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Low- and High-Frequency Transcutaneous Electrical Acupoint Stimulation Induces Different Effects on Cerebral μ-Opioid Receptor Availability in Rhesus Monkeys

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Although systematic studies have demonstrated that acupuncture or electroacupuncture (EA) analgesia is based on their accelerating endogenous opioid release to activate opioid receptors and that EA of different frequencies is mediated by different opioid receptors in specific areas of the central nervous system, there is little direct, real-time evidence to confirm this in vivo. The present study was designed to investigate the effects of transcutaneous electrical acupoint stimulation (TEAS), an analogue of EA, at low and high frequencies on μ-opioid receptor (MOR) availability in the brain of rhesus monkeys. Monkeys underwent 95-min positron emission tomography (PET) with ^{11}C-carfentanil three times randomly while receiving 0, 2, or 100 Hz TEAS, respectively. Each TEAS was administered in the middle 30 min during the 95-min PET scan, and each session of PET and TEAS was separated by at least 2 weeks. The results revealed that 2 Hz but not 100 Hz TEAS evoked a significant increase in MOR binding potential in the anterior cingulate cortex, the caudate nucleus, the putamen, the temporal lobe, the somatosensory cortex, and the amygdala compared with 0 Hz TEAS. The effect remained after the end of TEAS in the anterior cingulate cortex and the temporal lobe. The selective increase in MOR availability in multiple brain regions related to pain and sensory processes may play a role in mediating low-frequency TEAS efficacy.

Key words: μ-opioid receptor (MOR); positron emission tomography (PET); transcutaneous electrical acupoint stimulation (TEAS)

Acupuncture, which has been used to treat various disorders in China for thousands of years, has more recently been recognized in the West as a useful complementary medicinal approach. The methodology of acupuncture is ever changing along with the development of science and technology. The use of various types of thick needles made of silver has been followed by the use of fine needles made of stainless steel to puncture the skin. In recent decades, electroacupuncture (EA) has often been used to enhance mechanical stimulation. Furthermore, skin electrodes can be used to replace needle penetration (transcutaneous electrical acupoint stimulation [TEAS]) for the same purpose in a safe and time-saving manner (Han, 2011). Moreover, acupuncture can block upcoming pain, also known as pre-emptive analgesia (Gupta et al., 1999; Coura et al., 2011). Reports from China and elsewhere state that manual movements or low-frequency electrical stimulation via the acupuncture needles, or EA, can provide clinical analgesia, allowing surgery (Andersson et al., 1973). Pain alleviation via EA or TEAS is one of their most common applications, and the analgesic potency as well as the underlying neurobiological mechanisms have been proved to be very similar, if not identical (Wang et al., 1992; Han, 2003). However, the cellular and molecular pathways involved in EA- or TEAS-induced analgesia remain largely unknown.

Additional Supporting Information may be found in the online version of this article.

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TABLE I. Regions Displaying Changes in MORs DV During 2 Hz TEAS

	Coordinates[a]					2 Hz (DV mean ± SEM)		0 Hz (DV mean ± SEM)	
Region	x	y	z	Voxels	T[b]	Before	During	Before	During
Temporal lobe	22	21	5	44	5.23	2.38 ± 0.17	2.24 ± 0.19	2.85 ± 0.28	2.52 ± 0.29
Amygdala	17	7	3	35	4.94	2.50 ± 0.17	2.36 ± 0.18	2.99 ± 0.32	2.65 ± 0.31
Somatosensory cortex	21	11	19	50	4.78	2.42 ± 0.19	2.13 ± 0.21	2.91 ± 0.26	2.27 ± 0.24
Caudate nucleus	16	−1	18	44	4.76	1.66 ± 0.09	1.51 ± 0.11	1.99 ± 0.15	1.72 ± 0.16
ACC	−4	31	26	44	4.55	2.45 ± 0.15	2.30 ± 0.16	2.95 ± 0.27	2.57 ± 0.25
Putamen	14	22	18	29	4.13	2.16 ± 0.19	1.94 ± 0.20	2.60 ± 0.24	2.19 ± 024

[a]The coordinates are specifically used in template of rhesus monkey from Wisconsin University.
[b]All regions $P < 0.001$ uncorrected in voxel level with a minimum cluster size of 20 voxels.

Accumulating data reveal that the mechanisms of acupuncture or EA analgesia involve the activation of endogenous opioid antinociceptive systems in the central nervous system (CNS) (Pomeranz and Chiu, 1976; Mayer et al., 1977; He et al., 1985; Ho and Wen, 1989; Chen and Han, 1992b; Chen et al., 1996). Neurochemical studies conducted in humans and rodents revealed that 2-Hz stimulation preferentially increased the cerebrospinal fluid (CSF) content of enkephalins and endorphins, whereas 100-Hz stimulation favored the release of dynorphins (Han et al., 1991; Han, 2003), which was supported in pharmacological studies using type-specific opioid receptor blockers and cross-tolerance studies (Chen and Han, 1992a). Intrathecal injection of antibodies against enkephalin prevented the analgesic effect induced by low- but not high-frequency EA. In contrast, antibodies against dynorphin prevented high- but not low-frequency EA-induced analgesia.

