NEURAL REGENERATION RESEARCH
April 2016, Volume 11, Issue 4
www.nrronline.org

RESEARCH ARTICLE

Electroacupuncture at *Baihui* (DU20) acupoint up-regulates mRNA expression of NeuroD molecules in the brains of newborn rats suffering *in utero* fetal distress

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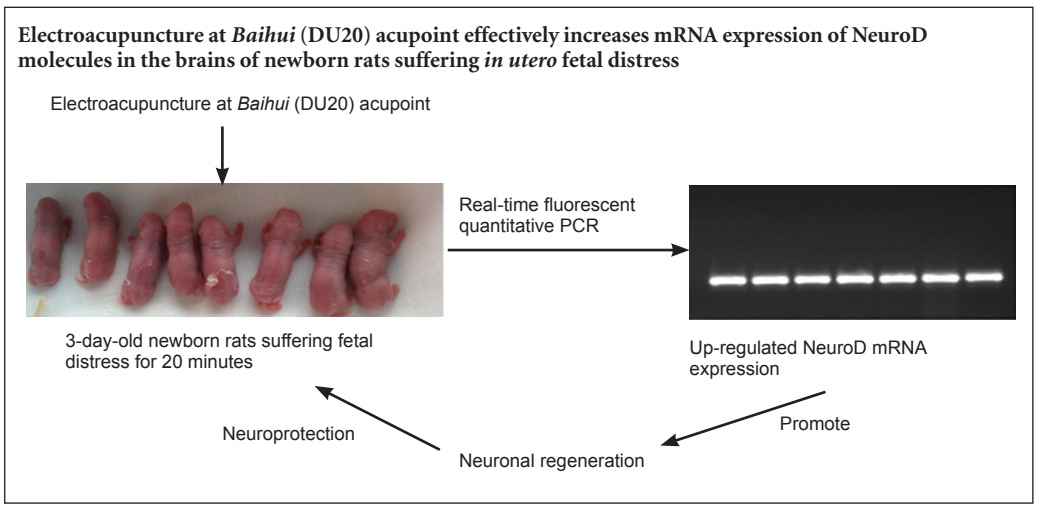
How to cite this article: *Chen L, Liu Y, Lin QM, Xue L, Wang W, Xu JW (2016) Electroacupuncture at Baihui (DU20) acupoint up-regulates mRNA expression of NeuroD molecules in the brains of newborn rats suffering in utero fetal distress. Neural Regen Res 11(4):604-609.*

Funding: *This research was supported by the Natural Science Foundation of Fujian Province of China, No. 2015J01133; the Professor Academic Development Foundation of Fujian Medical University of China, No. JS11003.*

Graphical Abstract



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doi: *10.4103/1673-5374.180745*
http:*//www.nrronline.org/*

Accepted: *2016-03-07*

Abstract

NeuroD plays a key regulatory effect on differentiation of neural stem cells into mature neurons in the brain. Thus, we assumed that electroacupuncture at *Baihui* (DU20) acupoint in newborn rats exposed to *in utero* fetal distress would influence expression of NeuroD. Electroacupuncture at *Baihui* was performed for 20 minutes on 3-day-old (Day 3) newborn Sprague-Dawley rats exposed to *in utero* fetal distress; electroacupuncture parameters consisted of sparse and dense waves at a frequency of 2–10 Hz. Real-time fluorescent quantitative PCR results demonstrated that mRNA expression of NeuroD, a molecule that indicates NeuroD, increased with prolonged time in brains of newborn rats, and peaked on Day 22. The level of mRNA expression was similar between Day 16 and Day 35. These findings suggest that electro acupuncture at *Baihui* acupoint could effectively increase mRNA expression of molecules involved in NeuroD in the brains of newborn rats exposed to *in utero* fetal distress.

Key Words: *nerve regeneration; brain injury; in utero fetal distress; hypoxic-ischemic brain injury; electroacupuncture; real-time fluorescent quantitative PCR; NeuroD; nerve repair; Baihui (DU20) acupoint; non-acupoint; neural regeneration*

Introduction

Fetal distress *in utero*, a major factor for hypoxic-ischemic encephalopathy, can cause permanent neurological disorders such as mental retardation, movement disorders, and even death (Group of Neonatology et al., 2005; Kadam et al., 2007; Pichiule et al., 2007). Previous transplantation studies have shown limited survival of neural stem cells. Those that do survive primarily differentiate into glial cells, as well as a small number of neurons (An et al., 2002; Jeong et al., 2003; Qun et al., 2010; Fan et al., 2013; Tu et al., 2014; Yan et al., 2014). The microenvironment greatly influences the proliferation and differentiation of neural stem cells (NSCs), and the manipulation of the microenvironment has become the focus of studies attempting to promote neuronal differentiation.

