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Differentiation of endogenous neural stem cells in adult *versus* neonatal rats after brachial plexus root avulsion injury☆

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Abstract

An experimental model of brachial plexus root avulsion injury of cervical dorsal C_{5-6} was established in adult and neonatal rats. Real-time PCR showed that the levels of brain-derived neurotrophic factor, nerve growth factor and neurotrophin-3 in adult rats increased rapidly 1 day after brachial plexus root avulsion injury, and then gradually decreased to normal levels by 21 days. In neonatal rats, levels of the three neurotrophic factors were decreased on the first day after injury, and then gradually increased from the seventh day and remained at high levels for an extended period of time. We observed that greater neural plasticity contributed to better functional recovery in neonatal rats after brachial plexus root avulsion injury compared with adult rats. Moreover, immunohistochemical staining showed that the number of bromodeoxyuridine/nestin-positive cells increased significantly in the spinal cords of the adult rats compared with neonatal rats after brachial plexus root avulsion injury. In addition, the number of bromodeoxyuridine/glial fibrillary acidic protein-positive cells in adult rats was significantly higher than in neonatal rats 14 and 35 days after brachial plexus injury. Bromodeoxyuridine/β-tubulin-positive cells were not found in either adult or neonatal rats. These results indicate that neural stem cells differentiate mainly into astrocytes after brachial plexus root avulsion injury. Furthermore, the degree of neural stem cell differentiation in neonatal rats was lower than in adult rats.

Key Words

neural stem cells; neurotrophic factors; brain-derived neurotrophic factor; neuroregeneration; brachial plexus; nerve root avulsion injury; neural regeneration

Research Highlights

After brachial plexus root avulsion injury, proliferating endogenous neural stem cells mainly differentiated into astrocytes. Neonatal rats did not exhibit a greater rate of differentiation of endogenous neural stem cells into neurons than adult rats.

Abbreviations

BrdU, bromodeoxyuridine; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; NT-3, neurotrophin-3; GFAP, glial fibrillary acidic protein

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INTRODUCTION

Brachial plexus root avulsion directly induces changes in the spinal cord microenvironment, resulting in apoptosis of anterior horn motor neurons. Newborns have greater nervous system plasticity, and are better able to adapt to neuronal disease or injury compared with adults. Among adult patients, functional regeneration is limited by time and distance. The greater distance that a regenerating axon must extend and impaired repair capacity affect functional

recovery[1]. The recovery process in neonates is also different from that in adults following brachial plexus root avulsion injury. The effective functional recovery time is longer for newborns and for children up to 5 years of age[2].

Clinical studies show that prognosis is better for children than adults, and children have better recovery of sensory function after nerve injury[3-4]. Some researchers believe that neural regeneration capacity is better in children than in adults[4-5], because children have relatively greater plasticity of the nervous system[3, 6]. Neuronal cell death in neonatal rats is far greater than in adult rats after spinal nerve root avulsion[7], and this higher death rate is associated with better functional recovery. There may be other factors that contribute to better functional recovery following brachial plexus root avulsion injury in children compared with adults. However, it is unclear why functional recovery is better in neonatal rats, which suffer more cell death, than in adult rats.

In the present study, we used real-time PCR to assess gene expression of three different neurotrophic factors in the spinal cord after brachial plexus root avulsion injury in adult and neonatal rats, and we used immunohistochemical staining to observe the proliferation and differentiation of endogenous neural stem cells. We aimed to determine whether neonatal rats have a greater rate of differentiation of endogenous neural stem cells into neurons and greater neurotrophin expression compared with adult rats after brachial plexus root avulsion injury.

RESULTS

Quantitative analysis of experimental animals

A total of 100 rats were randomly assigned to real-time PCR and double immunostaining groups, with 50 in each group. Rats from the real-time PCR group were selected at different time points (1 day before injury, and 1, 7, 14 and 21 days after injury), with 5 rats per time point. Rats from the double immunostaining group were selected at different time points (1 day before injury, and 1, 7, 14 and 35 days after bromodeoxyuridine (BrdU) labeling), with 5 rats per time point. The grouping of neonatal rats (n = 100) was the same as that of adult rats. No animals suffered infection. Two adult rats died due to vertebral artery injury during operation, and were supplemented. A total of 15 neonatal rats died; 8 died due to excessive bleeding during operation, and 7 were eaten by their mothers. In total, 180 rats were involved in the final analyses after supplementation.