Although systematic studies have demonstrated that acupuncture or EA analgesia is based on their amplifying the release of endogenous opioid peptides to activate opioid receptors and that EA of different frequencies functions via different opioid receptors in specific areas of the CNS (Han, 2003), there have been few direct, real-time, in vivo studies confirming these results (Harris et al., 2009). Positron emission tomography (PET) is the only currently available, real-time, in vivo method for studying μ-opioid receptor signaling (Henriksen and Willoch, 2008). Recently, Zubieta et al. directly explored the involvement of the endogenous opioid system during acupuncture treatment for chronic pain patients with fibromyalgia using ^{11}C-carfentanil (CFN) PET (Harris et al., 2009). However, no association was detected between a certain frequency of TEAS and any specific change in opioid receptor binding potential (BP). This study has successfully synthesized the μ-opioid receptor-selective radiotracer ^{11}C-CFN, and we investigated TEAS effects on ^{11}C-CFN distribution volume (DV) in anesthetized rhesus monkeys to determine whether any specific change in opioid receptor BP was correlated with a certain frequency of TEAS, as well as the relative importance between the release of endogenous opioid peptides and the change in opioid receptor activity.

MATERIALS AND METHODS

Animals

Eight adult male rhesus monkeys (*Macaca mulatta*, mean [SD] age 5.7 [1.2] years) weighing 6.9–9.0 kg were obtained from a commercial supplier (Institute of XieErXin Biology Resources, Beijing, China). Demographics of the sample population are presented in Supporting Information Table I. The housing conditions and the experimental procedures were in accordance with the Chinese law on humane care and use of laboratory animals and complied with the recommendations for the use of nonhuman primates in research. Specifically, monkeys were housed in individual cages in a temperature-controlled room and maintained on a 12-hr light and 12-hr dark cycle. Water was available ad libitum, and standard primate biscuits were supplied daily with fresh fruits and vegetables. We routinely introduced toys into the home cage environment to improve their condition of life. Monkeys were monitored daily by the researchers and the animal care staff, as well as every 3 days by the veterinarian, to check on their health and welfare. The animals' care was supervised by experienced veterinarians, and all study protocols were approved by the Chinese PLA General Hospital, Beijing, Animal Care and Use Committee and the Radioactive Drug Research Committee. All experiments adhered to the guidelines of the Committee for Research and Ethical Issues of the IASP (Zimmermann, 1983).

The monkeys could see their roommates and communicate with others during the experimental interval. To reduce potential stress related to the experiment, the researcher stayed in the housing room to interact with the animals at least 1 hr each day during the experiment. The monkeys were individually and quickly anesthetized to alleviate suffering and to reduce the others' fear. At the end of each experimental session, the researcher cared for the monkeys until they woke up from the anesthesia. No animals were sacrificed, because the worst injury in this study was blood vessel puncture.

Apparatus

The TEAS device (HANS-200E; Nanjing Gensun Medical Technology, Nanjing, China) is a battery-driven dual-channel acupoint nerve stimulator with two pairs of constant-current electric outputs that send electrical impulses through two adhesive electrode pads placed on the skin. Hegu (LI4,

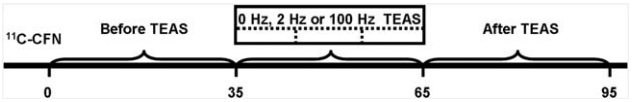

Fig. 1. PET scan protocol of experiments. PET with ^{11}C-carfentanil was performed three times (95 min/session) randomly, and each monkey received TEAS of 0 Hz, 2 Hz, or 100 Hz separated by at least 2 weeks. TEAS was administered in the middle 30 min (from 35 to 65 min) during the 95-min PET scan. Thus, each 95-min scan can be separated into three stages, 0–35 min, 35–65 min, and 60–95 min, which were defined as baseline, the TEAS immediate effect, and the TEAS after effect, respectively.

located at the midpoint of the second metacarpus on the radial side) and Laogong (PC8, midway between the second and third metacarpus on the palmar side, where the middle finger falls when the hand makes a fist) of the same hand (Cui et al., 2008), which together form an electric circuit, are two acupoints commonly used for TEAS (Han et al., 1994; Chao et al., 2007). The frequency of the output stimulation was adjusted to 2 Hz (0.6-msec pulse width), 100 Hz (0.2-msec pulse width), or 0 Hz (the two adhesive electrode pads were only placed on the same acupoints without switching on the current). The stimulation intensity administered to each subject was adjusted to 25 mA for 2 Hz, 15 mA for 100 Hz, which is a maximal but comfortable level that was obtained from the human experimental reports (Jiang et al., 2013). PET and computerized tomography (CT) were performed with a Siemens Biograph 64 TruePoint PET/CT scanner (Siemens, Knoxville, TN). Structural MRI scans were acquired from all subjects with a Siemens 1.5T Symphony whole-body scanner (Siemens, Erlangen, Germany).