The microenvironment described in modern medicine is analogous to the "sea of marrow" in traditional Chinese

medicine. Hypoxic-ischemic encephalopathy is considered a "deficiency of marrow-reservoir" in newborn infants. *Baihui* (DU20) acupoint is strongly associated with the brain, and its name, "One Hundred Meetings," refers to the convergence of all *yang* meridians in the body; acupuncture at *Baihui* acupoint tonifies the marrow and brain. Electroacupuncture (EA) is the combination of traditional acupuncture, moxibustion, and electrical stimulation, and is a form of acupuncture where a small electrical current is passed between pairs of acupuncture needles, which induces a needling sensation. Recent studies have focused on electroacupuncture-induced differentiation of NSCs into neurons, and the effect of EA has been affirmed (Kang et al., 1995; Fumagall et al., 2009; Stone et al., 2011). Acupuncture has been shown to effectively mitigate hypoxic-ischemic brain injury by resisting free radicals, improving electrical activity in the brain and cerebral microcirculation, and affecting neurotrophic factor secretion and angiogenesis (Liu et al., 2006; Chung et al., 2007; Yang et al., 2008; Stone et al., 2011). Several studies have shown that EA has an anti-apoptotic effect, and can promote the formation of new mature neurons (Lee et al., 1995; Wang et al., 2011; Guo et al., 2012).

NeuroD is expressed throughout the peripheral nervous system and central nervous system (Lawler et al., 2012; Mu et al., 2014; Xu et al., 2014), and plays an important role in regulating NSCs differentiation into mature neurons (Lee et al., 1997; Roybon et al., 2009; Hu et al., 2013). We presumed that EA could regulate NeuroD expression by affecting the brain microenvironment. This study measured the effects of EA on NeuroD mRNA expression in the brains of newborn rats exposed to *in utero* fetal distress by using real-time fluorescent quantitative PCR.

Materials and Methods

Experimental animals

Ten specific pathogen-free, male Sprague-Dawley rats weighing 200 ± 20 g and 20 female rats weighing 220 ± 20 g were provided by the Experimental Animal Center of Fujian Medical University of China [license No. SCXK (Min) 2012-0001]. All rats were allowed to acclimatize to their new environment for 2 weeks prior to placement in the same cage. All rats were maintained and housed under controlled conditions at 22°C in a 12-hour reversed light/dark cycle with free access to food and water. All surgeries were performed under anesthesia, and all efforts were made to minimize pain and distress of the experimental animals. All animal experiments were performed in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animal (NIH Publication No. 85-23, revised 1986). The experiments were approved by the Animal Ethics Committee of Fujian Medical University of China (Approval No. 2014-12).

Establishment of a rat model of fetal distress *in utero* and EA intervention

Male and female rats were placed in the same cage at 18:00. Vaginal smears were used to detect sperm every morning at 7:00 a.m. Under a light microscope (Olympus, Tokyo, Japan), the first day of observing sperm was considered embryonic day 1 (E1). On E22, newborn rats delivered by natural birth were considered as the blank control group ($n = 6$). Pregnant rats on E22 were continuously anesthetized with an appropriate amount of anhydrous diethyl ether. *Via* cesarean section, a median incision was made on the abdomen to expose the uterus. Bilateral uterine arteries and bilateral ovarian arteries were occluded with a bulldog clamp for 20 minutes. The fetuses were then removed.

