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Brain Research 689 (1995) 21–31

BRAIN RESEARCH

Research report

An electrophysiological characterization of the projection from the central nucleus of the amygdala to the periaqueductal gray of the rat: the role of opioid receptors

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Accepted 18 April 1995

Abstract

The midbrain periaqueductal gray (PAG) and the central nucleus of the amygdala (CNA) are both known to be involved in fear and anxiety, analgesia, vocalization, cardiovascular and respiratory changes, and freezing. Anatomical studies have shown that a connection between these two regions exists but little is known about the physiology or the neurochemical constituents of this pathway. The goals of this study were to characterize the projection from the CNA to the PAG using electrophysiological techniques and to determine whether μ- and/or δ-opioid receptors, which play a large role in a majority of the functions of the PAG, are involved in this pathway. Of the 38 PAG cells tested with single shock stimulation of the CNA, 44% responded; of those, 46% were excited and 54% were inhibited. The latency to onset of response for the inhibitory cells (12.71 ± 6.61 ms) was shorter than that of the excitatory cells (22.33 ± 4.04 ms). Forty-six percent of the 129 PAG cells tested with train electrical stimulation of the CNA responded; 44% were excited and 56% were inhibited. Chemical stimulation of the CNA (10 mM D,L-homocysteic acid) produced similar results; 48% (62/128) of PAG cells responded; 45% of cells were excited and 55% were inhibited. The baseline firing rate of the inhibitory cells was significantly higher compared to the excitatory cells. Chemical stimulation of the CNA produced an increase in blood pressure in 12 animals, a decrease in two animals, and had no effect on the blood pressure of 68 animals. The blood pressure changes ranged between 8.5 and 26.3 mmHg with a mean of 16.2 ± 2.2 mmHg. The effect of naloxone (given either on site in the PAG or systemically) on the response to CNA stimulation was tested in 21 cells. Twenty-five percent of the excitatory cells (2/8) and 77% (10/13) of the inhibitory cells were blocked by naloxone with the majority of the blocked cells located in the ventrolateral PAG. It is concluded that: (1) Approximately 50% of cells in the lateral and ventrolateral columns of the PAG respond to CNA stimulation; (2) the inhibitory response is mediated by a faster conducting or a more direct pathway than the pathway that mediates the excitatory response; (3) neurons that are inhibited by CNA stimulation have a significantly higher baseline firing rate than neurons that are excited, suggesting that they may be tonically active interneurons; and (4) at least one link in the CNA-PAG pathway utilizes μ- or δ-opioid receptors.

Keywords: Periaqueductal gray; Amygdala; Fear; Anxiety; Analgesia; Enkephalin

1. Introduction

The midbrain periaqueductal gray (PAG) is an important center for the integration of fear and anxiety [17], vocalization [29], pain modulation [4,20] and autonomic modulation [42,43]. Although stimulation of all regions of the PAG can alter some or all of the functions listed above, the effect of PAG stimulation is highly dependent on the location within the PAG [15]. For example, stimulation of the dorsal PAG produces fear and anxiety, increases in blood pressure and causes vocalizations that are normally associated with rage. In contrast, stimulation of the ventrolateral PAG produces analgesia and freezing.

Fear and anxiety have been described as a functional behavioral system evolved as an anti-predator defense mechanism [17]. This system involves behavioral responses such as freezing to avoid detection [10], a potentiated startle response which shortens reaction time [37], physiological changes such as the cardiovascular responses required for fight or flight [14] and analgesia [9,26]. Fear-induced analgesia prevents pain related behaviors from interrupting defensive maneuvers [11]. The amygdala is known to be a crucial center for fear and anxiety. Stimula-

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SSDI 0006-8993(95)00525-0

tion of the central nucleus of the amygdala (CNA) causes cardiovascular and respiratory changes [1,46] and also a disruption of ongoing behavior or freezing [2,30]. Electrical stimulation of the amygdala in humans is known to evoke feelings of fear and anxiety as well as the cardiovascular and respiratory responses listed above [13]. In contrast, lesions of the CNA have been found to eliminate freezing [9,26,33], cardiovascular changes [32], the fear-potentiated startle response [27] and analgesia [26]. The overlap in function between the PAG and the CNA and the fact that lesion of the PAG diminishes fear and freezing produced by CNA stimulation suggests that the PAG may mediate the behavioral and physiological responses produced by CNA stimulation.

The anatomical connection between the CNA and the PAG has been well documented [34,35,52]. However, little is known about the physiology of the connections between the PAG and the CNA or the neurochemical constituents involved in this pathway. The CNA contains a wealth of neurotransmitters including neurotensin, somatostatin, substance P and galanin [12,25] but has been especially noted for containing an abundance of enkephalin containing neurons [12,45]. The PAG contains the three opioid receptors μ, δ and κ [41] and the enkephalinergic system within the PAG is involved in the majority of its functions [5,8]. Therefore, it is possible that enkephalinergic afferents to the PAG arise from the CNA.

The goals of this study were (1) to characterize the pathway between the PAG and the CNA using electrophysiological and pharmacological techniques and (2) through the use of the enkephalin antagonist naloxone, determine whether μ- and/or δ-opioid receptors play a role in the connection between these two areas.

