Published in final edited form as:
Behav Brain Res. 2010 December 20; 215(1): 156–159. doi:10.1016/j.bbr.2010.07.004.

The modulatory effects of rostral ventromedial medulla on air-puff evoked microarousals in rats

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Abstract

This study tested whether the duration of microarousals from sleep evoked by innocuous air-puff is affected by intra-RVM administration of neurotensin and bicuculline, pharmacological manipulations that affect ON and OFF cell activity. Air-puff evoked microarousal duration was unaffected by 0.05 ng neurotensin, but decreased by 502 ng neurotensin, and 5 and 50 ng bicuculline. These results suggest a putative role for OFF cells in protecting sleep from interruption by non-noxious stimuli.

Keywords

ON cells; OFF cells; rostral ventromedial medulla; microarousals; neurotensin; bicuculline

Neurons in the rostral ventromedial medulla (RVM) are critical for the descending modulation of nociceptive transmission [4,19,31,42,50,51]. In the lightly anesthetized rat, the correlation between RVM neuronal activity and onset of tail flick withdrawal was used to classify two putative pain modulatory cell types – ON and OFF cells [13]. Both cell types are non-serotonergic and have reciprocal patterns of discharge in the anesthetized rat [3,18,34,40]. Several findings implicate ON and OFF cells in facilitating and inhibiting, respectively, nociceptive transmission [9,28]. For example, tail flick latencies are shorter during the peak of ON cell activity than during ON cell silence and longer during the peak of OFF cell activity than during OFF cell silence [25]. Furthermore, ON cells are inhibited and OFF cells are excited by analgesic doses of μ opioid receptor agonists [2,9,13,28]

However, neither noxious stimulation nor μ opioid administration is necessary for changes in ON and OFF cell activity, suggesting that these cells participate in functions beyond nociceptive modulation. Extracellular recordings in the unanesthetized rat demonstrate that

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innocuous stimulation, such as gentle brushing of the back, is associated with cellular changes in the same direction as those seen following noxious heat stimulation [33]. Additionally, changes in cellular activity are evident during spontaneous behaviors such as eating, drinking, and micturition [1,15–17,33]. Moreover, ON and OFF cells display state-dependent discharge across the sleep-wake cycle: ON cells are active during waking and inactive during slow wave sleep (SWS), whereas OFF cells are most active during SWS and sporadically active during waking [15,33]

The reciprocity in ON and OFF cell activity during SWS and waking provides an avenue to test whether these cell types modulate stimulus-evoked reactions in a state-dependent manner. Indeed, noxious heat elicits different reactions during SWS and waking: when heat was applied during SWS, the rat returned to sleep within 5 min, but took almost 3.5 times longer to adopt a sleep posture when stimulated during waking [36]. In humans, motor reactions to painful stimuli are suppressed, and pain perception likely depressed, during SWS compared to waking [5,10,32]. We have thus proposed that ON and OFF cells modulate arousals to external stimulation, whether noxious or non-noxious, in a state-dependent manner such that arousals are suppressed during sleep and promoted during waking [14,35,36].

Microarousals are brief transitions from sleep to wakefulness that occur spontaneously or evoked [7,12,23,41,43,44]. Here, we tested the idea that RVM cells modulate arousal by assessing the duration of microarousals evoked by an innocuous air-puff before and after pharmacological manipulations that are expected to influence ON and OFF cell activity. Henceforth, we will use the abbreviation APEM to refer to air-puff evoked microarousals. We expect that intra-RVM administration of a low dose of neurotensin (0.05 ng, N 0.05), which produces hyperalgesia [38,47,48], would lengthen the duration of APEM. In contrast, a higher dose of neurotensin (502 ng, N 502) or doses of bicuculline (5 ng and 50 ng, Bic 5 and Bic 50, respectively), that produce analgesia [11,21,26,27,29,47,48], are expected to shorten APEM duration. Rats were microinjected with 0.50 μl of saline (Sal) or neurotensin in Expt. 1, and with 0.25 μl of saline or bicuculline in Expt. 2. The drug doses were selected on the basis of their nociceptive effects in unanesthetized rats [11,21,47,48]. While microinjection of 0.5–0.7 μl of dye and/or drug into RVM has very little spread [6,37], we took a more conservative approach in Expt. 2 and used a smaller volume.

Male Sprague-Dawley rats (Sasco Kingston, NY) with an average weight of 416 g were used. They were housed individually in plastic cages (26.0 × 46.0 × 20.5 cm) in a vivarium maintained at 23–25°C on a reverse 12 h light/dark cycle. Rats were fed *ad lib* and tested during the light phase. All procedures used were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by IACUC of the University of Chicago.

Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), placed on a heated blanket, and in a stereotax. A 26 G guide cannula (Plastics One, Roanoke, VA) was implanted 3.0 mm above RVM (AP: −11.2, L: 0.0, V: −7.7 to bregma [39]), and kept patent by a dummy cannula. All rats in Expt. 1 and a subset in Expt. 2 were also prepared for chronic electroencephalographic (EEG) and electromyographic (EMG) recording. Stainless steel screws were placed in the frontal and parietal lobes, and microwires (Cooner Wire, Chatsworth, CA) were sutured into the nuchal muscles. EEG and EMG leads were attached to a microconnector affixed to the skull. Rats were allowed at least 4 days to recover.

Rats were handled and habituated to the apparatus and procedure before testing. On the test day, they were brought to the laboratory and briefly restrained in a towel to allow attachment of a 3.0 mm extension microinjector connected to a 1.0 μl Hamilton microsyringe (Instech Laboratories Inc., Plymouth Meeting, PA) via a swivel and PE 20

tubing. A cable was attached to the microconnector for acquisition of EEG and EMG signals, which were amplified 5-times by a pre-amplifier, and a further 1,000-times by a differential AC amplifier (A-M Systems, Carlsborg WA), and then digitized at 1 kHz by a Power1401 interface (Cambridge Electronic Design, Cambridge, UK).

When placed back into their home cages, rats usually fell asleep within 45 min. While rats were sleeping, air was puffed onto their face from a distance of ≈ 3.0 cm for 10.0 s. Air was delivered at 2.0 psi at a flow rate of 5.0 l/min via a plastic pipette (tip diameter = 2.0 mm) fitted to Tygon tubing. To the experimenters, this stimulus felt like a light tactile sensation. In response to air-puff, rats woke and typically made a slight shift in posture before returning to sleep. During testing, wake and sleep states were determined by behavioral observations. The rat was considered to be in SWS when he adopted a sleep posture and displayed primarily respiratory-related movements. The duration of APEM was calculated as the time between air-puff evoked waking and onset of SWS. None of the rats were observed to display behaviors indicative of rapid eye movement sleep. Three baseline measures of APEM duration were taken. To obtain relatively stable baselines, pre-injection trials were conducted until the data from three trials fell within a factor of two from each other. Trials with active behaviors such as eating and grooming were classified as awakenings, were clearly different from brief arousals, and were therefore excluded. Awakenings or full arousals (mean duration = 660.7 ± 427.4 s) following air-puff stimulation were rare, occurring in only 2 rats from Groups Sal and Bic 5 of Expt. 2 on 3 pre-injection trials. Data from these trials for these rats were excluded in the calculation of their baseline durations. At least 3 min after the last baseline trial, saline or drug was microinjected across 1 min. Rats were then tested for three post-drug trials. All inter-trial intervals were ≈ 15 min.

After testing, rats were euthanized with sodium pentobarbital (100 mg/kg), and perfused transcardially with saline followed by 10% phosphate buffered formalin. Brains were stored in formalin, and then in 30% sucrose formalin. Frozen coronal sections 40 μm thick were taken through the rostral-caudal extent of RVM, mounted onto slides, and stained with cresyl violet. Microinjection sites within RVM, for each of the groups in Expts. 1 and 2, are plotted on representative sections from the rat brain atlas [39] and illustrated in Figs. 1B and 2B, respectively.

EEG and EMG recordings were analyzed subsequent to testing and without knowledge of drug condition to determine sleep and wake states. During SWS, the EEG was synchronized and high in amplitude, while the nuchal EMG showed low tonic activity. Using the formula mentioned above, the duration of APEM for each trial was calculated and then averaged within pre-injection (Baseline) and post-injection (Post) trials for each subject. A repeated measures ANOVA, with α set at 0.05, was performed to test for significant differences between Baseline and Post durations for each group, followed by post-hoc SNK. EEG and EMG data were used in Expt.1, whereas behavioral observation data were used in Expt. 2. The accuracy of using behavioral observations to determine the duration of APEM will be discussed later.

Fig. 1A shows the mean Baseline and Post APEM durations for each of the three groups in Expt. 1 (Sal, n = 11; N 0.05, n = 14; and N 502, n = 10). The duration of APEM was unaffected by either Sal or N 0.05. However, the duration of APEM was significantly decreased by N 502 ($F = 5.5$, $p = 0.04$). Fig. 2A shows the mean Baseline and Post APEM duration for each of the three groups in Expt. 2 (Sal, n = 12; Bic 5, n = 13, and Bic 50, n = 4). The duration of APEM was unaffected by intra-RVM saline. Both Bic 5 and Bic 50 produced significant decreases in APEM durations ($F = 14.2$, $p = 0.00$, and $F = 40.0$, $p = 0.01$, respectively).