Newborn rats exposed to *in utero* fetal distress were randomly assigned to a 20-minute fetal distress group, 20-minute fetal distress + *Baihui* group, 20-minute fetal distress + non-acupoint group, and a blank control group, with six rats in each group. Newborn rats at the age of 3 days (day 3) in the 20-minute fetal distress + *Baihui* group and 20-minute fetal distress + non-acupoint group were treated with EA using an electroacupuncture stimulator (KWD808-I; Changzhou Yingdi Electronic Medical Device Co., Ltd., Changzhou, China). In accordance with an animal acupoint atlas from Experimental Acupuncture Science, the needle (0.5 cun, 1 *cun* = 3.33 cm; Suzhou Medical Sino-foreign Joint Venture Suzhou Hua Tuo Medical Instruments Co., Ltd., Suzhou, Jiangsu Province, China) was inserted into the *Baihui*, at a depth of 1 cm. The non-acupoint was 0.5–1.0 cm right to *Baihui*, with a needling depth of 1 cm. The EA parameters were as follows: sparse and dense waves with a frequency of 2–10 Hz; needle handle tremor verified that the electric current was stimulating muscle contraction and was used to determine the most effective intensity. The rats did not exhibit any stress. EA was performed at 17:00, once a day for 15 minutes, for 7 consecutive days.

Total brain RNA extraction

On Days 16, 22, and 35 (*i.e.*, 7, 13 and 26 days after treatment), rats from each group were anesthetized with 10% chloral hydrate and sterilized with 75% ethanol. Brain tissue, except the cerebellum, was frozen at −80°C, and then triturated with liquid nitrogen. Trizol (1 mL/100 mg) was added and incubated at room temperature for 15 minutes without stirring. Then, 200 μL of chloroform/1 mL Trizol was added and further incubated at room temperature for 10 minutes without stirring. The samples were centrifuged at 4°C and 12,000 r/min for 15 minutes. The upper aqueous layer was removed to a new RNase-free Eppendorf tube, and an equal volume of precooled isopropanol was added. After shaking, the mixture was incubated at room temperature for 10 minutes, and centrifuged at 4°C and 12,000 r/min for 15 minutes. After removal of the supernatant, the precipitate was washed with 1 mL 75% ethanol and centrifuged at 7,500 r/min for 5 minutes. After removal of the supernatant, the precipitate was allowed to rest without stirring for 30 seconds, and 30–60 μL of RNase-free water was added and mixed with a pipette, followed by incubation in a 65°C water bath for dissolution. A small amount of total RNA was detected by 1% nondenaturing agarose gel electrophoresis. The ratio of absorbance at 260 nm and 280 nm ($A_{260\ nm/280\ nm}$) was measured with an ultraviolet spectrophotometer (NanoDrop 2000; Thermo, Waltham, MA, USA).

cDNA preparation and PCR assay for determining primer specificity

RT-PCR was performed in accordance with a TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech, Beijing, China). Each reaction system contained 2 μg total RNA, 10 μL of 2× TS Reaction Mix, 1 μL of TransScript® RT/RI Enzyme Mix, 1 μL of genomic DNA. RNase-free water was added to a total volume of 20 μL. All reagents were incubated at 42°C for 30 minutes, followed by 85°C for 5 minutes. RNA was efficiently reverse transcribed into cDNA and stored in an iced bath. The cDNA was diluted five-fold and considered as a template. The system was as follows: 10 μL of 2× EasyTaq PCR SuperMix, 0.8 μL of upstream primer (10 μM), 0.8 μL of downstream primer (10 μM), and 1 μL of cDNA template. RNase-free water was added to a total volume of 20 μL. Reaction conditions were as follows: initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 20 seconds, followed by extension at 72°C for 7 minutes. Primer specificity was detected by agarose gel electrophoresis with 5 μL of PCR products.

The primers used are as follows:

Gene	Primer sequence (5'–3')
β-Actin	Forward: 5'-CGG TCA GGT CAT CAC TAT CG-3' Reverse: 5'-ACT GTG TTG GCA TAG AGG TCT T-3'
NeuroD	Forward: 5'-TCC AGG GTT ATG AGA TCG TCA CTA TTC-3' Reverse: 5'-TCC TTC TTG TCT GCC TCG TGT TCC-3'

Real-time fluorescent quantitative PCR for NeuroD gene expression

The reaction system was composed of 10 μL of TransStart Tip Green qPCR SuperMix (2×), 0.4 μL of PCR Forward Primer (10 μM), 0.4 μL of PCR Reverse Primer (10 μM), 0.4 μL of Rox Reference Dye II (50×), and 2 μL of cDNA template. Double-distilled water was added to a total volume of 20 μL. Three parallel wells of internal reference gene and target gene from each sample were set for each group. The total reaction solution was prepared, and then placed separately. With an Applied Biosystems 7500 Real-Time PCR System (ABI, Foster, CA, USA), the reaction conditions were as follows: initial denaturation at 94°C for 30 seconds, 40 cycles of denaturation at 94°C for 5 seconds, annealing and extension at 60°C for 34 seconds. Melting curves at 60–95°C were analyzed. Relative changes in gene expression were analyzed using the $2^{-\Delta\Delta Ct}$ method for relative quantitation.

