Induction of CRMP-4 in striatum of adult rat after transient brain ischemia[1]

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ABSTRACT

AIM: To study the expression of collapsing response mediated protein-4 (CRMP-4) and nestin in the ischemic adult rat brain following transient brain ischemia. **METHODS:** Brain ischemia was induced by transient left middle cerebral artery occlusion (MCAO) for 60 min in adult rats. The expression of CRMP-4, nestin and bromodeoxyuridine (BrdU) was analyzed by immunohistochemical method. The co-localization of CRMP-4 and nestin or BrdU was analyzed by double staining combined with confocal laser scanning microscopy. **RESULTS:** CRMP-4, a marker of immature neuron, could be expressed in the ipsilateral striatum and cerebral cortex at 1st and 2nd week after the ischemia-reperfusion; nestin, a marker of neural stem cell, occurred in above regions from several hours to 2 weeks. CRMP-4 costained with nestin and with BrdU incorporation. **CONCLUSION**: Neural stem cells may present in the striatum and cerebral cortex of adult rat and can be triggered to differentiate into newborn neuron there by ischemic brain trauma.

INTRODUCTION

Cerebral ischemia causes neuronal death through complicated mechanisms[1]. Traditionally, the loss of neuron in adult brain was regarded as irreversible because it had been believed that neurons were not replaced after death. However, increasing evidences indicate that neuronal progenitors do exist in the discrete brain regions in adult mammalian, including the subgranular zone of hippocampal dentate gyrus (DG) and the forebrain subventricular zone and ventricular zone (SVZ/VZ)[2]. Now, it has been well demonstrated that neurogenesis takes place in the adult brain throughout life[2] and can be modulated by several experimental variables[3]. Several lines of evidence demonstrate that bromodeoxyuridine (BrdU) incorporation significantly increases in the edge of injured cerebral cortex or DG under the models of photothrombotic stroke or transient cerebral ischemia, suggesting the existence of neurogenesis in the cerebral cortex[4,5]. This phenomenon exhibits that adult neurogenesis in the hippocampus and cortex can be triggered by ischemic brain injury and probably plays a very important role in the brain repair. However, research on whether neuro-genesis can be induced in any other non-neurogenitor regions of adult brain after the injury is scarce[6] and still

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needs to be illustrated.

Collapsing response mediated protein (CRMP) genes have been identified from the rat brain and each member is differentially expressed in the nervous system of rat brain[7]. It has been demonstrated and accepted that the antigen for antibody-25, formerly named the gene TOAD-64 (turned on after division 64), should be CRMP-4 (TUC-4)[8,9]. CRMP-4/TOAD-64 has been determined to express during discrete period of neuronal development and in the discrete regions of the adult nervous system[7,10]. The analysis of cellular distribution indicates that CRMP-4/TOAD-64 expresses in the newborn neurons, but not in the glial and progenitor cells[11]. A recent study showed that the expression of CRMP-4 can be upregalated in mature DG by transient global ischemia, indicating enhanced neurogenesis[12]. Thus, CRMP-4/TOAD-64 has been widely used as a good marker for neurogenesis.

Nestin, a member of the intermediate filament protein family, abundantly and transiently presents in the multipotential stem cells during the developing central nervous system (CNS)[13]. It can be used to indicate the proliferation of neuronal progenitor in the developing and adult mammalian CNS[14]. In addition, brain damage could upregulate the expression of nestin in adult rat brains[15]. Although the pattern of nestin expression is changeable in the brain based on the ischemic model[15,16], several studies have demonstrated that ischemia-induced nestin expression also exists in the neuron of ischemic brain regions including the striatum besides the cerebral cortex[16,17]. As mentioned above, neurogenesis occurs in the cerebral cortex of adult brain following the cerebral ischemia. To understand whether the neurogenesis exists in the striatum of the adult rat brain following the ischemic injury, we observed the changes of CRMP-4 expression in the ischemic injured brains and used double fluorescence staining combined with confocal microscopy to investigate the colocalization of CRMP-4 and nestin or BrdU in the striatum of rat following a transient middle cerebral artery occlusion (MCAO).

