Effects of Central Administration of An Enkephalin Analog ([D-Ala ^{2}]-Methionine Enkephalinamide) on the Development of Conditioned Heart Rate Responses

by

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A THESIS

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Abstract

The administration of the opioid compound D-ala-met-enkephalinamide (DALA) into the rostral portion of the fourth ventricle of the brain in rats prevented the development of an aversive classically conditioned heart rate (HR) conditioned response (CR) to a tone conditioned stimulus (CS) (i.e., CS+) paired with an electric shock unconditioned stimulus (UCS). A discrimination paradigm was used in which CS+ trials were randomly interspersed with nonreinforced trials involving a different CS (i.e., CS-) that was presented without the UCS. In this experiment, three groups of rats received cannula placements into the rostral region of the fourth ventricle, while a fourth group received a cannula in the brain stem ventral to the fourth ventricle infusion site. After a four day recovery period, all groups were exposed to the experimental apparatus for a one hour habituation session. During the next two days, the three ventricular groups were infused with either saline (SAL), 10µgs of DALA (DALA), or 10µgs of DALA + 5µgs of the opioid antagonist naltrexone (DALA-NAL) prior to conditioning. The brain stem group (DALA-BR.ST.) also received 10µgs of DALA prior to conditioning. After two days of conditioning, consisting of 12 CS+ and 12 CS- trials on each day, all groups remained in their home cage and received no treatment. Subsequently, all groups received four nonreinforced CS+ and CS- trials under a non-drugged condition to test for the presence of a HR CR.

The DALA-NAL and the DALA-BR.ST. groups showed the development of a normal HR CR, comparable to that seen in the SAL group. The DALA group, however, failed to develop a HR CR. The absence of a CR in this group

was evident even when tested 48 hours later in a non-drugged state. A significant reduction in the performance of HR orienting responses was seen in the DALA group only on the first CS presentation. No significant effects were seen on the HR UCR in the DALA group when compared to the SAL group. On day one of conditioning, the administration of DALA produced an immediate and profound bradycardia in the DALA group and to a lesser extent also in the DALA-BR.ST. group. On the second day of conditioning, the fall in baseline HR was significant only in the DALA group.

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 locus coeruleus or in the periaqueductal/periventricular gray regions. It is believed that the activation of opioid receptors in this area of the brain may have prevented the development of a learned association by decreasing emotional awareness of stimuli.

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Following the recent discovery of endogenous opioid peptides by Hughes and coworkers (1975), many attempts have been made to show how these peptides might influence physiological functions in the central and peripheral nervous systems. The initial investigations were based on the notion that endogenous opioid peptides might exert morphine-like effects, such as analgesia and euphoria. Current theories imply that the primary function of these endogenous peptides may be to modulate behavior (Bharagava, 1982). Because the synthesis and release of opioid peptides from neuronal terminals resemble that of traditional neurotransmitters (Hughes, Beaumont, Fuentes, Malfroy, & Unsworth, 1980), it has been suggested that opioid peptides could function as neurotransmitters, hormones, or neuromodulators (Bhargava, 1982).

Since their discovery, it has been suspected that opioid peptides may be involved in the physiological regulation of cardiovascular functions (Szekely, 1982). This assumption was based in part on the known vasodepressant effects of opioid administration (Holaday, 1983). The application of opioids intracerebrally, intravenously, or directly to the brain stem has been shown to produce potent cardiovascular responses (Feldberg & Wei, 1978; Florez & Mediavila, 1977; Lemaire, Tseng, & Lemaire, 1978). Additional evidence of a possible functional involvement between opioid systems and central cardiovascular control was accumulated when opioid receptors and opioid containing terminals were localized in brain regions known to control cardiovascular activity (Atweh & Kuhar, 1977). The continued investigation of the effects of opioid compounds on the cardiovascular system may assist our understanding of the neural mechanisms involved in cardiovascular

regulation.

More recently, opioid agents have been related to the neural systems which may contribute to memory formation (Martinez, Jensen, Messing, Rigter, & McGaugh, 1981). A number of investigations on the effects of various opioid agonists and antagonists, in different learning paradigms, have shown that under certain circumstances opioid agonists tend to impair learning while opioid antagonists may sometimes facilitate learning and memory processes (Castellano, 1980; Gallagher & Kapp, 1978; Izquierdo, 1979; Jenson et al., 1978; Martinez & Rigter, 1980). However, several studies have been reported which appear to disagree with the above findings (Ader, Weijnen, & Moleman, 1972; Belluzzi & Stein, 1977; Mondadori & Waser, 1979; Strabli & Huston, 1980). In these studies, the administration of low doses of opioids prior to training were actually found to facilitate rather than decrement performance in passive avoidance learning situations.

Close examination of studies dealing with the effects of opioids on learned behaviors, reveals that both the nature and direction of the drug effects may depend on the task being used, the drug dose, and the time of drug administration relative to training and test experience. Posttraining injections of opioid compounds can be seen as possibly altering the consolidation of recently formed memories by affecting the internal neurohumoral states that relate to arousal. Pretreatment injections of opioid compounds may alter acquisition of learned responses by affecting the neurohumoral states that relate to attention or perception of stimulus relationships (Gold & McGaugh, 1975) or in some cases by decreasing the effectiveness of aversive reinforcement (Mahalik & Fitzgerald, 1987). The basic question of what role opioids

may play in the physiology of learning has yet to be answered. Further investigation may lead to a greater understanding of the neural processes involved in memory acquisition and consolidation.

Traditionally, two major approaches have been used to study the effects of opioids in learning paradigms (Koob, Le Moal, & Bloom, 1981). The first approach employs administration of an opioid agonist to act directly on opioid receptors. The second approach involves the administration of an opioid antagonist, such as naloxone or naltrexone, to see what effect blocking endogenous opioid peptide activity may have on learning. The current study will focus on the first approach and will deal with the effects of a centrally administered opioid analogue on classically conditioned heart rate responses.

What follows is an historical account of the known effects of opioids on memory mechanisms. Studies involving both instrumental conditioning procedures and classical conditioning procedures are presented. The main emphasis will be on classical conditioning and only a very brief review will be presented on instrumental paradigms. Also included in the introduction will be pertinent information regarding the importance of opioids in regulating cardiovascular function and pain perception.

Instrumental Conditioning

Both pretreatment (Banerjee, 1971; Rigter et al., 1980) and posttreatment (Domino, Karoly, & Walker, 1963; Martinez & Rigter, 1980; Mondadori & Waser, 1979; Verhave, Owen, & Robbins, 1958) injections of various opioid agonists have been found to change responding in a variety of instrumental tasks. The vast majority of researchers using avoidance tasks, either passive (Martinez & Rigter, 1980) or active

(Banerjee, 1971; Domino, Karoly, & Walker, 1963; Izquierdo, 1979), have found opioid compounds to decrease retention of the task. The effective intraperitoneal (ip) doses were found to range from 0.1 to 20.0 µg/kg for Beta-endorphin or the enkephalins (Izquierdo & Dias, 1981; Izquierdo et al., 1980; Martinez & Rigter, 1980), and from 1.0 to 5.0 mg/kg for morphine (Izquierdo, 1979). When using intracerebroventicular (icv) administration, the effective doses were found to be in the range of 5.0 to 25.0 ng for Beta-endorphin or Met-enkephalin (Lucion et al., 1982) and from 0.3 to 20.0 µg for morphine (Jensen et al., 1978).

The first study to show opioid-induced amnesia in a shuttle avoidance task and an habituation task, was reported by Izquierdo, Paiva, and Elisabetsky (1980). They found that immediate post-training injections of Beta-endorphin or Leu-enkephalin (10 µg/kg ip) caused a significant retention deficit. Interestingly, higher doses of these substances were not found to be amnesic (Izquierdo, 1980), implying that there was an optimum dose for the production of amnesia.

The amnesic effect was believed to be centrally mediated since much smaller doses (5.0-25.0 ng) were required to produce the effect when given intracerebroventricularly (icv) (Lucion, Rosito, Sapper, Palmini, & Izquierdo, 1982). In a study by Lucion et al. (1982), icv injections of Beta-endorphin or Met-enkephalin given after training were found to cause retrograde amnesia for shuttle avoidance. This amnesia was found to be naloxone reversible (0.1 mg/kg ip) in a separate study done by Izquierdo et al. (1981).

Even though the opioid induced amnesia appeared to be a centrally mediated phenomenon, it was discovered that when opioids were injected peripherally, the opioid induced amnesia was found to require the

concomitant secretion of adrenaline by the adrenal medullary glands. It was reported that the amnesic effect of peripherally administered Met-enkephalin could be attenuated by adrenal medullectomy (Izquierdo et al., 1981). In addition, the amnesic effect was found to reappear when as little as 1.2 μg/kg of adrenaline was given ip together with the peptide. However, adrenaline administration alone was found to have no effect upon task retention.

In contrast to studies using opioid agonists, studies which administered naloxone, an opioid receptor antagonist, posttraining, have found it to facilitate learning in a variety of tasks. Facilitory effects of naloxone were found in passive avoidance (Messing et al., 1979), shuttle avoidance (Izquierdo, 1979), one-way active avoidance (Messing et al., 1979), and habituation of a rearing response to a tone (Izquierdo & Graudenz, 1980). The results of these studies were taken as further evidence for the position that opioids play some role in memory formation and retention.

Classical Conditioning

Opioids have also been found to influence responding in classical conditioning paradigms. Both peripheral (Mahalik & Fitzgerald, 1987; Mauk, Madden, Barchas, & Thompson, 1982; Mauk, Warren, & Thompson, 1982; Schindler, Gormezano, & Harvey, 1983, 1984) and central (Gallagher, Kapp, McNall, & Pascoe, 1981; Gallagher, Kapp, & Pascoe, 1982; Lavond, Mauk, Madden, Barchas, & Thompson, 1983) administration of various opioid agents were found to adversely affect classical conditioning of heart rate (HR) conditioned responses (CRs) (Gallagher et al., 1981; Gallagher, Kapp, & Pascoe, 1982; Mahalik & Fitzgerald, 1987; Lavond et al., 1983), leg flexion CRs (Stephens & Gant, 1955), and nictitating

membrane (NM) CRs (Mauk et al., 1982; Mauk, Warren, & Thompson, 1982; Schindler, Gormezano, & Harvey, 1983, 1984).

In terms of when opioids are administered, it has been found that pretraining injections of various opioid compounds impaired the acquisition of both HR and NM CRs (Gallagher et al., 1981; Gallagher, Kapp, & Pascoe, 1982; Mahalik & Fitzgerald, 1987; Schindler, Gormezano, & Harvey, 1983, 1984), and under certain circumstances posttraining injections of opioids abolished recently learned HR and NM CRs (Lavond et al., 1983; Mauk et al., 1982; Mauk, Warren, & Thompson, 1982). The magnitude of the decremental effect of posttraining injections of opioids on NM CRs, depends on the amount of prior training (Mauk, Castellano, Rideout, Madden, Barchas, & Thompson, (1983). If animals are overtrained the effects of opioids on the CR is not as strong (Mauk et al., 1983). In the following section, the studies which used peripheral administration will be separated from those studies employing central administration, since the present study deals with central administration.

Peripheral Administration

A major problem with studies dealing with possible drug effects on learning is that of determining whether an observed decrease in responding is due to a direct action of the drug on some part of a memory trace or associative process or whether it is due to a drug-induced decrement in the capacity to perform the response. There are three postulated ways in which a drug may decrement the occurrence of a CR (Schindler, Gormezano, & Harvey, 1984). One way would be to alter the sensory processing of either the CS or US. A second way would be to affect the neural mechanisms involved in the learning process, and

finally the third way would be to alter the motor functioning used to perform the CR. A study done by Schindler, Gormezano, and Harvey (1983) adressed the issue of learning versus performance by first training the animals in a drugged state and then testing them in a non-drugged state.

In this study, rabbits were assigned to various morphine dosage groups (0.0, 0.2, 1.0, 5.0, and 10.0 mg/kg) and then injected intravenously (iv) 20 to 30 minutes prior to the beginning of classical conditioning training of the NM CR. Both tone and light conditioned stimuli (CS) were separately paired with a 3-mA paraorbital eye shock unconditioned stimulus (UCS). After 10 days of conditioning, all groups were then left in their home cages for 5 days after which time they were brought back to receive 10 more days of conditioning in a non-drugged state.

It was found that morphine produced a dose dependent decrease in the original acquisition of NM CRs. Morphine, however, was not found to have an effect on the latency or amplitude of the unconditioned response (UCR). When the groups were again trained, 5 days later, in a non-drugged state, it was found that the high dose morphine groups, which had showed little if any evidence of a CR previously, were slow to develop a NM CR. Because the performance of these groups was similar to a naive group, it was suggested that it was not the performance of the CR that was interfered with originally, but that the development of an association between the CS and US was in some way retarded. There was no indication that learning in phase one aided the development of a CR in phase two. Because morphine appeared to have no effect on UCR performance, the authors suggested that morphine may have retarded NM conditioning by interfering with the sensory processing of the CS.

The effects of morphine and naloxone on the sensory processing of the CS and UCS were tested in a series of three experiments done by Schindler, Gormezano, and Harvey, (1984). In the first experiment, three groups of rabbits (5 mg/kg morphine, 5 mg/kg morphine + 1 mg/kg naloxone, and saline) were injected iv 30-40 minutes prior to classical conditioning of the NM CR. The conditioning procedure consisted of tone and light CSs which were separately paired with a 3-mA paraorbital eye shock. The two different CSs were incorporated to assess the effects of morphine on two distinctive sensory modalities. The group which received morphine prior to conditioning was found to be significantly retarded in the acquisition of NM CRs, while the group that received naloxone in conjunction with morphine showed normal acquisition of NM CRs to both CSs, indicating that the CR decrement was mediated by opioid receptor activity. Again, morphine was found to have no significant effect on NM UCRs.

In the second experiment, the effects of morphine and naloxone on the sensory processing of the UCS were determined by examining the effects of several morphine doses (0.2 - 10.0 mg/kg) on the UCRs produced by a range of UCS intensities (0.25 - 4.0 mA). Rabbits were assigned to one of 10 drug conditions and injected iv prior to test sessions. Test sessions consisted of 35 shock alone trials, with shock intensities randomly varied during the sessions. It was found that the 5- and 10-mg/kg groups showed a significant reduction in UCR magnitude at the two highest shock intensities when compared to saline controls. Prior injections of naloxone (1 mg/kg) effectively blocked the reduction in UCR magnitude produced by the 5- and 10-mg/kg doses of morphine, again indicating that the UCR losses related to opioid-receptor

activity.

The third experiment in this series was designed to examine the effects of morphine and naloxone on the sensory processing of tone CS's using a type of generalization paradigm. Rabbits were trained in a non-drugged state for 10 days and were then randomly assigned to 4 different drug conditions (5 mg/kg morphine, 1 mg/kg naloxone + 5 mg/kg morphine, 1 mg/kg naloxone, and saline). Drug injections were given iv prior to additional test sessions. These test sessions consisted of 54 tone-shock trials composed of the random presentation of nine tone intensities that were different from the intensity of the original CS.

It was found that while the saline group showed generalized NM CRs at tone intensities lower than the training CS intensity, the morphine group showed a significantly reduced frequency of CRs at all CS intensites. Naloxone given alone, was found to elevate responding to the two lowest tone intensities and when given in conjuction with morphine completely blocked the adverse effects of morphine on NM CRs. These authors concluded that because morphine had a weak effect on the UCR but produced a substantial reduction in the generalization of the CR, that the decremental effects of morphine may be due to decreased sensory processing of the CS which they felt may be equivalent to decreasing the physical intensity or salience of the CS.