Tracer

^{11}C-CFN was synthesized with a high specific activity (>2,000 Ci/mmol), as previously described (Jewett, 2001). During the scan, 4–6 mCi (148–222 MBq) ^{11}C-CFN was administered. Half of the ^{11}C-CFN dose was administered as a bolus, and the remaining 50% was administered via continuous infusion for the remainder of the study. Online arterial radiocount was performed to construct the input function during the 95-min PET scan. Procedures associated with measurement of the input function have previously been described in detail (Endres et al., 2003). Four samples (collected at 20, 40, 60, and 80 min) were analyzed via HPLC to determine the fraction of plasma activity representing nonmetabolized parent tracer.

Functional Image Acquisition

As depicted in Figure 1, monkeys received PET with ^{11}C-carfentanil three times (95 min/session) randomly while receiving one of the three frequencies of TEAS, 0, 2, or 100 Hz. Each session of PET and TEAS was separated by at least 2 weeks. Before each session, a venous catheter was inserted into the left median cubital vein for anesthetic infusion, tracer injection, and blood return. Monkeys starved for 12 hr were anesthetized with 2% sodium pentobarbital solution (20 mg/kg). An oral trachea cannula was inserted to prevent suffocation, and a heparinized catheter was inserted into the left popliteal artery. Heparin sodium (800–1,000 U per monkey) was injected to heparinize the blood, and extracorporeal circulation was established between the arterial and the venous catheters prior to tracer injection for online tracer radiocounting and arterial blood sampling. A thermoplastic mask was individually fitted to each monkey's face, which was created for the purpose of immobilization to eliminate intrascan head movement and to aid the structural localization via MRI during PET acquisition, as previously described (Bencherif et al., 2004). Emission data were collected in three-dimensional list mode for 95 min. Each TEAS treatment was administered in the middle 30 min (from 35 to 65 min) during the 95-min PET scan. Thus each 95-min scan can be separated into three stages, 0–35 min, 35–65 min, and 65–95 min, which were considered as the baseline, the TEAS immediate effect and the TEAS after effect, respectively. All puncture operations accorded with standard aseptic technique, and ceftriaxone sodium was administered to each monkey before withdrawal of the venous catheter. All animals received compression to prevent bleeding at the puncture site after the experiments.

Structural Image Acquisition

A CT scan was obtained prior to radiotracer injection for attenuation correction and location assistance. The CT imaging parameters were as follows: tube voltage 120 kV, tube current − exposure time product 88 mAs, section thickness 1.0 mm, FOV 25 cm, pitch 0.8, interval 1.5 mm, and scan time 1.0 sec. A three-dimensional spoiled gradient recalled acquisition in the steady state (SPGR) MRI sequence was used for scanning with the following parameters: repetition time 563 msec, echo time 3.67 msec, flip angle 20°, number of excitations 1, field of view 22.5 × 30 cm, slice thickness 1.5 mm, and reconstruction matrix 256 × 256, yielding an in-plane pixel size of 1 × 1 mm. MRI scans were applied for PET structural localization using a standard technique.

Image Processing

The PET data acquired in list mode were separated into 41 frames: 4 × 15 sec, 3 × 20 sec, 2 × 30 sec, 2 × 60 sec, and 30 × 180 sec. Images were reconstructed using iterative algorithms (brain mode; OSEM + PSF, four iterations, 16 subsets; no smoothing) into a 256 × 256 pixel matrix. Small head motions during the emission scans were corrected using an automated computer algorithm for each subject prior to analysis, and the images were coregistered to each other (Harris et al., 2009). Time points were decay-corrected during reconstruction of the PET data. The receptor-related measure (DV) of each stage was calculated using classical Logan plot with an input function (Logan et al., 2001). DV is an important parameter for measuring the receptor (B_{max}/K_d, the receptor-related measure, or binding potential) that corresponds to the tissue-to-plasma ratio of the concentration of tracer in each compartment. Image data were then transformed on a voxel-by-voxel basis into a parametric map of the DV. DV images for each experimental period and MR images were coregistered to each other and to the population-average MRI-based stereotactic atlas orientation collection of the rhesus macaque (McLaren et al., 2009), which was downloaded from a website (http://

