Effects and Mechanism of Action of Neonatal Versus Adult Astrocytes on Neural Stem Cell Proliferation After Traumatic Brain Injury

YONG DAI, a,b,* FEIFAN SUN, a,b,* HUI ZHU, a QIANQIAN LIU, a XIDE XU, a PEIPEI GONG, a RUJI JIANG, a GUOHUA JIN, c JIANBING QIN, c JIAN CHEN, a XINGHUA ZHANG, c WEI SHI G,a

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ABSTRACT

Due to the limited capacity of brain tissue to self-regenerate after traumatic brain injury (TBI), the mobilization of endogenous neural stem cells (NSCs) is a popular research topic. In the clinic, the neurogenic abilities of adults versus neonates vary greatly, which is likely related to functional differences in NSCs. Recent studies have demonstrated that the molecules secreted from astrocytes play important roles in NSC fate determination. In this study, conditioned media (CM) derived from neonatal or adult rat astrocytes, which were unstimulated or stimulated by lipopolysaccharide (LPS), were prepared to treat NSCs. Our results revealed that neonatal rat astrocytes can significantly promote the proliferation of NSCs, compared with adult rat astrocytes, regardless of whether or not they were stimulated by LPS. Furthermore, we used mass spectrometry to detect the constituents of the CM from each group. We analyzed and screened for a protein, Tenascin-C (TNC), which was highly expressed in the neonatal group but poorly expressed in the adult group. We found that TNC can bind to the NSC surface epidermal growth factor receptor and promote proliferation through the PI3K-AKT pathway in vitro. Additionally, we confirmed in vivo that TNC can promote damage repair in a rat model of TBI, through enhancing the proliferation of endogenous NSCs. We believe that these findings provide a mechanistic understanding of why neonates show better neuroregenerative abilities than adults. This also provides a potential future therapeutic target, TNC, for injury repair after TBI. STEM CELLS 2019;37:1344–1356

SIGNIFICANCE STATEMENT

It has been previously shown that neurogenic abilities of adults versus neonates after traumatic brain injury (TBI) vary greatly, which is likely related to functional differences in neural stem cells (NSCs). In this study, it was shown that neonatal rat astrocytes can significantly promote the proliferation of NSCs, compared with adult rat astrocytes. Furthermore, the study showed that Tenascin-C (TNC), which is highly expressed in the neonatal group but poorly expressed in the adult group, can promote NSC proliferation in vitro and in vivo. Thus, TNC significantly impacts NSC function as well as injury repair after TBI.

INTRODUCTION

Currently, the clinical treatment for traumatic brain injury (TBI) remains a serious challenge. Considerable past research has shown that, in adult mammals, central nervous system (CNS) regeneration occurs primarily in two regions: the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone of the dentate gyrus of the hippocampus [1, 2]. In these areas, neural regeneration relies on the existence and maintenance of endogenous neural stem cells (NSCs) [3–5]. NSCs have been shown to have the potential to both proliferate and differentiate, to generate the different cell types of the CNS [6–8]. Recently, however, a growing number of studies have demonstrated that a group of endogenous stem cells in the postnatal and adult cortex can be activated after TBI. These cells are capable of self-renewal, proliferation, and the production of new neurons, astrocytes, and oligodendrocytes, which can contribute to damage repair [9, 10].

Increasing research indicates that under normal physiological conditions, there are significant differences in the number and distribution of NSCs in the brains of neonatal versus adult rats [11]. In the developing brain, NSCs are distributed in many areas, such as the cerebral cortex, corpus striatum, hippocampus, olfactory bulb,
cerebellum, and spinal cord [12]. As the CNS matures, the number of NSCs rapidly declines [13]. Compared with neonates, NSC self-renewal, and the corresponding capacity for endogenous repair, in the adult brain is poor [14]. Though it has previously been demonstrated that a series of substances [15–17] can promote the proliferation of endogenous NSCs, it should be noted that the capacity for neural repair by endogenous NSCs postinjury remains severely limited. This results in irreversible damage and lasting neurological deficits after neuronal injury such as TBI. Some studies have shown that the neurogenic ability of the brain decreases as the body matures, suggesting that neonates have a greater capacity for damage repair than mature animals [18]. In clinical practice, it has also been found that the ability of the brain to conduct injury repair after TBI decreases with age [19], and that functional recovery from TBI during the neonatal period is significantly greater [20]. In order to overcome the limited neurogenic ability in adult patients after TBI, a first and crucial step is to determine whether the greater neural regeneration abilities seen in neonates is related to a particular characteristic of their NSCs, and to then determine the possible mechanisms involved.

