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EFFECTS OF FORMALIN AS A PERIPHERAL NOXIOUS STIMULUS ON THE NUCLEUS RETICULARIS PARAGIGANTOCELLULARIS NEURONS OF ANESTHETIZED RATS

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ABSTRACT

In the present study, the effects of formalin as a peripheral noxious stimulus on spontaneously active units of the nucleus reticularis paragigantocellularis (PGi), a narrow region of the ventral pontine reticular formation, was examined in urethane anesthetized rats. Spontaneous discharge of the PGi neurons was variable, ranging from 1 to 37 spikes per second. Formalin as a chemical irritant and prolonged noxious stimulus induced changes in the firing of spontaneously active PGi neurons. There were three neuronal subpopulations in the PGi that responded to formalin: excited (19.35 %), inhibited (45.16 %) and unchanged (35.48 %). Thus we suggest that PGi neurons may be involved in the processing of information related to formalin as a chemical irritant.

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Keywords: Nucleus reticularis paragigantocellularis; Formalin; Single-unit recording.

INTRODUCTION

The nucleus reticularis paragigantocellularis (PGi) covers a large portion of rostroventral medulla.[3] Neurons within the PGi have been implicated in a variety of functions including cardiovascular regulation,[8,27] respiratory control,[16] pain and analgesia[5,24] and arousal.[4] The rostral ventromedial medulla (RVM) receives a major input from the midbrain periaqueductal gray matter (PAG), another element within the nociceptive modulatory network.[1,6] PAG, PGi, and the nucleus raphe magnus (NRM) have convergent effects on morphine induced antinociception.[26] Previous studies have revealed that many PGi neurons are synaptically activated by noxious stimuli such as cutaneous stimulation or electrical stimulation of the contralateral hindpaw.[5,20]

The subcutaneous (s.c.) injection of diluted formalin is now a widely used model of nociception. Formalin provides a sustained noxious stimulus and induces a diffuse, long-lasting pain that mimics the same feature of post-injury pain in man.[9,11] This s.c. chemical irritant activates a spinal cord to brain and back to spinal cord loop.[28] Formalin also creates a tonic pain secondary to tissue injury, inflammation and central sensitization.[12]

As mentioned above, acute noxious stimuli evoke responses of the PGi neurons, but effects of the prolonged stimuli on the unit activity of this nucleus have not yet been investigated. Thus, this is the question that is addressed in this study: Does formalin as a noxious stimulus evoke neuronal activity in the PGi?

MATERIAL AND METHODS

Animals

Adult male NMRI rats (Razi institute, Iran, 300-450 g) were used in this study. Subjects were kept 5 per cage at an

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ambient temperature of 22-24°C, placed on a 12-h light-dark cycle and provided standard rat chow and water *ad libitum*.

Surgical preparation

Subjects were anesthetized with urethane (1.3 g/kg, intraperitoneally), tracheostomized, and placed in a stereotaxic instrument for surgery. Anesthesia was maintained by administering supplemental doses of urethane (0.15 g/kg) per hour. The skull was exposed and a hole was drilled above the PGi (11.8 mm caudal to the Bregma and 1.6-1.7 mm lateral to the midline), according to Paxinos and Watson's[22] coordinate system. Then the dura was reflected.

