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Electro-acupuncture stimulation protects dopaminergic neurons from inflammation-mediated damage in medial forebrain bundle-transected rats

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Abstract

Through producing a variety of cytotoxic factors upon activation, microglia are believed to participate in the mediation of neurodegeneration. Intervention against microglial activation may therefore exert a neuroprotective effect. Our previous study has shown that the electro-acupuncture (EA) stimulation at 100 Hz can protect axotomized dopaminergic neurons from degeneration. To explore the underlying mechanism, the effects of 100 Hz EA stimulation on medial forebrain bundle (MFB) axotomy-induced microglial activation were investigated. Complement receptor 3 (CR3) immunohistochemical staining revealed that 24 sessions of 100 Hz EA stimulation (28 days after MFB transection) significantly inhibited the activation of microglia in the substantia nigra pars compacta (SNpc) induced by MFB transection. Moreover, 100 Hz EA stimulation obviously inhibited the upregulation of the levels of tumor necrosis factor (TNF)-α and interleukin (IL)-1β mRNA in the ventral midbrains in MFB-transected rats, as revealed by reverse transcriptase polymerase chain reaction (RT-PCR). ED1 immunohistochemical staining showed that a large number of macrophages appeared in the substantia nigra (SN) 14 days after MFB transection. The number of macrophages decreased by 47% in the rats that received 12 sessions of EA simulation after MFB transection. These data indicate that the neuroprotective role of 100 Hz EA stimulation on dopaminergic neurons in MFB-transected rats is likely to be mediated by suppressing axotomy-induced inflammatory responses. Taken together with our previous results, this study suggests that the neuroprotective effect of EA on the dopaminergic neurons may stem from the collaboration of its anti-inflammatory and neurotrophic actions.

Keywords: Electro-acupuncture; Microglia; Inflammation; Proinflammatory cytokines; Neuroprotection; Axotomy

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Although the mechanism of neuronal degeneration has yet to be uncovered, accumulating evidence suggests that neuroinflammatory processes may account for the progressive death of dopaminergic neurons (Grunblatt et al., 2001; Hunot and Hirsch, 2003). One of the major characteristics of neuroinflammation is the activation of microglia, the resident immune cells in the central nervous system (Liu et al., 2002). Large numbers of reactive microglia are predominantly found near the remaining dopaminergic neurons in the SNpc in postmortem parkinsonian brains. In addition, microglial activation is highest in those subregions most affected by neurodegenerative process (McGeer et al., 1988).

Unilateral transection of the medial forebrain bundle (MFB) is often used to set up an animal model of PD. Axotomy of the MFB produces a precisely defined lesion of nigrostriatal dopaminergic afferents, which results in

Abbreviations: CR3, complement receptor 3; EA, electro-acupuncture; IL, interleukin; MFB, medial forebrain bundle; NO, nitric oxide; OD, optical density; PD, Parkinson's disease; RT-PCR, reverse transcriptase polymerase chain reaction; SN, substantia nigra; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase, TNF, tumor necrosis factor.

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degenerative changes in the dopaminergic cell bodies in the SNpc. Recent studies demonstrates that microglial activation is involved in the MFB axotomy-induced neurodegeneration (Revuelta et al., 1999; Sugama et al., 2003). Activated microglia are believed to contribute to neurodegeneration through the release of cytotoxic compounds, such as proinflammatory cytokines, nitric oxide (NO), reactive oxygen intermediates, arachidonic acid and its derivatives (Streit et al., 1999). Moreover, activated microglia were transformed into brain phagocytes which ingested dopaminergic neurons in the early, and thus, reversible stage of neuronal apoptosis. Previous studies have suggested that the blockade of phagocytic activity of microglial cells may lead to neuroprotection (Revuelta et al., 1999; Sugama et al., 2003).

