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Analgesia in Defeated Mice: Evidence for Mediation Via Central Rather Than Pituitary or Adrenal Endogenous Opioid Peptides[1]

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THOMPSON, M L , K A MICZEK, K NODA, L SHUSTER AND M S A KUMAR *Analgesia in defeated mice Evidence for mediation via central rather than pituitary or adrenal endogenous opioid peptides* PHARMACOL BIOCHEM BEHAV **29**(3) 451–456, 1988 —Mice subjected to defeat in a social conflict paradigm display an analgesic response that is apparently mediated by endogenous opioids It is blocked by naloxone and shows full cross-tolerance to and from morphine The present study investigated the contribution of sources of endogenous opioids outside of the central nervous system, namely the pituitary and adrenal glands Treatments known to enhance (metyrapone pretreatment), reduce (2% saline in the drinking water) or block (dexamethasone pretreatment) the release of β-endorphin from the anterior pituitary did not affect the display of analgesia in defeated mice Similarly, treatments known to enhance (reserpine pretreatment) or block release of enkephalins (removal of the adrenals or hexamethonium pretreatment) from the adrenal medulla also failed to influence defeat-induced analgesia in the expected manner If anything, adrenalectomy enhanced and reserpine pretreatment suppressed the analgesic response to defeat The data are discussed in terms of providing evidence that defeat-induced analgesia is mediated primarily by endogenous opioids released and acting within the central nervous system

Stress-induced analgesia Adrenalectomy Dexamethasone Reserpine Defeat Metyrapone
2% Saline Aggression Beta-endorphin

IN an effort to delineate the physiological roles of endogenous opioids, their involvement in the phenomenon of endogenously produced antinociception (stress-induced analgesia) has been actively investigated Evidence supports the active involvement of some of these substances in such phenomena as stimulation-produced analgesia and acupuncture The role of opioid peptides in stress-induced analgesia has been more difficult to demonstrate unequivocally Endogenous opioid systems may be activated under some conditions of stress, but other stressors apparently evoke non-opioid mediated forms of analgesia [1–3, 15, 21, 28, 32]

Previously we provided evidence for a critical role of endogenous opioids in analgesia due to defeat in social conflict [19] Mice that were exposed to attack and consequently defeated by another mouse showed a profound long-lasting analgesia This analgesia was fully blocked by pretreatment with naloxone and naltrexone and showed cross-tolerance to and from morphine These findings have since been replicated by other investigators [25, 26, 29]

The question arises as to the identity and source of the endogenous opioid(s) involved in the mediation of this response β-Endorphin, Met- and Leu-enkephalin, and even dynorphin, acting alone or in concert with one another, and released from sources either within or outside the CNS may mediate the analgesic response to stress [12,21] Our previous studies have suggested that defeat-induced analgesia is mediated centrally in that quaternary naltrexone, which does not enter the CNS, was ineffective in reducing the analgesic response to defeat [19], and naloxone infused directly into the CNS via cannulae implanted into the periaqueductal gray or arcuate nucleus regions blocked analgesia from defeat completely [20]

In this report we describe the effect of manipulations of

[1]The experimental protocols used in these studies were approved by the Tufts IACUC in accordance with the USDA guidelines for the use and care of animals in research

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the pituitary-adrenocortical opioid sources on the analgesia observed in defeated mice Some of these observations have been mentioned in preliminary form [17,18]

METHOD

Subjects

Male B6AF$_1$ mice aged 2–6 months obtained from Jackson Labs , Bar Harbor, ME and CFW male mice obtained from Charles River, Wilmington, MA were used in the present experiments Subjects were housed 6 to a cage and kept in a temperature controlled room (20±2°C) on a 12 hr on 12 hr off light-dark cycle, not reversed

Defeat Paradigm

Experimental mice were introduced singly as intruders into the home cage of a resident CFW male living with a female [16] The female and any pups were removed prior to testing Upon being placed in the cage, the intruders were attacked by the resident CFW males This paradigm produces aggressive and defensive reactions in the mice which closely resemble those that mice display normally when observed in the field, and as such does not require any special training or isolation of the animals, nor does it subject them to any abnormal forms of stressful, noxious stimuli Unless otherwise specified, after receiving 20 bites the intruders were removed, assessed for responsiveness to pain, then returned to a different resident's cage for the next bout of twenty bites This procedure was repeated five times, and lasted usually about 4–5 min Intruder mice were then returned to their home cage

