Different central manifestations in response to electroacupuncture at analgesic and nonanalgesic acupoints in rats: a manganese-enhanced functional magnetic resonance imaging study

Jen-Hwey Chiu, Mao-Shu Chung, Hui-Cheng Cheng, Tzu-Cheng Yeh, Jen-Chuen Hsieh, Chung-Yen Chang, Wan-Yo Kuo, Henrich Cheng, Low-Tone Ho

Abstract

Acupuncture analgesia is an important issue in veterinary medicine. This study was designed to elucidate central modulation effects in response to electroacupuncture (EA) at different acupoints. Manganese-enhanced functional magnetic resonance imaging was performed in Sprague-Dawley rats after sham acupuncture, sham EA, or true EA at somatic acupoints. The acupoints were divided into 3 groups: group 1, analgesic acupoints commonly used for pain relief, such as Hegu (LI 4); group 2, nonanalgesic acupoints rarely used for analgesic effect, such as Neiguan (PC 6); and group 3, acupoints occasionally used for analgesia, such as Zusanli (ST 36). Image acquisition was performed on a 1.5-T superconductive clinical scanner with a circular polarized extremity coil. The results showed that there was no neural activation caused by EA at a true acupoint with shallow needling and no electric current (sham acupuncture). When EA at a true acupoint was applied with true needling but no electric current (sham EA), there was only a slight increase in brain activity at the hypothalamus; when EA was applied at a true acupoint with true needling and an electric current (true EA), the primary response at the hypothalamus was enhanced. Also, there was a tendency for the early activation of pain-modulation areas to be prominent after EA at analgesic acupoints as compared with nonanalgesic acupoints. In conclusion, understanding the linkage between peripheral acupoint stimulation and central neural pathways provides not only an evidence-based approach for veterinary acupuncture but also a useful guide for clinical applications of acupuncture.

Résumé

L'analgésie par acupuncture est un sujet important en médecine vétérinaire. Cette étude avait pour but d'élucider les effets centraux de modulation en réponse à l'électro-acupuncture (EA) à différents points d'acupuncture. L'imagerie par résonance magnétique amplifiée par le manganèse a été effectuée chez des rats Sprague-Dawley soumis à un faux traitement par acupuncture, à un faux traitement par EA ou à un vrai traitement par EA sur des points somatiques d'acupuncture. Les points d'acupuncture ont été séparés en 3 groupes : groupe 1, points d'acupuncture analgésiques utilisés généralement pour apaiser la douleur, tel que Hegu (LI 4); groupe 2, points d'acupuncture non-analgésiques rarement utilisés pour apaiser la douleur, tel que Neiguan (PC 6); groupe 3, points d'acupuncture utilisés à l'occasion pour l'analgésie, tel que Zusanli (ST 36). Les résultats ont démontré qu'il n'y avait pas d'activation neurale produite par EA sur un vrai point d'acupuncture s'il n'y avait pas puncture et de courant électrique appliqué (fausse EA). Lors de puncture sur un vrai point d'acupuncture mais sans application de courant électrique (fausse EA), une légère augmentation de l'activité du cerveau au niveau de l'hypothalamus fut notée, comparativement au groupe ayant reçu un vrai traitement d'EA (EA sur un vrai point d'acupuncture) chez qui la réponse primaire au niveau de l'hypothalamus était beaucoup plus marquée. De plus, il y avait une tendance pour les régions d'activation précoce de modulation de la douleur d'être proéminente après EA sur les points d'acupuncture analgésiques comparativement aux points d'acupuncture non-analgésiques. En conclusion, la compréhension du lien entre la stimulation des points d'acupuncture périphériques et la voie neurale centrale fournie à l'acupuncture vétérinaire non seulement une approche basée sur les faits, mais également un guide utile pour les applications cliniques de l'acupuncture.

*(Traduit par D*r* Serge Messier)*

Introduction

In the past decade, functional magnetic resonance imaging (fMRI) as well as positron emission tomography (PET) have been generally accepted as strong tools for providing detailed information in the noninvasive mapping of brain function (1,2). For example, fMRI analysis clearly identifies the structures activated during the brain's processing of pain, and the intensity of the response in

Institute of Traditional Medicine, National Yang-Ming University, Number 155, Section 2, Li-Nong Street, Peitou, Taipei, 112, Taiwan, Republic of China (Chui); Division of General Surgery, Department of Surgery (Mao-Shu); Departments of Medical Research/Education (Cheng, Yeh, Hsieh); Department of Radiology, Veterans General Hospital, Taipei, Taiwan, Republic of China (Chang; Kuo); Neural Regeneration Laboratory, Department of Neurosurgery, Veterans General Hospital, Taipei, Taiwan, Republic of China (Cheng).

