Neuroscience 335 (2016) 1–8

ANALYSIS OF MORPHINE-INDUCED CHANGES IN THE ACTIVITY OF PERIAQUEDUCTAL GRAY NEURONS IN THE INTACT RAT

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Abstract—Microinjection of morphine into the periaqueductal gray (PAG) produces antinociception. *In vitro* slice recordings indicate that all PAG neurons are sensitive to morphine either by direct inhibition or indirect disinhibition. We tested the hypothesis that all PAG neurons respond to opioids *in vivo* by examining the extracellular activity of PAG neurons recorded in lightly anesthetized and awake rats. Spontaneous activity was less than 1 Hz in most neurons. Noxious stimuli (heat, pinch) caused an increase in activity in 57% and 75% of the neurons recorded in anesthetized and awake rats, respectively. The same noxious stimuli caused a decrease in activity in only 17% and 6% of neurons recorded in anesthetized and awake rats. Systemic administration of morphine caused approximately equal numbers of neurons to increase, decrease, or show no change in activity in lightly anesthetized rats. In contrast, administration of morphine caused an increase in the activity of 22 of the 27 neurons recorded in awake rats. No change in activity was evident in the remaining five neurons. Changes in activity caused by morphine were surprisingly modest (a median increase from 0.7 to 1.3 Hz). The small inconsistent effects of morphine are in stark contrast to the large changes produced by morphine on the activity of rostral ventromedial medulla (RVM) neurons or the widespread inhibition and excitation of PAG neurons treated with opioids in *in vitro* slice experiments. The relatively modest effects of morphine in the present study suggest that morphine produces antinociception by causing small changes in the activity of many PAG neurons.

Key words: opioid, antinociception, pain modulation, electrophysiology.

INTRODUCTION

The periaqueductal gray (PAG) is a complex midbrain structure involved in a wide range of behaviors (Depaulis and Bandler, 1991). Opioids are known to contribute to both the antinociceptive and locomotor effects mediated by the PAG (Morgan et al., 1998). Microinjection of morphine into any region of the PAG produces antinociception (Yaksh et al., 1976; Jensen and Yaksh, 1986), suggesting that a large population of PAG neurons is sensitive to opioids. *In vitro* slice recordings support this view by showing that all PAG neurons appear to respond to opioids (Chieng and Christie, 1994a,b; Vaughan and Christie, 1997). Opioids directly inhibit GABAergic neurons by binding to mu-opioid receptors and disinhibit PAG neurons receiving input from these GABAergic neurons (Vaughan et al., 1997). Despite the view that every PAG neuron is either directly inhibited or indirectly excited by opioids, there are no published reports examining the effects of opioids on PAG neurons in an intact rat.

There are a number of studies examining the activity of PAG neurons in response to noxious stimuli (Eickhoff et al., 1978; Sanders et al., 1980; Nakahama et al., 1981; Handwerker and Sack, 1982; Heinricher et al., 1987). These extracellular recordings reveal distinct subsets of PAG neurons that are excited and inhibited by noxious stimuli, although the number of responsive neurons is surprisingly low (< 20%) in some of these studies (Sanders et al., 1980; Heinricher et al., 1987).

There are also a number of studies examining the effect of opioids on neurons in the rostral ventromedial medulla (RVM), a major output target for PAG-mediated antinociception (Zambotti et al., 1982; Morgan et al., 1992, 2008; Heinricher and Tortorici, 1994). Three classes of RVM neuron have been identified based on changes in activity associated with nociceptive reflexes (On-, Off-, and Neutral cells) (Fields et al., 1983). Administration of opioids at doses that produce antinociception inhibits the activity of On-cells, enhances the activity of Off-cells, and has no effect on Neutral cells (Barbaro et al., 1986). The present study tested the hypothesis that morphine will inhibit or enhance the activity of every PAG neuron recorded. Surprisingly, administration of morphine produced relatively small changes in the activity of a subset of PAG neurons, suggesting that antinociception is caused by small changes in the activity of many PAG neurons.

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Abbreviations: PAG, periaqueductal gray; RVM, rostral ventromedial medulla.

http://dx.doi.org/10.1016/j.neuroscience.2016.08.025
0306-4522/

EXPERIMENTAL PROCEDURES

Experiment 1: anesthetized rats

Recordings of PAG neurons were conducted in 22 male Sprague–Dawley rats. All rats were anesthetized with methohexital throughout the experiment and euthanized immediately afterward. This and the subsequent experiment were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This experiment was approved by the Institutional Animal Care and Use Committee at Washington State University.

