Neurol Sci (2011) 32:369–374
DOI 10.1007/s10072-010-0402-6

ORIGINAL ARTICLE

Electroacupuncture enhances cell proliferation and neuronal differentiation in young rat brains

Jianfeng Gao · Sheng Wang · Xiaoyang Wang · Changlian Zhu

Received: 31 January 2010 / Accepted: 28 August 2010 / Published online: 18 September 2010

Abstract To investigate the effect of electroacupuncture on cell proliferation and differentiation in young rat hippocampus, postnatal day-14 rats were assigned randomly to control, ketamine anesthesia, nonacupoint, or acupoint electroacupuncture groups. Electroacupuncture was applied at bilateral acupoints (Quchi, Waiguan, Huantiao, and Zusanli) 30 min daily for 7 successive days. The proliferation and neuronal differentiation of proliferated surviving cells in the dentate gyrus were evaluated at 4 weeks after last stimulation. The stimulatory effect of electroacupuncture on cell proliferation had a long-lasting effect, as indicated by the increased phosphor-histone H3-positive cells. The number of proliferated and survival cells, indicated by BrdU labeling, was highest in the electroacupuncture group. The number of newly differentiated neurons, as indicated by BrdU/NeuN double labeling, was significantly higher in the electroacupuncture group than in any of the other groups. This finding provides a theoretical basis for the clinical application of acupuncture to cerebral injury rehabilitation in children.

Keywords Cell proliferation · Electroacupuncture · Hippocampus · Neurogenesis

J. Gao · S. Wang · X. Wang · C. Zhu (✉)
Department of Pediatrics, The Third Affiliated Hospital of Zhengzhou University, Kangfuqain Street 7, Zhengzhou 450052, China
e-mail: zhuc@zzu.edu.cn

X. Wang · C. Zhu
Henan Key Laboratory for Neonatal Brain Injury, Zhengzhou, China

J. Gao
Department of Physiology, Henan Traditional Chinese Medical College, Zhengzhou, China

Introduction

Acupuncture is utilized as a complement to clinical treatment for various diseases in Eastern medicine, and may improve symptoms in patients with brain injury [1–4]. It is now accepted as one of the most common complementary therapeutic techniques by the World Health Organization. The theory of acupuncture is based on a model of energy (Chi) balance, and acupuncture may correct imbalances of Chi flow at specific acupoints. The mechanisms by which acupuncture may influence disease are largely unknown, although several studies have demonstrated, using modern biomedical techniques, that acupuncture possesses many effects in the central nervous system, such as analgesia, promotion of homeostasis, improvement of brain circulation, and improvement of neuromodulatory function [5–8]. It has been reported that acupuncture may enhance cell proliferation in the neurogenic area (hippocampal dentate gyrus and the subventricular zone of the lateral ventricle walls) in pathological conditions [9–11], which is related to improve brain functioning [12, 13]. However, most acupuncture studies have either been on adult animal models or have involved cell proliferation only, without any further study of neuronal differentiation [9, 10, 14, 15]. Furthermore, most of the newly proliferated brain cells that have been stimulated by pathological or physiological conditions have died within 1 month without further differentiation into neurons [12, 13, 16–18]. Our previous studies demonstrated that cell proliferation and differentiation, as well as apoptotic cell death, decrease with age [16, 18, 19]. Furthermore, application of anesthesia to the developing brain could induce neuronal cell death and impair cell proliferation and brain function, effects that are more pronounced in the developing brain [20–23]. To clarify the effects of acupuncture on cell proliferation and survival,

neuronal differentiation, as well as the possible effects of anesthesia, rats were treated with electroacupuncture (EA) at postnatal day 14 (P14), during which the development of the rat brain is similar to that of human child, with the DG still continuing to grow. The effect of electroacupuncture on cell proliferation and differentiation in the DG was compared with that of nonacupoint stimulation and anesthesia with ketamine.

