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Behavioral Effects of D-Ala^2-β-Endorphin in Squirrel Monkeys[1]

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OLSON, G. A., R. D. OLSON, A. J. KASTIN, F. X. CASTELLANOS, M. T. KNEALE, D. H. COY AND R. H. WOLF. *Behavioral effects of D-Ala^2-β-endorphin in squirrel monkeys.* PHARMAC. BIOCHEM. BEHAV. **9**(5) 687–691, 1978.—The effects of D-Ala^2-β-endorphin administered either intravenously (IV) or intracisternally (IC) in squirrel monkeys were tested using a number of behavioral measures: general activity, eating, social behavior, aggression/distress, analgesia, and startle/escape. There were 10 groups (N=5) consisting of 4 dose levels administered IC (0, 4, 40, 400 μg/kg) and 6 dose levels injected IV (0, 4, 40, 80, 400, 800 μg/kg). Every monkey was tested with all tasks on each of 5 identical repeated trials, one pre-injection baseline trial and 4 post-injection trials. After IC administration, the 2 largest doses exerted toxic effects, which were partially reversed with naloxone, producing in 2 cases muscular rigidity and profound sedation. The smaller 4 μg/kg dose produced significant decreases in activity over trials but increased reactivity to noxious stimulation after the initial post-injection trial. With IV injection reliable changes in activity and approach to food were found. The results demonstrate significant behavioral effects of an endorphin analog in the squirrel monkey after both central and peripheral injection.

Endorphin analog Squirrel monkeys

ANALGESIA, immobilization, and catatonic-like behavior have been observed in rats after the central injection of the opiate neuropeptides. Both Bloom *et al.* [1] and Jacquet and Marks [7] found that IC administration of β-endorphin produced prolonged naloxone-reversible immobility together with muscular rigidity, waxy-flexibility, profound sedation, and analgesia. Izumi *et al.* [6] later reported akinesia and loss of corneal reflex after IC administration of β-endorphin in the rat. Other investigators have reported that β-endorphin produced analgesia after IC [2,14] as well as IV [15] administration. A more extensive review of the effects of endorphin is presented elsewhere [10].

Olson *et al.* compared IC and IP injections of D-Ala^2-β-endorphin [11] and other opiate peptides [12] in goldfish and found the decreased general activity to occur more rapidly after IC than IP injections, although the IP effects were pronounced. The few other reports of behavioral changes after peripheral administration of the opiate peptides have been reviewed elsewhere [8].

D-Ala^2-β-endorphin exerts very potent effects in rats [16] and goldfish [11]. Like the other opiate peptides, little is known of its effects in primates. We report here the systematic investigation of the behavioral effects of peripheral and central injections of several doses of D-Ala^2-β-endorphin in the squirrel monkey.

METHOD

Animals

A total of 46 nonpregnant female Bolivian squirrel monkeys (*Saimiri sciureus*) were used in this study. The monkeys were exported from Santa Cruz and were housed at the Delta Regional Primate Center, Covington, Louisiana, where the research was conducted. Their weights ranged from 486 to 800 gm, with a mean of 606.2 gm; there were no significant differences between the group means for weight, $F(9,36)=0.816$, $p>0.05$.

Drugs

D-Ala^2-β-endorphin was synthesized by solid phase methods [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. The vehicle solution also served as the 0-level condition. Besides the 0 condition, concentrations of 4, 40, 80, 400 and 800 μg/kg were injected in a volume of 250 μl, except at the 80 and 800 μg/kg concentrations, where 500 μl doses were used. Injections were administered either intravenously (IV) in the saphenous vein of the leg or via a cisternal tap (IC) in which the amount of drug injected was identical to the amount of spinal fluid that was removed. All animals, regard-

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less of treatment condition, were anesthetized with halothane for the injection period.