Expression of neurotrophic factors in neonatal and adult rats after brachial plexus root avulsion injury

Real-time PCR results showed that before injury, the expression levels of the three genes in neonatal rats were significantly higher than in adult rats (brain-derived neurotrophic factor, BDNF: P = 0.001 3; nerve growth factor, NGF: P = 0.000 3; neurotrophin-3, NT-3: P = 0.028 9). In the adult rat spinal cord, BDNF, NGF and NT-3 gene expression levels were significantly increased after injury (BDNF: P = 0.004 3; NGF: P = 0.004 2; NT-3: P = 0.001 6). The expression levels were highest at 7 days, and then gradually decreased to levels before injury at 21 days. BDNF, NGF and NT-3 gene expression levels were significantly lower than the pre-injury levels at 1 day after injury (BDNF: P = 0.000 1; NGF: P = 0.017 0; NT-3: P = 0.027 6). BDNF gene expression gradually recovered after the first 7 days, and reached a peak level at 21 days. NGF gene expression was the lowest at 7 days after injury, began to increase after 14 days, and peaked at 21 days. NT-3 gene expression increased on the 7th day after injury, and peaked on the 21st day (Table 1).

BrdU/nestin double immunostaining in the spinal cord of neonatal and adult rats after brachial plexus root avulsion injury (Figure 1, Table 2)

The number of BrdU/nestin-positive cells began to increase in adult rats from the 1st day after brachial plexus root avulsion injury (P < 0.05), peaked by 14 days, and gradually decreased.

Table 1 Neurotrophic factor mRNA levels in neonatal and adult rats after brachial plexus root avulsion injury

Neurotrophic factor	Rat	Before injury	Time after injury (day)			
			1	7	14	21
BDNF	Neonate	0.043 0±0.005 3[a]	0.008 0±0.001 0[a]	0.011 7±0.001 5[a]	0.011 3±0.003 1[a]	0.019 3±0.003 5[a]
	Adult	0.008 4±0.000 9	0.036 1±0.007 1	0.086 2±0.006 2	0.555 8±0.007 9	0.009 3±0.002 5
NGF	Neonate	0.004 9±0.000 4[a]	0.004 4±0.000 4[a]	0.002 4±0.000 2	0.002 3±0.000 3[a]	0.003 3±0.000 4[a]
	Adult	0.000 3±0.000 1	0.000 4±0.000 1	0.002 3±0.000 3	0.000 8±0.000 1	0.000 5±0.000 2
NT-3	Neonate	0.002 3±0.000 4[a]	0.001 7±0.000 5[a]	0.003 1±0.000 5[a]	0.004 4±0.000 6[a]	0.006 4±0.000 7[a]
	Adult	0.001 1±0.000 4	0.007 5±0.002 3	0.026 4±0.001 5	0.010 4±0.001 9	0.001 3±0.000 3

[a]P < 0.05, *vs*. rats at the same time point. Results are expressed as the absorbance ratio of the target gene to β-actin. Data are expressed as mean ± SD of 5 rats in each group at each time point (multivariate analysis of variance). BDNF: Brain-derived neurotrophic factor; NGF: nerve growth factor; NT-3: neurotrophin-3.