MATERIALS AND METHODS

Materials A mouse monoclonal antibody against nestin was purchased from PharMingen (Canada). A rabbit polyclonal antibody against CRMP-4 was kindly gifted from Dr Hockfield SUSAN (Yale University, USA). Vectastain Elite ABC kit and an anti-rabbit IgG-fluorescein were purchased from Vector Laboratories, Inc (Burlingame CA, USA). A mouse monoclonal antibody against BrdU, an anti-mouse IgG-fluorescein and an anti-rabbit IgG-rhodamine were purchased from Roche (Mannheim, Germany). An anti-mouse IgG-Cy5 was purchased from Amersham (UK).

Treatment of animals Experiments were performed on 35 male Sprague-Dawley rats (220-250 g, Department of Experiment Animals, Shanghai Medical College of Fudan University, Grade II, Certificate No 02-22-2). All rats were anesthetized by 10 % chloral hydrate (360 mg/kg, ip). During surgical operation, their rectal temperature was maintained at (37±0.5) ºC using a heating pad, tail artery was cannulated to monitor blood pH, p_{CO_2} and p_{O_2}, which were kept within the physiological range. Focal cerebral ischemia was induced by a transient MCAO largely based on previous report[18]. Briefly, a 4-0-nylon suture with an expanded tip was introduced into the left internal carotid artery through the stump of the external carotid artery and gently advanced from the common carotid artery bifurcation to block the origin of middle cerebral artery (MCA) for 60 min. Withdrawing the intraluminal suture from the internal carotid artery restored the MCA blood flow. Sham-operated animals (n=5) were subjected to the same surgical procedure but no suture was inserted.

For the time course observation on the expressions of CRMP-4 and nestin, the animals were sacrificed at the indicated time points in the Tab 1 (n=5 for each time point). For the observation of BrdU incorporation, rats were received a single injection of BrdU (30 mg/kg/day, ip) on the day after ischemic or sham operation, continuously given for 13 d and sacrificed after 2 weeks post ischemia-reperfusion (n=3 for each group) as follows.

Each rat was anesthetized and perfused intracardially with 0.9 % saline solution followed by 250 mL fixative (4 % paraformaldehyde in phosphate buffer, pH 7.4) 0.1 mol/L at desired time points after the operation as described above. Brains were then removed and postfixed in the same fixative overnight, dehydrated in 30 % sucrose and cut into 30-mm coronal serial sections from 0.70 to 0.48 mm Bregma levels[19] on freezing microtome (Jung Histocut, model 820-II, Leica, Germany). The sections were stored in cryoprotectant solution at -20 ºC for the histological analysis.

Nestin, CRMP-4, and BrdU immunohistochemical study Free-floating sections were fixed in

4 % paraformaldehyde for 10 min, blocked with 10 % normal goat serum with 0.3 % Triton X-100 for 30 min, incubated in 1:1000 dilution of a mouse monoclonal antibody against nestin (PharMingen, Canada) or 1:10,000 dilution of a rabbit polyclonal antibody against CRMP-4 (a gift from Dr Hockfield SUSAN, Yale University) in 1 % normal goat serum and 0.3 % Triton X-100 overnight at 4 ºC. Endogenous peroxidase activity was quenched by exposing the sections to 0.3 % H_2O_2. Then the sections were incubated with 1:200 dilution of biotinylated secondary antibodies for 30 min, 1:100 dilution of avidin-biotin-peroxidase (Vectastain Elite ABC kit, Vector Laboratories, Inc, Burlingame) for 45 min, developed with 0.05 % diaminobenzidine (DAB, Sigma, St Louis)/0.01 % H_2O_2. Control sections received identical treatment except exposure to the primary or secondary antibody and showed no specific staining.

For BrdU immuno-staining, sections were first incubated with 50 % formamide/2×SSC (NaCl 0.3 mol/L, sodium citrate 0.03 mol/L) and HCl 2 mol/L for DNA denaturation as described by Kuhn[20]. Sections were then rinsed three times in Tris buffer (0.1 mol/L, pH 7.6) and treated with 1 % H_2O_2. After washed for 15 min each in buffer A (Tris buffer 0.1 mol/L plus 0.1 % Triton X-100) and buffer B (Tris buffer 0.1 mol/L plus 0.1 % Triton X-100, 0.05 % BSA), sections were placed in 10 % normal goat serum in buffer B for 1 h and incubated with a mouse monoclonal antibody against BrdU (1:100, Roche, Germany) overnight at 4 ºC. After being rinsed consecutively in buffer A and buffer B for 15 min, sections were incubated with biotinylated secondary antibody for 45 min and avidin-biotin-peroxidase complex for 1 h, developed with 0.05 % DAB containing 0.03 % H_2O_2 in Tris buffer 0.1 mol/L (pH 7.6). Negative control received the same treatment except exposure to the primary antibody and showed no specific staining.

