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Responses of opioid and serotonin containing medullary raphe neurons to electroacupuncture

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Abstract

The midline medulla oblongata, which includes the nucleus raphe obscurus, raphe magnus and raphe pallidus (NRP), is involved in regulation of cardiovascular responses. Opioids and serotonin (5-HT) are thought to function as important neurotransmitters in this region. We previously have demonstrated that electroacupuncture (EA) at the Neiguan-Jianshi acupoints (P5-P6, overlying the median nerves) attenuates sympathoexcitatory blood pressure reflexes through its influence on several brain regions. However, the role of these three raphe nuclei in the acupuncture responses is unknown. In baroreceptor denervated and vagotomized cats, the present study evaluated c-Fos activation in the raphe nuclei induced by EA and examined its relationship to enkephalin and 5-HT. To enhance detection of perikarya containing enkephalin, colchicine (90–100 µg/kg) was administered into the subarachnoid space in anesthetized cats 28–30 hours before the placement of acupuncture needles at P5-P6 acupoints with or without electrical stimulation for 30 min. Perikarya containing the opioid and 5-HT were found in the raphe nuclei of all animals following application of colchicine. Compared to controls without electrical stimulation (n=5), c-Fos immunoreactivity and neurons double-labeled with c-Fos and either enkephalin or 5-HT were found more frequently in all three midline medullary nuclei, especially in NRP (n=6, all $P<0.05$) of EA-treated cats. Moreover, neurons triple-labeled with c-Fos, enkephalin and 5-HT were noted frequently in the NRP following EA stimulation. These results suggest that the medullary raphe nuclei, particularly the NRP, process somatic signals during EA and participate in EA-related modulation of cardiovascular function through an opioid or serotonergic mechanism.

Keywords

Acupuncture; raphe nuclei; enkephalin; serotonin; c-Fos

1. Introduction

Although acupuncture has been used for many years to treat a number of diseases, including cardiovascular dysfunction, the mechanisms underlying its effects on central regulation of cardiovascular function remain unclear. Acupuncture at the Neiguan-Jianshi acupoints (P5-P6, pericardial meridian overlying the median nerve) is applied commonly to manage cardiovascular diseases (Ho et al., 1999; Li et al., 1998; Lin et al., 2001). Our previous studies

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have demonstrated that electrical stimulation of the P5-P6 acupoints significantly attenuates visceral sympathoexcitatory pressor responses through an influence on μ and δ opioid receptors in the rostral ventrolateral medulla (rVLM), a site where visceral afferents converge on bulbospinal premotor sympathetic neurons (Li et al., 2001; Tjen-A-Looi et al., 2003). These results suggest that electroacupuncture (EA) has the potential to affect the rVLM through an opioid mechanism, perhaps involving endorphin and enkephalins, and thereby modulate sympathetic activity.

The midline medulla oblongata, including, in part, the nucleus raphe obscurus (NRO), nucleus raphe magnus (NRM) and nucleus raphe pallidus (NRP), modulates sympathetic outflow and cardiovascular responses through an influence on the rVLM (Coleman and Dampney, 1995; Bago et al., 2002; Orer et al., 2008). There are direct neuronal projections from the raphe nuclei to the rVLM (Bago et al., 2002). Furthermore, opioids and serotonin (5-HT) function as important neurotransmitters in this region (Arvidsson et al., 1992; Hunt and Lovick, 1982). It is unknown whether neurons, in particular, opioidergic and/or serotonergic neurons, in the raphe nuclei respond to EA stimulation. However, demonstration of their involvement would suggest a potential role for their participation in the physiological responses to EA.

Expression of c-Fos has been used widely as a marker of neuronal activation (Guo and Longhurst, 2003; Morgen et al., 1987). Cell bodies containing 5-HT and enkephalin, but not β -endorphin are distributed in the raphe nuclei (Arvidsson et al., 1992; Hunt and Lovick, 1982). There is anatomical evidence for co-existence of enkephalin and 5-HT in perikarya of the raphe nuclei (Arvidsson et al 1992), and for their involvement in mediating analgesic effects (Hunt and Lovick, 1982). Considering this background, the present study evaluated expression of c-Fos in the cat raphe nuclei during EA stimulation specifically with respect to neurons containing enkephalin and/or 5-HT. We hypothesized that EA applied at P5-P6 acupoints increases c-Fos expression in the midline medulla oblongata. Moreover, we proposed that neurons demonstrating c-Fos activity in this region co-localize with enkephalin and/or 5-HT.

2. Results

2.1. Blood pressure and heart rate

As described in our previous report (Guo and Longhurst, 2007), mean BP (MAP) decreased slightly (5–10 mmHg) in three of six cats demonstrating similar paw flexion of forelimbs during EA stimulation at P5-P6 as three animals whose MAP was not altered. Furthermore, MAP was not changed in any of five control animals. No changes in HR were observed during EA stimulation or control in any cat from either group.