Apparatus

Stop watches were used to time all measures of activity and latencies. A stuffed toy monkey, about 13 cm tall with a long shoelace pinned to the head for retrieval purposes, served as the social stimulus. Thin slices of freshly-cut apple placed on a round 7.5 cm yellow plastic lid, one slice per trial, were used to measure eating behavior. A syringe equipped with a stop exposing 1.5 mm of needle was used to prick the skin of the monkeys to test analgesia. An air puff produced by a can of pressurized air (Omit Plus) directed at the face and delivered through a 17 cm plastic tube and a light produced by a flashlight (Mallory Big Bruiser L57) and shone at the face were used to assess startle/escape.

During testing the monkeys were housed singly in 45.7 cm wide×60 cm deep×86.3 cm high cages. These contained a platform on the left side which stretched the entire depth of the cage front to back at about 30 cm from the top of the cage as well as 2 bars, the higher one extending the full width of the cage, about 15 cm below the platform and midway between the back and front, and the lower one situated front-to-back about 10 cm from the right side and about 30 cm from the floor.

Design

Each monkey was randomly assigned to 1 of the 10 coded treatments, with 5 animals per treatment. Four dose levels (0, 4, 40 and 400 μg/kg) were administered IC, and 6 levels (0, 4, 40, 80, 400 and 800 μg/kg) were injected IV. All monkeys received 5 identical, repeated trials, including one pre-injection trial to establish baseline behavior for each animal and, following a 15 min recovery period from the anesthetic, 4 post-injection trials to measure drug effects. For purposes of analysis, the scores on Trial 1 for each animal for each task were subtracted from each of the other 4 trial scores of that animal to normalize the data. The data collected for each measure were analyzed separately for the monkeys injected IC and those injected IV.

The first 2 animals receiving 40 μg/kg IC and 3 of the first 4 animals receiving 400 μg/kg IC suffered respiratory failure and in some cases accompanying heart failure. All such animals were treated with IV injections of naloxone hydrochloride (Narcan). One monkey died, but in the others the respiratory failure was reversed. Two to 3 injections of 0.4 mg naloxone at intervals of not more than 15 min were required to maintain the reversal effects. All animals receiving naloxone were assigned maximum latencies and minimum response scores, as if they had died. No further animals were tested under these conditions. Even though data are included in the analyses for all 50 monkeys, this meant that 46 monkeys were actually tested, with only 41 receiving all 5 trials without naloxone intervention. For Trial 1, the 4 remaining animals (3 from the 40 μg/kg condition and 1 from the 400 μg/kg condition) were assigned a score equal to the mean of the 46 tested on that trial.

Procedure

Each trial involved observations for 30 min, followed by a rest period of 30 min. Each period of observation included measurement of general activity for 10 min, eating behavior for 5 min, social behavior for 5 min, and tasks with various noxious stimuli, including measures of aggression, distress vocalization, analgesia, and startle/escape, for 10 min.

Activity. Activity was recorded every 5 sec for a total of 120 observations per trial, with a notation of the behavior and location of the animal at each time of observation. For purposes of analysis, these observations were later converted to a numerical scale, with 0 indicating no activity and 5 the most activity (locomotion).

Eating. Latency was recorded from the time of the placement of the plastic lid with a single slice of apple on the cage floor until the monkey picked up the apple. A second latency measure recorded the time required to totally consume the slice. A maximum of 5 min was allowed for eating, so that the score for an animal who did not approach the food was 300 sec. A third index of eating measured the total activity of the monkey relative to the apple on a 4 point scale: ignoring the apple (0 points), approaching it (1 point), manipulating it (2 points), and consuming it (3 points). A score was given for each activity noted, so that a maximum of 6 points was possible for any single monkey on each trial.

Social behavior. The toy monkey was placed on the cage floor just inside the door, with the shoestring outside the cage. Latency was recorded from the time of placement on the cage floor until the initial contact, with a duration of 5 min allowed for the period of observation. In addition, the types and number of contacts during the 5 min were recorded. A score was later assigned to the types of contacts on a 9 point scale ranging from no approach (0 points) through attack (8 points), with each score multiplied by the number of times that activity occurred.

Aggression and distress vocalization. An attempt was made by an observer protected with a thick leather glove to grab and hold the monkey while it was still in the cage. For each trial, the animal was rated on a 0–3 point scale for aggressiveness, with 0 indicating no aggression and 3 indicating biting. During the same task, each monkey was also rated on a 0–3 point scale for distress vocalizations, with a maximum of 3 points for continuous squealing during the attempt at grabbing.

