figure 24. As was seen in two of the LC groups, CS+ responding was greater than CS- responding. However, there were no PBN group differences. A 3 x 2 x 3 (Groups x CS Type x Measurement Intervals) ANOVA demonstrated only a significant CS type effect, $F(1,13) = 21.9$, $p < .01$.

<u>Conditioned Responses: Brain Location</u>

<u>Day 1 Conditioning in LC Groups.</u> Figure 25 illustrates the mean HR responses of each LC group to CS+ and CS- averaged over successive blocks of three trials on Day 1. It may be seen that the DAGO group exhibited very small responses to both CS+ and CS- relative to the Muscarine and Vehicle groups and showed no consistent differential responding to CS+. By contrast, the Muscarine and Vehicle groups showed a major bradycardia to CS+.

The reliability of the group differences was tested by means of a 3 x 2

Figure 24. Heart rate orienting responses during three 2-s CS measurement intervals for each of the PBN injected groups to CS+ and to CS- averaged over a two-trial block during the CS-alone phase that occurred prior to conditioning. Responses are plotted as CS minus pre-CS heart rate difference scores.

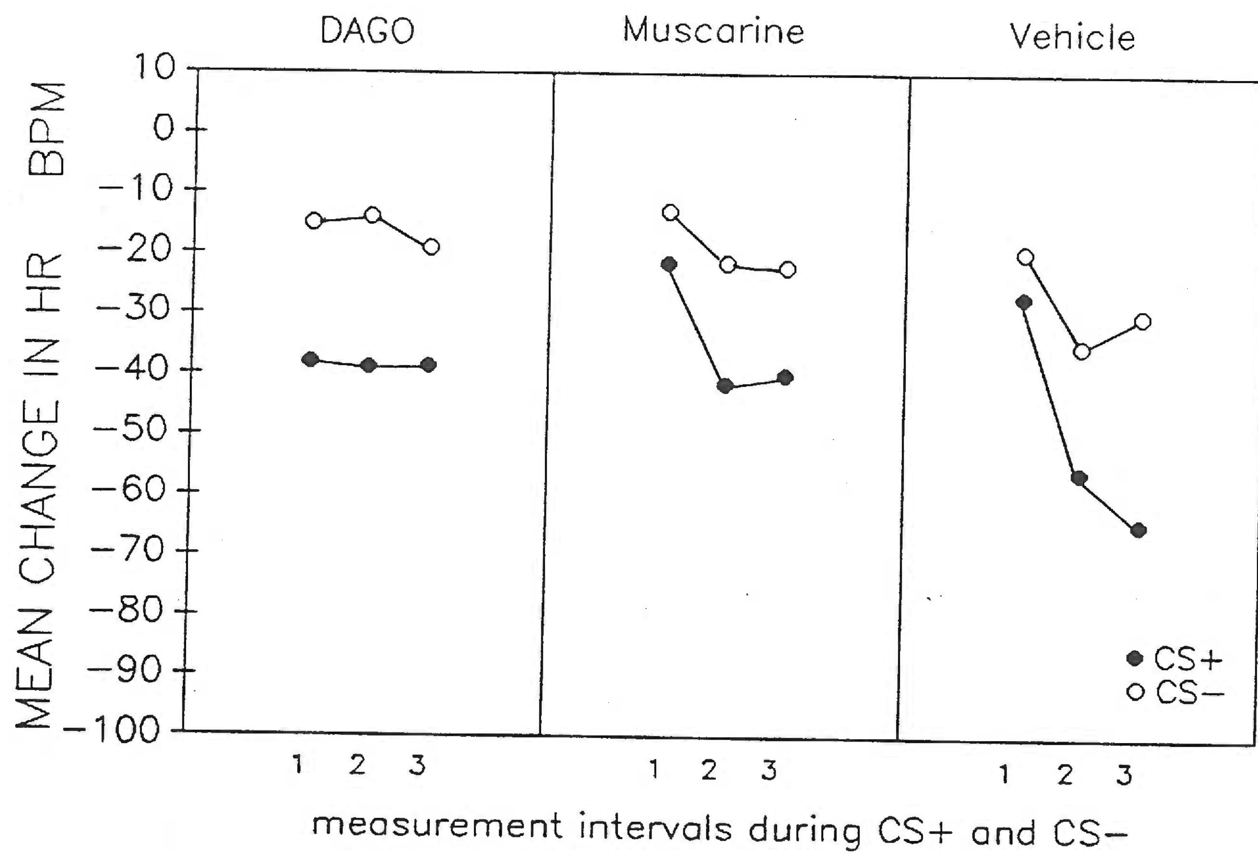
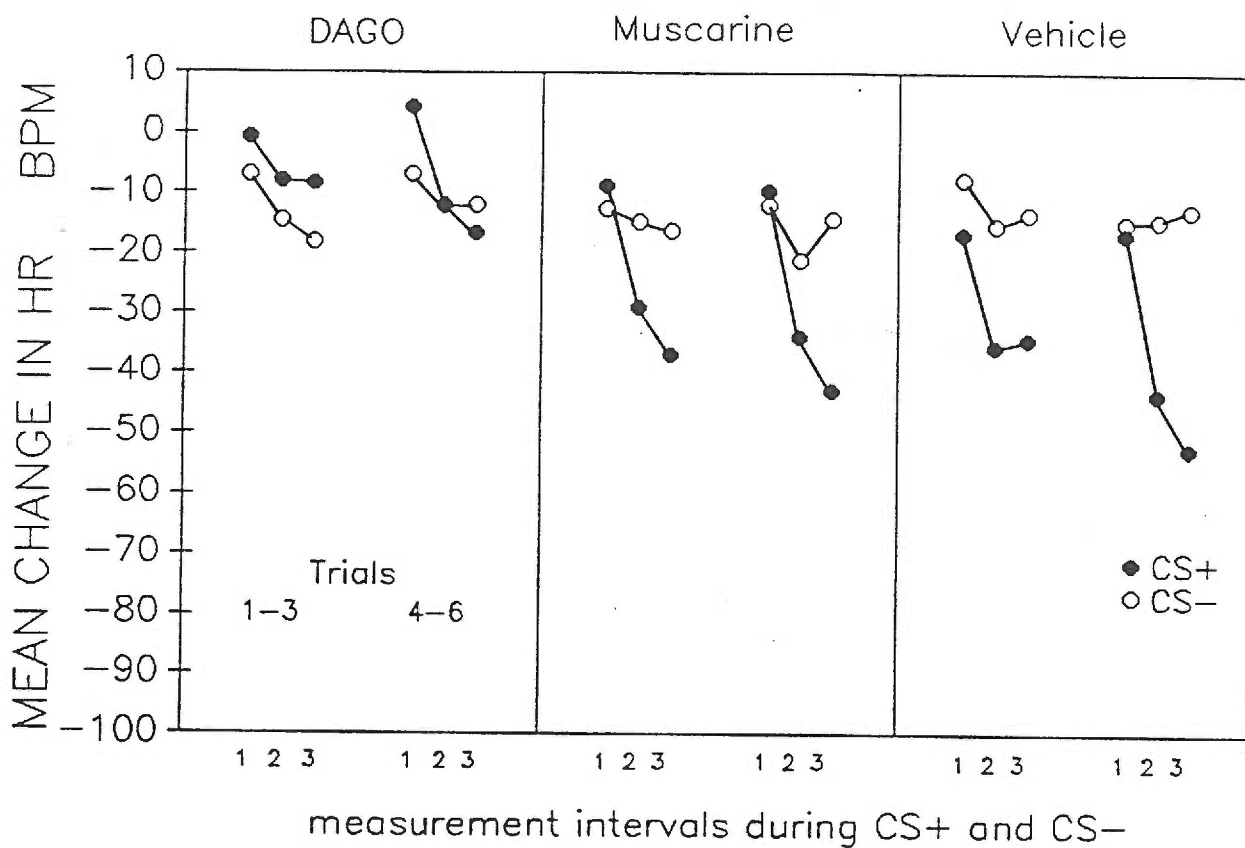

DAGO
Muscarine
Vehicle
MEAN CHANGE IN HR BPM
10
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
1 2 3
1 2 3
1 2 3
● CS+
○ CS−
measurement intervals during CS+ and CS−

Figure 25. Heart rate responses during three 2-s CS measurement intervals for each of the LC injected groups to CS+ and to CS- averaged over two blocks of three trials on Day 1 of conditioning. Responses are plotted as CS minus pre-CS difference scores.

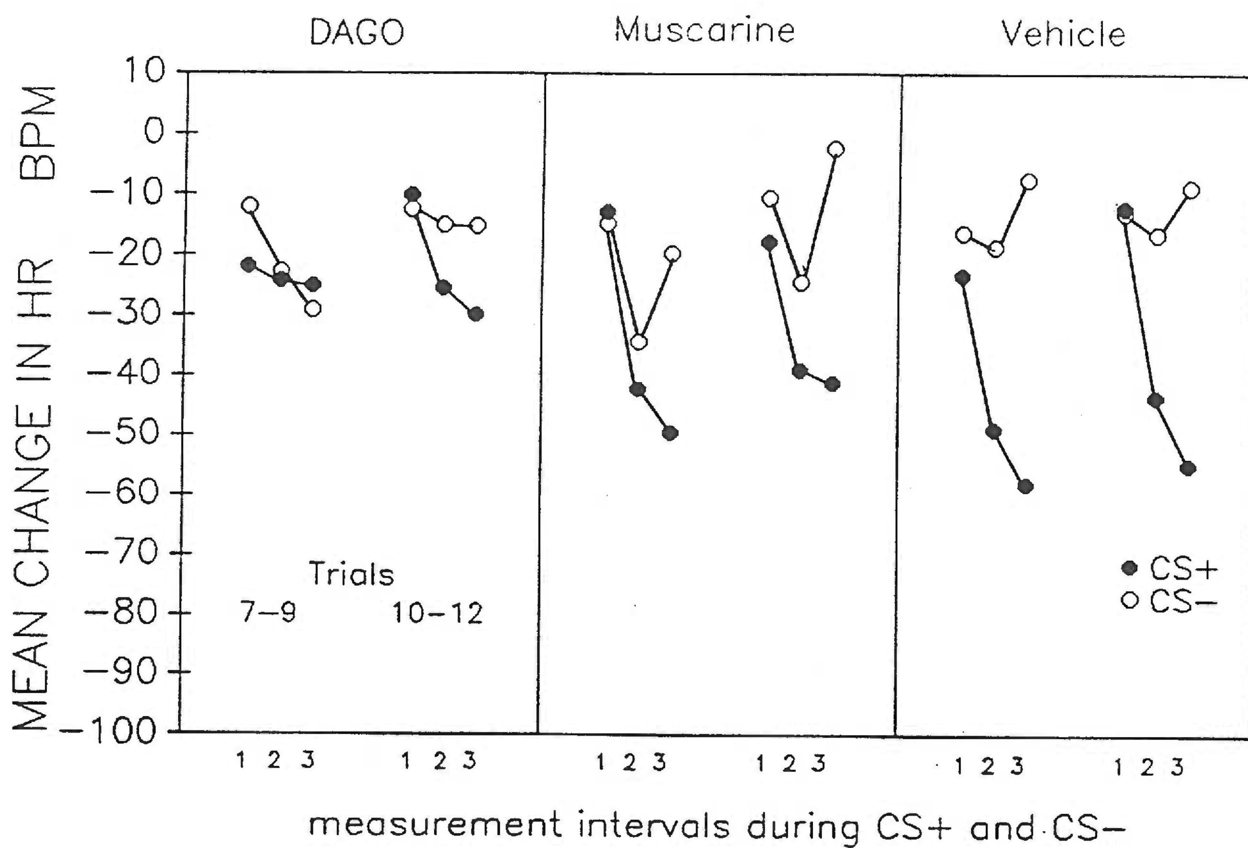

DAGO
Muscarine
Vehicle
MEAN CHANGE IN HR BPM
10
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
Trials
1−3
4−6
● CS+
○ CS−
1 2 3
1 2 3
1 2 3
1 2 3
1 2 3
1 2 3
measurement intervals during CS+ and CS−

x 2 x 3 (Groups x Trial Blocks x CS Type x Measurement Intervals) ANOVA. This test established a significant groups effect, $F\ (2,13) = 8.07$, $p <$ .01, and a significant groups x CS type interaction, $F\ (2,13) = 6.83$, $p <$ .01. In addition, there was a significant 3-way trial blocks x CS type x measurement intervals interaction, $F\ (2,26) = 6.29$, indicating that across the groups conditioning occurred over trials. Not significant was the 4-way interaction involving group, which would support the statement of differential conditioning among the groups.

A separate one-way ANOVA on just the CS+ data revealed a significant groups effect, $F\ (2,13) = 11.91$, $p < .01$. A follow-up test revealed that the DAGO group showed significantly smaller responses than the Vehicle group. A similar test on CS- data revealed no significant differences.

Day 2 Conditioning in LC Groups. Figure 26 displays the mean HR responses of each LC group during three 2-s CS measurement intervals averaged over successive blocks of three trials on Day 2. It may be noted in this figure that the CRs acquired on Day 1 by the Muscarine and Vehicle groups persisted on Day 2. The DAGO group, on the other hand, continued to show little differential responding to CS+ and CS- during the first trial block but by the second trial block did show more bradycardia to CS+ than to CS- suggesting the begining of a small CR to the CS+. A 3 x 2 x 2 x 3 (Groups x Trial Blocks x CS Type x Measurement Intervals) ANOVA produced a significant groups x CS type x measurement intervals interaction, $F\ (4,26) = 8.0$, $p < .01$. While CS- responding was not found to be significantly different among the groups, a follow-up 3 x 3 (Groups x Measurement Intervals) ANOVA on CS+ data provided a groups x measurement intervals interaction, $F\ (4,26) = 4.37$. The DAGO group was found to be significantly

Figure 26. Heart rate responses during three 2-s CS measurement intervals for each of the LC injected groups to CS+ and to CS- averaged over two blocks of three trials on Day 2 of conditioning. Responses are plotted as CS minus pre-CS difference scores.


DAGO
Muscarine
Vehicle
MEAN CHANGE IN HR BPM
10
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
Trials
7−9
10−12
● CS+
○ CS−
1 2 3
measurement intervals during CS+ and CS−

suppressed relative to the Vehicle group during measurement intervals 2 and 3, $p < .05$).

A separate 2 x 2 x 3 (Trial Blocks x CS Type x Measurement Intervals) ANOVA on just the LC-DAGO group provided a significant three-way interaction suggesting the growth of some differential responding between CS+ and CS-, F (2,10) = 14.4, $p < .01$. However, as will be noted below, this conditioning effect was very transitory.

Post-Conditioning CS Test in LC Groups. Figure 27 shows the HR responses of each group to CS+ and to CS- averaged over the four CS-alone test trials. The small CR-like bradycardia seen in the DAGO group at the end of conditioning on Day 2 (see Figure 26) was no longer evident when this group was tested 5 min later with no US present. The Muscarine and Vehicle groups, on the other hand, continued to show major bradycardias to CS+. A 3 x 2 x 3 (Groups x CS Type x Measurement Intervals) ANOVA provided a significant groups effect, F (2,13) = 5.54, $p < .05$, a significant groups x CS type interaction, F (2,13) = 16.30, $p < .01$, and a groups x CS type x measurement intervals interaction, F (4,26) = 4.48, $p < .01$. A follow-up 3 x 3 (Groups x Measurement Intervals) ANOVA on CS+ data produced a significant groups effect, F (2,13) = 18.63, $p < .01$, and a significant groups x measurement intervals interaction, F (4,26) = 4.2, $p < .01$. Responding to CS+ in the DAGO group was significantly suppressed relative to the other groups during measurement intervals 2 and 3, $p < .05$. No significant differences were found in CS- responding.

Non-Drug Test in LC Groups. Figure 28 shows the HR responding of each group collapsed across the 6 non-drug CS-alone test trials given 48 hours following Day 2 conditioning. The HR responses of the DAGO group to

Figure 27. Heart rate responses during three 2-s CS measurement intervals for each of the LC injected groups to CS+ and to CS- averaged over four trials during the CS test which occurred following conditioning on Day 2. Responses are plotted as CS minus pre-CS heart rate difference scores.