The signals of nestin, BrdU, and CRMP-4 immunopositive staining were analyzed in the striatum (Bregma level: A0.70-0.48, L4-5, H4-5,) and cerebral cortex (Bregma level: A0.70-0.48, L3-4, H1-2) of rat brain under Image Analyzer (Q500IW Image, Leica, Germany). The integrating optic density (IOD) in the ischemic region A was measured to express the amount of nestin positive staining. Each number of IOD per section was represented as an average of six measuring. To express the amount of BrdU and CRMP-4 positive staining, total number of positive cell was counted at the ipsilateral hemisphere to ischemia.

Double staining For double staining of CRMP-4 and BrdU, the sections were treated with the same procedure except pre-incubation in formamide. Sections were then treated with the antibody against BrdU overnight at 4 ºC, incubated for 1 h with anti-mouse IgG-Cy5 (1:100; Amersham, UK) to reveal BrdU signals. After washed for three times, sections were again treated with the antibody against CRMP-4 overnight at 4 ºC, followed by incubation for 1 h with anti-rabbit IgG-fluorescein (1:20; Vector Laboratories, Inc, Burlingame).

For double staining of CRMP-4 and nestin or GFAP, the sections were first incubated in 10 % normal goat serum for 30 min, then with the antibody against nestin and the antibody against CRMP-4 or GFAP (1:100, Dako, Denmark) overnight at 4 ºC. Then sections were incubated in 1:20 dilution of anti-mouse IgG-fluorescein (Roche, Germany) and anti-rabbit IgG-rhodamine (Roche, Germany) for 1 h at 37 ºC. Signals on the slides were examined under a confocal laser-scanning microscope (TCS NT, Leica, Germany).

Statistical analysis Data were presented as mean±SD and analyzed using a one-way ANOVA followed by unpaired *t* test. Difference among groups was considered statistical significant at $P<0.05$.

RESULTS

Induction of CRMP-4 in the ipsilateral striatum of ischemic brain of adult rat In the present study, immunostaining was used to observe CRMP-4 positive cell in the adult rat brain following MCAO. No signal for CRMP-4 positive staining could be detected at each side of hemisphere in the control rats. However, CRMP-4 positive staining cells and fibers could be observed in the ipsilateral striatum and cerebral cortex after 2 weeks post ischemia-reperfusion. The CRMP-4 staining was presented in the ipsilateral cortex and striatum of rat after 2 weeks following ischemia-reperfusion (Fig 1). Furthermore, time course of CRMP-4 induction was analyzed in the brain from d 1 to 2 weeks following the ischemia-reperfusion. The results showed that a few neurons in the ischemic hemisphere had indistinct staining with CRMP-4 at d 3. At 1 week after the ischemia-reperfusion, however, some neurons were stained with CRMP-4 showing dark brown positive cell body and long dendrite/fibber. To quantify the positive reaction, the number of dark-brown positive stained cell was counted at the different time after the ischemia.