2. Materials and methods

2.1. Animal preparation

All animal procedures were approved by the University of Cincinnati Institutional Animal Care and Use Committee. Male Sprague–Dawley rats (Zivic–Miller) ranging in weight 300–450 g were anesthetized with an initial dose of 40% chloral hydrate (0.4 g/kg, i.p.). The femoral vein was then cannulated with PE-50 tubing for intravenous injection of 4% chloral hydrate, given as needed to maintain deep anesthesia. The femoral artery was cannulated as well and attached to a transducer and a monitor for continuous recording of the blood pressure. Rats were then placed in a stereotaxic frame and maintained at a body temperature of 36–38°C with a temperature controlled heating pad. A 6 mm diameter hole was drilled into the skull starting 4 mm caudal to bregma along the midline to allow for electrode insertion in the PAG. A similar hole of 4 mm was drilled 2.5 mm caudal to bregma and 4.6 mm lateral to midline in order to stimulate the CNA. The sagittal sinus was sutured rostral and caudal to the study site in the PAG then cut to prevent a future rupture from electrode manipulation.

2.2. Electrophysiological recording

CNA stimulation was performed using a two part electrode. Electrical stimulation was done with a pair of twisted 50 μm insulated wires cut to a blunt end. Chemical stimulation was done with a glass electrode, tip diameter 15–25 μm, filled with 10 mM D,L-homocysteic acid (DLH) (pH 7.6). The two electrodes were glued together with dental wax to allow for both chemical and electrical stimulation at the same site. The electrode was lowered into the CNA using coordinates derived from the stereotaxic atlas of the rat by Paxinos and Watson [48] (2.5 mm caudal to bregma, 4.6 mm lateral to midline, 7.3 mm ventral). For a volume control, 25 PAG cells that responded to chemical stimulation were recorded during injection of an equal volume of 0.9% saline (pH 7.0) into the CNA. Because no PAG neurons responded to saline injection into the CNA, the control was removed from the stimulating electrode in order to minimize damage to the stimulation site. The recording electrode used in the PAG consisted of a tungsten electrode (A-M Systems, Everett, WA) attached to a glass electrode (tip diameter: 15–25 μm) filled with 10 mM naloxone. Both naloxone and DLH were pressure injected using 5–80 ms pulses at 20–35 kPa from a Picospritzer. Approximately 50–200 nl of each drug was released with each pressure application. For the PAG cells injected with naloxone, CNA stimulation would not continue until the cell firing rate returned to baseline to insure that resulting changes were due to CNA stimulation rather than the naloxone. Cells recorded in the PAG were located 5.8–7.0 mm caudal to bregma, 0.1–0.5 mm lateral to midline and 4.2–5.6 mm ventral to the surface of the brain.

Action potentials were recorded extracellularly and monitored with both an oscilloscope and an auditory monitor. Single cells were selected by using a window discriminator connected to an on-line computer. Cognitech Data Acquisition and Analysis software was used to record simultaneous firing and blood pressure rates and to construct peri-stimulus time histograms (PSTHs).

2.3. Experimental protocol

For each cell, the baseline firing rate and the response to peripheral stimulation (pinching of the tail or hind paws) were recorded. The touch stimulation consisted of brushing the skin of the hind legs and tail. Noxious pinch stimulus consisted of pinching the tail with forceps at an intensity that, when applied to the human hand, produced pain and, when applied to the tail of a lightly anesthetized rat, produced tail withdrawal. Cells were classified as either excited, inhibited or no response (NR) to peripheral stimulation. Next, the cell's response to constant current

train stimulation (100 Hz for 10 s, 50–200 μA intensity, 200 μs duration) of the CNA was determined. Current was set initially at 50 μA and increased at 50 μA intervals until a response or the upper limit was achieved. Current spread from stimulation above 200 μA was considered too large to be interpretable [50]. No attempts were made to determine the precise response threshold by varying the intensity of the stimulating current. An excitatory response was defined as an increase in the firing rate of 50% above the baseline for at least 15 bins (1 s per bin) and an inhibitory response was defined as a decrease in firing rate of 50% below the baseline for at least 15 bins. The cell was considered recovered when the firing rate returned to within 10% of the baseline firing rate. Latency of the response was measured starting from stimulation offset until the first three consecutive bins that showed a firing rate change of at least 50% to meet the excitatory/inhibitory criteria. The duration of the response was measured from the end of the latency period until the change in firing rate returned to 10% of the baseline for at least 10 consecutive bins. In order to determine approximate conduction velocities of cell responses, constant current single pulse stimulation (1 Hz, 50–200 μA intensity, 200 μs duration) was used and PSTHs were constructed. To discern whether the response was due to activation of CNA cell bodies rather than fibers of passage, the excitatory amino acid DLH (50–200 nl) was pressure injected into the CNA and the cells were characterized as described above. During the recording period, blood pressure was monitored simultaneously with the firing rate. Any changes in the blood pressure due to chemical stimulation were noted. The mean arterial blood pressure (MAP) was calculated as $MAP = 2/3\ DP + 1/3\ SP$, where DP is the diastolic and SP the systolic pressure. Blood pressure responses were considered significant if there was a change of 5 mmHg for at least 10 consecutive bins.

To determine the involvement of opioid receptors in the CNA-PAG projection, 10 mM naloxone was administered either on site in the PAG or systemically (0.2 ml i.v., approx. 220 μg/kg) and its effect on the response of the PAG cell to CNA stimulation was tested. If the response was blocked (the change in firing was no longer significant according to the standards set earlier), the cell was then stimulated every 10 minutes until the response was restored. Cells in which the initial response to stimulation was never restored were not considered in the final analysis.