An alternative explanation (Mahalik & Fitzgerald, 1987) for morphine-induced suppression of NM CRs is that morphine analgesia may have produced a loss in emotional responding to the UCS, and that this in turn decremented the development of the CR. Such a loss could occur without decrementing the NM UCR to the shock UCS. The NM UCR is elicited through reflex pathways located in the brain stem by either

tactual stimulation of the cornea or electrical stimulation of the skin just lateral to the eye (Schindler, Gormezano, & Harvey, 1985). Presumably the stimulus does not have to be painful in order to elicit extension of the nictitating membrane. Therefore, morphine induced analgesia might not be expected to affect the reflex nictitating membrane UCR. Schindler, Gormezano, and Harvey (1984) did not discuss the possibility that a morphine-produced reduction in the emotional state to the UCS could have contributed to the CR decrement.

There is currently evidence which indicates that the analgesic effects of opioids may be due in part to their ability to alter the negative emotional state which accompanies a painful experience (Sternback, 1968). In rats, fear responses (i.e. piloerection, trembling, shaking, micturition, etc.) normally elicited by stimuli previously associated with pain, are diminished by morphine administration (Morris & Gebhart, 1978). Even a subanalgesic dose (0.25 mg/kg) of morphine has been found to cause suppression of conditioned emotional responses to a tone associated with shock presentation (Banerjee, 1971). This finding suggests that the suppression of the alarm or fear responses by morphine may be independent of its analgesic effects. It is possible that opioid induced suppression of the emotional state of fear might affect responding in aversive classical conditioning studies.

The disruption in the development of a classically conditioned response has been found to be most severe when morphine is injected prior to training (Gallagher et al. 1981; Mahalik & Fitzgerald, 1987; Schindler, Gormezano, & Harvey, 1983, 1984), and least severe in highly overtrained animals when the drug is given subsequent to training (Mauk

et al., 1983).

Mauk et al. (1983) found that the more training an animal received, the less suceptible the NM CR was to iv morphine effects. In this study, rabbits were given classical conditioning training of the NM CR by pairing a tone CS with a corneal airpuff UCS. The animals were trained to a criterion of 8 CRs out of a 9 trial block and were then divided into three groups. One group received 2 additional blocks of training (18 trials), a second group received 9 additional blocks (81 trials), and a third group 15-26 additional blocks (135-234 trials). Two days later, all groups received 2 blocks (18 trials) of training prior to iv administration of 5 mg/kg of morphine.

The group which received the least overtraining showed an immediate abolition of the recently learned NM CR following morphine administration. The other two groups showed almost no loss of the CR. Increasing the amount of overtraining was found to greatly reduce the effect of morphine on NM CRs. These authors claimed that while initially the development of conditioned responses was susceptible to the mood altering effects of opioids, with increased training conditioned responses may develop functional autonomy and be uneffected by opioids.

As mentioned above, in an aversive classical conditioning situation, the association that develops between a CS and a US may be dependent upon the ability of the US to elicit an emotional response (Mowrer, 1947). Mauk et al. (1983) have emphasized a two process approach to the learning of a NM CR, one process being the conditioning of an emotional state (fear) and the second process being the development of the learned NM CR, that somehow depends upon fear.

Opioid induced abolition of a NM CR early in training reflects an action of opioids on conditioned fear. They further suggested that with sufficient training, NM CRs may become functionally autonomous and thereby become independent of the conditioned fear state. This was their explanation of why overtraining (Mauk et al., 1983) decreased the ability of morphine to reduce the CR.

In a series of experiments conducted by Mahalik and Fitzgerald (1987) the effects of morphine and naloxone on the development of conditioned HR responses in rats were explored. In the conditioned HR procedure, a tone (CS) paired with a electrical shock (UCS) comes to elicit a bradycardia HR response (CR) in rats in about 10 to 15 CS-UCS pairings. Since the initial response to a tone CS is also a bradycardia, many researchers give CS alone trials prior to conditioning in order to habituate the bradycardia orienting response (OR) to the CS.

In this series of experiments, a discrimination paradigm was used. The discrimination paradigm consists of the presentation of two different CSs, one of which is always paired with shock (CS+), while the other CS is never paired with shock (CS-). All animals are exposed to the random presentations of both CSs. This procedure allows for the distinction between CRs which are formed by associative mechanisms from responses which occur as a result of non-associative mechanisms (i.e., sensitization and pseudoconditioning).

In the first experiment, restrained rats received subcutaneous (sc) injections of various doses of morphine (0.0, 0.25, 5.0, or 10.0 mg/kg) or naloxone (0.0, 0.1, 1.0, or 10.0 mg/kg) 15 minutes prior to classical conditioning training. The conditioning procedure consisted of a tone

CS which was paired with shock UCS, delivered through the cardiac electrodes located on either side of the rat's chest.

It was found that all morphine groups showed attenuated responses to the CS on the preconditioning CS alone trials. During conditioning, the highest dose of morphine (10.0 mg/kg) produced a substantial decrease in the development of HR CRs and also decreased the appearance of HR UCRs. The 5-mg/kg dose group developed HR CRs, but showed decreased HR UCRs like the 10-mg/kg dose group. Administration of naloxone alone was found to have no effect on HR CRs or UCRs.

The design of this experiment did not allow for the determination of whether the adverse effect of the 10-mg/kg dose of morphine on the HR CR was due to interference with a learning mechanism or whether the animals simply could not perform the response due to morphine influences on the cardiovascular system. Morphine and other opioid compounds are known to have potent cardiovascular effects (Holaday, 1983). In general, opioids have been found to increase parasympathetic tone and to decrease sympathetic tone (Holaday, 1983). The characteristic response to peripherally administered morphine (Stein, 1976) and enkephalin analogs (Wei, Lee, & Chang, 1980) has been shown to be bradycardia. However, when HR is recorded long enough after iv morphine administration, it has been found that the cardiovascular response is biphasic with the bradycardia being followed by tachycardia (Schwarz, Peris, & Cunningham, 1987). Mahalik and Fitzgerald (1987) also reported a decrease in baseline HR following morphine administration, indicating the presence of drug-induced effects on HR.

In order to more fully examine the effects of morphine on the performance of HR CRs, a second experiment was conducted in which

naloxone was administered to morphine animals after conditioning to block the effects of morphine. It was expected that if the decrement in CR level found in the 10-mg/kg morphine group was due to direct cardiovascular effects of morphine and not due to a failure to learn, then the CR should appear after these effects were reversed by naloxone.

In this study, two groups of rats received sc injections of either 10-mg/kg of morphine or an equivalent volume of saline 15 minutes prior to classical conditioning. After training, both groups were divided into different subgroups, with all groups then receiving an additional sc injection. There were two morphine subgroups, one receiving a 1-mg/kg dose of naloxone (morph-nal) and the other saline (morph-sal). There were three saline subgroups receiving a 1-mg/kg dose of naloxone (sal-nal), a 10-mg/kg dose of morphine (sal-morph), or saline (sal-sal). After the injections, all groups were given four unreinforced test trials with CS+ and four trials with CS-. These trials were followed by reinforced reconditioning trials.

The results of this experiment showed that the decremental effects of morphine on the production of HR CRs and UCRs were not reversed by subsequent administration of naloxone. However, supression of baseline HR by morphine was reversed by naloxone; a finding which suggests that at least some of the cardiovascular effects of morphine were reversed by naloxone. Morphine given to animals trained under the saline condition produced only a slight decrement in HR CRs but greatly reduced the appearance of HR UCRs. Naloxone administration to the animals trained in the saline group had no effect on HR CRs or UCRs.

It was concluded from this experiment that the adverse effects of morphine on the development of HR CRs might be due to interference with

an associative mechanism involved in the learning of a HR CR and not due to the inability of the animals to perform CRs. The finding that the suppression of the UCR by morphine was not reversed by naloxone was surprising. The authors thought that, in general, the absence of UCRs during conditioning with morphine may be due in part to opioid-induced analgesia and that the 1-mg/kg dose of naloxone should have been sufficient to block the analgesia.

A third experiment was conducted to test this assumption. The reversal of analgesia induced by a 10-mg/kg dose of morphine by a 1-mg/kg dose of naloxone was measured in a tail-flick test. The tail-flick test involves measuring the latency of the rat's tail-flick response to a thermal stimulus. This experiment found that the 1-mg/kg dose of naloxone was sufficient to reverse morphine induced analgesia at least as far as the tail-flick test was concerned.

Mahalik and Fitzgerald (1987) concluded that morphine induced analgesia probably contributed to the decrease in UCRs seen during conditioning and could have possibly reduced the aversiveness or emotional impact of the UCS below levels needed to support conditioning. In addition, they felt that opioid induced analgesia did not completely abolish all the sensory consequences of the UCS, since an analysis of responses during the first conditioning trial showed that the morphine groups made orienting-like responses to the first presentation of shock. This provided some indication that at least some of the sensory qualities of the shock were not blocked by morphine.

Previous research has shown that opioids affect emotional states as well as pain (Jaffe & Martin, 1985, Julien, 1981). For instance, Davis (1979) found that morphine (10 mg/kg) attenuated conditioned fear in a

potentiated startle paradigm. It is possible that morphine, in the Mahalik and Fitzgerald study, decreased the emotional experience of fear that is necessary for the development of HR CRs.

An alternative mechanism by which opioids could have an adverse effect on emotional responding would be through their reported ability to produce inhibitory actions on neuronal functions (Ronai & Szekely, 1983). The inhibitory actions of opioids on cellular functions could play a role in opioid-induced abolition of classically conditioned responses. Evidence for this view was found when Mauk, Warren, and Thompson (1982) reported that 5- and 10-mg/kg doses of morphine, administered iv following conditioning, abolished the learning-induced hippocampal unit activity that develops during the learning of a NM CR, along with the recently learned NM CR. The action of morphine on the learning induced hippocampal response was found to parallel very closely its action on the behavioral response. The application of naloxone (0.1 mg/kg iv) immediately restored hippocampal activity and the NM CR. These results were interpreted as supporting the possibility that morphine disrupts the neuronal circuitry that develops when two stimuli become associated in a learning situation. It can not be determined from this study if the change in hippocampal neurons was due to a direct morphine effect or whether morphine was affecting other systems which provide neuronal input to the hippocampus.

Central Administration

All of the aforementioned studies have involved the systemic administration of opioid compounds. While peripheral administration of opioid compounds may show influences on memory that are not due to performance factors, the question of where these influences occur is

often difficult to answer. The injected compounds could exert their effects by acting at peripheral sites, central sites, or both. Recent research has indicated that peripherally administered opioid compounds are able to enter the brain (Izquierdo, 1983) either by crossing the blood brain barrier by active transport into the cerebral spinal fluid (Wang & Takemori, 1972), or by crossing at places where the blood brain barrier is not intact (i.e., the area postrema, the neurohypophysis, pineal body, intercolumnar tubercle, and the supraoptic crest) (Wislocki & Leduc, 1952).

Central administration of opioids allows for greater, though not complete localization of the effects of opioids. As long as volume is restricted, then location of effect in central administration can be pinpointed to a specfic area. Microinjections of compounds in volumes of 1 μl or less have been found to be retained within 1 mm of the site of injection (Lomax, 1966). It has also been found that only very small fractions of the chemical in solution ever reach specific receptor sites, when high concentrations of compounds are used in microinjection procedures (Myers, 1974).

Mauk, Madden, Barchas, and Thompson (1982) have conducted four experiments (two involving central administration of opioids and two involving peripheral) which were designed to assess the relative importance of peripheral and central sites in possibly mediating the opioid-induced abolition of aversive classical conditioned responses. The results of these experiments have indicated that the selective abolition of NM CRs by opioid compounds appeared to be due to the activation of opioid receptors located in the region of the fourth ventricle. In these studies, rabbits were trained to a criterion of 8

NM CRs out of a 9 trial block, by receiving paired presentations of a tone CS and a corneal air-puff UCS. Immediately following this training procedure, animals received injections of various compounds either into the rostral region of the fourth ventricle (icv) or into the marginal ear vein (iv).

In the first icv study, the animals received 20 µl injections of either 200 nmols of morphiceptin, 200 nmols of morphiceptin + 20 nmols naltrexone, an opioid receptor antagonist, 200 nmols of (D-Pro) morphiceptin, an analogue void of opioid agonist activity, or an equivalent volume of vehicle solution. Five minutes following the injections, all groups recieved three additional blocks of conditioning.

In the second icv study, animals were injected with a vehicle solution or varying amounts of morphiceptin (12 nmols, 6 nmols, or 3 nmols) following the initial conditioning session. After three blocks of training one of the animals in the 12 nmol group received naloxone (2.5 mg/kg iv), while the rest of the animals received saline injections. After this injection, all groups were exposed to three extra conditioning blocks.

In one iv study, animals were given a series of injections of morphiceptin following conditioning. The doses corresponded to 0.1, 1.0, and 10.0 times the doses given icv. A total of three blocks of conditioning were given following the initial injection. After these injections, all groups were given morphine (7.5 mg/kg iv) followed by a final two blocks of conditioning.

A second iv study was conducted in which animals were given 5 mg/kg doses of morphine, following the initial conditioning procedure, and three blocks later were given a series of of four iv injections of

naloxone methobromide, a quanternary derivative of naloxone which does not cross the blood brain barrier. The doses of naloxone methobromide used corresponded to 1.0, 25.0, 50.0, and 100.0 times the dosage of naloxone (0.1 mg/kg) previously shown to reverse morphine's effects on conditioned responding (Rigter et al., 1980). One block following the final injection of naloxone methobromide, an injection of naloxone (0.1 mg/kg) was given iv before two additional blocks of conditioning.

The results of these four experiments (Mauk et al., 1982) showed that icv administration of morphiceptin completely abolished recently learned NM CRs in a dose dependent fashion. The highest dose (12 nmol) was found to have a potent and long lasting effect on the abolition of NM CRs. In addition, morphiceptin was found to have no effects on the amplitude of NM UCRs. The concurrent administration of naltrexone with morphiceptin or the administration of the (D-Pro) analogue of morphiceptin, which has no opioid agonist properties, were found to have no effect on NM CRs.

The iv administration of morphiceptin, which does not cross the blood brain barrier, even in doses 10 times those given icv, was found to have no effect on NM CRs. Morphine, on the other hand, was found to abolish the CR when given following the iv morphiceptin. The administration of naloxone methobromide, unlike administration of naloxone, was not able to reverse the morphine-induced abolition of conditioned responding.

The results of these studies (Mauk et al., 1982) suggest that opioid-induced abolition of recently learned NM CRs is due to activation of opioid receptors located within the central nervous system. The finding that doses of morphiceptin when given iv, as opposed to icv,

were ineffective in abolishing NM CRs together with the finding that the quanternary naloxone derivative was unable to reverse the effects of morphine on NM CRs, support this assertion. The authors of these studies believe that opioids act on some critical component of the circuitry that is necessary for the production of the CR. It is not clear from these studies if the effects of opioids on NM CRs were due to an associative mechanism or if opioids were just interfering with the performance of the recently learned NM CR.

The authors of this study (Mauk et al., 1982) report that previous research has shown that the rostral region of the fourth ventricle is an important site for the production of opioid-induced analgesia in the rabbit. They suggest that it is possible that certain opioids exert their selective effects on NM CRs by inducing analgesia which may alter the "learned fear system" in the brain.