In China, acupuncture has been employed to treat PD for many years, and accumulated data have shown promising effects of electro-acupuncture (EA) in alleviating the suffering of PD patients (Cai and Hua, 1996; Cheng, 1996; Li, 2003; Liu et al., 1993; Wang, 2000; Wang et al., 1999; Zhuang and Wang, 2000). Our previous work demonstrated that EA stimulation at the frequency of 100 Hz, but not 2 or 0 Hz, protected dopaminergic neurons from degeneration following MFB axotomy (Liang et al., 2002). However, the mechanisms underlying the neuroprotective effect are still unknown. Son et al. (2002) reported that acupuncture stimulation was effective in suppressing the production of pro-inflammatory cytokines in the hypothalamus induced by lipopolysaccharide injection. We hypothesize that the neuroprotective effect of 100 Hz EA stimulation on dopaminergic neurons might be related to its anti-neuroinflammatory effect. The present study was conducted to evaluate the effects of 100 Hz EA stimulation on microglial activation and the transcription of proinflammatory cytokines in MFB-transected rats.

Materials and methods

Animals and surgery

Forty-five adult female Wistar rats weighing 180–200 g were supplied by the Laboratory Animal Center, Peking University, and housed in a standard 12-h on/off light cycle with food and water ad libitum in the home cage. They were allowed to acclimate to their environment for 10 days before experiments. The rats were anesthetized with 350 mg/kg chloral hydrate and positioned in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, U.S.A.) with the tooth bar set at −3.3 mm. MFB lesions were performed using a retractable wire knife (Scouten knife, Kopf Instruments) as described previously (Tseng et al., 1997). The experimental procedures were approved by the Committee on Animal Care and Usage of Peking University Health Science Center, and all effort was made to minimize animal suffering.

EA stimulation

Rats were divided randomly into three groups of 15: the normal group, MFB-lesioned control group and the MFB-lesioned group followed by 100 Hz of EA stimulation. The EA stimulation was administered from the second day following MFB lesion as described before (Liang et al., 2002, 2003). Two stainless-steel needles 0.25 mm in diameter and 5 mm long were inserted obliquely at the acupuncture point DAZHUI (Du 14, just below the spinous process of the vertebra prominens) and horizontally at BAIHUI (Du 21, at the midpoint of the line connecting the two ears). Bidirectional square wave electrical pulses (0.2 ms duration, 100 Hz), designated as EA, were given for a total of 30 min each day, 6 days per week. The intensity of the stimulation was increased stepwise from 1 to 2 mA and then to 3 mA, with each step lasting for 10 min. The animals stayed in the cage quietly during EA administration with no anesthetization. Animals were sacrificed at the indicated time points.

Tissue collection and processing

Five rats from each group were randomly selected 14 (for ED1 immunohistochemical staining) or 28 (for CR3 immunohistochemical staining) days after MFB transection, respectively. The rats were deeply anesthetized with 350 mg/kg chloral hydrate, and then transcardially perfused with 100 ml saline followed by 200 ml 4% paraformaldehyde in phosphate buffer. Brains were dissected and post-fixed in the same fixative and cryoprotected in 30% sucrose for 3–5 days. The brains were frozen on powdered dry ice and then arranged for frontal sectioning according to the rat brain atlas of Paxinos and Watson. Frozen sections (35 μm in thickness) were cut with a cryostat at −20°C and processed for immunohistochemistry. All the remaining rats were decapitated 28 days after MFB transection and the bilateral ventral midbrains were dissected quickly and stored at −80°C.

Immunohistochemistry and quantification of microglial activation

All sections spanning the substantia nigra (SN) (bregma, −4.80 to −6.30 mm) were collected for immunohistochemistry. Every sixth section through the compacta region was selected for immunohistochemical detection of monocyte/macrophage/microglial marker CR3 (OX-42 or CD11b, Chemicon, Temecula, CA, USA) and phagolysosomal marker (ED1, Chemicon). Adjacent sections were immunostained for detection of the dopaminergic neuronal marker tyrosine hydroxylase (TH, Sigma, St. Louis, MO, USA). The anti-CR3, anti-ED1 and anti-TH mouse monoclonal antibodies were used at 1:400, 1:200 and 1:3300 dilutions, respectively. Sections were incubated in primary antibodies for 24 h at 4°C. Diluted non-immune goat serum was substituted for the primary antibodies and served as a