2.2. c-Fos immunohistochemical staining in raphe nuclei

Fos immunoreactivity was noted to be distributed throughout the rostro-caudal extension of the raphe nuclei in both control and EA-treated cats following colchicine treatment, bilateral baroreceptor denervation and cervical vagotomy. Compared to the control animals, Fos-labeled neurons were found easily at multiple-levels throughout each of the three raphe nuclei in the EA-treated cats. In this regard, we observed that following EA stimulation, abundant c-Fos immunoreactivity was distributed in the NRP of all six cats, and in the NRO and NRM in two of six cats in which BP was slightly decreased. Photomicrographs in Fig. 1 demonstrate the distribution of Fos-labeled neurons in the raphe nuclei from a cat in the sham-operated control and EA-treated groups, respectively.

Similar patterns of c-Fos distribution appeared in the raphe nuclei in single c-Fos labeled sections and double-stained sections containing c-Fos and enkephalin or 5-HT. We quantitatively evaluated c-Fos immunoreactivity in the double-labeled sections. Compared to

controls (n=5), a significant increase in the number of Fos positive neurons was found in the NRP but not in the NRM and NRO of cats treated with EA (n=6; $P<0.05$, Tables 1 and 2).

2.3. Double-labeling with c-Fos and enkephalin in raphe nuclei

Compared to animals not treated with colchicine, we observed many more perikarya containing enkephalin in the raphe nuclei of both control and EA-treated cats following application of colchicine. Although there tended to be fewer enkephalin-labeled cells in the NRO and NRM, than the NRP, the differences were not significant. Also, we did not find a difference in distribution of enkephalin-labeled neurons in the NRO, NRM and NRP of EA and control colchicine-treated animals (Table 1).

Similar to of the observed increase in Fos immunoreactivity following EA, neurons double-labeled with c-Fos and enkephalin were observed more frequently in raphe nuclei, especially in the NRP of EA colchicine-treated compared to control cats. Compared to the control group (n=5), the number of double-labeled neurons, in relation to neurons stained with either enkephalin or c-Fos, were significantly increased by EA in the NRP (n=6; Table 1, Fig. 2). More cells in the NRM of the EA group showed co-localization of c-Fos with enkephalin, when the numbers were expressed relative to the total population of enkephalin or c-Fos positive cells (Table 1). However, we did not note a significant increase in neurons double-labeled with c-Fos and enkephalin in the NRO of EA-treated cats relative to controls. Fig. 3 provides examples of confocal images of neurons double-labeled with c-Fos and enkephalin in the NRP of an EA-treated cat following colchicine.

2.4. Double-labeling with c-Fos and 5-HT in raphe nuclei

We observed a robust population of cell bodies stained with 5-HT in the NRO, NRM and NRP, particularly in the latter nucleus. There were almost twice as many cell bodies containing 5-HT in the NRP vs. the NRO and NRM. The number of neurons labeled with 5-HT in the three raphe nuclei was not altered by colchicine treatment. Furthermore, we also did not note a difference in distribution or density of neurons containing 5-HT in any of the raphe nuclei comparing controls to EA-treated cats following colchicine (Table 2).

As mentioned above, c-Fos immunoreactivity was detected more often in the raphe nuclei of EA-treated cats compared to controls. Neurons co-labeled with 5-HT and c-Fos were found throughout the NRO, NRM and NRP, particularly in the NRP of EA-treated cats but rarely in control animals after colchicine treatment. Relative to controls (n=5), neurons double-labeled 5-HT and c-Fos and their number relative to c-Fos positive cells were significantly increased ($P<0.05$) in the NRP of EA-treated cats (n=6). In addition, in comparison to the control group, more neurons co-labeled with 5-HT and c-Fos were noted in the NRO and NRM of the intervention group, in relation to the total population of 5-HT or c-Fos positive cells (Table 2, Fig. 4). Photomicrographs in Fig. 5 provide an example of co-localization of Fos-like immunoreactive nuclei with perikarya containing 5-HT in the NRP of an EA-treated cat.

2.5. Triple-labeling with c-Fos + enkephalin + 5-HT in raphe nuclei

We found similar patterns of distribution of neurons double-labeled with c-Fos + enkephalin or 5-HT in sections evaluated for double and triple labeling. Specifically in the triple-labeled sections, we noted a similar distribution of neurons containing enkephalin + 5-HT in the NRO, NRM and NRP of both control (n=2) and EA-treated cats (n=3) following colchicine. The average number of double-labeled cells considering the entire raphe was similar in the EA and control groups (30 vs. 31 cells per section). Double-labeled neurons comprised two-thirds of the total population of neurons labeled with enkephalin and 40% of neurons stained with 5-HT in the three raphe nuclei of both control and EA cats. We also observed neurons triple-labeled with c-Fos + enkephalin + 5-HT in the raphe nuclei of EA-treated cats following

colchicine treatment. In this regard, we more frequently found triple-labeled neurons in the NRP (6 ± 2 cells per section) than in the NRM (2 ± 1 cells per section) or NRO (1 ± 1 cells per section). In contrast, in both control colchicine treated animals, triple-labeled neurons were found rarely in the NRP (0–1 cell per section), but none were observed in the NRM or NRO. Triple-labeled neurons represented 29% and 0–3% of neurons containing both peptide neurotransmitters in the three raphe nuclei of EA-treated and control cats, respectively. Photomicrographs in Fig. 6 show a neuron triple-labeled with c-Fos, enkephalin and 5-HT in the NRP of a cat following EA treatment.