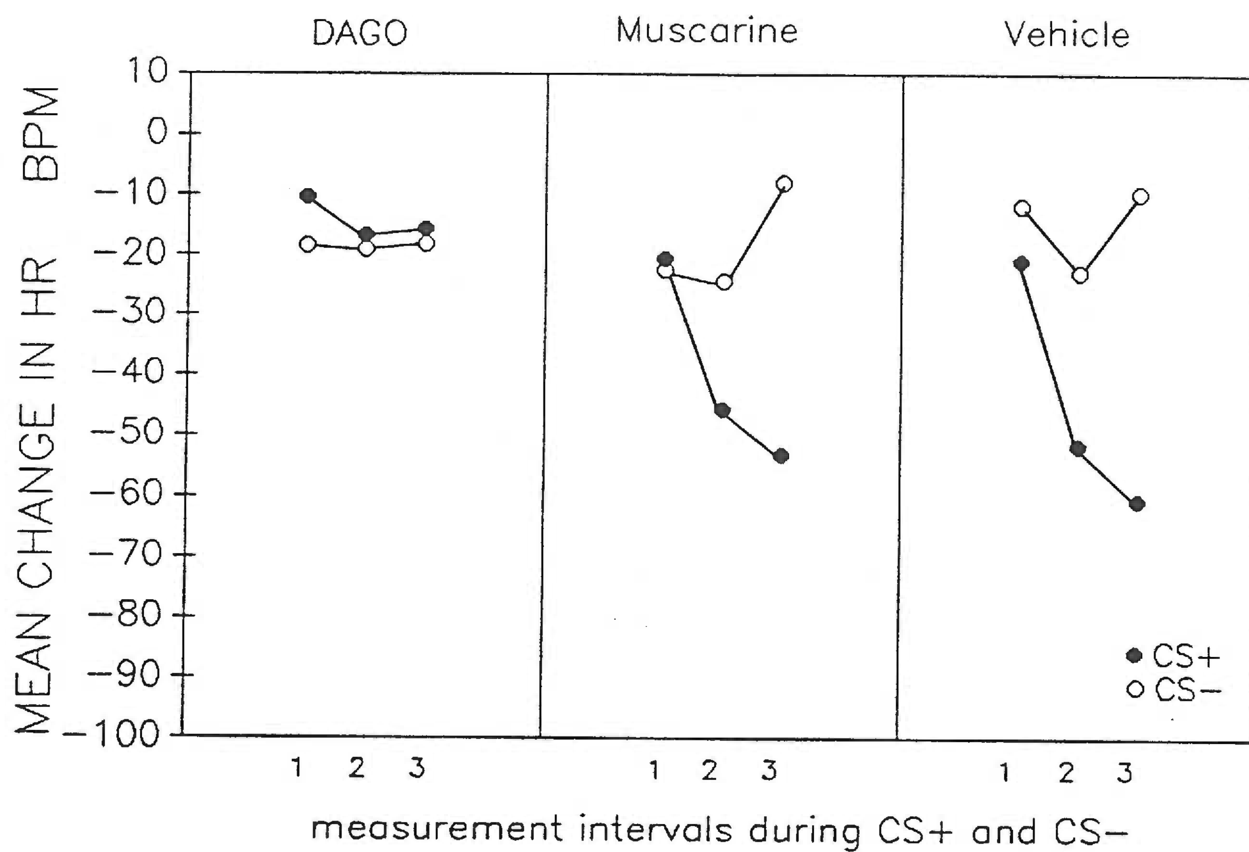
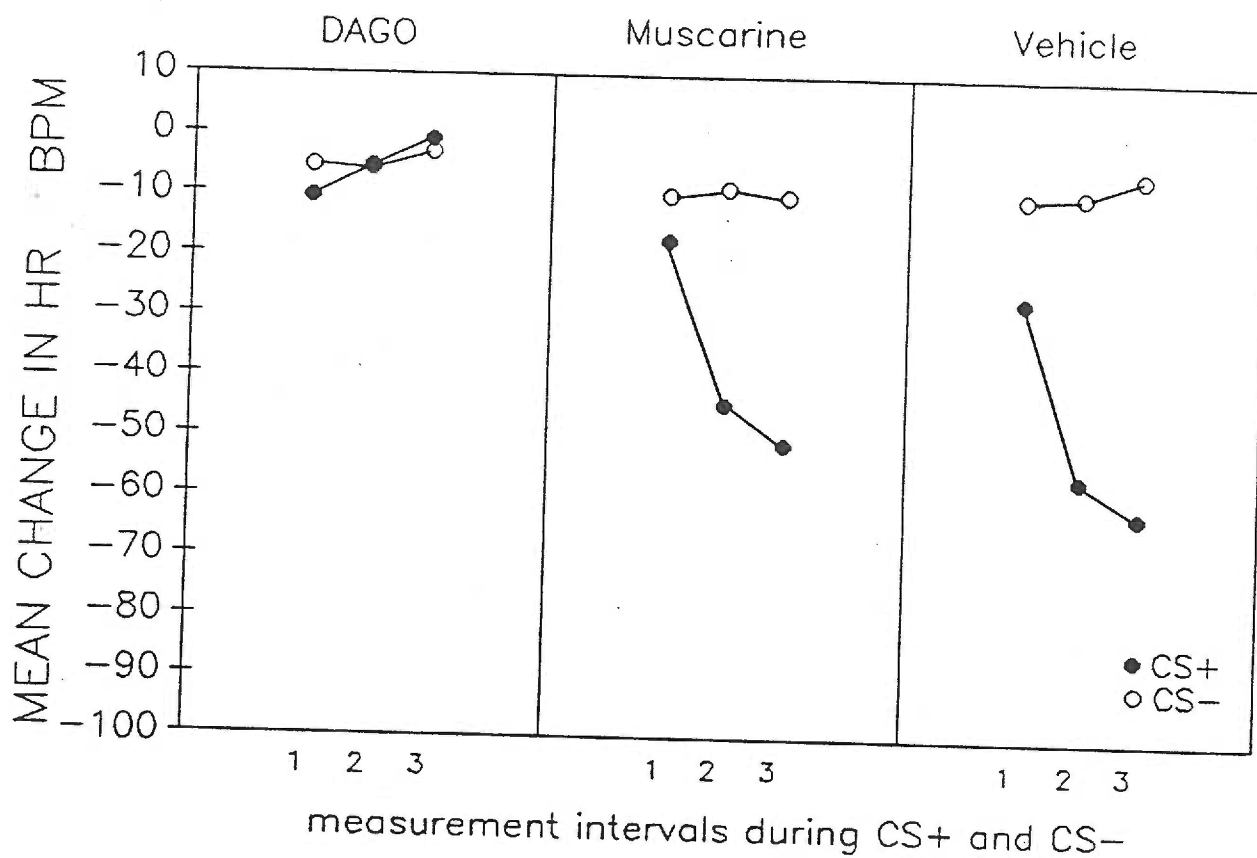

DAGO
Muscarine
Vehicle
MEAN CHANGE IN HR BPM
10
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
1 2 3
1 2 3
1 2 3
CS+
CS−
measurement intervals during CS+ and CS−

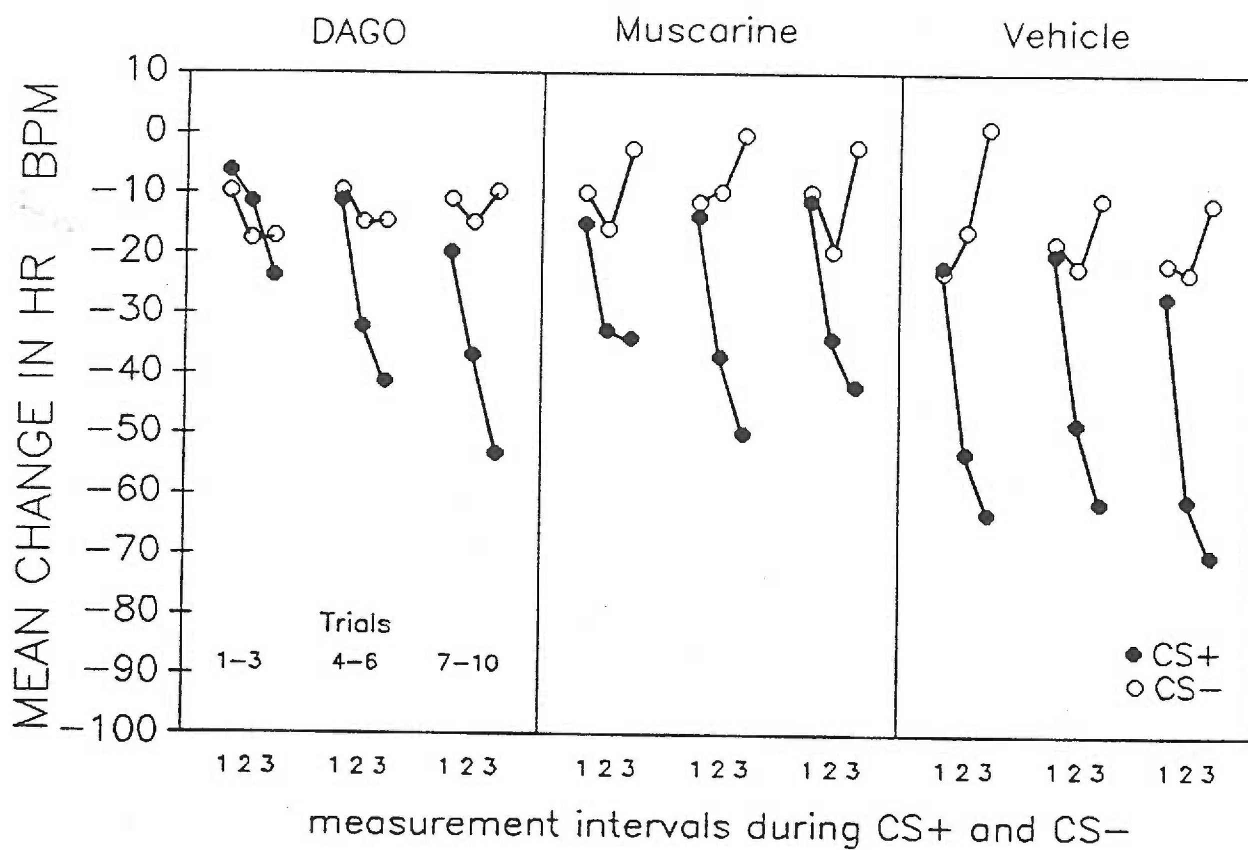
Figure 28. Heart rate responses during three 2-s CS measurement intervals for each of the LC injected groups to CS+ and to CS- averaged over six trials during the non-drug test. Responses are plotted as CS minus pre-CS heart rate differences scores.


DAGO
Muscarine
Vehicle
MEAN CHANGE IN HR BPM
10
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
1 2 3
1 2 3
1 2 3
CS+
CS−
measurement intervals during CS+ and CS−

CS+ were very small and indistiguishible from CS- responding, while the Muscarine and Vehicle groups continued to show large HR CRs to CS+. A 3 x 2 x 3 (Groups x CS Type x Measurement Intervals) ANOVA indicated a significant groups effect, F (2,13) = 16.34, $p < .01$, a significant groups x CS type interaction, F (2,13) = 32.36, $p < .01$, and a significant groups x CS type x measurement intervals interaction, F (4,26) = 12.36, $p < .01$. A follow-up 3 x 3 (Groups x Measurement Intervals) ANOVA on CS+ data affirmed a significant groups effect, F (2,13) = 104.64, $p < .01$, and a significant groups x measurement intervals interaction, F (4,26) = 13.78, $p < .01$. Responding to CS+ in the DAGO group was found to be significantly suppressed during all three measurement intervals, $p < .05$). No group differences were found in CS- responding.

Non-Drug Reconditioning Phase in LC Groups. Figure 29 depicts the HR CRs of each LC group averaged over trial blocks 1-3, 4-6, and 7-10 of reconditioning. As can be seen from this figure, the DAGO group shows the development over trials of a CR to the CS+. A 3 x 3 x 2 x 3 (Groups x Trial Blocks x CS Type x Measurement Intervals) ANOVA established a significant groups effect, F (2,13) = 5.23, $p < .05$, a significant groups x CS type interaction, F (2,13) = 4.25, $p < .05$, a significant groups x CS type x measurement intervals interaction, F (4,26) = 2.92, $p < .05$, and a significant groups x trial blocks x CS type interaction, F (4,26) = 3.82, $p < .05$ reflecting different levels of conditioning among the groups as a function of trials. A follow-up ANOVA on CS+ data revealed a significant groups effect, F (2,13) = 7.59, $p < .01$, and a significant groups x trial blocks interaction, F (4,26) = 3.08. Relative to the Vehicle group, CS+ responding in the DAGO group was found to be significantly suppressed at

Figure 29. Heart rate responses during three 2-s CS measurement intervals for each of the LC injected groups to CS+ and to CS- averaged over trials 1-3, 4-6, and 7-10 during reconditioning. Responses are plotted as CS minus pre-CS heart rate differences scores.


DAGO
Muscarine
Vehicle
MEAN CHANGE IN HR / BPM
10
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
Trials
1−3
4−6
7−10
● CS+
○ CS−
1 2 3
measurement intervals during CS+ and CS−

each trial block, $p < .05$). A similar analysis of CS- data produced no significant group effects.

LC Groups Drug-Test Phase. The responses of each LC group to CS+ and to CS- during three 2-s CS measurement intervals averaged over four CS-alone test trials that were presented following drug administration are shown in Figure 30. This figure illustrates that the CR previously established in the LC-DAGO group during reconditioning was abolished when DAGO was readministered. The CR in the LC-Muscarine group was not changed by muscarine. A 3 x 2 x 3 (Groups x CS Type x Measurement Intervals) ANOVA indicated a significant groups effect, $F(2,13) = 5.88$, $p < .05$, a significant groups x CS type interaction, $F(2,13) = 7.91$, and a significant groups x CS type x measurement interval ANOVA, $F(4,26) = 10.53$, $p < .01$. A follow-up 3 x 3 (Groups x Measurement Intervals) ANOVA on CS+ data revealed a significant groups effect, $F(2,13) = 10.71$, $p < .01$, and a significant groups x measurement intervals interaction, $F(4,26) = 9.0$, $p < .01$. The DAGO group was found to be significantly suppressed during measurement intervals 2 and 3, $p < .05$. There were no significant group differences in CS- responding.

Day 1 Conditioning in PBN Groups. The HR responses of each PBN group during three 2-s CS measurement intervals to CS+ and CS- are shown in Figure 31. In sharp contrast to the decremented CR that occurred when DAGO was placed in the LC, the magnitude of the bradycardia to CS+ in the PBN-DAGO group was actually enhanced by the placement of DAGO in the PBN. A 3 x 2 x 2 x 3 (Groups x Trial Blocks x CS Type x Measurement Intervals) ANOVA yielded a significant groups x CS type interaction, $F(2,13) = 4.51$, $p < .05$, and a significant groups x CS type x measurement intervals interaction,

Figure 30. Heart rate responses during three 2-s CS measurement intervals for each of the LC injected groups to CS+ and to CS- averaged over four trials during the drug test phase. Responses are plotted as CS minus pre-CS heart rate difference scores.

