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Naloxone injections into the periaqueductal grey area and arcuate nucleus block analgesia in defeated mice

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Abstract. In a situation of social conflict, mice that are defeated by an opponent exhibit a marked analgesia. Microinjections of naloxone (1 or 10 μg) into the periaqueductal grey area (PAG) or into the region of the arcuate nucleus prior to the defeat prevented the emergence of analgesia. Microinjections of morphine (5 μg) into these sites had previously been shown to produce profound analgesia. Mice whose adrenals were removed rapidly developed analgesia when attacked by a stimulus animal. Injection of naloxone into PAG also antagonized defeat-induced analgesia in adrenalectomized mice. These observations indicate that sites and processes in the brain rather than in the periphery are responsible for the development of analgesia in mice that are subjected to social defeat.

Key words: Analgesia – Aggression – Defeat – Pain – Naloxone – Morphine – Adrenal glands – Adrenalectomy – Microinjection

The role of endogenous opioid peptides in the mechanisms mediating analgesia that results from exposure to aversive stressful stimuli has been a focus of investigation since the discovery of these peptides. Yet, at present, an essential and indispensible function for endogenous opioid peptides in stress-induced analgesia has not been established (Millan 1981; Watkins and Mayer 1982). A large variety of noxious, aversive events can act as stressors that are capable of rendering an organism analgesic. Yet, it appears that only certain types of stressors, under circumscribed parameters, produce analgesia that can be blocked, at least in part, by opiate antagonists and that also show cross-tolerance to morphine (Chesher and Chan 1977; Lewis et al. 1980; Grau et al. 1981; Watkins and Mayer 1982). Many other types of stress-induced analgesia are insensitive to naloxone or naltrexone and fail to show cross-tolerance to morphine (Bodnar et al. 1978; Lewis et al. 1980; Chance and Rosecrans 1979).

Even in those cases of stress-induced analgesia that do appear to be mediated by endogenous opioid systems it is not clear which of these peptides is critical for the analgesia and from which source they originate. The arcuate nucleus of the hypothalamus contains cell bodies that project fibers to several limbic structures and, most significantly, to the periaqueductal grey area. These cells contain endogenous opioid peptides (Akil and Watson 1983). Cells in the anterior and intermediate lobes of the pituitary gland, and chromaffin cells in the adrenal medulla secrete opioid peptides (Guillemin et al. 1977; Viveros et al. 1979). It has not been shown how these peptides, when secreted from peripheral glands, penetrate into the central nervous system (CNS) and modulate transmission in the pain pathways. However, hypophysectomy or adrenalectomy may affect analgesia that is generated by some forms of stress (MacLennan et al. 1982; Lewis et al. 1982; Mousa et al. 1983).

Recently, we studied mice subjected to social conflict and observed a profound analgesia in those animals that were defeated (Miczek et al. 1982). The analgesia produced by this biologically relevant form of stress was completely blocked by naloxone or naltrexone. Mice that were tolerant to morphine did not become analgesic after defeat and, conversely, chronically defeated mice failed to show morphine analgesia. A single defeat experience decreased brain β-endorphin by about 40% as measured with radioimmunoassay (Thompson et al. 1981). Furthermore, when pituitary or adrenal secretions of endogenous opioid peptides were experimentally compromised, analgesia continued to be evident in defeated mice (Thompson and Miczek 1983).

We now report that analgesia in defeated mice is antagonized by microinjections of naloxone into the periaqueductal grey area or into the arcuate nucleus of the hypothalamus. Even after the adrenal source of endogenous peptides was eliminated, defeated mice became analgesic. Blockade of opiate receptors with naloxone microinjections into the periaqueductal grey area effectively prevented analgesia in adrenalectomized mice. These findings indicate that the analgesia in adrenalectomized mice is opioid mediated. However, the adrenals are not the source of the material that activates the receptors that are blocked by naloxone microinjections into brain.