2.4. Histology

At the end of each experiment, the animals were overdosed with chloral hydrate and perfused intracardially with 0.9% saline followed by 25% formalin. The brain was removed and stored for a minimum of 3 days in 25% formalin containing 20% sucrose as a cryoprotectant. For the PAG, 80 μm sections were cut using a freezing microtome, mounted onto gelatin-coated slides and stained using Neutral Red. In order to best delineate the CNA, however, 40 μm slices were cut and stained for detection of acetylcholinesterase. They too were then mounted on gelatin coated slides. Both were dehydrated, coverslipped and viewed under a microscope to verify electrode placement.

Table 1
Properties of the responses of PAG cells to single pulse stimulation of the CNA

Response to single-pulse stimulation	Average firing rate (spike/s)	Latency to onset (ms)	Duration (ms)	Change in firing rate (%)
Excited ($n = 6$)	2.9 ± 1.33	22.33 ± 4.04	64.16 ± 15.49	1590.8 ± 903
Inhibited ($n = 7$)	16.37 ± 3.4	12.71 ± 6.61	70.57 ± 28.93	87.16 ± 8.85
No response. ($n = 25$)				

2.5. Statistical analysis

Statistical analyses were done using χ^2-analysis and the Welch t-test because it does not assume equal variances. All analyses were computed using the Graphpad

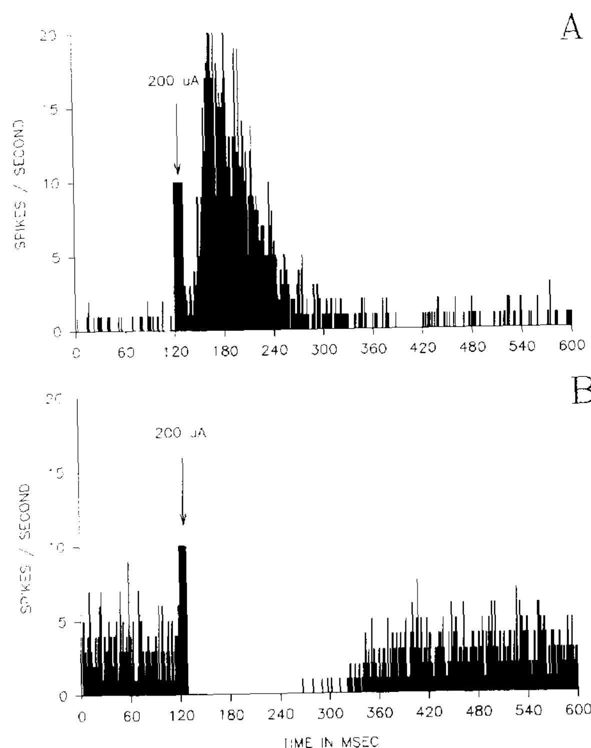


Fig. 1. A: typical excitatory response of a PAG cell to CNA single-pulse stimulation. The onset of excitation was measured as 12 ms from the end of the stimulation artifact and lasted for 131 ms. B: typical inhibitory response of a PAG cell to CNA single-pulse stimulation. The onset of response was immediately after the offset of the stimulus and lasted for 213 ms.

Table 2
Response properties of PAG neurons to 100-Hz train stimulation of the CNA.

Response to train electrical stimulation	Average firing rate (spikes/s)	Latency to onset (s)	Duration (s)	Response to peripheral stimulation	
Excited ($n=26$)	8.33 ± 1.60	4.54 ± 1.43	72.16 ± 13.68	excited	44%
				inhibited	12%
				NR	44%
Inhibited ($n=33$)	9.38 ± 1.35	2.72 ± 1.39	125.64 ± 21.8	excited	34%
				inhibited	34%
				NR	32%
No response ($n=70$)	8.93 ± 0.99			excited	26%
				inhibited	29%
				NR	45%

Table 3
Response properties of PAG cells to injection of 50–200 nl of 10 mM D,L-homocysteic acid into the CNA

Response to chemical stimulation	Average firing rate (spikes/s)	Latency to onset (s)	Duration (s)	Response to peripheral stimulation	
Excited ($n=28$)	5.29 ± 0.89	40.18 ± 7.6	151.39 ± 17.8	excited	37%
				inhibited	19%
				NR	44%
Inhibited ($n=34$)	9.19 ± 1.35	49.97 ± 7.49	231.82 ± 37.9	excited	30%
				inhibited	40%
				NR	30%
No response ($n=66$)	10.78 ± 1.06			excited	29%
				inhibited	28%
				NR	43%

Software package. A two-tailed P-value of less than 0.02 was considered significant.

3. Results

3.1. Characteristics of periaqueductal gray neuronal activity

Using 108 animals, recordings were made from 167 neurons located in all regions of the PAG. All of the neurons were spontaneously active and had a baseline firing rate ranging from 0.46 to 39.14 spikes/s with an average firing rate of 8.92 ± 0.61 spikes/s (mean ± S.E.M.). Of those tested, 60% of the neurons responded to tail pinch; of those, 57% were excited and 43% were inhibited.