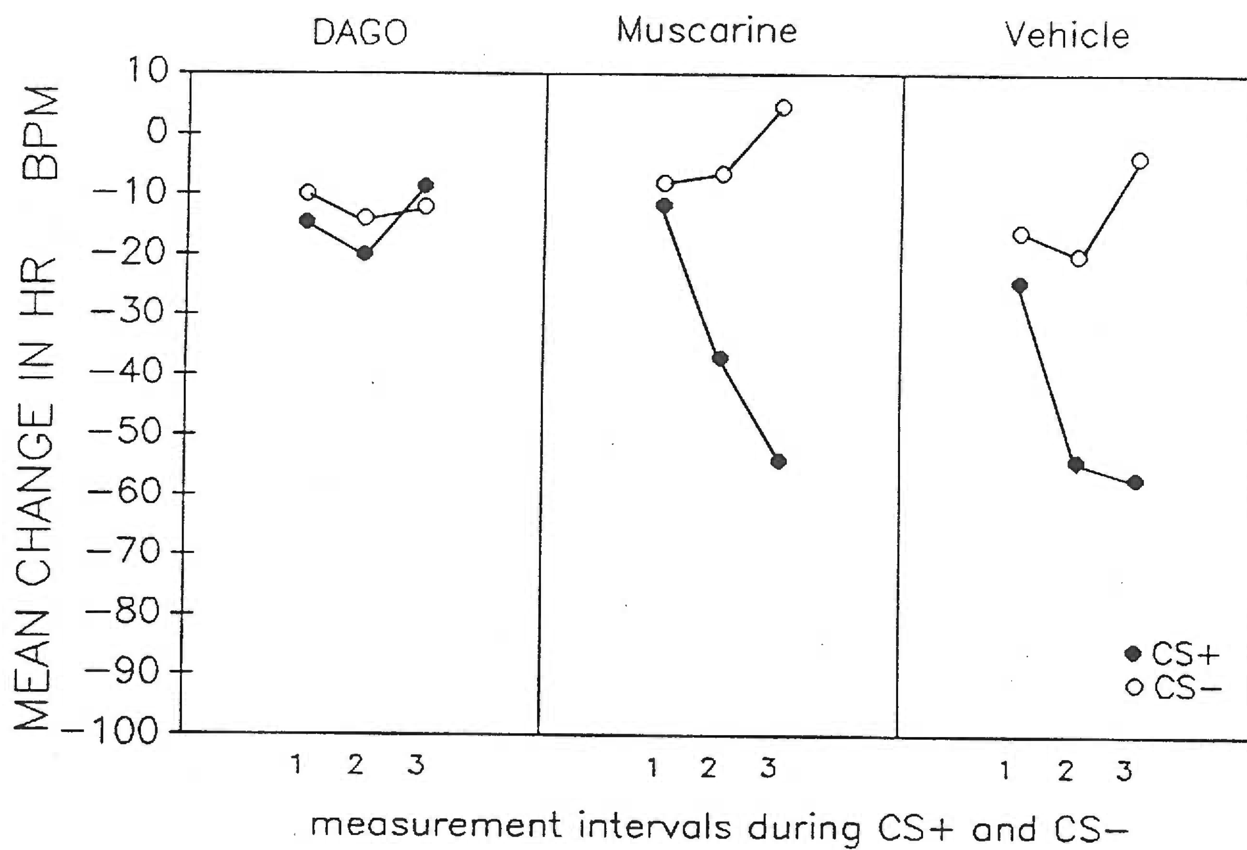
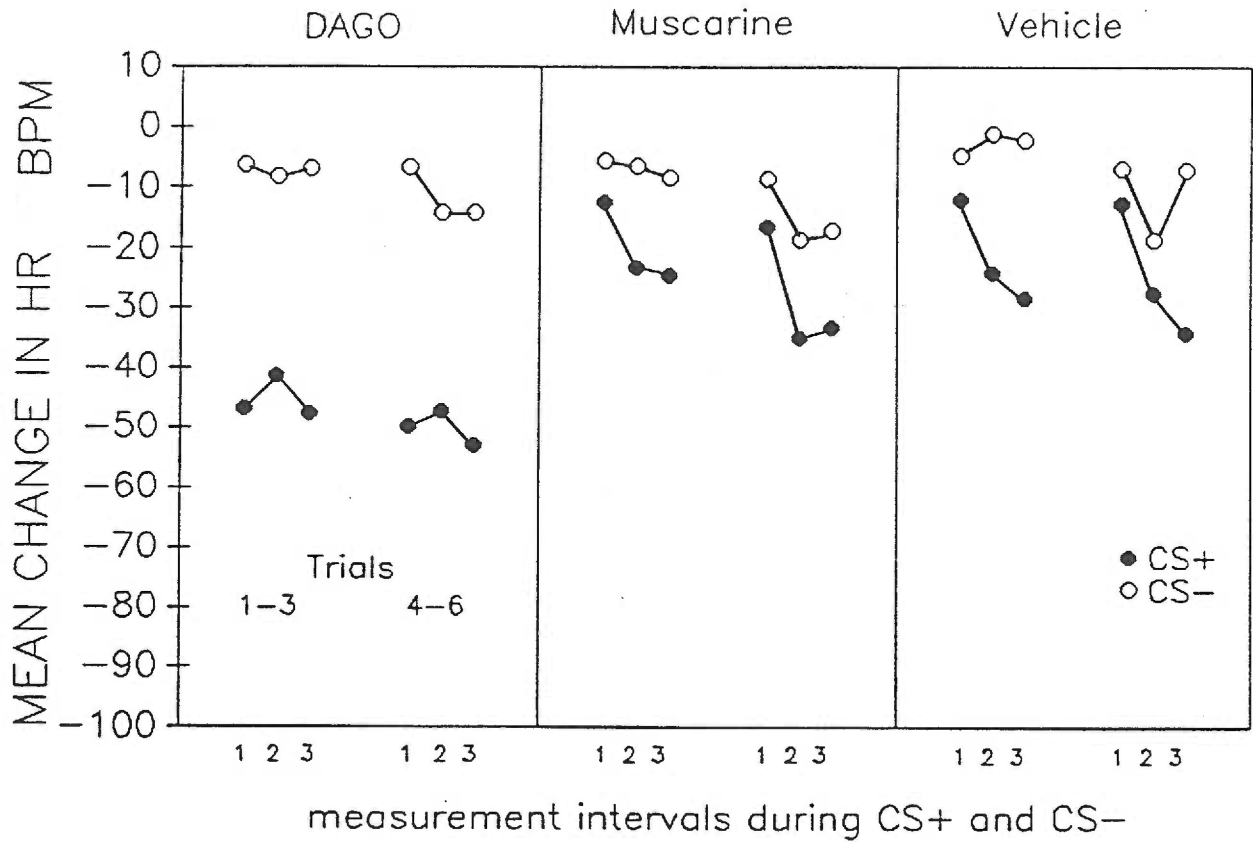

DAGO
Muscarine
Vehicle
MEAN CHANGE IN HR BPM
10
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
1 2 3
1 2 3
1 2 3
CS+
CS−
measurement intervals during CS+ and CS−

Figure 31. Heart rate responses during three 2-s CS measurement intervals for each of the PBN injected groups to CS+ and to CS- averaged over two blocks of three trials on Day 1 of conditioning. Responses are plotted as CS minus pre-CS difference scores.


DAGO
Muscarine
Vehicle
MEAN CHANGE IN HR BPM
10
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
Trials
1−3
4−6
CS+
CS−
1 2 3
measurement intervals during CS+ and CS−

F (4,26) = 2.90, $p < .05$, showing that the level of responding to CS+ and CS- was not the same among the groups. The CS type x trial blocks interaction, reflecting conditioning, was not significant due mainly to the robust bradycardia to CS+ shown by the PBN-DAGO group early in the conditioning session. A follow-up 3 x 3 (Groups x Measurement Interval) ANOVA on CS+ revealed a significant groups effect, F (2,13) = 5.61, $p < .05$, and a groups x measurement intervals interaction, F (4,26) = 2.8, $p < .05$. The bradycardia to CS+ in the DAGO group was found to be greater than that shown by the Vehicle group during measurement interval 1, $p < .05$). No significant differences were seen in CS- responding.

Day 2 Conditioning in PBN Groups. The HR responses of each group to CS+ and to CS- averaged over successive blocks of three trials during three 2-s CS measurement intervals are shown in Figure 32. It may be noted that the PBN-DAGO group continued to show enhanced bradycardia responding to the CS+, relative to the other two groups. In all groups, bradycardia to CS+ was visibly larger on Day 2 than on Day 1. A 3 x 2 x 2 x 3 (Groups x Trial Blocks x CS Type x Measurement Intervals) ANOVA revealed a significant groups x CS type interaction, F (2,13) = 11.37, $p < .01$. Separate one-way ANOVAs on CS+ and CS- data showed that while the groups were not different in CS- responding, overall responding to CS+ in the DAGO group was significantly enhanced; significant groups effect, F (2,13) = 5.86, $p < .05$), follow-up test, $p < .05$ compared to Vehicle group.

Post-Conditioning CS Test in PBN Groups. Heart rate responses for each group averaged over the four CS-alone trials given 5 min after conditioning are shown in Figure 33. As can be seen in this figure, although the bradycardia to CS+ in the PBN-DAGO group remains elevated, a 3

Figure 32. Heart rate responses during three 2-s CS measurement intervals for each of the PBN injected groups to CS+ and to CS- averaged over two blocks of three trials on Day 2 of conditioning. Responses are plotted as CS minus pre-CS differences scores.

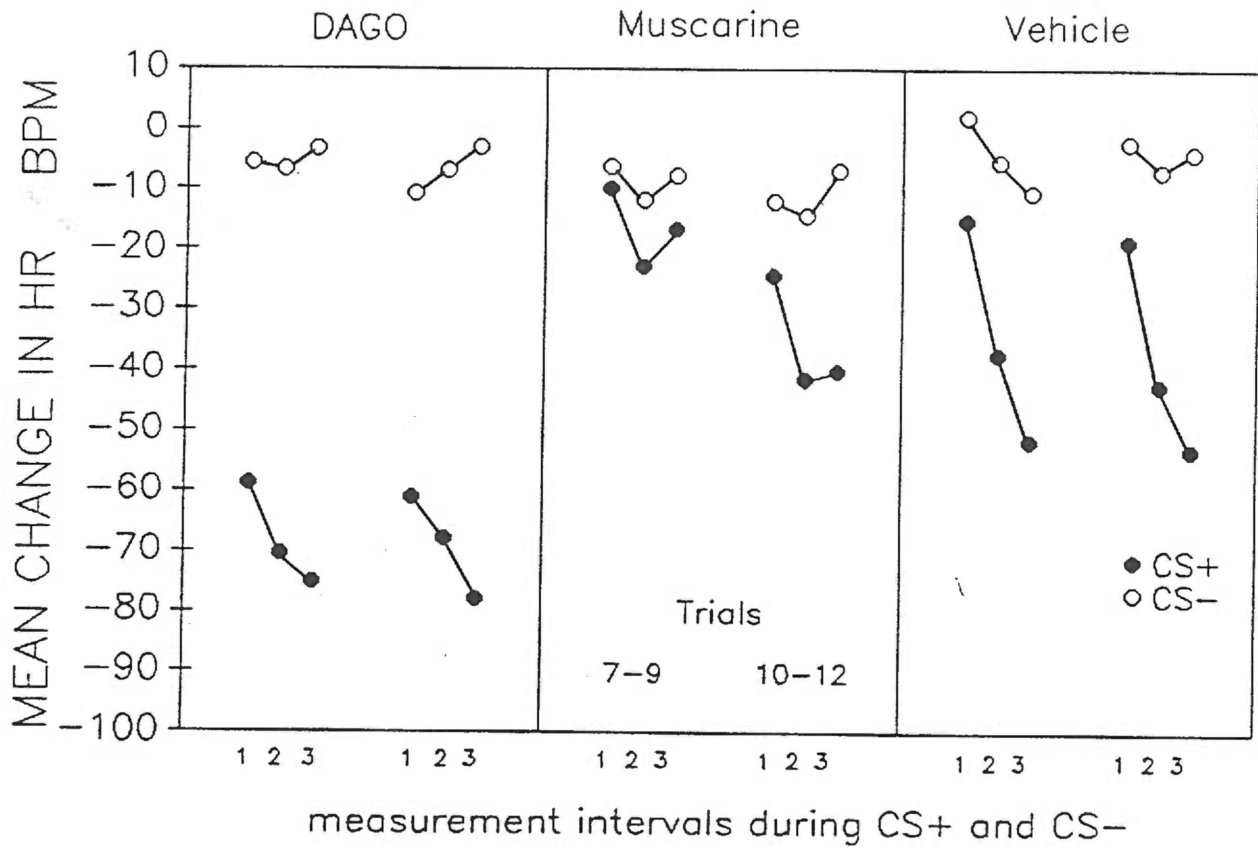
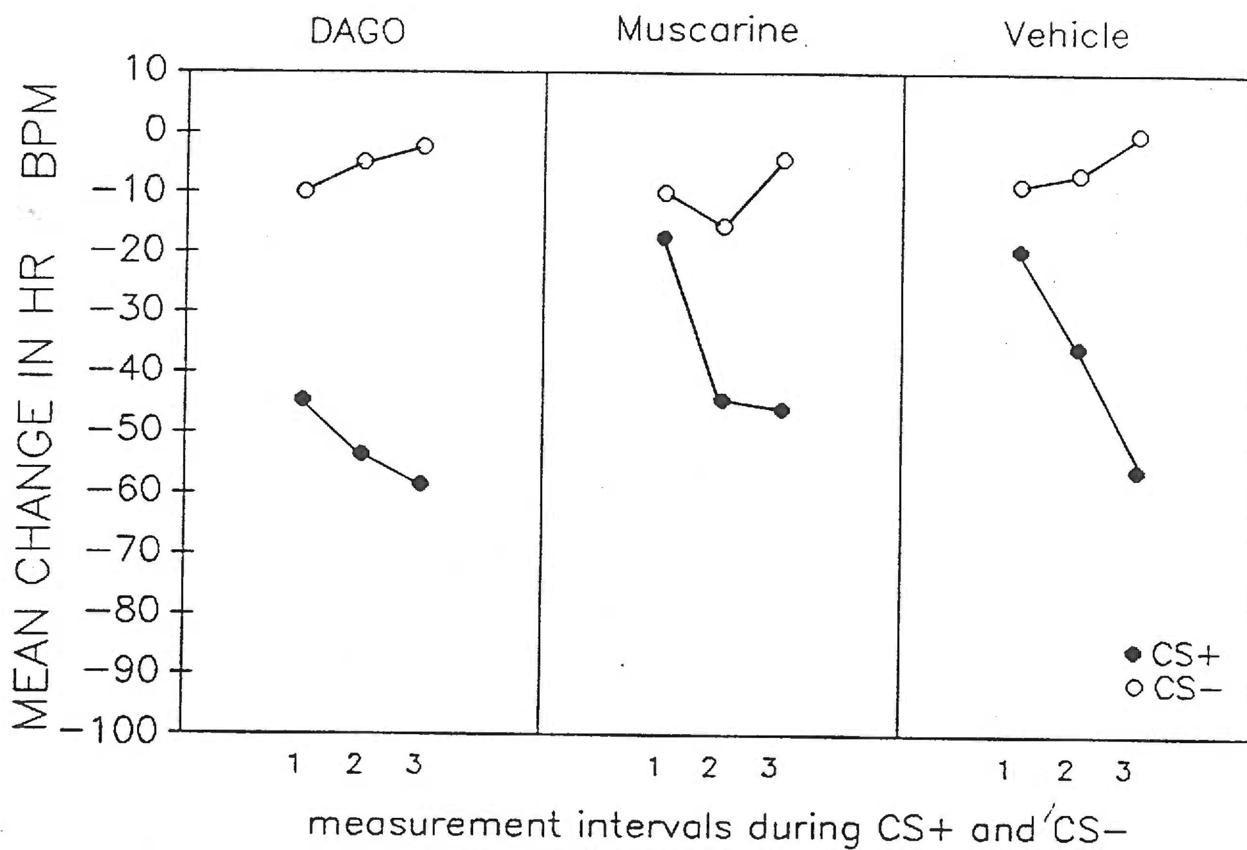

DAGO
Muscarine
Vehicle
MEAN CHANGE IN HR BPM
10
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
Trials
7−9
10−12
CS+
CS−
1 2 3
measurement intervals during CS+ and CS−

Figure 33. Heart rate responses during three 2-s CS measurement intervals for each of the PBN injected groups to CS+ and to CS- averaged over four trials during the CS test which occurred at the end of conditioning on Day 2. Responses are plotted as CS minus pre-CS heart rate difference scores.