Each rat was initially anesthetized with pentobarbital (55 mg/kg, i.p.). A cannula was implanted into the right jugular vein in order to maintain anesthesia with continuous infusion of methohexital. The rat was placed in a stereotaxic frame with the head held in a horizontal position. A section of the skull over the right caudal PAG was removed. The dura mater was retracted and a stainless steel recording electrode (Frederick Haer Inc.) was lowered into the right PAG. A metal needle in the trapezius muscle was used to ground the circuit. The rat's tail or hindpaw was pinched periodically to identify the presence of nociceptive reflexes as the pentobarbital wore off. The search for PAG neurons began 30 min after the return of nociceptive reflexes. Once a reflex was detected, continuous infusion of methohexital (15–30 mg/kg/h) was initiated to maintain the rat at a constant level of anesthesia that allowed for nociceptive reflexes, but no spontaneous movements.

The electrode was advanced in 3-μm steps until the activity of a single neuron could be distinguished from background activity. The waveform of the action potential was digitized and analyzed online during the experiment and subsequently offline to insure isolation (DataWave Technologies, Loveland, Colorado). Occasionally, the action potentials of two distinct neurons were recorded with the same electrode. One to five neurons were recorded in each rat in response to the tail withdrawal reflex. Morphine was applied during the last of these cell recordings. The tail withdrawal test consisted of placing the tail in 52 °C water and measuring the latency for a withdraw reflex. Antinociception was defined a priori as a latency exceeding 13 s because this value was sure to exceed any baseline tail withdrawal latency while also limiting exposure of the tail to prolonged heat. The tail was removed from the water by the experimenter if no withdrawal occurred within 13 s. A mechanical transducer was attached to the tail to synchronize the tail withdrawal reflex to neural activity. At least two tail withdrawal tests, separated by 3 min, were conducted for each neuron.

Morphine (5 mg/kg, s.c.) was administered following at least two baseline tail withdrawal trials. A second morphine injection was administered if the tail withdrawal reflex was not inhibited by the third tail withdrawal test (approximately 8 min after the first morphine injection). Antinociception was defined as inhibition of the tail withdrawal reflex on two consecutive tests. The opioid receptor antagonist naloxone (1 mg/kg, s.c.) was injected following the induction of antinociception, and two additional tail withdrawal tests were conducted to assess reinstatement of nociception.

Spontaneous firing rate was determined during the 30 s prior to placing the tail in hot water. To determine whether noxious heat altered cell activity, firing rate during the 2 s immediately prior to the tail withdrawal reflex (i.e., stimulus-evoked activity) was compared to the spontaneous firing rate. An increase or decrease in stimulus-evoked activity was defined as at least a 20% change on two consecutive tail withdrawal trials. Mean neural activity on the two morphine-induced antinociception trials were divided by mean neural activity during the last two baseline trials to determine the percent change in activity. A change in activity following morphine administration was defined as a 20% increase or decrease from baseline activity. Given that neuronal firing rates do not conform to a normal distribution, data are presented using medians, not means.

Following testing, an electrolytic lesion was made to mark the recording site. A lethal dose of methohexital was administered through the jugular cannula. The brain was removed and placed in formalin for at least 2 days. Coronal sections (100 μm) through the PAG were cut with a vibratome and placed on a slide. The location of the PAG lesion was visualized at 10× and plotted on coronal sections from the atlas of Paxinos and Watson (2005). The other recording sites along the electrode tract were reconstructed based on the distance between recording sites.

Experiment 2: awake rats

The activity of PAG neurons was recorded in six male Long-Evans rats implanted with 8–12 tetrodes per rat. Recording tetrodes, constructed from four twisted 20-μm lacquer-coated tungsten wires (California Fine Wire), were aligned in two rows of four or six with approximately 0.5 mm between tetrodes, and mounted on an array of four independently adjustable microdrives (2–3 tetrodes/microdrive; custom made). Tetrode tips were gold-plated to reduce impedance to 0.2–0.4 MΩ (tested at 1 kHz). The number of rats used was kept to a minimum by simultaneously recording from multiple neurons in each rat. This experiment was approved by the Institutional Animal Care and Use Committee at the University of Washington.

Each rat was placed in an induction chamber and deeply anesthetized under isoflurane (4% mix with oxygen at a flow rate of 1 L/min). The rat was placed in a stereotaxic instrument (David Kopf Instruments) with the skull in a horizontal position, and anesthesia was maintained throughout surgery by isoflurane (1–2.5%) delivered via a nosecone. A hole was drilled through the right side of the skull and the dura mater retracted. The tetrode array was unilaterally implanted targeting the right PAG (7.0–7.5 mm posterior from Bregma, 1.5 mm lateral to the midsagittal suture, and 5.5 mm ventral from the dural surface). Tetrodes were implanted at a 10-degree angle from the sagittal plane in order to avoid the midsagittal sinus. A ground wire was inserted