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Effects of Endorphin and Enkephalin Analogs on Fear Habituation in Goldfish[1]

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OLSON, R. D., A. J. KASTIN, G. F. MICHELL, G. A. OLSON, D. H. COY AND D. M. MONTALBANO. *Effects of endorphin and enkephalin analogs on fear habituation in goldfish.* PHARMAC. BIOCHEM. BEHAV. 9(1) 111–114, 1978.—The effects of two opiate peptides administered by two routes were tested in an auditory habituation paradigm presenting goldfish with a 108 db buzzer and using the fish's ascending latency as a measure of fear. Twelve groups of goldfish (N=10) received a 5 μl (8 μg/kg) IP or ICV injection of either D-Ala²-β-endorphin, D-Ala²-Met-enkephalin, or the diluent control-solution. Half of each group experienced the buzzer during testing and the other half (non-buzzer) served as controls. Fish injected with the endorphin analog appeared to be immobilized since they had much longer latencies than the other groups. Latencies were shorter after presentation of the buzzer which appeared to disinhibit the swimming response of the fish receiving D-Ala²-β-endorphin. Effects occurred more rapidly after ICV than IP injections with the larger D-Ala²-β-endorphin where the effects were similar after ICV and IP injections of the smaller but less potent D-Ala²-Met-enkephalin, which was not significantly different from diluent. In all cases, effects were reliable only in the absence of the buzzer. Analyses of activity measures and total testing time yielded data comparable to those obtained for latencies. The results indicate a profound behavioral effect of D-Ala²-β-endorphin in fish for both central and peripheral administration.

Endorphin Enkephalin Habituation Goldfish

DATA collected in this laboratory over the past several years have supported the value of studying habituation to assess the behavioral effects of pharmacological agents in goldfish ([13], Olson, unpublished observations). When placed in a tall, narrow tank, fish tend to swim up and down at a fairly constant rate. If, however, a loud buzzer is presented during the ascent, a fish will either become immobile and remain near the bottom or actively swim to the bottom of the tank. After repeated experience with the buzzer, this response habituates readily, with latencies for the rate of ascent rapidly approaching baseline. Accordingly, this habituation paradigm produces increasing mobility and faster latencies over time with repeated presentations of the stimulus, and thus nullifies the problem of fatigue typically encountered in other habituation paradigms.

Further, the goldfish seems to be an excellent subject for evaluating the effects of opiate-like compounds. Not only have opiate receptor binding sites in the brain of the goldfish been demonstrated [14], but the blood-brain barrier is similar to that found in all higher vertebrates that have been studied, both with regard to site [2] and action [11]. Finally, the ease of a central (ICV) injection through the cartilagenous covering of the brain makes it an excellent preparation.

It has been shown that endorphin, especially β-endorphin, will influence behavior. Bloom *et al.*[1] and Jacquet and Marks [6] demonstrated decreased activity and immobilization accompanied by rigidity. In both cases, only rats injected with β-endorphin exhibited the pronounced immobilization. Little evidence exists, however, to document the effects of either enkephalin or endorphin on the acquisition of new behavior. Kastin *et al.* [8] found that IP administration of Met-enkephalin and D-Ala²-Met-enkephalin-NH_2 produced faster running and fewer errors in a maze than did diluent controls. Lichtblau *et al.* [10] found that centrally administered β-endorphin suppressed fixed-ratio 15, food-reinforced responding by rats in a dose-dependent manner. Moreover, D-Ala²-Met-enkephalin [4] and D-Ala²-β-endorphin [17] have been shown to be more potent opiates than their parent compounds. Accordingly, the purpose of the present study was to compare the effects of

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D-Ala^2-Met-enkephalin and D-Ala^2-β-endorphin on activity and learning, as well as their ability to cross the blood-brain barrier.

METHOD

Animals

Naive goldfish *(Carassius auratus)* were obtained from Ozark Fisheries, Stoutland, Missouri. The average weight of the 120 fish was 50 g.

Drugs

D-Ala^2-Met-enkephalin and D-Ala^2-β-endorphin were synthesized by solid phase methods [3,4] and dissolved in a vehicle consisting of 0.9% saline acidified with acetic acid to 0.01 M, with a pH of 4.1, which also served as control.