Materials and methods

Adult male $B6AF_1$ mice were housed in standard clear polycarbonate cages (28 cm long, 18 cm wide, 12 cm high) with unrestricted access to Purina rodent chow and water. These and additional mice of the Swiss-Webster strain were kept in a vivarium with controlled 12-h light-dark cycle, temperature (21° ± 1° C), and humidity (40%–50%).

Using 75 mg/kg sodium pentobarbital, IP, as anesthetic, the $B6AF_1$ mice were stereotaxically implanted with a 3- or 4-mm-long 26-gauge guide cannula (inside diameter

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0.254 mm, outside diameter 0.457 mm). The cannula was permanently affixed to the skull with acrylic cement. The tip of the guide cannula was positioned exactly 1 mm ventral to dura and directly dorsal to the target site of injection according to the stereotaxic coordinates of Slotnick and Leonard (1975). The sagittal sinus and dura were visualized unter a dissecting microscope. A stylus, which was removed during injections, was inserted into the guide cannula.

Solutions were injected into the periaqueductal grey area or the arcuate nucleus through a 33-gauge cannula (inside diameter 0.102 mm, outside diameter 0.203 mm), which was inserted into the guide cannula. The length of the injection cannula was adjusted so that its tip was in the dorsal portion of the target structure. A 25-mm length of Intramedic PE-10 tubing connected the injection cannula to a 1-µl Hamilton syringe. A Sage syringe pump (Model 341 A) was set so that 0.5 µl was dispensed over 56 s and 0.25 µl was dispensed over 28 s. To prepare sterile, pyrogen-free solutions for injection 2-µm Gelman polysulfone filters were used.

The injection procedure consisted of removing the stylus, inserting the injection cannula while the animal was hand-held, injecting the solution for 28 or 56 s in the freely moving mouse, leaving the injection cannula in place 1 min longer, and then removing the injection cannula. The patency of the injection cannula was checked before and after each injection. Morphine sulfate was injected at one of two concentrations, 2.5 or 5.0 µg/µl, and naloxone concentrations were either 1.0 or 10 µg/µl.

The animals were tested for responsiveness to pain with the tailflick assay (D'Amour and Smith 1941). Radiant heat was focused on the mouse's tail. A flick of the tail terminated the heat stimulus, and the latency to flick was displayed by a digital timer. During control tests all mice flicked their tail within 1.2–1.7 s. The heat stimulus was discontinued at 8.0 s if no response occurred. To avoid damage to the cannula implant and to reduce restraint by the experimenter, the mice were gently wrapped in a soft cloth during tailflick tests.

Defeat behavior was induced in the experimental mice during resident-intruder tests (Miczek and O'Donnell 1978). When the experimental mice were placed individually into the home cage of a mouse of the Swiss-Webster strain, the resident mouse threatened, pursued, and bit the intruder. After initial retaliatory bites, the intruder quickly engaged in defensive postures and escape attempts. Eventually, the intruder showed a pattern of submission or defeat behavior; it assumed an upright body posture, its forepaws were limp, the head was angled upward, and the ears were retracted (Miczek et al. 1982). Most mice display defeat behavior after being bitten 50–70 times. The cannula implant had no discernable effect on the fighting behavior of the stimulus or experimental animals.

The experimental protocol consisted of stereotaxically implanting the mice with a unilateral intracranial guide cannula. After a 4–7-day recovery period, the animals were injected through the intracranial cannula with either saline or morphine and tested for responsiveness to pain with the tailflick assay. The animals that showed morphine analgesia were injected a second time 5–7 days later; either naloxone or saline was injected immediately before the intruders were subjected to attack bites by resident stimulus animals. After being bitten 20 times, the experimental mouse was removed from the resident's cage and a tailflick test was performed. This sequence of 20 attack bites followed by a tailflick assay was repeated five times for a total of 100 bites and five analgesia determinations. The entire procedure lasted 6–12 min.