Statistical analysis

Measurement data are expressed at the mean ± SD, and analyzed using SPSS 21.0 software (IBM, New York, NY, USA). One-way analysis of variance and the least significant difference *t*-test were used to compare differences in intergroup data. A value of $P < 0.05$ was considered statistically significant.

Results

Identification of total RNA in the brains of newborn rats exposed to *in utero* fetal distress

Agarose gel electrophoresis results revealed clear 28S rRNA and 18S rRNA bands. The brightness of 28S bands was one- or two-folds greater than the 18S bands (**Figure 1**). Spectrophotometry results demonstrated that the ratio of $A_{260\ nm/280\ nm}$ was 1.9–2.1. The results indicated intact RNA of a high purity from the brain material.

Primer specificity as detected by real-time fluorescent quantitative PCR for *NeuroD*

Gel electrophoresis results showed single bands of products amplified by β-actin primers on the left and products amplified by NeuroD primers on the right visible at 200 bp. The background was clear. Nonspecific bands and primer dimmers were not found (**Figure 2A**). Melting curve analysis results showed a single peak of each product (**Figure 2C**). S-shaped amplification curves presented with good fitting, and Ct values were similar (**Figure 2B**). These results confirmed good primer design, stable reaction system, uniform template allocation, and reliable outcomes.

On Day 16, NeuroD mRNA expression was higher in the 20-minute fetal distress + *Baihui* group and 20-minute fetal distress + non-acupoint group compared with the blank control group ($P < 0.01$), and NeuroD mRNA expression was highest in the 20-minute fetal distress + *Baihui* group ($P < 0.05$). On Day 22, NeuroD mRNA expression reached a peak in each group. NeuroD mRNA expression was significantly different among groups: the blank control group < 20-minute fetal distress group < 20-minute fetal distress + non-acupoint group < 20-minute fetal distress + *Baihui* group ($P < 0.01$ or $P < 0.05$). On Day 35, NeuroD mRNA expression was significantly higher than in the blank control group ($P < 0.05$). NeuroD mRNA expression was highest in the 20-minute fetal distress + *Baihui* group ($P < 0.01$; **Figure 2D**). These findings suggested that EA induced a trend of increased NeuroD mRNA expression, which peaked and then decreased again. EA had a significant effect on the 20-minute fetal distress + *Baihui* group.

Discussion

This study established models of hypoxic-ischemic encephalopathy based on results from a previous study (Bjelke et al., 1991). In traditional Chinese medicine, the key for treating hypoxic-ischemic encephalopathy is *Qi* and blood support in the brain and spinal cord (Lei et al., 2011). In accordance with the animal acupoint atlas in Experimental Acupuncture Science and Science of Acupuncture and Moxibustion, *Baihui*, which is located at the right midpoint of the parietal bone, was selected in this study; the non-acupoint was 0.5–1.0 cm right to *Baihui* acupoint (Huang et al., 2013).

EA was used to treat 3-day-old newborn rats, and the effect of EA on NeuroD mRNA expression was examined. EA has been previously shown to improve collateral circulation, reduce tissue swelling, regulate cell metabolism, affect synthesis and release of neurotransmitters, signaling molecules, and

apoptosis-related proteins, adjust phosphorylation levels, and induce neuronal recovery after hypoxia and ischemia (Kaneto et al., 2009; Ohira, 2011; Zhang et al., 2011). EA is used to rhythmically stimulate a specific acupoint by pulse current to accelerate meridian-*QI* and blood circulation (Wu, 2013).