Gallagher, Kapp, McNall and Pascoe (1981) have indentified what they believe is a specific brain structure possibly involved in the opioid induced attenuation of HR CRs in rabbits. Previous research done in their laboratory has shown that lesions made in the central nucleus of the amygdala resulted in an attenuated development of HR CRs (Kapp, Frysinger, Gallagher, & Haselton, 1979). Because the central nucleus is known to contain opioid receptors (Simantov, Kuhar, Uhl, & Snyder, 1977), these authors examined whether administration of opioids into this area would also impair performance in the aversive conditioned HR paradigm.

In this experiment (Gallagher, Kapp, McNall, & Pascoe, 1981) rabbits were divided into seven conditioning and three pseudoconditioning groups. The conditioning procedure consisted of

paired presentations of a tone CS and a 2-mA eyelid shock UCS, while the pseudoconditioning procedure consisted of the same CS and UCS presented in an unpaired manner. The seven conditioning groups consisted of two control groups, an unoperated group and a vehicle injected group, plus four drug injected groups. The drug injected groups received either 5.0 nmols of levorphanol, a mu opioid receptor agonist, 2.5 nmols of naloxone, 5.0 nmols of dextrorphan, an inactive enantiomer of levorphanol, or 5.0 nmols of levorphanol + 2.5 nmols of naloxone. Drug injections were made bilaterally into the central nucleus of the amygdala in 1 μl volumes prior to classical conditioning training. A separate group of animals also received a 5.0 nmols dose of levorphanol but the injections were made 1-2 mm. dorsal to the central nucleus. The three pseudoconditioning groups consisted of an unoperated control group, a 5.0 nmol of levorphanol dose group, and a 2.5 nmol of naloxone dose group.

It was found that paired presentations of the CS and UCS resulted in bradycardic HR CRs in the unoperated control animals and vehicle injected animals. Administration of levorphanol into the central nucleus significantly decreased the acquisition of HR CRs but produced no effect on baseline HR or on orienting responses to the CS. No information was provided on HR UCRs. Injections of naloxone were found to increase the magnitude of HR CRs when compared to vehicle injected groups, while injections of dextrorphan were found to have no effect on HR CRs. The group which received a combination injection of levorphanol + naloxone was found to respond in a manner similar to the vehicle injected group. Finally, injections of levorphanol outside of the central nucleus were found to be ineffective in changing the acquisition

of HR CRs.

The fact that naloxone effectively blocked the effects of levorphanol and that dextrorphan administration was ineffective in changing HR CRs led these authors to conclude that the detrimental effects of levorphanol administration on the development of HR CRs was due to the activation of opioid receptors located in the central nucleus. It was also suggested that since the levorphanol group displayed normal ORs to the CS, that interference with CS sensory processing could be effectively ruled out as a reason why levorphanol decreased HR CR acquisition. In addition, it was suggested that interference with UCS sensory processing could also be ruled out since previous research has shown that opioid administration into the central nucleus does not alter shock induced pain thresholds (Rodgers, 1978).

These authors believe that the effect of levorphanol administration probably reflects a decrease or alteration in a conditioned state of fear. These authors have accepted the operational definition of fear as being indexed by numerous autonomic, endocrine, and skeletal responses that accompany emotional responding (Kapp & Gallagher, 1979). In support of their conclusions about attenuation of fear responding, they cite a study done by File and Rodgers (1979) in which opioid administration into the central nucleus of the amygdala resulted in decreased emotional reactivity in rats.

There is current evidence which suggests that there are three major functional groups of opioid receptor subtypes located in the brain. These different subtypes include mμ, delta, and kappa (Wood, 1982). The study done by Gallagher et al. (1981) looked at the effects of the mμ receptor agonist, levorphanol, but did not explore the possibilities of

the effects of agonists for other receptor subtypes located in the central nucleus of the amygdala. Delta receptors have also been found in the central nucleus (Ninkovic, Hunt, Emson, & Iversen, 1981) and could also have an effect on the development of HR CRs.

A study conducted by Gallagher, Kapp, and Pascoe (1982) explored the effects of both a mμ and delta receptor agonist administered into the central nucleus on the development of HR CRs. In this study, rabbits were assigned to either one of two control groups (unoperated or vehicle injected) or one of six drug injected groups. The conditioning procedure consisted of a tone CS paired with a 2-mA eyelid shock UCS. Prior to the conditioning procedure, animals in the drug injected groups received either D-alanine-methionine-enkephalinamide (DALA), a mμ receptor agonist (0.5, 1.0, or 2.0 μgs), or they received injections of D-alanine, D-luecine enkephalin (DADL), a delta receptor agonist (1.0 or 2.0 μgs). A group which received both DALA (2.0 ug) plus naloxone (1.0 μg) was also included. All injections were made bilaterally into the central nucleus of the amygdala in 1 ul volumes.

It was reported that administration of DALA or DADL produced no change in baseline HR or in ORs to the CS on the CS alone trials. During the conditioning phase, it was found that the 1.0 and 2.0 μg doses of DALA impaired the acquisition of HR CRs. No information was provided on HR UCRs. Groups which received DADL responded similarly to vehicle injected animals. It was concluded that only activation of mμ receptors in the central nucleus of the amygdala has an effect on the development of HR CRs.

The fourth ventricle of the rabbit has been found to be an important location for opioid-induced abolition of a recently learned HR CR. The

only experiment involving the administration of opioids into the fourth ventricle on HR CRs was done by Lavond, Mauk, Madden, Barchas and Thompson (1983). They have found that the most immediate and reliable decremental effects on the HR CR were obtained with the administration of opioids in the rostral portion of the fourth ventricle, suggesting a primary action on structures rich in mu opioid receptors in the vicinity of the periaqueductal gray and the periventricular region of the brain.

Structures which surround the fourth ventricle of the brain have been found to be rich in opioid receptors (Atweh & Kuhar, 1977). In addition these structures have also been found to be involved in central cardiovascular control (Holaday, 1983). Enkephalin containing neurons have been found within the nucleus of the solitary tract (NTS), nucleus ambiguus (NA), and the dorsal motor nucleus of the vagus (DVN) (Sar, Stumpf, Miller, Chang, & Cuatrecasas, 1978). Opioid receptors have similarly been found in the brain stem near the enkephalin containing terminals (Atweh & Kuhar, 1977). The region around the obex of the fourth ventricle is particularly rich in opioid receptors (Young, Wamsley, Zarbin, & Kuhar, 1980). This location is crucial to cardiovascular control since all known afferent baroreceptor fibers pass through this region (Korner, 1971).

In the afferent parasympathic pathways, the NTS is the primary CNS synapse of the baroreflex and chemoreflex arcs (Holaday, 1983). This nucleus directly innervates the NA and the DVN (Holaday, 1983). It is from the NA that the vagal efferent component of the baroreflex arc originates, and it is suggested that it might be the primary site for intergration of reflex bradycardia (Holaday, 1983). The DVN has also been found to transmit efferent information via the vagus (Holaday,

1983). Taken together, the NTS, NA, and the DVN can be seen to occupy the role of the major parasympathic components of the brain stem that modulate general parasympathic outflow (Galosy, Clarke, Vasko, & Crawford, 1981). Injections of opioids into these areas have been shown to produce hypotension, bradycardia, and modification of cardiovascular reflexes (Punnen, Willette, Krieger, & Sapru, 1984).

In the study by Lavond et al. (1983) involving the fourth ventricle, rabbits were trained in an aversive classical conditioned HR paradigm using paired presentations of a tone CS and a 2-mA faceshock UCS. After 15 conditioning trials, the animals who showed CRs were divided into three groups and injected icv prior to additional conditioning trials. The groups were injected with either 12 nmols of morphiceptin, 12 nmols of morphiceptin + 10 nmols of naltrexone, or with a vehicle solution. All injections were made into the fourth ventricle in 20 µl volumes.

In this study it was reported that the administration of morphiceptin into the fourth ventricle caused an immediate abolition of the recently learned HR CR. In contrast, the group given both morphiceptin and naltrexone continued to show HR CRs. It was reported that both the agonist group and the agonist-antagonist group showed a decrease in baseline HR responding after injection. None of the treatments had any effect on tachacardia HR UCRs, although specific data on this point were not provided.

It was suggested that the decremental effect of morphiceptin on an established HR CR was due to the activation of mu opioid receptors located within the rostral region of the fourth ventricle. It was further indicated that the application of opioids in this region may act

to inhibit some part of a conditioned fear circuitry that was necessary for the learning of the HR CR.

It is not possible to determine if the decremental effects of morphiceptin found in this experiment were caused by a reduction in the animal's emotional state or whether the activation of opioid receptors in the region surrounding the injection site prevented the performance of the HR CR. As previously mentioned, many of the opioid receptors in the region of the fourth ventricle are contained within structures responsible for the production of the parasympathetically mediated HR CR (Gallagher et al. 1981). It could be that the inhibitory actions of morphiceptin on neuronal functions simply prevented the performance of the HR CR.

Summary

So far it has been found that the application of morphine iv (Schindler et al., 1983) or sc (Mahalik & Fitzgerald, 1987) or enkephalin analogs centrally (Gallagher et al., 1981), impair the development of both NM and HR CRs when given prior to training. In addition, it has also been found that when given after training, morphiceptin administered centrally abolished a recently learned HR CR (Lavond et al., 1983), while morphine administered iv (Mauk et al., 1982) abolished a recently learned NM CR. In the future it will be important for this area of research to be concerned with the localization of the opioid-induced effect on learning. Is the brain the main target organ for the abolition of classically conditioned responses by opioids? If so, then what are the most susceptible structures to the influence of opioids?

Other important issues to be addressed will be the mechanism of

action of opioid compounds on classical conditioned responses. Is the effect of opioid compounds due to changes in the sensory processing of stimuli, changes in the neuronal circuits responsible for associations between stimuli, changes in the emotional states which create the climate of arousal sufficient for associations to be formed or retrieved, or are changes created in the circuits involving the motor output of the CR. It is hoped that future research in this area will begin to provide answers to these questions, and expand on our current knowledge base about the physiology of memory formation and consolidation.

Rationale

The aim of the present study was to acquire more information concerning the effects of centrally administered opioid compounds on the development of HR changes occurring during aversive classical conditioning. This study was an attempt to distinguish between the learning and performance effects of opioid compound administration on HR CRs. The classically conditioned HR paradigm was choosen as the model system to be studied because many of the factors that can influence the development of the HR CR have been well characterized (Fitzgerald, 1976; Fitzgerald & Hoffman, 1976; Fitzgerald, Martin, & O'Brien, 1973; Hatton, Foutz, Fitzgerald, Gilden, & Martinsen, 1984).

In addition, the HR CR has been used by many theorists (Kapp, Gallagher, Applegate, & Frysinger, 1982; Schneiderman, Smith, Smith, & Gormezano, 1966) as a measure of conditioned fear. This was considered an important factor since the proposed opioid-induced attenuation of fear responses is an issue of concern in the determination of the mechanism of action of opioid compounds on HR CRs (Gallagher et al.,

1981; Lavond et al., 1983; Mahalik & Fitzgerald, 1987).

To date only two specific brain regions have been identified to play a role in opiate induced abolition of the HR CR, one being the central nucleus of the amygdala and the other being the fourth ventricle. The fourth ventricle was chosen as the site of injection for this study for two reasons. Structures located in the walls of the fourth ventricle are not only populated with opiate receptors but are also known to play a role in cardiovascular control. Second, in the paper reported on by Lavond et al. (1983) the fourth ventricle was found to be a location particularly sensitive to opioid-induced abolition of an established HR CR.

In contrast to the Lavond et al. study (1983), the present experiment proposed to inject the opioid compound prior to classical conditioning training and later to test for evidence of a HR CR in a non-drugged state. It was hoped that this design would allow a determination to be made between opioid effects on the HR CR that are due to actions on associative mechanisms from those that are due to actions on performance mechanisms. Also, by giving the opioid prior to conditioning, the possible effects of the opioid on the HR UCR could be assessed throughout the conditioning session.

The opioid compound chosen to be tested was a highly potent analog of methionine-enkephalin, D-ala2-met-enkephalinamide (DALA). The compound DALA (Pert, Pert, Chang, & Fong, 1976) was chosen for this experiment due to its specificity for the mu receptor, and for its conformational protection from enzymatic attack (Pert et al., 1976). These are important qualities, which allow for receptor subtype identification and longer lasting drug effects. While naturally

occuring enkephalins prefer the delta receptor (Szekely, 1982), amidation of the carboxy terminal of enkephalin, as occurs in the synthetic analog D-ala2-met-enkephalinamide, results in a compound with a greater affinity for the morphine mu receptor and less affinity for the enkephalin delta receptor (Oliverio, Castellano, & Puglisi-Allegra, 1984).

In addition to a group given DALA (DALA) (n=6), three other groups (n=6 each) were included in the study. One group was the control group for the surgical procedure and received saline injections (SAL), while a second group received DALA plus the opioid antagonist naltrexone (DALA-NAL). The agonist-antagonist group was included to control for opioid effects that are not due to opioid receptor activation. It was anticipated that naltrexone would block all receptor effects of DALA administration (Gallagher et al., 1982).

A third group was composed of animals with cannulas located in the brain stem area directly ventral to the rostral fourth ventricle infusion site in the region of the medial longitudinal fasciculus. Due to a surgical error a number of animals (n=8) all received cannulas in the brain stem at the same time and were assigned to the DALA condition. These animals, though not randomly assigned, were included as a separate group (DALA-BR.ST.) in this study following histological examination to provide some information on the localization of DALA's effects. Two of the animals in this group were discarded because their data were incomplete. A list of surgical errors made in each group and other sources of attrition occurring in each group are listed in the appendix.

Methods

Subjects

The subjects were 24 male Sprague-Dawley albino rats (Simonsen), weighing between 350 and 425 gm. 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. The experiment took place during the light part of the cycle.

Surgical Procedure

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 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. Inside the cannula was an obturator made from a piece of 30-ga. stainless-steel tubing that was used to isolate the ventricular space from the external environment. The length of the obturator was adjusted so that it extended 1-mm beyond the end of the guide cannula. The part of the obturator which extended

outside the external surface of the guide cannula was bent at a 90 degree angle to prevent it from falling through the cannula and damaging the brain.

Anesthesia

A 0.4-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. A booster dose of 0.2 ml was given during surgery.

Surgical Procedure . As soon as the rat was fully anesthetized, 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. Bactofura wound powder was sprinkled over the cuts in the skin made by the suture needle to prevent infections. At this time, a 0.1-ml dose of Crysticillin (penicillin G in procaine suspension, 600,000 units/ml) was injected intramuscular into the left hind leg and a booster dose of anesthetic was given.

The rat was then placed in a Kopf stereotaxic apparatus and the skull exposed. A burr hole was made using a high speed dentist's 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: -11.0 (posterior to bregma), L: 0.0 (lateral to the midline), V: -8.0 (verticle to the dura) (Pellegrino, Pellegrino, and Crushman, 1979). Coordinates were referenced from sterotaxic zero and the incisor bar was set so 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 longer than the guide cannula. The length of the infusion needle was important since the scar tissue which forms around the ventricular end of the guide cannula might have hindered the flow of solution from the infusion needle. The infusion needle was connected to a 10-µl Hamilton microsyringe by a 1-cm length of PE-10 tubing. The infusions were given by opening the experimental chambers and placing the infusion needle into the guide cannula without picking up the rat. The infusion needle was left in place for one 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.

