NEURAL REGENERATION RESEARCH
February 2016, Volume 11, Issue 2
www.nrronline.org

RESEARCH ARTICLE

Manual acupuncture at the SJ5 (*Waiguan*) acupoint shows neuroprotective effects by regulating expression of the anti-apoptotic gene *Bcl-2*

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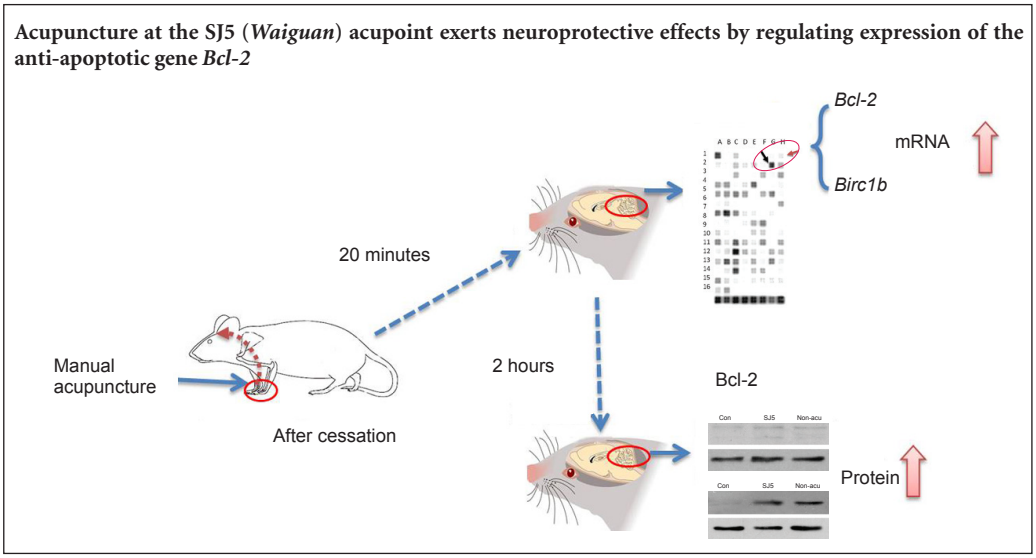
How to cite this article: *Lin D, Lin LL, Sutherland K, Cao CH (2016) Manual acupuncture at the SJ5 (Waiguan) acupoint shows neuroprotective effects by regulating expression of the anti-apoptotic gene Bcl-2. Neural Regen Res 11(2):305-311.*

Funding: *This work was supported by the National Natural Science Foundation of China, No. 81273672, 81373720; the Natural Science Foundation of Fujian Province of China, No. 2014J01353; and a grant from Fujian Province Health Planning Commission Project (2014) for Training Young Talents, No. 2014-ZQN-JC-28.*

Graphical Abstract



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doi: *10.4103/1673-5374.177740*
http:*//www.nrronline.org/*

Accepted: *2015-07-30*

Abstract

Acupuncture at the SJ5 (*Waiguan*) acupoint has neuroprotective effects in cerebral infarction, but the underlying mechanism remains poorly understood. Here, we analyzed gene expression in healthy rat cerebellum using a pathway-focused DNA microarray to screen 113 genes associated with 18 signal transduction pathways. After 20 minutes of acupuncture at SJ5, the expression of *Bcl-2* and *Birc1b* mRNA was markedly increased. This was confirmed by real-time reverse transcription PCR. Furthermore, western blot analysis showed that Bcl-2 protein expression remained high in the cerebellum until at least 2 hours after cessation of acupuncture. These findings indicate that acupuncture at SJ5 exerts neuroprotective effects by regulating the expression of anti-apoptotic gene *Bcl-2*.

Key Words: *nerve regeneration; neuroprotection; acupuncture; SJ5; cerebellum; apoptosis; signal transduction; Bcl-2; neural regeneration*

Introduction

Acupuncture is gaining widespread popularity. Its effects on brain function are well documented (Kim et al., 2011; Bai et al., 2014), and recent reports have demonstrated its therapeutic effects in Parkinson's disease (Arankalle and Nair, 2013), epilepsy (Cakmak, 2006), stroke (Cho et al., 2013) and a number of neurological disorders (Soligo et al., 2013). Acupuncture has also shown promising effects in the cerebellum in experimental and clinical studies (Chen et al., 2013; Jin et al., 2014; Leung et al., 2014; Xie et al., 2014). A recent functional magnetic resonance imaging study showed that cerebellar activity was elevated after acupuncture (Bai et al., 2014). Furthermore, mounting evidence suggests that acupuncture stimulation affects brain activation by increasing blood oxygen level-dependent signal intensity in the cerebellum (Zhang et al., 2012; Lee et al., 2013). Early functional neuroimaging data indicate that acupuncture stimulation modulates specific motor-related networks in different

brain regions, and in particular activates the cerebellum (Quah-Smith et al., 2013). Acupuncture can significantly enhance sensorimotor integration, particularly by increasing the functional connectivity of the cerebellum (Chen et al., 2013; Leung et al., 2014; Romoli et al., 2014; Xie et al., 2014). Moreover, the cerebellum shows significant activation after manual acupuncture, even after local anesthesia at the acupoint (Jin et al., 2014). The impact of acupuncture on the functional connectivity between the cerebellum and the cerebrum might also be significant.