In the present study, real-time fluorescent quantitative PCR results demonstrated that NeuroD mRNA expression was greatest in the 20-minute fetal distress + *Baihui* group on Days 16, 22, and 35. NeuroD mRNA expression was higher in the 20-minute fetal distress + non-acupoint group than in the 20-minute fetal distress group on Day 22. The effect of acupuncture at non-acupoint is not theoretically identical to that at an acupoint (such as *Baihui*). In the present study, the position of the non-acupoint (0.5–1.0 cm right to *Baihui*) was close to *Baihui*. The EA frequency at the non-acupoint was thought to affect *Baihui acupoint*, thereby exerting a weak effect on reinforcing marrow and benefiting brain. However, the precise mechanism remains unclear. Zhao et al. (2012) considered that two regions far from each other in the body (an acupoint and a special region of the body) had a functional connection. For example, acupuncture at *Baihui* acupoint could tonify the marrow and brain (Kuhn et al., 1999; Song et al., 2002; Jia et al., 2005).

EA has been shown to improve brain injury by changing the microenvironment, inhibiting apoptosis, and promoting neural regeneration, and early intervention results in better outcomes (Rui et al., 2011; Song et al., 2013; Zhu et al., 2013; Huang et al., 2014; Tao et al., 2014; You et al., 2014). Thus, we began treatment of rats at 3 days of age. Results showed that NeuroD mRNA expression was higher in the 20-minute fetal distress + *Baihui* group and 20-minute fetal distress + non-acupoint group than in the blank control group on Day 35. NeuroD plays a positive regulatory role in neuronal differentiation of NSCs (Lee et al., 1997; Roybon et al., 2009; Hu et al., 2013).

In summary, EA might effectively improve the brain microenvironment by affecting mechanisms related to neural regeneration and neuroprotection. Previous studies have shown that NeuroD contribute to neural regeneration and neuroprotection against hypoxic-ischemic brain injury, and results from the previous study showed that EA increased NeuroD mRNA expression. This study provided an important basis for clinical studies regarding the effects of EA on hypoxic-ischemic brain injury.

Acknowledgments: *We are very grateful to Wei Guo from the Department of Human Anatomy, Histology and Embryology, School of Basic Medical Sciences, Fujian Medical University, China for guiding experimental procedure, and every teacher from the Department of Human Anatomy, Histology and Embryology, School of Basic Medical Sciences, Fujian Medical University, China for their suggestions and assistance on experiments.*

Author contributions: *LC conceived and designed the study, performed experiments, ensured the integrity of the data, analyzed data, and wrote the paper. YL and LX performed experiments, and collected data. QML provided assistance in experimental technique. JWX conceived the study, provided experimental guidance and technical support, and obtained funding. All authors approved the final version of the 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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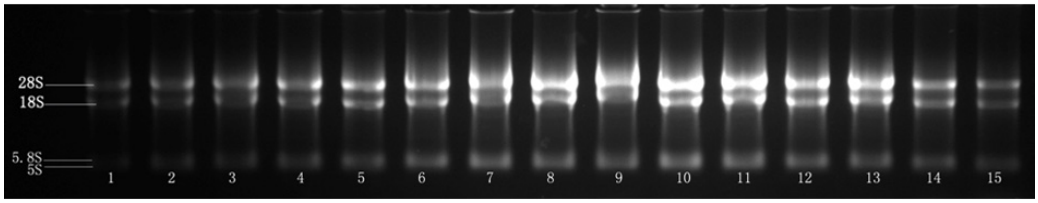

Figure 1 Agarose gel electrophoresis results of total RNA in the brains of newborn rats exposed to *in utero* fetal distress.
1–3: Blank control group; 4–7: 20-minute fetal distress group; 8–11: 20-minute fetal distress + *Baihui* group; 12–15: 20-minute fetal distress + non-acupoint group. Gel imaging system shows distinct bands of total RNA from each sample.

