Neuroscience 151 (2008) 564–571

ARE OPIOID-SENSITIVE NEURONS IN THE ROSTRAL VENTROMEDIAL MEDULLA INHIBITORY INTERNEURONS?

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Abstract—μ-Opioid agonists frequently activate output neurons in the brain via disinhibition, that is, by inhibiting "secondary cells," which results in disinhibition of "primary cells," considered to be output neurons. Secondary cells are generally presumed to be inhibitory interneurons that serve only to regulate the activity of the output neurons. However, studies of the opioid-sensitive neurons in the rostral ventromedial medulla, a region with a well-documented role in nociceptive modulation, indicate that the opioid-inhibited neurons in this region (termed "on-cells" when recorded *in vivo*) have a distinct functional role that parallels and opposes the output of the subset of RVM neurons that are activated following opioid administration, the "off-cells."

The aim of the present study was to analyze the relative timing of on- and off-cell reflex-related firing in the rostral ventromedial medulla to help determine whether on-cells are likely to function as inhibitory interneurons in this region. On- and off-cells display complementary firing patterns during noxious-evoked withdrawal: off-cells stop firing and on-cells show a burst of activity. If on-cells are inhibitory interneurons mediating the off-cell pause, the on-cells would be expected to begin their reflex-related discharge before the off-cells cease firing. To examine this we recorded activity of on- and off-cell pairs during heat-evoked paw or tail withdrawal in lightly anesthetized rats. For each cell pair, we measured the onsets of the off-cell pause and the on-cell burst. Contrary to what would be expected if on-cells were inhibitory interneurons, off-cells typically ceased firing before on-cells began reflex-related firing, with a mean 481 (±69) ms lag between the final off-cell spike and the first on-cell spike. This suggests that on-cells do not mediate the off-cell pause, and points instead to presynaptic mechanisms in opioid-mediated disinhibition of medullary output neurons. These data also support an independent role for on-cells in pain modulation.

Key words: pain modulation, descending control, disinhibition, nucleus raphe magnus, ON cells, OFF cells.

μ-Opioid agonists frequently activate output neurons in the brain via disinhibition. Thus, direct inhibition of "secondary cells" disinhibits "primary cells" or output neurons, allowing them to become active (Zieglgänsberger et al., 1979; Duggan and North, 1983; Madison and Nicoll, 1988; Johnson and North, 1992; Pan et al., 2004). It is generally assumed that the secondary cells are inhibitory interneurons, serving only to regulate the activity of the output neurons.

One region in which this opioid disinhibition model has been applied is the rostral ventromedial medulla (RVM), which includes the nucleus raphe magnus and adjacent reticular formation. Consistent with the model, μ-opioid agonists hyperpolarize secondary cells in the RVM, and reduce GABA-mediated inhibition of primary cells (Pan et al., 1990). At a behavioral level, focal application of morphine or μ-opioid agonists in this region produces antinociception via activation of a class of neurons referred to as "off-cells." Off-cells become continuously active, presumably via disinhibition, following administration of morphine or μ-opioid agonists (Fields et al., 1983b; Heinricher et al., 1994), and off-cell activation is necessary for the analgesic actions of systemically administered morphine (Heinricher et al., 1999, 2001a,b). A second class of RVM neurons, referred to as "on-cells" is inhibited by μ-opioid agonists, and presumably correspond to secondary cells recorded *in vitro* (Barbaro et al., 1986; Heinricher et al., 1992). Although at least a subset of both on- and off-cells projects to the dorsal horn (Vanegas et al., 1984; Fields et al., 1995) and anatomical evidence suggests that on-cell axons do not arborize within the RVM (Mason and Fields, 1989), on-cells have nevertheless been widely assumed to function as opioid-sensitive inhibitory interneurons in the RVM. In this model, inhibition of on-cells by opioids in turn disinhibits off-cells to produce antinociception (Fields and Heinricher, 1985; Barbaro et al., 1986; Heinricher et al., 1994).

In addition to their opposing responses to μ-opioid agonists, the firing patterns of the off- and on-cell classes are generally consistent with a role for the latter as inhibitory interneurons. The two populations show reciprocal firing in lightly anesthetized preparations, with both on-cells and off-cells alternating between phases of silence and activity. Activity within each class is in phase, and the two classes fire out of phase (Barbaro et al., 1989). Moreover, on-cells are defined by a burst of activity that begins just before nocifensor reflexes such as the tail flick, while off-cells cease firing at that time (Fields et al., 1983a). That is, on-cells show a characteristic reflex-related "burst," and off-cells a characteristic GABA-mediated reflex-related "pause," and it was therefore reasonable to suggest that on-cells might serve as the GABA-containing inhibitory interneurons mediating the off-cell pause (Heinricher et al., 1991).

However, despite the complementary firing patterns of the on- and off-cell classes, there is now accumulating evidence that on-cells have an independent functional role that parallels and opposes the output of off-cells. Whereas off-cells have a net antinociceptive effect, on-cells are now

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Abbreviations: CI, confidence interval; RVM, rostral ventromedial medulla.

