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Dark rearing maintains tyrosine hydroxylase expression in retinal amacrine cells following optic nerve transection☆

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Abstract

The present study examined changes in retinal tyrosine hydroxylase (TH) expression in rats having undergone optic nerve transection and housed under a normal day/night cycle or in the dark. The aim was to investigate the effects of amacrine cells on axonal regeneration in retinal ganglion cells and on the synapses that transmit visual signals. The results revealed that retinal TH expression gradually decreased following optic nerve transection in rats housed under a normal day/night cycle, reaching a minimum at 5 days. In contrast, retinal TH expression decreased to a minimum at 1 day following optic nerve transection in dark reared rats, gradually increasing afterward and reaching a normal level at 5–7 days. The number of TH-positive synaptic particles correlated with the TH levels, indicating that dark rearing can help maintain TH expression during the synaptic degeneration stage (5–7 days after optic nerve injury) in retinal amacrine cells.

Key Words: optic nerve transection; tyrosine hydroxylase; dark rearing; amacrine cells; peripheral nerve injury; neural regeneration

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INTRODUCTION

Previous studies examining optic nerve regeneration following injury have mainly focused on the effects of trophic factors on retinal ganglial cell (RGC) survival[1-8]. Very little information is available on the changes in the microenvironment or on neuronal number or synaptic structure in the retina following optic nerve injury. It has been reported that visual function cannot completely recover after optic nerve injury[9-11]. Changes in synaptic structure and number play major roles in the regeneration of the optic nerve[11]. The internal structures of the retina remain normal in the early stage following optic nerve transection, and a light stimulus can be transmitted to bipolar cells through the photoreceptors. It was reported that a light stimulus could delay RGC apoptosis[12], but that bipolar cells were excessively activated due to blocking of RGC-derived trophic support and optical signal transduction, resulting in disorders in synaptic transmission between RGCs and bipolar cells[13]. Dopaminergic amacrine cells are sensitive to light stimulation and can synthesize and release dopamine (DA) to promote the formation of synaptic connections with bipolar cells and RGCs[14-15], helping to improve retinal visual connectivity. Dopamine may modulate the response of bipolar cells to light stimulation following optic nerve transection, but the mechanism remains poorly understood[16-18]. Increasing or inhibiting tyrosine hydroxylase (TH) activity can significantly influence dopamine production and alter its biological effects[19-22]. Insufficient dopamine in the retina is one of pathological features of visual dysfunction associated with Parkinson's disease[23]. TH is a specific marker of dopaminergic amacrine cells in the retina, expressed in the somata and processes of these cells. Therefore, changes in TH expression likely reflect functional changes in dopaminergic amacrine cells[22-23].

To investigate the protective effects of dark rearing on retinal visual circuitry and the influence of light stimulation on TH expression, we used the rat model of optic nerve transection and subjected the animals to a dark or normal day/night cycle. TH-positive amacrine cells and TH protein expression were assessed using immunohistochemistry and western blotting.

RESULTS

Quantitative analysis of experimental animals

A total of 144 Sprague-Dawley rats were

randomly assigned to normal (n = 12), sham-surgery (n = 12) and injury (optic nerve transection; n = 120) groups. Half of the animals in each group were reared in the dark and the other half in a normal day/night cycle. The injury group was sampled at 1, 3, 5, 7 and 14 days following optic nerve transection, with 12 animals at each time point. The optic nerve of animals in the sham-surgery group was not transected, and the normal group was untreated. A total of 144 rats were included in the final analysis.

Effect of rearing condition on retinal TH expression

Immunohistochemistry showed TH immunoreactivity in the inner plexiform layer and in the inner nuclear layer of the normal retina. TH-positive amacrine cells were few and distributed in the inner nuclear layer (Figures 1A, H). The pattern of TH expression was similar among the sham-surgery and normal groups (Figures 1B, I).

The pattern of TH expression in animals from the injury group reared in the normal day/night cycle for 1, 3, 5, 7 and 14 days is shown in Figures 1C–G. Compared with the normal group, retinal TH expression was reduced following optic nerve transection in the injury group reared in the normal day/night cycle, reaching a minimum at 5 days, increased to the normal level at 7 days, and declined at 14 days (P < 0.05; Figure 1O, supplementary Table 1 online).