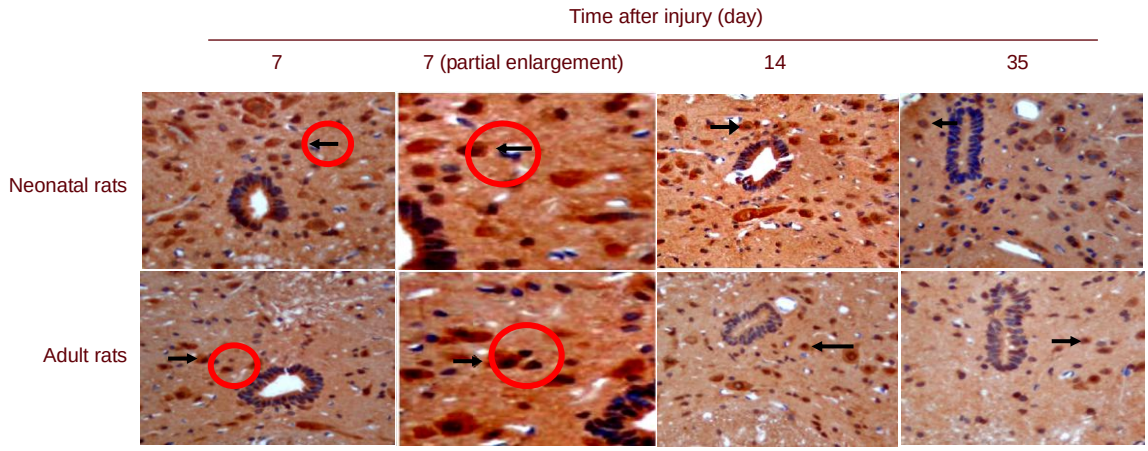

Figure 1 Double-labeling for bromodeoxyuridine (BrdU)/nestin in neonatal and adult rats after brachial plexus root avulsion injury (immunostaining, × 200).

Arrows represent BrdU/nestin positive cells. The number of positive cells reached a peak at 14 days in adult rats, which is different from that in neonatal rats (on the 7th day). The circle represents the magnified area.

Table 2 Number of bromodeoxyuridine/nestin-positive cells in the spinal cord at different time points

Group	Before injury	Time after injury (day)	
		1	7
Adult control	1.66±0.57	1.67±1.15	2.33±0.58
Adult injury	1.67±1.53	10.33±2.52[a]	18.33±3.06[a]
Neonate control	4.66±1.53	4.33±1.52	3.67±1.53
Neonate injury	4.67±1.15[a]	7.33±1.53[a]	14.67±1.53[a]

Group	Time after injury (day)	
	14	35
Adult control	2.00±1.73	2.67±1.53
Adult injury	19.33±2.08[a]	11.00±2.65[a]
Neonate control	2.33±0.58	1.33±1.15
Neonate injury	10.67±2.08[a]	4.67±1.53[a]

[a]$P < 0.05$, *vs*. control group of neonatal and adult rats at the same time point. Data are expressed as mean ± SD of five rats in each group at each time point (multivariate analysis of variance).

The positive nucleus was blue-black, and the cytoplasm was brown. At 400 × magnification under the optical microscope, the number of positive cells per 100 cells was quantified for each specimen. Five consecutive counts were averaged.

Most of the positive cells were observed near the ependyma and the gray matter. The number of BrdU/nestin-positive cells began to increase starting on the 1st day after injury in neonatal rats ($P < 0.05$), peaking on the 7th day. Positive cells were mainly observed near the ependyma and the gray matter in neonatal rats. The number of positive cells was greater in adult rats compared with neonatal rats at the same time point.

BrdU/glial fibrillary acidic protein (GFAP) double immunostaining in the spinal cord of neonatal and adult rats after brachial plexus root avulsion injury

The number of BrdU/GFAP-positive cells began to increase in adult and neonatal rats starting 14 days after injury, and peaked on day 35. These cells were mainly found near the central canal and spinal cord gray matter. At 14 and 35 days after injury, the number of positive cells significantly increased ($P < 0.05$) in adult and neonatal rats. The number of BrdU/GFAP-positive cells in adult rats was significantly higher than in neonatal rats (Figure 2, Table 3).

BrdU/β-tubulin double immunostaining in the spinal cord of neonatal and adult rats after brachial plexus root avulsion injury

BrdU/β-tubulin-positive cells were not observed.