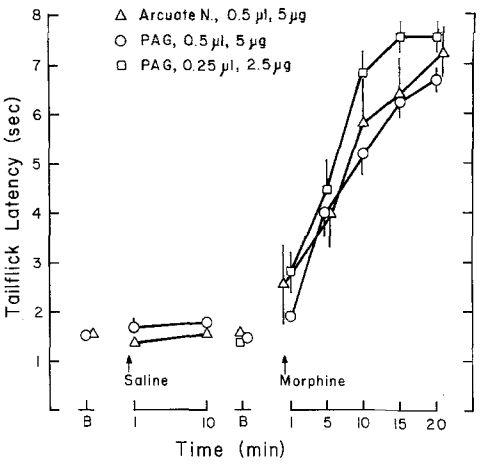

Fig. 1. Time course for the effects of saline (*left*) and morphine (*right*) injected into arcuate nucleus (*triangles*) or into periaqueductal grey area (PAG, *circles*: 5 µg in 0.5 µl; *squares*: 2.5 µg in 0.25 µl). Vertical lines in data points indicate ±1 SEM. The heat stimulus was automatically terminated at 8 s if no flick occurred

In the initial experiments the injection volume was 0.5 µl, and the drug doses were 5 and 10 µg for morphine and naloxone, respectively. In an additional set of mice the volume was reduced to 0.25 µl, and the drug doses were lowered (2.5 µg morphine, 1 µg naloxone).

Treatment effects were evaluating using nonparametric statistical methods. The Wilcoxon signed-ranks test was used within treatment groups to compare tailflick latencies after each block of attack bites with baseline, and between-group comparisons were made with the Mann-Whitney *U* test. The two-tailed criterion of significance was used.

Results

Injections of 5 µg morphine sulfate in a 0.5 µl volume into the dorsal portion of the periaqueductal grey area ($n=22$) or the arcuate nucleus ($n=8$) resulted in analgesia. Within 5–15 min after injection, tailflick latencies greater than 6 s were observed (Fig. 1). When the injection sites were near, but outside these target regions ($n=29$), morphine produced only a modest increase in tailflick latencies, i.e., between 3 and 6 s. Saline injections into morphine-sensitive sites within the periaqueductal grey area or the arcuate nucleus failed to alter tailflick latencies immediately after the injection or 10 min later (Fig. 1). In additional mice ($n=19$) 2.5 µg morphine sulfate in a 0.25 µl volume produced maximal analgesia within 5–15 min after injection into the periaqueductal grey area (Fig. 1).

Exposure to bites from the attacking animal led to analgesia in saline-injected mice (Fig. 2). In the initial experiments, saline (0.5 µl) was injected into the periaqueductal grey area ($n=10$) before the animal was exposed to the attacking mice (Fig. 2, left); after the mouse had been bitten at least 40 times, tailflick latencies were significantly elevated and after 100 bites six of the ten mice failed to react to the heat stimulus within the allotted 8 s. In an additional group of seven mice, very similar results were obtained