The pattern of TH expression in animals from the injury group reared in the dark for 1, 3, 5, 7 and 14 days is shown in Figures 1J–N. Compared with the normal group, retinal TH expression reached a minimum following optic nerve transection in the injury group reared in the dark, increased gradually afterward, reached a normal level by 5–7 days, and declined at 14 days (P < 0.05; Figure 1O, supplementary Table 1 online).

Compared with rats reared in a normal day/night cycle, TH expression in animals from each group reared in the dark was reduced at 1 day, but increased at 3 and 5 days (P < 0.05; Figure 1O, supplementary Table 1 online).

Effect of rearing condition on the number of retinal TH-positive synaptic particles

Immunofluorescence double-labeling showed that TH-positive synapses were mainly distributed in the inner plexiform layer, forming synaptic connections with protein kinase C alpha (PKC-α)-positive bipolar cells (Figure 2).

Changes in the number of TH-positive synaptic particles were consistent with the area of TH-positive expression in rats reared under different conditions, except that the number of TH-positive synaptic particles remained elevated at 14 days following optic nerve injury in rats reared in the dark (Figure 2, supplementary Table 2 online).

Compared with rats reared in a normal day/night cycle, the number of TH-positive synaptic particles in animals from each group reared in the dark increased at 5 days (P < 0.05), but there was no significant difference between the two conditions at other time points (P > 0.05; Figure 2N, supplementary Table 2 online).

Effect of retinal TH protein expression in rats reared in normal and dark conditions following optic nerve injury

The results of western blot analysis of retinal homogenates were consistent with the immunohistochemical observations (Figure 3).

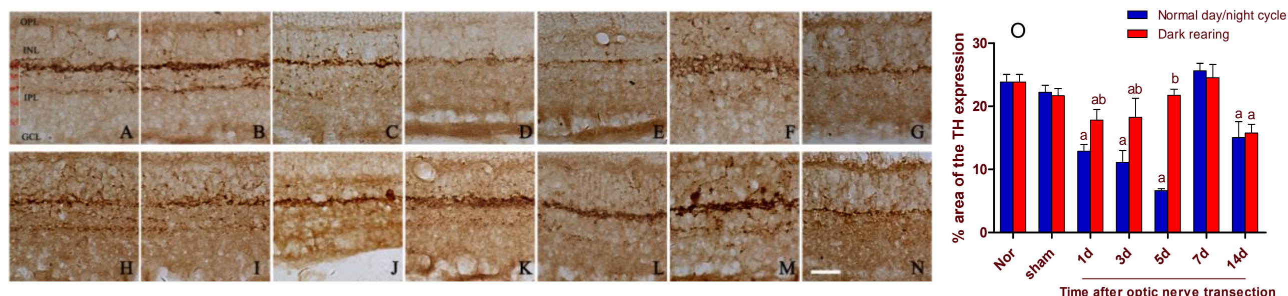

Figure 1 Retinal tyrosine hydroxylase (TH) expression in rats having undergone optic nerve transection and reared in a normal light/dark cycle or in the dark at different time points (immunohistochemical staining).

(A, H) Normal group; (B, I) sham-surgery group; (C, J) injury group at 1 day; (D, K) injury group at 3 days; (E, L) injury group at 5 days; F, M: injury group at 7 days; G, N: injury group at 14 days (scale bar = 100 μm in A–N).

(A–F) rats reared in a normal day/night cycle; (G–L) rats reared in the dark. Compared with the normal group, retinal TH expression was reduced in rats reared in a normal day/night cycle following optic nerve injury. TH expression reached a minimum at 5 days, and recovered to the normal level at 7 days. Retinal TH expression decreased to a minimum at 1 day in dark reared rats, gradually increasing afterward and reaching a normal level at 5–7 days. S1–S5: first to fifth layers of the inner plexiform layer.