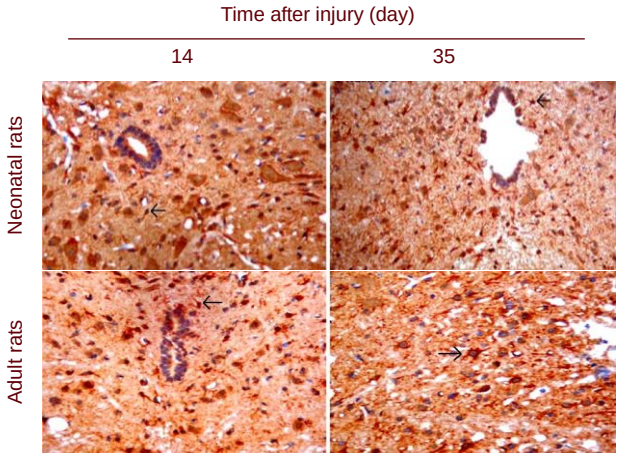

Figure 2 Bromodeoxyuridine (BrdU)/glial fibrillary acidic protein (GFAP) double immunostaining in neonatal and adult rats after brachial plexus root avulsion injury (immunostaining, × 200).

The number of BrdU/GFAP-positive cells in adult and neonatal rats reached peak values on the 35th day, and the quantity was greater in adult rats compared with neonatal rats. Arrows represent BrdU/GFAP-positive cells.

Table 3 Number of bromodeoxyuridine/glial fibrillary acidic protein-positive cells at different time points

Group	Before injury	14 days after injury	35 days after injury
Adult injury	3.33±1.53	14.33±22.52[a]	23.67±3.51[a]
Adult control	3.67±2.08	3.33±0.58	3.00±1.73
Neonatal injury	3.00±1.73	7.67±3.06[a]	21.67±2.51[a]
Neonatal control	3.40±2.30	3.67±1.15	3.60±1.34

[a]$P < 0.05$, *vs*. control group of neonatal and adult rats at the same time point (multivariate analysis of variance). At 400 × magnification under the optical microscope, the number of positive cells per 100 cells was quantified for each specimen. Five consecutive counts were averaged.

DISCUSSION

Neurotrophic factors play a crucial role in the development of the nervous system–the maintenance of normal function and plasticity[8]. Immature neurons are more dependent on trophic support from the target tissue, and are more vulnerable to axonal injury compared with mature neurons[9]. In the present study, expression of neurotrophic factors increased rapidly after brachial plexus root avulsion injury over a short period of time, and then gradually decreased to normal levels in adult rats. This indicates that in adult rats, neurons are less dependent on trophic support from the target. The expression of neurotrophic factors in the spinal cord rapidly increased to protect the damaged neurons and to promote functional recovery in response to external injury. The expression of neurotrophic factors in neonatal rats was different from that in adult rats. Levels initially decreased, and then gradually increased and remained at high levels for an extended period of time. Neurons in neonatal rats are more dependent on the target tissue for trophic support than neurons in adult rats. When trophic support was cut off, expression of neurotrophic factors decreased in the spinal cord; however, the immature nervous system has excellent plasticity[10], and this plasticity requires support from neurotrophic factors. Thus, while expression levels were low in the early phase, they later increased and remained at high levels. Enhanced neural plasticity is the reason why neonatal rats have better functional recovery after brachial plexus root avulsion compared with adult rats.

In the present study, BrdU/β-tubulin, BrdU/GFAP and BrdU/nestin were used to label proliferating neurons, proliferating astrocytes and proliferating neural stem cells, respectively. We found that BrdU/nestin-positive cells increased significantly in the spinal cord of adult and neonatal rats after brachial plexus root avulsion injury. However, the number of these cells in adult rats was higher than in neonatal rats, suggesting that while brachial plexus root avulsion injury induces proliferation of neural stem cells in both adult and neonatal rats, it does so to a lesser extent in neonatal rats. We also found that the quantity of BrdU/GFAP-positive cells in adult rats was significantly higher than in neonatal rats after 14 and 35 days of brachial plexus root avulsion injury. BrdU/β-tubulin-positive cells were not found in adult rats or neonatal rats. These results indicate that endogenous neural stem cells differentiate mainly into astrocytes. The differentiation of neural stem cells in neonatal rats did not occur to the same extent as in adult rats. Therefore, these results suggest that there is no greater neural stem cell regeneration in neonatal rats after brachial plexus root avulsion injury than in adult rats.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

Performed at the Laboratory of Histology and Embryology, Basic Medical College, and Department of Experimental Pharmacology and Toxicology, Jilin University, China, from October 2009 to January 2011.