The CS+, which was paired with shock, consisted of a 6-sec electronically generated click that occurred at a frequency of 3 Hz.

The CS-, which was not paired with shock, was a continuous 6-sec 1-kHz tone. The UCS was a 0.5-sec train of 0.5-msec, 250-Vdc pulses of shock, delivered through the ECG electrodes.

An Apple II Plus microcomputer controlled the data acquisition and experimental programs. Experimental events were timed to a resolution of 10 msec 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 relayed through a Massey Dickinson logic circuit. This circuit converted each heart beat to a +5-volt square wave pulse which was then transfered 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 sec. In order to reduce the influences of movement artifacts, IBIs that were less than 90 msec or greater than 300 msec were automatically scored as errors as were IBIs that differed from the previous interval by more than 30 msec. 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 offline BASIC program.

At the beginning of each trial HR measurements were taken for a 6-sec period just prior to the CS. An average HR in BPM was generated from this 6-sec pre-CS period and this measure represented baseline HR for that trial. Difference scores were calculated by the offline BASIC

program for each trial by subtracting the pre-CS BPM rate from the BPM rate during three 2-sec measurement intervals for the CS period and during three 2-sec measurement intervals following the UCS.

Experimental Procedure

Before the start of the experiment, 18 of the animals were randomly assigned to one of three treatment groups (n = 6 for each group). One group received a 10 µg dose of DALA. A second group received a 10 µg dose of DALA in combination with a 5 µg dose of naltrexone. The third group received an equivalent volume of 0.9% saline. A fourth group (n=6) which was not randomly assigned consisted of rats in which DALA (10 µg) was infused in the brain stem below the fourth ventricle. All drugs were dissolved in 0.9% saline and infused in a 1-µl volume. Because little information was available on the pharmacokinetics of DALA, pilot studies were conducted to establish the effective dose and the time course of drug efficacy. This pilot work established that a 10 µg dose of DALA was effective in decrementing the development of HR CRs when administered into the fourth ventricle as long as the conditioning session did not exceed one hour. In addition, this pilot work showed that a 5 µg dose of naltrexone was sufficient to block the decremental effects of DALA administration on HR CRs. The experimental procedure covered a span of 6 consecutive days. Table 1 summarizes the procedures 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 1 hr.

Day 2 . Once the animals were placed in the experimental chamber on Day 2, they were given a 15-min habituation period prior to the start

Table 1. Experimental Procedure

DAY 1	Habituation (1 hr)			
DAY 2	Habituation (15 min)	Drug Infusion (5 min)	CS Alone 2CS+, 2CS-	Conditioning 12CS+, 12CS- (52 min)
DAY 3	Habituation (15 min)	Drug Infusion (5 min)	Conditioning 12CS+, 12CS- (45 min)	
DAY 4	All groups left in home cages			
DAY 5				
DAY 6	Habituation (15 min)	Nonreinforced trials 4CS+, 4CS- (22 min)		

of the experiment. Heart rate was recorded during this period to provide information on preinjection baseline HR.

Following this period, each group was infused with the appropriate compound. Heart rate was recorded for 4 trials with a 30 sec intertrial interval (ITI) beginning 1 min following the infusion. Including the duration of each trial, this time of approximately 5 min, allowed for drug diffusion to receptor sites. All groups were then given two presentations of each CS to assess the HR orienting responses to the novel CSs.

The conditioning phase began after the CS alone phase. In the conditioning phase only 12 CS+ and 12 CS- trials were presented to ensure that the drug effects would not dissipate while the conditioning trials were being given. The trials were given in a quasi-random order with the stipulation that no more than three trials of a given type could occur consecutively. The intertrial interval was varied randomly between 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 following drug infusion was approximately 52 min.

Day 3 . The procedure on Day 3 was similar to that used on Day 2. Baseline heart rate was recorded prior to and following drug infusion. Again only 12 CS+ and 12 CS- conditioning trials were given immediately following the post-injection period. The timing of the conditioning trials was identical to that given on Day 2. The length of the conditioning session was approximately 45 min.

Days 4 and 5 . After Day 3, the groups were left in their home cage for 2 days and received no treatments. It was felt that this two day period would allow for the dissipation of any residual drug effects of

DALA.

Day 6 . Following the 2-day rest period, 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-drugged state. The test trials included the random presentations of four CS+ and four 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 DALA group. It was expected that if the animals in the DALA group had learned the CR earlier during conditioning, but were unable to perform it due to the presence of DALA, then the CR should appear when DALA was no longer present.

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 in to the cannula. The brains of those animals in the DALA, SAL, and DALA-NAL groups with dye in the ventricle were saved for sectioning. Brains from the animals in the DALA-BR.ST. group were also saved for sectioning. The brains were blocked so that the section of brain which contained the cannula track was separated from the rest of the brain. Frozen 100-mμ sections were made of the tissue block on a cryostat. The sections were then mounted on glass slides and stained with thionin. The slides were examined microscopically to determine precise location of the cannula in each rat. The stereotaxic atlas of Pellegrino et al. (1979) was consulted to determine actual location.

Data Analyses

The HR responses in this experiment were analysed by a 3-way

analysis of variance (ANOVA). There was one between groups factor and two within groups factors. The factors compared were groups (4) x type of CS (2) x trial blocks (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

All of the animals in the DALA, DALA-NAL, and SAL groups had cannulas located in the roof of the rostral portion of the fourth ventricle. An example of cannula locations in these groups are shown in figure 1. Animals in the DALA-BR.ST. group had cannulas in the brain stem directly ventral to the rostral fourth ventricle infusion site in the region of the medial longitudinal fasciculus. There were 6 subjects in each of these groups.

Baseline Heart Rate

Figures 2 and 3 show the 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 group fell immediately following drug administration and reached a low point during the first block of conditioning trials. During the rest of the conditioning phase on each day, the baseline HR of the DALA group recovered and returned toward but did not reach the saline level. The DALA-BR.ST. group also showed a depression of baseline HR following drug

Figure 1. Photomicrograph of a brain section showing the location of the cannula track in the roof of the rostral fourth ventricle.

Figure 2. Baseline HR for each group during the first day of conditioning. Scores were calculated from mean HR during the pre-CS period of each trial and are averaged over 4 trial blocks during the pre-drug phase, drug-absorption phase, CS-alone phase, and conditioning trials 1-24.

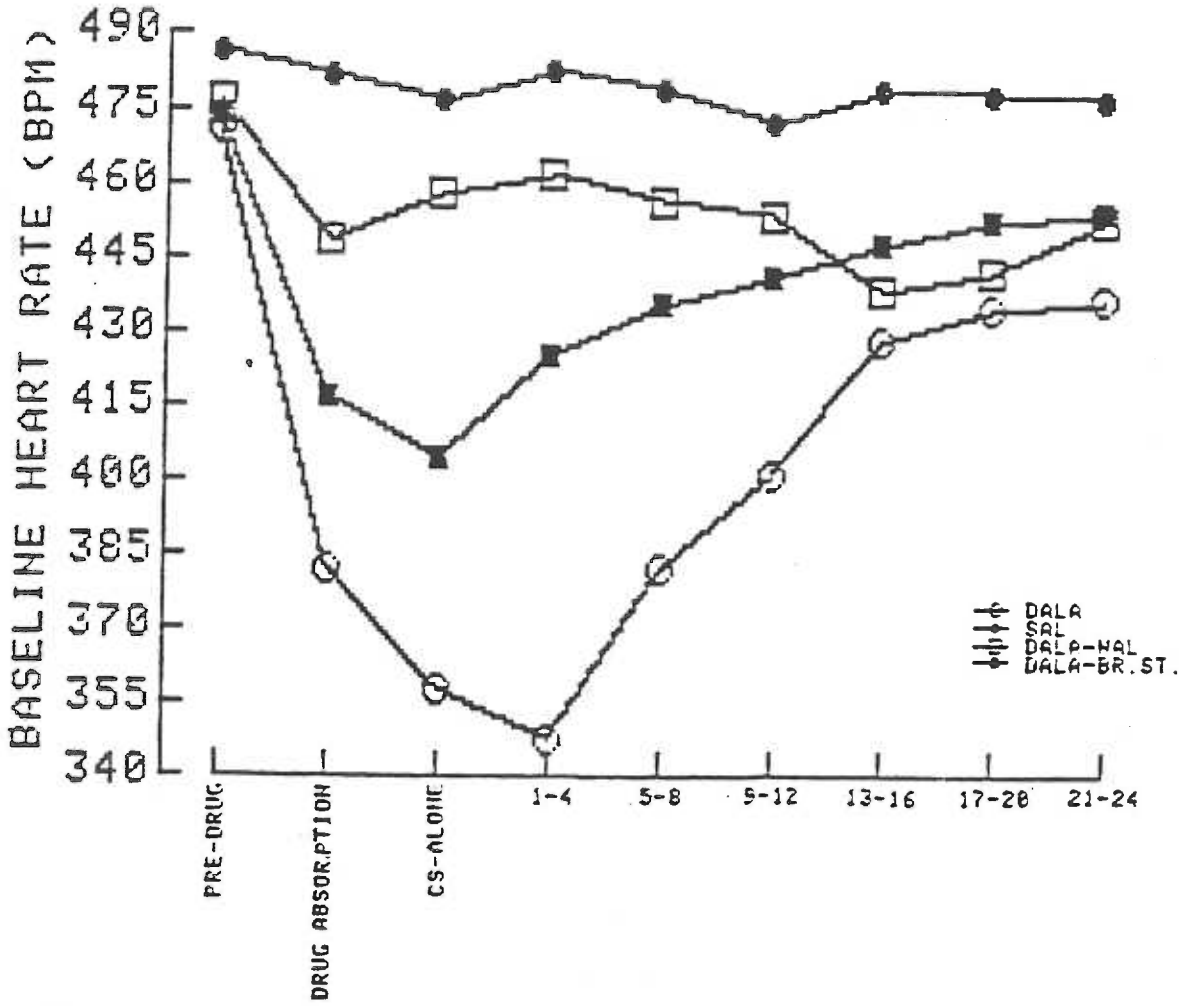
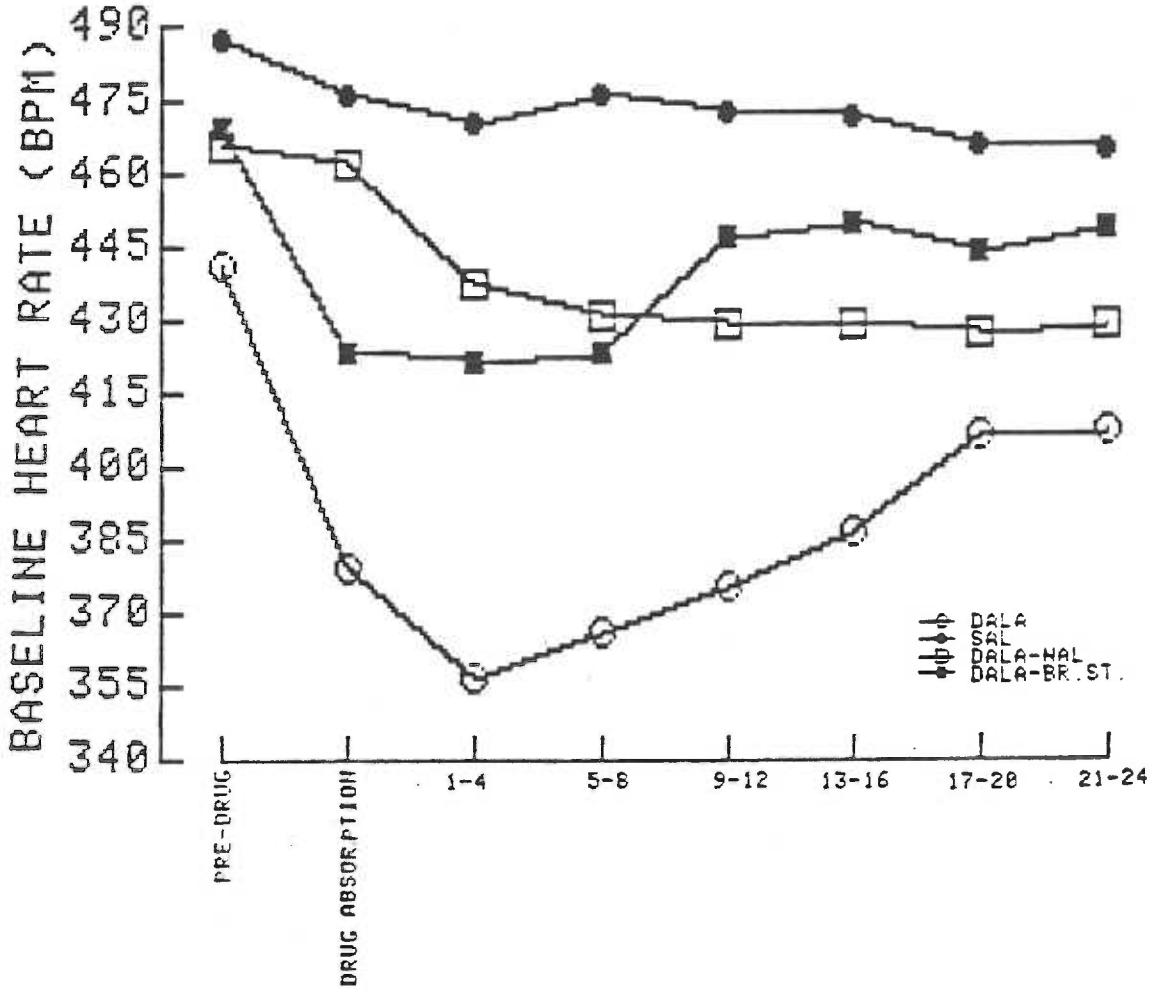

BASELINE HEART RATE (BPM)
490
475
460
445
430
415
400
385
370
355
340
PRE-DRUG
DRUG ABSORPTION
CS-ALONE
1-4
5-8
9-12
13-16
17-20
21-24
DALA
SAL
DALA-NAL
DALA-BR.ST.

Figure 3. Baseline HR for each group during the second day of conditioning. Scores were calculated from mean HR during the pre-CS period of each trial and are averaged over 4 trial blocks during the pre-drug phase, drug-absorption phase, and conditioning trials 1-24.


BASELINE HEART RATE (BPM)
490
475
460
445
430
415
400
385
370
355
340
PRE-DRUG
DRUG ABSORPTION
1-4
5-8
9-12
13-16
17-20
21-24
DALA
SAL
DALA-NAL
DALA-BR.ST.

administration on each day. However, the magnitude of the response was not as great as that seen in the DALA group. The DALA-NAL group showed only a gradual decline in HR on Day 1 and on Day 2. A small decline in baseline HR over trials on each day was also seen in the SAL group.