Neuronal recordings

Extracellular recordings from individual neurons in the PGi were obtained using glass micropipettes (2-4 µm tip diameter, 2-12 MΩ impedance), filled with 2% pontamine sky blue dye in 0.5 M sodium acetate. Micropipettes were guided towards the PGi (8.5-8.7 mm ventral from dura) with the assistance of a dissecting microscope and were advanced using a micromanipulator. Micropipette recordings were amplified by a microelectrode amplifier (Nihon Kohden), displayed continuously on a storage oscilloscope (Textronix) as unfiltered and filtered (100 Hz - 10 KHz bandpass) signals, and also monitored with an audio monitor. Action potentials were isolated from background activity with a discriminator (WPI) which generated output pulses for signals that crossed a lower voltage gate, peaked below an upper voltage gate. The discriminator output was led to the computer for on-line data collection. The subject's body temperature was maintained at 35.5 - 36.5°C with a thermistor-controlled heating pad. All units described in this report were spontaneously active. The spontaneous firing rate was defined as the mean firing rate (in spikes per second). After isolating a cell and determining that its firing rate was stable (20-30 min), recording was begun. Baseline activity of neurons was recorded for 60-180 min. In control subjects pre-injection spontaneous firing rate was determined for 60 min, then 50µL sterile saline was subcutaneously injected into the plantar region of the contralateral hindpaw and post-injection single unit recording continued for 60 min. For assessment of the neuronal responsiveness to formalin, the same experimental procedure was repeated with s.c. injection of 50 µL diluted formalin (2.5%).

Histological verification

The recording site in the PGi was marked by deposition of pontamine sky blue dye from the electrode tip. Following experimental sessions, subjects were deeply anesthetized and perfused with saline (0.9%), followed by formalin solution (10%), and the brains were removed for subsequent histologic analysis. All data reported here are from rats in which recording sites were histologically verified in the 80-100 µm unstained transverse sections of the PGi.

Data analysis

In these experiments a computer saved the number of output signals as spikes in time and was set manually (100 msec - 3600 msec). Data collection was carried out for 360 sec intervals with 500 msec bin size, and unit activity was calculated as average frequency (spikes per sec). Peri-stimulus time histograms (PSTHs) were used for data presentation of the spontaneous discharges and unit responses to formalin injection. A responsiveness index in % (percent of possible response-RES) was calculated for each neuron according to the following equation:

$$\text{RES}\ (\%) = \frac{\text{Postinjection - Preinjection}}{\text{Preinjection}} \times 100$$

The onset of significant responsiveness was defined as firing rate (spikes/sec) after formalin injection (post-injection) whose value exceeded or decreased mean baseline activity (pre-injection) by two standard deviations (baseline ± 2SD). Firing rate of the pre- and post-injection was compared by Student's paired t-test. Differences were considered significant at a level of $p < 0.05$.

RESULTS

A total of 91 cells were studied in 71 rats. To obtain useful data, stable recordings from isolated single units in the PGi were required in all experiments for periods ranging from about 20-30 min.

Baseline activity of the PGi neurons

Baseline activity of the 50 isolated units in 30 rats were recorded for 1 to over 3 hours. Their spontaneous discharges were variable, ranging from 1 to 37 spikes per second (12 ± 1.27 spikes/sec; mean ± SE). Their impulse waveforms were heterogeneous but the majority exhibited negative waveforms (60%) in unfiltered records with amplitudes less than 500 µV. Others had positive, or biphasic impulse waveforms, beginning with a negative phase of 70-400 µV amplitude and then the positive phase, with a 50-250 µV amplitude. We observed neurons which exhibited rhythmic discharge in dorsal PGi. These neurons were related to respiration, silent in inspiration and activated in expiration. They have positive waveforms with an amplitude above 250µV, and firing about 15 spikes per second in the period of expiration.

Neuronal recordings in the control group

The control group consisted of 10 animals (10 neurons) that were assessed by saline injection. Spontaneous discharge of these neurons did not change after saline injection (Table I). Statistical analysis was made by paired t-test and there was no significant difference between the mean firing rate of one hour pre-, and one hour post-injection of saline (Fig-

