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Enkephalin Analogue Effects in the Amygdala Central Nucleus on Conditioned Heart Rate[1]

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GALLAGHER, M., B. S. KAPP AND J. P. PASCOE. *Enkephalin analogue effects in the amygdala central nucleus on conditioned heart rate.* PHARMAC. BIOCHEM. BEHAV. 17(2)217–222, 1982.—The experiment was conducted to assess the effects of enkephalin analogue administration into the amygdala central nucleus on the acquisition of classically conditioned heart rate responding in rabbits. Bilateral injections of either D-Ala^2, Met^5-enkephalinamide (DALA) or D-Ala^2, D-Leu^5-enkephalin (DADL) were administered in a 1.0 μl volume into the central nucleus immediately prior to the conditioning session. Administration of DALA significantly attenuated the acquisition of conditioned heart rate responding whereas groups which received comparable doses of DADL exhibited conditioned responding which did not differ from the vehicle-injected control group. The effect on conditioned responding produced by DALA administration was blocked by concurrent administration of the opiate antagonist naloxone. These results provide some support for the involvement of mu receptor activity within the central nucleus region of the amygdala in conditioning processes.

Amygdala central nucleus Heart rate conditioning Enkephalin analogues Opiate receptors Rabbits

CONSIDERABLE evidence has accumulated implicating opioid peptides in some aspect of learning and memory processes (see [19] and [23] for recent reviews). However, it is unlikely that a common mode of action underlies all of the effects of opiate and opioid peptide administration which have been reported in these numerous investigations. For example, while a number of the effects of opiate and opioid peptide administration on learning and memory are blocked by low dose opiate antagonist administration [1, 5, 6, 11, 15], others are not [17,18]. One approach to understanding the mechanisms by which opioid peptide systems may participate in learning and memory processes, at least within the context of selected behavioral testing procedures, is to focus on the contribution of specific neural systems which contain opioid peptides. We have chosen in previous investigations to focus on the amygdala complex which possesses high concentrations of opioid peptides [8,21].

Research conducted in our laboratory demonstrated that opiate manipulations within the amygdala complex altered memory processes in rats [5]. Post-training administration of the opiate agonist levorphanol into the amygdala produced stereospecific decreases in retention of passive avoidance conditioning. Administration of the opiate antagonist naloxone was observed to block retention deficits produced by levorphanol, and naloxone administration, by itself, produced a dose-dependent increase in retention. The increased passive avoidance retention observed following naloxone administration into the amygdala may be due to blocking endogenous opioid peptide activity in this region. Furthermore, since comparable opiate manipulations within other brain regions which possess high concentrations of opiate receptors and opioid peptides (i.e., basal ganglia and periaqueductal grey region of the midbrain) have been reported to have no significant effect on retention of passive avoidance conditioning [5,14], it appears that opiate sensitive systems in different brain regions do not serve a comparable memory function, and that at least a component of the opiate sensitive system which is involved in memory processes is located within the amygdala complex.

More recent research from our laboratory has demonstrated that opiate manipulations within the central nucleus region of the amygdala complex alter the acquisition of classically conditioned heart rate responding in the rabbit [6]. Levorphanol administration into the central nucleus significantly impaired the acquisition of a conditioned bradycardia response. The effect was observed to be stereospecific and

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blocked by concurrent administration of the opiate antagonist naloxone. Naloxone administration, by itself, significantly increased the magnitude of the conditioned bradycardia response. Effects comparable to those produced by opiate administration into the central nucleus were not observed following injection into adjacent sites within the amygdala. Since opioid peptides particularly the pentapeptide enkephalins, are highly concentrated within the central nucleus of the amygdala complex [8,21], these findings suggest that enkephalin activity at opiate receptors within this region may be involved in conditioning processes.