Analgesia Testing

Analgesia was assessed in the tailflick assay [4] Mice were placed on a small platform on the tailflick apparatus and gently restrained under the cupped hand of the experimenter An intense light from a tungsten-halogen lamp was focussed onto the distal tip of their tail The time from onset of the lamp until the tailflick response occurred was measured by a digital timer activated by a switch and automatically cutoff by a photocell mounted over the lamp The lamp, unless specified, was adjusted to yield baseline responses between 1 5–2 5 sec and had an automatic cutoff of 8 sec in order to avoid tissue damage in the absence of a tailflick response In several of the experiments, analgesic testing was continued at 5 min intervals for an additional thirty (30) minutes after the end of the defeat test to assess the effect, if any, of drug treatment on the duration over time of the analgesic response induced by defeat Mice were housed in their home cage during this period

Drugs

Dexamethasone (dexamethasone sodium phosphate—Decadron, Merck, Sharp and Dohme), hexamethonium and metyrapone (Sigma) were dissolved in 0 15 M saline Reserpine (Serpasil, Ciba) was dissolved in oil All drugs were administered IP

Data Analysis

Tailflick latencies were subjected to analysis of variance [33] Dunnett's t was used for post-hoc testing to compare treatment means to a control For significance testing, $p<0$ 05, two-tailed

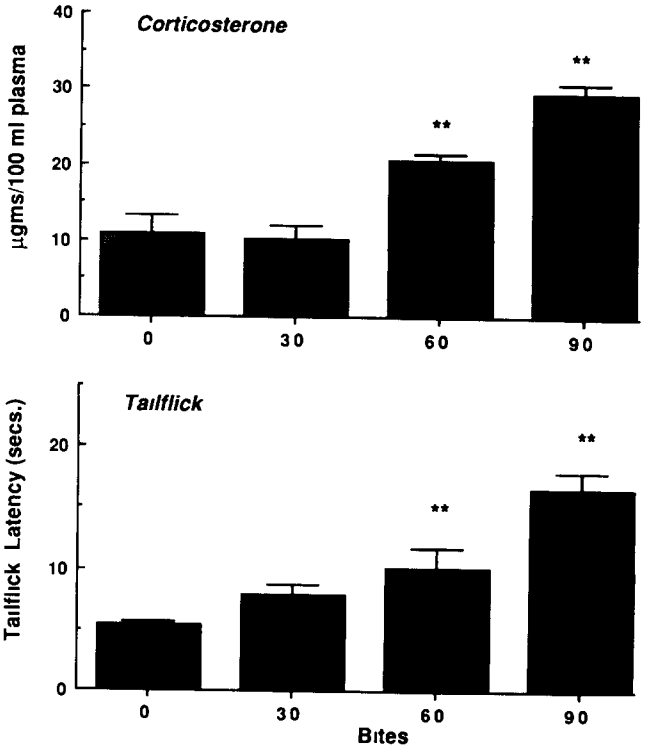

FIG 1 (Top) Mean plasma corticosterone values (µg/100 ml) in mice that were exposed to either 0, 30, 60, or 90 bites from resident mice (Bottom) Mean tail flick latencies (seconds) in mice that were exposed to either 0, 30, 60, or 90 bites from resident mice Vertical lines in each bar represent ±1 SEM