Address all correspondence and reprint requests to Dr. Jen-Hwey Chiu; telephone: 886-2-2826-7178; fax: 886-2-2875-7537; e-mail: chiujh@mailsrv.ym.edu.tw

regional cerebral blood flow correlates parametrically with perceived pain intensity (3,4). Nevertheless, the informative data about pain-modulation mechanisms in animals remain to be elucidated.

The recognition and alleviation of animal pain is a growing veterinary and public concern. Previously, much effort was devoted to studies of pain management in both large and small animals, such as dogs and cats (5). Accumulating knowledge of the physiologic and pharmacologic features of pain from human imaging has had a significant impact on clinical veterinary medicine (6,7). Several lines of evidence suggest that acupuncture is effective for treating not only human diseases (8) but also animal disorders (9–11). For example, Hegu (LI 4) is an acupoint commonly chosen for acupuncture analgesia (9), and electroacupuncture (EA) at acupoint Neiguan (PC 6) inhibits the bradykinin-induced cardiac pressor response and consequently improves the function of ischemic myocardium in rats (12). The better outcome with EA is postulated to depend on the activation of opioid receptors, especially those in the rostral ventrolateral medulla, which is responsible for maintaining blood pressure and integrating cardiovascular reflexes (13). In addition, EA stimulation of acupoint Riyue (GB 24) regulates the motility of the sphincter of Oddi (SO) in rabbits and cats through a somatovisceral reflex mediated by the secretion of cholecystokinin (14). Although the brain plays important roles in modulating body responses to peripheral stimulation, the link between acupuncture effects and central cortical modulation remains unclear.

Many studies have demonstrated the relationship between acupuncture stimulation and cortical activation in the brain (15,16). The involvement of the hypothalamic–limbic system has been postulated to be critical in the mechanism of acupuncture analgesia (17). It is generally accepted that manganese, a paramagnetic contrast agent, can be used in vivo to provide higher functional resolution for fMRI in static states (18). By using Mn^{2+}-fMRI in our previous study, we found brain activation to be primarily in the hippocampus when EA was applied at acupoint Zusanli (ST 36) and in the hypothalamus, insula, and motor cortex when EA was applied at acupoint Yanglingquan (GB 34) (19). Recently we found that EA at Hegu (LI 4), an acupoint known to be effective in acupuncture analgesia, induced central neural activation in pain-modulation areas, such as periaqueduct grey matter (PAG) and the median raphe nucleus (MnR), whereas EA at Riyue (GB 24), an acupoint known to be effective in SO relaxation, induced less or no activation in such areas.

Since information about the correlation between central effects and EA stimulation at different acupoints (analgesic or nonanalgesic) is lacking, we investigated the correlation between acupuncture effects, target specificity, and brain modulatory mechanisms with the aid of Mn^{2+}-fMRI.

Materials and methods

Animals and experimental protocol

Thirty-eight male Sprague-Dawley rats weighing 250 to 300 g were obtained from the animal centre of the National Science Council or National Yang-Ming University. They were treated according to the principles outlined by the US National Institutes of Health (20). The experiment was performed as described previously (19), with some modification. Briefly, after food was withheld overnight but free access to water was allowed, the animals were anesthetized with ketamine hydrochloride (35 mg/kg body weight (BW), administered intramuscularly), anesthesia was maintained with urethrane (125 mg/100 g BW, administered intraperitoneally) (Sigma Chemical Company, St. Louis, Missouri, USA), and additional low doses of ketamine (5 to 10 mg/kg) were given as needed. The depth of anesthesia was kept steady, such that heart rate and arterial blood pressure were not affected and there was no pain response to skin traction. Usually the rectal temperature was kept around 38°C with the use of an intermittent heating pad throughout the procedure. Catheters were placed in a femoral artery and vein for monitoring of heart rate and blood pressure. A catheter was also placed in a carotid artery for administration of drugs. A polyethylene tube (PE-50) was positioned in the external carotid artery (ECA), which was ligated distal to the entrance of the tube. The tip of the catheter was located at the junction between the ECA and the internal carotid artery (ICA), so that blood flowed to the brain through the ICA during periods when drugs or saline (0.9% NaCl) solution was not being infused. Hypertonic (20%) D-mannitol solution (5 mL/kg) was administered via the ICA to break down the blood–brain barrier, and $MnCl_2$ (120 mM in isotonic saline solution, 0.5 mg/kg) was administered for manganese-enhanced fMRI.