DAGO
Muscarine
Vehicle
MEAN CHANGE IN HR BPM
10
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
1 2 3
1 2 3
1 2 3
CS+
CS−
measurement intervals during CS+ and CS−

x 2 x 3 (Groups x CS Type x Measurement Intervals) ANOVA, indicated that there were no significant group differences during this phase of the experiment. Contributing to the absence of a significant groups effect here was the increase in bradycardia to CS+ shown by the Muscarine and Vehicle groups relative to Day 2, and the slight loss of bradycardia in the PBN-DAGO group.

Non-Drug Test Phase in PBN Groups. Heart rate responses to CS+ and to CS- averaged over the six post-conditioning non-drug test trials are shown in Figure 34. During this test, all groups showed HR CRs to CS+ that were of comparable magnitude. The enhanced CR that was evident in the PBN-DAGO group during conditioning on Days 1 and 2 in the presence of DAGO is clearly absent in the non-drug state. A 3 x 2 x 3 (Groups x CS Type x Measurement Intervals) ANOVA supported the fact that there were no significant group differences.

Non-Drug Reconditioning Phase in PBN Groups. Figure 35 shows the mean HR responses for the PBN groups during the reconditioning phase. As can be seen in this figure, all groups showed a similar level bradycardia to CS+. A 3 x 3 x 2 x 3 (Groups x Trial Blocks x CS Type x Measurement Intervals) ANOVA produced no significant group differences.

Drug Test Phase in PBN Groups. The HR responses of the PBN groups following drug injections are shown in figure 36. It can be noted from this figure that DAGO administration into the PBN slightly increased the HR CR, although the magnitude of this effect was smaller than when the drug was present during conditioning. A 3 x 2 x 3 (Groups x CS Type x Measurement Intervals) ANOVA indicated there were no significant group effects. A separate ANOVA on just the PBN-DAGO group comparing the end of

Figure 34. Heart rate responses during three 2-s CS measurement intervals for each of the PBN injected groups to CS+ and to CS- averaged over six trials during the non-drug test. Responses are plotted as CS minus pre-CS heart rate differences scores.

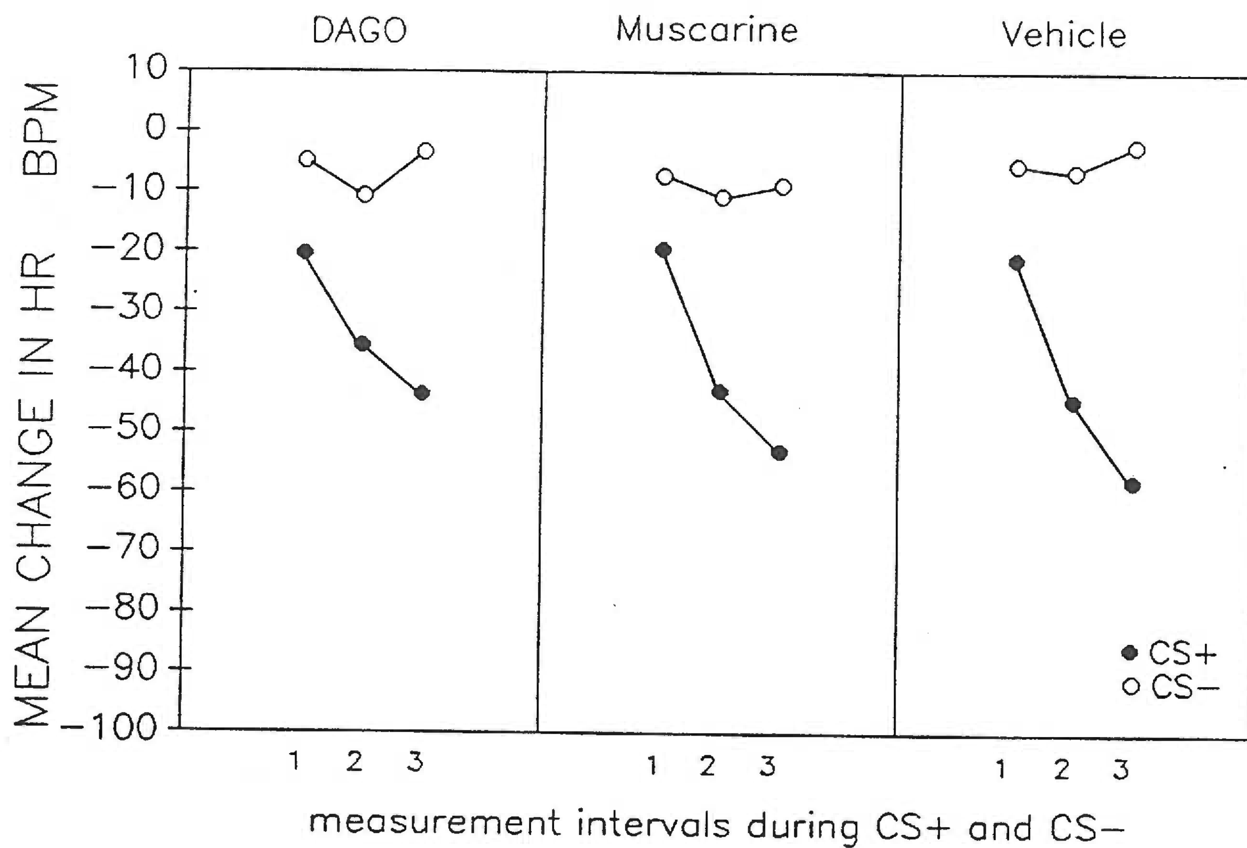

DAGO
Muscarine
Vehicle
MEAN CHANGE IN HR BPM
10
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
1 2 3
1 2 3
1 2 3
● CS+
○ CS−
measurement intervals during CS+ and CS−

Figure 35. Heart rate responses during three 2-s CS measurement intervals for each of the PBN injected groups to CS+ and to CS- averaged over trials 1-3, 4-6, and 7-10 during reconditioning. Responses are plotted as CS minus pre-CS heart rate difference scores.

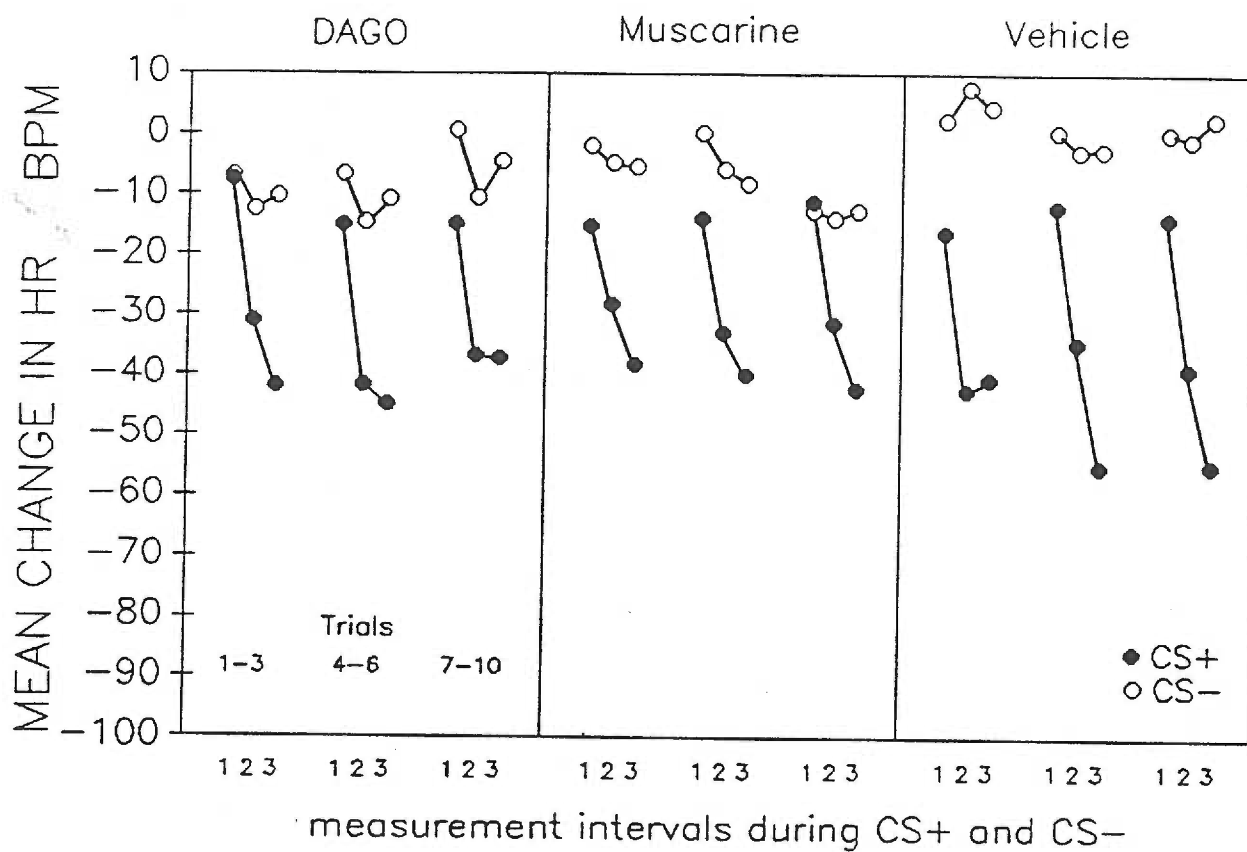
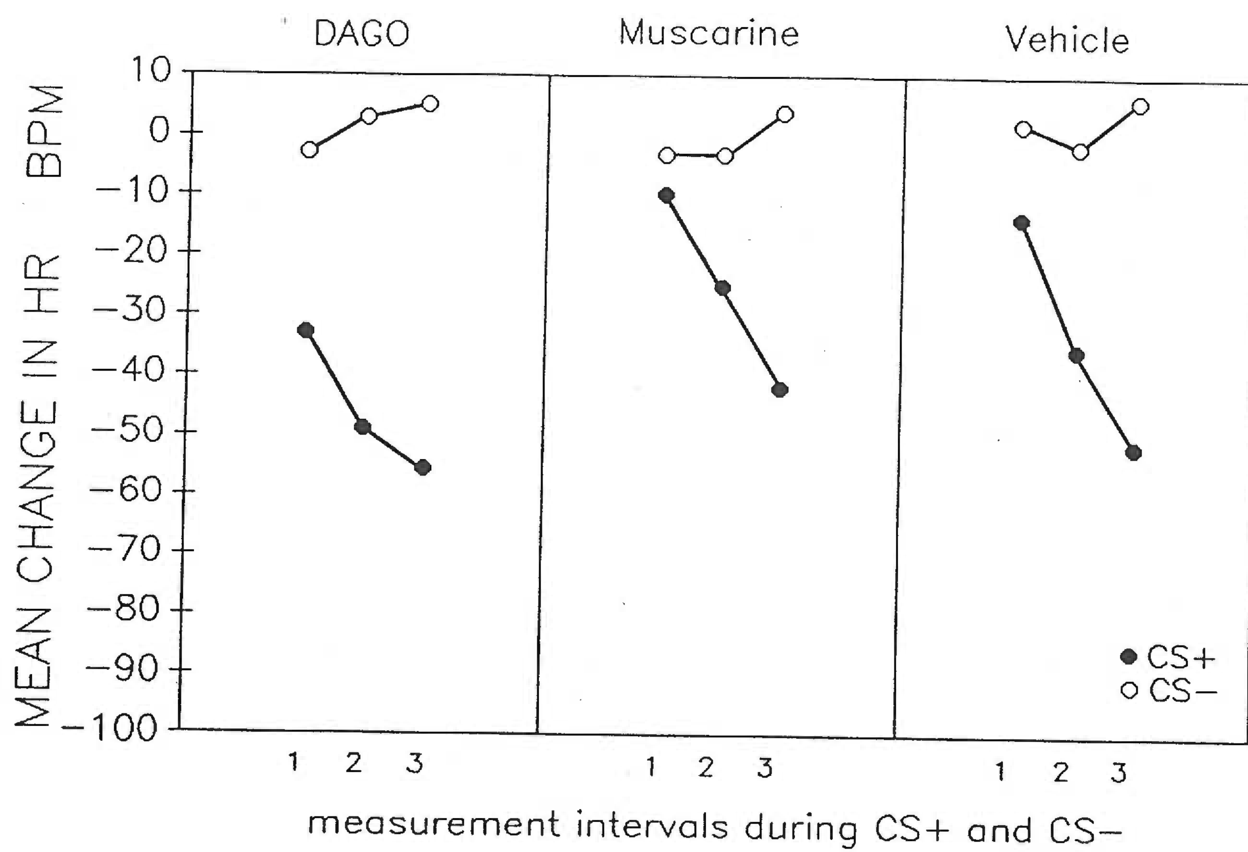

DAGO
Muscarine
Vehicle
MEAN CHANGE IN HR BPM
10
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
Trials
1−3
4−6
7−10
CS+
CS−
1 2 3
1 2 3
1 2 3
1 2 3
1 2 3
1 2 3
1 2 3
1 2 3
1 2 3
measurement intervals during CS+ and CS−

Figure 36. Heart rate responses during three 2-s CS measurement intervals for each of the LC injected groups to CS+ and to CS- averaged over four trials during the drug test phase. Responses are plotted as CS minus pre-CS heart rate difference scores.


DAGO
Muscarine
Vehicle
MEAN CHANGE IN HR BPM
10
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
1 2 3
1 2 3
1 2 3
● CS+
○ CS−
measurement intervals during CS+ and CS−

reconditioning (trials 7-10, Figure 36) with the drug-test responses gave a significant phase x CS type interaction, $F\ (1,5) = 10.42$, $p < .05$. A follow-up test established that CS+ responding during the drug test phase was significantly enhanced by DAGO administration relative to reconditioning, $p < .05$.

Unconditioned Responses

The HR URs of each of the LC injected groups to the shock US averaged over trials for Days 1 and 2 of conditioning and reconditioning are shown in Figure 37. It can be seen from this graph that all LC groups exhibited a consistent tachycardia UR to the US. A 3 x 2 (Groups x Trial Blocks) ANOVA on Day 1 revealed a significant groups effect, $F\ (2,13) = 14.0$, $p < .01$ (LC-DAGO responding was significantly less than the other groups, $p < .05$). A similar analysis on Day 2 also indicated that the LC-DAGO group was significantly supressed only with respect to the Muscarine group ($F\ (2,13) = 4.72$, $p < .05$, DAGO < Muscarine, $p < .05$). An identical analysis of reconditioning data indicated that the URs in the DAGO group were elevated relative to the Vehicle group (significant groups: $F\ (2,13) = 12.3$, $p < .01$, DAGO > Vehicle, $p < .05$).

Figure 38 shows the tachycardia URs of the PBN groups during each day. Separate 3 x 2 (Groups x Trial Blocks) ANOVAs revealed a significant groups effect only on Day 2, $F\ (2,13) = 5.0$, $p < .05$ (DAGO group responding was significantly suppressed relative to the Vehicle group, $p < .05$).

Conditioned Responses: LC vs PBN drug effects

In the above analyses, separate tests of the effects of the drugs were done at the LC and PBN locations. Listed below are the outcomes from analyses (Brain Location x Drug Type x Trial Blocks x CS Type x Measurement

Figure 37. Heart rate responses for each of the LC injected groups averaged over the 6-s post-US period and over six trials during conditioning on Days 1 and 2 and over ten trials during reconditioning. Responses are graphed as post-US minus pre-CS difference scores.