3. Discussion

EA at the P5-P6 acupoints is used frequently to manage cardiovascular disease (Ho et al., 1999; Li et al., 1998; Lin et al., 2001). Our previous studies have demonstrated that activation of these acupoints reduces myocardial ischemia and sympathoexcitatory pressor reflexes caused by stimulation of abdominal viscera through modulation of sympathetic activity (Tjen-A-Looi et al., 2004; Li et al., 1998 and 2002). Moreover, EA at the P5-P6 acupoints overlying the median nerve specifically inhibits sympathoexcitatory cardiovascular responses, compared to the absence of responses to stimulation of acupoints overlying other nerves (e.g., superficial radial nerve; Tjen-A-Looi et al., 2004). The raphe nuclei in the midline of the medulla oblongata are considered to be important brain stem regions that modulate autonomic activity (Barman and Gebber, 1997; Coleman and Dampney, 1995; Larsen et al., 2000). The present study identified neuronal activation in those nuclei during acupuncture by detecting c-Fos expression following pretreatment with colchicine. Moreover, we also showed that neurons activated by EA co-localize with enkephalin and 5-HT. As such, the present study provides anatomical data suggesting that the medullary raphe nuclei participate in EA-related regulation of neuronal activity and cardiovascular responses, as well as the potential involvement of enkephalin and 5-HT neurotransmitters.

C-Fos, an immediate early gene, is expressed rapidly but transiently after cellular stimulation. Mapping c-Fos expression is a valuable tool for identifying neuronal activation in the central nervous system in response to peripheral sensory neuronal stimulation (Morgen et al., 1987). We and other investigators have applied Fos immunohistochemical labeling to locate specific brain regions that respond to acupuncture (Guo et al., 2004; Guo and Longhurst, 2007; Lee and Beitz, 1993). In the present study, care was taken to minimize the expression of c-Fos in the raphe nuclei induced by non-specific stimuli, including treatment with the smallest possible dose of colchicine, anesthesia, surgical procedures and input from baroreceptors resulting from changes in blood pressure (Guo et al., 2004; Guo and Longhurst, 2007). In particular, the colchicine treated sham-operated controls were performed with only one difference between control and EA, i.e., electrical stimulation of acupuncture needles at P5-P6 was not applied in the control group. Thus, compared to controls, the pattern of increased c-Fos in the EA-treated cat was caused exclusively by electrical stimulation of needles placed in the P5-P6 acupoints rather than by colchicine or other non-specific stimuli.

A slight decrease in blood pressure (5–10 mmHg) was noted during the EA stimulation in three of the six cats. In two of these three cats, we observed relatively more c-Fos expression in the NRO, NRM and NRP, compared to the three cats that did not demonstrate a change in BP following EA stimulation. It is possible that activation of more neurons in all three raphe nuclei may have contributed to a decrease in BP in these two cats. In addition, individual differences in the type of afferent fiber activation during stimulation of P5-P6 acupoints may lead to different cardiovascular responses, including changes in BP. In this regard, other investigators have demonstrated that depressor responses can be caused by activation of myelinated somatic afferents (Johansson, 1962; Koizumi et al., 1970; Lee and Beitz, 1993). We have shown that stimulation of median nerves with EA at the P5-P6 acupoints activates more myelinated than

unmyelinated fibers (Li et al., 1998), although our data also show that both fiber types contribute to the EA-related modulation of reflex excitatory responses (Tjen-A-Looi et al., 2005). As such, a slight decrease in blood pressure was not an unexpected finding. It is unlikely, however, that the small decreases in blood pressure during EA contributed to our observations since barodenervation eliminated any secondary baroreflex influence on expression of c-Fos in the raphe nuclei (Guo and Longhurst, 2003; Potts et al., 1997).