Recent studies using selective radioligands have provided evidence for the presence of two distinct populations of opiate receptors which are distributed in different relative concentrations in various brain regions [2, 7, 16]. Classic opiate agents possess greater affinity for the mu receptor population whereas enkephalins, and in particular the opioid peptide analogue D-Ala^2 D-Leu^5-enkephalin, possess greater affinity for the delta receptor population [2,7]. The possibility has been raised that mu and delta receptors may represent sites which are differentially sensitive to met- and leu-enkephalin respectively [22]. Both mu and delta receptors appear to be located within the amygdala. Although differential labeling of these populations using autoradiographical techniques revealed a predominance of delta receptors in the amygdala of the rat [7], binding assays performed on dissected regions of bovine brain have revealed a comparatively high concentration of mu receptor binding sites relative to delta sites within the amygdala [16]. The results of our previous research using opiate agents would suggest that mu receptors within the amygdala complex, at least in part, contribute to learning and memory processes. In the present investigation we examined the effects of opioid peptide administration into the central nucleus on acquisition of conditioned heart rate responding in the rabbit. Two peptide analogues, D-Ala^2 D-Leu^5-enkephalin (DADL) and D-Ala^2 Met^5-enkephalinamide (DALA), were used in this study. Since DADL has the greater affinity for delta receptors, comparing the effects of DADL and DALA administration may provide some additional information regarding the relative contribution of delta and mu receptor activity within the central nucleus to conditioning processes.

METHOD

Animals

Eighty seven experimentally naive New Zealand Albino rabbits (Canadian Breeding Farms and Laboratories, Ltd.) weighing from 2.2 to 2.7 kg at the beginning of the experiment were used. All animals were maintained on a 12 hr light-dark cycle (lights on from 7:00 a.m. to 7:00 p.m.) and were provided with food and water ad lib.

Surgery and Histology

All animals, with the exception of those in unoperated control groups, were pretreated with chlorpromazine hydrochloride (20 mg in 0.8 cc saline, IV) and anesthetized with Nembutal (30–75 mg, IV). They were mounted in a Kopf stereotaxic instrument fitted with a rabbit headholder, and bregma was adjusted 1.5 mm above the plane of lambda. Bilateral 23 g cannulae were implanted using the following coordinates: 0.1 mm anterior to bregma, 5.7 mm lateral to the midline and 11.8 mm ventral to dura. Immediately following surgery all animals received intramuscular injections of Crysticillin (30,000 units, Squibb and Sons).

Following behavioral testing all animals were sacrificed and perfused with physiological saline followed by 10% formal-saline. Frozen sections (50 μ) were taken through the amygdala and stained with Thionin. Cannula tip placement was determined microscopically with the aid of the stereotaxic atlas of Urban and Richard [25]. Cannula tip placements for all groups, were rated as unacceptable if they were (1) more than 0.5 mm dorsal or ventral to the dorsal surface of the central nucleus; (2) anterior to the central nucleus as represented on plate A 18.5 mm of the Urban and Richard atlas [25]; (3) posterior to the central nucleus as represented on plate A 15.5 mm of the Urban and Richard atlas [25]. Only animals with bilaterally acceptable cannula placements were included in the data analysis.

Apparatus

The apparatus employed in this experiment was identical to that used previously [12]. During conditioning each animal was placed in a Plexiglas rabbit restrainer and positioned in one of four sound attenuating chambers within a shielded, soundproof, IAC room. Shock was delivered through stainless steel dresshooks attached to the upper and lower left eyelids. Stainless steel wire loops positioned subcutaneously, one dorsomedial to the left shoulder and one dorsomedial to the right haunch, were inserted shortly before the conditioning session to serve as EKG recording electrodes. The presentation of stimuli and recording of the EKG on a Grass Instruments Model 78 Polygraph were controlled by solid state programming equipment.