Analgesia. Responsiveness to pain was tested by pricking the skin with a needle on 3 parts of the body: tail, paw, and torso. An attempt was made to hold the monkey during this test. Flinching, squealing, and movement away from the needle were used as indications that pain was perceived. The behavior was scored on an all-or-none basis; the presence or absence of analgesia was recorded at each of the 3 sites tested, with the scores summed for each trial so that a maximum of 3 points was earned for responsiveness on a given trial.

Startle/escape. Two independent measures were used to assess startle/escape. One involved shining a bright light suddenly at the face of the monkey from a distance of about 45 cm. Five presentations of the light were made on each trial, and the latencies of turning from the light were summed so that a single score for each trial resulted. A maximum of 30 sec was allotted for each presentation. The second measure was the latency of escape from an air puff directed at the face from a distance of about 45 cm. One air puff was presented per trial, with a maximum latency of 5 sec allowed.

RESULTS

The results were reported separately for each behavioral measure. Table 1 presents a summary of all significant dose-

TABLE 1
SUMMARY OF DOSE-RELATED EFFECTS BY TASK AND INJECTION SITE

Task	Injection Site IC	IV
Activity	+++*†	+++†
Eating Approach Latency	+++†	+++†
Eating Consumption Latency	—	—
Eating Behavior Score	—	—
Social Approach Latency	—	—
Social Behavior Score	—	—
Aggression	+++*	—
Distress Vocalization	+++†	—
Analgesia	+++*†	—
Startle/Escape (Light)	+++*	—
Startle/Escape (Air)	+++*†	—

*=Significant dose effect
†=Significant dose × trials interaction

related effects by task and injection site, including dose effects and dose×trials interactions.

Activity

A mixed analysis of variance performed on the activity scores for the monkeys injected IC yielded a highly significant drug-dose effect, $F(3,16)=11.61$, $p<0.001$, with decreased activity at the higher doses. Sheffé's tests showed no significant differences in effects between the 0 and 4 μg/kg treatments or between the 40 and 400 μg/kg conditions, but there were reliable differences between 0 and 40 μg/kg, $F'(3,76)=25.53$, $p<0.01$, 0 and 400 μg/kg, $F'(3,76)=12.88$, $p<0.05$, 4 and 40 μg/kg, $F'(3,76)=23.92$, $p<0.01$, and 4 and 400 μg/kg, $F'(3,76)=11.267$, $p<0.05$. A significant effect of trials was also found, $F(3,48)=4.45$, $p<0.01$. Subsequent Sheffé's tests for multiple comparisons indicated that level of activity increased significantly from post-injection Trial 1 to Trial 2, $F'(3,76)=8.385$, $p<0.05$ and from Trial 1 to Trial 3, $F'(3,76)=10.34$, $p<0.05$ and that activity did not differ reliably among any of the other trials. There was, however, a significant difference between Trial 1 and the other three trials combined, $F'(3,76)=13.15$, $p<0.01$. A significant trials ×dose interaction also existed, $F(9,48)=3.57$, $p<0.01$, with reliable differences between the performances of animals injected with 4 μg/kg and each of the other groups: $F(3,48)=58.1$, $p<0.01$ for 0 vs. 4 μg/kg, $F(3,48)=7,37$, $p<0.01$ for 40 vs. 4 μg/kg, and $F(3,48)=6.01$, $p<0.01$ for 400 vs. 4 μg/kg. The group receiving 4 μg/kg showed an initial decline in activity, followed by a slight increase, while the other 3 conditions exhibited no changes across trials.