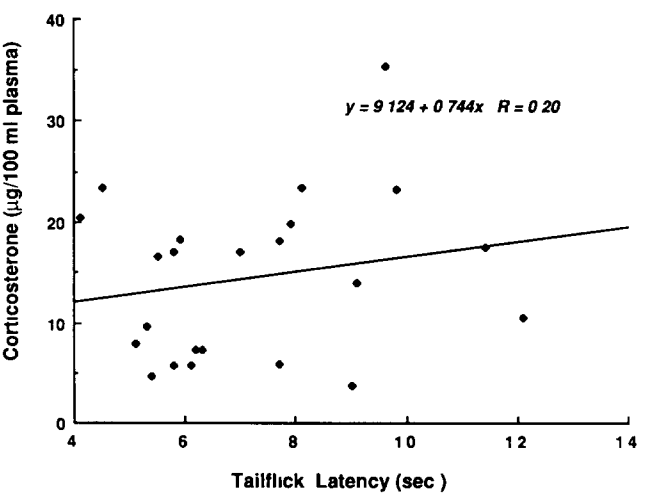

FIG 2 Mean corticosterone values (µg/100 ml plasma) in mice exposed to attack as a function of latency to flick the tail in reaction to a heat stimulus Each point represents data from a single mouse

RESULTS

Experiment I Correlations Between Pituitary-Adrenal Activation and Analgesia

As a first test of the importance of pituitary-adrenal activation, we asked whether or not plasma corticosterone levels are correlated with the magnitude of analgesia ob-

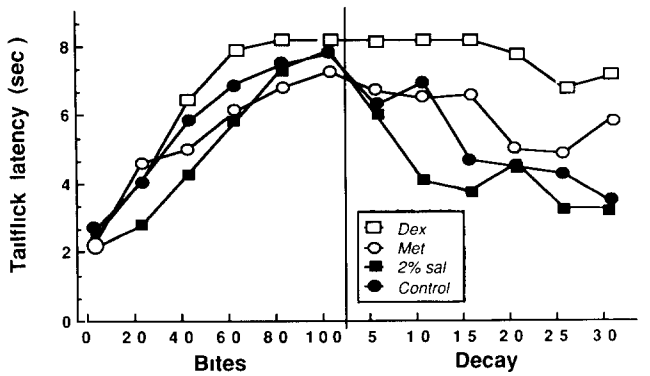

FIG 3 Tailflick latencies in intruder mice as a function of being bitten (left side of panel) or time from the cessation of social conflict (right side of panel) Intruder mice were either injected with dexamethasone (400 μg/kg at 24 hours prior to testing and 200 μg/kg two hours prior to testing), metyrapone (80 mg/kg SC at 6 p m on Day 1, at 10 a m and 6 p m on Day 2 and 3, and again at 10 a m on Day 4, with mice being tested 1 hr later) A third group of mice was given a 2% saline solution as drinking water for three days prior to testing A fourth group of mice received control injections of saline (0 9%) Each point reflects mean tailflick latency ±SEM for at least 6 mice

served in individual mice exposed to different numbers of bites from an opponent CFW mice were used in this instance rather than the $B6AF_1$ mice which we have used previously for two specific reasons CFW mice are not as sensitive to the effects of defeat as are $B6AF_1$ mice [17], and it is possible to obtain mice that show a more graded analgesic response Secondly, we also sought to compare corticosterone levels in the resident mice to those in the intruder mice This comparison, it was hoped, would demonstrate that although both the intruder and resident show large pituitary-adrenal activation, only the defeated mice develop analgesia

Male CFW mice, housed in groups of 10, were individually exposed to either 0, 30, 60, or 90 bites by resident stimulus CFW mice Before and after the introduction into the cage of the stimulus animal their response to pain was determined with the tailflick assay For this experiment, the intensity of the heat stimulus was adjusted so that baseline reaction times were 5–6 sec in duration The heat stimulus intensity was adjusted to allow for the possibility that the resident, attacking males might show hyperalgesia following attack [25] Following the test, the animals were killed by decapitation and trunk blood was collected in a heparinized microcentrifuge tube and centrifuged at 13,000×g for 10 minutes The supernatant was used for determination of corticosterone levels via radioimmunoassay [7]