Despite mounting evidence pertaining to the benefits of acupuncture in different neurological and neurodegenerative diseases, few studies have addressed the link between acupuncture and biological action in specific brain regions in healthy animals (Manni et al., 2010). San Jiao 5 (SJ5), also known as *Waiguan*, is an important traditional acupuncture point commonly used in the treatment of stroke-related motor, neurological and autonomic nerve problems. Acupuncture at SJ5 specifically influences functional networks of the central nervous system, including sensorimotor areas, to improve sensorimotor function (Liu et al., 2009; Chen et al., 2014). In a recent study using ^{18}F-fluorodeoxyglucose positron-emission computed tomography (PET/CT), needling at SJ5 in patients with cerebral infarction increased glucose metabolism in local cerebellar regions (Liu et al., 2013). Furthermore, continuous wavelet transform analysis of electroencephalography signals showed that during SJ5 acupuncture stimulation, θ power (4–8 Hz) was significantly elevated after 20 minutes of acupuncture (Hsu et al., 2011). Additionally, studies have shown a strong correlation between acupuncture at SJ5 acupoint and the function of corresponding brain regions (Small et al., 2002; Wang et al., 2010).

In the present study, we examined the effects of acupuncture on normal brain function, to elucidate the neural mechanisms underlying its therapeutic action. We hypothesized that acupuncture at SJ5 would modulate certain biological activities in the cerebellum, as suggested in a previous study (Liu et al., 2013). To investigate the effect of acupuncture on specific gene expression, we used a pathway-focused DNA microarray to identify signal transduction pathways activated after acupuncture, and real-time reverse transcription (RT)-PCR and western blot assays to determine mRNA and protein expression of Bcl-2 in the cerebellum.

Materials and Methods

Animals

All experiments were approved by the Institutional Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine, China, and performed according to the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal discomfort and reduce the number of animals used. Thirty-nine adult male Sprague-Dawley rats (Shang Hai SLAC Laboratory Ltd., License No. SCXK (Hu) 2007-0005), weighing 180–220 g, were randomly assigned to three groups. The control group (9 animals; 3 for the microarray and 6 for western blot/RT-PCR) received no treatment, but the animals were immobilized by hand for 20 minutes. The SJ5 acupuncture group (15 animals; 3 for the microarray and 12 for western blot/RT-PCR) received acupuncture at SJ5. In the non-acupoint group (15 animals; 3 for the microarray and 12 for western blot/RT-PCR), a needle was inserted 3 mm medial to SJ5 (**Figure 1**). All rats were given free access to water and food, and were housed under a 12 hour light/dark cycle and standard vivarium conditions at 25 ± 3°C.

Acupuncture stimulation

To determine approximate acupoints in rats, we used the transpositional method, which transforms human acupoints onto animal anatomy (Yin et al., 2008). Acupuncture stimulation was performed by inserting a pair of stainless steel pins (0.3 mm in diameter, 13 mm in length; batch No. 120380, Suzhou HuaTuo Medicine Instrument Co., Ltd, China) into the SJ5 acupoint to a depth of 2–3 mm (No author listed, 2009). SJ5 is located at the midpoint between the ulna and the radius, about 2 cun (67 mm) above the crease of the wrist (Hsu et al., 2011) (**Figure 1A**). To rule out non-specific effects of acupuncture, the same stimulation was given at a "non-acupoint", 3 mm medial to SJ5 (Chen et al., 2014) at the midpoint between the San Jiao (triple energizer) and small intestine meridians (**Figure 1A**). All rats were immobilized by hand during acupuncture to reduce stress. Acupuncture was performed manually by an experienced acupuncturist. The needles were twisted twice per second for 30 seconds, and the stimulation was applied every 5 minutes for a total of 20 minutes. The rats were observed while the acupuncture was performed, and if a rat looked uncomfortable, it was stroked gently on the back until it became calm again.