negative control. Sections were incubated in biotinylated goat anti-mouse antibody and then in the avidin–biotin–peroxidase complex for 30 min at 37°C, respectively. The bound complex was visualized by incubating sections in a solution containing 0.1% 3, 3′-diaminobenzidine (Sigma), 1% H_2O_2 and 8% ammonium nickel sulfate (Fluka Chemie GmbH, Switzerland).

The OX42-stained sections were used to establish the extent of microglial activation in the SNpc. The outline of the compacta region was determined in the adjacent TH-stained sections by the distribution of the dopaminergic neurons and by well-established landmarks. The SNpc on both the lesioned and non-lesioned side were analyzed by an investigator blind to the animal treatment using an advanced image-analysis system (Metmorph). Acquisition of the average optical density (OD) was accomplished by using a 50× objective under normal bright-field illumination (Olympus, Japan). Data were resolved relative to a 255-level gray scale and the value was converted to OD units. Background values derived from areas where positive signal was absent were subtracted from the OD values. Six sections from each animal were recorded. ED1-positive cells were counted in six sections throughout the entire rostrocaudal extent of the SNpc. All sections were coded and examined blind.

RT-PCR analysis of TNF-α and IL-1β gene expression

For these studies, the ventral midbrains, processed and stored at −80°C as described above, were used. A semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay was used to determine the mRNA levels of tumor necrosis factor (TNF)-α or interleukin (IL)-1β in relation to GAPDH message as previously described (Bhat et al., 1998; Caivano and Cohen, 2000). Briefly, total RNA was extracted from the ventral midbrains by TRIZOL reagent (Invitrogen Corporation, Carlsbad, CA, USA). A 2-μg sample of total RNA was used for cDNA synthesis by reverse transcription with 200 U of M-MLV reverse transcriptase (Invitrogen Corporation) in a RT buffer in the presence of 0.5 mM of dNTPs, 30 U of RNase inhibiter, and 0.5 μg of oligo dT as primers. The thermal cycler was programmed for 60 min at 42°C, 5 min at 95°C. A 4-μl sample of cDNA synthesized in the RT reaction was used for PCR amplification in the presence of 1 U of Taq DNA polymerase (Invitrogen Corporation) in Taq buffer, 0.2 mM each of dNTPs and 1 μM of each primer. TNF-α and IL-1β were amplified for 30 cycles using a three-step program (30 s at 94 °C, 30 s at 60°C, 1 min at 72°C for TNF-α and 30 s at 94°C, 1 min at 60°C, 1 min at 68°C for IL-1β). GAPDH was amplified for 18 cycles using the same program as that for TNF-α or IL-1β. After amplification, equal volumes of the target and GAPDH product were mixed and separated on an agarose gel in the presence of ethidium bromide and visualized under UV light. DL2000 was used as a DNA marker to measure the molecular weight. The following sequences of the primers were adopted from previously published reports (Bhat et al., 1998; Caivano and Cohen, 2000). TNF-α (upstream), 5′-CAC GCT CTT CTG TCT ACT GA-3′, TNF-α (downstream), 5′-GGA CTC CGT GAT GTC TAA GT-3′ (616 bp). IL-1β (upstream), 5′-AAG CTC TCC ACC TCA ATG GAC AG-3′, IL-1β (downstream), 5′-CTC AAA CTC CAC TTT GCT CTT GA-3′ (260 bp). GAPDH (upstream), 5′-TCC CTC AAG ATT GCT AGC AA-3′, GAPDH (downstream), 5′-AGA TCC ACA ACG GAT ACA TT-3′ (306 bp). GAPDH (upstream), 5′-GGG TGG TGC CAA AAG GGT C-3′, GAPDH (downstream), 5′-GGA GTT GCT GTT GAA GTC ACA-3′ (532 bp).