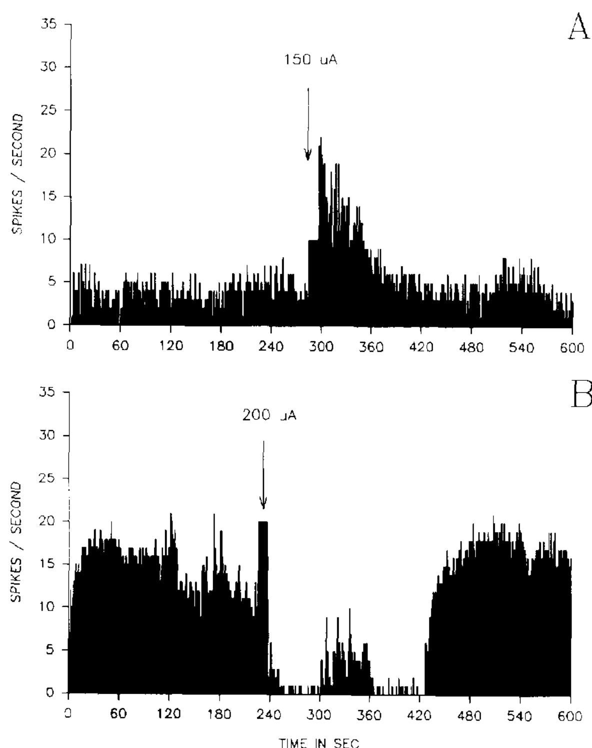


Fig. 2. A: typical excitatory response of a PAG cell to CNA train electrical stimulation (100 Hz, 10 s, 150 μA). The onset of excitation was immediately following the stimulus artifact and lasted for 59 s. B: typical inhibitory response of a PAG cell to CNA train electrical stimulation (100 Hz, 10 s, 200 μA). The onset of excitation was immediately following [illegible]

3.2. Response of periaqueductal gray neurons to single pulse stimulation of the central nucleus of the amygdala

Single pulse stimulation of the CNA was used to determine the latency of the response of PAG cells to CNA stimulation. Of the 38 cells tested, 13 cells responded. This small percentage of responsive cells is comparable to other studies on PSTHs involving much greater sample sizes [6]. The response characteristics of these cells can bee seen in Table 1.

Of the cells that responded to single pulse stimulation, 46% (6/13) were excited with an average firing rate of 2.9 ± Hz. The latency to onset of response was 22.33 ± 4.04 ms with a mean duration of 64.16 ± 15.49 ms. Fifty-four percent (7/13) of the cells that responded to single pulse stimulation were inhibited with a baseline firing rate of 16.4 ± 3.4 Hz. This was significantly higher than the baseline firing rate of the excited cells ($t=3.458$, df = 11, $P=0.0078$). The latency to onset of the inhibitory response was shorter than that of the excited cells (12.71 ± 6.61 ms) but they had a similar duration of 70.57 ± 28.93 ms. Fig. 1 shows an example of an excitatory and an inhibitory response.

3.3. Periaqueductal gray responses to train electrical stimulation of the central nucleus of the amygdala

The effects of train electrical stimulation of the CNA was tested on 129 neurons in the PAG. Of those cells recorded, 59 cells (46%) responded. The baseline firing rates of all of the cells ranged from 0.5 to 39.14 spikes/s [illegible]

cells responded to peripheral stimulation; 54% were excited and 46% were inhibited. Table 2 lists the response characteristics of these cells.

Of the cells that responded to train electrical stimulation of CNA, 44% (26/59) were excited. The average firing rate of the excitatory cells was 8.33 ± 1.60 Hz. Latency to onset of response was 4.54 ± 1.43 s and the duration was measured to be 72.16 ± 13.68 s. Fifty-six percent of these cells responded to peripheral stimulation with 79% excited and 21% inhibited. Fifty-six percent (33/59) of the cells responding to CNA train electrical stimulation were inhibited. The average firing rate (9.38 ± 1.35 Hz) was similar to that of the excitatory cells. The latency was similar as well (2.72 ± 1.39 Hz) but the inhibitory cells had a slightly greater duration of response (125.64 ± 21.80 s). Sixty-eight percent of these cells responded to peripheral stimulation with 50% excited and 50% inhibited. Fig. 2 shows an example of an excitatory and inhibitory response.

Table 4
Comparison of the response of PAG cells to chemical and electrical stimulation of the CNA

Chemical stimulation	Electrical train stimulation			
	excited	inhibited	no response	total
Excited	6	0	9	15
Inhibited	0	10	5	15
No response	6	7	46	59
Total	12	17	60	89

Out of the total number of cells recorded, 54% (70/129) did not respond to train stimulation. They were similar in firing rate (8.92 ± 0.99 Hz) to the responsive cells. Fifty-