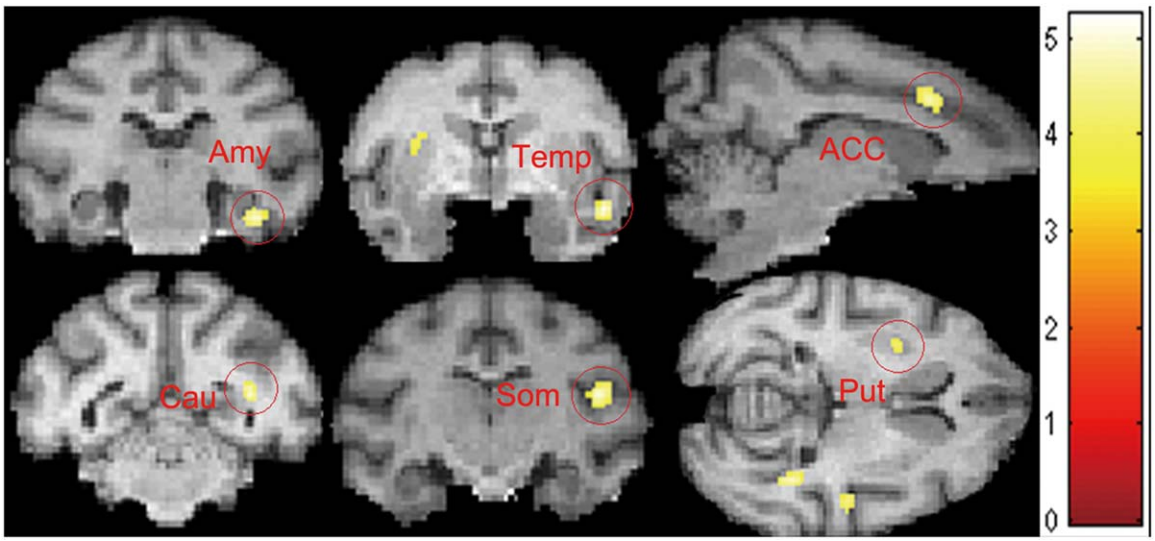

Fig. 2. During stimulation, 2-Hz TEAS could increase the MOR BP significantly in the ipsilateral temporal lobe, the ipsilateral amygdale, the contralateral ACC, the ipsilateral caudate nucleus, the ipsilateral somatosensory cortex, and the contralateral putamen compared with 0-Hz TEAS. Ipsilateral or contralateral brain regions are relative to the side of TEAS stimulation. See also Figures 3–5. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

www.brainmap.wisc.edu; Oregon Health and Science University and University of Wisconsin—Madison) using SPM 8. Group differences were mapped into stereotactic space by using *t* maps of statistical significance. No global normalization was applied to the data, so the calculations presented are based on absolute B_{max}/K_d estimates. To compensate for small residual anatomic variations across subjects and to improve signal-to-noise ratios, a three-dimensional Gaussian filter (FWHM 3 mm) was applied to each scan.

Image Analysis

The immediate effects of TEAS on MOR BP were detected using two-sample *t*-tests between the subjects receiving 2 Hz (or 100 Hz) and 0 Hz TEAS on a voxel-by-voxel basis in SPM8: comparison I = (2 Hz [during TEAS − before TEAS] > 0 Hz [during TEAS − before TEAS]), comparison II = (0 Hz [during TEAS − before TEAS] > 2 Hz [during TEAS − before TEAS]), comparison III = (100 Hz [during TEAS − before TEAS] > 0 Hz [during TEAS − before TEAS]), and comparison IV = (0 Hz [during TEAS − before TEAS] > 100 Hz [during TEAS − before TEAS]). The after effects of TEAS on MOR BP were detected using two-sample *t*-tests between subjects receiving 2 Hz (or 100 Hz) and 0 Hz on a voxel-by-voxel basis in SPM8: comparison V = (2 Hz [after TEAS − before TEAS] > 0 Hz [after TEAS − before TEAS]), comparison VI = (0 Hz [after TEAS − before TEAS] > 2 Hz [after TEAS − before TEAS]), comparison VII = (100 Hz [after TEAS − before TEAS] > 0 Hz [after TEAS − before TEAS]), and comparison VIII = (0 Hz [after TEAS − before TEAS] > 100 Hz [after TEAS − before TEAS]). Significant effects were detected for each comparison using two separate approaches: 1) an entire image-wide search unconstrained by regional predictions and 2) a regional approach based on a priori hypotheses. For the latter approach, a priori regions that had been previously identified as involved in MOR-mediated antinociception in humans (Zubieta et al., 2001, 2005; Harris et al., 2009) or PET trials using acupuncture (Biella et al., 2001) were determined by using a standard brain atlas. These regions included the cingulate cortex, insula, nucleus accumbens, caudate, putamen, thalamus, hypothalamus, amygdala, temporal lobe, and periaqueductal gray. When effects of treatment were observed in these regions, we employed an uncorrected statistical threshold of $P < 0.001$ with a minimum cluster size of 20 voxels. For brain regions not previously hypothesized, significant regions were identified based on a threshold of $P < 0.05$ after correction of multiple comparisons using the familywise error approach (Friston et al., 1995a,b). Numerical values of MOR binding were extracted from the image data by averaging the values of voxels contained in areas in which significant effects were detected via voxel-by-voxel analyses using MRIcro software (Columbia, SC). These values were then entered into GraphPad Prism 5.0 for plotting and assessment of possible differences. To illustrate the anatomical location of regions with significant effects, three-dimensional images of significant regions were displayed and overlaid onto the population-average MRI-based stereotactic atlas orientation collection of the rhesus macaque, which was downloaded from the website of the University of Wisconsin.