For Day 1, a 4 x 9 (Groups x Trial Blocks) ANOVA provided a significant groups effect, $F(3,20) = 19.11$, $p < .01$, a significant trial blocks effect, $F(8,160) = 8.70$, $p < .01$, and a significant groups x trial blocks interaction, $F(24,160) = 3.47$, $p < .01$. Newman-Keuls tests at each trial block showed that the groups were not significantly different from each other during the pre-drug phase. Subsequently, the DALA group was found to be significantly lower than the SAL group during the drug absorption phase, CS-alone phase, and conditioning trials 1-16, $p < .01$. In addition, the DALA group was found to be significantly lower than the DALA-NAL group during the drug absorption phase, CS-alone phase, and conditioning trials 1-12, and the DALA-BR.ST. group during the CS-alone phase and conditioning trials 1-12, $p < .01$. The DALA-BR.ST. group was found to be significantly lower then the SAL group during the drug absorption phase, the CS-alone phase, and conditioning trials 1-4 and was significantly lower than the DALA-NAL group during the CS-alone phase, $p < .05$. The DALA-NAL group did not differ from the SAL group on any trial block.

On Day 2, a 4 x 8 (Groups x Trial Blocks) ANOVA produced a significant groups effect, $F(3,20) = 12.04$, $p < .01$ and a significant trial blocks effect, $F(7,140) = 6.54$, $p < .01$. A follow-up Newman-Keuls test showed that overall, the DALA group had a significantly lower baseline HR than the other groups, $p < .01$. No other group differences were significant.

Baseline HR during the test phase is shown in Figure 4. All groups showed similar baseline HR levels at this time. A 4 x 8 (Groups x Trial Blocks) ANOVA proved that there were no significant differences in baseline HR between groups on this day. This outcome indicates that there were no residual effects on baseline HR due to previous drug exposures.

Orienting Responses

Figure 5 depicts HR orienting responses (ORs) to CS+ and CS- for each group at each trial. This figure shows that in all of the groups the bradycardia OR to the CS+ was greater than that to CS-, especially on the first CS alone trial. A 4 x 2 x 2 (Groups x CS Type x Trials) ANOVA revealed a significant effect for CS type, F (1,20) = 16.34, $p < .01$, a significant effect for trials, F (1,20) = 20.34, $p < .01$, and a significant CS type x trials interaction, F (1,20) = 5.33, $p < .05$, however, no significant group differences were found. The failure to find a group effect was attributed to the fact that a few animals in the SAL, DALA-NAL, and DALA-BR.ST. groups showed very large ORs (-100 BPM) in response to the first CS+ presentation, creating large variability within these groups.

A F max test for homogeneity of variance on these data reached significance at $p < .05$, indicating that group variances were not homgeneous. Consequently, a Kruskal-Wallis one-way ANOVA was run on each trial. This test indicated that the groups were significantly different in responding to CS+ on trial 1, H (3) = 8.38, $p < .05$. No other significant group differences were found. A follow-up Mann-Whitney U test showed that the DALA group had a lower magnitude OR

Figure 4. Baseline HR for each group during the non-drug test day. Scores were calculated from mean HR during the pre-CS period of each trial and are shown at each trial during the pre-test habituation phase and averaged over 2 trial blocks during the CS-alone test phase.

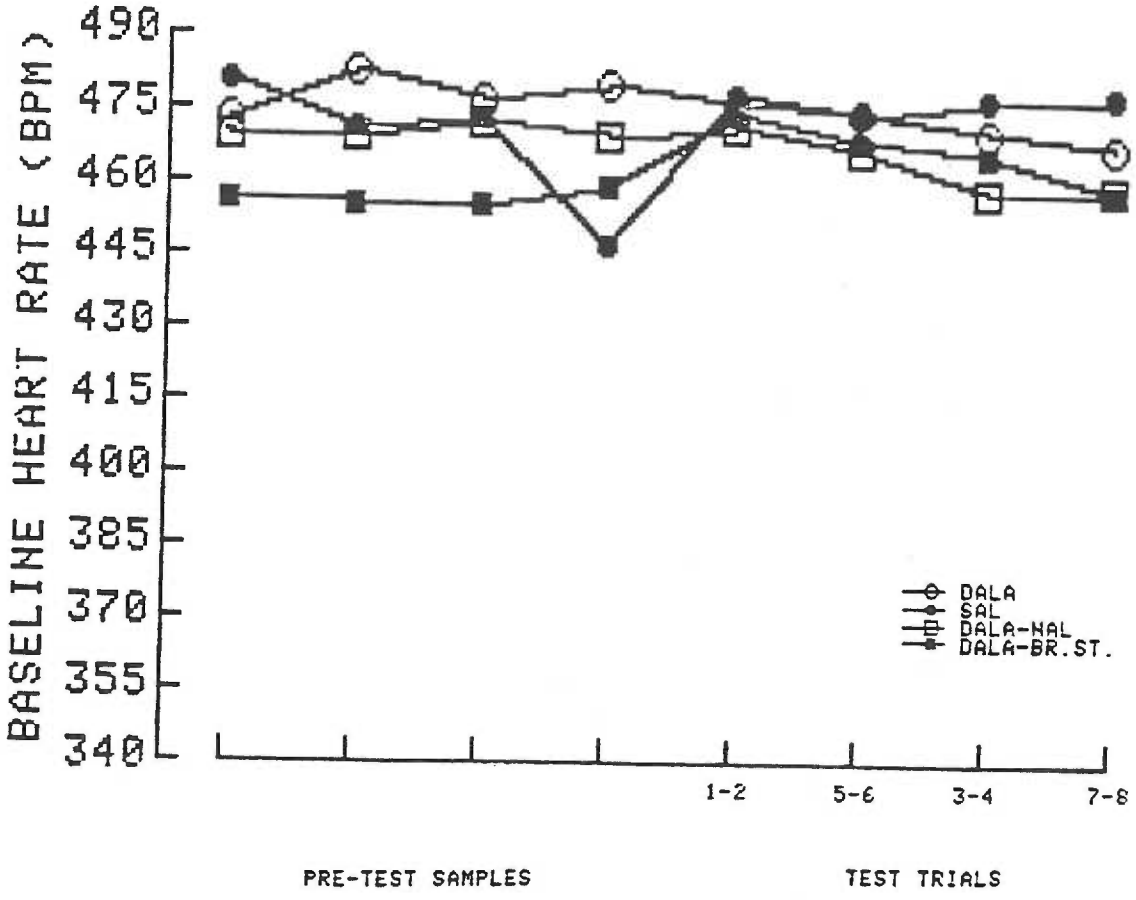
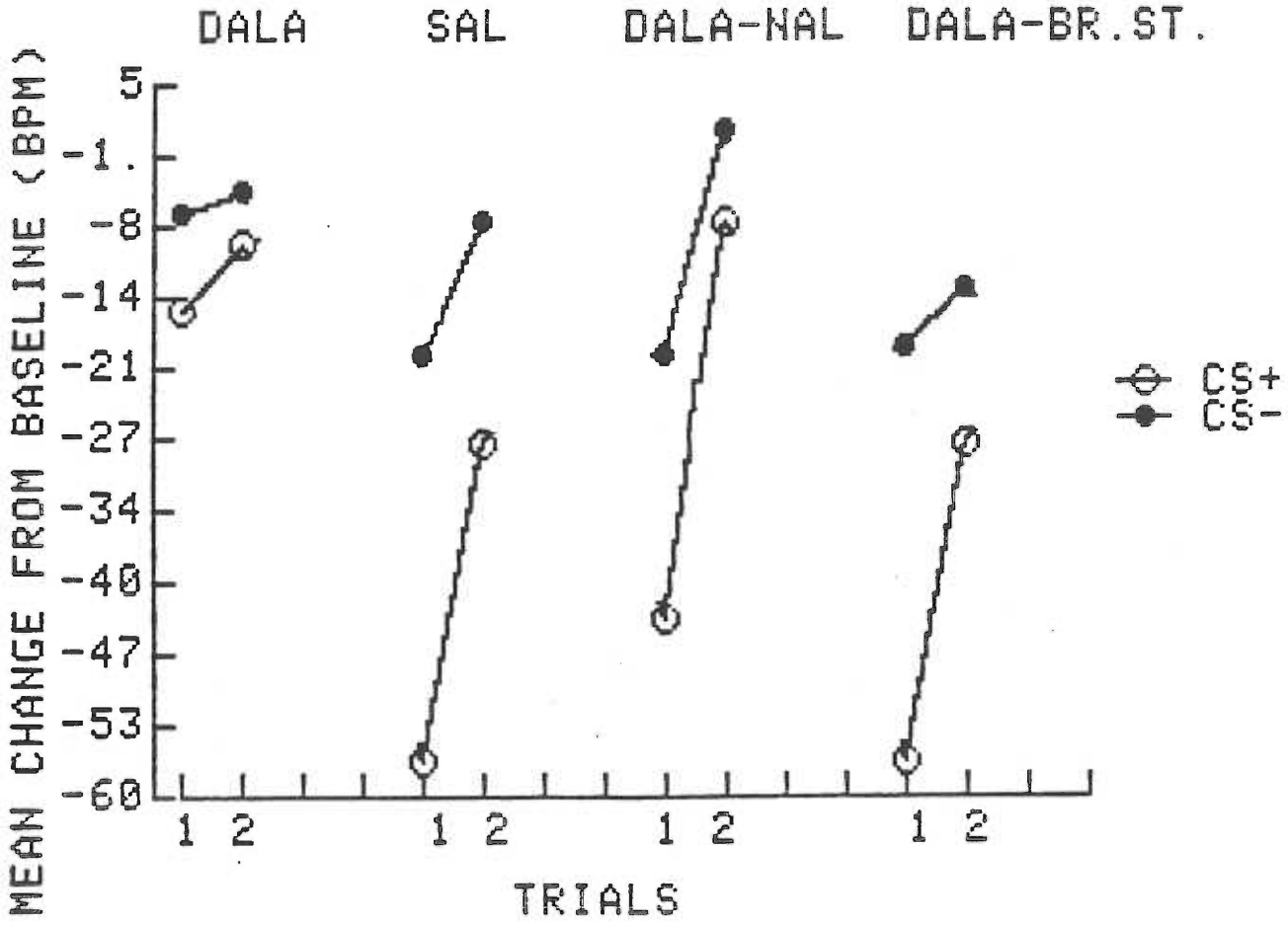

BASELINE HEART RATE (BPM)
490
475
460
445
430
415
400
385
370
355
340
DALA
SAL
DALA-NAL
DALA-BR.ST.
1-2
5-6
3-4
7-8
PRE-TEST SAMPLES
TEST TRIALS

Figure 5. Heart rate orienting responses displayed as mean CS HR change from pre-CS HR during each CS-alone trial. Separate responses for CS+ and CS- are shown for each group.


DALA
SAL
DALA-NAL
DALA-BR.ST.
MEAN CHANGE FROM BASELINE (BPM)
5
-1.
-8
-14
-21
-27
-34
-40
-47
-53
-60
1 2
1 2
1 2
1 2
TRIALS
CS+
CS-

to CS+ on the first trial when compared to the SAL group, U (6,2), $p < .05$ and the DALA-BR.ST. group, U (6,4), $p < .05$.

A t test showed that the ORs in the DALA group were significantly below baseline, t (10) = 2.58, $p < .05$ for CS+ and t (10) = 3.21, $p < .05$ for CS-. A Wilcoxon test on CS+ and CS- responding in all groups revealed CS+ responding was significantly different from CS- responding during both trials, $z = 3.53$, $p < .01$, one-tailed for trial 1 and $z = 2.14$, $p < .01$, one-tailed for trial 2.

Conditioned Responses

Day 1 Conditioning. Figure 6 shows the mean HR responses of each group to CS+ and CS- averaged over successive blocks of 4 trials on Day 1. It may be seen that the DALA group showed very small bradycardia responses to CS+ which were only slightly larger than the HR decreases to CS-. By contrast, the DALA-NAL and DALA-BR.ST. groups showed the development over trials of a major decelerative HR CR to CS+ that was comparable to each other and to that displayed by the saline group. All three of these groups also showed considerable bradycardias to CS-.

The reliability of the conditioning outcomes was tested by means of a 4 x 2 x 3 (Groups x CS Type x Trial Blocks) ANOVA. There was a significant groups effect, F (3,20) = 8.21, $p < .01$, a significant CS-type effect, F (1,20) = 24.82, $p < .01$, and a significant trial blocks effect, F (2,40) = 8.68, $p < .01$. In addition, there was a significant groups x CS-type interaction, F (3,20) = 3.68, $p < .05$, a significant CS type x trials interaction, F (2,40) = 11.55, $p < .01$, indicating that conditioning occurred over trials, and a significant groups x CS type x trial blocks interaction, F (2,40) = 2.80, $p < .05$, demonstrating that the level of conditioning was not the same in all

groups.

The three-way interaction was tested with separate 4 x 3 (Groups x Trial Blocks) ANOVA's on the CS+ and the CS- data. For the CS+ data, the ANOVA revealed a significant groups effect, F (3,20) = 10.46, p < .01, a significant trials effect, F (2,40) = 20.20, p < .01, and a significant groups x trial blocks interaction, F (6,40) = 2.73, p < .05. A subsequent Newman-Keuls test revealed that the decelerative HR responses to CS+ in the DALA group were significantly smaller than those of each of the other groups at each trial block, p < .01. The CS- analysis showed a significant groups effect, F (3,20) = 5.57, p < .01. A follow up Newman-Keuls test again revealed that the DALA group showed a significantly smaller overall response to CS- than did the other groups, p <.05.

Day 2 Conditioning. Figure 7 depicts the mean HR responses of each group to CS+ and CS- averaged over successive blocks of 4 trials on Day 2 of conditioning. It may be seen from this figure that the CRs acquired on Day 1 by the DALA-NAL, DALA-BR.ST., and SAL groups persisted on Day 2 with the DALA group continuing to display no evidence of a HR CR. In the three groups displaying a CR, responding to CS- was generally lower on Day 2 than on Day 1, suggesting that discrimination learning improved on Day 2.

A 4 x 2 x 3 (Groups x CS Type x Trial Blocks) ANOVA revealed a significant groups effect, F (3,20) = 11.48, p < .01, a significant CS type effect, F (1,20) = 41.20, p < .01, a significant trials effect, F (2,40) = 8.39, p < .01, and a significant groups x CS type interaction, F (3,20) = 4.35, p < .05. A follow-up one-way ANOVA on

Figure 6. Heart rate conditioned responses are displayed as mean CS HR change from pre-CS HR averaged over 4 trial blocks. Separate responses for CS+ and CS- are shown for each group during Day 1 of conditioning.