Materials and methods

Fourteen-day-old male Sprague–Dawley rats (weight 50–75 g) purchased from Animal Resources Center, Zhengzhou University of Henan Province, were randomly assigned to one of the four groups: (1) Control (Cont) (rats without anesthesia or EA, $n = 7$); (2) Anesthesia (Anes) (rats with anesthesia but without EA, $n = 6$); (3) Nonacupoint (NA) (rats with anesthesia and EA stimulation on the tail, $n = 4$); and (4) Electroacupuncture (EA) (rats with anesthesia and EA stimulation on Quchi, Waiguan, Huantiao, Zusanli, $n = 6$). All experimental procedures involving the use of animals were approved by the Animal Use and Care Committee of Zhengzhou University.

Electroacupuncture stimulation

A 30-min session of EA was applied by an acupunctoscope device (Model G6805-2, Smeif, Shanghai, China) to rats in the nonacupoint and electroacupuncture groups. Anesthesia was induced with an intraperitoneal injection of ketamine at a dose of 10 mg/kg in the animals, with the exception of the normal control group. In the acupuncture group, EA was applied on bilateral acupoints. Stainless acupuncture needles of 0.3-mm diameter were bilaterally inserted at a depth of approximately 2–4 mm into the locus of the Zusanli acupoint, 5 mm below the knee joint of the hindlimb and 2 mm lateral to the anterior tubercle of the tibia. The Huantiao acupoint is located at the junction of the lateral one-third and the medial two-thirds of the distance between the greater trochanter and the hiatus of the sacrum; below are the sciatic nerve, inferior gluteal nerve, and gluteal muscles. The Huantiao acupoint was located on the rat's hindlimbs using the comparable anatomical landmarks. The Waiguan acupoint is located dorsally between the radius and ulna, 2 units (based on the standard acupuncture measurement of 12 units between the transverse cubital crease and the transverse wrist crease) above the transverse crease of the wrist; it was located on the rat's forepaw using the comparable anatomical landmarks. The Quchi acupoint is anatomically located in the depression lateral to the anterior aspect of the radius joint of the forelimb in rats. A frequency of 2 Hz with a square waveform at a pulse duration of 0.5 ms was used. The applied voltage was 0.7 mV, an intensity just below the threshold of muscle contraction. Stimulation was applied 30 min/day for 7 days (Fig. 1).

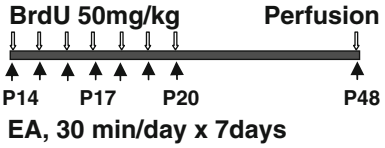

Fig. 1 Experimental design. P14 rat pups were randomly assigned to one of the four groups. All pups were injected one BrdU daily for 7 days and killed 4 weeks after the final BrdU injection

Bromodeoxyuridine administration

The thymidine analog bromodeoxyuridine (BrdU) (5 mg/mL, dissolved in 0.9% saline) (Sigma-Aldrich, USA) was injected intraperitoneally at a dose of 50 mg/kg once daily for 7 days, starting at the time of electroacupuncture treatment. All animals were killed 4 weeks after last BrdU injection (Fig. 1).

Immunohistochemistry

The animals were deeply anesthetized with 50 mg/mL phenobarbital and perfusion-fixed with 5% formaldehyde in 0.1 mol/L PBS followed by immersion fixation in the same fixative for 24 h at 4°C. After dehydration with graded ethanol and xylene, the brains were embedded in paraffin and serial 5-μm coronal sections were cut and mounted on glass slides. Every 50th section was stained for either BrdU or phosphor-histone H3 (P-HH3). Antigen recovery was performed by boiling the sections in 10 mmol/L citrate buffer (pH 6.0) for 10 min. Nonspecific binding was blocked for 30 min with 4% donkey or goat serum in PBS. A monoclonal rat anti-BrdU primary antibody (1:100, 5 μg/mL; clone: BU1/75, Oxford Biotechnology Ltd. Oxfordshire, UK) or rabbit anti-phosphohistone H3 (ser10) (1:500, 2 μg/mL, Upstate, Temecula, CA, USA) was applied and incubated at 20°C for 60 min, followed by a biotinylated donkey anti-rat IgG (H + L) secondary antibody (1:200, 5.5 μg/mL; Jackson ImmunoResearch Lab, PA, USA) or goat anti-rabbit (1:200, Vector Laboratories, Burlingame, CA, USA) for 60 min at 20°C. Endogenous peroxidase activity was blocked with 3% H_2O_2 in PBS for 10 min. Visualization was performed using Vectastain ABC Elite (Vector Laboratories, Burlingame, CA, USA) with 0.5 mg/mL 3,3-diaminobenzidine enhanced with 15 mg/mL ammonium nickel sulfate, 2 mg/mL beta-D glucose, 0.4 mg/mL ammonium chloride, and 0.01 mg/mL b-glucose oxidase (all from Sigma).