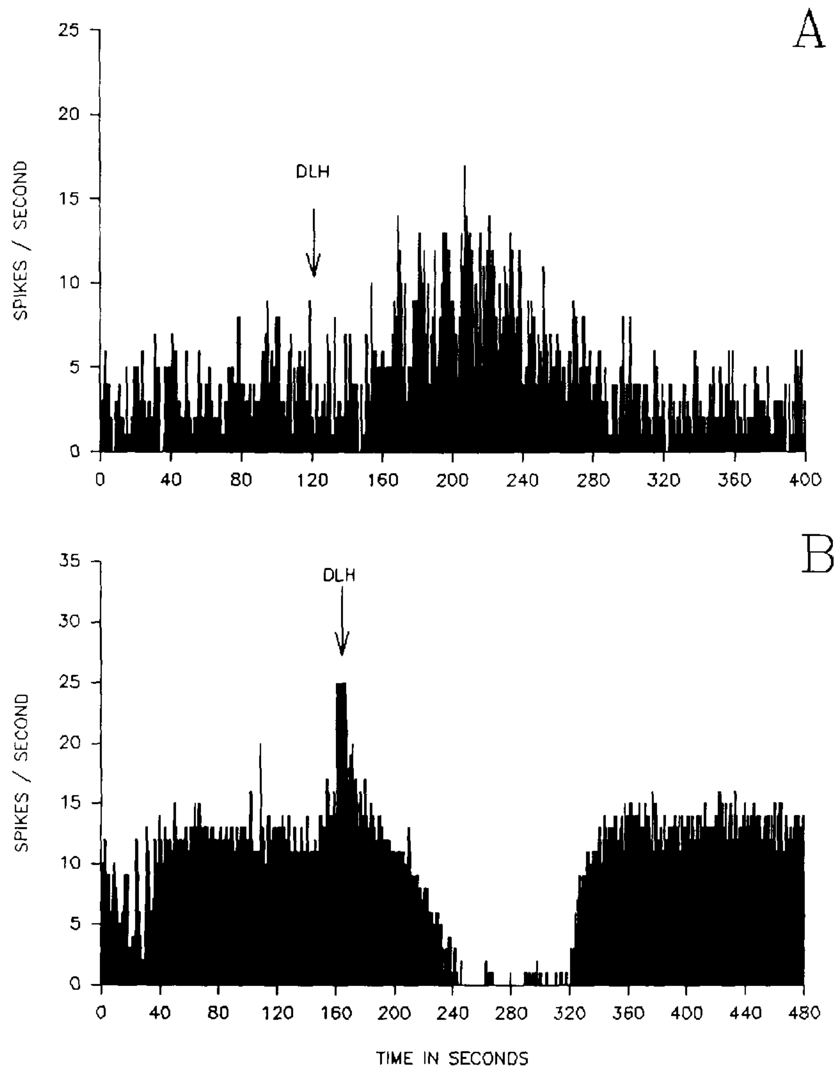


Fig. 3. A: typical excitatory response of a PAG cell to CNA chemical stimulation with 100 nl of 10 mM DLH. Latency to onset of response was 45 s measured from stimulation artifact and lasted for 194 s. B: typical inhibitory response of a PAG cell to CNA chemical stimulation with 100 nl of 10 mM DLH. Latency to onset of response was 51 s after the end of the stimulation artifact and the response lasted for 193 s.

five percent responded to peripheral stimulation with 47% excited and 53% inhibited.

3.4. *Periaqueductal gray responses to D,L-homocysteic acid stimulation of the central nucleus of the amygdala*

One hundred twenty eight cells were tested for response to microinjection of DLH into the CNA. Of the cells recorded, 62 cells (48%) responded to CNA stimulation. Their baseline firing rate ranged from 0.46 to 39.14 spikes/s with an average of 9.16 ± 0.71 Hz. Of the total, 59% responded to peripheral stimulation with 52% of the responding cells excited and 48% inhibited. See Table 3 for the characterization of cell responses.

Of the cells responding to DLH stimulation of the CNA, 45% (28/62) were excited. The mean baseline firing rate was 5.29 ± 0.89 Hz. Latency to onset of response was 40.18 ± 7.6 s with a mean duration of 151.39 ± 17.8 s. Fifty-six percent of the cells responded to peripheral stimulation with 66% excited and 34% inhibited. Fifty-five percent of the cells responding to DLH stimulation were inhibited. They had a mean baseline firing rate of 9.19 ± 1.35 Hz which was significantly greater than that of the excitatory cells ($t = 2.412$, df = 55, $P = 0.0192$). Both latency and duration were slightly greater in the inhibited cells at 49.97 ± 7.49 s and 231.82 ± 37.9 s respectively. Seventy percent of the cells responded to peripheral stimulation and, of those, 43% were excited and 57% were inhibited. An example of an excitatory and an inhibitory response is shown in Fig. 3.

Of the total number of cells tested with DLH, 52% (66/128) did not respond. The firing rate was similar to