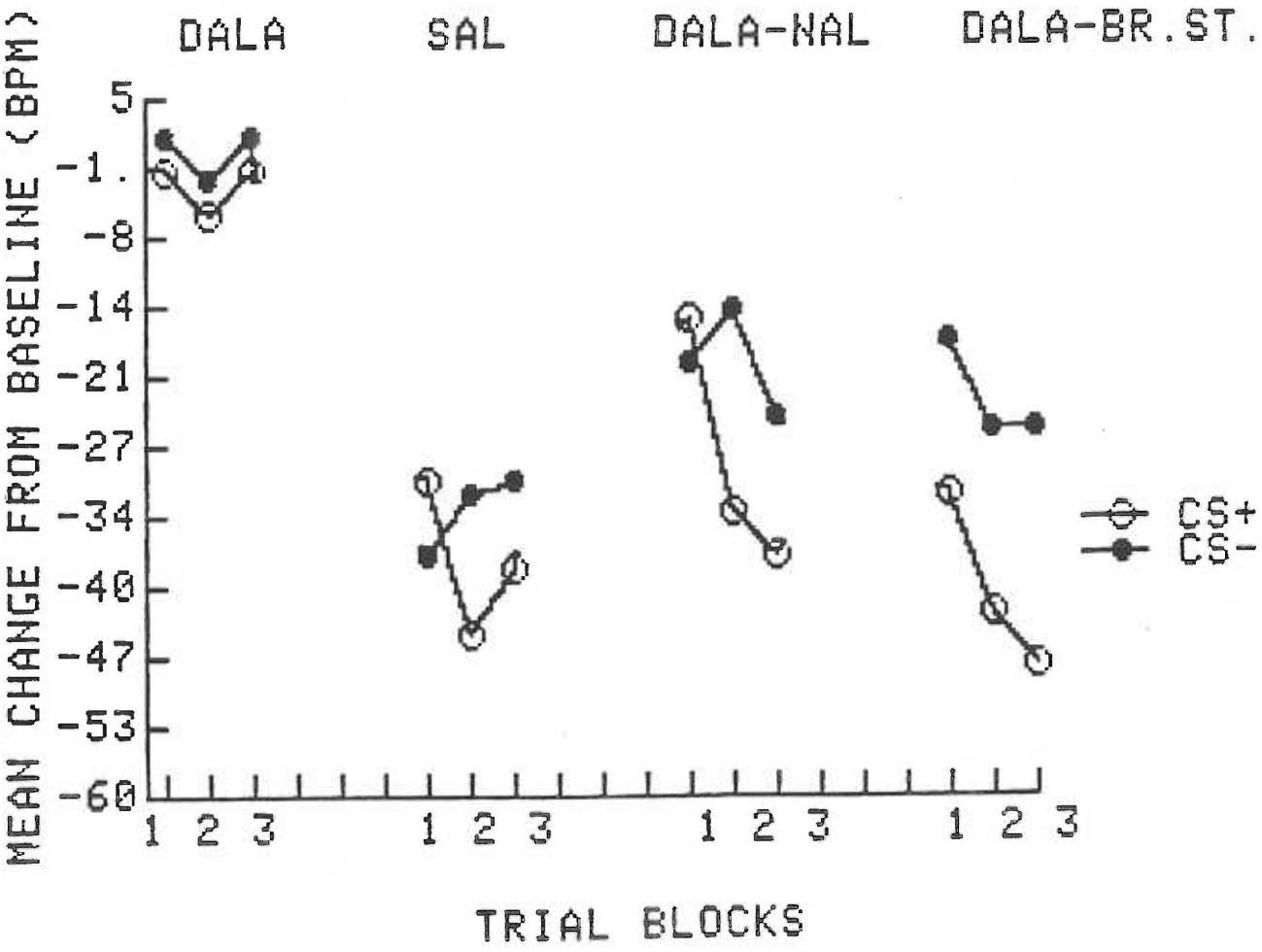
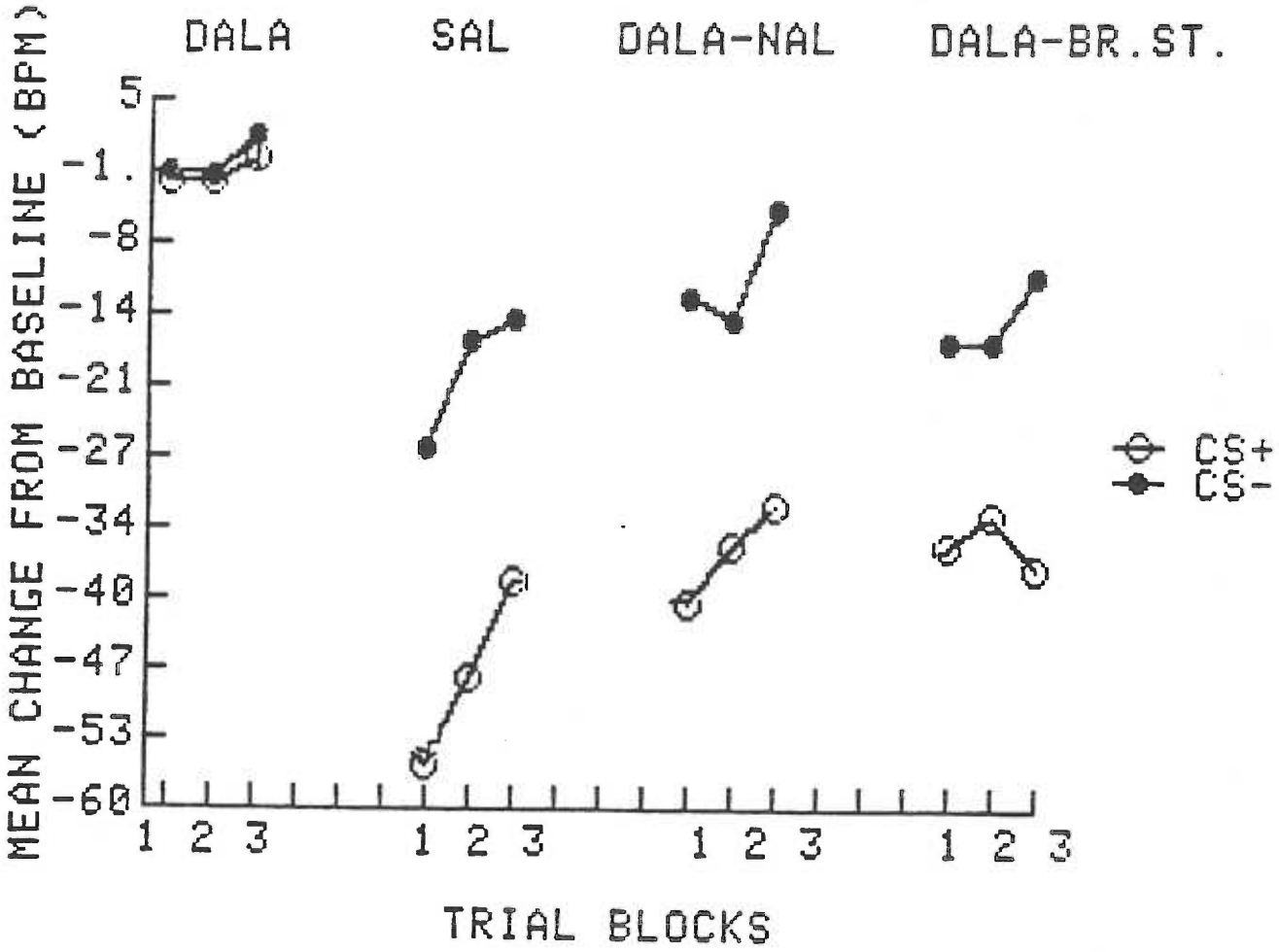

DALA
SAL
DALA-NAL
DALA-BR.ST.
MEAN CHANGE FROM BASELINE (BPM)
5
-1.
-8
-14
-21
-27
-34
-40
-47
-53
-60
1 2 3
1 2 3
1 2 3
1 2 3
TRIAL BLOCKS
CS+
CS-

Figure 7. Heart rate conditioned responses are displayed as mean CS HR change from pre-CS HR averaged over 4 trial blocks. Separate responses for CS+ and CS- are shown for each group during Day 2 of conditioning.

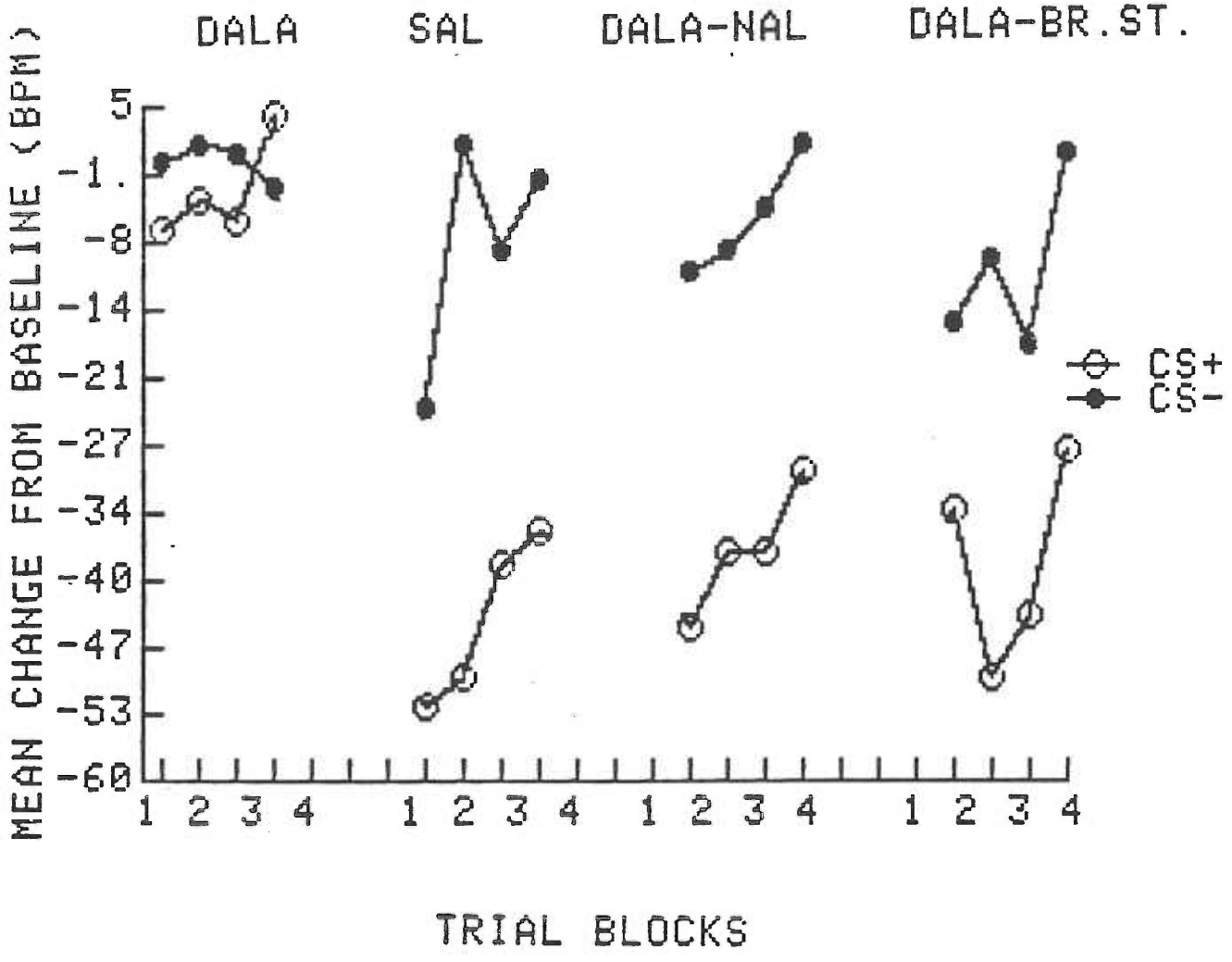

DALA
SAL
DALA-NAL
DALA-BR.ST.
MEAN CHANGE FROM BASELINE (BPM)
5
-1.
-8
-14
-21
-27
-34
-40
-47
-53
-60
1 2 3
1 2 3
1 2 3
1 2 3
TRIAL BLOCKS
CS+
CS-

just the CS+ data provided a significant groups effect, F (3,20) = 16.06, $p < .01$. A subsequent Newman-Keuls test showed that the DALA group was significantly suppressed in their responding to CS+ relative to the other three groups, $p < .01$. A one-way ANOVA on CS- data produced no significant effects.

Test Phase. Heart rate responding on the test trials given two days after the second day of conditioning is shown in Figure 8. The HR responses of the DALA group to CS+ were again small in magnitude, although some increase in size relative to the conditioning days may be seen in the first three test trials. The SAL, DALA-NAL, and DALA-BR.ST. groups continued to show HR CRs to CS+ throughout the test phase, with the magnitude of the CRs decreasing over trials.

A 4 x 2 x 3 (Groups x CS Type x Trial Blocks) ANOVA revealed a significant groups effect, F (3,20) = 16.10, $p < .01$, a significant CS type effect, F (1,20) = 80.22, $p < .01$, a significant trials effect, F (3,60) = 6.50, $p < .01$, and a significant groups x CS type interaction, F (3,20) = 7.31, $p < .01$. A follow-up one-way ANOVA on the CS+ data demonstrated a significant groups effect, F (3,20) = 16.46, $p < .01$. A Newman Keuls test revealed that the responses of the DALA group were significantly attenuated when compared to all other groups, $p < .01$. An analysis of the CS- data showed that the groups were not significantly different in their responses to CS-. Examination of the individual ANOVAs run on each group separately showed a significant conditioning effect (i.e., type of CS) in each group ($p < .01$ in each case), except the DALA group.

Figure 8. Heart rate conditioned responses are displayed as mean CS HR change from pre-CS HR at each trial. Separate responses for CS+ and CS- are shown for each group during the non-drug test day.


DALA
SAL
DALA-NAL
DALA-BR.ST.
MEAN CHANGE FROM BASELINE (BPM)
5
-1.
-8
-14
-21
-27
-34
-40
-47
-53
-60
1 2 3 4
1 2 3 4
1 2 3 4
1 2 3 4
CS+
CS-
TRIAL BLOCKS

Unconditioned Responses

The HR UCRs of each group averaged over trials for Days 1 and 2 of conditioning are shown in Figures 9 and 10 respectively. It can be seen from these graphs that all groups, including the DALA group, exhibted tachycardia UCRs to the UCS. There is some indication that the UCR of the DALA group was slightly depressed relative to the SAL group whereas, the UCR of the DALA-NAL group may have been slightly enhanced. However, a 4 x 3 (Groups x Trial Blocks) ANOVA revealed no significant group differences for Day 1. A separate analysis for Day 2 did show a significant group effect, F (3,20) = 6.12, $p < .01$. No other significant effects were found. A Newman-Keuls test demonstrated that the UCR of the DALA-NAL group was significantly larger than that seen in the DALA-BR.ST. and the DALA groups, but not different from the SAL group, $p < .05$. Inspection of the UCRs of the DALA-NAL group in successive trial blocks indicated that they were consistently greater than the UCRs of the other groups across the entire conditioning session.

A separate one-way ANOVA was run on just the last four trials of the conditioning sessions on Day 1 and Day 2. This was a time when baseline HR did not differ significantly among the groups. No significant group differences were found in UCRs during this time period.

A t Test was used to determine whether the UCR of the DALA group was significantly different from baseline. It was found that the UCRs of the DALA group were significantly greater than baseline on Day 1, t (10) = 3.96, $p < .01$ and on Day 2, t (10) = 2.70, $p < .05$. Another test was done to look at the UCRs of each group on the first shock trial

Figure 9. Heart rate unconditioned responses for each group are shown as mean post-UCS HR change from pre-CS HR averaged over the entire conditioning session on day one.