Functional MRI

The fMRI studies were performed with a circular polarized extremity coil in a 1.5-T superconductive magnet (Siemens, Erlangen, Germany). Multislice, T1WI conventional spin-echo images (TR, 400 ms; TE, 12 ms) were acquired. A 116 × 128 matrix with a 5-cm field of view was used. Slice thickness was 4 mm. For better resolution and registration of anatomic structures, images were acquired with the 3D Fast Low Angle Shot (FLASH) (TR, 50 ms; TE, 10 ms; flip angle, 50°; matrix, 128 × 128; field of view, 64 mm; slice thickness, 0.5 mm) after completion of EA.

To separate nonspecific and specific signal enhancement, we obtained 5 series of images. Baseline fMRI was performed at 0 min. At 5 min, mannitol was infused and a 2nd series of images obtained. At 10 min, $MnCl_2$ was infused and a 3rd series obtained. At 15 min, EA was begun at the acupoints to be studied. A 4th series of images was obtained 5 min after initiation of EA stimulation. To study the aftereffect of acupuncture treatment, we administered $MnCl_2$ at the end of EA or after removal of the needles and obtained images 5 min later. The experimental setup is shown in Figure 1.

Electroacupuncture

The EA was performed by applying an electric current with an electrical nerve stimulator (Han Acutens, LH 202H, Taipei, Taiwan, Republic of China) to 2 fine needles (1 and 1.5 mm) positioned 2 to 3 mm apart at 1 acupoint to prevent a short circuit. The nomenclature of the acupoints was standardized (21,22). Stimulation was pulse-waved with alternate frequencies of 2 and 15 Hz. Wave width was 300 μs, stimulation duration 20 min, and intensity of stimulation between 1 and 2 mA.
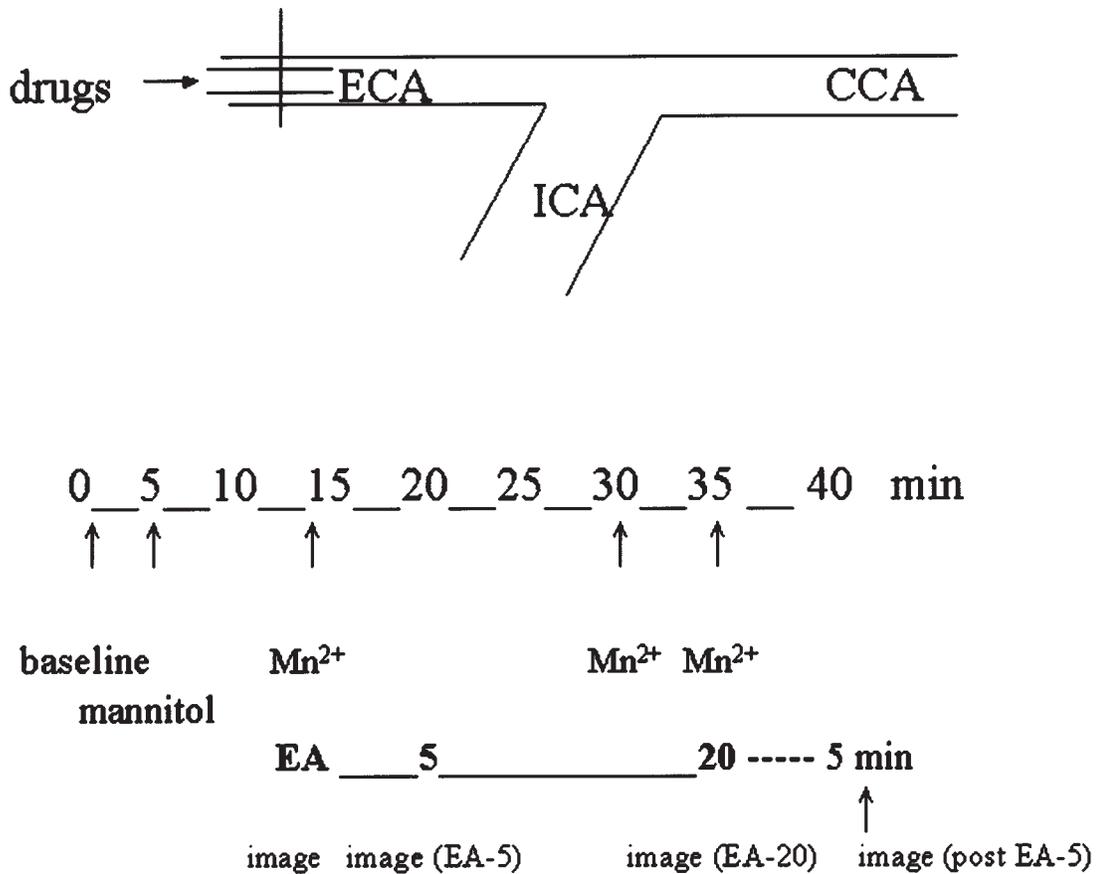
Figure 1. Experimental setup. Functional magnetic resonance imaging (fMRI) was performed at baseline and the indicated number of minutes after mannitol administration, $MnCl_2$ injection, electroacupuncture (EA), and the end of EA stimulation or removal of the needles. ECA — external carotid artery; ICA — internal carotid artery; CCA — common carotid artery.
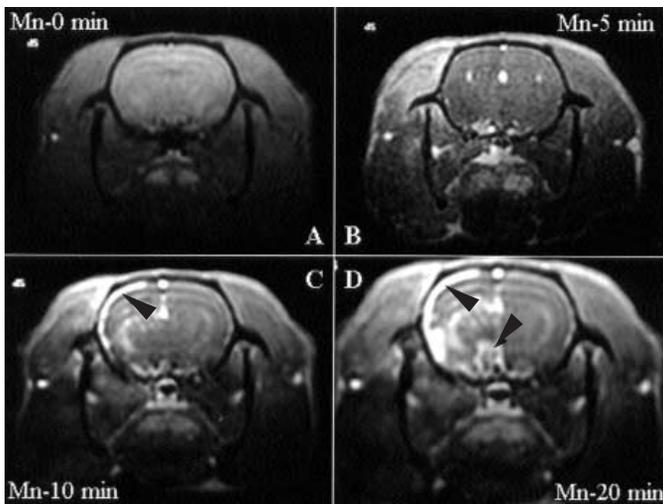