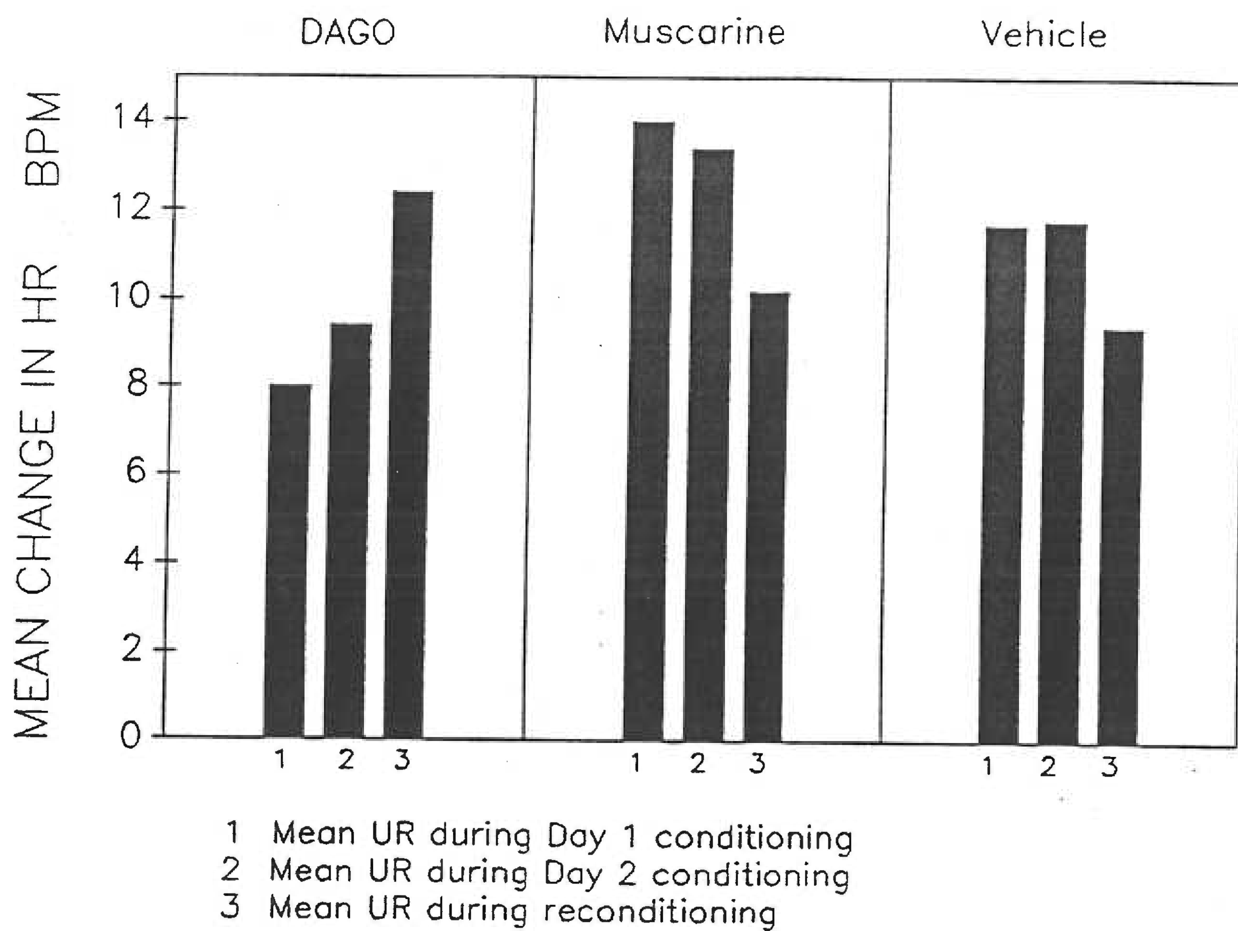
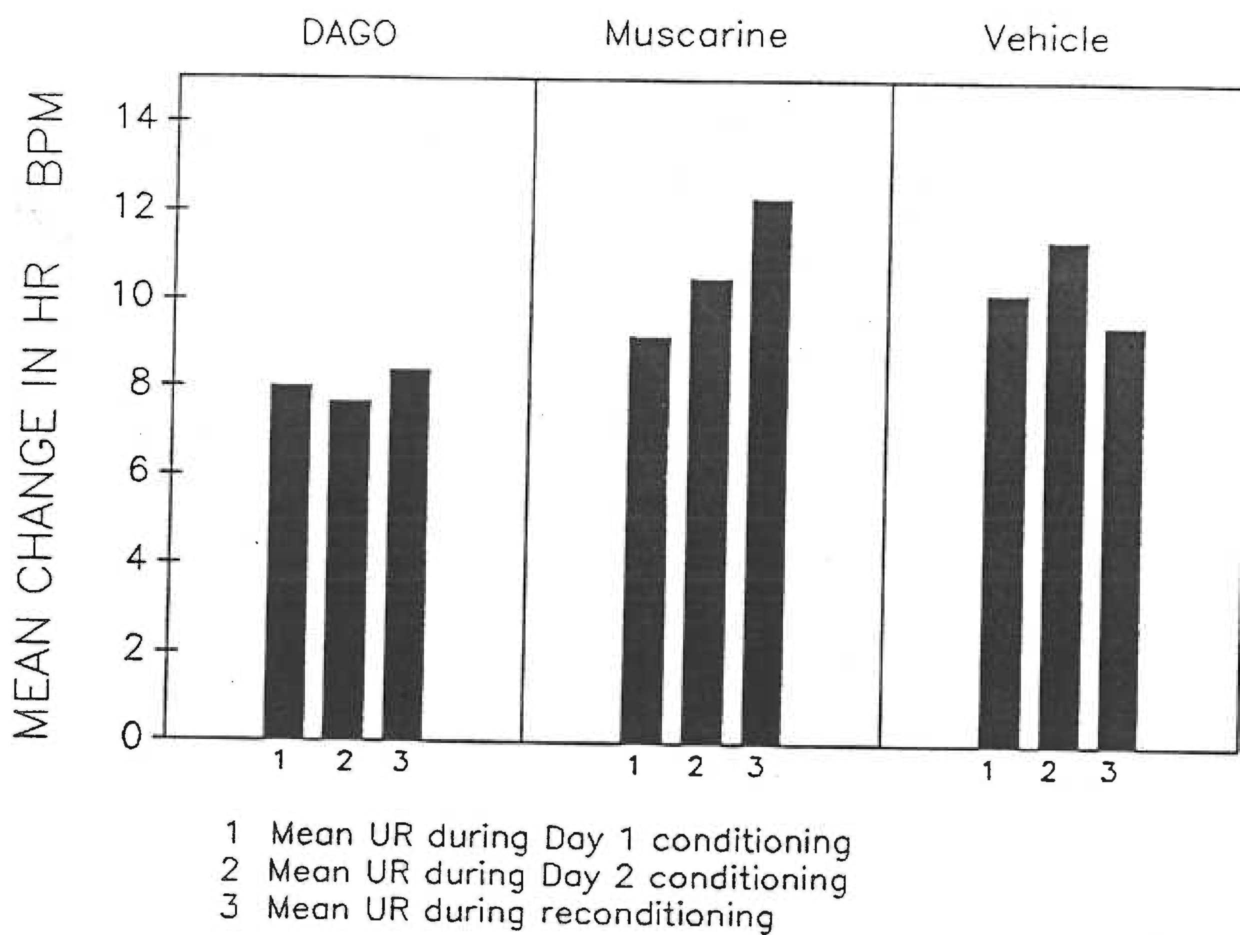

DAGO
Muscarine
Vehicle
MEAN CHANGE IN HR BPM
0
2
4
6
8
10
12
14
1 2 3
1 2 3
1 2 3
1 Mean UR during Day 1 conditioning
2 Mean UR during Day 2 conditioning
3 Mean UR during reconditioning

Figure 38. Heart rate responses for each of the PBN injected groups averaged over the 6-s post-US period and over six trials during conditioning on Days 1 and 2 and over ten trials during reconditioning. Responses are graphed as post-US minus pre-CS difference scores.


DAGO
Muscarine
Vehicle
MEAN CHANGE IN HR BPM
14
12
10
8
6
4
2
0
1 2 3
1 2 3
1 2 3
1 Mean UR during Day 1 conditioning
2 Mean UR during Day 2 conditioning
3 Mean UR during reconditioning

Intervals) comparing drug effects at the two brain locations. Initially, brain location and drug type were between group factors. Given a significant interaction involving these two factors, separate follow-up ANOVA's were used contrasting the effects of each drug at the two brain locations.

Conditioned Responses For DAGO Injected Groups

Day 1. (main 5-way ANOVA interaction: F (2,26) = 7.9, $p < .01$) A follow-up 2 x 2 x 2 x 3 (Brain Location x Trial Blocks x CS Type x Measurement Intervals) ANOVA comparing the LC-DAGO and PBN-DAGO groups produced a significant groups effect, F (1,10) = 15.69, $p < .01$, a significant groups x trial blocks interaction, F (1,10) = 10.76, $p < .01$, and a significant groups x CS type interaction, F (1,10) = 63.6, $p < .01$. A follow-up test revealed that the LC-DAGO group was significantly suppressed in its responding to CS+ but not to CS-, relative to the PBN-DAGO group, $p < .05$.

Day 2. (main 5-way ANOVA interaction: F (2,26) = 11.66, $p < .01$) A 3 x 2 x 2 x 3 (Groups x Trial Blocks x CS Type x Measurement Intervals) ANOVA on the two DAGO groups indicated a significant groups x CS type interaction, F (1,10) = 33.2, $p < .01$, and a significant groups x CS type x measurement intervals interaction, F (2,20) = 3.85, $p < .05$. Follow-up comparisons between the two groups indicated that the LC-DAGO group was significantly suppressed in their responding to CS+ at each measurement interval, $p < .05$. No differences were found in CS- responding.

Post-Conditioning CS Test. (main 4-way ANOVA interaction: F (2,26) = 4.9, $p < .05$) A 2 x 2 x 3 (Groups x CS Type x Measurement Intervals) ANOVA on the LC-DAGO and PBN-DAGO groups provided a significant groups effect, F

$(1,10) = 7.37$, $p < .05$, and a significant groups x CS type interaction, F $(1,10) = 31.37$, $p < .01$. A follow-up test affirmed that the LC-DAGO group was significantly suppressed in CS+ responding, $p < .05$. No differences were found in CS- responding.

Non-Drug Test. (main 4-way ANOVA interaction: $F\ (2,26) = 4.7$, $p < .05$) A 2 x 2 x 3 (Groups x CS Type x Measurement Intervals) ANOVA produced a significant groups effect, $F\ (1,10) = 16.0$, $p < .01$, a significant groups x CS type interaction, $F\ (1,10) = 62.55$, and a significant groups x CS type x measurement intervals interaction, $F\ (2,20) = 8.2$, $p < .01$. The LC-DAGO group was found to be significantly suppressed in their responding to CS+ during measurement intervals 2 and 3, $p < .05$. No differences were found in CS- responding.

Non-Drug Reconditioning Phase. (main 5-way ANOVA interaction: F $(2,26) = 4.1$, $p < .05$) A 2 x 3 x 2 x 3 (Groups x Trial Blocks x CS Type x Measurement Intervals) ANOVA provided a significant groups x CS type interaction, $F\ (1,10) = 5.5$, $p < .05$, and a significant groups x trial blocks x CS type x measurement intervals interaction, $F\ (4,40) = 7.0$, $p < .01$. A subsequent follow-up test indicated that the LC DAGO group was suppressed in CS+ responding only during measurement intervals 2 and 3 on trial block 1, $p < .05$.

Drug Test Phase. (main 4-way ANOVA interaction: $F\ (2,26) = 4.7$, $p < .05$) A 2 x 2 x 3 (Groups x CS Type x Measurement Intervals) ANOVA produced a significant groups x CS interaction, $F\ (1,10) = 21.3$, $p < .01$, and a significant groups x CS type x measurement intervals interaction, $F\ (2,20) = 7.4$, $p < .01$. LC-DAGO responding to CS+ was found to be significantly suppressed during all three measurement intervals, $p < .05$.

Conditioned Responses for Muscarine Injected Groups.

Analyses similar to those run on DAGO-injected groups were performed on Muscarine injected groups. No significant Muscarine group differences were found.

Experiments III and IV

Rationale

The findings of Experiment II indicated that the administration of the mu opioid agonist DAGO into the LC decremented the development of an original bradycardia CR and the performance of a recently established CR. The decremental effects on the HR CR of giving DAGO in the LC cannot be explained by the spread of the drug to the neighboring cardiovascular nucleus, the PBN, because injections of DAGO directly into this nucleus actually seemed to enhance the performance of the HR CR. Certainly there was no sign of a CR loss with DAGO present in the PBN. However, despite the clear evidence of the behavioral specificity of the DAGO injections into the the LC as opposed to DAGO injections into the PBN, it is possible that DAGO may have affected brain regions in addition to the LC and the PBN. Also, Experiment II did not establish that DAGO's effects in the LC were in fact mediated by opioid receptor activity. Experiments III and IV were designed to address the location of DAGO effects and opioid receptor involvement in those effects.

Experiment III: Pharmacological Specificity. Microinjections of drugs into brain tissue can cause a number of changes in the neuronal environment that are not specific to the pharmacological actions of the drug (Bozarth, 1987). For example, drug injections can change the pH, osmolarity, and regional ion balance leading to nonspecific activation or inactivation of cells proximal to the injection site. It is important, therefore, to establish that the behavioral effects of a microinjected drug are due to the pharmacological actions of the drug at its receptor site and not due to nonspecific alterations in the cellular environment. Tests for

the pharmacological specificity of DAGO involves pretreating animals with an opiate antagonist such as naltrexone to block the actions of DAGO that are dependent upon the activation of opioid receptors.

In Experiment III, two groups of animals were treated the same as the LC-DAGO and LC-Vehicle groups in the previous experiment except that they were pretreated with the opiate antagonist naltrexone (3 mg/kg, i.p.) prior to being placed into the experimental chambers on two conditioning days. The 3-mg/kg dose of naltrexone was based on pilot data showing that the gross behavioral effects of LC DAGO injections, consisting of a calming and taming of the animals accompanied by a fixed and vacant stare, were completely reversed by 3 mg/kg of naltrexone but not by lower doses. The moderately high dose of naltrexone required to block central DAGO in this experiment may be explained by the fact that local administration of a drug into a specific brain area may lead to the "wash out" of the peripherally administered antagonist, and therefore higher concentrations of the antagonist may be needed to completely block the agonist effects.

Experiment IV: Anatomical Specificity Anatomical specificity relates to the question of whether the behavioral effects of a microinjected drug are due to the activation of neural mechanisms proximal to the site of injection or due to the diffusion of the drug to distal sites of action. Experiment IV focused on functional tests of the spread of DAGO as opposed to physical tests. What is important in this experiment is not how far, or where, DAGO may have spread but whether the concentration of the drug in other sites is behaviorally effective and relevant to the learning of the bradycardia CR. The most likely places for excess DAGO to have diffused is along the pathways that provide the least resistance to solution flow; these

include the cannula tract and areas within the fourth ventricle (Bozarth & Wise, 1984).

Experiment IV encompassed the use of two additional experimental groups to test both of these brain locations. One group was designed to control for drug diffusion up the cannula tract. In this group (PAG-DAGO), the bilateral cannulae were placed in locations identical to the ones in the LC-DAGO group in Experiment II, with the one exception that the injection site was moved to 1-mm above the LC in an area of the periaqueductal gray (PAG). In this case, structures that might have been effected in the LC-DAGO group by the diffusion of drug up the cannula tract and into surrounding tissue were contacted directly by DAGO. The LC itself should not have been affected by the microinjections in the PAG-DAGO group.

The second group was designed to control for diffusion of the drug into the fourth ventricle. In this group (VENT-DAGO) a single cannula was placed into the fourth ventricle slightly caudal to the location of the LC and the same volume and concentration of DAGO used in the LC-DAGO group were given. In this way, the structures that could have been affected by diffusion of DAGO out of the LC and into the ventricle would receive the full dose or at least a higher dose of DAGO.

Methods Experiment III

Subjects

The subjects were 8 male Sprague-Dawley albino rats the same age and weight as those used in Experiment II.

Surgical Procedure

All animals had bilateral cannulae in the LC. The coordinates used for cannulae placement were identical to those used for LC groups in experiment II. All other other surgical procedures were also identical.

Apparatus

The same apparatus was used in Experiment III as was used in Experiment II.

Experimental Procedure

The only difference in the experimental procedure of Experiment III from that of Experiment II was the fact that prior to being placed in the plastic restrainers on Days 1 and 2 of conditioning the animals were given i.p. injections of naltrexone (3 mg/kg). The experimental procedure after the naltrexone injections was identical to that shown in Table 3. The animals were randomly divided into 2 groups and received microinjections (0.5 ul) of either DAGO (1.6 uM) (NAL-DAGO) or the artificial CSF solution (NAL-Vehicle). On Day 4 of the experiment, the groups were not pretreated with naltrexone. This was done to allow an evaluation to be made of the effects of DAGO on the established CRs in the NAL-DAGO group during the drug test phase. All other aspects of this experiment such as histology and data analyses were identical to those used in Experiment II.