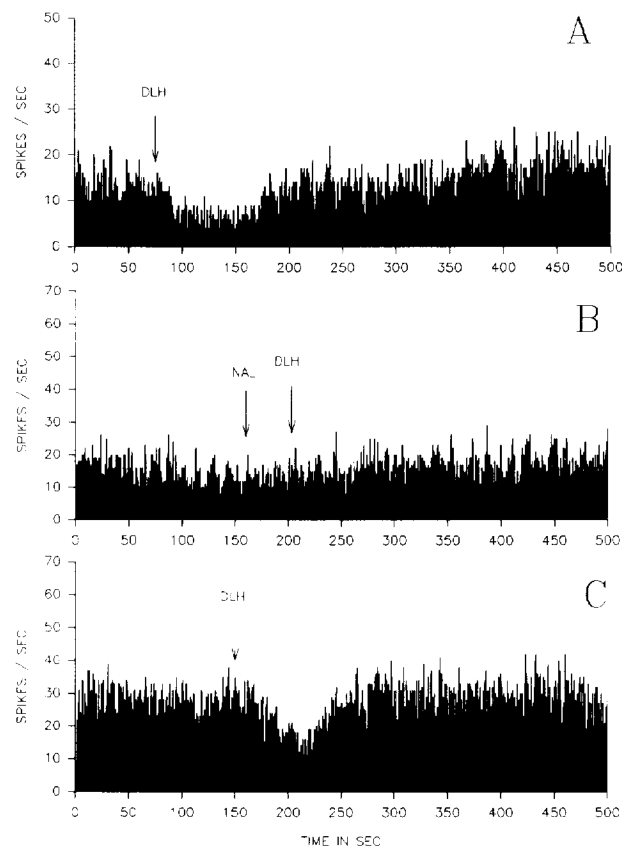


Fig. 5. A: first initial run showing the inhibitory response of a PAG cell caused by microinjection of 150 nl of DLH into the CNA. Latency to onset of response was 12 s and the response lasted for 81 s. B: response of the PAG cell to CNA stimulation with 150 nl DLH following naloxone microinjected next to the cell in the PAG. The initial inhibitory response is blocked. C: response of the PAG cell to 150 nl DLH microinjected into the CNA. This run was taken approximately 20 minutes after the naloxone was given. Latency to onset of response was 28 s and the response lasted for 59 s.

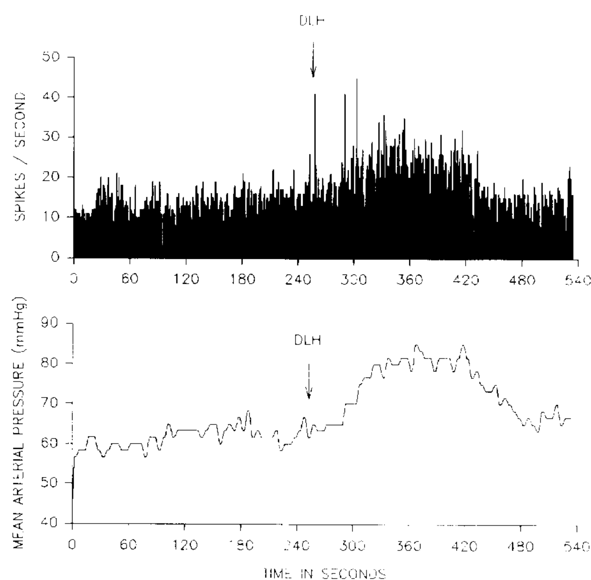


Fig. 4. An example of an increase in blood pressure from chemical stimulation of the CNA.

that of the inhibitory cells at 10.78 ± 1.06 Hz. Fifty-seven percent responded to peripheral stimulation with 51% of those excited and 49% inhibited.

3.5. *Comparison data of cells stimulated both electrically and chemically*

In order to determine if there was a correlation between electrical and chemical stimulation responses, 89 cells were tested for both. An overview of this data is seen in Table 4.

Of the 12 cells excited by electrical stimulation, 50% (6/12) were excited by DLH (six cells did not respond). Of the 17 cells inhibited by electrical stimulation, 59% (10/17) were inhibited by DLH with seven not responding. Finally, of the 60 cells that did not respond to electrical stimulation, 77% (46/60) did not respond to chemical stimulation. From this data we can assume that

the electrical stimulation is comparable to the chemical stimulation and that the majority of the responses noted are from cell bodies in the CNA rather than from stimulation of fibers of passage. Further, χ^2-analysis of the two main sets of data show no statistical difference between the two stimulation methods ($\chi^2 = 0.2$; df = 2; $P > 0.5$).

3.6. The effects of central nucleus of the amygdala stimulation on blood pressure

The blood pressure was monitored simultaneously with cell firing rate. Overall, the animals recorded had a mean arterial pressure (MAP) of 69.15 ± 1.75 mmHg. Out of 84 animals in which chemical stimulation of CNA was used, 12 animals showed a pressor effect from CNA stimulation, two animals showed a depressor effect and the remaining animals showed no change in blood pressure. The changes in the blood pressure ranged between 5.15 and 26.25 mmHg. There was no difference in the baseline MAP of animals that responded by a pressor and those that showed depressor response. An example of a pressor response can be seen in Fig. 4.

3.7. Pharmacology of the central nucleus of the amygdala-periaqueductal gray projection

Twenty-one cells were tested to determine whether their responses could be blocked by naloxone given systemically or through microinjection into the PAG. Six animals were given intravenous naloxone and in 15 animals naloxone was injected directly into the PAG next to the recording electrode. Similar results were achieved with both injection methods. In addition, electrical and chemical stimulation also produced similar results. For these reasons, all of the data was pooled together. Injection of naloxone did not alter the baseline firing rate of any neuron or change the spike height of any cell. Since injection of a large volume causes movement of tissue and thereby either changes the spike height or firing rate, the fact that neither of these parameters were affected by naloxone indicates that naloxone's effect was not a volume artifact.