Conditioning Procedure

Following 10–14 days of postoperative recovery, animals in the conditioning groups were habituated to the Plexiglas restrainers for four daily one-half hour sessions followed on the fifth day by a one hour habituation session to the experimental chamber. Two days later the animals were placed into one of the four experimental chambers for the Pavlovian conditioning session. Fifteen presentations of the conditioned stimulus (CS), a 5.0 sec, 1000 Hz, 92 dB tone, were first presented using a random, variable, intertrial interval (80, 90, 100 sec; mean=90 sec). The presentation of fifteen CS alone trials, prior to the onset of paired Pavlovian conditioning trials, was used to habituate the decelerative heart rate orienting response. Without this habituation, any decelerative heart rate changes to the CS during the initial paired conditioning trials could represent, at least in part, orienting responses to a novel stimulus rather than true conditioned responses. Immediately following the 15 CS alone trials, 20 paried conditioning trials were presented, again using a random, variable, 90 sec intertrial interval. The offset of the CS was coincident with the onset of the unconditioned stimulus (US), a 500 msec, 2.0 mA eyelid shock.

Experimental Groups and Injection Procedure

The vehicle used for all injections was a Krebs-Ringer phosphate solution [24]. In addition to the unoperated (UNOP) and vehicle-injected (VEHICLE) control groups, five separate drug-injected groups received administration of either DALA (0.5, 1.0, or 2.0 μg) or DADL (1.0 or 2.0 μg). In addition, a group receiving combined administration of DALA (2.0 μg) and the opiate antagonist naloxone (1.0 μg)

was included in this experiment. Each injection consisted of the dose identified for each group delivered in a 1.0 μl volume. For the group receiving combined injections of DALA and naloxone, each injection consisted of both drugs administered in a 1.0 μl volume. Bilateral injections were administered approximately 5 min prior to behavioral testing. The DALA and DADL analogues were obtained from Peninsula Laboratories, Inc.

Data Analysis

In order to provide a measure of the heart rate response to the tone CS, heart rate was recorded for 5 sec preceding the presentation of the CS and for the duration of the 5 sec CS. The magnitude of heart rate change to the CS for each trial was computed by subtracting the number of beats occurring during the 5 sec CS period from the number of beats occurring during the 5 sec pre-CS baseline period. For each animal the mean heart rate change to the CS was computed for each block of 5 trials. Statistical analyses of heart rate responding during CS alone presentation and during conditioning were performed using these 5 trial means.

A measure of baseline heart rate was provided by the number of beats occurring during the 5 sec pre-CS baseline period. Analyses among groups were performed on baseline heart rate during the 15 CS tone alone trials in order to determine whether drug administration altered baseline heart rate.

A two factor (Groups × Trials) mixed design analysis of variance and orthogonal comparisons were used to analyze the heart rate data.

RESULTS

Histology

Histological inspection of cannula placements yielded bilaterally acceptable placements for seven animals in each group. The data for these animals were included in the data analysis. Representative cannula placements for an animal included in the DADL 1.0 μg group are presented in Fig. 1.

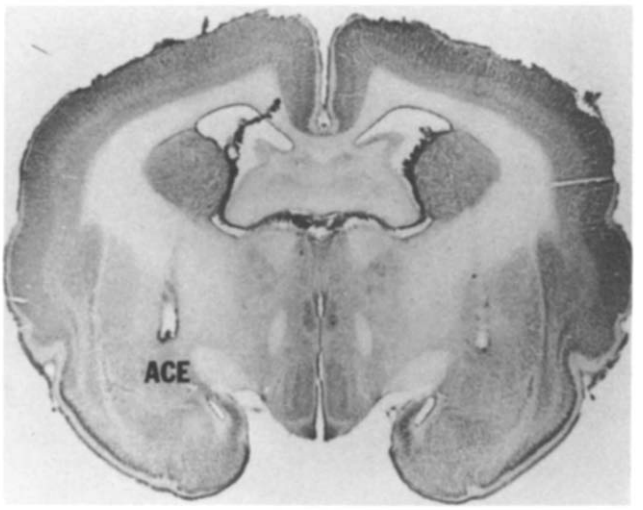

FIG. 1. Cannula placements for an animal in the DADL 1.0 μg group. ACE, amygdala central nucleus.