RESULTS

Immediate Effect of 2-Hz and 100-Hz TEAS on MOR BP

The immediate effect of 2-Hz TEAS on MOR BP was examined with two separate comparisons: comparison I = (2 Hz [during TEAS − before TEAS] > 0 Hz [during TEAS − before TEAS]) and comparison II = (0 Hz

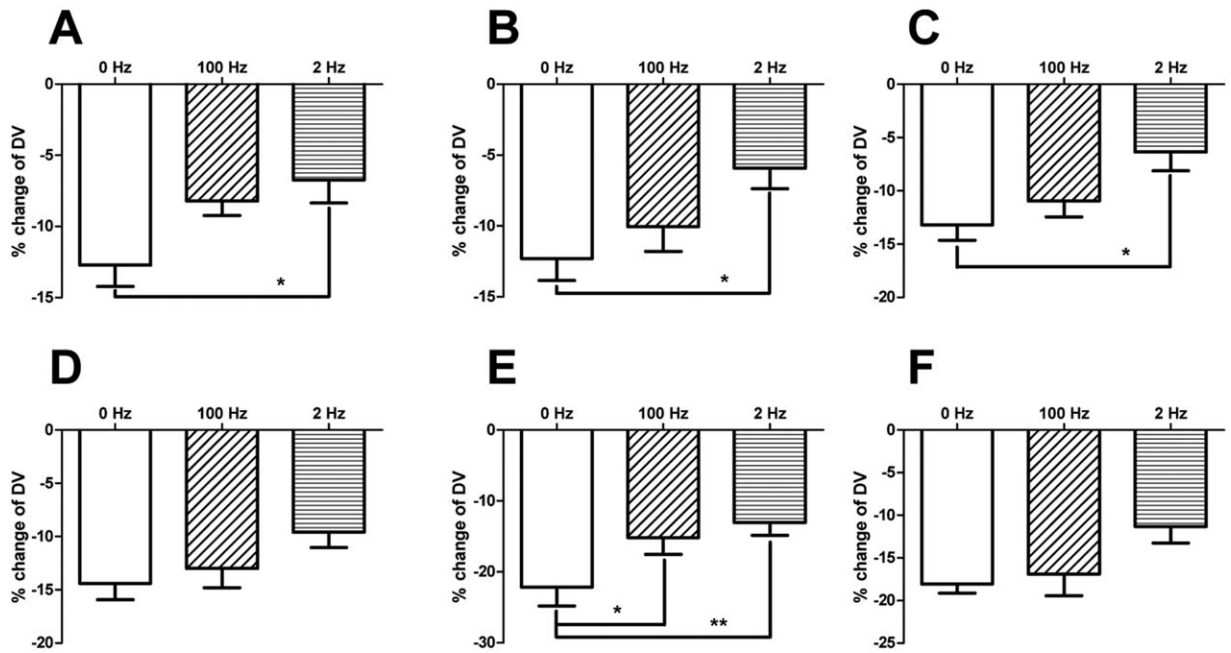

Fig. 3. Numerical values of MOR BP in the TEAS stage of 0 Hz, 100 Hz, and 2 Hz stimulation were extracted from the image data by averaging the values of voxels contained in areas in which significant effects were detected (ipsilateral temporal lobe, **A**; ipsilateral amygdala, **B**; contralateral ACC, **C**; ipsilateral caudate nucleus, **D**; ipsilateral somatosensory cortex, **E**; and contralateral putamen, **F**) and were then analyzed and plotted in the bar diagrams. $\star P < 0.05$, $\star\star P < 0.01$.