Results/Discussion

Histology

Examination of injection sites resulted in the selection of four animals within each group. A total of three animals were discarded because of misplaced cannulae, two from the NAL-DAGO group and one from the NAL-Vehicle group. Figure 39 depicts the location of the injection sites for each individual animal in both groups.

Baseline Heart Rate

A 2 x 5 (Groups x Trial Blocks) ANOVA on HR baselines of each day of the experiment produced no significant effects. The mean BPM baseline HR for the NAL-Vehicle and NAL-DAGO groups on Day 1 of conditioning was 474 and 442, respectively. On Day 2 the baselines were 458 and 457, and on Day 4 460 and 445. These outcomes are consistent with the absence of a downward shift in baseline HR shown by LC-DAGO group in Experiemnt II.

Orienting Responses

A 2 x 2 x 3 (Groups x CS Type x Measurement Intervals) ANOVA provided a significant CS type effect, $F\ (1,6) = 32.9$, $p < .01$. No significant group differences were found. The mean BPM ORs to CS+ and CS- for the NAL-Vehicle group were -56.0 and -29, while those for the NAL-DAGO group were -51 and -28, respectively. Here, naloxone eliminated the OR suppression by the LC-DAGO group in Experiment II.

Conditioned Responses

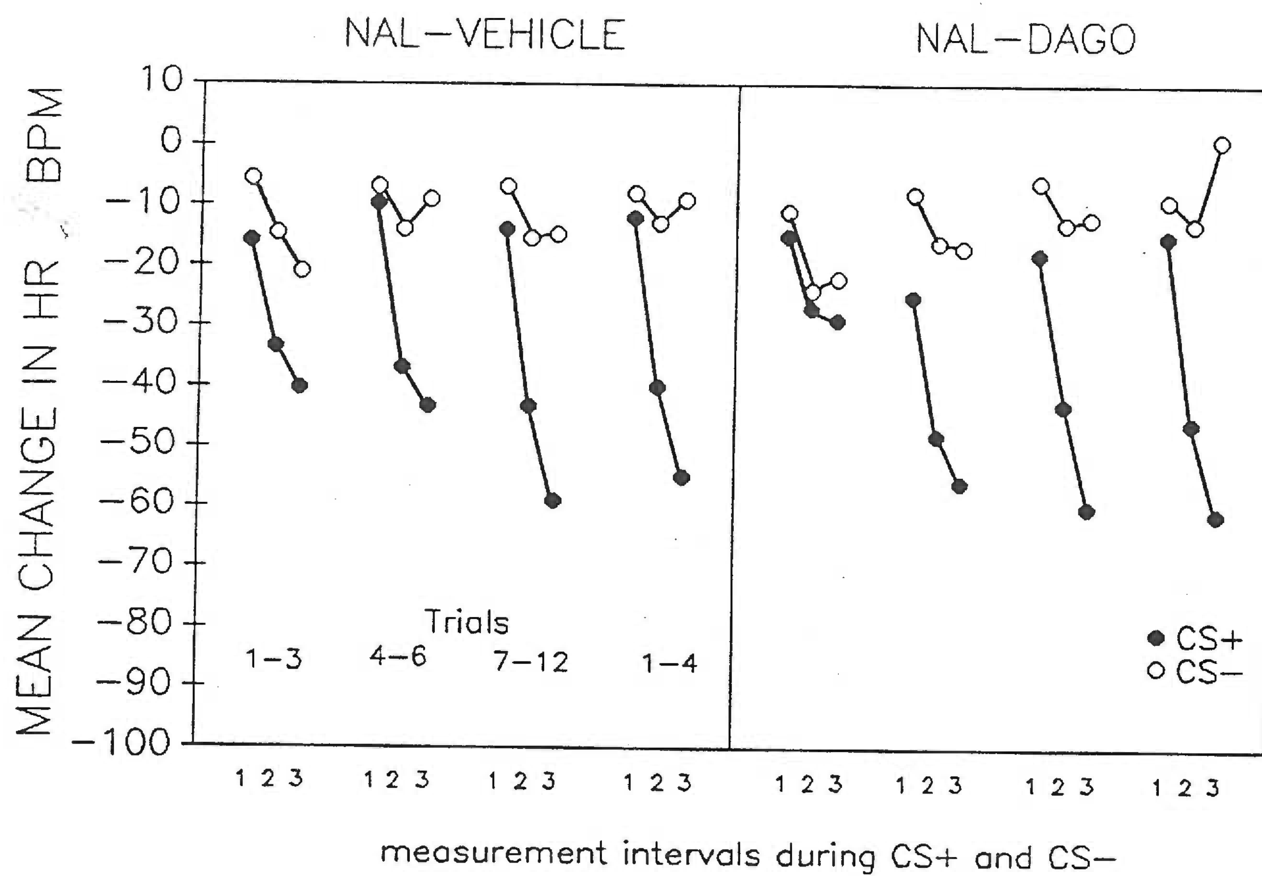
Figure 40 shows the HR responses of each group to CS+ and to CS- during trial blocks 1-3 and 4-6 on Day 1 of conditioning, 7-12 on Day 2 of conditioning, and 1-4 during the CS test which occurred at the end of Day 2. Both groups show the development over trials of a major decelerative CR to

Figure 39. The triangles in this figure depict the location of the infusion sites for individual animals in the NAL-Vehicle group, while the stars represent the individual placements for animals in the NAL-DAGO group.

▲NAL–VEHICLE

* NAL–DAGO

Figure 40. Heart rate responses during three 2-s CS measurement intervals for the NAL-Vehicle and NAL-DAGO groups to CS+ and to CS- averaged over conditioning trials 1-3, 4-6 (Day 1 conditioning), and 7-12 (Day 2 conditioning), and over trials 1-4 of the CS test that occurred at the end of conditioning on Day 2. Responses are plotted as CS minus pre-CS heart rate difference scores.


NAL-VEHICLE
NAL-DAGO
MEAN CHANGE IN HR BPM
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
Trials
1-3
4-6
7-12
1-4
CS+
CS-
1 2 3
measurement intervals during CS+ and CS-

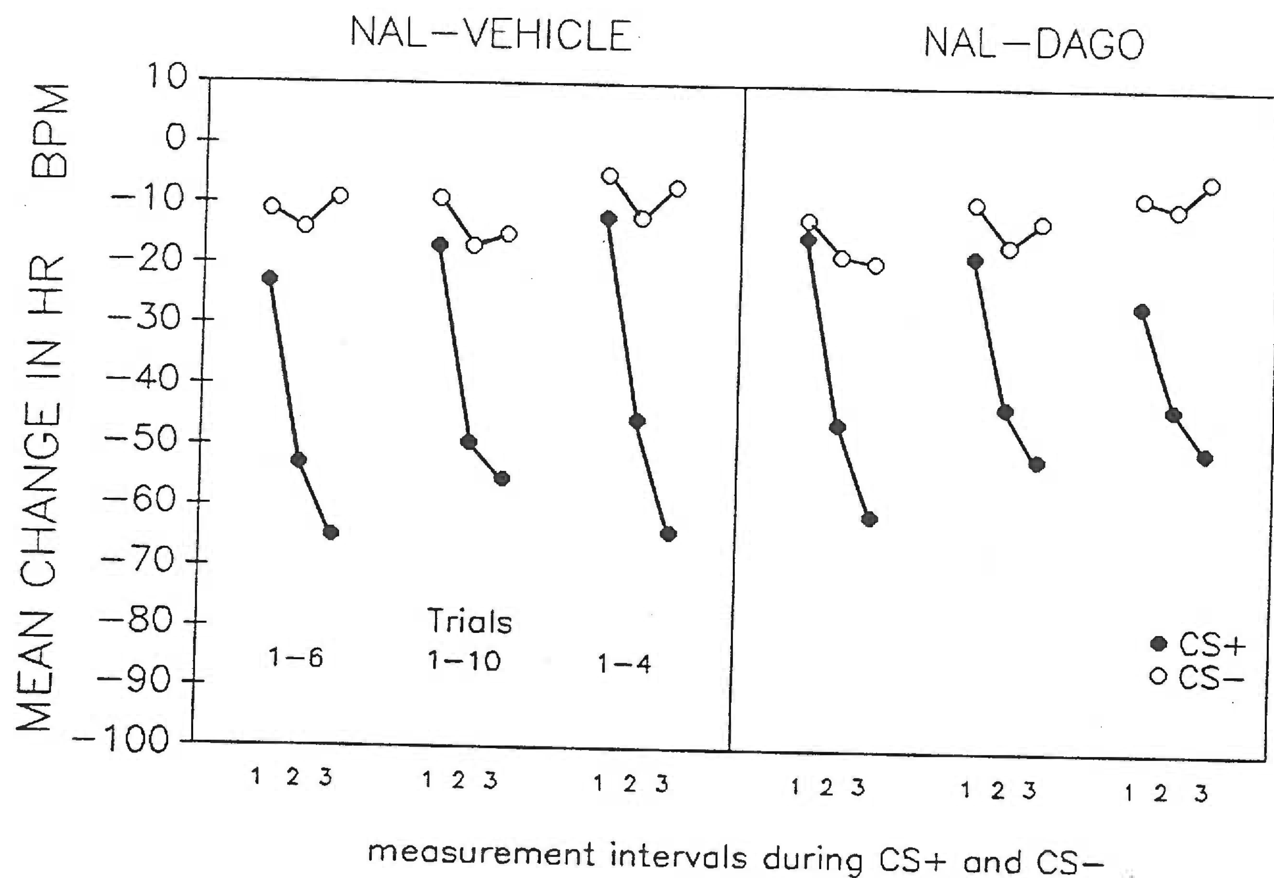
the CS+. A 2 x 2 x 2 x 3 (Groups x Trial Blocks x CS Type x Measurement Intervals) ANOVA run on each day of conditioning revealed no significant group effects. On Day 1 there was a significant trial blocks x CS type x measurement intervals interaction, F (2,12) = 4.41, $p < .05$, indicating that conditioning developed over trials. A 2 x 2 x 3 (Groups x CS Type x Measurement Intervals) ANOVA run on the CS test data also affirmed no significant group differences.

Figure 41 shows the HR responses of both groups to CS+ and to CS- averaged over trials 1-6 of the non-drug test, 1-10 of reconditioning, and 1-4 of the drug test phase. Both groups show comparable levels of HR CRs on this day, and the subsequent administration of DAGO in the NAL-DAGO group during the drug test (trials 1-4) did not produce any noticeable impairment in the HR CR. (Recall that in Experiment II, DAGO placed in the LC following reconditioning did block the performance of the just established HR CR.) An ANOVA run on each individual phase of the experiment on this day produced no significant group effects. These results clearly show that the CR decrement exhibited by the LC-DAGO group in Experiment II was reversed by pretreating with naloxone.

Unconditioned Responses

A 2 x 2 (Groups x Trial Blocks) ANOVA indicated no significant group differences during any phase of the experiment. The mean UR during conditioning on Days 1 and 2 and during reconditioning for the NAL-Vehicle group was +10 BPM, while that for the NAL-DAGO group was +9.6 BPM. Although the LC-DAGO group in Experiment II showed some evidence of a slight UR decrement, this effect was completely absent in the NAL-DAGO group.

Figure 41. Heart rate responses during three 2-s CS measurement intervals for the NAL-Vehicle and NAL-DAGO groups to CS+ and to CS- averaged over non-drug test trials 1-6, reconditioning trials 1-10, and drug test trials 1-4. Responses are plotted as CS minus pre-CS heart rate difference scores.


NAL-VEHICLE
NAL-DAGO
MEAN CHANGE IN HR BPM
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
Trials
1-6
1-10
1-4
CS+
CS-
1 2 3
measurement intervals during CS+ and CS-

Methods Experiment IV

Subjects

The subjects were 6 male rats of the same strain, age, and weight as those used in the other experiments.

Surgical Procedure

The animals in the PAG group received bilateral cannulae implanted 1 mm above the LC placements used in experiment II, V: -3.2 (ventral to the dura). Animals in the fourth ventricle group received a single cannula (identical to those described in Experiment I) in the fourth ventricle caudal to the LC (1 mm posterior to the placements used in Experiment I, AP: -2.5, posterior to lamda). All other surgical procedures were identical to those previously described.

Apparatus and Experimental Procedure

The apparatus and experimental procedures were identical to those used for the LC-DAGO group in Experiment II (see Table 4). Prior to conditioning on Days 1 and 2, the PAG group (PAG-DAGO) received bilateral 0.5-ul injections of 1.6 uM of DAGO, while the ventricle group (VENT-DAGO) received a single 0.5-ul infusion of 1.6 uM of DAGO.

Histology

The histology procedures for the PAG-DAGO group were identical to those used for the microinjection groups in Experiment II, while those for the VENT-DAGO group were identical to those used for the other ventricle injected groups in Experiment I (i.e., the presence of dye in the ventricle was sufficient to be included in this group).

Data Analysis

Although not shown again, data from the LC-Vehicle group in Experiment

II were included with the data from the PAG-DAGO and VENT-DAGO groups as part of the statistical analyses to provide a normal HR CR baseline for evaluating the potential decrementing effects of DAGO in the two control groups. Analyses of variance similar to those described for the other experiments were performed. Newman-Keuls test were used to compare group means when the overall ANOVA indicated significant group main effects or interactions.

Results

Histology

Examination of brains from animals in the PAG-DAGO and VENT-DAGO groups resulted in the selection of three animals for each group (one animal in the VENT-DAGO group had to be discarded due to cannula misplacement). The localization of injection sites for individual animals in the PAG-DAGO group are depicted in Figure 42.

Baseline Heart Rate

While the VENT-DAGO group showed a slight suppression of baseline HR after drug administration on each day (-60 - -40 BPM from pre-injection baseline) no significant group differences in baseline HR were seen from any 3 x 5 (Groups x Trial Blocks) ANOVAs run on any day.

Orienting Responses

A 3 x 2 x 3 (Groups x CS Type x Measurement Intervals) ANOVA revealed a significant CS type effect, $F\ (1,7) = 225.39$, $p < .01$ reflecting greater bradycardia to CS+ and a significant groups x CS type interaction, $F\ (2,7) = 43.77$, $p < .01$. A follow-up showed that the CS+ OR in the VENT-DAGO group was suppressed relative to the LC-Vehicle group. Mean ORs to CS+ and to CS- were -65 and -28 in the PAG-DAGO group and -37 and -33 in the VENT-DAGO group.