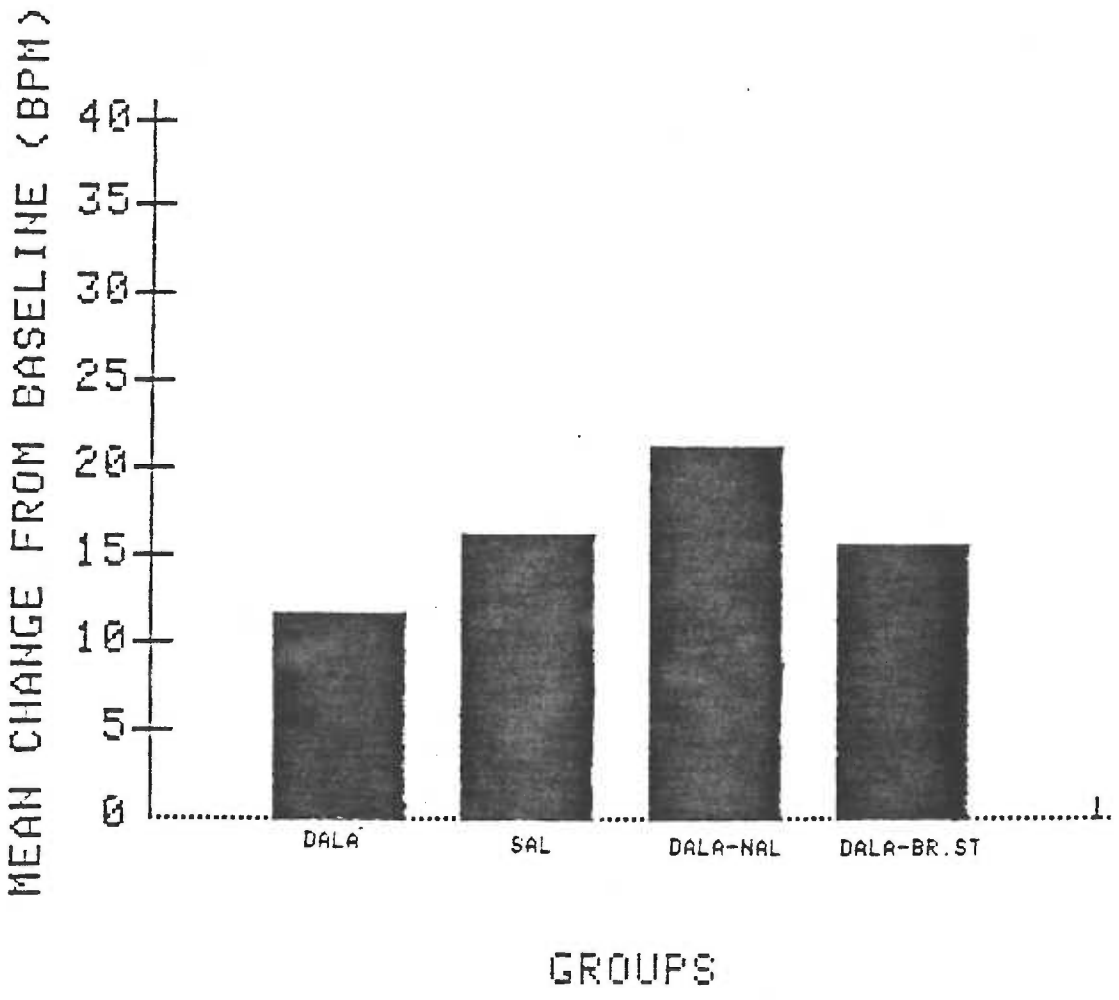
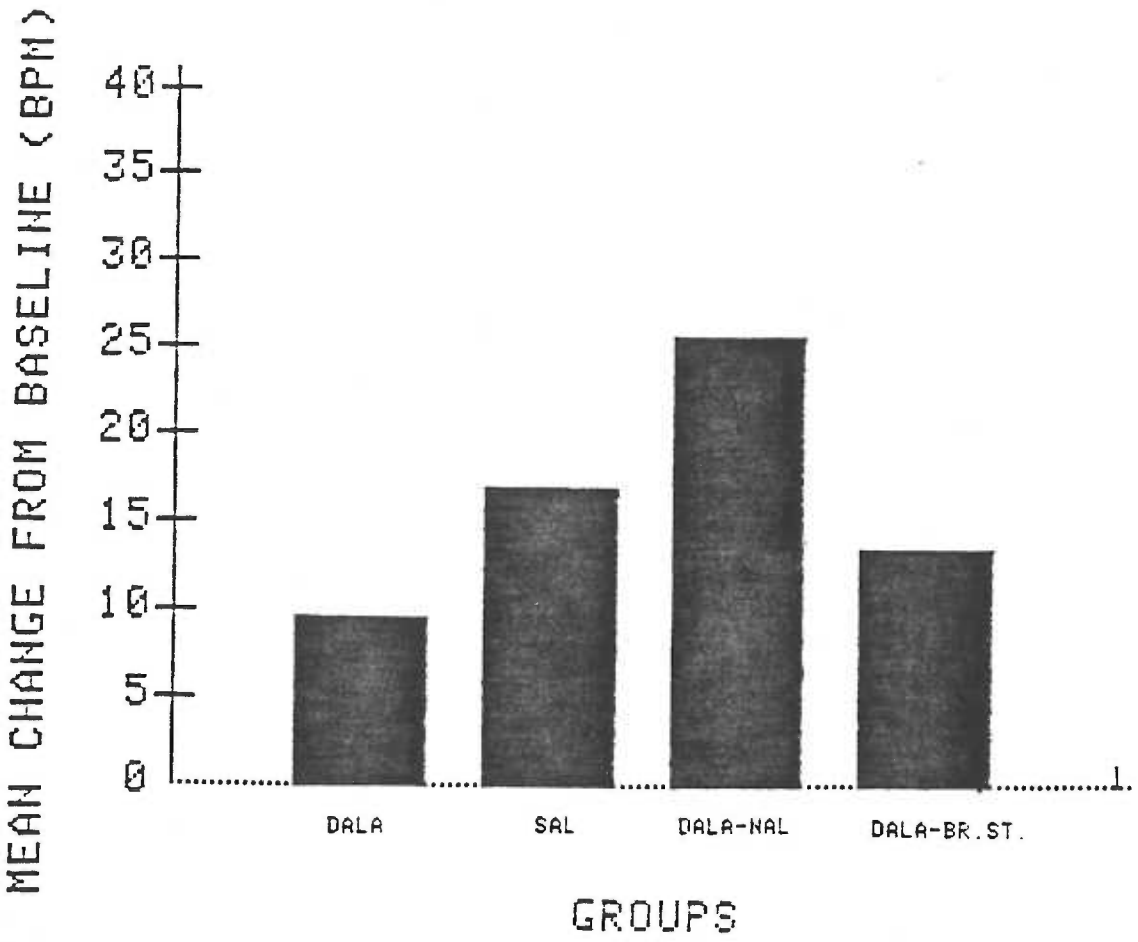

MEAN CHANGE FROM BASELINE (BPM)
40
35
30
25
20
15
10
5
0
DALA
SAL
DALA-NAL
DALA-BR.ST
GROUPS

Figure 10. Heart rate unconditioned responses for each group are shown as mean post-UCS HR change from pre-CS HR averaged over the entire conditioning session on Day 2.


MEAN CHANGE FROM BASELINE (BPM)
40
35
30
25
20
15
10
5
0
DALA
SAL
DALA-NAL
DALA-BR.ST.
GROUPS

of Day 1 and Day 2. The characteristic response to shock on the first trial on Day 1 was a bradycardia. The group means for the DALA, SAL, DALA-NAL, and DALA-BR.ST. groups were -7.08, -24.33, -12.33, -20.92 respectively. A one-way ANOVA revealed no significant group effect. The responses to shock on the first trial on Day 2 were smaller tachycardias and again no significant group effects were found following a one-way ANOVA.

Discussion

In this study, the administration of the opioid compound DALA, into the rostral region of the fourth ventricle prior to conditioning resulted in the complete absence of the development of a HR CR during conditioning. This loss of the CR was evidenced even when the DALA group was tested 48 hrs later in a non-drugged state, suggesting that prior conditioning trials failed to instill a HR CR. It seems unlikely that the absence of conditioning in the DALA group was due to the destruction of neural tissue necessary for the learning or performance of a HR CR because the DALA-NAL and SAL groups had lesions comparable to those in the DALA group and yet both of these groups showed normal HR conditioning. It is more probable that the absence of conditioning in the DALA group was due to activation of opioid receptors which are known to line the ventricular space, particularly the periventricular or periaqueductal gray regions (PVG, PAG) of the brain (Sar et. al., 1978). This conclusion is supported by two findings. First, the concominant administration of the opioid antagonist naltrexone in the DALA-NAL group blocked the adverse effects of DALA on the CR. Second, DALA delivered into brainstem regions below the fourth ventricular space produced no effects on the HR CR, suggesting that the critical location of opioid

receptor activation was in the fourth ventricle.

The bradycardia CR that developed to the CS+ in the SAL, DALA-NAL, and DALA-BR.ST. groups was comparable to those seen in previous experiments using a discrimination paradigm (Hatton et al., 1984; Mahalik & Fitzgerald, 1987). In addition, the responses shown to CS- in these groups was similar to responses that develop to tones using other control procedures (explicitly unpaired or truly random tone shock presentations) (Cunningham, Fitzgerald, & Francisco, 1977). This suggests that the CR shown by these groups in this study was probably due to the pairing of the CS+ with the UCS.

The administration of DALA had a pronounced affect on baseline HR in the DALA group and to a lesser extent in the DALA-BR.ST. group. On Day 1, this change in baseline was seen as an immediate bradycardia occurring after drug infusion with HR showing recovery toward the end of the session. On the second day of conditioning, baseline HR fell only in the DALA group. The reductions in baseline HR are in agreement with the results of several other studies which also have found that opioid administration into the fourth ventricular region of the brain produced bradycardia (Bolme, Fuxe, Agnati, Bradley, & Smythies, 1978; Feldberg & Wei, 1978; Florez & Mediavilla, 1977; Laubie, Schmitt, & Vincent, 1979; Punnen et al., 1984). Here, as in other studies (Bolme et al., 1978; Feldberg & Wei, 1978; Florez & Mediavilla, 1977; Laubie et al., 1979) naltrexone administration was effective in blocking the fall in HR that was seen following DALA administration suggesting that like the CR, this response was also mediated through opioid receptor activation.

It is believed that the loss of the CR occurred independently of the change in baseline HR. This is evidenced by the fact that the

baseline HR of the DALA group begins to recover at the start of the conditioning phase on both days, but no CR develops during that recovery process. The fact that a CR does not develop after baseline HR recovers, suggests that these two effects of the drug may be mediated by the activation of different populations of opioid receptors. One possibility is that the change in baseline HR seen in the DALA group may have involved pathways to nucleus ambiguus (NA) or the drug may have diffused directly to the NA (Laubie, Schmitt, & Vincent, 1979), while the loss of the CR could be mediated by activation of opioid receptors in the PAG or PVG (Lavond et al., 1983). The recovery of baseline HR could have been due to compensatory adjustments in the cardiovascular system which work to raise cardiac output following a fall in HR. The presence of shock during the conditioning phase may also have worked to bring baseline HR back toward its original level because shock has been shown to activate the sympathetic nervous system (Fitzgerald, 1976). The activation of the sympathetic nervous system would counteract the effects of the opioid compound which works to lower HR by activation of the parasympathetic nervous system (Holaday, 1983).

The administration of DALA in the fourth ventricle only affected performance of the bradycardia OR to the CS+ on the first presentation. A previous study (Mahalik and Fitzgerald, 1987), which used sc administration of morphine, found that various doses (0.25, 5.0, 10.0 mg/kg) had a small but reliable effect on the performance of bradycardia ORs. The larger ORs found in general to the CS+ in the current study, probably reflects the fact that intermittent tones are more salient. Some evidence for this view comes from a study done by Papsdorf, Fishbein, and Gormezano (1964). In their study, it was shown that the

acquisition of a NM CR was faster if the CS was an intermittent tone than if it was a continuous tone. It may be that a small component of the OR to an intermittent tone involves emotional arousal. If this is true, then it might be assumed that the DALA group could have been decremented in their initial responses to the CS+ due to a drug induced depression of emotionality.

The administration of DALA had no significant effects on the performance of tachycardia UCRs relative to the SAL group. The UCRs of the DALA group and the DALA-BR.ST. group were, however, significantly depressed relative to those of the DALA-NAL group on Day 2. The UCRs of the DALA-NAL group, though not significantly elevated above the SAL group, might have been increased by naltrexone blockade of endogenous opioid receptors in the fourth ventricle of the brain. The endogenous activation of these receptors might normally work to dampen the actions of the sympathetic nervous system through regulation of parasympathetic outflow. It has previously been shown that opioid administration into the fourth ventricle of the brain increases vagal outflow (Holaday, 1983). Inhibition of this opioid-mediated increase through the administration of naltrexone may lead to enhanced sympathetic outflow. Some evidence for this possibility comes from a study which showed that the response to sympathetic nerve stimulation was enhanced following peripheral naloxone administration (Montastruc, Montastruc, Morales-Olivas, 1981). Because the HR UCR has been shown to be regulated by the level of sympathetic activity (Fitzgerald, 1979; Pappas, DiCara, & Miller, 1972), an increase in sympathetic outflow would conceivably increase the magnitude of the HR UCR.

There are several possible ways in which DALA could have blocked

the appearance of a HR CR in the current study. One way would be if DALA blocked the performance of the CR. Because DALA lowered baseline HR, it could be assumed that DALA prevented the performance of the HR CR during the training phase because of the Law of Initial Values (Wilder, 1956, 1967). This law implies that the lower the prestimulus level of the response, the smaller will be the change to stimuli which call for a further decrease. From this law, one could assume that the lower baseline HR seen in the DALA group would decrease the magnitude of bradycardia shown to the CS relative to the other groups which had higher baseline HRs. However, the fact that the DALA group failed to show a HR CR when tested without the drug, at a time when baseline HR was comparable to the other groups, argues against the law of initial values as a possible mechanism for the abolition of a HR CR.

The non-drug test data also rule out other possible performance-type explanations for the absence of a HR CR. Once the drug was removed, the animals should have been able to perform the previously learned response had one in fact been learned. A related point is that on the first day of conditioning, the DALA BR.ST. group showed a decrease in baseline HR, but was still able to perform HR CRs. Although the magnitude of change in baseline HR seen in the DALA-BR.ST. was not as great as that seen in the DALA group, the appearence of a HR CR in this group adds strength to the view that DALA's blockade of the CR was not tied to the baseline effects of the drug on HR.

A second explanation for the lack of evidence of a HR CR in the DALA group involves the notion of state dependent learning (SDL). At a general level, the acquisition of a response under a drug condition that fails to occur in a subsequent non-drug test may be the result of SDL

(Overton, 1968). It is the change in the state of the organism from the drug state to the non-drug state that is assumed to produce the loss of the previously learned response. It is possible that the DALA group learned a CR during conditioning, but did not display it because of the direct effects of DALA on the cardiovascular system. Further, because of the change in drug state that occurred between the training phase and the test phase, the CR was also not shown on the non-drug test trials.

Some support for this state dependent viewpoint is given by the fact that opioid compounds have strong stimulus properties that can affect the nature of the conditioning situation by changing either the stimulus conditions, the perception of stimulus events, or the affective state of the animal (Hill, Jones, & Bell, 1971; Hirschhorn & Rosecrans, 1974). However, in the present study the failure of the DALA group to show a CR during the training phase makes it difficult to treat the absence of a CR in the test phase as being due to state dependent affects. According to Overton (1968), demonstrations of SDL can only be determined in those tasks where there is acquisition of the learned response in spite of the generally disabling effects of the drug (Overton, 1968), and this did not occur in the present study. While the possibility of the occurrence of SDL in this study cannot be ruled out, neither can it be conclusively proven to exist.

In this connection, it is interesting to note that the DALA-BR.ST. group which experienced a similar, although not identical, change in drug state as the DALA group, showed no decrement in performance during the test phase. This observation suggests that the alteration in drug-produced stimulus events from the training to the test phase was not a major factor controlling the appearance of a HR CR.