The phenotype of BrdU-labeled cells was determined using antibodies against NeuN to detect mature neurons. Antigen recovery was performed as above, followed by incubation with rat anti-BrdU (1:100, 5 μg/mL; clone: BU1/75, Oxford Biotechnology Ltd. Oxfordshire, UK) together with mouse anti-NeuN monoclonal antibody (1:200, 5 μg/mL; clone: MAB377, Chemicon, Temecula, CA, USA) in PBS at 20°C for 60 min. After washing, the sections were incubated with secondary antibodies: Alexa Fluor 488 donkey anti-rat IgG (H + L) combined with Alexa Fluor 555 donkey anti-mouse IgG (H + L) at 20°C for 60 min. All secondary antibodies were from Jackson ImmunoResearch Lab, and were diluted 1:1,000. After washing, the sections were mounted using Vectashield mounting medium.

Cell counting

Area contours were created and measured, and BrdU-positive and P-HH3-positive cells were counted in the granular cell layer of the DG, including the subgranular zone, in every 50th section by using stereology microscopy. Positive cells were expressed as the average number of counted cells per mm^3. For phenotypic determination, at least 50 BrdU-positive cells were counted using a confocal laser scanning microscope and the ratio of BrdU/NeuN double-labeled cells to counted BrdU-positive cells was calculated separately. The total number of neuronal lineages was calculated by applying the ratio of BrdU/NeuN double positive cells to the total number of BrdU-positive cells to calculate the total number of new neurons per DG.

Statistics

All data are expressed as mean ± s.e.m. Student *t* test was used to compare two groups with equal variance. Results were considered statistically significant different at 5% level and indicated as $p < 0.05$.

Results

Effect on cell proliferation

To evaluate the effects of EA on cell proliferation and survival in the hippocampus, P14 rats were administered one BrdU injections daily for 7 consecutive days (Fig. 1). Cell proliferation or survival of the proliferated cells at 4 weeks after the last EA session was evaluated by using phospho-histone H3 (P-HH3) or BrdU immunostaining. The number of P-HH3-positive cells was 4,802 ± 754/mm^3 in the control group, 5,541 ± 1,474/mm^3 in anesthesia group, 3,790 ± 865/mm^3 in NA group, and 8,735 ± 2,475/mm^3 in the EA group, representing a 1.82-, 1.58-, and 2.31-fold increase compared to the control, anesthesia, or NA group (Fig. 2), indicating that EA has a persistent effect on cell proliferation.

The number of BrdU-labeled cells was 2,384 ± 506/mm^3 in the control group, 2,655 ± 575/mm^3 in the anesthesia group, 2,851 ± 285/mm^3 in the NA group, and 4,209 ± 331/mm^3 in the EA group, representing a 1.77-, 1.59-, 1.48-fold increase compared to control, anesthesia, or NA group. The significant difference was only seen between EA and control ($p = 0.023$), but not between the other groups (Fig. 3).