(O) Quantitative results of TH expression in rats reared in different conditions at each time point and expressed as mean ± SD. There were three rats in the normal and sham-surgery groups, and six rats in the injury group at each time point. ^{a}P < 0.05, *vs.* normal group under the same rearing condition; ^{b}P < 0.05, *vs.* rats reared in a normal day/night cycle at the same time point (one-way analysis of variance was performed for intergroup comparison under the same rearing condition at different time points; paired sample t-test was used for intergroup comparison under different rearing conditions).

GCL: Ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; Nor: normal group; sham: sham-surgery group; 1, 3, 5, 7, 14 days: 1, 3, 5, 7 and 14 days after optic nerve injury.

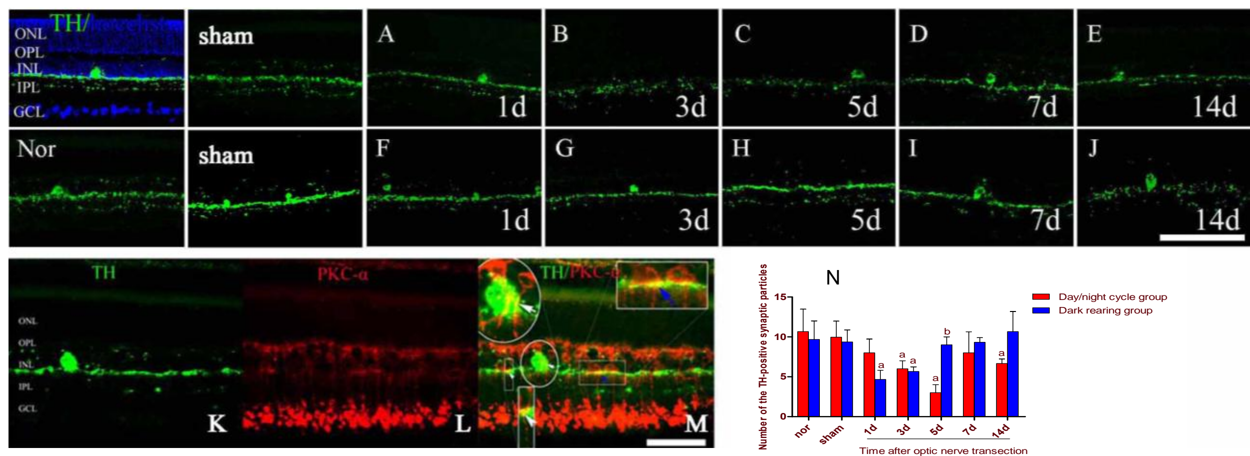

Figure 2 Retinal tyrosine hydroxylase (TH)-positive synaptic particles (immunofluorescence double-labeling).

Scale bars = 100 μm in A–M. Hoechst staining demonstrating the layers of the retina; (A–E) retinal TH-staining results (Alexa-488 labeling, green) at 1, 3, 5, 7 and 14 days in rats reared in a normal day/night lighting cycle following optic nerve injury; (F–J) retinal TH-staining results at 1, 3, 5, 7 and 14 days in rats reared in the dark following optic nerve injury. TH was expressed in dopaminergic amacrine cells (green, K), which formed synaptic connections with ON-type bipolar cells (Alexa-594 labeling, red, L) in the inner nuclear layer and inner plexiform layer. Arrow represents co-labeling of amacrine cells and bipolar cells (M). Co-labeling of an amacrine cell body and a bipolar cell process is shown in the ellipse; the blue arrow represents co-labeling of an amacrine cell synapse and a bipolar cell body; the white arrow represents co-labeling of an amacrine cell synapse and a bipolar cell process.

(N) comparison of TH-positive particle quantity between normal and dark rearing following optic nerve injury (× 400). Results are expressed as mean ± SD. There were three rats in the normal and sham-surgery groups, and six rats in the injury group at each time point. $^aP < 0.05$, *vs.* normal group under the same rearing condition; $^bP < 0.05$, *vs.* rats reared in a normal day/night cycle at the same time point (one-way analysis of variance was performed for intergroup comparison under the same rearing condition at different time points; paired sample *t*-test was used for intergroup comparison under different rearing conditions).

Nor: Normal group; sham: sham-surgery group; 1, 3, 5, 7, 14 days: 1, 3, 5, 7 and 14 days after optic nerve injury. GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer.