Gene expression profiling

Rats were sacrificed immediately after acupuncture stimulation, the brains removed, and the cerebellum dissected out and frozen rapidly. The frozen samples were homogenized in TRIzol Reagent (Invitrogen, Rockville, MA, USA), and total RNA was extracted using the ArrayGrade™ Total RNA Isolation kit (SABiosciences, Frederic, MA, USA) according to the manufacturer's protocol. RNA integrity was assessed by electrophoresis in 1.5% agarose gel with ethidium bromide. Structural integrity, yield, and purity of RNA were confirmed using a spectrophotometer. For the conversion of RNA into cDNA and its further transcription, biotin-16-dUTP (Roche Cat. No. 1-093-070) labeling was performed using the True Labeling-AMP™ Linear RNA Amplification Kit (SABiosciences). The reaction mixture was incubated for 5 hours at 37°C. The cRNA obtained was purified with the SuperArray ArrayGrade cRNA Cleanup Kit (SABiosciences). After rapid amplification and labeling of the antisense RNA, we used the Oligo GEArray® DNA Microarray Rat Signal Transduction Pathway Finder™ (SABiosciences) to profile the expression of 113 genes, representative of 18 signal transduction pathways (**Table 1**). Gene expression profiling was then conducted from the pooled total RNA (5 μg) using an Oligo GEArray® (SABiosciences) according to the manufacturer's instructions. Before prehybridization, the array membrane was wetted by adding 5 mL of deionized water to the hybridization tube, and the tube was allowed to sit inverted for at least 5 minutes. GEAhyb Hybridization Solution (SABiosciences) was warmed to 60°C and the bottle was inverted several times to allow complete dissolution of the buffer components. Hybridization solution was prepared by adding 4 μg cRNA target to a 2.0-mL aliquot

Table 1 Functional gene grouping in the Oligo GEArray® DNA Microarray: Rat Signal Transduction Pathway Finder™

Signal transduction pathway	Studied genes
Mitogenic	*EGR1, FOS, JUN, NAB2*
Wnt	*BIRC5, CCND1, JUN, LEF1, MYC, PPARG, TCF7, VEGF, WISP1, WISP2, WISP3*
Hedgehog	*BMP2, BMP4, EN1, FOXA2* (*HNF3B*), *HHIP, PTCH, PTCH2, WNT1, WNT2, WSB1*
TGF-beta	*CDKN1A, CDKN1B* (*p27*), *CDKN1C* (*p57Kip2*), *CDKN2A* (*p16Ink4*), *CDKN2B* (*p15 Ink2b*), *CDKN2C, CDKN2D* (*p19*)
Survival	PI3 Kinase/AKT pathway: *BCL2, CCND1, FN1, JUN, MMP-7* (*matrilysin*), *MYC. Jak / Src Pathway: BCL-2, BCL-2 L1.* NF-κB pathway: *BCL-2 A1* (*Bfl-1/A1*), *BIRC1* (*NAIP*), *BIRC2* (*c-IAP2*), *BIRC3* (*c-IAP1*), *TERT*
P53	*BAX, CDKN1A* (*p21Waf1/p21Cip1*), *GADD45A, IGFBP3, MDM2, TNFRSF6* (*Fas*), *TNFRSF10B* (TrailR/DR5), *TP53I3* (*PIG3*)
Stress	*ATF2, FOS, HSF1* (*tcf5*), *HSPB1* (*hsp27*), *HSPCA* (*hsp90*), *MYC, TP53*
NF-κB	*CCL20, ICAM1, IKBKB, IL1A, IL2, IL8, LTA* (*TNFb*), *NFKB1* (*NFkB*), *NFKBIA* (*IkBa*), *NOS2A* (*iNOS*), *PECAM1, TANK, TNF, VCAM1*
NFAT	*CD5, IL2, TNFSF6* (*FasL*)
CREB	*CYP19A1, EGR1, FOS*
Jak-Stat	*A2M, CSN2, CXCL9* (*MIG*), *IL4, IL4R, IRF1, MMP10* (*stromelysin-2*), *NOS2A*
Estrogen	*BCL2, BRCA1, CTSD, CXCL12, EGFR, GREB1, IGFBP4, NRIP1, PGR, RBBP8, TRIM25* (*ZNF147*)
Androgen	*CDK2, CDKN1A* (*p21Waf1/p21Cip1*), *EGFR, KLK2* (*hGK2*), *KLK3* (*PSA*), *TMEPA1*
Calcium and protein kinase C	*CSF2* (*GM-CSF*), *FOS, IL2, IL2RA, JUN, MYC, ODC1, PRKCA, PRKCB1, PRKCE, TFRC*
Phospholipase C	*BCL2, EGR1, FOS, ICAM1, JUN, JUNB, NOS2A, PTGS2* (*COX-2*), *VCAM1*
Insulin	*CEBPB* (*C/EBP-beta*), *FASN, GYS1, HK2, LEP* (*Ob*)
LDL	*CCL2, CSF2* (*GM-CSF*), *SELE, SELPLG* (*P-selectin*), *VCAM1*
Retinoic acid	*CDX1, CTSD, EN1, FLJ12541* (*Stra6*), *HOXA1, HOXB1, RBP1* (*CRBPI*), *RBP2* (*CRABPII*)

Wnt: Wingless-type MMTV integration site family; TGF-beta: transforming growth factor beta; NF-κB: nuclear factor kappaB; NFAT: nuclear factor of activated T cells; CREB: cAMP-response element binding protein; LDL: low-density lipoprotein.