Figure 2. Transverse images obtained by manganese-enhanced functional magnetic resonance imaging (fMRI) (Mn^{2+}-fMRI) of the brain of rats at baseline (A) and 5, 10, and 20 min after infusion of $MnCl_2$ into the internal carotid artery (ICA). No electroacupuncture (EA) was done. Images were obtained at the level of the hypothalamus and thalamus. Nonspecific increases in signal intensity are apparent in the cortex (arrow) and in the hypothalamus (arrowhead).
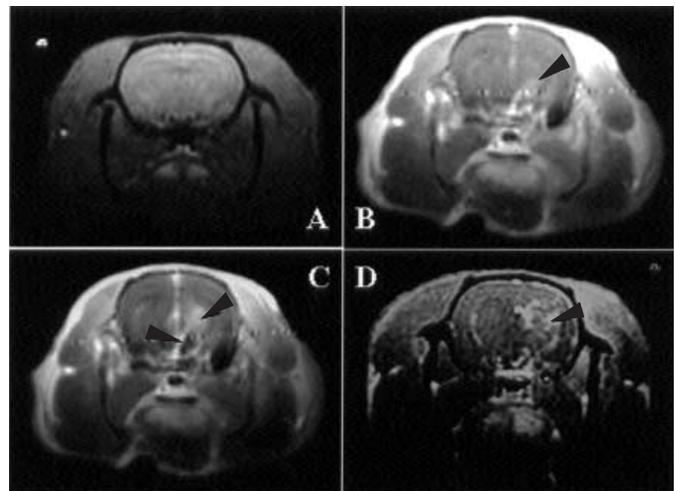

Figure 3. Transverse images obtained by Mn^{2+}-functional magnetic resonance imaging (fMRI) of the brain of rats 5 min after the start of acupuncture stimulation. A: Sham acupuncture (stimulation at a true acupoint [Hegu (LI 4)], with shallow needling and no electric current). B: Sham electroacupuncture (EA) (stimulation at Hegu with true needling but no electric current). C: True EA (stimulation at Hegu with true needling and an electric current). D: Control EA (stimulation at a control acupoint 2 mm from a true acupoint, with true needling and an electric current). Increased signal intensity is apparent in the hypothalamus (arrow) and in the thalamus (arrowhead).

Study design

The rats' acupoints were arbitrarily divided into 3 groups (19,22): group 1, analgesic acupoints commonly used in humans or in animals, such as Hegu (LI 4), Sanyinjiao (SP 6), and Yanglingquan (GB 34); group 2, nonanalgesic acupoints rarely used for analgesic effect, such as Neiguan (PC 6), Riyue (GB 24), and Feishu (BL 13); and group 3, acupoints occasionally used for analgesia, such as Zusanli (ST 36) and Zhiyin (BL 67). These choices were based on scientific studies (9,12,13,19,22), the ancient Chinese literature, and advice of senior acupuncturists at the Veterans General Hospital, Taipei.