Statistical analysis

Data are expressed as means ± SEM. Statistical significance was assessed using a one-way ANOVA followed by Newman–Keuls post hoc test of difference between groups. A value of $P < 0.05$ was considered statistically significant.

Results

EA improves the survival of dopaminergic neurons after MFB axotomy

TH immunohistochemical staining was performed 28 days after MFB lesion, and the number of dopaminergic neurons in the SNpc was counted as previously described (Liang et al., 2002). In the MFB-lesioned rats, the number of TH-positive neurons on the lesioned side was about 40.68 ± 7.78% of that on the non-lesioned side. In animals that received 100 Hz EA after MFB transection, the survival rate of the TH-positive neurons was 67.76 ± 8.91%, which increased significantly compared to that of the MFB axotomy group ($P < 0.05$). The neuroprotective effect of 100 Hz EA stimulation was consistent with our previous study (Liang et al., 2002).

EA inhibits microglial activation induced by MFB axotomy

As shown in Fig. 1D, in the SN of the normal control animals, OX42-positive cells were ramified resting microglia with two or three fine processes. In the lesioned side of MFB axotomy group, activated microglia were readily identifiable throughout the SN by their thicker processes and more rounded cell bodies. In addition, many amoeboid microglia could be found in the SN (Fig. 1E). In animals that received EA treatment after MFB axotomy, the activation of microglia was inhibited significantly (Fig. 1F). More microglia in this group retained ramified morphology than in the MFB axotomy group. As shown in Fig. 2, the content of CR3 on the lesioned side increased significantly in the MFB axotomy group, compared to the naive group ($P < 0.001$). After EA treatment for 24 sessions, the

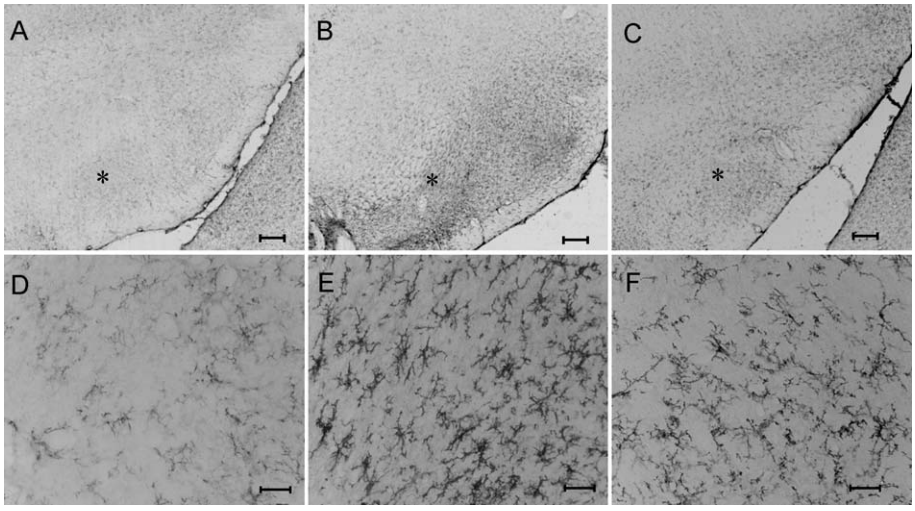

Fig. 1. CR3 immunohistochemical staining of the microglia in the SN 28 days after MFB axotomy. A, normal control group; B, MFB-lesioned group; C, MFB-lesioned group followed by 100 Hz EA stimulation; D, E and F are higher-magnification views of the areas labeled with asterisks in A, B, and C, respectively. Scale bar represents 200 μm in A, B and C, and 30 μm in D, E, and F.

content of CR3 in the SN was greatly reduced compared to the MFB axotomy group ($P < 0.001$) (Fig. 2).