The analysis of variance of activity for the IV treatment revealed only a reliable dose by trials interaction, $F(15,72)=2.28$, $p<0.05$, indicating the differential effect of the peptide over time. Subsequent F tests for simple effects revealed that both the 4 and 400 μg/kg doses, which produced increases in activity over trials, differed from 40 and 800 μg/kg doses, which produced decreases in activity over trials: for 4 vs. 40 μg/kg, $F(3,72)=7.08$, $p<0.01$; for 4 vs. 800 μg/kg, $F(3,72)=4.05$, $p<0.05$; for 400 vs. 40 μg/kg, $F(3,72)=5.92$, $p<0.01$; for 400 vs. 800 μg/kg, $F(3,72)=3.1$, $p<0.05$. In addition, Condition 0, which decreased slightly over trials, differed significantly from the 4 μg/kg dose, $F(3,72)=2.99$, $p<0.05$. No other results were significant.

Eating and Social Behavior

Analyses of the latency scores for eating, which measure the time until the apple was picked up, after both the IC and IV injections produced no significant main effects, but each did yield a reliable trials×drug interaction, $F(9,48)=2.095$, $p<0.05$ after IC injection and $F(15,72)=2.115$, $p<0.05$ after IV injection. For the IC treatments, F tests for simple effects yielded one significant comparison for the monkeys injected with diluent (0) vs. monkeys injected with 4 μg/kg, $F(3,72)=5.37$, $p<0.01$; the diluent group became quicker over trials in grabbing the apple, while the group receiving 4 μg/kg decreased in speed over trials. After the IV injections, changes over trials for the dose of 800 μg/kg were significantly different from those for all of the other doses except 4 μg/kg: for 0 vs. 800 μg/kg, $F(3,72)=5.06$, $p<0.01$; for 40 vs. 800 μg/kg, $F(3,72)=6.93$, $p<0.01$; for 80 vs. 800 μg/kg, $F(3,72)=3.54$, $p<0.05$; for 400 vs. 800 μg/kg, $F(3,72)=9.08$, $p<0.01$. Monkeys injected with 800 μg/kg IV became slower over trials, while the other doses resulted in faster picking up of the apple. The other two measures of eating behavior and the two social behavior scores yielded no significant findings after either the IC or IV injections.

Aggression

Measures of aggression after IC administration showed a significant main effect for dose level, $F(3,16)=3.84$, $p<0.05$, with decreased aggression after higher dose levels. The only reliable Sheffé's comparison was 0 vs. 400 μg/kg, $F'(3,76)=9.07$, $p<0.05$, with animals receiving the lower dose showing greater aggression. Also, there was a reliable effect for trials, $F(3,48)=3.16$, $p<0.05$, with the least aggression being demonstrated on the second trial and the most on the last one, $F(3,76)=8.32$, $p<0.05$. No clear-cut effects on aggression were found after IV injections.

Distress Vocalization

Distress vocalizations increased over trials after the IV injections, $F(3,72)=4.66$, $p<0.01$. Sheffé's tests revealed reliable differences only between Trials 1 and 2, $F'(3,116)=10.16$, $p<0.05$, and between Trials 1 and 3, $F'(3,116)=10.16$, $p<0.05$. The fewest vocalizations were recorded on Trial 1. After IC injection, the only reliable effect was a trials×drug interaction, $F(9,48)=3.20$, $p<0.01$, with the group receiving 4 μg/kg increasing in vocalizations over trials but the other conditions remaining essentially constant over trials: for 0 vs. 4 μg/kg, $F(3,48)=6.36$, $p<0.01$; for 40 vs. 4 μg/kg, $F(3,48)=4.47$, $p<0.01$; for 400 vs. 4 μg/kg, $F(3,48)=4.85$, $p<0.01$.

Analgesia

On the analgesia tasks after IC injections, there were significant main effects for doses, $F(3,16)=13.65$, $p<0.001$, and for trials, $F(3,48)=27.58$, $p<0.00001$, and a significant interaction of those variables, $F(9,48)=9.25$, $p<0.00001$. For the effect of drug dosage, there was a significant increase in analgesia with increases in dosage. Reliable comparisons existed between the control and the 2 highest doses, for 0 vs. 40 μg/kg, $F'(3,76)=33.36$, $p<0.01$ and for 0 vs. 400 μg/kg, $F'(3,76)=17.38$, $p<0.01$, and between the 4 and 40 μg/kg

doses, $F'(3,76)=19.00$, $p<0.01$. For the effect of trials, there was increased responsiveness and decreased analgesia over trials, with reliable differences occurring between Trial 1 and each of the succeeding trials: compared against Trial 2, $F'(3,76)=52.04$, $p<0.01$, against Trial 3, $F'(3,76)=64.07$, $p<0.01$, and against Trial 4, $F'(3,76)=52.04$, $p<0.01$. The interaction resulted from the fact that the monkeys receiving 40 and 400 μg/kg demonstrated basically unchanged behavior over trials, while those injected with 4 μg/kg changed the most over trials. All comparisons were significant except that between the 40 and 400 μg/kg groups.