The raphe nuclei mainly encompass the NRO, NRM and NRP (Taber et al., 1960; Jacobs et al 1984). These regions are involved in regulation of blood pressure during cardiovascular activation (e.g., defense reaction), but do not exert tonic control of blood pressure (Coleman and Dampney, 1995). Stimulation of different raphe nuclei may increase or decrease blood pressure in different species, likely due to predominant activation of excitatory or inhibitory groups of neurons (Blessing and Nalivaiko, 2000; Coleman and Dampney, 1995; McCall, 1988; McCall and Clement, 1989; Silva et al., 2002). The medullary raphe has been shown to modulate blood pressure through its effects on sympathetic outflow in the rostral ventrolateral medulla (rVLM) as well as through its direct projections to the spinal cord (Larsen et al., 2000; Coleman and Dampney, 1995; Dean and Bago, 2002; Loewy, 1981). The rVLM, where many premotor sympathetic neurons originate, receives significant serotonergic input from all three of the midline raphe structures (Bago et al, 2002). Previous studies have suggested that NRP, but not NRO and NRM, participates in the analgesic effect of acupuncture at the Zusanli acupoint (S36, stomach meridian, located over the deep peroneal nerve in the hind leg; Lee and Beitz, 1993) in rats. We observed an increase in c-Fos expression in the raphe nuclei, particularly in NRP of cats, induced by EA at P5-P6, two acupoints on the forelimb that frequently are used to treat cardiovascular disorders. Activation of neurons in the caudal NRO and NRP of cats evokes powerful depressor and sympathoinhibitory responses (Coleman and Dampney, 1995). Thus, the medullary raphe receives somatic input from P5-P6 stimulation and potentially may play a role in EA-modulation of sympathoexcitatory cardiovascular responses (Li et al., 1998 and 2001).

The raphe nuclei contain neurons that synthesize and release a variety of neurotransmitters and neuropeptides (Arvidsson et al., 1992; Bago et al, 2002). EA's attenuation of sympathoexcitatory responses has been tied closely to the opioid system. In fact, we have shown that enkephalin acting through stimulation of δ opioid receptors is a prominent neurotransmitter system that contributes significantly to control of sympathetic tone by EA (Li et al, 2001). Although in preliminary experiments without colchicine, we observed fibers but few perikarya containing enkephalin in the raphe nuclei, after treatment with colchicine, a number of cell bodies containing enkephalin were found in all three midline medullary nuclei. This distribution is consistent with previous studies (Arvidsson et al., 1992; Conrath-Verrier et al., 1983). Using colchicine to enhance opioid immunostaining in cell bodies (Ceccatelli et al., 1989; Cirriello and Caverson, 1989; Guo et al., 2004) thus allowed demonstration of frequent c-Fos immunoreactive co-localization with perikarya containing enkephalin in the three raphe nuclei following EA at P5-P6. Moreover, a significant increase in neurons co-labeled with c-Fos and enkephalin was identified in the NRP after EA, indicating that some NRP neurons activated by EA at P5-P6 acupoints synthesize enkephalin. Our findings support a role for the opioid NRP system in EA regulation of autonomic function.

Some of serotonergic neurons in the raphe modulate sympathetic outflow through a 5-HT_{1A} receptor mechanism in the rVLM (Dean and Bago, 2002; Dean, 2005; Orer et al., 2008). In the present study, we found that Fos co-localized with 5-HT in the raphe nuclei following EA at P5-P6. Most neurons double-stained with the two labels were observed in the NRP. Thus, some serotonergic neurons in the raphe nuclei, especially the NRP, respond to somatic nerve stimulation during EA. Serotonergic projections from the midline raphe inhibit activity in the rVLM and spinal cord (Cao and Morrison, 2003; Dean and Bago, 2002; Macrae et al., 1986).

In light of our data, we suggest that EA modulates sympathetic activity in rVLM and spinal cord through activation of inhibitory serotonergic neurons in the raphe nuclei, particularly NRP. This hypothesis requires further testing.

We noted co-localization of enkephalin and 5-HT in some raphe neurons, which expressed c-Fos following EA. These finding implies potential interactions between enkephalin and 5-HT in the raphe during somatic stimulation by EA.

In summary, the present study provides the first demonstration that EA activates midline medullary nuclei during stimulation of P5-P6 acupoints. Many activated neurons in this area contain opioids and/or serotonin. The results imply that through serotonergic and opioidergic mechanisms, the raphe nuclei, particularly the NRP participates in EA-modulation of neuronal responses in the rVLM and spinal cord to ultimately inhibit sympathetic outflow and excitatory cardiovascular responses.

4. Experimental Procedure

4.1. Surgical Preparation

All procedures were carried out in accordance with the US Society for Neuroscience and the National Institutes of Health guidelines. Surgical and experimental protocols of this study were approved by the animal use and care committee at the University of California, Irvine. The minimum possible numbers of adult cats ($n=11$, 3–4 kg) of both sexes were used to obtain reproducible and statistically significant results. Throughout the study, steps were taken to minimize discomfort and suffering of the animals.

Similar to other previous studies (Arvidsson et al., 1992), in a preliminary study, we observed neuronal fibers and a few weakly labeled perikarya containing enkephalin in the raphe nuclei of the cat. Colchicine enhanced the content of enkephalin in perikarya in these nuclei by disrupting microtubular transport (Ceccatelli et al., 1989; Ciriello and Caverson, 1989). Thus, to more comprehensively demonstrate perikarya containing enkephalin in the midline medullary nuclei, we treated all cats with colchicine as in our previous investigation (Guo et al., 2004).