ED1 immunohistochemistry was performed to detect the activated microglia with cytophagic activity. We did not find any ED1-positive cells in the normal control group (Figs. 3A and 4) or the non-lesioned side of the other groups. There were many ED1-positive cells in the SN on the lesioned side in the MFB axotomy group (Fig. 3B). Most of the ED1-positive cells were amoeboid microglia with round cell bodies (Fig. 3D). In animals that received EA stimulation after MFB axotomy, the number of ED1-positive cells decreased by 47% compared to the axotomy group ($P < 0.001$)) (Figs. 3C, E and 4).

EA inhibits TNF-α and IL-1β gene expression induced by MFB axotomy

MFB axotomy induces microglial activation (Revuelta et al., 1999; Sugama et al., 2003). To determine functional change of microglia after EA treatment, we examined the levels of TNF-α and IL-1β mRNA in the ventral midbrain using semi-quantitative RT-PCR. Since the levels of GAPDH were invariable in all groups (Figs. 5A and 6A), the levels of the target mRNAs were normalized against those of GAPDH mRNA. While low, but detectable, levels of TNF-α and IL-1β mRNA were present in the normal control group, MFB axotomy significantly elevated the levels of TNF-α (Fig. 5) and IL-1β (Fig. 6) mRNA in the ventral midbrain. EA reduced MFB axotomy-induced increases in TNF-α and IL-1β mRNA significantly ($P < 0.01$) (Figs. 5 and 6).

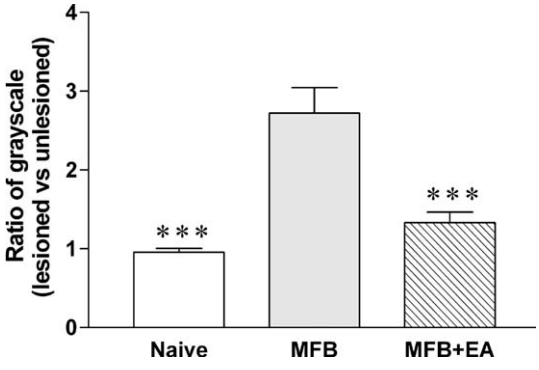

Fig. 2. Effect of EA stimulation on the average optical density of CR3 in the SN. The outlines of the compacta region were determined by the distribution of dopaminergic neurons in the adjacent TH-stained sections. $n = 3–4$. ***$P < 0.001$ versus the MFB-lesioned group.

Discussion

Our results demonstrate that microglial activation may be involved in the dopaminergic neuronal injury in MFB-transected rats. EA at 100 Hz significantly inhibits MFB axotomy-induced activation of microglia and the resultant increase in the levels of TNF-α and IL-1β mRNA. This may underlie the mechanism of neuroprotective effect of 100 Hz EA stimulation on dopaminergic neurons.

It has been well documented that neuroinflammation, characterized by microglial activation, may be involved in the degeneration of dopaminergic neurons in PD. Under normal physiological conditions, microglia are involved in immune surveillance and host defense against infectious agents. However, microglia readily become activated in response to injury or immunological challenges, as indicated by a change in morphology, from a ramified resting state to an amoeboid appearance. These modifications are accompanied by immunophenotypic changes characterized by increased expression of CR3, as determined by OX-42 immunohistochemical staining (Kreutzberg, 1996; Streit et al., 1999). In addition to the morphological changes, acti-