Table 2 Oligo GEArray density analysis

	GenBank ID	Symbol	Description	Gene ontology term	Ratio[a]	*P*[b]
SJ5 acupuncture group *vs.* control group	NM_016993	*Bcl-2*	B-cell leukemia/ lymphoma 2	Integral to membrane; protein binding; apoptosis; anti-apoptosis; mitochondrion; cytosol; membrane	2.2362	0.0353
	XM_226742	*Birc1b*	Baculoviral IAP repeat-containing 1b	Anti-apoptosis	5.3376	0.0114
Non-acupoint group *vs.* control group	NM_133416	*Bcl2a-1*	B-cell leukemia/ lymphoma 2 related protein A1	Regulation of apoptosis; apoptosis; anti-apoptosis; intracellular	3.477898	0.00

Gene array analysis was performed using the Oligo GEArray DNA Microarray Rat Signal Transduction Pathway Finder™. Acupuncture at SJ5 or the non-acupoint upregulated the expression of the three genes listed. The normalized value for each gene was calculated as the ratio of the mean value for each gene to the mean value for GAPDH. a, Ratio: fold-change in SJ5 acupuncture group or non-acupoint group *vs.* control group; b, $P < 0.05$ was considered significant (nonparametric Student's *t*-test, $n = 3$).

of warm GEAhyb Hybridization Solution. The mixture was left overnight at 60°C with continuous agitation. A chemiluminescent array image was captured using X-ray film and a flatbed desktop scanner. The pictures were saved in grayscale 16-bit TIFF files and analyzed using the GEArray Expression Analysis Suite (SABiosciences, Frederic, MA, USA). The mean signal intensity was calculated from duplicates of each gene. Genes were considered to be differentially expressed if their expression level was at least twofold higher or lower than that in the control group.

Western blot assay

The frozen cerebellum samples were obtained after 20 minutes of acupuncture or 2 hours after acupuncture and lysed with RIPA buffer supplemented with a protease inhibitor cocktail. Protein concentrations were identified using the bicinchoninic acid assay method. Equivalent amounts of protein (30 mg/lane) were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis, on a 10% gel, and transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% (w/v) bovine serum albumin in Tris-buffered saline with Tween 20 for 1 hour, then incubated with rabbit anti-rat Bcl-2 and mouse anti-rat GAPDH antibodies (Santa Cruz Biotechnology) overnight at 4°C (1:1,000; Thermo Fisher Scientific Inc, MA, USA). The immunoblots were then incubated with goat anti-rabbit/mouse horseradish peroxidase-conjugated IgG for 2 hours at room temperature (1:1,000), and labeled proteins were detected using the Electrochemiluminescence Plus Kit (Thermo Fisher Scientific Inc, MA, USA). Band intensities obtained by western blot assay were determined using ImageJ open source software (http://rsb.info.nih.gov/ij/) and protein levels were

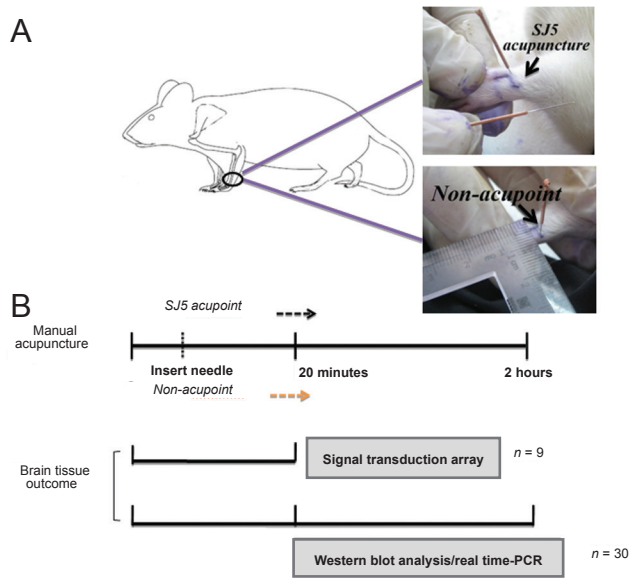

Figure 1 Acupuncture treatment and experimental procedure.
(A) Location of the San Jiao 5 acupoint (SJ5, or *Waiguan*; black arrow, upper image) and the non-acupoint used for sham acupuncture (black arrow, lower image). The non-acupoint was approximately 3 mm away from SJ5 towards the ulna. (B) Experimental schedule. Rats in the SJ5 acupuncture and non-acupoint groups received manual acupuncture for 20 minutes; control rats were restrained manually for the same duration. At cessation of acupuncture, 3 rats per group were sacrificed for gene expression analysis (signal transduction array), and 6 rats per group for western blot analysis/real time-PCR. Two hours after cessation of acupuncture, 6 further rats per group in the SJ5 acupuncture and non-acupoint groups were sacrificed for western blot/PCR.