TABLE II. Regions Displaying Changes in MORs DV After 2 Hz TEAS

	Coordinates[a]					2 Hz TEAS (DV mean ± SEM)		0 Hz TEAS (DV mean ± SEM)	
Region	x	y	z	Voxels	T[b]	Before	After	Before	After
ACC	6	69	20	44	5.15	2.53 ± 0.16	2.02 ± 0.14	2.92 ± 0.25	2.13 ± 0.22
Temporal lobe	16	23	0	19	4.36	3.30 ± 0.30	3.01 ± 0.28	3.84 ± 0.35	3.37 ± 0.40

[a]The coordinates are specifically used in template of rhesus monkey from Wisconsin University.
[b]All regions $P < 0.001$ uncorrected in voxel level with a minimum cluster size of 19 voxels.

[during TEAS − before TEAS] > 2 Hz [during TEAS − before TEAS]). Six regions were identified as exhibiting differences in MOR BP between groups based on comparison I (Table I, Figs. 2, 3). TEAS at 2 Hz significantly increased the MOR BP in the contralateral anterior cingulate cortex (ACC) and putamen and in the ipsilateral caudate nucleus, temporal lobe, somatosensory cortex, and amygdala during TEAS. No regions were detected based on comparison II that met significance after correction for multiple comparisons (data not shown). Inspection of Table I and Figures 2, 3 indicates that 2-Hz TEAS could increase MOR BP compared with 0-Hz stimulation. To investigate whether the observed differences between 2-Hz TEAS and 0-Hz TEAS could be due to baseline differences between treatment groups, we compared the baseline MOR BP values of these six regions. None of the regions of interest (ROIs) displayed significant baseline differences between groups with repect to MOR BP (all $P > 0.08$), confirming that the changes were due largely to treatment.

The immediate effect of 100-Hz TEAS in MOR BP was examined by using two separate comparisons: comparison III = (100 Hz [during TEAS − before TEAS] > 0 Hz [during TEAS − before TEAS]) and comparison IV = (0 Hz [during TEAS − before TEAS] > 100 Hz [during TEAS − before TEAS]). No significant difference was observed between the effect of 100-Hz TEAS and that of 0-Hz stimulation.

After effect of 2-Hz and 100-Hz TEAS on MOR BP

The after effect of 2-Hz TEAS on MOR BP was examined using two separate comparisons: comparison V = (2 Hz [after TEAS − before TEAS] > 0 Hz [after TEAS − before TEAS]) and comparison VI = (0 Hz [after

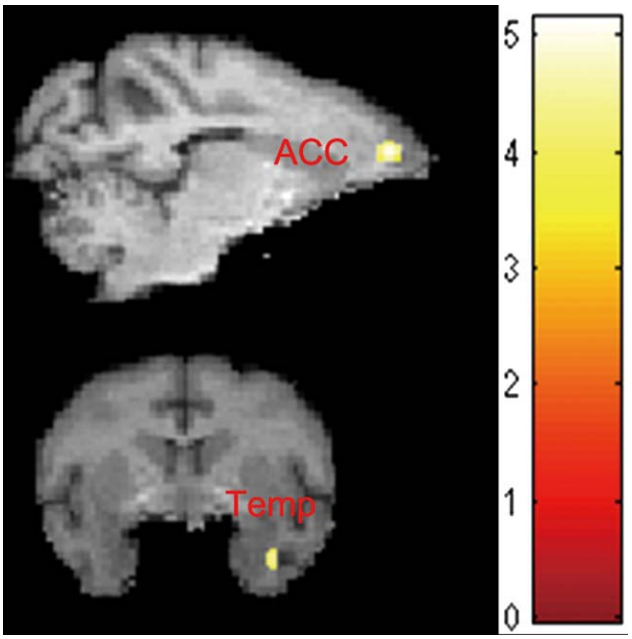

Fig. 4. The MOR BP in the 2-Hz session remained significantly higher in the anterior cingulate cortex and the temporal lobe compared with 0-Hz TEAS in the after stage. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

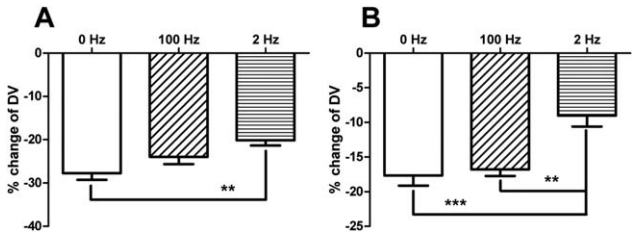

Fig. 5. Numerical values of MOR BP in the after TEAS stage of 0 Hz, 100 Hz, and 2 Hz stimulation were extracted from the image data by averaging the values of voxels contained in areas in which significant effects were detected (anterior cingulate cortex, **A**; temporal lobe, **B**) and were then analyzed and plotted in the bar diagrams. $^{**}P < 0.01$, $^{***}P < 0.001$.