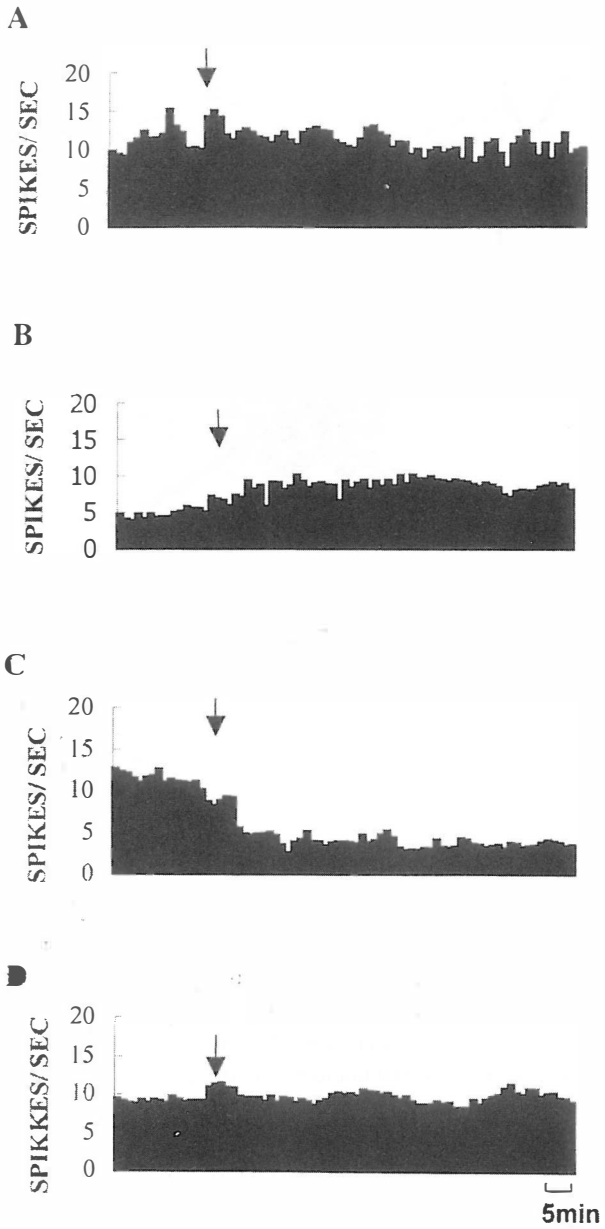

Fig. 1. Typical examples from peristimulus time histograms of the recording PGi neurons: 1-A, -B, -C, and -D show control, excited, inhibited and unchanged neurons respectively. Firing patterns represented 15 min pre-injection and 60 min post-injection (filled arrows) of saline in the control group and formalin in others.

ure 2). A typical example of control neurons is shown in Fig.1-A, which shows it's firing pattern 15 min before and 1 hour following saline injection.

Neuronal responsiveness to formalin injection

In 31 formalin injected rats, the firing rate of 31 units was assessed by comparing between 1 hour pre- and post-injection of formalin. On the basis of the above mentioned statistical definition and equation, three different types of neuronal responsiveness to formalin were observed (Table I). The first type (n = 6, *excited neurons*) showed an increase in their mean firing rates. The spontaneous discharges of these neurons were increased after formalin injection. The second type (n = 14, *inhibited neurons*) showed a decrease in the mean firing rate. In these neurons spontaneous discharge was decreased after formalin injection. In the third type (n = 11, *unchanged neurons*) there was no change in the mean firing rate following formalin injection. In the excited and inhibited neurons responses to formalin persisted for at least 1h, their latency of onset determined about 1-5 min for inhibited and 1 min for excited neurons. The magnitude of responses ranged from 24 to 135% for excited and -31 to -81% for the inhibited neurons. Inhibited neurons had fast and regular spontaneous discharge (10 -37 spikes/sec) against irregular firing of the excited neurons (1 -12 spikes/sec) and intermediate unchanged neurons (5 -15 spikes/sec).

