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THE ROLE OF MU-OPIOID RECEPTOR SIGNALING IN THE DORSOLATERAL PERIAQUEDUCTAL GRAY ON CONDITIONAL AND UNCONDITIONAL RESPONDING TO THREATENING AND AVERSIVE STIMULI

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Abstract—Here we examined how mu-opioid receptor signaling in the periaqueductal gray (PAG) mediates conditional and unconditional responses to aversive stimuli. The mu-opioid agonist morphine (MOR) and/or the partially mu-selective antagonist naltrexone (NAL) were infused into dorsolateral PAG (dlPAG) during a fear conditioning task, in which rats were trained to fear an auditory conditional stimulus (CS) by pairing it with a unilateral eyelid shock unconditional stimulus (US). During drug-free test sessions, the CS elicited movement suppression responses (indicative of freezing) from trained rats that had not recently encountered the US. In trained rats that had recently encountered the US, the CS elicited flight behavior characterized by turning in the direction away from the eyelid where US delivery was anticipated. Infusions of MOR (30 nmol/side) into dlPAG prior to the test session did not impair CS-evoked movement suppression, but did impair CS-evoked turning behaviors. MOR infusions also reduced baseline motor movement, but US-evoked reflex movements remained largely intact. NAL was infused at two dosages, denoted 1x (26 nmol/side) and 10x (260 nmol/side). Infusions of NAL into dlPAG did not affect CS- or US-evoked behavioral responses at the 1x dosage, but impaired CS-evoked movement suppression at the 10x dosage, both in the presence and absence of MOR. When rats were co-infused with MOR and NAL, MOR-induced effects were not reversed by either dosage of NAL, and some measures of MOR-induced movement suppression were enhanced by NAL at the 1x dosage. Based on these findings, we conclude that mu-opioid receptors in dlPAG may selectively regulate descending supraspinal motor pathways that drive active movement behaviors, and that interactions between MOR and NAL in dlPAG may be more complex than simple competition for binding at the mu receptor.

Key words: opioids, periaqueductal gray, morphine, naltrexone, fear conditioning.

INTRODUCTION

When faced with a threat, an animal's defensive responses are triggered so that it can escape from or ward off the danger (Bolles and Fanselow, 1980; Fanselow and Sigmundi, 1986; Fanselow et al., 1994). A standard model of the brain's defensive circuitry proposes that the amygdala mediates emotional fear responses to threatening stimuli, and sends efferent projections to other brain areas that govern specific defensive responses to such stimuli (Kluver and Bucy, 1937; Weiskrantz, 1956; LeDoux et al., 1988, 1990; Davis, 1992; Blair et al., 2005; Seymour and Dolan, 2008). One important target of the amygdala's output projections is the midbrain periaqueductal gray (PAG), which mediates behavioral defenses such as flight and freezing (Fanselow, 1991; Depaulis et al., 1992; Kim et al., 1993; De Oca et al., 1998; Morgan et al., 1998; Vianna et al., 2001; Leman et al., 2003), as well as antinociceptive responses that are triggered by encounters with painful or threatening stimuli (Basbaum and Fields, 1984; Watkins and Mayer, 1986; Fanselow, 1991). Among the many receptor subtypes in PAG, it has been well established that mu-opioid receptors are high in density (Goodman et al., 1980; Mansour et al., 1988; Brodsky et al., 1995) and play a significant role in pain modulation (Pert and Yaksh, 1975; Yaksh et al., 1976; Smith et al., 1988; Manning et al., 1994), as well as defensive responding and aversion (Motta and Brandao, 1993; Morgan et al., 1998; Anseloni et al., 1999).

Here, we investigated how mu-opioid receptor signaling in PAG mediates conditional and unconditional responses to aversive stimuli during a Pavlovian fear conditioning task. Freely behaving rats were trained to fear an auditory conditional stimulus (CS) by pairing it with a shock unconditional stimulus (US) delivered to one of their eyelids. When the CS was subsequently presented to trained rats, they exhibited movement suppression responses (indicative of ''freezing'') when they had not encountered a shock within the past 24 h. However, when the CS was presented to trained rats that had recently encountered a shock, the rats exhibited a specific flight behavior during the CS: turning away from the eyelid where the shock US was anticipated. Hence, the recent encounter with shock induced a change in the mode of

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Abbreviations: CS, conditional stimulus; dlPAG, dorsolateral PAG; MOR, morphine; NAL, naltrexone; NAL1x, low dosage of NAL; NAL10x, high dosage of NAL; PAG, periaqueductal gray; US, unconditional stimulus; VEH, vehicle

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the rat's defensive responses to the CS, as reported previously (Tarpley et al., 2010).

To investigate the role of PAG mu-opioid receptors in these distinct modes of defensive behavior, we analyzed responses to the CS and US in trained rats following infusions of the mu-opioid agonist morphine (MOR) and/or the partially mu-selective antagonist naltrexone (NAL) into the dorsal and lateral columns of PAG (dlPAG). Results are discussed in the context of clinical and pharmacological evidence that interactions between MOR and NAL may be more complex than simple competition for binding at the mu receptor.