Data analyses

Data analyses were managed by a biomedical statistical group, including statisticians from National Yang-Ming University, Veterans General Hospital (Taipei), and Academia Sinica, Republic of China. The fMRI signals in 4 series of images (after mannitol infusion, after the beginning of the Mn^{2+} infusion, after EA, and after the end of the Mn^{2+} infusion) were compared to separate the non-specific signal enhancement in Mn^{2+}-fMRI. High-resolution 3-dimensional MRI images were analyzed by subtraction. All images were made with the same grey scale, and signal intensity of neural activation in the brain was graded as negative ($-$), borderline ($\pm$, defined as $< 10\%$ of vascular or intraventricular intensity), slightly increased ($+$, defined as 10% to 50% of vascular or intra-ventricular intensity), or markedly increased ($++$, defined as $> 50\%$ of vascular or intraventricular intensity). The neural activation was localized according to the stereotaxic reference system described previously (24).

Results

There was no obvious brain activation immediately after infusion of mannitol or $MnCl_2$ (data not shown). Since Mn^{2+} is neurotoxic, prolonged exposure to $MnCl_2$ resulted in time-dependent brain activation (Figure 2; $n = 4$); therefore, we reduced the infusion time to 5 min. In addition, since $MnCl_2$ interference was noticed in rats receiving repeated or sequential treatment at different acupoints, each rat was given only 1 treatment at 1 acupoint.

To elucidate the specificity of EA stimulation, changes in brain activity were evaluated by Mn^{2+}-fMRI in different control manip-ulations (2 to 3 rats per group). When the stimulation was at a true acupoint with shallow needling and no electric current (sham acupuncture), no change in central neural activity was noticed (Figure 3A). When the stimulation was at a true acupoint with true needling but no electric current (sham EA), there was a slight increase in brain activity in the area of the hypothalamus (Figure 3B). However, the primary response in the hypothalamic region was enhanced with EA at a true acupoint (true EA) (Figure 3C). Interestingly, an increase in brain activity was also noticed with EA stimulation at a control acupoint (EA at sham point) 3 mm from the true acupoint [Hegu (LI 4)] (Figure 3D).

Since acupuncture analgesia is the most widely accepted function of acupuncture treatment, Hegu (LI 4), an acupoint known to be effective in pain relief, was chosen to correlate the brain-activation areas with EA stimulation. There was consistent neural activation in the hypothalamus (Figure 4, panels B–D; $n = 4$), PAG (Figure 5,

panels B–D; $n = 4$), and MnR (Figure 5, panels C and D; $n = 3$) after EA at Hegu (LI 4) for 5 min. After EA at acupoint Riyue (GB 24) for 5 min, there was an increase in brain activity in the somatosensory area (Figure 6A) and the hypothalamus (Figure 6B) but not in the PAG (Figure 6C). Table I summarizes the areas of brain activation by EA applied for 5 min at different acupoints known to have different functions, such as analgesia or modulation of visceral functions. A tendency to activation of pain-modulation areas such as PAG and MnR was noticed with EA at group-1 (analgesic) acupoints, whereas little neural activation was noticed in these areas with EA at group-2 (nonanalgesic) acupoints.

To study the change in brain neural activity after the end of EA (post-EA effect), brain images were taken 5 min after the completion of EA stimulation and the removal of the needles from the acupoints. There was an increase in signal changes in the hypothalamus, insula, and sensory cortex 5 min after the end of EA at Hegu (LI 4), whereas the signal changes were persistent in the hypothalamic and thalamic areas 5 min after the end of EA at Zhiyin (BL 67) (Figure 7).

Discussion

In this study, we demonstrated, using Mn^{2+}-fMRI, a correlation between acupoint-specific EA stimulation and central activation of pain-modulation areas in the brain. To our knowledge, these are novel findings.

Because depth of anesthesia may affect brain activity (for example, there is nonspecific activation with minimal anesthesia and hypoactivity with deep anesthesia), a steady depth is mandatory to allow measurement of specific brain activity. Several lines of evidence suggest that anesthetized small animals are good models for studies of acupuncture effects (6,9,25). We maintained the animals at a surgical plane of anesthesia without affecting heart rate or arterial blood pressure and without inducing signs of pain in response to skin traction. Furthermore, results of this and a previous study indicated that fMRI of the brain could be performed in anesthetized rats (26).

Intravenous administration of mannitol followed by manganese is a useful fMRI technique to separate stimulation-specific signal enhancement from nonspecific enhancement (18,27). Manganese, a paramagnetic calcium analog, is a good contrast agent, entering cells through voltage-gated calcium channels. Moreover, manganese-induced activity-specific contrast is independent of cerebral blood flow. Our previous study indicated that manganese could be used as a contrast agent to monitor brain activation by use of fMRI (19). Since Mn^{2+} is neurotoxic, and prolonged exposure to $MnCl_2$ results in time-dependent brain activation similar to primary responses induced by EA stimulation (Figure 2), we designed our experiments to avoid this confounding effect, limiting the infusion time for $MnCl_2$ to 5 min.