Significantly elevated plasma corticosterone was found in mice that were exposed to 60 and 90 attack bites [Fig 1 (top), $F(3,26)=17\ 01$, $p<0.0001$], this change was paralleled by significant increases in tailflick latencies in the same groups [Fig 1 (bottom), $F(3,26)=13\ 39$, $p<0\ 0001$]. When these effects are expressed as a function of the number of bites, i e , the amount of stress stimulation, the data suggest a correlation between pituitary-adrenal activation and analgesia However, inspection of each individual's values for corticosterone and analgesia reveals large variations When each mouse's values for corticosterone is plotted as a function of its tailflick latency, the wide scatter of the points demonstrates the absence of a clear systematic relationship (Fig 2) Excluding the scores from mice which went to cutoff time, the Pearson product-moment correlation was $r=0\ 20$, $t(21)=0\ 81$, $p>0\ 05$ Corticosterone values of less than 5 micrograms/100 ml plasma to up to more than 23 micrograms/100 ml plasma are associated with similar tailflick latencies At the highest possible tailflick latencies, 18 sec being the cutoff at the currently used low intensity stimulus, many corticosterone values are higher than 30 micrograms/100 ml of plasma It is possible that a threshold level of corticosterone, unique to each individual, has to be exceeded in order for a large analgesia to develop However, this issue remains unresolved, because the animals that showed these high corticosterone values were also exposed to the greatest number of bites It would be informative to measure corticosterone in animals that fail to show analgesia after being exposed to a large number of bites

Direct manipulations of pituitary and adrenal activity should provide more compelling evidence concerning the contributions of these two glands to the development of analgesia in defeated mice than correlational data Therefore, in our next experiment we utilized a combination of pharmacological and surgical treatments to manipulate or block the output from either gland

Experiment II Investigation of the Contribution of Pituitary β-Endorphin to Defeat-Induced Analgesia

One group of $B6AF_1$ mice received dexamethasone pretreatment, 400 μg/kg at 24 hours prior to testing and 200 μg/kg two hours prior to testing A second group of mice was given a 2% saline solution as drinking water for three days prior to testing These regimens have been used in rats to block stress-induced release of ACTH and β-endorphin from the pituitary [27] A third group of mice received pretreatment with metyrapone according to a schedule which has been shown to increase the pituitary content of β-endorphin in rats (80 mg/kg SC at 6 p m. on Day 1, at 10 a m and 6 p m on Day 2 and 3, and again at 10 a m on Day 4, with mice being tested 1 hr later [9]) A fourth group of mice received control injections of saline

Neither dexamethasone, metyrapone, nor 2% saline pretreatment significantly affected baseline tailflick latencies [Fig 3, $F(3,53)=0\ 74$, n s] Furthermore, none of the treatments diminished the increase in tailflick latency observed after varying amounts of bites, $F(3,53)=1\ 78$, n s Duration of analgesia following the cessation of defeat testing was affected by the various pretreatments, $F(3,19)=8\ 87$, $p<0\ 001$ Further analysis showed this effect to be attributable to the dexamethasone pretreated mice This group of mice showed very little decline in their analgesic response over the thirty min test period Duration over time of the analgesic response in metyrapone and 2% saline pretreated mice did not differ from that observed in control mice

Experiment III Investigation of the Possible Contribution to Defeat Analgesia of Adrenal Enkephalins

Several studies have suggested that adrenal corticosteroids [13] or enkephalins [11] may be involved in the analgesic response produced by footshock stress Recently, it has been reported that stimulation produced analgesia is attenuated by adrenalectomy [30] The earlier demonstration by Leshner [10] that corticosterone pretreatment increases submissiveness in mice exposed to attack further suggests that adrenal contributions, either hormonal or opioid, may be crucial to defeat induced analgesia, even though the re-

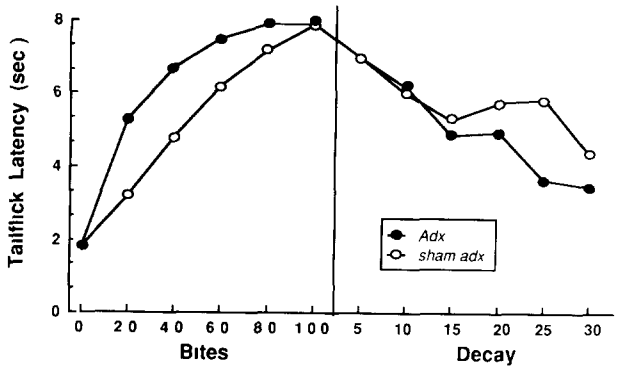

FIG 4 Tailflick latencies in intruder mice as a function of being bitten (left side of panel) or time from the cessation of social conflict (right side of panel) Intruder mice were either adrenalectomized or sham-operated one week prior to testing Each point reflects mean tailflick latency ±SEM for at least 6 mice