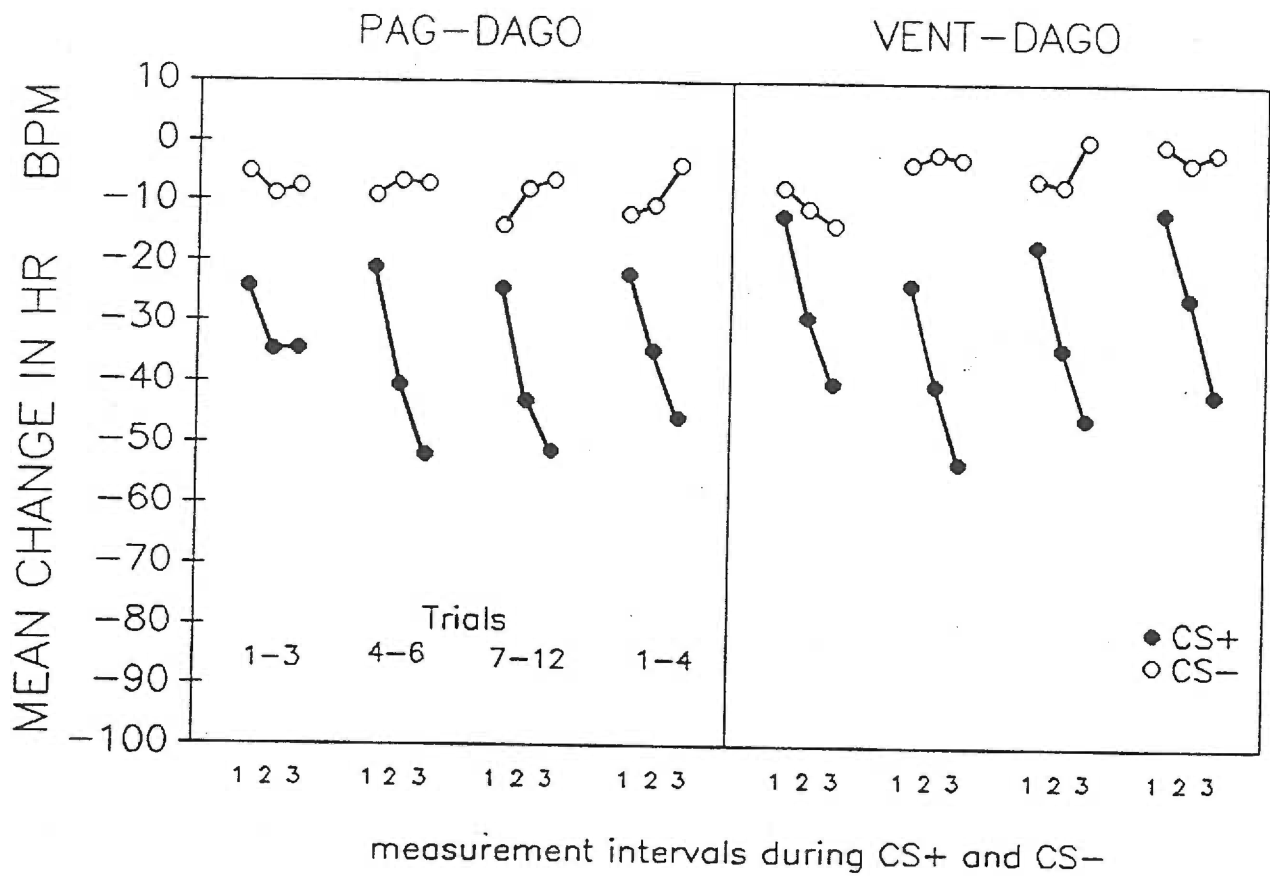
Conditioned Responses

The mean HR responses of the PAG-DAGO and VENT-DAGO groups to CS+ and to CS- during trial blocks 1-3, 4-6, and 7-12 of conditioning and during trials 1-4 of the CS test are shown in Figure 43. Both groups show the development over trials of a HR CR to the CS+ that was highly similar to that shown by the LC-Vehicle group (see Figures 25 and 26).

Figure 42. The diamonds in this figure depict the location of the infusion sites for individual animals in the PAG-DAGO group.

PAG

Figure 43. Heart rate responses during three 2-s measurement intervals for the PAG-DAGO and VENT-DAGO groups to CS+ and to CS- averaged over conditioning trials 1-3, 4-6 (Day 1 conditioning), and 7-12 (Day 2 conditioning), and over trials 1-4 of the CS test that occurred at the end of conditioning on Day 2. Responses are plotted as CS minus pre-CS difference scores.


PAG-DAGO
VENT-DAGO
MEAN CHANGE IN HR BPM
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
Trials
1-3
4-6
7-12
1-4
CS+
CS-
1 2 3
measurement intervals during CS+ and CS-

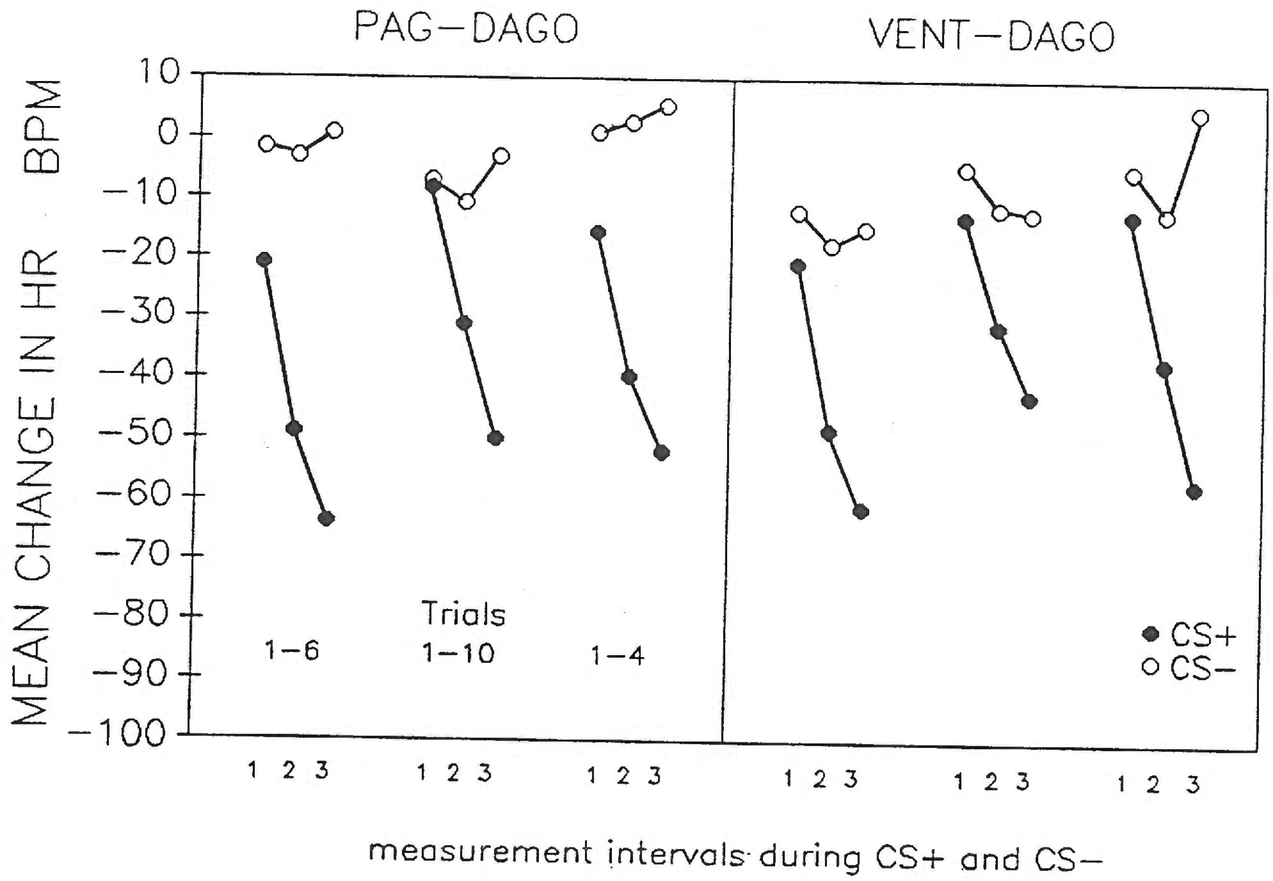
A 3 x 2 x 2 x 3 (Groups x Trial Blocks x CS Type x Measurement Intervals) ANOVA run on each day of conditioning revealed no significant group effects. A 3 x 2 x 3 (Groups x CS Type x Measurement Intervals) ANOVA run on the CS test data also affirmed no significant group differences.

Figure 44 shows the HR responses of the PAG-DAGO and VENT-DAGO groups to CS+ and to CS- averaged over trial blocks 1-6 of the non-drug test, 1-10 of reconditioning, and 1-4 of the drug test. Both groups showed consistent HR CRs throughout each phase on this day. An ANOVA run on each individual phase of the experiment on this day produced no significant group effects.

Unconditioned Responses

A 4 x 2 (Groups x Trial Blocks) ANOVA indicated no significant group differences during any phase of the experiment. The mean UR during conditioning on Days 1 and 2 and during reconditioning for the PAG-DAGO was +9.7 BPM, while in the VENT-DAGO group it was +10.0 BPM.

Figure 44. Heart rate responses during three 2-s CS measurement intervals for the PAG-DAGO and VENT-DAGO groups to CS+ and to CS- averaged over non-drug test trials 1-6, reconditioning trials 1-10, and drug test trials 1-4. Responses are plotted as CS minus pre-CS difference scores.


PAG—DAGO
VENT—DAGO
MEAN CHANGE IN HR BPM
10
0
−10
−20
−30
−40
−50
−60
−70
−80
−90
−100
Trials
1—6
1—10
1—4
● CS+
○ CS−
1 2 3
measurement intervals during CS+ and CS−

Discussion of Experiments II, III, & IV

In keeping with the findings of Experiment I, the present series of studies provides compelling evidence indicating that inhibition of LC output has the capacity to profoundly decrement the process of HR conditioning. The administration of DAGO into the LC produced a naloxone reversible decrement in the development of the bradycardia CR without producing any changes in baseline HR. The administration of DAGO into the LC also severely attenuated a recently-learned HR CR (Experiment II) but had no effects on a well-established CR (Experiment III). By contrast, the infusion of DAGO into a region of the PAG directly above the LC or into the fourth ventricle caudal to the LC had no effects on the development of the HR CR (Experiment IV). Furthermore, DAGO administration into the PBN, very near the LC, actually enhanced the performance of the bradycardia CR while significantly reducing baseline HR. It was noted on the basis of informal unquantified observations that immediately following DAGO infusion the LC animals were visible suppressed in their activity, whereas PBN animals showed an increase in activity. The changes in the behavioral activity level of these two groups of animals could also be observed on the polygraph records following drug infusion. Taken together, these outcomes make it very unlikely that LC injections of DAGO had significant direct effects on brain regions other than the LC.

Muscarine administration into either the LC or PBN had no effects on the HR CR but had opposite effects on baseline HR. In the LC, muscarine significantly elevated baseline HR, while in the PBN it significantly suppressed baseline HR. These differential baseline effects also suggest

Table 5. Summary of the behavioral effects of DAGO and Muscarine administration in the LC and PBN during Experiment II.

Table 5.

HR CR

	Muscarine	DAGO
LC	No change	Decreased
PBN	No change	Increased

Baseline HR

	Muscarine	DAGO
LC	Increased	No change
PBN	Decreased	Decreased

Behavioral Activation

	Muscarine	DAGO
LC	Increased	Decreased
PBN	No change	Increased

that there was little movement of muscarine between the LC and PBN, once it was injected. In addition, it was observed that LC-Muscarine animals exhibited an increase in movement following drug infusion, whereas PBN-Muscarine animals did not. Table 5 depicts the major effects of DAGO and muscarine in the LC and PBN on HR CRs, baseline HR, and general behavioral activity.

The decremental effects of LC DAGO administration on the development of the bradycardia CR was evidenced by the fact that the LC-DAGO group failed to show any consistent differential responding to CS+ on Day 1 of conditioning relative to that shown by the Vehicle control group. Responding to CS+ in this group during the last trial block on Day 2 of conditioning was somewhat larger than responding to CS-, suggesting that some learning may have developed at this point. However, this differential response was no longer present when the animals were tested 5 min later on nonreinforced test trials, suggesting that the CR was very weak. It might be argued that because CS+ responding in the LC-DAGO group was very weak, the CR extinguished rapidly during the CS-alone test trials. To check this possibility individual CS+ test trial responses for each animal in this group were examined. This examination indicated that the CR in the LC-DAGO group was severely decremented on the first CS test trial before any extinction could occur. The fact that the LC-DAGO group showed no noticeable changes in baseline HR and only a minor attenuation of the OR response to CS+ argues against impaired cardiovascular performance and decreased CS sensory processing, repectively, as explaining the CR decrement.

During the non-drug test, given 48 hrs after conditioning on Day 2, the

LC-DAGO group continued to show no CR, providing additional evidence that a major HR CR was not learned earlier. When the animals in the LC-DAGO group were subsequently retrained with no drugs present, they showed the acquisition over trials of a normal decelerative HR CR to the CS+. The presence of a CR at this point indicates that the animals in this group had the capacity to learn and that damage to neural tissue from either the cannula placements or the microinjections were not responsible for the decrement in CR acquisition. The normal rate of acquisition seen in this group during reconditioning suggests that any learning that did occur during the initial training did not carry over in a major way into the retraining phase.

In Experiment III, it was shown that the decremental effects of DAGO in the LC on the learning of the HR CR could be completely reversed by pretreating the animals with the opiate antagonist naltrexone (i.e., the NAL-DAGO group). This indicates that the decremental effects of DAGO administration into the LC was opioid receptor mediated. In addition, Experiment IV showed that the administration of DAGO into a region of the PAG above the LC or into the fourth ventricle caudal to the LC had no effects on the learning of the HR CR, implying that the decremental effects of LC DAGO administration could not be accounted for by the leakage of the drug up the cannula tract or into the ventricle. The outcome of Experiment IV strongly argues for the involvement of the LC/NE system in mediating the CR loss seen in the LC-DAGO group.

The readministration of DAGO after reconditioning in the drug-free state severely diminished the recently acquired CR in the LC-DAGO group (Experiment II) but had no noticeable effects on the well-established CR in

the NAL-DAGO group (Experiment III). This implies that the weaker, recently learned, HR CR in the LC-DAGO group was more susceptible to the attenuating effects of DAGO than was the stronger, well-trained, CR in the NAL-DAGO group. This finding supports several previous reports indicating that opiate administration has very little effect on the performance of well established, overtrained, CRs (Mahalik & Fitzgerald, 1987; Mauk et al., 1983).

The administration of DAGO into the PBN, in contrast to the LC, significantly enhanced the performance of the CR at the time of the initial training (Days 1 and 2), particularly during the first 2-seconds after CS onset. The administration of DAGO into the PBN also decreased baseline HR. The fact that baseline HR was significantly lowered in this group while bradycardia CR performance was augmented, lends further support for the notion that reductions in baseline HR can occur independently of decrements in HR CR performance.

The enhancement of the CR in the PBN-DAGO group led to a significant alteration in the topography of the CR relative to the CRs seen in the PBN-Vehicle group. The bradycardia response to the CS+ in the vehicle injected group was found to gradually increase in magnitude over the 6-second CS+ period and reach a maximum during the last 2 seconds that occurred just before shock (US) onset. In the PBN-DAGO group, on the other hand, a large bradycardia occurred to CS+ onset (i.e., first 2-s of the CS) and was maintained throughout the 6-second CS+ period. This effect of DAGO in enhancing the CR did not carry over into the non-drug state and, therefore, was very likely only an effect on HR CR performance and not learning. The absence of the drug cue during the non-drug test,

alternatively, could have produced a state-dependent decrement in the magnitude of the CR relative to that seen during the initial training. Although, the administration of DAGO in the PBN at the end of reconditioning did not significantly enhance the magnitude of the CR relative to the PBN-Vehicle group it did elevate responding with respect to CR performance of the PBN-DAGO group during the preceding non-drug reconditioning phase.

Previously, the PBN has been proposed to be a part of the pathway involved in the performance of conditioned bradycardia in rabbits (Schneiderman et al., 1987). The PBN has been found to have reciprocal anatomical projections to and from many regions of the forebrain previously implicated in the mediation of the conditioned bradycardia response (Schneiderman et al., 1987). These regions include the ACE, lateral preoptic region, medial forebrain bundle, bed nucleus of stria terminalis, anterior and lateral hypothalamus, and lateral zona incerta. In addition, the PBN projects to and has been shown to activate barosensory-sensitive neurons in the nucleus tractus solitarius and cardioinhibitory vagal preganglionic neurons in the dorsal motor nucleus of the vagus and nucleus ambiguus (Liskowsky, Ellenberger, Haselton, Schneiderman, and Hamilton, 1981). Given these interconnections, opioid administration into the PBN could have enhanced the performance of the predominantly vagally mediated bradycardia CR (Fitzgerald, Martin, & O'Brien, 1973) by increasing vagal outflow.