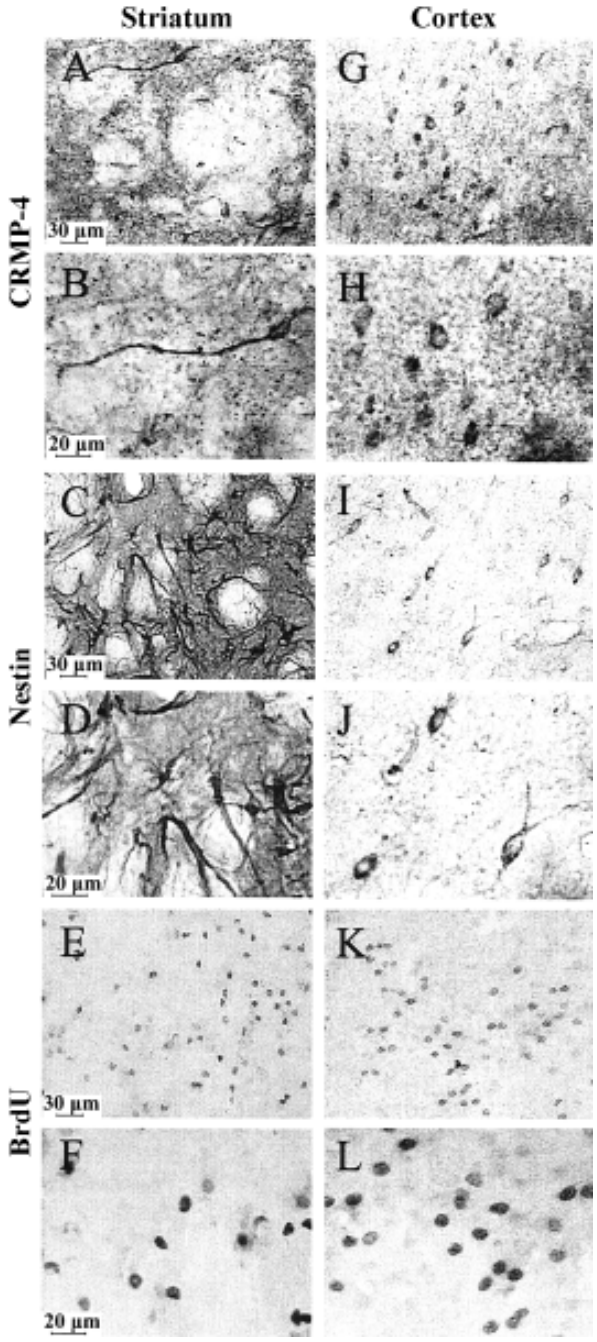

Fig 1. Representative photographs of CRMP-4, nestin, and BrdU-positive cells in the rat brain at 2 weeks of reperfusion after ischemia. Photographs B, D, F, H, J, and L were high power magnification corresponding to the low power photographs A, C, E, G, I, and K, respectively.

As shown in Tab 1, we could not count the number until 1 week, and still could observe them at 2nd week after the ischemia-reperfusion. Meanwhile, we could not find any CRMP-4 signal in the contralateral hemisphere at all time points.

Induction of nestin in the ipsilateral striatum of ischemic brain of adult rat Nestin immuno-positive staining was performed on adjacent brain sections. The results showed that, in sham-operated rats, nestin positive staining was almost exclusively localized in the blood vessel, choroid plexus epithelium, epidermal cell and subependymal area lining SVZ/VZ. Besides, labeled neurons were occasionally seen in the region of the medial septum, diagonal band of Broca and cingulate cortex. Those observations were consistent with previous report[21]. In the ischemic injured rats, the induction of nestin was analyzed in both hemispheres from 3 h to 2 weeks following the ischemia-reperfusion. In the contralateral hemisphere to ischemia, the distribution of nestin positive cell was the same as that in sham-operated rats. However, in the ipsilateral hemisphere, very strong nestin positive signals were detected especially in the ischemic regions, mainly including the ipsilateral striatum and cortex. Based on morphological characteristics, some positive cells showed large star-sharp neuralgia with numerous long cytoplasmic processes; others showed small neuronal body with short process or even only with long filament (Fig 1). Therefore, the optical density was used to measure the amount of the nestin positive staining instead of cell counting. The results showed that optical density was significantly increased in the ipsilateral ischemic region at d 1 ($P<0.05$), peaked at 1st week and persisted to 2nd week after ischemia-reperfusion (Tab 1).

Co-localization of CRMP-4 with nestin and BrdU To analyze the co-localization, double fluorescent immunostaining was used to label CRMP-4 and nestin in adjacent sections from rat brain after 2 weeks following ischemia-reperfusion. As seen in Fig 1 and 2, each single staining signal for nestin or CRMP-4 could be observed in the cortex and striatum of ipsilateral hemisphere. Confocal laser scanning showed that CRMP-4 positive cells mostly costained with nestin.

To determine whether the neurogenesis occurred in the ischemic injured striatum, BrdU incorporation was observed in the brain sections from adult rat at 2nd week after ischemia-reperfusion. The results showed that BrdU positive cells were barely detected in sham-operated group. However, cerebral ischemia significantly increased the number of BrdU positive cells in the ipsilateral hemisphere (130±57 cells/ hemisphere, $n=3$, $P<0.05$ *vs* control). It is noted that BrdU positive cells could be observed in ipsilateral striatum (Fig 1) besides periventricle, and frontoparietal cortex.