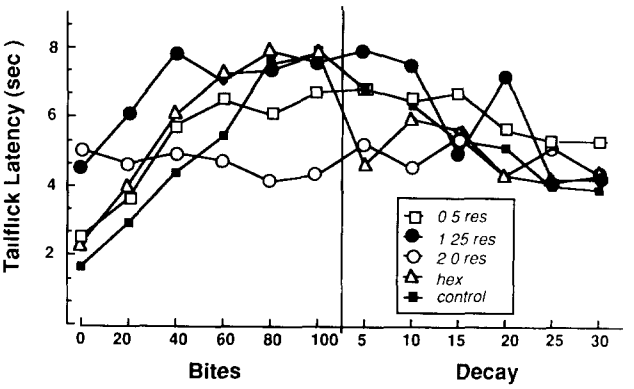

FIG 5 Tailflick latencies in intruder mice as a function of being bitten (left side of panel) or time from the cessation of social conflict (right side of panel) Intruder mice were pretreated with reserpine (0 5, 1 25, or 2 mg/kg IP 48 and 24 hr prior to testing), or hexamethonium, 10 mg/kg, 20 min prior to testing Each group contained at least 6 mice Control animals were injected with vehicle Each point reflects mean tailflick latency ±SEM for at least 6 mice

sults of our first experiment suggested that plasma corticosterone levels did not correlate directly with analgesia In order to test for the involvement of the adrenals in defeat-induced analgesia, one group of intruder males was subjected to bilateral adrenalectomy under pentobarbital anesthesia and maintained on 0 15 M saline until testing one week later Control mice were subjected to similar operative procedures with the exception that the adrenals were exposed but not removed Adrenalectomized mice were sacrificed and checked for completeness of tissue removal following the end of testing

Additional groups were pretreated with varying doses of reserpine (0 5, 1 25, or 2 mg/kg IP) for two consecutive days prior to testing This latter treatment has been reported to enhance stress-induced analgesia in rats, presumably by enhancing the storage and release of adrenal enkephalins [11] A final group was pretreated with hexamethonium, 10 mg/kg IP, 20 min prior to testing [11] This peripheral ganglionic blocker has been shown to reduce adrenal enkephalin secretion *in vivo*

Adrenalectomy did not block defeat-induced analgesia (Fig 4) In fact, the analgesic response of the adrenalectomized mice was significantly enhanced over that observed in the sham-operated mice, $F(1,34)=4\ 23$, $p<0\ 05$ Adrenalectomy did not affect the duration of the analgesia, with these mice showing a decline similar to that observed in the sham-operated mice, $F(1,9)=0\ 47$, n s The results for reserpine were somewhat unclear (Fig 5), in that reserpine at the two higher doses (1 25 and 2.0 mg/kg) significantly elevated baseline latencies, $F(3,26)=14\ 98$, $p<0\ 001$ It is clear that reserpine did not *enhance* the analgesic response to defeat If anything, it appeared to produce a dose-related attenuation of the response At the highest dose, 2 mg/kg, mice showed no increase in response to defeat above the elevated baseline These mice showed marked ptosis and were somewhat ataxic The lowest dose, 0 5 mg/kg, did not affect analgesic responsiveness to defeat The duration of analgesic response following defeat was also not affected by reserpine pretreatment Hexamethonium pretreated mice did not differ from controls, either in terms of induction of analgesia or duration Analyses of variance supported these observations Data for the 2 mg/kg reserpine group were not included in the calculations Overall treatment F was significant, $F(3,19)=3\ 16$, $p<0\ 05$, but this was readily attributable to the elevated baselines of the mice in the 1 25 reserpine group No other point during the defeat testing phase was found to be different Duration of analgesia also did not differ between the groups, $F(3,19)=1\ 09$, n s