Other possibilities which might explain the absence of a HR CR in the DALA group include changes in CS sensory processing. It has previously been proposed that peripherally administered opioid compounds may retard the development of CRs by altering the sensory processing of a tone CS (Schindler, Gormezano, & Harvey, 1984). Some evidence which has been cited in support of this hypothesis is the fact that morphine has been shown not to affect UCR amplitude as much as it does NM CR amplitude (Schindler, Gormezano, & Harvey, 1983, 1984). And second, that morphine given after training decreases the percentage of CRs performed at a variety of different tone intensities (Schindler, Harvey, & Gormezano, 1984). While it is possible that morphine could affect the sensory processing of a tone CS, current theories of opiate action do not lend much support to this view. For instance, it has been reported that morphine relieves pain without depressing vision, hearing, touch, or pressure sense (Julien, 1981). In the current study, the lack of known opioid containing auditory pathways in the rostral fourth ventricle suggests that DALA could not have a direct effect on CS sensory processing. However, it is possible that DALA could have indirect influences on CS sensory processing. If DALA were found to decrease arousal levels in the brain, then it could potentially affect the processing of CS information.

Other evidence has indicated that opioids have greater effects on the processing of aversive sensory events than on neutral sensory events (Warren and Ison, 1982). In this case, iv morphine was found to have a dose related depressive affect on the response amplitude to shock induced startle but not to tone induced startle. It would be expected that if iv morphine decreases the subjective intensity or salience of a

tone CS then this would be reflected in a reduced startle response to a tone. In addition, when a shock immediately precedes a tone presentation, startle to the tone decreases. Morphine was found to have no effect on the ability of shock to inhibit tone startle (Warren & Ison, 1982). The fact that morphine decreased the startle response to shock and not to a tone, and the fact that morphine had little effect on reflex inhibition produced by shock, together suggest that opioid compounds may interfere with the nociceptive properties of shock while leaving sensory processes of other more neutral stimuli intact.

A likely possibility which might explain how opioid compounds interfere with learning would be through a decrease in UCS sensory processing resulting from the antinociceptive properties of these compounds. Although analgesia is widely observed (Martin & Jaffe, 1985) with the administration of opioids, the mechanisms by which it occurs are not entirely understood. It is presently believed that the analgesic actions of opioid compounds result not only from inhibition of primary pain afferents at the level of the spinal cord but also from an altered emotional perception of the painful stimulus (Kelly, 1985). Distinctions can be made between pain as a specific sensation subserved by distinct neurophysiological structures and pain as emotional suffering (i.e. the original sensation plus the emotional reactions evoked by that sensation) (Sternback, 1978). There is general agreement that all types of painful experiences include both the original sensation and the emotional reaction to that sensation (Sternback, 1978). It has been reported that opiate compounds in humans are characterized by a less specific blunting of pain sensation and a more specific production of a state of indifference or emotional detachment

from the experience of suffering (i.e., frequently patients receiving opiates report that the pain is still present but they are no longer concerned about it) (Martin & Jaffe, 1985). Thus, it would appear that opioids can work not only through altering the sensory perception of painful events but also may decrease the emotional suffering associated with painful events.

Opioid analgesia could decrement conditioned emotionality which in turn could explain opioid-produced losses in aversively mediated CRs (Lavond et al., 1983; Mauk et al., 1982). Although the role of fear in the development of various CRs is not known, it seems plausible to suggest that opioid administration could affect HR conditioning by decreasing the aversiveness of the UCS thereby decreasing the ability of the UCS to elicit an emotional response. Previously, HR CR magnitude has been shown to relate positively to the intensity and probably to the noxiousness of the UCS (Fitzgerald & Teyler, 1970; Kamin & Brimer, 1963; Smith, 1969).

The associative strength that underlies the development of a HR CR may be dependent upon the aversiveness of the UCS. It could also be assumed that the amplitude of the HR UCR would provide information on UCS aversiveness. However, in the study by Mahalik and Fitzgerald (1987), it was found that while both 5 and 10 mg/kg doses of sc morphine disrupted HR UCR performance, only the 10 mg/kg dose had a decremental affect on the development of a HR CR. The authors suggested that perhaps UCR amplitude may not be a good measure of aversiveness. Thus, the ability of different doses of morphine to alter UCR aversiveness may not be reflected in HR UCR amplitude when a near zero level of responding is produced by all of the doses. The lack of variation in

UCR amplitude between the 5 and 10 mg/kg groups in the Mahalik and Fitzgerald (1987) study was thought to be due to a kind of floor effect where a further decrease in UCR amplitude in the 10 mg/kg group could not be measured (Mahalik & Fitzgerald, 1987).

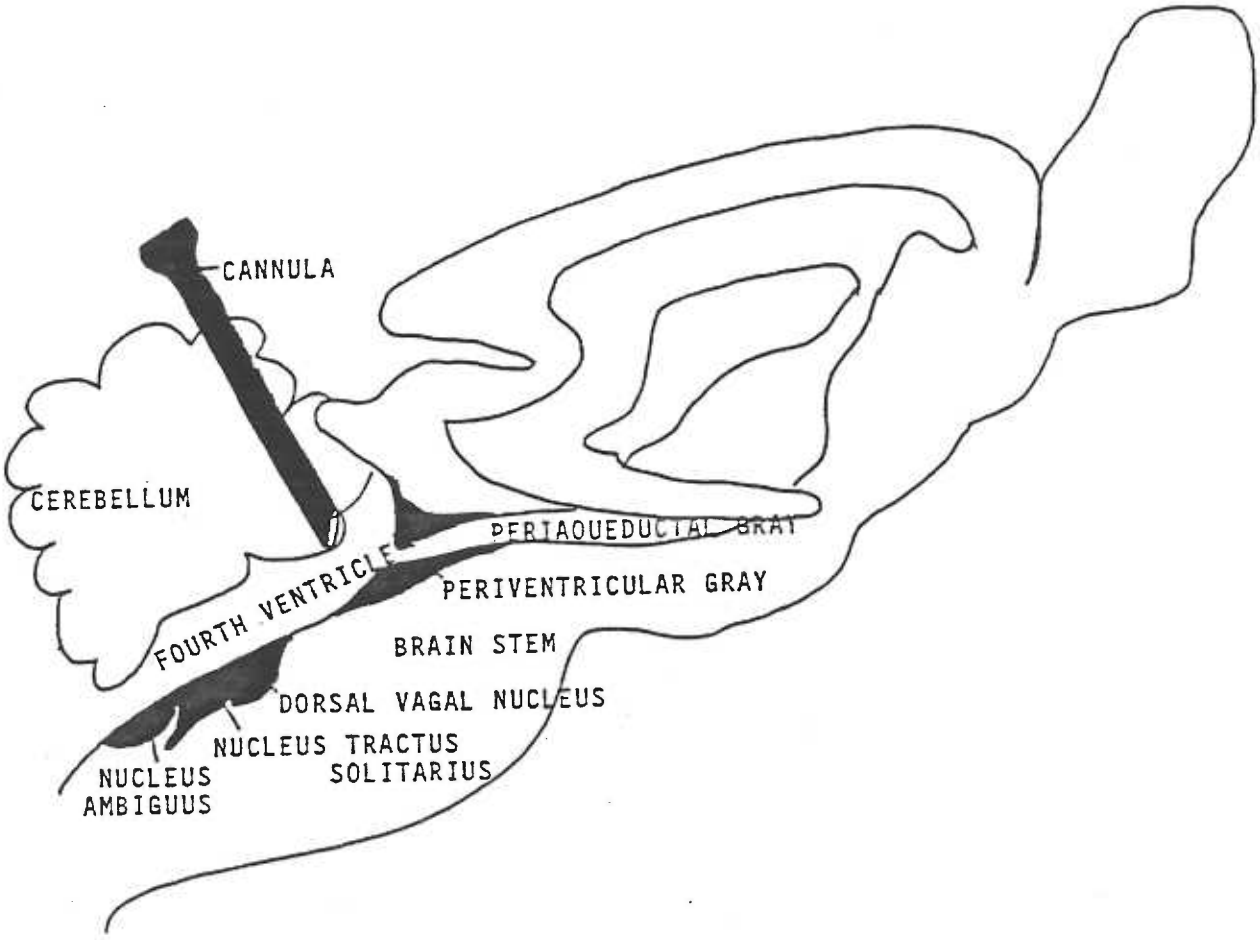
In the present study and in the study by Lavond et al. (1983), no significant decrements in the HR UCR were seen after central opioid administration. This finding, which is contrary to the findings of Mahalik and Fitzgerald (1987), could be due to differences in the route of administration. Peripherally administered compounds may have effects on the cardiovascular system that are not seen when the compounds are administered into the rostral portion of the fourth ventricle. For instance, perpherially administered opioids are known to promote the release of histamine from mast cells which then causes vasodilation and hypotension to occur (Evans, Nasmyth, & Stewart, 1952). Previously it has been shown that drugs which produce hypotension will decrease HR UCRs (Fitzgerald, Hatton, & Foutz, 1984). In addition, the baroreceptor reflex which has been shown to be important in mediating the tachycardia UCR to shock (Hatton, Buchholz, & Fitzgerald, 1981; Hatton, Foutz, & Fitzgerald, 1984; Hoffman & Fitzgerald, 1978), is known to be inhibited by opioid administration (Petty & Reid, 1980; 1981; Punnen, Willette, Krieger, & Sapru, 1983). Thus, peripheral administration could decrease HR UCRs by inhibiting the baroreceptor reflex. Finally, because opioid receptors have been found on the heart (Gautret & Schmitt, 1985; Lang et al., 1983), peripherally administered compounds might have direct effects on the heart which would inhibit the production of UCRs. Therefore, the loss of the UCR in the Mahalik and Fitzgerald study may have been due to the interactions of morphine with a wide variety of

systems which would not be encountered with central administration.

In the study done by Lavond et al. (1983), it was suggested that the administration of opioid compounds into the rostral region of the fourth ventricle abolished previously learned HR CRs by interfering with some part of the conditioned fear circuitry that may be necessary for the maintenance of the newly learned HR CR. This view would be consistent with the fact that the PAG and PVG areas adjacent to the fourth ventricle are rich in opioid receptors (Sar et al., 1978) and are thought to help mediate opioid induced analgesia (Jaffe & Martin, 1985). Also, previous research has suggested that both of these areas of the brain may be involved in emotional responding (Adams, 1979; De Molina & Hunsperger, 1959; Olds & Olds, 1962). In this light it has been proposed that the PAG may be important in modulating pain input on the basis of emotional state (Kelly, 1985).

In the present study, the effective site of DALA administration for the abolition of a HR CR was also the rostral region of the fourth ventricle. The position of the cannulas was in an area of the ventricle that bordered the PAG and PVG (see figure 11), which could mean that DALA diffused from the infusion site and abolished the HR CR through activation of receptors in these areas. The fact that the infusion of DALA into certain brain stem regions, as occurred in the DALA-BR.ST. group, failed to decrement the HR CR, suggests that DALA's adverse effects in the DALA group were not mediated by opioid receptor occupation in this region. Additional evidence mentioned above supporting the possible involvement of the PAG and PVG in the loss of the HR CR seen in this study comes from current knowledge showing that both structures are involved in regulating pain and emotional behavior

Figure 11. Diagram of a rats brain with a cannula located in the rostral fourth ventricle. This diagram shows the relationship of the cannula to the PVG and the PAG.


CANNULA
CEREBELLUM
PERIAQUEDUCTAL GRAY
FOURTH VENTRICLE
PERIVENTRICULAR GRAY
BRAIN STEM
DORSAL VAGAL NUCLEUS
NUCLEUS TRACTUS SOLITARIUS
NUCLEUS AMBIGUUS

(Liebman, Mayer, & Liebeskind, 1970; Lewis & Gebhart, 1977; Mayer & Price, 1976).

The PAG and PVG have been shown to have a well-documented role in the supraspinal control of nociceptive transmission (Mayer & Price, 1976). Electrical stimulation of the PAG and opioid microinjection into the PAG have both been shown to inhibit nociceptive reflexes and dorsal horn neurons (Lewis & Gebhart, 1977; Reynolds, 1969; Yaksh & Rudy, 1978; Yaksh, Yeung, & Rudy, 1976) by way of projections to medullary nuclei (Cheng, Fields, & Heinricher, 1986). Microinjections of DALA into the PAG have been shown to produce analgesia responses as measured in a tail flick latency task (Pert, Pert, Chang, & Fong, 1976; Pert, 1976). In addition to the regulation of nociceptive perception, the PAG has also been found to be critical for the normal expression of fear in rats in terms of shock tolerance, shock avoidance, and open field activity (Liebman, Mayer, & Liebeskind, 1970). It would seem, based on this research, that the PAG may play an important role in cardiovascular conditioning through its ability to alter the perception of painful stimuli and perhaps even to alter the fear responses that normally develop to aversive stimuli.

Recently, evidence has been found showing fiber pathways from the central nucleus of the amygdala (CE) to the PAG (Kapp, Wilson, Schwaber, & Bilyk-Spafford, 1986). The CE is known to play an important role in the development of HR CRs (Gallagher et al., 1981; Gallagher, Kapp, and Pascoe, 1982) and is known to be involved in emotional responding (Kaada, 1972). It may be that the result of opioid diffusion into the PAG might be to alter input from the CE and thereby alter the normal circuitry involved in the development of emotional responding. It is

plausible to suggest that DALA administration in this study, could have affected neuronal responding in this area of the brain and attenuated the emotional responses that might be necessary for learning to occur in a classically conditioned aversive HR paradigm.

Another structure rich in opioid receptors (Sar et al., 1978) located in the rostral fourth ventricle which could potentially affect the development of HR CRs is the locus coeruleus (LC). The LC provides the major noradrenergic input to many brain structures, including the CE (Pickel, Segal, & Bloom, 1981), the PAG, the cerebellum, and cerebral cortex (Redmond & Krystal, 1984). Because it receives input from primary pain afferents, it has been proposed to play a critical role in initating feelings of alarm, panic, fear, and anxiety associated with aversive events (Redmond & Krystal, 1984). Electrical stimulation of the LC in primates elicits fear-like behaviors, which can be suppressed by systemic administration of drugs which decrease noradrenergic transmission, such as propranolol, and morphine (Kapp & Gallagher, 1979). Direct applications of opioid compounds to neurons in the LC have been shown to reduce both basal activity and neuronal responses evoked by noxious stimuli (Duggan & North, 1983). The location of the LC with respect to the cannula placement in the rostral fourth ventricle is shown in figure 12. It is possible that if DALA activates opioid receptors in the LC, then it could have prevented the UCS from activating brain structures necessary for HR conditioning. Therefore, it is conceivable the DALA could effect UCS processing through the activation of opioid receptors located in either the PAG, PVG, or LC.

Figure 12. Photomicrograph of a rat brain slice showing the location of the locus coeruleus.

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Appendix: Subject Attrition

DALA group:

Eight animals had the wrong cannula placements (now called the DALA-BR.ST. group).

One animal lost its cannula.

Two animals were lost due to equipment failure.

SAL group:

Four animals were lost because of misplaced cannulas.

Three animals were lost due to a poor HR signal.

DALA-NAL group:

Three animals had wrong cannula placements.

One animal lost its cannula.

Two animals were lost due to equipment failure.

DALA-BR.ST. group:

One animal lost its cannula.

One animal lost due to a poor HR signal.