Recent evidence in humans as well as in animals indicates that fMRI is able to detect brain activation in response to pain, thereby identifying regions involved in the central processing of pain (3). Following episodes of intense electrical stimulation on the forepaw of rats, activation was observed consistently in the contralateral sensory–motor cortex and frontal cortical regions and frequently in the anterior cingulate cortex and the ipsilateral sensory–motor cortex (28,29). In addition to these regions, the claustrum, ventral
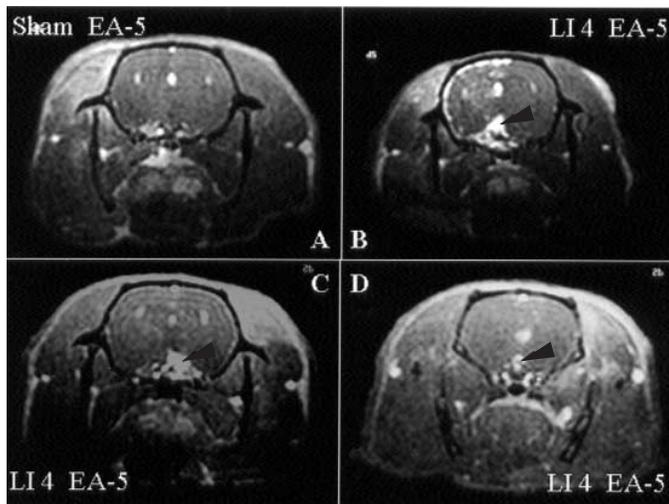
Figure 4. Transverse images obtained by Mn^{2+}-functional magnetic resonance imaging (fMRI) of the brain of rats 5 min after the start of electroacupuncture (EA) at Hegu (LI 4) in different animals (B–D), at the level of the hypothalamus and thalamus. As compared with sham EA (A), true EA results in an increase in signal intensity in the hypothalamus (arrowhead).
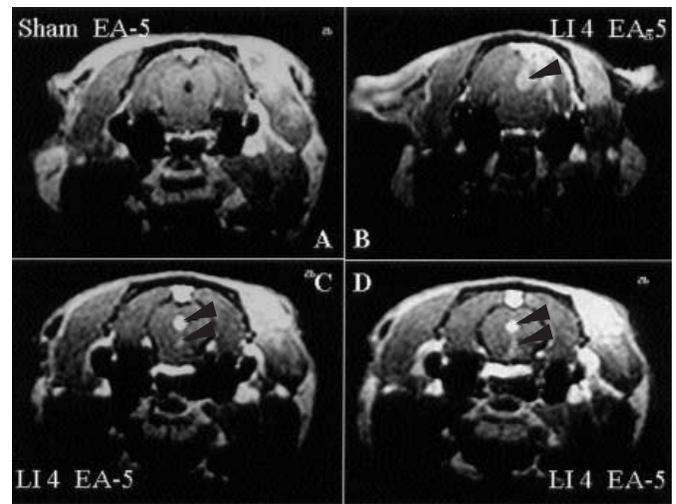

Figure 5. Transverse images obtained as with Figure 4 but at the level of the midbrain. As compared with sham electroacupuncture (EA) (A), true EA results in an increase in signal intensity in the periaqueduct grey (PAG) matter (arrowhead) and in the median raphe nucleus (MnR) (arrow).
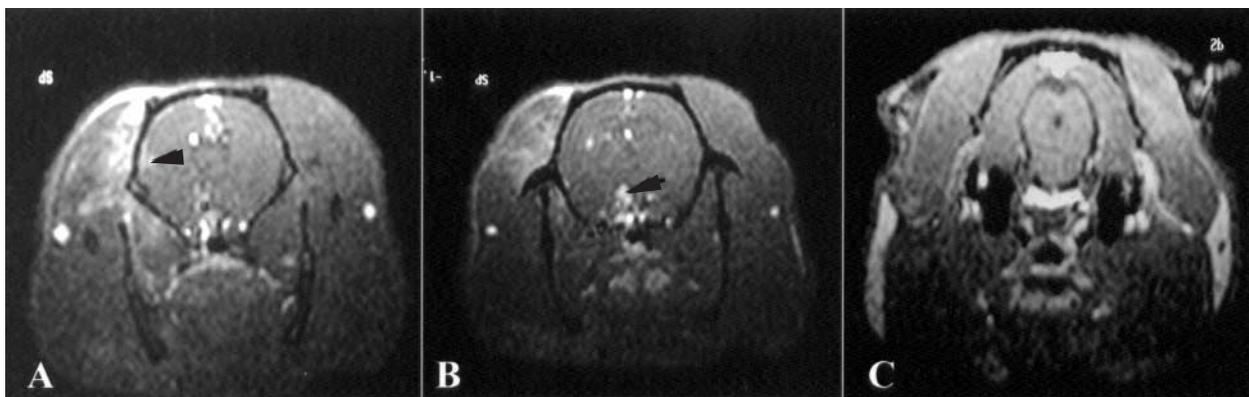