Of the cells tested, 38% were excited and 62% were inhibited by CNA stimulation. Naloxone blocked the excitatory response in 25% of the cells (2/8; one chemically

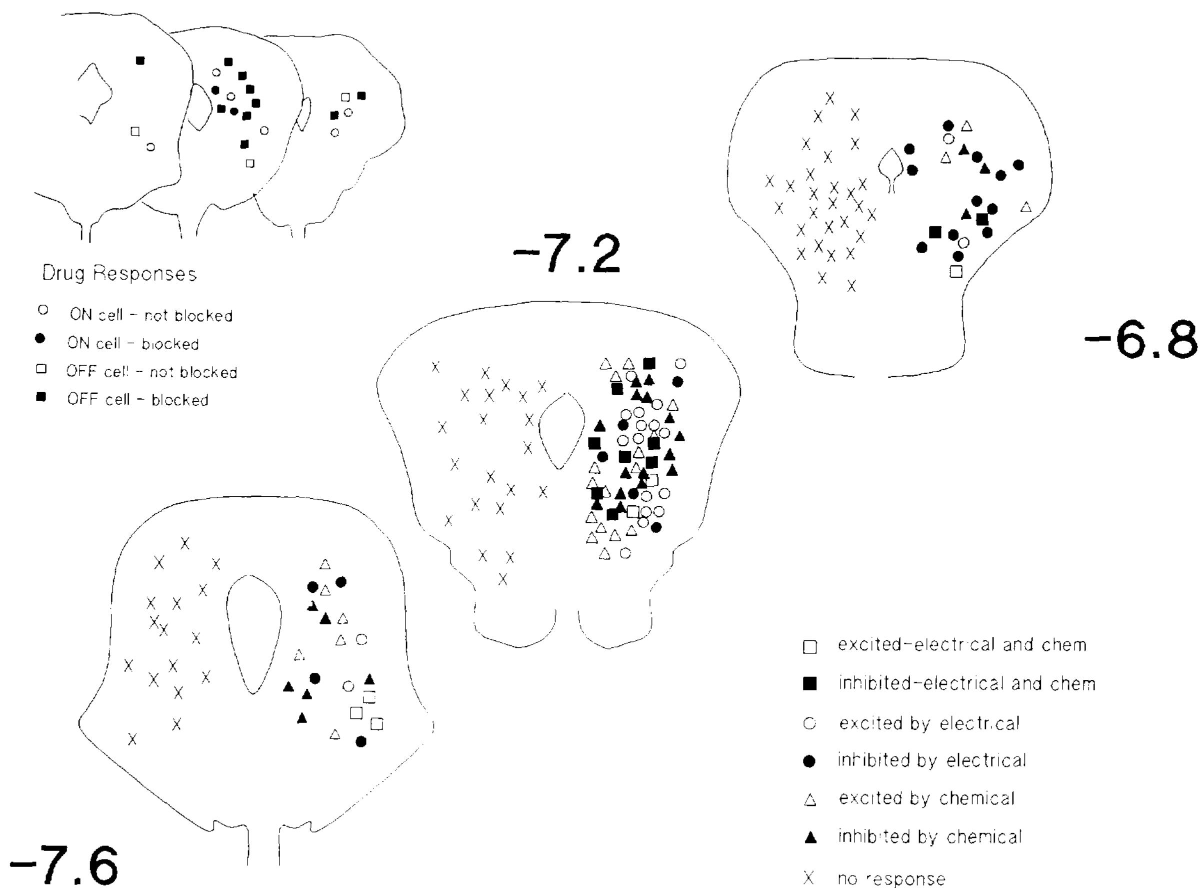
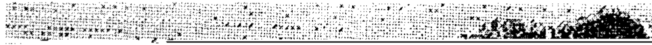
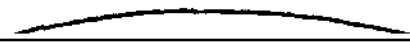


Fig. 6. Location of cells recorded in the PAG ($n = 142$). 'No response' cells were moved to the left side of the figure so that responsive cells could be more easily viewed. In the upper left corner, the location and reponse of PAG cells to injection of naloxone either on site in the PAG or systemically are shown ($n = 21$). ON cells, cells excited by CNA stimulation; OFF cells, cells inhibited by CNA stimulation.





recorded were located in the lateral and ventrolateral PAG columns. However, the responsive and non-responsive cells were homogeneously distributed within these regions. In addition, there was no correlation between the location of a cell and the nature (inhibitory or excitatory) of its response to CNA stimulation. Fig. 7A shows an example of a typical CNA recording site.

3.9. Sites outside the central nucleus of the amygdala

We separated the locations outside the CNA into the basolateral nucleus of the amygdala (BLA) and other sites outside both the CNA and the BLA. The reason for this division is because the BLA also projects to the PAG and therefore its stimulation can produce an effect that is related to its projection properties. The location of these sites can be seen in Fig. 7B. As shown in Table 5, the responses of 110 cells to electrical (67 cells) and chemical (43 cells) were examined. The majority of these stimulation sites were ventrolateral to CNA and were closer to the basolateral nucleus than to the CNA. In response to electrical stimulation, 7% of cells (5/67) were excited; 16% (11/67) were inhibited and 76% (51/67) did not respond. Responses of 43 cells to chemical stimulation outside both the CNA and the BLA were examined as well. In this population, one cell (2%) was excited, five cells (11%) were inhibited and 37 (86%) did not respond. Responses of 14 cells to stimulation (13 electrically, 7 both electrically and chemically and one only chemically) of the BLA were examined. Electrical stimulation excited 15% (2/13), inhibited 54% (7/13) and had no effect on 31% (4/13) cells. Chemical stimulation excited 25% (2/8), inhibited 37.5% (3/8) and produced no effect in 37.5% (3/8) of cells.