Tab 1. Time-dependent changes of nestin and CRMP-4 expression in the ischemic injured brain following a lateral transient middle cerebral artery occlusion. *n*=5. Mean±SD. [b]*P*<0.05 *vs* control.

	Time after ischemia-reperfusion						
	Control	3 h	6 h	1 d	3 d	1 weeks	2 weeks
Nestin (*OD*/μm^2)	0.9±0.4	1.8±1.8	3±4	21±55	49±46[b]	60±46[b]	59±31[b]
CRMP-4 (cells/hemisphere)	0	-	-	0	0	38±24[b]	21±15[b]

"-" means no detection in the present study.

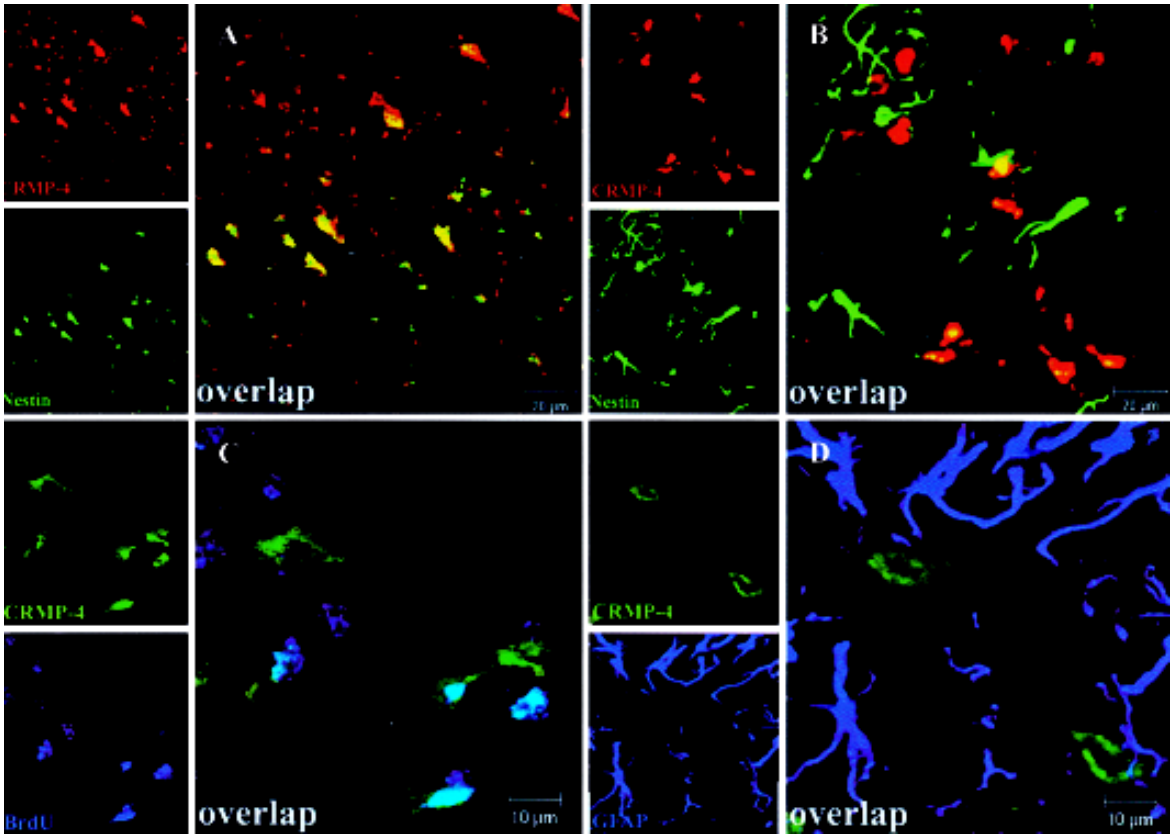

Fig 2. Photographs of double staining of CRMP-4 and nestin or BrdU or GFAP in the ipsilateral brain of adult rat at 2 w after ischemia-reperfusion. Overlapping of green color and red color produced yellow color, indicating that CRMP-4 costained with nestin in frontoparietal cortex (A) and striatum (B). Overlapping of green color and blue color produced light blue color, denoting CRMP-4 costained with BrdU (C), but not with GFAP (D) in striatum.