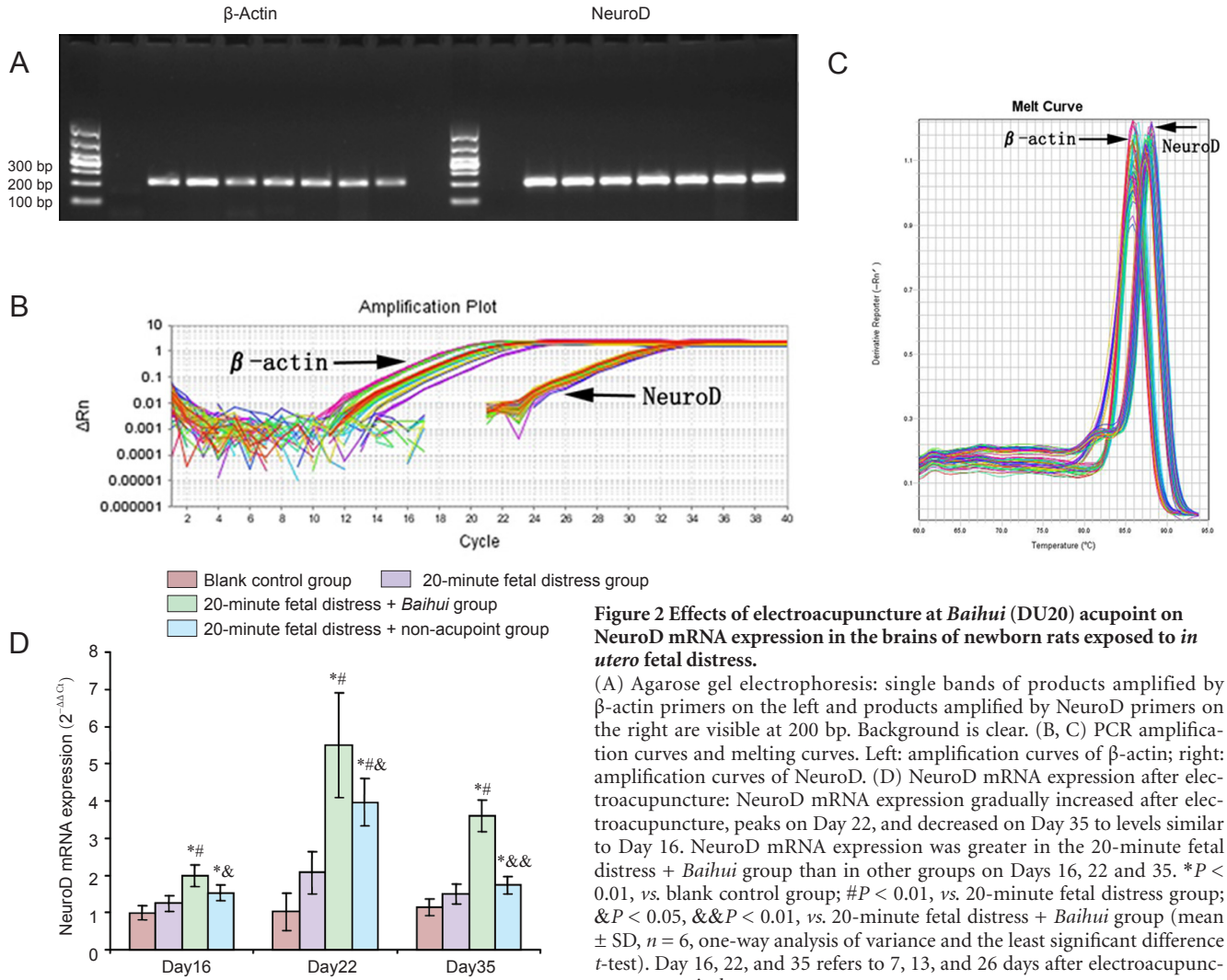

Figure 2 Effects of electroacupuncture at *Baihui* (DU20) acupoint on NeuroD mRNA expression in the brains of newborn rats exposed to *in utero* fetal distress.
(A) Agarose gel electrophoresis: single bands of products amplified by β-actin primers on the left and products amplified by NeuroD primers on the right are visible at 200 bp. Background is clear. (B, C) PCR amplification curves and melting curves. Left: amplification curves of β-actin; right: amplification curves of NeuroD. (D) NeuroD mRNA expression after electroacupuncture: NeuroD mRNA expression gradually increased after electroacupuncture, peaks on Day 22, and decreased on Day 35 to levels similar to Day 16. NeuroD mRNA expression was greater in the 20-minute fetal distress + *Baihui* group than in other groups on Days 16, 22 and 35. *$P < 0.01$, *vs.* blank control group; #$P < 0.01$, *vs.* 20-minute fetal distress group; &$P < 0.05$, &&$P < 0.01$, *vs.* 20-minute fetal distress + *Baihui* group (mean ± SD, $n = 6$, one-way analysis of variance and the least significant difference *t*-test). Day 16, 22, and 35 refers to 7, 13, and 26 days after electroacupuncture, respectively.

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