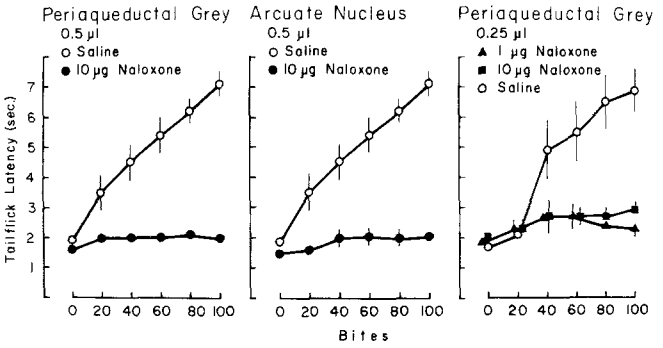

Fig. 2. The latency to flick the tail away from the heat stimulus (in seconds) as a function of exposure to attack bites from a stimulus animal. *Left*: Saline (*open circles*) or 10 µg naloxone (*solid circles*) were injected in a volume of 0.5 µl into the periaqueductal grey area. *Center*: Saline (*open circles*) or 10 µg naloxone (*solid circles*) were injected in a volume of 0.5 µl into the region of the arcuate nucleus. *Right*: Saline (*open circles*), 1 µg naloxone (*solid triangles*), or 10 µg naloxone (*solid squares*) in a volume of 0.25 µl were injected into the periaqueductal grey area. Vertical lines in data points indicate ±1 SEM. The heat stimulus was automatically terminated at 8 s if no flick occurred

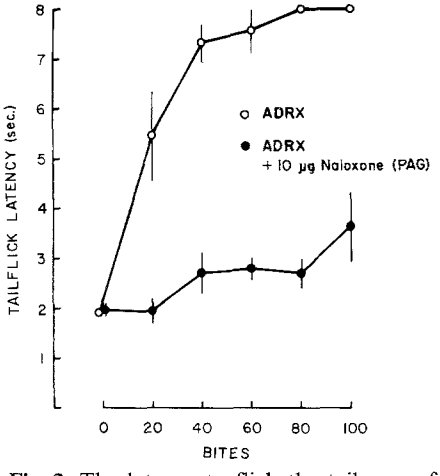

Fig. 3. The latency to flick the tail away from the heat stimulus (in seconds) as a function of exposure to attack bites from a stimulus animal. All experimental mice were adrenalectomized. Data from the control group are represented by *open circles*, and *solid circles* show those from the group injected with 10 µg naloxone into the periaqueductal grey area. Vertical lines in data points indicate ±1 SEM. The heat stimulus was automatically terminated at 8 s if no flick occurred

when the injection volume was halved (0.25 µl; Fig. 2, right). Naloxone (10 µg in 0.5 µl), injected into the periaqueductal grey area ($n=18$) or into the arcuate nucleus ($n=8$), blocked the emergence of analgesia in mice that were exposed to as many as 100 attack bites (Fig. 2, left and center). Further experiments with the 0.25-µl volume indicated that naloxone injections into the periaqueductal grey area either at the 10 µg ($n=7$) or 1 µg ($n=6$) dose effectively blocked analgesia in mice that were bitten up to 100 times.

Additionally, the effects of naloxone (10 µg in 0.5 µl) were examined in mice that were exposed to bites from stimulus animals after adrenalectomy. In comparison to adrenalectomized controls ($n=10$), naloxone injections into the periaqueductal grey area significantly blocked the development of analgesia in adrenalectomized mice ($n=7$; Fig. 3).

A total of 104 injection sites were examined histologically. Injections aimed for the periaqueductal grey area with a 5-µg morphine dose produced a marked analgesic response in 22 cases (i.e., tailflick latency greater than 6 s), moderate analgesia in 25 cases (i.e., tailflick latency between 3 and 6 s), and a negligible response (less than 3 s) in 12 cases. Additionally, the lower 2.5-µg morphine dose resulted in a marked analgesia in 19 cases with periaqueductal grey placements, and in moderate analgesia in two more animals; six injections with 2.5 µg morphine had negligible effects. The most effective sites for morphine analgesia were located medially in the periaqueductal grey area; when the injection sites were more than 0.5 mm from the midline, only two analgesia-producing sites were recorded. Three sites producing morphine analgesia were located in the parafascicular nucleus of the thalamus and one in the bed nucleus of the posterior commissure.

The actual placements for injections that were aimed for the arcuate nucleus of the hypothalamus were scattered more than those for periaqueductal grey area; in eight cases 5 µg morphine produced a marked analgesic response, and in four cases a moderate response. Of these twelve cases five placements were actually located posterior to the arcuate nucleus in the dorsal and ventral premammillary and in the mammillary nuclei. In six mice the arcuate nucleus was missed entirely and no morphine analgesia was seen.