Apparatus

Habituation was studied using a tall, narrow aquarium originally described by Olson and May [12]. Photoelectric units were located at the top and bottom of the chamber to measure activity and ascending latencies. The entire apparatus was housed in a sound attenuated chamber and all data were recorded automatically by appropriate programming modules.

Procedure

Each fish was randomly assigned to one of the twelve treatments, ten fish per treatment, in a 3 × 2 × 2 design consisting of peptides (enkephalin analog, endorphin analog, diluent) by injection site (IP, ICV) by stimulus (buzzer, no buzzer). All fish were given a 5 μl injection (8 μg//kg) of the appropriate coded solution.

Prior to testing, each fish was placed in the apparatus for a 15-min acclimation period. During this interval and the experimental session, a measure of activity was made by counting the number of times the top and bottom beams were broken. After acclimation, the programming equipment was activated so that each time the fish broke the bottom beam a 108 db buzzer was sounded for 10 sec and a printout counter was activated; each successive breaking of the lower beam resulted in the presentation of the buzzer again but did not affect the operation of the printout counter. When the fish broke the top photocell beam, the printout counter recorded the latency in tenths of a second. This constituted one trial. After 30 trials, a 100 W light was activated continuously for the remaining trials (31–40) in order to further assess sensitization effects. The fish was removed after 40 trials. The operation of the apparatus was identical for fish in the absence of the buzzer (no-buzzer group).

RESULTS

The data collected from each fish during testing were presented as mean ascending latencies by blocks of five trials. The latencies for all groups for the first block of five trials are shown in Fig. 1. A mixed analysis of variance performed on the first six blocks of pre-light trials yielded a significant peptide effect, $F(2,108)=14.15$, $p<0.0001$. Scheffé's test for multiple comparisons was used to further analyze the effect and yielded reliable differences between diluent and D-Ala^2-β-endorphin: $F(2,108)=17.69$, $p<0.001$; and D-Ala^2-Met-enkephalin and D-Ala^2-β-endorphin: $F(2,108)=24.24$, $p<0.001$, but not between D-Ala^2-Met-enkephalin and diluent. The groups receiving D-Ala^2-β-endorphin had the longest latencies followed by the groups receiving diluent and D-Ala^2-Met-enkephalin. A comparison of latencies after injection (ICV vs. IP) also showed significant differences, $F(1,108)=5.21$, $p<0.05$, which reflected the longer latencies experienced by the groups injected ICV. The presence or absence of the stimulus did not produce a reliable difference in latencies.

A significant trials effect, $F(5,540)=50.68$, $p<0.0001$, suggested that habituation occurred during the first six blocks of trials. A reliable peptides by trials interaction, $F(10,540)=7.40$, $p<0.0001$, initially suggested a differential rate of habituation for the three groups across trials; however, a Winer test for simple effects revealed that the three groups differed significantly only during the first block of trials, $F(2,648)=50.19$, $p<0.0001$, indicating that the differential effects were to differences in sensitization rather than in habituation. A significant injection site by trials interaction, $F(5,540)=2.31$, $p<0.05$, suggested that the magnitude of the injection site difference decreased over trials. The groups differed reliably only on the first block of trials, $F(1,648)=15.81$, $p<0.0001$, again indicative of the initial sensitization effect.

A significant stimulus by injection site interaction, $F(1,108)=5.32$, $p<0.05$, statistically supported the observation that fish in the IP-buzzer and ICV-buzzer groups had virtually identical mean latencies while the corresponding no-buzzer groups showed a reliable difference, with the ICV fish having much longer mean latencies, especially when injected with D-Ala^2-β-endorphin.

A second analysis was performed on trial blocks 6 and 7 to evaluate the effects of the light after 30 buzzer habituation trials. It revealed a significant peptide effect, $F(2,108)=4.52$, $p<0.02$. A Scheffé test indicated that fish receiving D-Ala^2-β-endorphin had significantly longer latencies than diluent controls, $F(2,108)=7.63$, $p<0.05$, but no other comparisons were reliable. A significant difference between trials existed, $F(1,108)=4.79$, $p<0.05$, indicative of longer latencies due to sensitization to the onset of the light prior to block 7. No other results were significant.