Sterile surgical procedures were conducted for administration of colchicine in the surgical operating room of the vivarium at the University of California, Irvine. Cats were pre-anesthetized with ketamine (25 mg/kg, im) and valium (5 mg/kg, im). Anesthesia was maintained with isoflurane (1–2%, inhalation). The head was placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) and flexed approximately 30° forward in the frame. A one-inch midline incision was made from the external occipital protuberance located at the base of the skull. After exposing the foramen magnum near the brainstem, a 27×1.25-gauge hypodermic needle attached to a 1.0 ml syringe was inserted into the subarachnoid space through the atlanto-occipital membrane overlying the fourth ventricle. We injected colchicine (90–100 µg/kg, Sigma, St. Louis, MO, USA) in 0.08~0.13 ml of solution (3000 µg/ml) dissolved in 0.9% normal saline. The dose of colchicine used in this study was determined based on our and previous experience of others (Guo et al., 2004; Ciriello and Caverson, 1989). Following administration of colchicine, the incision was closed and the cats were allowed to recover.

Cats were re-anesthetized with ketamine (40–50 mg/kg, im) and α -chloralose (50–60 mg/kg, iv) following the 22–24 hour post-operative period after treatment with colchicine. Supplemental α -chloralose (5–10 mg/kg, iv) was applied to maintain an adequate depth of anesthesia as judged by stability of respiration, blood pressure and heart rate and the lack of a withdrawal response to toe pinch. A femoral artery and vein were cannulated for measuring

arterial blood pressure (BP, Statham P 23 ID, Oxnard, CA, USA) and administering drugs and fluids, respectively. Heart rate (HR) was derived from the arterial pressure pulse with a biotach (Gould Instrument, Cleveland, OH, USA). The animal was ventilated artificially through a cuffed endotracheal tube after incubation. Arterial blood gases and pH were monitored with a blood gas analyzer (Radiometer, Inc., Model ABL-3, Westlake, OH, USA). They were kept within normal limits (PO₂, 100–150 mmHg; PCO₂, 28–35 mmHg; pH, 7.35–7.45) by adjusting the volume and/or ventilatory rate, enriching the inspired O₂ supply and administration of 1 M NaHCO₃. Body temperature was maintained at 36–38°C by a water heating pad and a heat lamp.

Cardiovascular hemodynamic changes lead to secondary baro- and cardiopulmonary reflex responses, which can induce c-Fos expression in the brain (Guo and Longhurst, 2003; Potts et al., 1997). To control for input from this secondary activation of neural pathways consequent to EA at the P5-P6 acupoints (Johansson, 1962; Koizumi et al., 1970; Li et al., 1998), bilateral sino-aortic denervations and cervical vagotomies were performed. The carotid sinus nerves and cervical vagus were isolated and transected from the internal and common carotid artery, respectively. Subsequently, the carotid bifurcations were stripped of adventitial tissue and painted with 10% phenol (Sigma; St. Louis, MO). Barodenervation was verified by the absence of a normal decrease of heart rate in response to elevated arterial blood pressure elicited by intravenous administration of phenylephrine (10 µg/kg, Gensia Sicor Pharmaceuticals, Irvine, CA, USA).

Similar to procedures in our previous studies (Guo et al., 2004, Guo and Longhurst, 2007), cats were stabilized for 4 hours after surgical preparation. Approximately 28–30 hours following administration of colchicine, pairs of stainless steel, 32 ga acupuncture needles were inserted bilaterally at the P5-P6 acupoints, overlying the median nerves. The P5-P6 acupoints on both forelimbs of small animals are analogous to those in humans (Hua, 1994). The needles were connected to a constant current stimulator with a stimulus isolation unit and stimulator (Grass, model S88, W. Warwick, RI, USA).

4.2. Experimental Protocols

Cats were divided randomly into an EA-treated group (n=6) and a sham-operated control group (n=5). Our previous studies have shown that low frequency EA for 30 min attenuates rVLM premotor sympathetic neuronal and reflex sympathetic responses in anesthetized cats (Li et al., 1998 and 2001; Tjen-A-Looi et al., 2003). Thus, in the present study, low frequency EA (0.5 ms pulses, 2 Hz, 2–5 V, 1–4 mA) was administered for 30 min. This stimulation was sufficient to produce moderate, repeated paw flexion in each forelimb. Each set of electrodes was stimulated separately so that current did not flow from one location to the contralateral side. In control animals, acupuncture needles were put into the P5-P6 acupoints for 30 min without electrical stimulation. We have demonstrated that this form of stimulation in controls does not evoke input to the rVLM or modulate sympathetic reflexes and serves as an adequate control for EA (Zhou et al., 2005; Tjen-A-Looi et al., 2004).