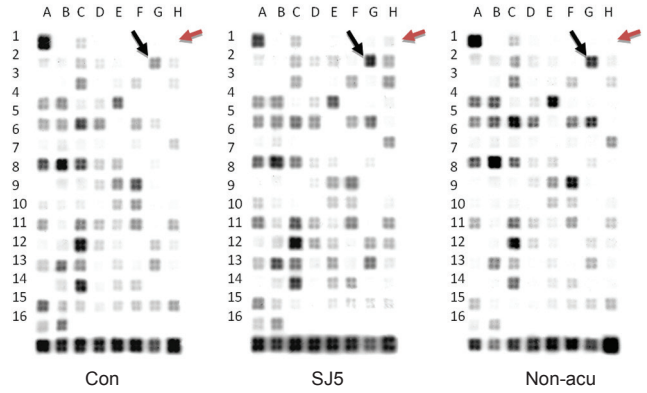

Figure 2 Oligo GEArray microarray.
Raw images of the gene arrays after hybridization with biotin-labeled cDNA probes, detected by chemiluminescence. Densitometry revealed an effect of acupuncture at SJ5 on rat cerebellum compared with controls. The upregulated genes and their corresponding positions on the microarrays are as follows: Bcl-2, red arrows; Birc1b, black arrowheads. Con: Control (handling only); SJ5: acupuncture at SJ5; Non-acu: needling at a non-acupoint 3 mm medial to SJ5.

normalized to GAPDH.

Real-time RT-PCR

Total RNA (500 ng) of the frozen cerebellum samples from animals in each group, obtained 20 minutes or 2 hours after acupuncture, was reverse-transcribed using the AMV First Strand cDNA Synthesis Kit, according to the manufacturer's

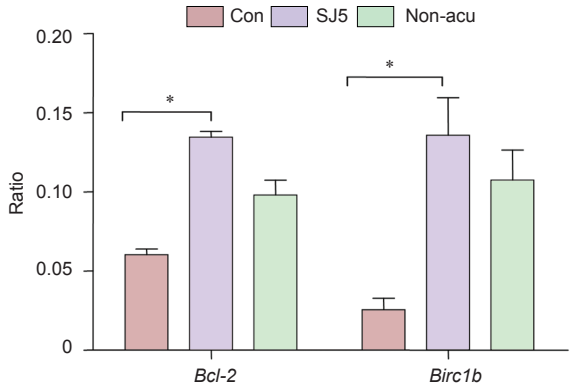

Figure 4 mRNA expression of *Bcl-2* and *Birc1b* in the cerebellum 2 hours after acupuncture (RT-PCR).
Data are presented as the mean ± SEM (n = 6 rats per group). All samples were tested at least in triplicate. Ratio represents the relative expression of the target gene in each group to that of β-actin; $^*P < 0.05$ (one-way analysis of variance followed by Tukey's honestly significant difference *post hoc* test). Con: Control (handling only); SJ5: acupuncture at SJ5; Non-acu: needling at a non-acupoint 3 mm medial to SJ5.

instructions (Thermo Fisher Scientific Inc, Waltham, MA USA; catalog No. LSK1200, USA). Complementary DNA was first synthesized from total RNA using reverse transcriptase (Takara Co., Shiga, Japan). Advanced relative expression levels of the representative genes, showing differential expressions, were monitored with the ABI StepOne Plus RT-PCR instrument (Applied Biosystems, Foster City, CA, USA) under the following conditions: 40 cycles of denaturation at 94°C for 10 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 30 seconds. Primers for Bcl-2, *Birc1b* and β-actin were designed with Primer Premier 5.0 software for the rat (Premier Biosoft International, Palo Alto, CA, USA) as follows: β-actin (229 bp), 5′-CGT AAA GAC CTC TAT GCC AAC A-3′ (forward) and 5′-CGG ACT CAT CGT ACT CCT GCT-3′ (reverse); Bcl-2 (219 bp), 5′-GCA AAG CAC ATC CAA TAA AAG C-3′ (forward) and 5′-CAT CTC CAG TAT CCC ACT CGT AG-3′ (reverse); Birc1b (110 bp), 5′-GGC TGA AGA CTT TTG TGA CCT AT-3′ (forward) and 5′-GCA GAA GCA CTG AAC CCC AT-3′ (reverse). For each reaction, 1 μL of each diluted cDNA sample was added to a mixture containing 10 μL of SYBR Green PCR Master Mix (2X) (Life Technologies, Carlsbad, CA, USA), 1 μL of each primer (10 μM), and 7 μL of ddH_2O. Mean crossing point values were obtained, and the advanced relative expression level of the target gene in each experimental group was normalized by that of β-actin. The fold change of target gene cDNA relative to the internal control (β-actin) was determined as follows: fold change = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{target} - Ct_{\beta\text{-actin}})$ test − $(Ct_{target} - Ct_{\beta\text{-actin}})$ control. Ct values were defined as the number of PCR cycles at which fluorescence signals were detected.