Effect on neuronal differentiation

Neuronal differentiation was detected by BrdU/NeuN co-labeling. The majority of surviving, newly proliferated cells (>90%) were differentiated into neurons in the control and EA groups. However, in the anesthesia group, only

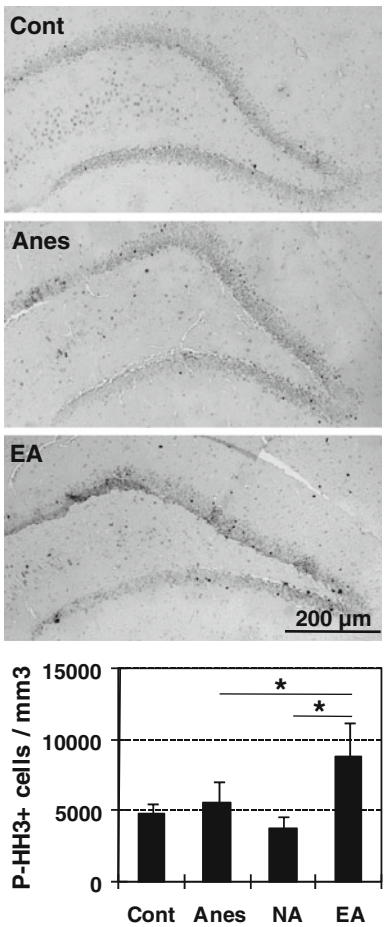

Fig. 2 Cell proliferation in the dentate gyrus. Representative micrographs of phospho-histone H3 (P-HH3) staining in the DG of P48 pups from the control (Cont), anesthesia (Anes), and EA groups. The number of P-HH3-positive cells in the DG (GCL + SGZ) increased significantly in the EA group compared to anesthesia or NA group. * $p < 0.05$

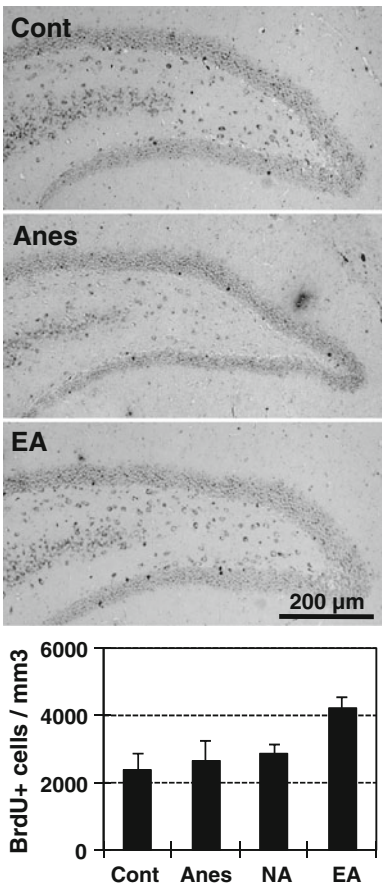

Fig. 3 BrdU incorporation. Representative microphotographs of BrdU staining in the hippocampal DG of pups from the control, anesthesia, and EA groups. The bar graph shows the number of BrdU-labeled cells

around 70% of the surviving cells were differentiated into neurons. The number of newly formed neurons was 2,230 ± 482/mm^3 in the control group, 1,864 ± 401/mm^3 in the anesthesia group, 2,554 ± 265/mm^3 in NA group, and 3,784 ± 244/mm^3 in the EA group. The number was significantly higher in EA group than that of other groups (Fig. 4).