4.3. Immunohistochemical staining

4.3.1. Tissue preparation—As described in our previous studies (Guo and Longhurst, 2003; Guo et al., 2004), 90 min after termination of EA stimulation or control procedures, deep anesthesia was induced by another larger dose of α -chloralose (100 mg/kg, iv). The animal then was perfused transcardially with 0.9% saline and cold 4% paraformaldehyde in phosphate buffer (PB, pH 7.2). The medulla oblongata was removed and stored in 4% paraformaldehyde for 2 hours and subsequently in 30% sucrose for 48 hours to prevent ice crystallization.

Coronal sections of the brain (30 μ m) were collected on a cryostat microtome (Leica CM1850 Heidelberg Strasse, Nussloch, Germany) and placed serially in cold cryoprotectant solution (Chan and Sawchenko, 1994). Brain sections were used for performing immunohistochemical labels as described below, or stained with Nissl to reveal the cellular architecture (Guo and Longhurst, 2003). In this study, free-floating sections were used for labeling.

4.3.2. C-Fos immunohistochemical staining—The c-Fos protein was stained using the avidin-biotin-peroxidase complex (ABC) method (Guo and Longhurst, 2003). Briefly, after rinsing three times (10 min $\times$ 3) in 0.1 M PB (pH 7.2) containing 0.3% Triton X-100 (PBT), brain sections were placed in 0.5% hydrogen peroxide for 10 min to quench endogenous peroxidase activity. The sections then were placed in 1% normal goat serum (Vector ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 20 min. They were incubated with a primary polyclonal rabbit anti-Fos antibody (1:20,000 dilution, Oncogene research product, Calbiochem, San Diego, CA, USA) at 4°C for 48 hours. This antibody was raised specifically against amino acid 4–17 of human Fos. Subsequently, sections were washed in 0.1 M PBT three times and incubated with biotinylated goat anti-rabbit IgG (Vector Kit, 1:200) for 60 min. Following three rinses in 0.1 M PBT, brain tissues were placed in ABC solution (Vector Kit, 1:50) for 30 min. Sections were washed twice, each for 10 min, in 0.1 M PB and were incubated in a solution containing hydrogen peroxide and 3,3'-diaminobenzidine (DAB; Vector laboratory) for 5–8 min. In the ABC complex, DAB is reduced by hydrogen peroxide and deposited in brain tissue as a brown reaction product. The DAB reaction was terminated by rinsing sections in distilled water. Sections were mounted on slides in 0.1 M PB. Slides were allowed to air-dry, cleared in alcohol and xylene baths and covered by glass slips with permount (Fisher Scientific, Fair Lawn, New Jersey, USA). The c-Fos immunoreactivity was visualized as dark-brown staining. In an immunohistochemical control study, no labeling was detected when the primary antibody was omitted.

4.3.3. Double-fluorescent immunohistochemical labeling for c-Fos + enkephalin or 5-HT—After rinsing three times (10 min each) with phosphate buffered saline containing 0.3% Triton X-100 (PBST, pH=7.4), brain sections were placed in 1% normal donkey serum (Jackson immunoresearch laboratories, Inc., West Grove, PA, USA) for 1 hour and incubated with primary antibodies at 4°C for 48 hours. PBST solution containing the two primary antibodies included a rabbit polyclonal anti-Fos antibody (1:2,000 dilution, Oncogene research product) and either a mouse anti-met- and leu-enkephalin (1:200, Chemicon International, Inc. Temecula, CA, USA), or a goat anti-5-HT (ImmunoStar, Inc., Hudson, WI, USA). Sections then were incubated with rhodamine-conjugated donkey anti-mouse and fluorescein-conjugated donkey anti-rabbit antibodies or rhodamine-conjugated donkey anti-rabbit and fluorescein-conjugated donkey anti-goat antibodies (all 1:100; Jackson immunoresearch laboratories, Inc.) in PBST at 4°C for 24 hours. These secondary antibodies raised in the donkey are made for multiple labels. They have minimal cross-reactivity to other nonspecific species (Catalog, specializing in second antibodies, Jackson immunoresearch laboratories, Inc., 2007). After washing in phosphate buffered saline (PBS, pH=7.4) for 30 min (10 min $\times$ 3 times), the sections were mounted on slides and air dried. The slides were coverslipped using mounting medium (Vector Laboratories). Immunohistochemical control studies were performed by omission of the primary or secondary antibodies and by preabsorption with excess met- and leu-enkephalin (10 μ g/ml; Bachem Peninsula Labs. San Carlos, CA). No labeling was detected under these conditions.

4.3.4. Triple-fluorescent immunohistochemical labeling for c-Fos, enkephalin and 5-HT—The staining procedures were similar to those used for double-fluorescent immunohistochemical labeling described above. Briefly, after treating with PBST and 1% normal donkey serum, brain sections were incubated with three primary antibodies, i.e., a goat

anti-5-HT, a mouse anti-enkephalin and a rabbit anti-c-Fos (1:1000 dilution) for 48 hours at 4°C. Sections then were incubated with fluorescein-conjugated anti-goat, rhodamine-conjugated anti-mouse and coumarin-conjugated anti-rabbit antibodies (all 1:100; Jackson immunoresearch laboratories, Inc.) at 4°C for 24 hours. The sections were mounted on slides and covered by glass slips with mounting medium. No staining was detected when the corresponding primary or secondary antibody was omitted in immunohistochemical control studies.