Two methodological issues in Expt. 2 warrant discussion. Only a subset of rats in two of the groups (Sal, n = 6; Bic 5, n = 7) had physiological recordings. Consequently, wake and sleep states, and hence the duration of APEMs, were determined by behavioral observation. In the instrumented sub-groups, APEM durations determined using this method correlated highly (Rs > 0.9) with those measured using physiological data, confirming that the behavior observational technique was very accurate. Secondly, baseline APEM durations differed between groups, with Group Bic 50 having shorter overall baselines than those in Groups Sal and Bic 5 (Fig. 2A). This discrepancy occurred because non-instrumented rats had lower baselines than instrumented rats and none of the rats receiving Bic 50 were instrumented. Given that the recording cable created a small amount of torque, and therefore stress, it is unsurprising that rats with physiological recordings had longer baselines. While between group differences in baseline APEM durations are noteworthy, it is important to emphasize that the critical comparisons in this study were the within group differences that tested for the drug's effects on APEM durations.

In anesthetized rats, cortical processing of noxious paw heat, evidenced by EEG desynchronization, is blocked by intra-RVM bicuculline [37]. Here, we demonstrate for the first time that pharmacological manipulations of RVM modulate responses to non-noxious stimulation in rats under normal physiological conditions. Previous research has shown that air-puff (12.5 psi for 100 ms) which elicits startle has both tactile and acoustic components [46]. While our air puff stimulus (2.0 psi at 5 l/m for 10 s) was parametrically different, it is likely to have an acoustic component. Thus, rats were likely aroused from sleep by tactile or acoustic stimulation or both. In air-puff startle studies, air-puff is directed at the rat's back [8,20,46]. Because pilot studies indicated that our air-puff stimulus did not consistently arouse rats from sleep when directed at the back, whereas it did so when directed at the face, we chose to use the latter procedure. Air-puff directed at the rat's face stimulates facial skin receptors and the whiskers. Vibrissae information is important for tasks such as depth perception and proprioception [22,45]. We do not know how whisker stimulation affects APEMs. However, it must be noted that APEM durations were brief and rats moved very little during APEMs, suggesting that vibrissae-mediated information have a negligible role. Moreover, RVM is not activated during whisker movement [24,49].

We speculate that neurotensin and bicuculline decreased APEM durations by modulating ON and OFF cell activity. Due to the technical challenges of simultaneously recording and microinjecting drugs into RVM cells in unanesthetized rats, the effective volumes and doses necessary to alter ON and OFF cell activity in unanesthetized rats are unknown. However, data from anesthetized rats show that neurotensin and bicuculline modulate ON and OFF cell activity. At an extremely low dose (0.5 fg), neurotensin selectively activates ON cells and produces hyperalgesia, whereas higher doses (0.5 – 167.3 ng) activates both ON and OFF cells and produces analgesia [38]. Thus, it is likely that 502 ng neurotensin, which produces analgesia in unanesthetized rats [47,48], lengthened the duration of air-puff evoked microarousals by activating both ON and OFF cells. The effects of bicuculline are likely due to the disinhibition of OFF cells by the $GABA_A$ receptor antagonist, since bicuculline excites OFF cells in anesthetized rats across a large dose range (12.5 – 80 ng) and produces analgesia [11,21,27,29]. While 0.05 ng neurotensin did not increase duration of APEMs, it is possible that a lower dose would have done so.

In conclusion, this study demonstrates that pharmacological manipulation of RVM decreased stimulus-evoked microarousals. Future research might investigate the mechanisms by which ON and OFF cells modulate microarousals. Since cardiorespiratory changes are associated with microarousals [12,30,41], it may be possible that ON and OFF cells work in a coordinated manner to readjust homeostasis and restore sleep.

Acknowledgments

This work was supported by grants from NIDA, NINDS, and La Trobe University.

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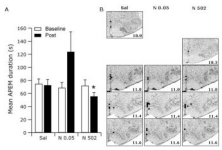

Figure 1.

(**A**) Mean air-puff evoked microarousal (APEM) durations before (Baseline) and after (Post) intra-RVM saline (Sal), 0.05 ng (N 0.05) or 502 ng (N 502) neurotensin. APEM durations were unchanged by Sal or N 0.05 but significantly decreased by N 502 (*, $p < 0.05$). (**B**). Location of microinjection sites (●) for rats in Groups Sal, N 0.05, and N 502.

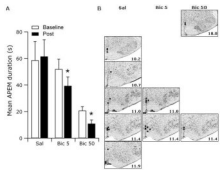

Figure 2.

(**A**) Mean APEM durations before (Baseline) and after (Post) intra-RVM saline (Sal), 5 ng (Bic 5), and 50 ng (Bic 50) bicuculline. APEM durations were unchanged by Sal but significantly decreased by Bic 5 and Bic 50 (*, $p < 0.05$). (**B**). Location of microinjection sites (●) for rats in Groups Sal, Bic 5, and Bic 50.