Heart Rate Conditioning

The heart rate responses to the CS for the control groups (UNOP and VEHICLE) and for the groups receiving DALA (0.5–2.0 μg) injections into the central nucleus prior to the conditioning procedure are presented in Fig. 2. The heart rate responses to the CS for the same control groups and the groups receiving injections of DADL (1.0 and 2.0 μg) into the central nucleus are presented in Fig. 3. Statistical analyses were conducted on baseline heart rate, heart rate responses to the 15 CS alone presentations, and heart rate responses to the CS during paried presentations of the CS and US for each peptide analogue.

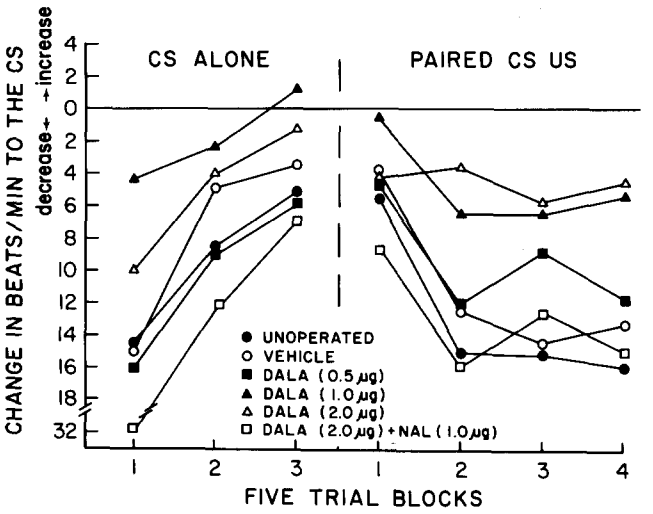

FIG. 2. Mean change in heart rate to the CS from pre-CS baseline for groups. Data points represent means for 5 trial blocks.

Effects of DALA Administration

No significant effects were obtained in the analysis of variance performed on baseline heart rate which included the UNOP, VEHICLE, DALA (0.5, 1.0 and 2.0) and DALA + NALOXONE groups. The analysis performed on heart rate responses to the CS alone presentations for the same groups revealed significant effects for both Groups, $F(5,36)=7.33$, $p<0.0001$, and Trials, $F(2,105)=12.17$, $p<0.0001$, with no significant Groups × Trials interaction. Subsequent orthogonal comparisons revealed no significant difference between the two control groups (UNOP and VEHICLE). Comparisons performed between each drug-injected group and the two control groups revealed that the 1.0 μg DALA group and the DALA + NALOXONE differed from the control groups, $F(1,105)=10.61$, $p<0.002$ and $F(1,105)=7.61$, $p<0.01$, respectively. No significant differences were obtained in the comparisons of the 2.0 and 0.5 DALA groups with the control groups.

Analysis of variance performed on the heart rate responses to the CS during conditioning for the UNOP, VEHICLE, DALA (0.5, 1.0, and 2.0 μg) and DALA + NALOXONE revealed significant effects for Groups, $F(5,36)=11.27$, $p<0.0001$, and for Trials, $F(3,144)=10.97$, $p<0.0001$, but no significant Groups × Trials interaction. Subsequent orthogonal comparisons revealed no signif-

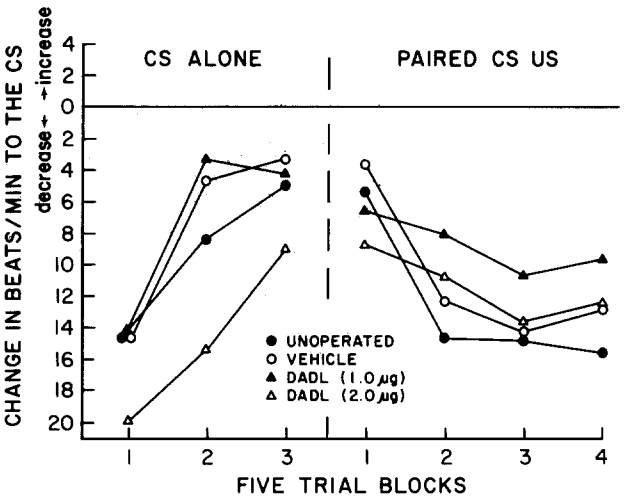

FIG. 3. Mean change in heart rate to the CS from pre-CS baseline for groups. The UNOPERATED and VEHICLE control groups are the same as those represented in Fig. 2. Data points represent means for 5 trial blocks.