4.4. Data Analysis

Brain sections were scanned and examined with a light and fluorescent microscope (Nikon, E400, Melville, NY, USA). Labeling of c-Fos appeared as round dots of approximate 7–12 µm in diameter and was obviously distinguishable from background staining at 40x magnification. Three epi-fluorescence filters (B-2A, G-2A, or UV-2A) equipped in a fluorescent microscope were used to identify single stains appearing as green (fluorescein), red (rhodamine) or blue (coumarin) in brain sections. Two or three single fluorescent images were captured with a Spot digital camera (RT color v3.0, Spot Diagnostic Instruments, Inc., Sterling Heights, MI, USA) from the same site of the brain section. The images were merged to identify double- or triple-labeled markers using the software provided with the Spot digital camera (Guo et al., 2005; Guo and Longhurst, 2006 and 2007). In every animal, two sections were selected for each of four representative planes of the medulla oblongata, which closely matched the standard stereotaxic planes of Berman's atlas (P 13.5, P 12.1, P 11.6, P 10.0; Berman, 1968). The numbers of single-, double- or triple-labeled cells in the same section were counted in each animal. The average number of labeled neurons in the four representative levels taken from the rostro-caudal extension of the raphe nuclei (Figs. 2 and 4) was obtained by dividing the total number of neurons by eight, representing the number of sections used for cell counting (Guo and Longhurst, 2003; Guo et al., 2004).

To confirm co-localization of two or three labels in the same neuron, selected sections that had been examined with a fluorescent microscope were evaluated further with a laser scanning confocal microscope (Zeiss LSM 510, Meta system, Thornwood, NY, USA). This apparatus was equipped with HeNe and Argon lasers and allowed operation of multiple channels. Lasers of 488 and 543 nm wavelengths were used to excite fluorescein (green) and rhodamine (red), respectively. A 790 nm laser was applied for two-photon excitation of coumarin (blue). Digital images of the labels were captured and analyzed with software (Zeiss LSM) provided with this microscope. Images in two or three colors in the same plane were merged for revealing the relationship between two or three labels (Figs. 3, 5 and 6). Single-, double- and triple-labeled neurons were evaluated.

Statistical Analysis—All statistical analyses were conducted with statistical software (SigmaStat, Version 3.0, Jandel Scientific Software, San Rafael, CA, USA). The Kolmogorov-Smirnoff test was used to determine if data were normally distributed. Comparisons between two groups were analyzed with the Student's t-test or Mann-Whitney Rank Sum Test. Values were considered to be significantly different when $P < 0.05$. Data are expressed as means $\pm$ SE.

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Abbreviations

BP

	Blood pressure
ABC	the avidin-biotin-peroxidase complex
DAB	3', 3' -diaminobenzidine
EA	Electroacupuncture
HR	Heart rate
NRM	Nucleus raphe magnus
NRO	Nucleus raphe obscurus
NRP	Nucleus raphe pallidus
P5-P6	Neiguan-Jianshi acupoints
PB	Phosphate buffer
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing Triton X-100
PBT	Phosphate buffer containing Triton X-100
rVLM	Rostral ventrolateral medulla
5-HT	Serotonin

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Figure 1.

Photomicrographs demonstrating Fos-like immunoreactive cells in raphe nuclei of the cat. A: a low-power photomicrograph showing midline medulla including nucleus raphe obscurus (NRO, at the level of P 13.5) in a control cat. B: magnified region shown within box in A. C: same area shown in B in a cat treated with electroacupuncture (EA). D: low-power photomicrograph displaying nucleus raphe magnus (NRM, the top box) and nucleus raphe pallidus (NRP, bottom box, P 10.0) in an EA-treated cat. E, F: magnified regions shown in top (NRM) and bottom (NRP) boxes in D. In B, C, E, and F, magnified area in small box is shown in large box in upper right corner. Arrows indicate dots representing c-Fos labeled cells. IO, inferior olive. Note: the raphe nuclei, including NRO, NRM and NRP, are located in midline medulla.

Figure 2.

Distribution of enkephalin (Enk) labeled cells and c-Fos reactivity in raphe nuclei of a control animal and a cat subjected to electroacupuncture (EA). Four coronal sections were selected from each animal. Each symbol, ●, □, or ★ represents one cell labeled with c-Fos, enkephalin or c-Fos + enkephalin, respectively. Levels of sections are consistent with those shown in Berman's atlas [Berman, 1968].

Figure 3.