Figure 6. Transverse images obtained by Mn^{2+}-functional magnetic resonance imaging (fMRI) of the brain of rats at the level of the hypothalamus and thalamus (A and B) and the midbrain (C). Increased signal intensity 5 min after the start of electroacupuncture (EA) at Riyue (GB 24) is apparent in the sensory cortex (A, arrowhead) and in the hypothalamus (B, arrow) but not in the PAG and MnR regions (C).

thalamic nucleus, interpeduncular nucleus, PAG, hippocampus, and insular and pyriform cortices have been implicated in the response of the central nervous system to pain in the rat (28,29). Interestingly, the activation seemed to appear sequentially in these regions. Accumulating evidence suggests that fMRI is playing an important role in elucidating the central mechanism of acupuncture effects. Cho et al (15) found a correlation between visual cortical activation and acupuncture at acupoint BL 67 as well as between auditory cortical activation and acupuncture at acupoint SJ 5. Furthermore, findings suggest a central nervous mechanism for acupuncture analgesia and a critical role for the hypothalamic–limbic system in this pathway (17). Since acupuncture is a manipulation evoking a sensory stimulation similar to peripheral stimulation (pain, touch, etc.), it is not surprising to find overlapping activation regions for acupuncture stimulation and pain stimulation.

It is generally accepted that clinically relevant acupuncture-induced body responses usually require prolonged and multiple stimulation sessions. The needed duration of acupuncture stimulation is within minutes for De-Qi (pronounced De-Chi) (16), 15–20 min for cholecystokinin-8 secretion (14), and 20–40 min for antinociceptive effect (9). Because of ethical considerations, it has been difficult to assess the analgesic effect of acupuncture in humans by the use of fMRI. The results of this study showed consistent neural activation in the hypothalamus by EA stimulation for 5 min at nearly all acupoints, whereas pain-modulation areas such as PAG and MnR seemed to be activated early by EA only at analgesic acupoints. These findings are consistent with previous reports that the descending antinociceptive systems, including the hypothalamus, the nucleus accumbens, and the mesencephalon (PAG and raphe nuclei), are important central pathways mediating acupuncture analgesia (17). Our previous studies designed to investigate acupuncture effects in a time-dependent manner showed that 5 min of EA stimulation at different acupoints induced more specific brain activation than 20 min of stimulation at these acupoints.

Table I. Correlation of stimulated acupoints and central neural activation, as determined by manganese-enhanced functional magnetic resonance imaging (fMRI) of the brains of rats

Acupoints[a]	Brain region; intensity of fMRI signals[b]								
	PAG	MnR	Motor	Sensory	Pyriform	Hypothalamus	Thalamus	PO	CIC
Group 1									
LI 4	++	++	+	+	±	++	+	+	++
LI 4	+	+	−	−	−	++	+	−	−
LI 4	+	+	+	−	−	++	+	+	−
LI 4	++	++	−	−	−	++	++	−	−
SP 6	+	+	−	−	−	+	−	+	−
GB 34	+	−	−	−	−	+	+	−	−
GB 34	+	+	+	+	+	+	+	+	−
Group 2									
BL 13	−	−	−	−	−	+	−	−	−
BL 13	−	−	−	−	−	+	−	−	−
BL 15	−	−	−	−	−	±	−	−	−
BL 15	−	−	+	+	−	+	−	−	−
GB 24	−	−	−	+	−	+	−	−	−
PC 6	++	−	+	+	−	++	+	+	+
PC 6	++	−	+	−	+	+	−	−	−
PC 9	++	−	−	−	−	±	−	−	−
Group 3									
GB 41	±	±	+	+	+	+	−	+	+
GB 41	+	+	−	−	−	++	+	+	−
BL 17	+	+	+	+	+	+	−	−	−
BL 17	±	−	−	−	−	+	−	−	−
LI 11	+	+	−	−	−	+	+	+	+
BL 67	−	−	−	−	−	±	±	−	±
BL 67	−	−	−	−	−	±	±	−	±
ST 36	+	+	+	−	−	+	++	+	+

PAG — periaqueduct grey matter; MnR — median raphe nucleus; PO — preoptic nucleus; CIC — central nucleus of interior colliculus