icant difference between the two control groups. Comparisons performed between each drug injected group and the two control groups revealed that the 1.0 μg and 2.0 μg DALA groups each differed significantly from the control groups, $F(1,144)=22.61$, $p<0.0001$ and $F(1,144)=30.78$, $p<0.0001$, respectively. The 0.5 μg DALA group did not differ significantly from the control group, $F(1,144)=3.83$, $p<0.052$. In addition, no significant difference was obtained in the comparison of the DALA + NALOXONE group with the control groups. A separate analysis of variance performed on the DALA + NALOXONE and 2.0 DALA groups revealed a significant Groups effects, $F(1,12)=44.02$, $p<0.0001$.

Effects of DADL Administration

No significant effects were obtained in an analysis of variance performed on baseline heart rate which included the UNOP, VEHICLE and DADL (1.0 and 2.0 μg) groups. The analysis of variance performed on heart rate responses to CS alone presentations for these same groups revealed significant effects for Groups, $F(3,24)=5.25$, $p<0.01$, and for Trials, $F(2,72)=13.25$, $p<0.0001$, but no significant Groups × Trials interaction. Subsequent orthogonal comparisons revealed that whereas the 1.0 μg DADL group did not differ significantly from the control groups, a significant difference was obtained in the comparison between the 2.0 μg DADL group and the control groups, $F(1,72)=12.07$, $p<0.01$. The analysis of variance performed on the heart rate responses to the CS during conditioning which included the DADL and control groups revealed no significant effects for either Groups or Groups × Trial interaction.

DISCUSSION

In the present investigation statistical analyses revealed no significant differences between the UNOP and VEHICLE groups for baseline heart rate, heart rate responses to CS alone presentations, or heart rate responding during conditioning. The heart rate data obtained from the control groups in this experiment are similar to those obtained in previous experiments conducted in a number of laboratories including our own [6, 12, 13, 20]. The heart rate orienting response to initial presentations of the CS consisted of a bradycardia which habituated with repeated CS presentations. During paired presentations of the CS and US, bradycardia responses developed rapidly to the CS. Previous studies conducted in our laboratory using the identical stimulus parameters have consistently failed to reveal pseudoconditioned heart rate responses to the CS during unpaired presentations of the CS and US [6,12]. Therefore, the heart rate responses which developed during paired presentations of the CS and US in this investigation would appear to reflect associative conditioning processes.

We observed that administration of the opioid peptide analogue DALA into the central nucleus impaired acquisition of conditioned heart rate responding. Compared to the VEHICLE group, groups injected with either the 2.0 μg or 1.0 μg dose of DALA exhibited significantly decreased conditioned responding. This effect obtained with DALA parallels the effect which we observed in a previous investigation following central nucleus administration of the opiate agonist levorphanol [6]. Since the DALA effect on conditioning in this experiment, as well as the levorphanol effect reported previously, are both blocked by concurrent low dose administration of the opiate antagonist naloxone, it would appear that both agents may alter conditioning by activation of mu opiate receptors in this region. In further support of this suggestion are the results obtained with the peptide analogue DADL in this experiment. Conditioned heart rate responding in groups injected with either the 2.0 μg or 1.0 μg doses of DADL into the central nucleus did not differ significantly from the VEHICLE group. Because the DADL analogue has greater selectivity for delta receptors, these results lend support to the suggestion that delta and mu receptors within the central nucleus region may serve different functions, and that the mu population may play a more critical role in conditioning processes. The proposed involvement of amygdala mu receptors in conditioning processes is consistent with our earlier observation that administration of naloxone, by itself, into the central nucleus produces a significant increase in the magnitude of conditioned heart rate responses, an effect which may reflect blocking endogenous activation of mu receptor activity [6].