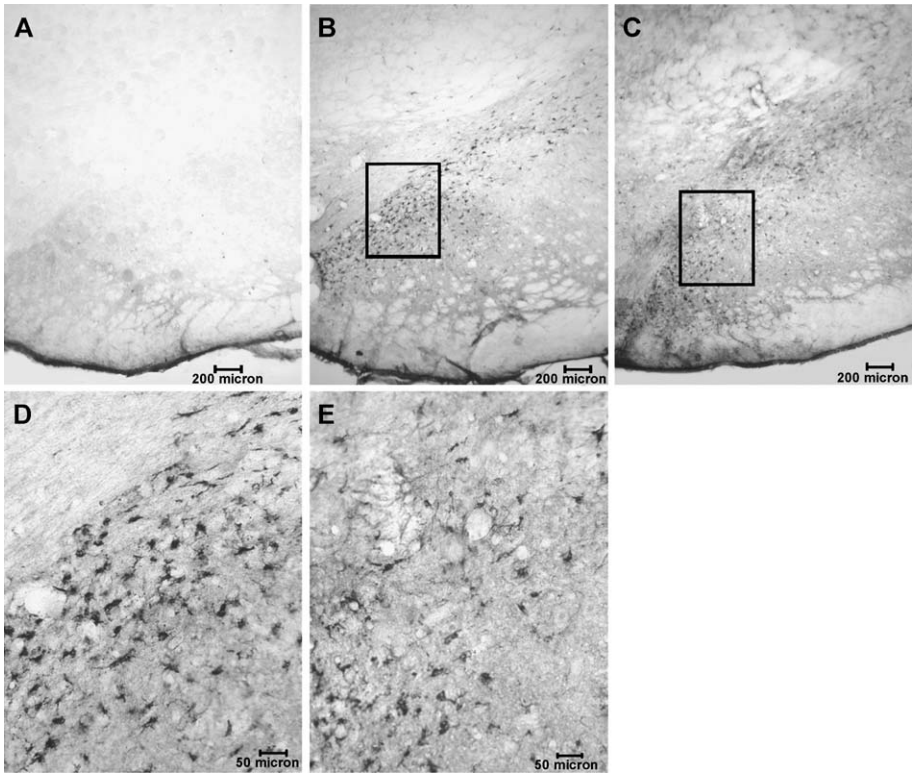

Fig. 3. ED1 immunohistochemical staining of phagocytic microglia in the SN 14 days after MFB axotomy. A, normal control group; B, MFB-lesioned group; C, MFB-lesioned group followed by 100 Hz EA stimulation; D and E, a higher-magnification view of the areas boxed in B and C, respectively. Scale bar represents 200 μm in A, B and C, and 50 μm in D and E.

vated microglia release a wide spectrum of proinflammatory cytokines, including TNF-α and IL-1β. Although the role of the proinflammatory cytokines in the pathogenesis of neurodegeneration is still controversial, most evidence indicates that cytokines may mediate neuronal degeneration through the activation of iNOS. Alternatively, cytokines may mediate dopaminergic cell death through a more direct cytotoxic mechanism, namely the activation of cytokine receptors localized on the dopaminergic neurons and coupled to intracellular death-related signaling pathways (Hengartner, 2000; Hunot et al., 1997; Testi, 1996). Therefore, the neuroprotective effect of 100 Hz EA on the dopaminergic neurons may stem from the inhibition of microglial activation and the synthesis of TNF-α and IL-1β.

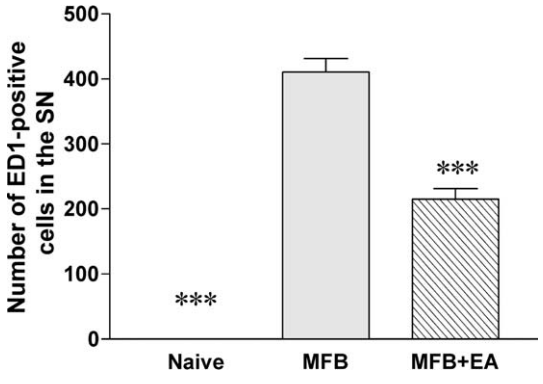

Fig. 4. Effect of 100 Hz EA stimulation on the number of macrophages in the SN. ED1-positive cells in the SN were counted as described in Materials and methods. $n = 5$. ***$P < 0.001$ versus the MFB-lesioned group.