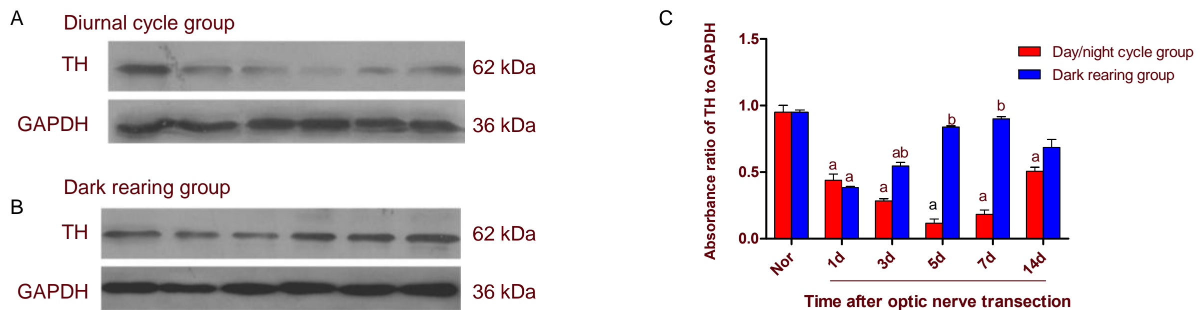

Figure 3 Western blot results of retinal tyrosine hydroxylase (TH) expression under different rearing conditions.

Western blot results of retinal TH expression in rats reared in a normal day/night cycle (A) and the dark (B).

(C) Quantitative results of retinal TH expression in rats reared in different conditions at each time point and expressed as mean ± SD of absorbance ratio of target gene to GAPDH. There were three rats in the normal and sham-surgery groups, and six rats in the injury group at each time point. $^aP < 0.05$, *vs.* normal group under the same rearing condition; $^bP < 0.05$, *vs.* rats reared in a normal day/night cycle at the same time point (one-way analysis of variance was performed for intergroup comparison under the same rearing condition at different time points; paired sample *t*-test was used for intergroup comparison under different rearing conditions). Nor: Normal group; sham: sham-surgery group.

TH protein expression was reduced in rats reared in a normal day/night cycle 1, 3 and 5 days following optic nerve injury, reaching a minimum at 5 days, and increasing at 7 and 14 days. However, these levels were lower than normal. TH protein expression reached a minimum at 1 day in rats reared in the dark, increased at 3, 5 and 7 days, and were restored to normal levels at 5, 7 and 14 days.

Compared with rats reared in a normal day/night cycle, TH protein expression was significantly decreased at 1 day in animals of each group reared in the dark ($P < 0.05$), but it increased at 3, 5 and 7 days ($P < 0.05$; Figure 3).

DISCUSSION

Dopaminergic amacrine cells are categorized into types I

and II in the normal rat retina, and both can specifically express TH[24-25]. TH expression is mainly localized to the cell body and dendritic branches of dopaminergic amacrine cells[24]. Type I cell processes are located in stratum S1 of the inner plexiform layer, which is associated with the regulation of OFF-cone bipolar cells; type II cell processes are located in stratum S3 of the inner plexiform layer[25], which is associated with the regulation of ON-cone bipolar cells[26]. Consistent with these observations, in the present study, TH-positive synapses were found to be primarily localized to the inner plexiform layer, and were occasionally observed in the inner nuclear layer. They receive visual signals from bipolar cells and input from intrinsically photosensitive retinal ganglion cells[27-28], to stimulate dopamine release from dopaminergic amacrine cells. They also receive feedback from target cells in the retina through their dendrites.

TH phosphorylation is important for regulating TH activity, and light stimulates TH phosphorylation[29]. In the present study, the area of TH-positive immunoreactivity, the number of TH-positive synaptic particles, and TH protein levels were initially reduced, but then increased gradually, in rats reared in both light conditions.