Although baseline heart rate was not altered by administration of either peptide analogue in this study, drug effects were observed during orienting/habituation trials. However, the effect on heart rate responding during CS alone presentations obtained with DALA administration was neither dose-dependent nor consistently related to the effect of DALA upon the acquisition of conditioned responding. Whereas the DALA 1.0 μg group exhibited significantly decreased initial orienting responses and significantly decreased responses during conditioning, the DALA 2. 0 μg group exhibited decreased responses during conditioning in the absence of any significant effect on CS alone responding. The effect of DADL on orienting/habituation which occurred at the 2.0 μg dose reflected a normal orienting response but an apparent failure to habituate. Although the presence of heart rate responses to the CS during conditioning trials in this group could reflect a persistence of orienting responses to the CS rather than the development of true conditioned responses, the 1.0 μg DADL group exhibited both normal responding

during the CS alone trials and a normal development of conditioned responding during paired CS and US presentations. Since both the 1.0 and 2.0 μg DALA groups exhibited conditioning deficits and both the 1.0 and 2.0 μg DADL groups exhibited heart rate responses during conditioning which did not differ significantly from the control groups, the isolated effects of these peptide analogues upon CS alone responding do not appear to account for the data obtained during conditioning. Although our results suggest that the effects of opioid peptide administration into the amygdala central nucleus upon orienting and habituation may warrant further investigation, it is worth noting that in our previous research, administration of either the opiate agonist levorphanol or the antagonist naloxone into the central nucleus was not observed to alter heart rate responding in rabbits during an identical habituation procedure [6].

The possibility raised by our findings that delta and mu receptors within the central nucleus of the amygdala complex may serve different functions is interesting in light of recent research implicating delta receptor activity within the limbic system in seizure phenomena [4, 9, 26]. Although intraventricular (IVT) injections of opiates and opioid peptides have been reported to produce seizure activity recorded from a number of limbic system structures including the amygdala and hippocampus, amygdala lesions have been reported to have no effect on the development of opioid peptide-induced seizures [10]. The possibility that the hippocampus may be sensitive to the seizure-inducing effects of opiates and opioid peptides is supported by research demonstrating that direct administration of opioid peptides into the hippocampus produces seizure activity [3]. However, we have recently observed that administration of DALA into the amygdala central nucleus in rabbits at the doses used in this experiment failed to induce seizures recorded from either the injection site within the amygdala or the hippocampus, (manuscript in preparation). Therefore, within the context of this investigation it would appear that the effects of opioid peptide administration into the amygdala central nucleus on heart rate conditioning are not secondary to the production of seizure activity at the dose used in this experiment.

The results of this investigation, in combination with our previous findings regarding the effects of intracerebral administration of opiate agents into the amygdala complex on the acquisition and the retention of conditioning, support a role for amygdala opioid peptide activity in learning and memory processes. The results of these investigations raise many basic issues ranging from questions concerning the exact function this neurochemical system may play in learning and memory to the synaptic mechanisms involved. As a prelude to further exploration of these questions, we have attempted to define a more precise nuclear grouping within the amygdala complex which is sensitive to the behavioral effects of opiate and opioid peptide manipulations. The results of our studies using classical conditioning of heart rate responding in the rabbit suggest that the central nucleus region of the amygdala may provide a suitable neural system in which to explore the contribution of opioid peptide function to conditioning processes. Not only does this subregion of the amygdala possess a high concentration of endogenous enkephalins, but the results of this investigation demonstrate that opioid peptide administration into the central nucleus alters the acquisition of conditioned heart rate. This effect closely parallels that previously observed following opiate agonist administration into the same region [6]. Furthermore, the results obtained in this experiment are consistent with our previous findings using opiate agonist and antagonist agents in implicating mu receptor activity within the amygdala central nucleus region in conditioning processes.

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