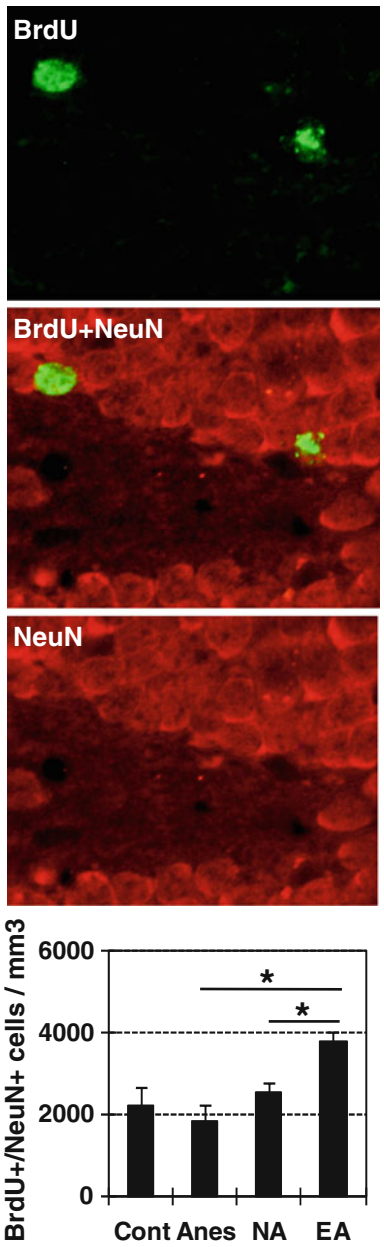

Fig. 4 Neuronal differentiation in the dentate gyrus. Representative microphotographs of BrdU (*green*), NeuN (*red*), and overlay staining. The bar graph shows that the number of BrdU/NeuN double-positive cells in the DG was increased significantly in the EA group compared to anesthesia and EA groups. * $p < 0.05$ (color figure online)

Discussion

Acupuncture has been used in Eastern medicine to treat various diseases, and is relatively safe [24, 25]. The ultimate goal of different acupuncture techniques is to restore the internal balance and harmony by restoring the flow of Chi [26]. Acupoints are points located along the skin that have properties of lower electrical impedance, higher electrical potential, and more peripheral nerves compared to other areas of the skin. The Quchi and Waiguan acupoints have been selected for brain injury rehabilitation. The Zusanli and Huantiao acupoints are commonly selected for various diseases. It has been shown that EA at these acupoints can stimulate cell proliferation and reduce brain injury [9, 10, 27].

Cell proliferation and neurogenesis occur primarily in two areas of the adult brain of humans and other mammals: the subgranular zone (SGZ) of the DG, and the anterior part of the subventricular zone along the ventricle [28]. Cell proliferation and neurogenesis are regulated by various

physiological and pathological conditions [16]. They decrease with brain development and are increased by environmental enrichment and physical activity [13]. Acupuncture as a form of stimulation has been shown to promote cell proliferation in pathological and physiological conditions [9, 10, 14]. However, there has been no evaluation of acupuncture's long-term effects on cell proliferation and differentiation. In this study, we found that EA could promote the proliferated cell survival; more than 90% the survival cells differentiated into neuron in the young rats (Fig. 4). The nonacupoint stimulation at the tail has no effect when compared with the stimulation of specific, defined acupoints, suggesting that incorrectly locating the acupoints may render EA therapy ineffective.

General anesthesia in children (ranging from premature babies to adolescents) is a common practice in modern anesthesiology, for surgery or for relief from procedural pain. Ketamine is a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, and reduces neuronal injury after cerebral ischemia by blocking the excitotoxic effects of glutamate. Recent findings indicate that the excitation of NMDA receptors may induce widespread neurodegeneration in the developing brain [29, 30]. Cerebral regeneration may be impaired by an NMDA receptor blockade [31]. Ketamine has been reported to have no obvious effect on cell proliferation or differentiation in adult brain [20], and none were found in this study in the developing brain. The number of newly formed neurons did not differ significantly compared with the control group. However, the percentage of neuronal differentiation was around 70% in the anesthesia group, compared to 90% in the control group, indicating a different effect of ketamine on the developing brain.

In summary, our study demonstrates that EA stimulation has a long effect on progenitor cell proliferation and promotes proliferated cells differentiated into neurons, reveals the cellular protective mechanism of EA stimulation, and provides the theoretical basis for pediatric clinical applications.

Acknowledgments This work was supported by the Medical Science Academy of Henan, a Grant from the Department of Education of Henan Province, the National Natural Science Foundation of China, and the Ministry of Education of China (the third period 211 project).