TEAS − before TEAS] > 2 Hz [after TEAS − before TEAS]). Two regions were identified as containing differences in MOR BP between groups based on comparison V (Table II, Figs. 4, 5). The sustained after effects of 2-Hz TEAS resulted in increased MOR BP in the ipsilateral ACC and temporal lobe. To investigate whether the observed differences between 2-Hz TEAS and 0-Hz TEAS could be due to baseline differences between the treatment groups, we compared the baseline MOR BP values of these two regions. None of the ROIs displayed significant baseline differences between groups with respect to MOR BP (all $P > 0.2$), confirming that the observed binding changes were largely due to treatment.

The after effects of 100-Hz TEAS on MOR BP were examined by using two separate comparisons: comparison VII = (100 Hz [after TEAS − before TEAS] > 0 Hz [after TEAS − before TEAS]) and comparison VIII = (0 Hz [after TEAS − before TEAS] > 100 Hz [after TEAS − before TEAS]). No significant difference was detected between the after effect of 100-Hz TEAS and that of 0-Hz stimulation.

DISCUSSION

By measuring the changes in MOR availability in the brain evoked by different frequencies of TEAS using PET with ^{11}C-carfentanil, we found that 30 min of 2-Hz TEAS significantly increased MOR BP in multiple brain regions related to pain and sensation, including the anterior cingulate cortex, the caudate nucleus, the putamen, the temporal lobe, the somatosensory cortex, and the amygdala. The effect persisted after the end of TEAS in the anterior cingulate cortex and the temporal lobe. These results are in accordance with results reported previously (Harris et al., 2009). The increased MOR BP in the cingulate cortex, the amygdala, and the temporal pole indicate the activation of an endogenous opioid circuit known to participate in the regulation of sensory and affective qualities of pain in humans (Zubieta et al., 2001, 2003; Kennedy et al., 2006). These regions, including the caudate and the putamen, have also been shown to be involved in responses to acupuncture (Hui et al., 2009; Napadow et al., 2012; Jiang et al., 2013; Kim et al., 2013), pain, and other salient stimuli (Gear and Levine, 1995; Scott et al., 2006; Fang et al., 2012). The cingulate cortex is a region that has been identified previously that exhibits reduced BP of MOR in fibromyalgia patients (Harris et al., 2007). The temporal pole has also been identified as displaying responsiveness to negative mood (Zubieta et al., 2003; Kennedy et al., 2006) as well as acupuncture treatment (Hui et al., 2000; Napadow et al., 2005).

In the present work, no increase in MOR BP was detected during and after the 100-Hz TEAS compared with 0-Hz TEAS, and no effect in MOR BP was found during and after 100-Hz TEAS compared with 2-Hz TEAS either. These results can help explain the following problems.

One issue that we sought to resolve in this experiment is to demonstrate directly the frequency specificity of TEAS effects on opioid receptor availability in the brain. Prevailing theories, arising largely from studies in animals, suggest that endogenous opioids and their corresponding receptors are involved in acupuncture treatment (Harris et al., 2009). Our previous studies have demonstrated that different frequencies of TEAS can induce analgesia by accelerating the release of specific opioid peptides in the CNS (Han, 2003). In this study, we revealed that 2-Hz but not 100-Hz TEAS could increase the MOR BP in multiple pain and sensory processing brain regions in rhesus monkeys. That is, the increases in MOR BP in multiple pain and sensory processing brain regions may play a role in mediating the effect of low- but not high-frequency TEAS. These results further

verify divergent opioid receptor mechanisms of low- and high-frequency TEAS.