DISCUSSION

Previously, we reported defeat-induced analgesia appeared to be mediated by endogenous opioids, in that it is fully antagonized by naloxone and shows full cross-tolerance with morphine Furthermore, quaternary naltrexone did not block this response, suggesting that brain, rather than peripheral stores of opioids were involved The present experiments are consistent with this hypothesis Neither stress-induced release of pituitary β-endorphin nor adrenal enkephalins were found to be necessary for the observed analgesic response Furthermore, plasma corticosterone levels did not correlate directly with the magnitude of the analgesic response It may be that this measure can only reflect the occurrence, rather than the degree of stress experienced by the subject, as suggested by Natelson *et al* [22] These results reinforce our previous suggestion [8] that stress itself does not appear to be the critical factor underlying the analgesic response observed in mice subjected to defeat A similar conclusion regarding stress and foot shock-induced analgesia has been reached by Watkins *et al* [31]

These results correspond remarkably well with data regarding the effects of these treatments on morphine analgesia [5,8], but differ from those reported by MacLennan *et al* [13] and Lewis *et al* [11] These latter studies found marked attenuation of analgesia in both hypophysectomized and in adrenalectomized rats, although the stress paradigms used in the two studies for generating naloxone-reversible analgesia differed In explaining their results, MacLennan *et al* [13] proposed a model for stress analgesia which was based on the permissive action of corticosterone on midbrain pain centers, rather than pituitary β-endorphin Alternatively, Lewis *et al* [11] suggested that their results indicated a primary role for adrenal medullary enkephalin-like peptides These results were difficult to interpret, however, given that circulating enkephalins would be unlikely to cross the blood brain barrier and would also be subject to rapid degradation [23,24] (It should be noted, however, that in a later report [6], these authors, using different parameters of shock deliv-

ery to induce analgesia, also failed to see an effect of adrenal removal) Maixner and Randich [14] have recently proposed that enkephalins released from peripheral sources may stimulate peripheral rather than central opioid receptors to induce analgesia by reflexively engaging vagal afferents linked to endogenous pain inhibition systems of the CNS, thereby obviating the need for passage of opioids into the CNS They argue that experimentally-induced analgesias may result from either the physiological activation of vagal afferents by increases in central venous pressure or from the resulting secretion of humoral substances into the circulation, which in turn stimulates vagal afferents

A procedural difference which may lead to discrepancies in interpretation of results from different laboratories involves the test paradigms used to measure the experimentally-induced analgesia Some investigators define their antinociceptive effect in terms of "peak response," or a change in the latency of response (either tailflick, pawlick, or flinch jump) immediately after termination of the stressor Others take into account not only the initial analgesic response, but also its decay over time, defining analgesia in terms of "area under the curve " These procedural differences become especially critical when deciding whether or not an experimentally-induced analgesia is opioid or nonopioid in nature Using the first definition, naloxone pretreatment could be said to be without effect if the analgesic response immediately after the stress did not differ from that of the control group However, using the second definition, naloxone is said to have significantly reduced the analgesic response if the duration of the analgesic response of the naloxone pretreated subjects is reduced over the course of some prolonged time period compared to the control group, even when there might not be a reduction in the magnitude of the analgesic response observed immediately after the cessation of stress Unfortunately, these quite different interpretations of naloxone antagonism are usually lost whenever research is summarized for review purposes

The response of an organism to stress is multifaceted, with the capacity to modulate pain being but one part. Different species have most likely evolved somewhat different ways for reacting to and dealing with the type of stresses they are likely to encounter Both peripheral and central mechanisms are activated by stress, and hormones released in response to stress have both central and peripheral actions It seems possible that central and peripheral reactions to stress may be separable, at least in terms of the analgesic response It may perhaps have been too easy to attribute an important role for pituitary β-endorphin or adrenal enkephalins because they are readily released into the blood in response to stress We would argue that, at least in regard to the paradigm employed here, these peripherally released opioids are relevant to peripheral reactions to stress We postulate that analgesic mechanisms existing in the CNS can be activated as part of a response to an environmental event independently of the classical pituitary-adrenal sympathetic nervous system stress response pathway Opioid peptide(s) released from and acting at sites within the CNS may be the actual analgesic agent(s) involved

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