References

1. Pei J, Sun L, Chen R, Zhu T, Qian Y, Yuan D (2001) The effect of electro-acupuncture on motor function recovery in patients with acute cerebral infarction: a randomly controlled trial. J Tradit Chin Med 21:270–272
2. Wu Y, Zou LP, Han TL, Zheng H, Caspi O, Wong V, Su Y, Shen KL (2008) Randomized controlled trial of traditional Chinese medicine (acupuncture and tuina) in cerebral palsy: part 1—any increase in seizure in integrated acupuncture and rehabilitation group versus rehabilitation group? J Altern Complem Med 14:1005–1009
3. Sun JG, Ko CH, Wong V, Sun XR (2004) Randomised control trial of tongue acupuncture versus sham acupuncture in improving functional outcome in cerebral palsy. J Neurol Neurosurg Psychiatry 75:1054–1057
4. Liu SY, Hsieh CL, Wei TS, Liu PT, Chang YJ, Li TC (2009) Acupuncture stimulation improves balance function in stroke patients: a single-blinded controlled, randomized study. Am J Chin Med 37:483–494
5. Cabyoglu MT, Ergene N, Tan U (2006) The mechanism of acupuncture and clinical applications. Int J Neurosci 116:115–125
6. Wu MT, Sheen JM, Chuang KH, Yang P, Chin SL, Tsai CY, Chen CJ, Liao JR, Lai PH, Chu KA, Pan HB, Yang CF (2002) Neuronal specificity of acupuncture response: a fMRI study with electroacupuncture. Neuroimage 16:1028–1037
7. Cho ZH, Chung SC, Jones JP, Park JB, Park HJ, Lee HJ, Wong EK, Min BI (1998) New findings of the correlation between acupoints and corresponding brain cortices using functional MRI. Proc Natl Acad Sci USA 95:2670–2673
8. Wong VC, Sun JG, Yeung DW (2006) Pilot study of positron emission tomography (PET) brain glucose metabolism to assess the efficacy of tongue and body acupuncture in cerebral palsy. J Child Neurol 21:456–462
9. Kim EH, Jang MH, Shin MC, Lim BV, Kim HB, Kim YJ, Chung JH, Kim CJ (2002) Acupuncture increases cell proliferation and neuropeptide Y expression in dentate gyrus of streptozotocin-induced diabetic rats. Neurosci Lett 327:33–36
10. Kim EH, Kim YJ, Lee HJ, Huh Y, Chung JH, Seo JC, Kang JE, Yim SV, Kim CJ (2001) Acupuncture increases cell proliferation in dentate gyrus after transient global ischemia in gerbils. Neurosci Lett 297:21–24
11. Liu Q, Yu J, Mi WL, Mao-Ying QL, Yang R, Wang YQ, Wu GC (2007) Electroacupuncture attenuates the decrease of hippocampal progenitor cell proliferation in the adult rats exposed to chronic unpredictable stress. Life Sci 81:1489–1495
12. Cheng H, Yu J, Jiang Z, Zhang X, Liu C, Peng Y, Chen F, Qu Y, Jia Y, Tian Q, Xiao C, Chu Q, Nie K, Kan B, Hu X, Han J (2008) Acupuncture improves cognitive deficits and regulates the brain cell proliferation of SAMP8 mice. Neurosci Lett 432:111–116
13. Naylor AS, Bull C, Nilsson MK, Zhu C, Bjork-Eriksson T, Eriksson PS, Blomgren K, Kuhn HG (2008) Voluntary running rescues adult hippocampal neurogenesis after irradiation of the young mouse brain. Proc Natl Acad Sci USA 105:14632–14637
14. Park HJ, Lim S, Lee HS, Lee HJ, Yoo YM, Kim SA, Yin CS, Seo JC, Chung JH (2002) Acupuncture enhances cell proliferation in dentate gyrus of maternally-separated rats. Neurosci Lett 319:153–156
15. Siu FK, Lo SC, Leung MC (2004) Effectiveness of multiple pre-ischemia electro-acupuncture on attenuating lipid peroxidation induced by cerebral ischemia in adult rats. Life Sci 75:1323–1332
16. Qiu L, Zhu C, Wang X, Xu F, Eriksson PS, Nilsson M, Cooper-Kuhn CM, Kuhn HG, Blomgren K (2007) Less neurogenesis and inflammation in the immature than in the juvenile brain after cerebral hypoxia-ischemia. J Cereb Blood Flow Metab 27:785–794
17. Bingham B, Liu D, Wood A, Cho S (2005) Ischemia-stimulated neurogenesis is regulated by proliferation, migration, differentiation and caspase activation of hippocampal precursor cells. Brain Res 1058:167–177
18. Zhu C, Qiu L, Wang X, Xu F, Nilsson M, Cooper-Kuhn C, Kuhn HG, Blomgren K (2009) Age-dependent regenerative responses in the striatum and cortex after hypoxia-ischemia. J Cereb Blood Flow Metab 29:342–354