A third analysis run on the last two blocks of trials to evaluate habituation to the light yielded a significant peptide effect, $F(2,108)=3.59$, $p<0.05$. The effects of neither injection nor trials were significant.

A mixed analysis of variance was performed on activity scores obtained during the entire experimental session. A significant difference existed among peptide groups, $F(2,108)=3.75$, $p<0.05$, with fish injected with D-Ala^2-β-endorphin being most active followed by fish injected with diluent and D-Ala^2-Met-enkephalin. The injection site was a reliable source of difference, $F(1,108)=5.99$, $p<0.02$, with the fish injected IP exhibiting less activity.

Finally, a simple analysis of total experimental testing time produced a reliable peptide effect, $F(2,108)=12.83$, $p<0.0001$, indicating that the three groups differed in the length of time required to complete testing. A Scheffé test indicated a significant difference between diluent and D-Ala^2-β-endorphin, $F(2,108)=16.03$, $p<0.001$, with the group injected with D-Ala^2-β-endorphin taking more time, as well as between D-Ala^2-Met-enkephalin and D-Ala^2-β-endorphin, $F(2,108)=21.98$, $p<0.001$, with D-Ala^2-β-endorphin again taking more time. Although the time for D-Ala^2-Met-enkephalin was faster than for diluent, the difference was not reliable.

A significant interaction between injection site and

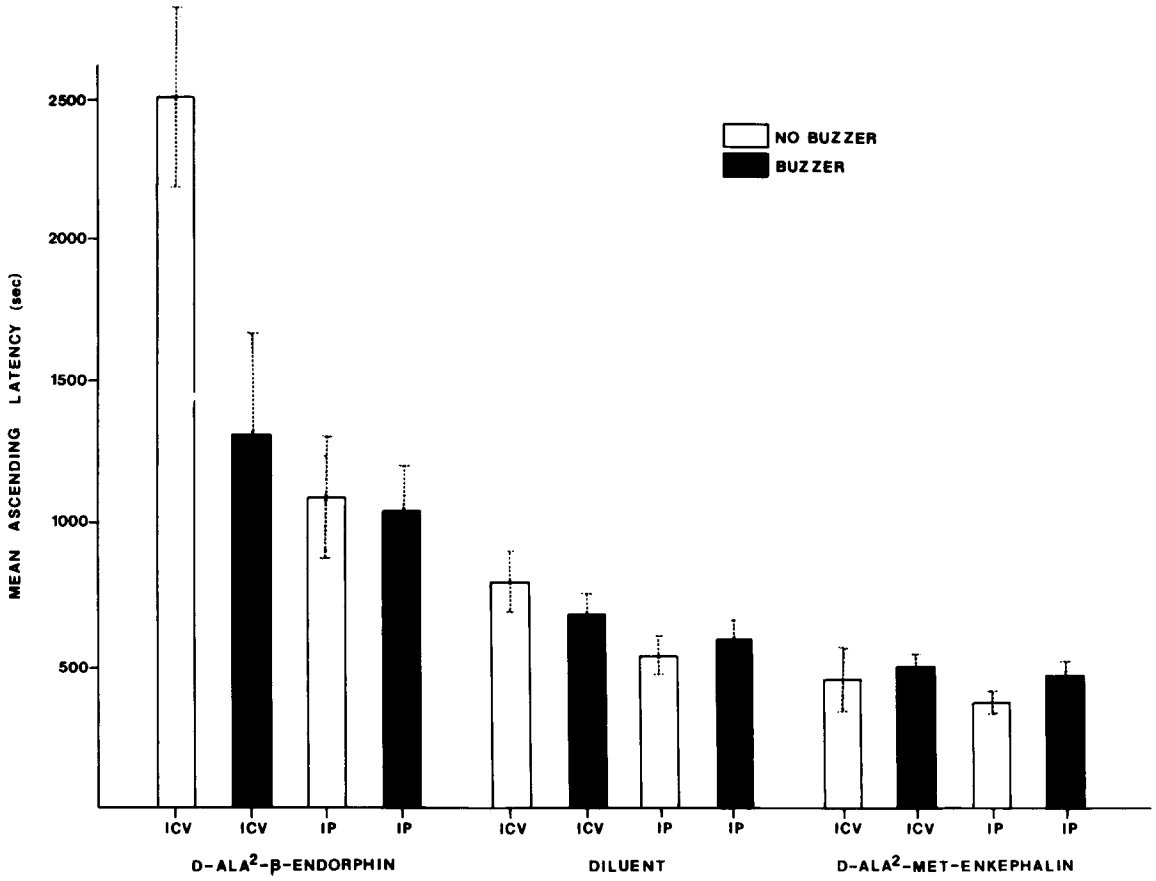

FIG. 1. Mean ascending latencies for trials 1–5 as a function of peptide, injection site, and stimulus.