A typical example of each type is presented in Figures 1-B, C, and D, respectively. Firing patterns are shown 15 min before and 60 min following formalin injection. Comparison of mean firing rate between pre- and post-injection in each different type is shown in Figure 2. This figure shows that formalin injection has resulted in a significant increase ($p < 0.05$) of firing rate from pre-injection values in excited neurons, but a significant decrease ($p < 0.01$) for the inhibited neurons. In unchanged neurons, no significant difference was seen in the mean firing rate between pre and post-injection. Histologically verified locations of these three neuronal types are shown in Figure 3.

DISCUSSION

Our findings about spontaneously active PGi neurons

Table I. Comparison of mean firing rate between pre- and post-injection of formalin in three neuronal types.

		Excited neurons			Inhibited neurons			Unchanged neurons		
Experimental groups	$n_{(total)}$	**(n)**	**Pre**	**Post**	**(n)**	**Pre**	**Post**	**(n)**	**Pre**	**Post**
Formalin	31	6	7.67 ±0.52	13.81 ±1.06*	15	15.02 ±0.49	5.76 ±0.11**	10	10.54 ±0.49	11.31 ±0.42
Control	10	-	-	-	-	-	-	10	15.79 ±0.4	16.44 ±0.41

Pre and Post abbreviations are related to the pre-injection or post-injection of formalin and saline in formalin and control groups, respectively. Analysis was carried out by Student's paired t-test (two tails). *p<0.05; **p<0.01.

were consistent with those of Ennis and Aston-Jones[4] and Azami et al.[5] These neurons were heterogenous in their waveforms, the majority had negative waveforms (60 %) and others were positive or biphasic. Andrezic et al.[3] have shown that PGi neurons are diverse in cellular morphology. This heterogeneity was also observed in their waveforms and velocities.[15] In addition, we observed neurons in the dorsal PGi that exhibited rhythmic discharge related to respiration.

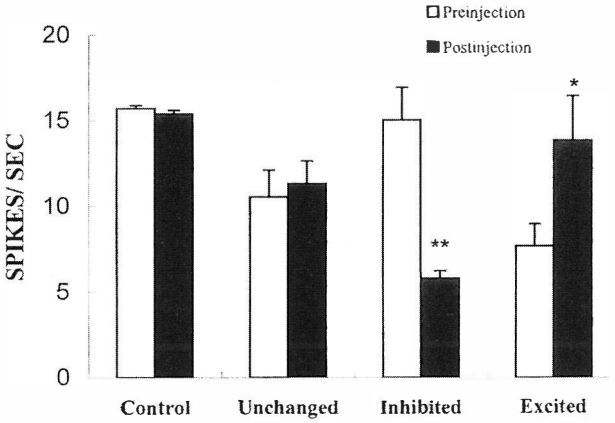

Fig. 2. Change in firing rate (mean ± SEM) measured pre- and post-injection of saline for control neurons and formalin for the other three types. There are no significant differences between pre- and post-injection in control and unchanged neurons, but for inhibited and excited neurons the differences were significant. $*p < 0.05$; $**p < 0.01$.

It has been reported that formalin nociception occurs in 2 phases; the 1st phase starts immediately after formalin injection and continues for 5 min, after which nociception appears to diminish. The 2nd phase is marked by a return to high levels of nociception beginning 10 -15 min after formalin injection and continuing for about 1 h. The first phase is probably a direct result of stimulation of nociceptors in the paw, while the 2nd phase may reflect, at least to some degree, sensitization of central nociceptive neurons.[10] Such biphasic responses to formalin have also been observed during electrophysiological recordings of convergent spinal dorsal horn and trigeminal brainstem neurons.[13,14]

We used diluted formalin (2.5%) as a useful chemical irritant to induce chronic pain, because it is more sensitive if concentrations between 0.5 and 2.5% are used.[9] Our results showed that most of the PGi neurons responded to formalin as a peripheral noxious stimulus. These responses persisted for at least 1h, differing significantly from that of acute nociceptive tests.[2,12]

Fields et al.[18] reported that there are three neuronal classes in RVM based on the correlation of their activity with nociffensor reflexes: noci-excited (on-cells), noci-inhibited (off-cells) and neutral. Moreover, on- and off-cells have been