For the IV conditions on the measure of analgesia, the only significant effect was a decrease in analgesia across trials, $F(3,72)=4.53$, $p<0.01$. The largest decrease was observed after the first post-injection trial, with Trial 1 reliably different from Trials 2 and 4, $F'(3,116)=10.94$, $p<0.05$ and $F'(3,116)=8.76$, $p<0.05$, respectively.

Startle/Escape

Analysis of startle/escape as measured by the latency of the reaction to the air puff after IC injection yielded significant effect of drug dosage, $F(3,16)=18.49$, $p<0.0001$. The 400 μg/kg dose produced the longest latencies, for 0 vs. 400 μg/kg, $F'(3,76)=27.63$, $p<0.01$, for 4 vs. 400 μg/kg, $F'(3,76)=24.12$, $p<0.01$, and for 40 vs. 400 μg/kg, $F'(3,76)=52.56$, $p<0.01$, but no differences were found among the other groups. In addition a significant effect of trials was found, $F(3,48)=7.00$, $p<0.0001$, indicating decreasing latencies after Trial 1 with the only reliable comparison existing between Trials 1 and 3, $F'(3,76)=20.66$, $p<0.01$. A significant interaction between dose and trials, $F(9,48)=2.10$, $p<0.05$, indicated that while the monkeys receiving 40 μg/kg did not change over trials, those receiving 4 and 400 μg/kg demonstrated decreased latencies over trials; for 4 vs. 40 μg/kg, $F(3,48)=3.82$, $p<0.05$, and for 400 vs. 40 μg/kg, $F(3,48)=4.12$, $p<0.05$. Measures of air puff latency after IV injection showed a reliable decrease in latencies over trials, $F(3,72)=6.07$, $p<0.001$, with latencies on Trial 1 significantly longer than those on Trial 3, $F'(3,116)=13.35$, $p<0.01$.

Finally, the analysis of variance of the latency of reaction to the light yielded a reliable effect for dosage, $F(3,16)=8.03$, $p<0.01$. The 400 μg/kg dose produced the longest latencies and the 40 μg/kg dose produced the shortest; for 40 vs. 400 μg/kg, $F'(3,76)=23.75$, $p<0.01$. There was also a significant effect of trials after IC injection, $F(3,48)=6.67$, $p<0.001$, with a decrease in latency over trials. There were long latencies on the first trial, followed by quicker latencies on the next three trials, which were not different from one another; for Trial 1 vs. 2, $F'(3,76)=8.76$, $p<0.05$, for Trial 1 vs. 3, $F'(3,76)=16.38$, $p<0.01$, for Trial 1 vs. 4, $F'(3,76)=13.08$, $p<0.01$. In the IV conditions, there was a significant monotonic decrease in latencies over trials, $F(3,72)=2.99$, $p<0.05$, with the only reliable comparison being Trial 1 vs. 3 and 4, $F'(3,116)=8.47$, $p<0.05$.

DISCUSSION

Strong, dose-related effects were noted after IC administration of D-Ala^2-β-endorphin in squirrel monkeys, but weaker effects also resulted after IV injection. The lowest IC dose (4 μg/kg) produced a number of effects different from the control condition, and the higher 2 doses (40 and 400 μg/kg, IC) were toxic to all but 1 of the monkeys tested. Although there were no significant main effects between the 0 and 4 μg/kg groups, the fact that there were significant differences over trials in monkeys injected with the control and 4 μg/kg solutions indicates that even the lowest dose produced behavioral changes in the monkeys. These results are consistent with previous reports of IC effects of endorphins and endorphin analogs in mammals [2, 14, 16] and with those in goldfish [11,12]. The findings for monkeys injected IV are in general agreement with those reported for rats [15], especially with the measure of analgesia. However, they do not replicate those for goldfish [11,12], in which no significant differences in behavior after central and peripheral administration of D-Ala^2-β-endorphin were found, even though the blood-brain barrier and opiate receptors in these two species are reported to be similar [3, 9, 13].