stimulus was also found, $F(1,108)=4.36$, $p<0.05$, as with the measures of latency showing that the differential effect of injection site on total testing time was a function of the presence of the buzzer. A Winer test confirmed that the groups injected IP and ICV groups differed significantly when comparisons were made with fish which did not experience the buzzer during testing, $F(1,108)=5.51$, $p<0.05$, although the two groups did not differ reliably if the buzzer was presented.

DISCUSSION

The results of the present study with goldfish support and extend previous research with mammals. As was anticipated from earlier data on the parent β-endorphin [1,6], the group receiving D-Ala²-β-endorphin showed increased latencies and/or immobilization. The mean ascending latencies for the groups injected with the β-endorphin analog were reliably longer than those injected with either D-Ala²-Met-enkephalin or diluent. Further, the total testing time of the group receiving D-Ala²-β-endorphin was significantly greater than that of the other groups, again indicative of the immobilizing effect associated with β-endorphin.

That fish exposed to the buzzer had shorter latencies than fish not exposed to the buzzer is also consistent with previous findings [16]. Apparently the buzzer serves as a disinhibitor and terminates the immobilized state produced by β-endorphin. Such a finding has also already been observed in studies of tonic immobility. It was initially demonstrated by Liberson [9] that after an immobilized state had been induced, an intense stimulus would tend to terminate the immobility and the organism would right itself. It is possible that β-endorphin may be involved in tonic immobility, and that fear may be a stimulus to some species for the release of that peptide.

Although the significant peptides by trial interaction would normally imply differential habituation, the finding that the three groups differed only at trial block one suggested that the interactions could be due only to initial differences in sensitizations as reflected by immobilization and long latencies. Such a distinction between sensitization and habituation has previously been proposed [5] and the present data support the existing model. Although no statistical evidence of differential habituation was obtained in this study, it was of interest to note that D-Ala²-Met-enkephalin tended to result in consistently shorter latencies as well as a shorter total testing time than did the diluent. This is consistent with a maze performance study [8] in which rats given IP injections of enkephalin or an analog ran faster than controls and a neuropharmacological model [15] in which mice receiving IP enkephalin and an analog were more active than controls. It should be mentioned that slightly different analogs were used in the strictly behavioral studies even though the results were very similar. The paradigm used in the current study has considerable variability and would not be sensitive to mild influences. The slightly faster latencies and testing times after the enkephalin analog were observed in fish injected both ICV and IP.

It has not been completely established at this time whether the opiate peptides and their analogs penetrate the blood-brain barrier. Although some evidence [6] in addition to that already mentioned [7,13] exists for enkephalin crossing the blood-brain barrier, additional work with a more direct method suggests that the percent passed may be smaller and more dependent upon concentration than previously realized (Wade, Kastin and Coy, unpublished observations). Data from the present study indicate an effect on the behavior of goldfish independent of whether administered peripherally or centrally. This behavioral effect was greater when injections were made ICV as opposed to IP, but the effect of the IP injections was significant.

This study provided behavioral confirmation of an effect of an opiate peptide. The location and mechanism for the effect is still uncertain. It was observed, however, that goldfish injected IP and ICV performed in a very similar fashion and differed in magnitude rather than type of response. If IP injections acted only peripherally, it would seem surprising to find an effect so similar to that observed rapidly after ICV injection. The longer trial latencies after ICV than IP injection of the D-Ala^2-β-endorphin suggests the possibility of some difficulty with penetration of the blood-brain barrier by this large peptide at the dose used. Based on our findings, it would appear that peripherally injected peptides, either in their injected or fragmented forms, enter the brain and produce a central effect.

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