Statistical analysis

Scanned density differences on the Oligo GEArray® Rat Signal Transduction PathwayFinder™ Microarray between the SJ5 or non-acupoint group *vs.* control group were analyzed by nonparametric Student's *t*-test. RT-PCR and western blot assay data are presented as the mean ± SEM and analyzed by one-way analysis of variance followed by Tukey's honestly significant difference (HSD) *post hoc* test using Prism 6.0

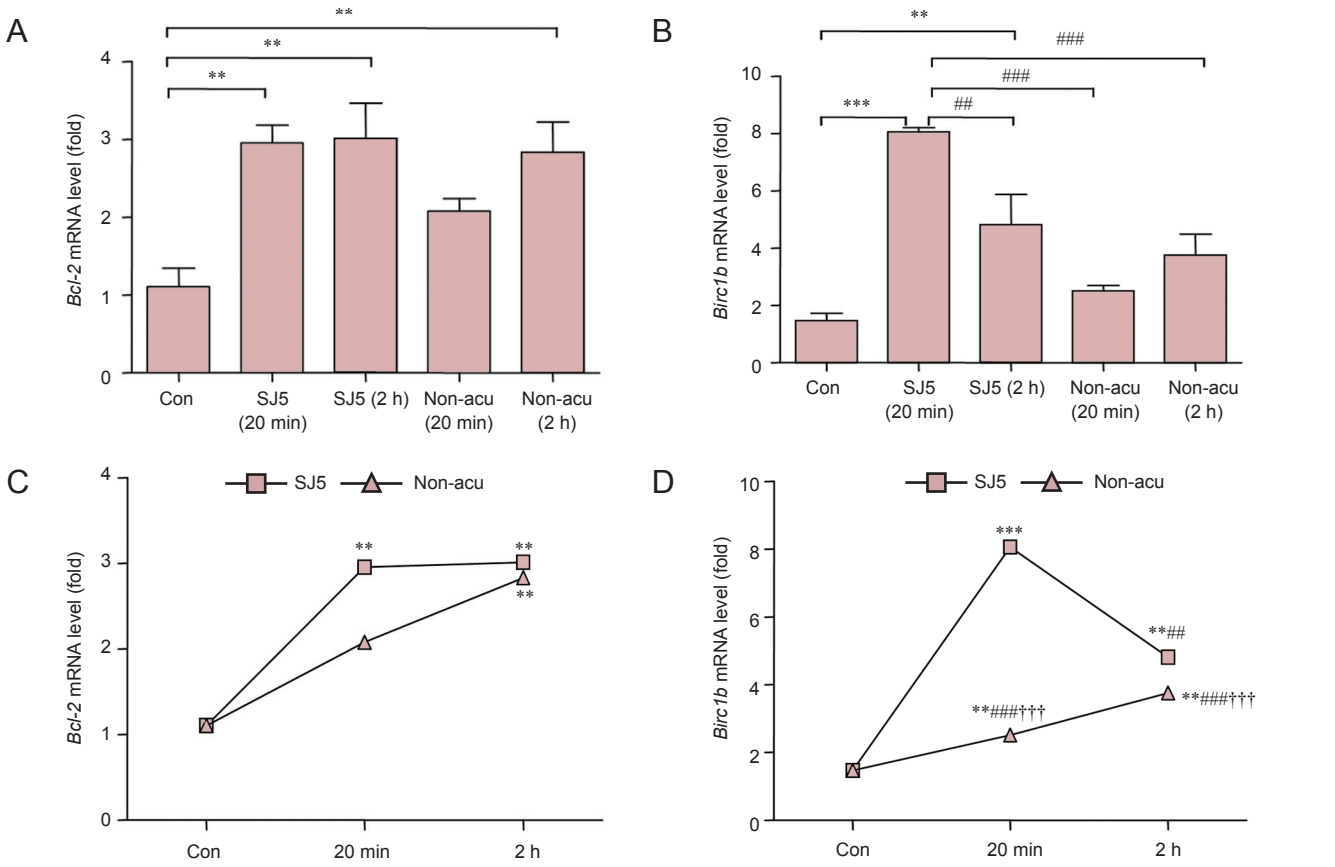

Figure 3 *Bcl-2* and *Birc1b* mRNA expression in the cerebellum after acupuncture stimulation.
Bcl-2 and *Birc1b* mRNA was detected by real-time reverse transcription PCR, and expression levels were normalized to those of the housekeeping gene, *β-actin*. All samples were tested at least in triplicate. (A, C) At 20 minutes, *Bcl-2* mRNA expression was significantly elevated in the SJ5 group (**$P < 0.01$, *vs.* control), but there was no significant difference between the non-acupoint (Non-acu) and control (Con) groups. Interestingly, after 2 hours, *Bcl-2* mRNA expression was upregulated in both the SJ5 and Non-acu groups (**$P < 0.01$, *vs.* Con). (B, D) *Birc1b* mRNA expression showed a marked increase after 20 minutes of acupuncture at SJ5 (***$P < 0.001$, *vs.* Con), but decreased after 2 hours (**$P < 0.001$, *vs.* Con; ##$P < 0.01$, ###$P < 0.001$, *vs.* SJ5 acupuncture). Moreover, *Birc1b* mRNA expression in the SJ5 acupuncture group was higher than that in the Non-acu group at both time points (†††$P < 0.001$, *vs.* SJ5 acupuncture). Data are presented as the mean ± SEM; one-way analysis of variance followed by Tukey's honestly significant difference *post hoc* test. ST5 (20 min), SJ5 (2 h): Acupuncture at the SJ5 acupoint for 20 minutes and 2 hours respectively; Non-acu (20 min), Non-acu (2 h): acupuncture at a non-acupoint 3 mm medial to SJ5 for 20 minutes and 2 hours respectively.