The activated microglia further transform into fully phagocytic brain macrophages. Although it is generally considered that microglial phagocytic activity near neuronal injury is involved in the clearance of cell debris from degenerating neurons, recent studies performed in in vitro systems have demonstrated that neurons were phagocytosed by activated microglia at an early, and thus, reversible stage of apoptosis (Adayev et al., 1998; Witting et al., 2000). In vivo experiments also demonstrated that phagocytic microglia ingested axotomized dopaminergic neurons that were morphologically and biochemically intact (Sugama et al., 2003). Thus, it is particularly important for neuroprotection to suppress early phagocytic action of microglia. The blockade of phagocytic activity of microglial cells by 100 Hz EA stimulation may afford an opportunity for the axotomized neurons to recover from injury in the presence of efficient neuroprotectants. As we have shown previously, 100 Hz EA stimulation up-regulated BDNF mRNA in the SN. The neuroprotective effect of EA on the dopaminergic neurons may stem from the collaboration of its anti-inflammatory and neurotrophic actions.

Evidence is accumulating that acupuncture has anti-inflammatory effects (Ceccherelli et al., 2002; Tian et al., 2003; Zhang et al., 2004). But the underlying mechanism

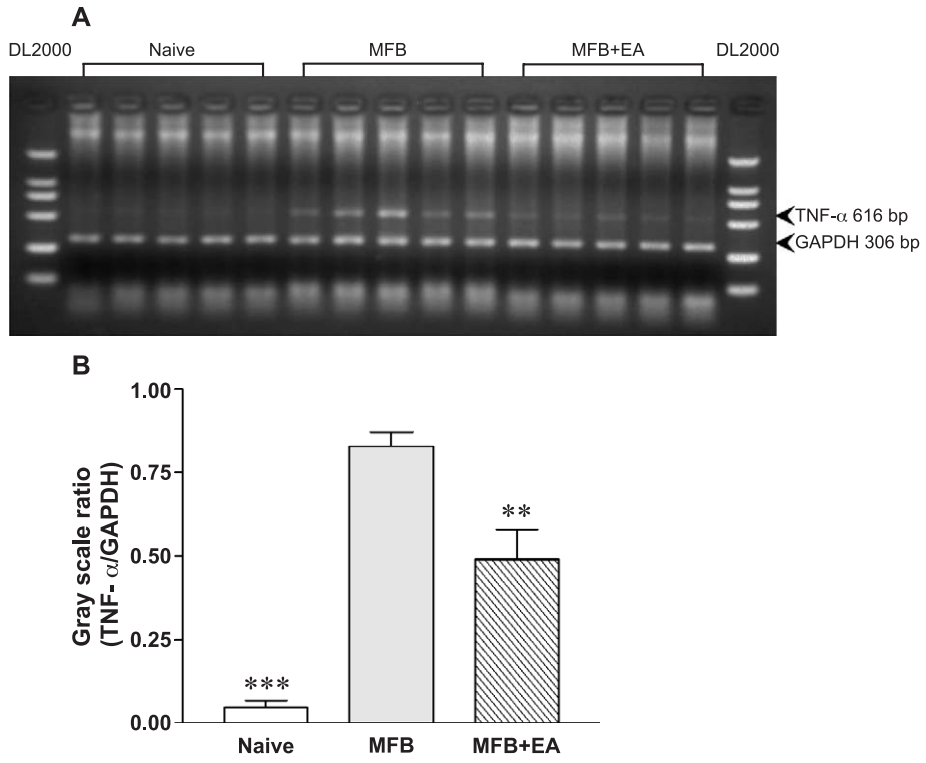

Fig. 5. Effect of 100 Hz EA stimulation on MFB axotomy-induced TNF-α transcription 28 days after MFB axotomy. The level of TNF-α mRNA in the ventral midbrain was detected by semi-quantitative RT-PCR. The PCR products were separated on an agarose gel in the presence of ethidium bromide and visualized under UV light (A). The grey ratio of TNF-α to GAPDH was calculated (B). $n = 5$. $^{**}P < 0.01$, $^{***}P < 0.001$ versus the MFB-lesioned group.