Although an increased vagal outflow coupled to a decrease in sympathetic outflow could help account for the larger bradycardia CR shown by the PBN-DAGO group, the general behavior of the animals in this group was typical of a state of increased sympathetic outflow. Thus, these animals

showed a great deal of agitation and activity after DAGO administration and in fact seemed hypersensitive to stimulus presentations. On the first day of conditioning, CS+ onset was accompanied by movement artifacts in this group that seemed to reflect a hightened startle response to CS+. What was most unusual about the PBN-DAGO group was the combination of a reduction in baseline HR and enhanced bradycardia CR performance on the one hand, and increased behavioral agitation, and startle-like responses to CS+ onset on the other. Because the PBN serves as a neural interface between limbic structures involved in emotionality and brain stem structures involved in autonomic regulation, it is possible that PBN DAGO administration altered activity in many divergent cell types, thereby leading to these seemingly paradoxical cardiac and behavioral manifestations. The strikingly different effects of DAGO in the LC and PBN on the HR CR, baseline HR, and general activity strongly suggest that DAGO did not diffuse from one nucleus into the other.

On Day 2 of conditioning, the tachycardia URs of the PBN-DAGO group were slightly suppressed relative to the vehicle group. However, this suppression may have been due to the fact that the PBN-DAGO group showed large bradycardia CRs just before the US was given, making it difficult for the URs in this group to reach the same level as in the PBN-Vehicle group. Also, if DAGO did enhance the PBN-DAGO groups CR by increasing vagal outflow then this might be expected to decrease the ability of the sympathetics to produce a large UR.

Like DAGO, muscarine administration in the PBN lowered baseline HR, although the decrease was not as great with muscarine. However, in contrast to DAGO, muscarine did not alter the performance of the HR CR. In addition,

muscarine administration into the PBN did not cause the behavioral agitation or startle-like responses to CS+ onset that were seen in the PBN-DAGO group. Because muscarine and mu opioid receptors have been reported to be co-localized on certain cells of the PBN (Christie & North, 1989), it was expected that DAGO and muscarine would have similar effects when placed in the PBN. The failure of muscarine to produce effects comparable to DAGO both behaviorally and in terms of the CR, might be explained by differences in receptor localization within the PBN. The neurons of the PBN, unlike those in the LC, are quite heterogenous. While there is evidence that a certain subpopulation of PBN cells have co-localized muscarine and mu opioid receptors (Christie & North, 1989) there is no evidence to indicate that all the muscarine and mu opioid receptors in the entire nucleus are co-localized. Furthermore, there could potentially be differences in the localization of these receptor types on the presynaptic nerve terminals that innervate the PBN that could lead to the differences that were seen with the two drugs.

In contrast to the suppression of baseline HR seen in the PBN-Muscarine group, muscarine administration into the LC caused a significant elevation in baseline HR. This was accompanied by increased behavioral agitation, which was not present in the PBN-Muscarine group. The opposing effects of muscarine in the LC and PBN on baseline HR and general behavior provide further support for the position that neither DAGO nor muscarine diffused from the LC into the PBN. Unlike DAGO in the LC, muscarine in the LC did not produce a significant decrement in learning nor did it affect the performance of an established CR. Because muscarine increases tonic LC activity, the level of NE released in LC target areas should also have been

increased by muscarine administration. It might be argued that increasing NE release should facilitate the learning of a response (McGaugh, 1973; Stein, Belluzzi, & Wise, 1975). One possible explanation for the failure of muscarine to enhance the HR CR in the current study could be that when baseline tonic activity in the LC was increased, CS evoked activity was decremented by the decrease in the signal to noise ratio. In support of this notion, it has been shown that intraventricular administration of the cholinergic agonist carbachol increases LC tonic activity, while decreasing sensory evoked responding (Valentino & Aulisi, 1987). The effects of muscarine in the LC should be the same as those found for carbachol, and therefore, muscarine probably reduced CS evoked LC activity. However, the potential impairment of CS processing in the current study was not sufficient to cause a major decrease in the HR CR. The URs in the LC-Muscarine group were somewhat elevated relative to those seen in the LC-DAGO group on both days of conditioning, perhaps reflecting a greater level of arousal in the muscarine animals.

Pilot data collected prior to the start of this experiment indicated that a higher dose of muscarine (500 uM) than was used in this study, when injected into the LC, caused intense emotional reactions. These responses occurred within the first 0.5 s of the injection when a volume of only .05 ul was given, and were characterized by intense and persistent struggling in the restrainer and continous vocalization lasting 5 - 10 min. This observation validates previous reports that drugs which increase tonic LC activity can evoke fear and rage in animals (Redmond & Huang, 1979).

General Discussion

The results of the present series of experiments provide compelling evidence indicating the importance of LC activity during the acquisition of a classically conditioned HR CR. In the present studies, robust learning deficits were shown by animals pretreated with drugs known to inhibit LC output, while other studies (Mason & Fibiger, 1983; Ogren & Fuxe, 1977; Pisa & Fibiger, 1983; Robbins et al., 1982; Robbins et al., 1985) using neurotoxic lesions of the LC system, have failed to find major deficits in learning. These opposing outcomes are difficult to reconcile because in both cases the treatment is associated with decreased NE activity in target areas. It might be that because the LC system is so critical in maintaining cortical arousal, the LC-NE system manages to compensate for the damage induced by neurotoxic lesions by enhancing activity in spared terminals or by allowing other systems to take over its function (Sara, 1985).

Currently, there is a great deal of evidence to support the notion that functional recovery can occur following neurotoxic lesions (Acheson et al., 1980; Harik et al., 1981; U'Prichard et al., 1980) and this could account for the absence of robust and reliable learning deficits in studies using neurotoxic lesions. A clear recognition of the potential for NE activity to recover following LC lesions is needed to properly evaluate the current controversy regarding the role of the LC in learning. It would seem that in order to adequately test the role of the LC in a learning task, LC inhibition should occur just prior to training before compensatory mechanisms can develop. The pharmacological technique of inhibiting LC activity that was used in the current studies meets that criterion.

The administration of DAGO in the LC produced a number of effects similar to those seen using fourth ventricle opioid administration, making it highly likely that these effects were mediated at the LC. These effects included the impairment of the HR CR both during acquisition and shortly after reconditioning, and the depression of behavioral responsiveness. The potent behavioral depressant effects seen here following LC DAGO administration suggest that the LC may mediate some of the reported effects of peripheral opiate administration. These opiate effects occur without a loss of consciousness, or of sensory and motor functioning and encompass drowsiness, inability to concentrate, apathy, and lethargy, in addition, to the well known effect of decreasing the panic, fear, and anxiety associated with pain.

The finding that fourth ventricle opioid administration (Experiment I) resulted in a more severe blockade of HR CR learning than did the direct microinjections of DAGO into the LC (Experiment II) could be attributed to fact that more brain structures would be affected by placing the drug into the ventricle. These additional structures might have contributed in some way to the CR loss. Alternatively, the ventricle route of administration may have resulted in a more complete blockade of LC activity than the microinjections because with microinjections the drug may not have spread to the entire nucleus, leaving some LC cells active during conditioning.

The administration of DAGO into the PBN, in contrast to the LC, produced some effects that were opposite to fourth ventricle opioid administration. For example, DAGO administration in the PBN enhanced rather than decremented HR CR performance, and increased rather than decreased behavioral responsiveness. The major differences found with respect to the

HR CR and behavior, suggest that opioid administration into the ventricle probably did not reach the PBN. Opioid administration into the PBN did, however, decrease baseline HR, as did fourth ventricle infusions. While the similarity of these baseline HR effects could mean that ventricle opioid infusions reached the PBN, there are other cardiovascular nuclei in the caudal fourth ventricle with opioid receptors that could have decreased baseline HR. The fact that fourth ventricle administration of alpha-2 agonists, which are reported to have little or no effects on PBN neurons (Christie & North, 1988), also decremented baseline HR could be explained by alpha-2 binding at cardiovascular nuclei.

While there are a number of ways in which LC DAGO administration could have decremented the HR CR, it seems very unlikely that the failure of a HR CR to appear in the LC-DAGO group (Experiment II) on the non-drug test day can be explained by a state dependent stimulus generalization decrement. In keeping with the LC-DAGO group, the PAG-DAGO, VENT-DAGO, NAL-DAGO, LC-Muscarine, and PBN-Muscarine groups all experienced what was no doubt a major change in drug state between training and testing and yet none of these groups showed any significant decrements in CR performance. The only drug state that was associated with the loss of the HR CR was the state of decreased LC activity and decreased NE release that would be expected to occur during HR CR acquisition in the DALA, UK 14,304, clonidine, and LC-DAGO pretreated groups. It would seem unlikely, given all of the potent drug states generated in the current studies, that only those associated with DALA or alpha-2 agonists in the fourth ventricle or DAGO in the LC would produce a state-dependent loss of the HR CR.

The conditioning decrement in the opioid and alpha-2 ventricle groups

(Experiment I) and in the LC-DAGO group (Experiment II) was not due to a failure to withhold responding to the CS-, as has occurred in studies using neurotoxic lesions of the LC (Berthier & Moore, 1980; McCormick & Thompson, 1982; Robbins et al., 1985). The CR deficits seen in the current studies were generally characterized by a failure of any response to develop to the CS+. This was evidenced by the fact that the opioid and alpha-2 groups were significantly different from the controls in CS+ responding but not significantly different in CS- responding. The failure of conditioned bradycardia to develop to CS+ could be interpreted both in terms of the arousal-attention hypothesis (Aston-Jones, 1985) and in terms of the anxiety hypothesis (Gray, 1982; Redmond, 1979) of LC function.

According to the arousal-attention hypothesis put forward by Aston-Jones (1985), decreasing LC activity by administering opioids or alpha-2 drugs would be expected to decrement the ability of animals to respond and attend to an environmental stimulus. The impaired HR CR in the current series of experiments might be interpreted according to this hypothesis as an inability of the animals to attend to either the CS or the US or to both events. Evidence bearing on this hypothesis in the current studies is mixed. Support for the involvement of a CS attention decrement in the loss of the CR was provided by the severe loss of the preconditioning OR in the alpha-2 ventricle groups (Experiment I). However, while the OR to the CS+ and the UR to the shock US in the LC-DAGO group (Experiment II) were also suppressed, both responses were still relatively large indicating that there was not a complete loss of attention to the CS and US. Also, the administration of DALA in the fourth ventricle in Experiment I, had no significant effects on either the OR or the UR and yet CR formation was

completely blocked. Finally, although the newly-established CRs in the DALA, alpha-2, and LC-DAGO groups were decremented on the drug test following reconditioning in a manner consistent with the loss of attention to the CS, the performance of the well established CR in the NAL-DAGO group (Experiment III) was not. This could indicate that decreasing arousal and attention to the CS only decrements learning during the early phases of acquisition. Once a response is established, then arousal and attention probably play only minor roles in maintaining responding.

According to the anxiety hypothesis (Gray, 1982; Redmond & Huang, 1979) the impaired HR CRs seen here could have been due to the fact that inhibiting LC activity decreases fear and anxiety. This inhibition of LC output may have specifically blocked the fear response to the US thereby, preventing the development of the conditioned fear/HR reaction. In the case of the loss of the established HR CR, LC inhibition could have blunted the conditional fear supporting the HR CR. The inability of the CS+ to inhibit startle responding in the DALA and alpha-2 ventricle groups supports the notion that fear responding was decremented in these groups. In addition, the informal observation that a high dose of muscarine in the LC, a drug which significantly increases LC activity, was able to elicit major fear-like responses, provides further support for the notion that LC activity is involved in fear responding.

The fact that DAGO administration in the LC had very little effect on the performance of a well-established response in the NAL-DAGO group (Experiment III) supports findings in lesion studies (Cole & Robbins, 1987; Robbins, et al., 1985) showing that the most severe effects of LC lesions are on the acquisition of responses, while little or no effects of lesions

are seen on established responses. Many researchers have proposed that the catecholamine, NE, may act directly to modulate processes involved in memory storage (Dismukes & Rake, 1972; Kety, 1970; McGaugh, 1973; Stein, Belluzzi, & Wise, 1975), which might explain why NE activity is important for the learning of new responses. Kety (1972) proposed that NE released during learning at recently activated synapses, favors the consolidation of learning by stimulating protein synthesis and other processes involved in long-term memory formation. The findings in the present studies and in previous studies mentioned above, that the presence of NE is only important during the acquisition of learning supports Kety's assertion that NE is important for memory consolidation. The slow rate of HR CR acquisition in the LC-DAGO group and the subsequent loss of what may have been a small CR after a 5 min interval could easily be interpreted within the framework of Kety's proposal. Thus, the administration of DAGO into the LC could have decreased sensory evoked NE release in target areas by the CS and US and thereby decreased the ability of the target cell activity to be permanently altered by the conditioning process. The finding that alpha-helical CRF, a drug that would be expected to increase NE release in response to a stimulus (i.e., the ratio of tonic to evoked LC activity is increased), was able to enhance the magnitude of the HR CR, while a drug like muscarine, which might be expected to decrease NE release in response to a stimulus, did not enhance the CR also provides support for Kety's NE theory of consolidation.

Because activation of the LC system results in a beta-adrenergic mediated inhibition of ongoing target cell activity that permits an enhancement of target cell activity evoked by projections from other brain sites, it has been proposed that the LC system functions to enable other

systems converging on the same target neuron to transmit more effectively during the period of simultaneous activity (Moore & Bloom, 1979). This proposal suggests that the activation of the LC by a CS and US during a conditioning trial should facilitate the responding of a target cell that is also activated by the CS and US and that this facilitation may strengthen the learning of a CS-US association. In direct support of this notion, Moore & Bloom (1976) have reported that LC activity can facilitate an excitatory neuronal response to a behaviorally significant CS.

The role of the LC in both fear responding and HR conditioning may be related to its dense innervation of both the ACE and lateral amygdaloid nucleus (LAN) (Fallon, Koziella, & Moore, 1978). The ACE has a well-established role in mediating fear and rage responses (Kaada, 1972), and learned fear responses in many different paradigms (Blanchard & Blanchard, 1972; Hilton & Zbrozyna, 1963; Hitchcock & Davis, 1986; Kapp, Pascoe, & Bixler, 1984; Spevak, Campbell, & Drake, 1975), including classically conditioned bradycardia CRs (Gentile, Jarrell, Teich, McCabe, & Schneiderman, 1986; Gallagher et al., 1981; Kapp et al., 1979). In addition, the LAN also appears to be important in learning the HR CR and may serve as an innerface between sensory systems of the thalamus and the emotional systems of the ACE (Cicchetti, LeDoux, & Reis, 1987). It could be that blockade of NE input to the amygdala complex may decrement conditioned emotionality by interfering with the neural processes responsible for the formation or preservation of the association made between environmental cues and the emotional state. Fear-motivated behaviors may be especially affected by changes in LC activity because of the dependency of fear on LC-induced NE activity within the ACE. While it has been shown that

blocking beta-adrenergic activity within the ACE can disrupt the learning of a HR CR (Gallagher et al., 1980), the LC also projects to and may modulate activity in other brain areas (Lindvall & Bjorklund, 1974) known to be critically involved in the learning of the HR CR. These include the medial geniculate nucleus (Romanski, Jarroll, Gentile, McCabe, & Schneiderman, 1986; Supple & Kapp, 1987), and the cerebellar vermis (Supple & Kapp, 1988; Supple & Leaton, 1986). Further work is needed to identify the relative contributions of LC projection areas in the HR conditioning process.

The primary excitatory afferent to the LC comes from nucleus paragigantocellularis (Ennis & Aston-Jones, 1987). The activity in this nucleus has been reported to vary with changes in autonomic activity, in addition to being activiated by a variety of noxious and non-noxious stimuli (Ennis & AstOn-Jones, 1987) and, therefore, can account for polymodal sensory activation of LC neurons. Paragigantocellularis neurons also have been reported to innervate preganglionic sympathetic neurons, which could explain why LC neurons and sympathetic nerves exhibit similar activity profiles in some circumstances.

The results of the current studies provide some support for the assertion by Amaral and Sinnamon (1977) that the LC may serve as a central analogue of a sympathetic ganglion that alerts and arouses an organism in the face of a perceived threat. However, the LC fires not only in situations of immediate stress or novelty but is also active in response to rather specific stimuli that predict later stress by virtue of previous learned associations (Rasmussen & Jacobs, 1986; Segal & Bloom, 1976). Therefore, the function of the LC in learning may be to maintain levels of arousal and emotionality optimal for information processing and to aid

target cells in the processing of relevant environmental stimuli, both by decreasing background activity, and by enhancing synaptic transmission.

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