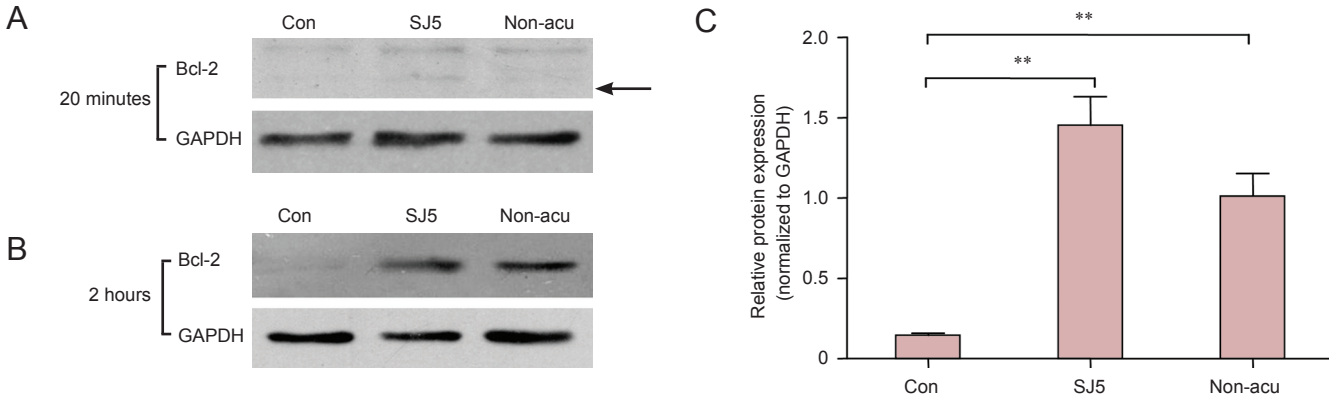

Figure 5 Bcl-2 protein expression in the cerebellum (western blot assay).
(A) After 20 minutes of acupuncture, there was no difference between the three groups in Bcl-2 protein expression in the cerebellum ($n = 6$ rats per group). (B, C) Two hours after cessation of acupuncture, Bcl-2 expression in the SJ5 and non-acupoint groups ($n = 6$ rats per group) was significantly greater than in the control group (**$P < 0.01$, *vs.* control; one-way analysis of variance followed by Tukey's honestly significant difference *post hoc* test). Data are presented as the mean ± SEM ($n = 6$ rats per group). All samples were tested at least in triplicate. Con: Control (handling only); SJ5: acupuncture at SJ5; Non-acu: needling at a non-acupoint 3 mm medial to SJ5.

(GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Identification of differential gene expression between acupuncture at SJ5 and at the non-acupoint in rat cerebellum

113 genes associated with 18 signal transduction pathways were screened by the Oligo GEArray™ system (**Table 1**). The hybridized signal detected for each gene was normalized to the signal obtained for GAPDH in the same gene array to derive the expression value for each gene. Expression of *Bcl-2* and *Birc1b* was elevated in distinct survival signaling pathways in the SJ5 acupuncture group compared with the control group (**Figure 2**; **Table 2**). The remaining candidate genes were excluded because the confirmatory analyses (n = 3) did not show a statistically significant difference in their expression levels between the two groups (SJ5 acupuncture *vs.* control, and non-acupoint *vs.* control), even if gene expression showed a twofold difference from the control group. These results suggest that after 20 minutes, stimulation at SJ5 and at the non-acupoint upregulated gene expression in distinct survival signaling pathways.

Acupuncture stimulation at SJ5 increased mRNA expression of *Bcl-2* and *Birc1b* in the cerebellum 2 hours after cessation

To validate the gene expression data obtained from the microarray, we analyzed *Bcl-2* and *Birc1b* mRNA expression using real-time PCR. Normalized mRNA levels of *Bcl-2* and *Birc1b* in the SJ5 and non-acupoint groups were significantly higher than those in the control group ($P < 0.01$; **Figure 3A**, **B**). At 20 minutes, *Bcl-2* and *Birc1b* expression was greater in the SJ5 and non-acupoint groups than in the control group ($P < 0.001$), confirming the microarray data. After 2 hours, *Bcl-2* expression remained elevated ($P < 0.01$), but *Birc1b* expression had decreased. Interestingly, the non-acupoint group showed the same tendency as the SJ5 group (**Figure 3C**, **D**), but the expression of these two genes remained lower than that in the SJ5 group ($P < 0.001$). The relative expression of *Bcl-2* and *Birc1b* mRNA, normalized against β-actin, are shown in **Figure 4**.