EXPERIMENTAL PROCEDURES

Subjects and surgery

Male Long–Evans rats weighing 350–400 g were housed singly and reduced to 85% of *ad-lib* weight through limited daily feeding. While it is possible that housing rats singly could influence their selection of defensive strategies, it has been shown that cagemate number does not significantly affect physiological variables that would indicate changes in anxiety (Giralt and Armario, 1989; Brown and Grunberg, 1995). Under deep isoflurane anesthesia, all rats were implanted with a pair of insulated stainless steel wires (75 μm diameter) threaded into the skin of each eyelid for delivering the periorbital shock US. Rats were also implanted with a pair of 26-gauge microinjector guide cannulae (Plastics One, Roanoake, VA) targeted bilaterally in the PAG (7.5 mm posterior, 0.75 mm lateral and 5.2 mm ventral to bregma). A total of 39 rats underwent surgery, of which 9 were later dropped from the study because of misplacement of cannula tips identified during histology ($n = 4$), or failure to reach behavioral criterion for progressing to the drug delivery phase of the study ($n = 5$, see section Experimental design below). The remaining 30 rats were included in the data analysis. All experimental procedures were approved by the UCLA Animal Research Committee and were conducted in accordance with USA federal guidelines. All efforts were made to minimize the number of animals used and their suffering.

Fear conditioning experiments

Throughout pre-exposure and fear conditioning sessions, rats constantly foraged on a 70 × 70-cm platform for 20-mg purified food pellets (Bioserv, Frenchtown, NJ) dropped from an overhead dispenser at ~30-s intervals, to provide a baseline of motor activity against which stimulus-evoked movement and turning behavior could be measured. The CS for fear conditioning was a train of 70-dB white noise pips, each lasting 250 ms, delivered at 1 Hz for 20 s through an overhead speaker. The US was a train of 2.0-mA shock pulses, each lasting 2.0 ms, delivered to one eyelid at a rate of 6.66 Hz for 2 s. During CS–US pairing trials, the first shock pulse was always delivered 300 ms after the offset of the final (20th) CS pip. The inter-trial interval was uniformly random between 180 and 240 s.

Experimental design

After recovery from surgery, rats were pre-exposed (i.e., habituated) for 5 days (20 min/day) to the experimental platform before any fear conditioning sessions were conducted. During this time, the animals learned to chase food pellets and became habituated to the test environment. Following pre-exposure, rats received 4–7 consecutive days of training, each consisting of six CS-alone presentations (test sessions) and 16 CS–US paired presentations (training sessions). The first intracranial infusion was administered 1 day after the second consecutive training session during which the rat's turning bias exceeded 4 degrees/s in the direction away from the trained eyelid (five rats that failed to meet this criterion by the 7th day of training were dropped from the study and did not receive drug infusions). For rats that reached behavioral criterion, different drugs were infused over multiple days, with a rest day given after each infusion day, followed by a day of drug-free retraining before the next infusion. Infusion conditions included MOR alone, NAL alone, combination of MOR and NAL, and vehicle (VEH). The order of drug infusions was counterbalanced across rats. This repeated measures design was beneficial because it reduced the number of animals required for the study, and because it eliminated variability of injection sites among drug conditions, since all drugs were infused at the same set of injection sites.

Behavioral scoring

The rat's moment-to-moment position on the platform was sampled at 30 Hz by an overhead video tracking system (Neuralynx Corporation, Bozeman, MT), which monitored the location of three light-emitting diodes (red, blue, and green) attached to the animal's headstage for automated scoring of freezing, movement, and turning behavior using software developed in our laboratory. The algorithm for scoring freezing behavior has been described elsewhere (Moita et al., 2003, 2004). The algorithm for scoring movement and turning behavior first performed one iteration of smoothing (5-point adjacent averaging) upon the position data for each of the three colored LEDs. The center point of the three LEDs was obtained by averaging their x and y coordinates, and the displacement distance of this center position between each successive video frame gave the rat's linear movement speed. The angles of the axes passing through each pair of tracking LEDs (red–green, red–blue, blue–green) were measured with respect to the horizontal axis of the video screen. Two of the angles (red–blue and blue–green) were rotated by the appropriate amount to align them with the third angle (red–green), and the mean of these transformed angular measurements was computed using circular averaging to obtain the rat's directional heading for each video frame (if one of the LEDs was occluded, then only one of the three color axes was used to estimate the directional heading). The change in directional heading angle between each successive video frame gave the rat's angular head-turning velocity.

Infusions

All drugs were dissolved in a VEH solution of 0.9% sterile saline containing 2% Tween. Drugs were infused intracranially into both hemispheres of PAG at a volume of 0.25 μl/side, through 33-gauge injectors at a rate of 0.2 μl/min. MOR was dissolved at a concentration of 30 nmol (Anseloni et al., 1999). A low dosage of NAL (NAL1x) was dissolved at 26 nmol (Coimbra and Brandao, 1997; de Luca et al., 2003), and a high dosage of NAL (NAL10x) was dissolved at 260 nmol. Cocktail drugs (MOR + NAL1x and MOR + NAL10x) were also infused at a volume of 0.25 μl per side. Prior to drug infusion, dummy cannulae (which were in place at all times except during infusions to prevent clogging of the guide cannulae) were removed and injector cannulae were inserted in their place. After drug infusions, the injectors were left in place for an additional 2 min to allow diffusion of the drug away from the cannulae tip, after which the injectors were removed and replaced with dummy cannulae. Throughout the infusion process, the animal was held gently on the experimenter's lap. After the infusion was complete, the rat was returned to its home cage for 15 min to allow time for the drug to take effect before the experiment resumed.