Discussion

Our observations focus on sites and processes in the brain as opposed to peripheral endocrine responses to stress as the critical mechanism for the development of analgesia in mice subjected to defeat. The principal finding of the present experiments is that naloxone injections into the periaqueductal grey area or into the region of the arcuate nucleus completely blocked defeat analgesia.

The antinociceptive effects of intracerebral microinjections of morphine described here extend previous observations in primates, rabbits and rats (for review see Yaksh and Rudy 1978). We confirmed in mice the periaqueductal grey area as a region that is very sensitive to morphine's antinociceptive effects (Criswell 1976). In addition, we identified the region of the arcuate nucleus of the hypothalamus as an area from which morphine analgesia could be produced. It was considerably more difficult to achieve accuracy with this ventral target as compared to the more dorsally located periaqueductal grey area. Morphine injections into areas outside of the two target structures resulted in partial or no analgesia indicating anatomical specificity for the effect. We have manipulated the volume and dose of morphine microinjections to explore the minimal conditions under which antinociceptive effects may be produced in the mouse. A significant elevation in tailflick latency was noted within 5 min after 2.5 µg morphine in 0.25 µl, indicating that even smaller morphine doses may be effective. It is difficult to verify absence or presence of analgesic effects that may be produced by nanoliter volumes of morphine solutions without independent confirmation of drug delivery. Injection with a syringe pump and tubing may not be the most satisfactory method for such small volumes.

The blockade of analgesia in defeated mice after naloxone injections into the periaqueductal grey area on the ar-

cuate nucleus parallel similar observations with morphine analgesia. The analgesic action of systemically administered morphine can be reversed by naloxone injections into brainstem sites (Azami et al. 1982). However, intracerebrally injected naloxone may be anatomically less discretely localized than morphine. Since naloxone is more lipid-soluble than morphine, it may be expected to diffuse more readily from the injection site. Yet, a marked anatomical site-specificity for naloxone's analgesia-blocking effect was seen. Of course, only sites that previously produced morphine analgesia were subsequently investigated with naloxone. Similarly, because of the high lipid solubility of naloxone, a rapid onset and short duration characterizes the action of this drug. With the presently used naloxone doses the analgesia-blocking effect was evident throughout the relatively short defeat procedure.

The term "stress-induced analgesia" is misleading in the sense that it attempts to encompass not only a variety of environmental stimulus situations and behavioral adaptations, but also appears to refer to stress physiology as critical for analgesia (Watkins et al. 1982). Because β-endorphin and ACTH are derived from the same precursor molecule, and because both are released by stress, it has been suggested that β-endorphin mediates stress-induced analgesia. Indeed, analgesia produced by certain types of electric foot shock and electro-acupuncture is attenuated by eliminating or reducing β-endorphin release from the pituitary (Cheng et al. 1979; Lewis et al. 1980; MacLennan et al. 1982; Mousa et al. 1983). Moreover, enkephalins from the adrenal medulla may be critical for at least one type of foot-shock analgesia (Lewis et al. 1982). Other types of naloxone-reversible stress-induced analgesia, like morphine analgesia, are actually enhanced by adrenalectomy or hypophysectomy (Kasson and George 1983; Watkins et al. 1982; Wei 1973). We confirmed that opioid peptides from adrenal or pituitary glands are not involved in analgesia of defeated mice (Thompson and Miczek 1983). Specifically, dexamethasone pretreatment, which blocks β-endorphin release from the pituitary in response to stress (Rossier et al. 1979), did not reduce the analgesic response in defeated mice. Removal of the adrenal glands prior to exposure to defeat also failed to diminish the analgesic response. The blockade of analgesia by naloxone injected into the periaqueductal grey area confirms our previous conclusion that opioid peptides acting on CNS receptors are responsible for the analgesia in defeated mice.

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