19. Zhu C, Wang X, Xu F, Bahr BA, Shibata M, Uchiyama Y, Hagberg H, Blomgren K (2005) The influence of age on apoptotic and other mechanisms of cell death after cerebral hypoxia-ischemia. Cell Death Differ 12:162–176
20. Tung A, Herrera S, Fornal CA, Jacobs BL (2008) The effect of prolonged anesthesia with isoflurane, propofol, dexmedetomidine, or ketamine on neural cell proliferation in the adult rat. Anesth Analg 106:1772–1777
21. Wilder RT, Flick RP, Sprung J, Katusic SK, Barbaresi WJ, Mickelson C, Gleich SJ, Schroeder DR, Weaver AL, Warner DO (2009) Early exposure to anesthesia and learning disabilities in a population-based birth cohort. Anesthesiology 110:796–804
22. Zou X, Patterson TA, Divine RL, Sadovova N, Zhang X, Hanig JP, Paule MG, Slikker W Jr, Wang C (2009) Prolonged exposure to ketamine increases neurodegeneration in the developing monkey brain. Int J Dev Neurosci 27:727–731
23. Zhu C, Gao J, Karlsson N, Li Q, Zhang Y, Huang Z, Li H, Kuhn HG, Blomgren K (2010) Isoflurane anesthesia induced persistent, progressive memory impairment, caused a loss of neural stem cells, and reduced neurogenesis in young, but not adult, rodents. J Cereb Blood Flow Metab 30:1017–1030
24. Witt CM, Pach D, Brinkhaus B, Wruck K, Tag B, Mank S, Willich SN (2009) Safety of acupuncture: results of a prospective observational study with 229, 230 patients and introduction of a medical information and consent form. Forsch Komplementmed 16:91–97
25. Ernst G, Strzyz H, Hagmeister H (2003) Incidence of adverse effects during acupuncture therapy-a multicentre survey. Complem Ther Med 11:93–97
26. Li A, Zhang RX, Wang Y, Zhang H, Ren K, Berman BM, Tan M, Lao L (2007) Corticosterone mediates electroacupuncture-produced anti-edema in a rat model of inflammation. BMC Complement Altern Med 7:27
27. Jang MH, Shin MC, Lee TH, Lim BV, Shin MS, Min BI, Kim H, Cho S, Kim EH, Kim CJ (2003) Acupuncture suppresses ischemia-induced increase in c-Fos expression and apoptosis in the hippocampal CA1 region in gerbils. Neurosci Lett 347:5–8
28. Taupin P, Gage FH (2002) Adult neurogenesis and neural stem cells of the central nervous system in mammals. J Neurosci Res 69:745–749
29. Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vockler J, Dikranian K, Tenkova TI, Stefovska V, Turski L, Olney JW (1999) Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 283:70–74
30. Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, Olney JW, Wozniak DF (2003) Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. J Neurosci 23:876–882
31. Winkelheide U, Lasarzik I, Kaeppel B, Winkler J, Werner C, Kochs E, Engelhard K (2009) Dose-dependent effect of S(+) ketamine on post-ischemic endogenous neurogenesis in rats. Acta Anaesthesiol Scand 53:528–533