Acupuncture at SJ5 increased Bcl-2 protein expression in the rat cerebellum 2 hours after acupuncture

Western blot assay showed that, 2 hours after acupuncture, protein expression of Bcl-2 in the SJ5 acupuncture group was significantly greater than that in the control group ($P < 0.01$; **Figure 5**). Compared with the control group, needling at the non-acupoint upregulated Bcl-2 protein expression after 2 hours ($P < 0.01$). No significant difference was observed between the SJ5 and non-acupoint groups ($P > 0.05$).

Discussion

It was recently reported that the effects of acupuncture start 15 minutes after the treatment, and are maintained for at least 30 minutes after completion of the treatment (Jiang et al., 2014). A study of the post-stimulation effects of electroacupuncture at the *Yintang* (EX-HN3) and *Baihui* (GV20) acupoints revealed that the cerebellum was activated 15 minutes after stimulation by electroacupuncture, and that this level of brain activation was higher at 15 minutes after treatment than at 5 minutes after treatment (Zheng et al., 2012). In the present study, after needle withdrawal, acupuncture modulation lasted up to 20 minutes in the cerebellum, manifesting as elevated *Bcl-2* and *Birc1b* expression. The Bcl-2 gene was maintained at this high level of expression for 2 hours after treatment, and was significantly higher in the SJ5 acupuncture group than in the control group. Our data provides further evidence that the effects of acupuncture start several minutes after treatment, and last beyond its cessation. At least a 30-minute treatment period might be needed for the full expression of the analgesic effects of acupuncture, and no less than 15 minutes may be needed to produce the initial treatment effect (Jiang et al., 2014). All of these findings are consistent with the conventional clinical practice of acupuncture (Han, 2011). A distinguishing aspect of acupuncture is its ability to trigger significant modulation in different areas of the brain, in addition to changes in blood pressure and cardiovascular excitatory reflex responses (Li et al., 2010, 2012). Our present findings suggest that acupuncture at SJ5 can initiate protein changes in the cerebellum related to neuroprotection, and this effect may last from 20 minutes to 2 hours after treatment. This result is consistent with previous clinical and experimental studies (Zhou et al., 2005; Li and Longhurst, 2010), such as the prolonged influence of acupuncture in hypertension (Li et al., 2010, 2012). Together, this evidence indicates that acupuncture stimulation has strong and lasting after-effects on cerebellar function.

As a consequence of acupuncture-induced variations in neural activity, the expression of Bcl-2 and *Birc1b* were elevated compared with the control group. Bcl-2 is the prototype member of the anti-apoptotic Bcl-2 family (Korsmeyer, 1999). Bcl-2 mRNA and protein are present at relatively high levels in the developing nervous system, and decline sharply in the postnatal brain (Abe-Dohmae et al., 1993; Merry et al., 1994; Akhtar et al., 2004). In the adult brain, neuronal overexpression of Bcl-2 in transgenic mice promotes neuronal survival in many brain regions by inhibiting naturally occurring cell death (Farlie et al., 1995). This means that by regulating cell death in the mature nervous system, Bcl-2 may be able to reverse neurodegenerative pathologies (Marshall et al., 1997). In the present study, microarray analysis revealed upregulation of the survival signaling pathway members *Bcl-2* and *Birc1b* in the SJ5 acupuncture group, and of Bcl-2 in the non-acupoint group, compared with the control group. Just being consistent with the gene expression array, we also found that there was no significant difference between the SJ5 and the non-acupoint acupuncture. This suggests that acupuncture stimulation (including the insertion of a needle) may play an important role in the effects of acupuncture. Our data support a recent study in which it was shown that acupuncture at the *Baihui* (GV20) and *Dazhui* (GV14) acupoints increased Bcl-2 expression, as well as reducing neuronal loss in the hippocampus and frontal lobe, and attenuating ultrastructural damage in ischemic rat brain (Hou et al., 2014). Together, this evidence supports the theory that acupuncture stimulation has neuroprotective effects and can mediate anti-apoptosis.

In conclusion, our results indicate that acupuncture

mediates brain function by regulating the expression of the anti-apoptotic factors *Bcl-2* and *Birc1b*, in turn affecting the survival signal pathway. Our study suggests that acupuncture has a promising role in the protection of the brain and central nervous system from injury.

Author contributions: *DL performed acupuncture, data analysis, fundraising, and wrote the paper. LLL performed PCR and western blots. KS provided critical revision of the paper for intellectual content. CHC designed the study and performed parts of the experiment. All authors approved the final version of this paper.*

Conflicts of interest: *None declared.*

Plagiarism check: *This paper was screened twice using Cross-Check to verify originality before publication.*

Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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Copyedited by Slone-Murphy J, Robens J, Li CH, Song LP, Zhao M