Materials

100 healthy neonatal 7-day-old Wistar rats and 100 healthy 3-month-old Wistar rats, male or female, were provided by the Laboratory Animal Center, Basic Medical Institute, Jilin University, China (license No. SCXK (Ji) 2007-0001). All experimental protocols 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[11].

Methods

Establishment of a brachial plexus root avulsion injury model

A model of brachial plexus root avulsion injury was established using classic brachial plexus root avulsion injury on the cervical dorsal C_{5-6}, based on a previously described method[12]. Briefly, adult rats were anesthetized by intraperitoneal injection with 5% ketamine (300–350 mg), and neonatal rats were anesthetized by diethyl ether inhalation. With C_6 as the center, a median incision, 1–3 cm, was made at the back to expose the dorsal muscle and the C_{4-6} vertebral plate. Arcuate ligaments between C_{4-5} and C_{5-6} were dissected using microscissors, and the zygapophysial joints of C_{4-5} and C_{5-6} were resected. The C_5–T_1 nerve root was clearly observed. The C_{5-6} dorsal nerve root was gently avulsed from the spinal cord. The C_{5-6} ventral nerve root was also avulsed using the same method.

BrdU labeling of proliferating cells

The rats were intraperitoneally injected with BrdU (each 50 mg/kg, prepared with 0.007 M NaOH + 0.01 M PBS before injection) 24 hours after brachial plexus root avulsion injury, twice per day, for 7 days.

Real-time PCR detection of BDNF, NGF and NT-3 gene expression

Spinal cord tissues, 20 mg, were harvested from each group of animals to extract RNA using total RNA extraction kit. The cDNA was obtained by reverse transcription. The primers are as follows:

Primer	Sequence (5'–3')	Product size (bp)
BDNF	Sense: GAG CTG AGC GTG TGT GAC AG Antisense: CGC CAG CCA ATT CTC TTT TTG C	278
NT-3	Sense: GAG CAT AAG AGT CAC CGA GG Antisense: AGT CAG TGC TCG GAC GTA GG	264
NGF	Sense: CTT CAG CAT TCC CTT GAC AC Antisense: AGC CTT CCT GCT GAG CAC ACA	594
β-actin	Sense: TAC AGC TTC ACC ACC ACG G Antisense: TCC ACG TCA CAC TTC ATG ATG	272

Each sample was amplified with three parallel real-time PCR reactions. Reaction conditions were as follows: pre-denaturation at 95°C for 10 minutes; and multiple cycles of melting at 95°C for 30 seconds, annealing at 58°C for 20 seconds, and extension at 72°C for 30 seconds. MxPro software was used to analyze data. Results were expressed as the absorbance ratio of the target gene to β-actin.

Immunohistochemical double staining for BrdU/nestin, BrdU/GFAP and BrdU/β-tubulin in the spinal cord

At 1, 7, 14 and 35 days following brachial plexus root avulsion injury, rats were perfused with 4% paraformaldehyde into the left ventricle, until the body became stiff. The C_5 and C_6 spinal cord segments were placed in 4% paraformaldehyde at 4°C for fixation. After routine paraffin imbedding, the sample was sliced into 4-μm-thick sections. After hematoxylin-eosin staining, the section was attached to a polylysine-coated slide for subsequent immunohistochemical staining. Labeling was visualized using the streptavidin-peroxidase-diaminobenzidine method, and was followed by hematoxylin staining. Positive nuclei were stained blue-black, and the cytoplasm stained brown. Using an optical microscope (Olympus, Tokyo, Japan) at 400 × magnification, the number of positive cells per 100 cells was quantified for each specimen. Five consecutive counts were averaged.

Statistical analysis

Experimental data were expressed as mean ± SD and analyzed using multivariate analysis of variance with SPSS 11.5 software (SPSS, Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

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Author contributions: Lei Chen and Bin Liu designed the study. Bingqi Wang conducted experiments and wrote the manuscript. Zhigang Liu evaluated the experimental results. Zhixin Zhang, Liangsong Song, Laijin Lu and Yuehai Pan collected experimental data.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee of Jilin University, China.

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