In rats reared in a normal day/night cycle following optic nerve injury, the area of TH-positive expression was significantly reduced 1 day after surgery, reached a minimum at 5 days, and was restored to the normal level by 7 days. The number of TH-positive synaptic particles exhibited a similar trend. This indicates that TH expression is sensitive to optic nerve injury. The injury-induced decline in TH expression may due to reduced substrate synthesis and diminished neurotrophic factor support[30-31]. The increased TH expression at 7 days may be a consequence of synaptic reorganization—the formation of new synapses by dopaminergic amacrine cells on different cell types in the retina in an effort to re-establish visual circuitry[32-33].

Previous studies by our group demonstrated that rats have a short shock stage after optic nerve transection, followed by a compensation stage at 3–14 days[32, 34-35]. Products associated with stress gradually increase in the inner and outer plexiform layers, but the number of RGCs and recoverin-positive bipolar cells gradually decrease after the fifth day, and the number of retinal neurons decrease after entering the degeneration stage at 14 days. Furthermore, there is a diminished number of synapses available for compensation.

Recoverin-labeled OFF-cone bipolar cells begin to decrease 7 days following optic nerve injury, and cell activity is attenuated. The results revealed that the period 5–7 days following optic nerve injury is a critical window during which retinal synapses participating in compensation begin to degenerate. Stress-induced changes in retinal nerve cells 7 days after injury may induce TH production in dopaminergic amacrine cells, thereby promoting dopamine synthesis, enhancing retinal visual circuitry and improving connectivity among the cells.

In the present study, TH expression began to decrease after the shock stage, reaching a minimum at 5 days in rats reared in a normal day/night cycle, which was consistent with the time during which retinal neurons decreased in number in the compensation stage. TH expression reached a minimum following the shock stage and gradually increased during the compensation stage in rats reared in the dark. In addition, synapses were orderly at 5 days in rats reared in the dark, compared with those reared in a normal day/night cycle, indicating that dark rearing protects retinal neurons during the compensation stage.

In the present study, we found that TH expression had a tendency to decrease and then increase under both rearing conditions at each time point, but TH expression was higher in rats reared in the dark compared to those reared in a normal day/night cycle at 5, 7 and 14 days. Light stimulation is important in regulating retinal dopamine synthesis. TH-catalyzed DOPA accumulation was three times higher in the vitreous body exposed to light for 30 minutes compared to that kept in the dark[33]. This explains why TH expression was significantly decreased in rats reared in the dark compared with those reared in a normal day/night cycle for 1 day. At 5 days after optic nerve injury, TH expression reached the minimal level in rats exposed to a normal day/night cycle, revealing decreased dopamine synthesis. This would likely lead to diminished regulation of target cells, possibly exacerbated by a reduction in the number of OFF-cone bipolar cells, which are tightly associated with the synapse[32]. Dark rearing accelerates the reduction in TH expression and diminishes consumption of the TH-synthesized substrate. TH expression gradually increased at 3, 5 and 7 days, but returned to normal levels at 7 and 14 days in response to the day/night cycle, pituitary adenylate cyclase activating peptide and brain-derived neurotrophic factor[30-31], which increase dopamine levels and protect visual pathways. However, the mechanisms behind the neuroprotective effects are unknown and require further investigation.

In conclusion, dark rearing modulates TH expression following optic nerve transection. TH expression was minimal during a stage in which bipolar cells function normally. With increasing TH expression, dopamine concentrations increased and were maintained for a long period of time after injury. This modulates target cell function, suppressing the overactivation of bipolar cells, benefitting visual circuitry and promoting visual recovery following axonal regeneration.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

The experiment was performed at the Laboratory of Human Anatomy and Neurobiology, Xiangya Medical

College of Central South University, China, from March to December 2010.

Materials

A total of 144 healthy Sprague-Dawley rats, of either gender, aged 6–8 weeks, weighing 200–250 g, were provided by the Laboratory Animal Department of Central South University (license No. SCXK (Xiang) 2006-0002). The animals had equal-sized eyes, clear corneas and normal pupils. They were housed at 18–28°C, with a humidity of 40–60%. All experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[36].

Methods

Generation of the optic nerve injury model and interventions

The rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.4 mL/100 g). The head was fixed in the stereotaxis instrument (Jingyi, Shanghai, China). Following administration of 0.25% chloromycetin eye drops, the skin at the right supraorbital margin was cut, and the optic nerve was transected, 0.5 cm posterior to the eyeball. The rats were reared in a normal day/night cycle (12 hours/12 hours) or in the dark at 24°C and allowed free access to food and water.