4. Discussion

The results of these experiments show that a large percentage of PAG neurons (46% by electrical and 36% by chemical) respond to CNA stimulation. This observation and anatomical studies showing a strong and highly site specific projection from the CNA to the PAG indicate that the CNA-PAG projection is an important pathway that modulates PAG functions. Although the number of cells excited by CNA stimulation was similar to the number of cells that were inhibited by stimulation of the CNA, there was a significant difference between the latency of these effects. The latency of the inhibitory response was 50% shorter than the latency of the excitatory response. This observation suggests that the inhibitory effect of CNA stimulation is produced through activation of either a faster conducting system or a more direct pathway than the system that serves the excitatory response. In addition to this latency difference, these studies show that neurons inhibited by CNA stimulation had a significantly higher mean baseline firing rate than that of the excitatory cells, indicating that two differing cells types are responding to the CNA stimulation.

The results of these studies show a very small and inconsistent effect of CNA stimulation on arterial pressure. Stimulation of amygdala has been reported to cause an increase [21,22], a decrease [30,31] or no effect [24] on systemic blood pressure. The difference between these studies can be related to the type of anesthetics that have been used, level of anesthesia [28] and species differences. In these studies the pressor effect was observed in approximately 15% of animals and only 2% of animals showed a depressor response to CNA stimulation. These observations are in agreement with other studies of the effect of amygdala stimulation on blood pressure that have used a deeply anesthetized preparation [21,22,30,31].

The fact that PAG stimulation produces analgesia has been well documented [4,18,19]. However, the mechanisms by which this system is activated under natural conditions remains unknown. One natural condition that activates the descending analgesic system is fear, such as exposure to a predator. It has been suggested that the CNA-PAG pathway is an important network in fear-induced analgesia [38]. Several factors support this hypothesis. First, in the rat, stimulation of the CNA produces freezing [2,30] and analgesia [53] that is identical to the behavioral response of the rat to a predator such as a cat [39]. Secondly, the freezing response evoked by CNA stimulation is blocked or significantly diminished by lesioning of the ventrolateral PAG [17]. Finally, stimulation of the ventrolateral PAG produces analgesia and immobility [3,40,42]. Although these behavioral studies establish a role for the PAG in CNA mediated fear and anxiety, the cellular mechanisms by which the CNA influences the PAG's neuronal activity is not clear. The PAG mediated analgesic system can be activated by electrical stimulation [51], injection of excitatory amino acids or their analogs [40,44] and by opioid agonists such as morphine and enkephalin [23,47]. It has been documented that there are tonically active inhibitory neurons in the PAG and there is significant evidence that enkephalin produces its analgesic effect by inhibiting these tonically active inhibitory interneurons within the PAG [5,54]. Since the CNA contains a large number of enkephalin containing neurons, it is possible that stimulation of the CNA releases enkephalin in the PAG which leads to analgesia by a disinhibition process. Our results indicate that CNA stimulation can excite or inhibit PAG neurons. However, the response type is dependent on the baseline firing rate of PAG cells and cells that have a high baseline firing rate are most likely to be inhibited by CNA stimulation. In addition, the inhibitory effect of CNA stimulation was blocked by naloxone in the majority of cases suggesting that an opioidergic system is involved in the CNA-PAG pathway.

Stimulation of the CNA also excited a large number of PAG neurons. However, the latency to this effect was

longer than the latency of the inhibitory effect of CNA stimulation. It is possible that the excitatory effect of CNA stimulation on PAG neurons is through activation of hypothalamic regions that project to the PAG. The projection from the amygdala to the hypothalamus, in particular, the lateral, medial and preoptic nuclei are well established [16,34–36,49]. In addition, these nuclei have strong projections to the PAG [7,55] and activation of these regions can produce analgesia.

Considering the above information, we propose the following model for a CNA-PAG network that mediates fear-induced analgesia and freezing. Exposure to a fearful condition excites CNA neurons that follow a fast conducting pathway to the PAG and also neurons that indirectly project to the PAG via the hypothalamus. The pathway with the faster conduction velocity involves an enkephalinergic link and its activation causes the release of enkephalin. Enkephalin then acts on tonically active neurons that inhibit PAG projecting neurons. The indirect pathway causes activation of hypothalamic cells that directly or indirectly excite neurons in the PAG.

The fact that the CNA contains a large number of enkephalinergic cells and our observation that naloxone blocks the inhibitory response to CNA stimulation supports one aspect of this model. In addition, the observation that neurons that were inhibited by CNA stimulation had a high baseline firing rate, suggesting their role as a tonically active cells, further strengthens this model. However, we do realize that this model is highly speculative and further experiments will be needed to test its validity. In particular, it will be necessary to obtain a physiological signature of inhibitory and tonically active neurons and establish that they are inhibited by CNA stimulation. In addition, the role of the hypothalamus in the CNA-PAG pathway must be directly tested by injection of local anesthetic in the hypothalamus.

In summary, the results of the electrophysiological studies show that a large projection from the CNA to the PAG exists but that the excitatory and inhibitory projections follow separate pathways and involve cells of differing characteristics. Both pathways involve μ- and/or δ-opioid receptors but the inhibitory pathway appears to be under stronger enkephalinergic control.

Acknowledgements

This study was supported by NIH Grant NS20743.

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