Confocal microscopic images of neurons double-labeled with enkephalin and c-Fos in nucleus raphe pallidus (level P 11.6) following stimulation with electroacupuncture. A: low-power photomicrograph; B: magnified region shown within box in A. Arrow indicates an example of co-localization of enkephalin-labeled neuron containing a c-Fos immunoreactive nucleus. B is merged image from C and D. Arrows in C and D respectively indicate c-Fos positive nucleus and cytoplasm of a neuron stained with enkephalin. Scale bars in A and B–D represent 50 and 20 μm , respectively.

Figure 4.

Distribution of serotonin (5-HT) labeled cells and c-Fos immunoreactivity in raphe nuclei following electroacupuncture (EA) and in a sham-operated control. Four coronal sections (Berman's atlas) were selected from one animal in each experimental group. Each symbol, •, Δ, + or represents one labeled cell with c-Fos, 5-HT or c-Fos + 5-HT, respectively.

Figure 5.

Confocal microscopic images of neurons double-stained with serotonin and c-Fos in nucleus raphe pallidus (level P 11.6) of cat treated with electroacupuncture. A: low-power photomicrograph; B: magnified region shown within box in A. Arrow indicates a neuron double-labeled with c-Fos and serotonin. B is merged image from C and D. Arrows in C and D respectively indicate a neuron containing serotonin and c-Fos positive nucleus. Scale bars in A and B–D represent 50 and 20 μm , respectively.

Figure 6. Confocal microscopic images of serotonin, enkephalin and c-Fos immunoreactivity in nucleus raphe pallidus (level P 10.0) in a cat treated with electroacupuncture. Panels A, B and C show immunostaining of serotonin (green), enkephalin (red) and c-Fos (blue). Panel D demonstrates merged images from Panels A–C. Arrows in Panel A–D indicate neurons containing serotonin, enkephalin, c-Fos, and serotonin + enkephalin + c-Fos. Scale bars in A–D represent 20 μm.

C-Fos immunoreactivity and co-location with enkephalin-labeled neurons in the raphe nucleus of cats following application of colchicine

Table 1

		Fos cells(#)	Enk cells(#)	Enk+Fos cells(#)	Enk + Fos cells Enk cells (%)	Enk + Fos cells Fos cells (%)
NRO	Control (n=5)	4 ± 1	15 ± 3	1 ± 0	3 ± 2	12 ± 7
	EA-treated (n=6)	8 ± 3	17 ± 7	2 ± 1	9 ± 4	22 ± 11
NRM	Control (n=5)	7 ± 2	13 ± 5	1 ± 0	5 ± 3	5 ± 3
	EA-treated (n=6)	14 ± 4	19 ± 5	4 ± 1	18 ± 4 *	25 ± 6 *
NRP	Control (n=5)	12 ± 5	26 ± 7	2 ± 1	12 ± 3	19 ± 6 *
	EA-treated (n=6)	27 ± 4 *	35 ± 3	10 ± 2 *	28 ± 6 *	35 ± 4 *

Means ± SE. Average number (#) of c-Fos positive cells, enkephalin-containing neurons and cells co-localized with both stains in the raphe nucleus expressed per section. Also shown are percentages (%) of neurons double-labeled with enkephalin and c-Fos relative to number of enkephalin-containing neurons or Fos positive cells. Enk, enkephalin; EA, electroacupuncture stimulation. NRO, nucleus raphe obscurus; NRM, nucleus raphe magnus; NRP, nucleus raphe pallidus.

* P<0.05,

** P<0.01; EA-treated group vs. control group after colchicine treatment.

C-Fos immunoreactivity and co-location with neurons labeled with serotonin in the raphe nucleus of cats following application of colchicine

Table 2

	Fos cells(#)	5-HT cells(#)	5-HT+Fos cells(#)	$\frac{5-HT+Fos\ cells}{5-HT\ cells}(\%)$	$\frac{5-HT+Fos\ cells}{Fos\ cells}(\%)$
NRO	Control (n=5)	3 ± 1	25 ± 3	1 ± 0	8 ± 8
	EA-treated (n=6)	9 ± 4	20 ± 4	3 ± 1	29 ± 11
NRM	Control (n=5)	6 ± 3	27 ± 3	1 ± 1	8 ± 5 *
	EA-treated (n=6)	12 ± 3	24 ± 5	3 ± 1	26 ± 4 *
NRP	Control (n=5)	13 ± 5	46 ± 6	4 ± 2 *	21 ± 7
	EA-treated (n=6)	30 ± 5 *	60 ± 3	15 ± 3 *	48 ± 5 **

Means ± SE. Average number (#) of c-Fos positive cells, neurons containing serotonin (5-HT) and cells co-labeled with both stains, expressed per section, in the raphe nucleus. Also shown are percentages (%) of neurons double-labeled with 5-HT and c-Fos, relative to neurons containing 5-HT or Fos positive cells. EA, electroacupuncture stimulation; NRO, nucleus raphe obscurus; NRM, nucleus raphe magnus; NRP, nucleus raphe pallidus.

* P<0.05,

** P<0.01; EA-treated group vs. control group after colchicine treatment.