Preparation of retinal tissues

The rats were anesthetized by intraperitoneal injection with 10% chloral hydrate and fixed by left ventricular perfusion with 2% paraformaldehyde and 0.2% p-benzoquinone at 9: 00 a.m. The eye balls at the injured side were harvested, fixed with 4% paraformaldehyde for 3 hours, followed by serial sectioning using a freezing microtome to obtain 20-μm-thick sections. The retinal sections adjacent to the optic nerve were stored at −20°C for immunohistochemistry and immunofluorescence double-staining. For western blotting, the animals were anesthetized and sacrificed. The eye balls were harvested on ice, the retina was separated, weighed and stored at −80°C.

Detection of TH expression using immunohistochemistry

The frozen retinal sections were subjected to immunohistochemical staining using the routine ABC method[37]. Sections were incubated with primary antibody, rabbit anti-TH (polyclonal, AB152, 1: 1 000; Abcam, Cambridge, UK), overnight at 4°C on a shaker. The sections were then incubated with secondary antibody, biotinylated goat anti-rabbit IgG (1: 200; Invitrogen, Carlsbad, CA, USA), at room temperature for 2 hours. The positive sections provided by the kit served as the positive control, and normal serum was used as the negative control. Sections were observed by confocal microscopy (Nikon, Tokyo, Japan), and the area of TH immunoreactivity was determined using Image J (National Institutes of Health, Rockville, Maryland, USA). The mean value of the area of TH immunoreactivity from six sections from one rat was defined as TH expression intensity.

Detection of TH and PKC-α expression using immunofluorescence double-labeling

The frozen retinal sections were subjected to immunofluorescence staining. Incubation with the primary antibodies—rabbit anti-TH (polyclonal, AB152, 1: 1 000; Abcam) and mouse anti-PKC-α (polyclonal, 1: 500; Abcam)—was performed at room temperature for 2 hours. Incubation with the secondary antibody—Alexa-488 or Alexa-594-conjugated donkey anti-mouse or rabbit IgG (1: 200; Invitrogen)—was performed at room temperature for 2 hours. Nuclei were stained with Hoechst (Hoechst33342, 1: 1 000; Invitrogen). Sections were observed by confocal microscopy (Nikon), and the number of TH-positive synaptic particles in a unit area of the S1 layer was quantified under 400 × magnification[38].

Detection of retinal TH protein expression using western blot

Retinal homogenates were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (constant 100 V) and protein was electrotransferred to a nitrocellulose membrane at 250 mA for 120 minutes. The blots were incubated with rabbit anti-TH (polyclonal, AB152, 1: 1 000; Abcam) or mouse anti-GAPDH (monoclonal, AB9484, 0.5 μg/mL; Abcam) primary antibody, followed by horseradish peroxidase-labeled goat anti-rabbit IgG (1: 10 000; Bio-Rad, Hercules, CA, USA) or goat anti-mouse IgG (1: 10 000; Bio-Rad) secondary antibody, respectively, at room temperature for 1 hour. The blots were then subjected to ECL chemiluminescence detection. The absorbance of the scanned bands was determined using Image J. The specific positive value was calculated by subtracting the nitrocellulose membrane absorbance from the band absorbance and dividing by the GAPDH absorbance.

Statistical analysis

Data were analyzed using SPSS version 18.0 (SPSS, Chicago, IL, USA) and was expressed as mean ± SD. One-way analysis of variance was performed for intergroup comparisons for the same rearing condition at different time points. The paired sample *t*-test was used for intergroup comparison for different rearing conditions. A value of $P < 0.05$ was considered statistically significant.

Author contributions: Xuegang Luo designed this study. Wei Wan, Zhenghai Liu and Xiaosheng Wang conducted the experiments.

Conflicts of interest: None declared.

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Ethical approval: This study received permission from the Animal Ethics Committee of Xiangya Medical College of Central South University, China.

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Supplementary information: Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org, and entering Vol. 7, No. 1, 2012 after selecting the “NRR Current Issue” button on the page.

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