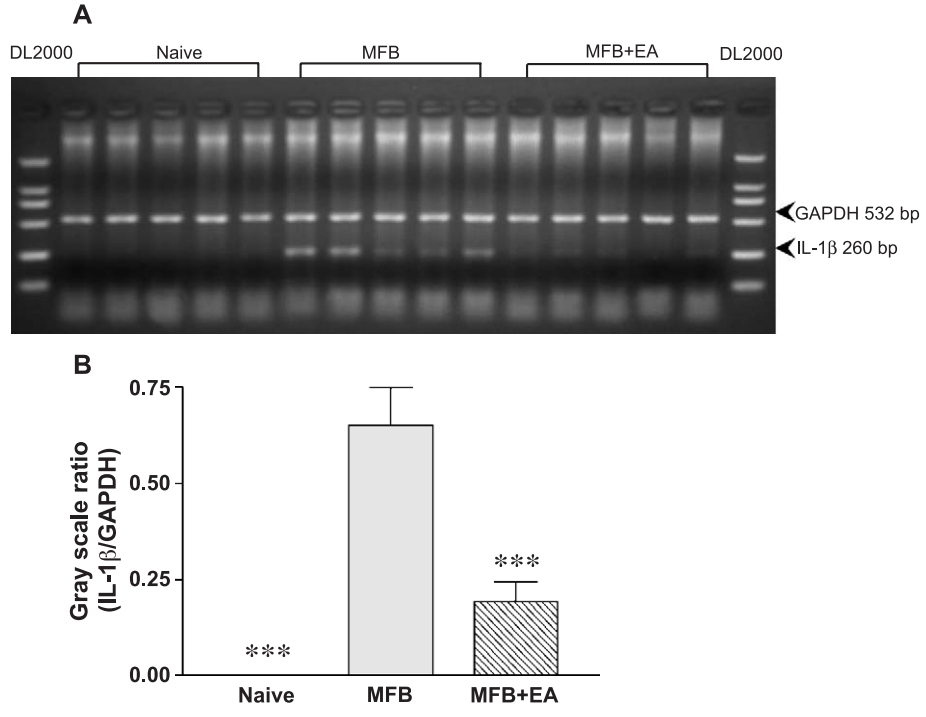

Fig. 6. Effect of 100 Hz EA stimulation on MFB axotomy-induced IL-1β transcription 28 days after MFB axotomy. The level of IL-1β mRNA in the ventral midbrain was detected by semi-quantitative RT-PCR. The PCR products were separated on an agarose gel in the presence of ethidium bromide and visualized under UV light (A). The grey ratio of IL-1β to GAPDH was calculated (B). $n = 5$. $^{***}P < 0.001$ versus the MFB-lesioned group.

still remains to be uncovered. Recent studies indicated that both opioid-dependent (Ceccherelli et al., 2002) and non-opioid-dependent (Zhang et al., 2004) mechanisms may be involved in its anti-inflammatory effect. It was reported that EA stimulation strongly inhibited the inflammation induced by carrageenan; and the inhibitory effect was not affected by intraperitoneal injection of naloxone (Zhang et al., 2004). This indicated that EA stimulation is capable of inhibiting inflammation via a non-opioid-dependent mechanism, or on the other hand, the non-receptor pathway of opioid system. Ceccherelli et al. (2002) reported that acupunctural stimulation alleviated the inflammation induced by capsaicin; and the administration of naloxone reversed the anti-inflammatory effect. These results indicated that the mechanism underlying the anti-inflammatory effect of acupuncture may be largely depending on the opioid system.

Our previous studies demonstrated that EA at 100 Hz enhanced the synthesis and release of dynorphin in the brain (He and Han, 1990; Guo et al., 1997). Recently, it has been shown that dynorphin protects dopaminergic neurons from inflammation-mediated damage (Liu et al., 2001). Therefore, it is plausible to speculate that the anti-inflammatory effect of 100 Hz EA may be mediated by dynorphin. This hypothesis can be examined further by applying an antagonist of dynorphin and then investigating if the effect of EA can be eliminated.

Acknowledgments

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