[a] The nomenclature was standardized (21,22). Group 1, analgesic acupoints commonly used in humans or animals; group 2, nonanalgesic acupoints rarely used for analgesic effect; group 3, acupoints occasionally used for analgesia

[b] Graded as negative (−), borderline (±, defined as < 10% of vascular or intraventricular intensity), slightly increased (+, defined as 10% to 50% of vascular or intraventricular intensity), or markedly increased (++, defined as > 50% of vascular or intraventricular intensity)

This phenomenon might explain the clinical observation that an analgesic effect can be induced by EA at several acupoints (19). EA at acupoint PC 6 leads to neural activation in the hypothalamus and primary sensorimotor areas but not in pain-modulating areas (PAG and MnR). Since acupoint PC 6 is supplied by the median nerve, this observation is in agreement with the finding that electrical stimulation of the median nerve leads to functional activation of the S1 and M1 cortex (30,31). Taking these findings together with our preliminary data, we assume that EA at acupoints causing early activation (within 5 min) of pain-modulation areas might result in better pain relief. However, this postulation requires further investigation, both experimental and clinical.

The advantages of using Mn^{2+}-fMRI to interpret acupuncture effects are 2-fold. First, the linkage of acupoints with cerebral function can be demonstrated in vivo. Second, manganese-enhanced fMRI requires less time for image processing than conventional fMRI. However, there are some drawbacks to Mn^{2+}-fMRI. First, unilateral injection of mannitol and $MnCl_2$ into the ICA precludes detection of cerebellar and contralateral brain activity. Second, change in brain activity detected by the use of Mn^{2+}-fMRI indicates the mobilization of cellular ions as a result of activation or deactivation of central neurons; therefore, it is unclear whether the actual change in brain activity is activation or deactivation in conditions in which EA stimulation at different acupoints results in similar fMRI manifestations. Third, Mn^{2+}-fMRI allows only qualitative or semiquantitative evaluation of brain images (18). In this study, the signal intensity in regions of interest was compared objectively with that in the intravascular or intraventricular space and was graded semi-quantitatively. Recently, monocrystalline iron oxide nanocolloid (MION) has been reported as a good contrast agent for quantitative fMRI analyses (32), and we are now investigating its use. In the West, acupuncture has been largely considered as having a psychological or hypnotic effect or a placebo effect rather than being a physiologically based treatment. A number of significant correlations between acupuncture stimulation and cortical activation have recently been observed. In addition, marked changes have been noted in central neural activation with true EA (EA at a true acupoint with true needling and an electric current), whereas no activation
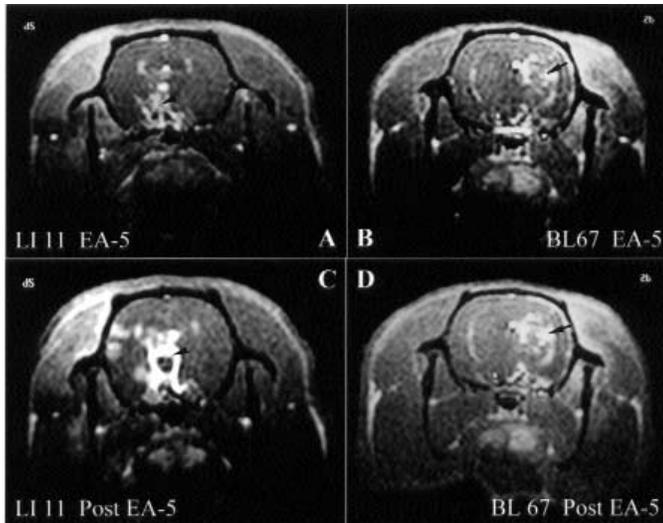
Figure 7. Transverse images obtained by Mn^{2+}-functional magnetic resonance imaging (fMRI) of the brain of rats at the level of the hypothalamus and thalamus 5 min after the start of electroacupuncture (EA) at Quchi (LI 11) (A) and Zhiyin (BL 67) (B) and 5 min after the end of EA or the removal of the needles from the Quchi (C) and Zhiyin (D) acupoints. Increased signal change is apparent in the hypothalamus, insula, and sensory cortex 5 min after the end of EA at Hegu (LI 4), whereas the signal changes are persistent in the hypothalamus (arrow) and thalamus (arrowhead) 5 min after the end of EA at Zhiyin.

was observed with sham acupuncture (stimulation at a true acupoint with shallow needling and no electric current), and very little activation was observed with sham EA (EA at a true acupoint with true needling but no electric current). Although the data are very preliminary, they bring into perspective the application of acupuncture analgesia in clinical veterinary medicine.

Acknowledgments

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