However, a few BrdU-positive cells could be detected in the contralateral hemisphere. Furthermore, double staining of BrdU or GFAP and CRMP-4 combined with confocal laser scanning microscopy was used to further analyze whether CRMP-4 cells were newborn neurons in the ischemic striatum. The results indicated that BrdU or CRMP-4 positive staining could be observed in the ipsilateral striatum and some CRMP-4 positive cells costained with BrdU, a marker of proliferation cell, but not with GFAP, a marker of astrocyte (Fig 2).

DISCUSSION

Present study first reported time-related induction of CRMP-4, the marker of immature neuron[11,12], in the striatum and cerebral cortex of ipsilateral hemisphere in adult rat following a transient MCAO. Nestin immuno-

like positive reaction was observed in the neuronal and glial cells among the regions, which confirmed the previous reports[16,21]. Confocal laser scanning microscopy revealed that CRMP-4 coexisted with nestin, a marker of neural stem cell, in the ipsilateral striatum and cortex. Besides, BrdU incorporation cells could be observed and costained with CRMP-4 in the ipsilateral striatum. Our results suggest that neuronal progenitor may subsist in the striatum of adult rat brain *in vivo* and neurogenesis there may be activated under ischemic brain trauma in the adult. This result is consistent with a recent study indicating stroke-generated neurons existed in the striatum[6].

Neurogenesis has been generally considered to be limited to the SVZ/VZ[2,3] and it can be promoted by cerebral ischemia in those regions even in adult animals[5]. More and more evidences indicate that adult-generated cortical neurons can be up-regulated by brain trauma[4]. In the present study, single and multiple CRMP-4 immunostaining were used to analyze whether neurogenesis occurred in the striatum of adult rat following cerebral ischemia. We observed that CRMP-4 positive signals mainly existed in ipsilateral striatum and cortex of ischemic brain, but not in the striatum or cortex of control brain. The results were consistent with most other studies[7,11,22] but not with Nacher's report[10]. Those differences may be due to different experiment conditions and protocols. BrdU, a thymidine analog, can be incorporated into newly synthesized DNA and used as marker for neurogenesis and gliogenesis and for DNA repair in the brain[23]. It may explain why part of BrdU positive cells do not costain with CRMP-4 in Fig 2, since astrocyte proliferation and DNA repair were activated during the pathophysiological process of brain following the ischemic injury[24]. To exclude whether CRMP-4 present in astrocyte, we further observed the double staining of CRMP-4 and GFAP, a specific marker of astrocyte. As shown in Fig 2, we could not see the double staining signal, which indicates CRMP-4 does not exist in the astrocyte in the rat brain. Putting together, CRMP-4 positive cells in the present were immature neurons generated from neuronal precursors in the ischemic striatum. In fact, several reports have arisen the possibility of neurogenesis in the striatum of adult mammalians. Such as, multipotential stem cells have been isolated from the striatum of adult mouse, which exhibits self-maintenance and generates large numbers of neuronal and glial cells in the presence of epidermal growth factor (EGF)[14]. In addition, treatment of basic fibroblast growth factor (bFGF) improves proliferation of some EGF-generated progenitors from the striatum in adult mouse[25]. Moreover, Lundberg and his colleagues found survival, integration and differentiation of neural stem cell lines after transplanting them into the adult rat striatum[26]. Therefore, it suggests the presence of internal environments for neurogenesis in the striatum of adult rat.

Present results further showed that the expression of nestin, a marker of neural precursor[11], appeared in the ischemic injured brain regions as early as several hours after ischemia-reperfusion, then increased the intensity of staining and clearly demarcated the ischemic boundary. Such a temporary profile of nestin expression (Tab 1) is in agreement with the previous publication[21]. As we mentioned before, nestin has been commonly used as an indicator of neuronal progenitor in mammalian brain[14]. Combined with the present of nestin positive reaction in the astrocyte as well as neurons[17,21] and colocalization of nestin and CRMP-4, a specific immature neuronal marker[11], it suggests that ischemic brain damage may trigger the proliferation of neuronal progenitor, which may participate in the generation of newborn neuron in the striatum of adult rats. Although it has been believed that active glial cell may become the precursor of newborn neurons, whether nestin positive glia in the striatum is a transient precursor in the formation of new neuron *in situ* and whether the newborn neurons is from SVZ/VZ still need to be illustrated in the future. In conclusion, our results suggest that neural progenitors might present in the striatum of the adult brain and ischemic injury could trigger neurogenesis there.

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