There were a number of significant interactions between the 0 and 4 μg/kg groups injected IC. Activity decreased over trials for the 4 μg/kg group, but remained constant for the control animals, in agreement with previous studies reporting decreased activity after injections of the opiate peptides [1, 6, 7, 11, 12]. Measures of eating behavior were similar to those of activity, with slower eating over trials after 4 μg/kg injections but somewhat faster over trials after 0 μg/kg injections. Measures of aggression, distress vocalization, and analgesia all indicated increased reactivity to noxious stimulation over trials for monkeys receiving 4 μg/kg but indicated no change (vocalization and analgesia) or opposite effects (aggression) over trials for the higher doses. For most of the measures after injection of 4 μg/kg, the largest changes in behavior occurred between Trials 1 and 2, suggesting a possible decrease of the effects of the drug with time.

The fact that there were toxic doses of D-Ala^2-β-endorphin is consistent with its other opiate properties. Of those monkeys whose drug effects were partially reversed by naloxone, two exhibited the immobility and profound sedation reported by Bloom *et al.* [1] and Jacquet and Marks [7] in rats and noted previously in squirrel monkeys by Olson, Olson, Wolf, Coy and Kastin in an unpublished observation. One of the squirrel monkeys slept on her side, with her neck rigidly holding her head about 1 cm off the cage floor. Both were essentially unresponsive to environmental stimuli and were analgesic, which was consistent with previous reports [1,7]. Toward the end of the 4 hr of observation, they exhibited uncoordinated movement, as reported by Izumi *et al.* [6]. Thus, even though the animals had received two to three large doses of naloxone, the effects were not completely reversed due to the extreme potency of the drugs being tested.

In agreement with Tseng *et al.* [15], who found analgesic effects with IV injections of β-endorphin in rats, the present study also found some analgesia after IV administration of D-Ala^2-β-endorphin. On the first post-injection trial, there was a low level of responsiveness to the pin prick, but on later trials the response increased. There were no differences in effects among the dose levels in the current study, in contrast to the findings of Tseng *et al.* [15]. However, their doses were much higher than ours; the lowest dose they found to produce analgesia (8.2 mg/kg) was more than ten times larger than our highest dose (800 μg/kg). In addition, their test of analgesia was given 10 min after the injection of the drug, whereas our first test was not administered until 35 min after the injection. Possible metabolic degradation and inactivation of the drug [5] in this study may have occurred during this time to account for the discrepancy. The significant effects after IC injection of D-Ala^2-β-endorphin in the

tests of analgesia are consistent with previous reports with the parent β-endorphin [2,14] as well as this analog [16].

The changing effects with trials after IV injections for the responses to the noxious stimuli, except analgesia, might be accounted for in part by learning, since there were no main effects for dosage. The increased responsiveness to the air puffs and the light could be due to learned escape responses or possibly anticipatory responses to the stimuli. The increased aggression and distress vocalizations could be partially due to acquired aversiveness of stimuli associated with the experimenter who consistently presented the noxious stimuli. This experimenter, indeed, noted that many of the monkeys made aggressive responses toward him when he walked in the room, while the experimenter who tested for eating and social behavior reported approach behavior by the monkeys when they saw her.

The social responses to the toy monkey were very low in number, probably because the animals immediately recognized the inanimate nature of the toy. Although some monkeys did try to groom it, just as many other monkeys were more interested in the shoelace pinned to the head of the toy than in the toy itself. After the initial preinjection trial with the toy, most monkeys ignored it.

Overall, strong effects were produced by central administration of D-Ala^2-β-endorphin. Much weaker and limited effects were noted after its peripheral administration in squirrel monkeys.

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