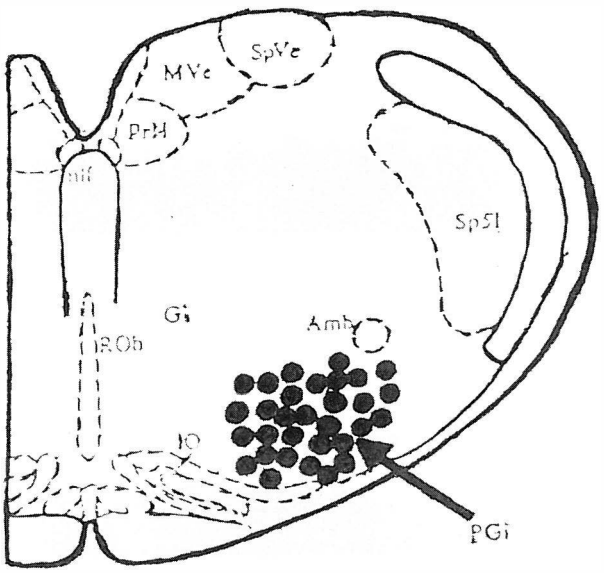

Fig. 3. Reconstruction of a coronal section taken through the rostral medulla showing anatomical maps of recording sites (filled circles) of the PGi neurons in formalin injected rats. Abbreviations: Amb, nucleous ambiguous; Gi, gigantocellular reticular nucleus; IO, inferior olive; MVe, medial vestibular nucleus; mlf, medial longitudinal fasciculus; PGi, paragigantocellular reticular nucleus; PrH, prepositus hypoglossal nucleus; ROb, raphe obscurus nucleus; Sp5I, spinal trigeminal nucleus, interpolar part; SpVe, spinal vestibular nucleus.

reported in PAG.[7] Our results show that there are also different subpopulations of the PGi neurons that respond to formalin: excited, inhibited and unchanged neurons. These three proposed types could be compared with on-cells, off-cells and neutral, respectively. Off- and on-cells each contribute to modulation of nociception; off-cells have a diverse distribution in the RVM and appear to be output modulators.[18] In this study inhibited neurons (off-like cells) were more frequent than the other two types and it may be consistent with the hypothesis that activity of the bulbospinal neurons results in supression of formalin-induced nociception as a model of unremitting pain. It is important to note that there are many relationships between PGi neurons and other brain regions. Porro et al's.[23] observation showed that in the unanesthetized, formalin injected rats, metabolic activity in the ventrolateral PAG was maximum in percentage (24 %) increase over control values. Thus PAG acts as a formalin-sensitive region. Moreover anatomical evidence showed that dorsal and ventrolateral PAG transmit different aspects of the PGi.[29,30] There is also an important contribution of GABAergic mechanisms in the RVM to the antinociceptive control generated by electrical stimulation in the PAG.[17] There are numerous GABA containing interneurons in the RVM that are in contact with off-cells.[21] They may be derived from outside of the RVM or be local interneurons. Morphine administered systemically, is involved at in least

two components within the RVM itself: direct inhibition of on-cells, and indirect activation of off-cells.[19] The above stated evidence supports the hypothesis that PAG on-cells may affect the RVM (part of the PGi as a subregion) off-cells. Thus, it could be probable that formalin, as a peripheral noxious stimulus, triggers supraspinal nociceptive circuits which lead to inhibition of PGi off-like cells, through direct activation of PAG on-cells. But excitation of the PGi excited neurons (on-like cells) is independently affected from the formalin injection.

In summary, our observations show three neuronal subpopulations in the PGi, affected from formalin, acting as a prolonged noxious stimulus: excited, inhibited and unchanged neurons, termed as on-like cells, off-like cells and neutral, respectively. Thus, PGi neurons may be involved in chronic nociception.

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