0306-4522/08$32.00+0.00
doi:10.1016/j.neuroscience.2007.10.023

known to promote nociception, an effect that does not require inhibition of off-cells. Moreover, on-cell firing can be suppressed directly without producing apparent disinhibition of off-cells (Heinricher and McGaraughty, 1998; Heinricher and Neubert, 2004; Neubert et al., 2004). A more detailed analysis is therefore needed to determine the nature of the interactions between these cell classes. If on-cells inhibit off-cells directly and mediate the reflex-related off-cell pause, it would be expected that on-cells should begin reflex-related firing *before* off-cells cease firing. The aim of the present study was to analyze the timing of the reflex-related on-cell burst and off-cell pause to help determine whether on-cell activity could account for the off-cell pause. Using simultaneous recordings from on-cell/off-cell pairs, we now show that the reflex-related off-cell pause most often precedes the onset of the on-cell burst.

EXPERIMENTAL PROCEDURES

Animals and surgical preparation

All experimental procedures followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Committee for Research and Ethical Issues of the International Association for the Study of Pain, and were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University. Every effort was made to minimize the number of animals used and their suffering. Male Sprague–Dawley rats (Sasco, Omaha, NE, USA, 250–350 g) were anesthetized with pentobarbital (60 mg/kg, i.p.), and a catheter was inserted into an external jugular vein for administration of anesthetic. The rat was placed in a stereotaxic apparatus, a hole drilled in the skull over the cerebellum, and the dura removed to allow placement of an electrode or pair of electrodes in the RVM. Body temperature was maintained at approximately 37 °C by a circulating water pad.

Following surgery, the anesthetic level was reduced until a withdrawal reflex could be elicited by application of noxious heat using a feedback-controlled projector lamp focused on the blackened ventral surface of the tail or on the plantar surface of the hind paw. The animals were then maintained in this lightly anesthetized state using a continuous infusion of methohexital at a rate (15–30 mg/kg per hour, i.v.) that allowed a stable withdrawal latency and that prevented any signs of discomfort. The animals did not move spontaneously, nor did they vocalize or produce vigorous or prolonged withdrawal reflexes following noxious pinch. The protocol was begun after a stabilization period of at least 30 min, and infusion rate was not altered during the protocol.

Recording

RVM neurons were recorded using stainless steel microelectrodes (FHC, Bowdoinham, ME) and classified as previously described (Fields et al., 1983a; Neubert et al., 2004). Spike waveforms were monitored and stored for off-line analysis (Datawave Systems, Thornton, CO, USA, or Spike 2, CED) to ensure that the units under study were unambiguously discriminated throughout the experiment. Spike times were stored with a temporal resolution of 0.1 ms. Each reflex trial consisted of a linear increase in temperature at approximately 1.8 °C/s from a holding temperature of 34 °C until the withdrawal reflex occurred. Off-cells were characterized by an abrupt pause in ongoing activity beginning just prior to the occurrence of the withdrawal. On-cells were identified by a sudden burst of activity beginning just prior to the occurrence of the reflex. Cells of a third class, "neutral cells," were identified by the absence of change in activity associated with withdrawal and were not studied further.

We studied pairs of neurons (one off-cell and one on-cell) fortuitously encountered with a single electrode or recorded with two independently movable electrodes (about half of the pairs were recorded with each method). Trials were separated by a minimum of 5 min, and only initiated or included in the analysis when the off-cell was active and the on-cell silent at the stimulus onset. All recording sites were verified to be in the RVM.

Population analysis

The parameters of class-specific firing patterns were determined as follows. To study the population dynamics of the two cell classes, we combined the accumulated spike data for all the cells of each class. Histograms showing total number of spikes at any time relative to either the withdrawal or heat onset were constructed using the spike times for each trial aligned with these parameters. The number of spikes per bin and the time values from the histogram then provided the x–y points to which a curve could be fit (GraphPad Prism). Interpolating from the equations describing these curves, we then determined the timing for a given percentage change in population discharge (10%, 50% and 90%) relative to withdrawal.

Single trial analysis

The focus of the single-trial analysis was on the timing of the off-cell pause and on-cell burst, measured as the time between the final action potential of an off-cell before the withdrawal and the initial spike of the on-cell as part of a reflex-related burst. A positive value indicates that the off-cell stopped firing before the on-cell started firing. A negative value arises from an overlap in firing times, that is, trials on which there was at least one spike attributed to the on-cell burst before the final off-cell spike preceding the reflex. The time of withdrawal and the beginning of the heat stimulus were also marked, and the time between these events and the beginning of the burst and pause was calculated. "Pause duration" was defined for off-cells as the time between the final spike before a withdrawal and the succeeding spike after the reflex. Similarly, for on-cells the "burst duration" was the time between the first and last spike of the reflex-related burst, with the end of the burst considered a silent period lasting at least 2 s. Burst/pause parameters for the two populations were compared using Student's *t*-test for independent means and an *F* ratio for comparison of variances.

RESULTS

Population analysis

We first created a summed histogram of cell activity for a sample of 69 trials recorded from 28 on-cell/off-cell pairs, summing data obtained over all trials and aligning with either the heat onset or the reflex. As indicated in the early description of on- and off-cell firing (Fields et al., 1983a), changes in cell activity were more closely linked to the reflex than to the heat stimulus (Fig. 1). This was confirmed using single-trial analyses of the timing of the burst and pause. Variances in the timing of the burst and pause were significantly greater when calculated relative to the heat onset vs. the reflex for both on- and off-cell populations (*F* ratio, $P<0.0001$ for both classes). The withdrawal, not the heat stimulus, is thus the relevant variable defining these cell populations and their patterns of activity.

If on-cells function primarily as inhibitory interneurons, the reflex-related activation of the on-cell population should precede the inhibition of the off-cell population.