Another issue that we wanted to resolve in this experiment was to determine the relative importance between the release of endogenous opioid peptides and the regulation of opioid receptors in mediating TEAS. Previous reports regarding EA analgesia paid more attention to the release of endogenous opioid peptides than to the regulation of opioid receptors (Gao et al., 1997; Harris et al., 2009). If EA analgesia is mediated only by release of the endogenous opioid peptides, the MOR BP would decrease when receiving acupuncture. Our results revealed that 2-Hz TEAS could increase the MOR BP compared with 0-Hz TEAS, suggesting that upregulation of MORs might occur as a result of TEAS. Recently, direct real-time evidence acquired from PET has been used to demonstrate the involvement of the opioid system during acupuncture treatment in vivo. Harris et al. (2009) compared the effects of traditional Chinese acupuncture (TA) with those of sham acupuncture (SA) treatment on MOR binding availability in chronic pain patients with fibromyalgia. One session of acupuncture evoked short-term increases in MOR binding potential in multiple pain and sensory processing regions, including the cingulate cortex, the insula, the caudate, the thalamus, and the amygdala. Multiple sessions of acupuncture therapy (eight treatments in 4 weeks) evoked long-term increases in MOR BP in some of the same structures associated with greater reductions in clinical pain, including the cingulate cortex, caudate, and amygdala. These short- and long-term effects were absent in the sham group, in which small reductions were observed. This is an important contribution that demonstrates the involvement of opioid receptors in these identified brain areas to mediate the analgesic effect of acupuncture (Harris et al., 2009). Our data indicated that the immediate effects of 2-Hz TEAS could increase MOR BP in multiple pain and sensory processing brain regions, including the anterior cingulate cortex, the caudate nucleus, the putamen, the temporal lobe, the somatosensory cortex, and the amygdala, and the effect persisted after TEAS in the anterior cingulate cortex and the temporal lobe, which is very similar to the results of Harris et al. Additionally, these results do not conflict with our previous conclusion that EA analgesia is mediated by the release of endogenous opioid peptides, because neither opioid receptor antagonists nor antibodies of endogenous opioid peptides could distinguish whether the reduction in EA analgesia occurred as a result of decreased endogenous opioid peptides or inhibition of opioid receptors. The relative decreases in BP could be due to competition between the PET ligand and the endogenously released opioid peptides, opioid receptor internalization, downregulation of receptor expression, or neuronal loss (Henriksen and Willoch, 2008). In contrast, increases in binding potentials could be due to upregulation of receptor expression (Sprenger et al., 2005; Henriksen and Willoch, 2008). In fact, we found that 2-Hz TEAS increased MOR BP, suggesting that upregulation of MORs is involved in the function of TEAS. The reductions in MOR BP may be mediated by the release of endogenous opioid peptides during the 2-Hz TEAS session; however, these effects may be masked by increases in receptor binding availability, as mentioned above.

The third issue that we sought resolve in this experiment was to rule out the placebo effect of TEAS. In a clinical trial, when an active treatment does not exhibit superior efficacy to a sham or placebo, the active treatment is assumed to be ineffective and operating only via a placebo effect (Han and Ho, 2011). However, this judgment was not supported in the study by Harris et al. (2009), in which the authors found that TA and SA were similarly effective in reducing clinical pain, although MOR BP levels were differentially altered by TA and SA via a different mechanism. The analgesic effects of SA could be due to regional reductions in MOR BP, consistent with the activation of MORs during placebo treatment (Zubieta et al., 2005), whereas TA could have evoked an increase in receptor binding availability and receptor activation. This interpretation is entirely consistent with the observed positive correlation between decreased MOR BP in the dorsolateral prefrontal cortex and decreased pain in the SA group (Harris et al., 2009). Because the TEAS treatment in the present work was carried out in unconscious, anesthetized monkeys, the placebo was no longer a confounding factor in the experiment.

Furthermore, the results also demonstrated the feasibility of the acupuncture anesthesia because we investigated the effect of TEAS on ^{11}C-CFN binding potential in anesthetized rhesus monkeys. The clinical practice of acupuncture anesthesia in China was used as a technique to induce an analgesic effect in place of anesthetics during surgical procedures, which began in the late-1950s (Wu, 2007). This nonconventional practice raised the interest of not only medical professionals around the world but also basic researchers who would like to explore its possible mechanisms (Han and Ho, 2011). This study shows that 2-Hz TEAS evoked a significant increase in MOR BP in multiple brain regions related to pain and sensory processing compared with 0-Hz TEAS, which indicates that the TEAS-induced ascending signal could induce a general anesthetic state in the brain.

Because the MOR-selective radiotracer ^{11}C-CFN was successfully synthesized in China for the first time (Zhang et al., 2011) and the present experiment was carried out in unconscious, anesthetized monkeys, there were some inevitable limitations. First, we could not measure the pain threshold in anesthetized monkeys. As a result, the changes in MOR BP could not be correlated with any change in the pain threshold. Second, to obtain a high-quality PET image, 4–6 mCi ^{11}C-CFN was administered to each monkey weighing only approximately 8 kg, which was too large compared with the dosage administered to human subjects. This might be the reason why the DV value in the ROIs tended to decrease from the beginning to the end of the each experiment. Additionally, small brain volume in adult rhesus monkey, approximately 80 ml, equivalent to 1/16 of the adult human brain, is an

impediment to PET resolution. Moreover, the lack of a rhesus monkey brain template and the anesthetic condition also contributed to the difficulty of this experiment. Therefore, further study using human subjects should be performed to verify these results in the future.

CONCLUSIONS

This study demonstrates that 2-Hz but not 100-Hz TEAS evoked a significant increase in MOR BP in the anterior cingulate cortex, the caudate nucleus, the putamen, the temporal lobe, the somatosensory cortex, and the amygdala. The effect persisted after the end of TEAS in the anterior cingulate cortex and the temporal lobe. The selective increase in MOR availability in multiple brain regions related to pain and sensory processing might play a role in mediating low-frequency TEAS efficacy. Additionally, these findings provide direct evidence in support of previous reports that specific frequencies of EA are mediated by different opioid receptors in the CNS.

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