It is well known that the ability of NSCs to proliferate and differentiate depends largely on the neuro-microenvironment [21]. An increasing number of studies have shown that, with age, the neuro-microenvironment under physiological or pathological conditions differs significantly [22]. The CNS is composed of different types of nerve cells which interact with each other to form a precise and complex neural network [23]. It is well accepted that astrocytes constitute a major component of the CNS neuro-microenvironment [24–26]. In addition to providing nutrition, support, and protection to neurons under physiological conditions, astrocytes also play crucial roles in CNS pathologies [27]. Additionally, it has been reported that various bioactive molecules produced by astrocytes can participate in regulating the behavior of NSCs [28–30]. Moreover, it should be noted that the astrocytes obtained from the perinatal rodent brain are able to influence other cells in the CNS, including NSCs [31], whereas astrocytes from the adult brain tend to be relatively “inactive” toward other CNS cells [32–34]. In particular, the effect of adult astrocytes on NSCs remains unknown, but we suspect that it may be an interaction that contributes to the more serious post-TBI neurologic deficits seen in adult patients relative to those in young patients.

In this study, we focused on neonatal and adult rat astrocytes to determine their differential effects on the proliferation of NSCs both in vitro and in an in vivo TBI model. Additionally, we explored the possible molecular signals involved in this process. We believe these findings help to explain the observed difference in injury repair between adults and neonates, which may provide new molecular targets and a promising future direction for the promotion of NSC proliferation after TBI in adults.

**Materials and Methods**

**Animals**

Sprague–Dawley (SD) rats were obtained from the Laboratory Animal Centre of Nantong University (Nantong, China). Animal experiments were approved by the Nantong University Animal Experimentation Committee, and were conducted in accordance with their ethical guidelines, which were in compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

**Extraction and Culture of Neonatal and Adult Rat Astrocytes and Extraction of Corresponding Conditioned Medium**

Neonatal astrocytes were extracted from 1- to 2-day-old postnatal SD rats and adult astrocytes were extracted from 8-week-old SD rats. Preparation of the astrocytes has been described previously [15]. When the neonatal and adult astrocytes reached approximately 80% confluence at the third passage, the cells were treated with fresh DMEM/F12 plus 5 μg/ml lipopolysaccharide (LPS) (Sigma Aldrich, St. Louis, MO, www.sigmaaldrich.com). After stimulation, the supernatant was replaced with fresh DMEM/F12 and collected to obtain inflammatory and non-inflammatory stimulated astrocyte conditioned media (CM), respectively, in cultures from both neonatal and adult rats. The CM were centrifuged, filtered, and stored at −80°C for testing and later use.

**Neural Stem Cell Cultures**

Primary NSC cultures were isolated from embryonic (E) day 14.5 SD rats. After removal of the meninges, the tissue was dissociated mechanically into a single-cell suspension. After centrifugation and resuspension, the cell suspensions were plated into flasks with DMEM/F12 containing 2% B27 (Gibco, Waltham, MA, www.thermofisher.com), 20 ng/ml epidermal growth factor (EGF, Sigma Aldrich, ) and 20 ng/ml fibroblast growth factor 2 (bFGF-2, Sigma Aldrich); together this constituted the NSC expansion medium. The culture medium was replaced by fresh NSC expansion medium every 3 days. After 7 days culture in vitro, the cells grew into floating neurospheres. After another 5–7 days, the newly formed passage (P) 1 neurospheres were incubated in accutase (Sigma Aldrich, ), and subsequently triturated into single-cell suspensions. For Nestin identification, cell suspensions were plated at a density of 1 × 10^5 cells/ml on poly-lysin-coated coverslips in 24-well plates (adherent conditions), containing NSC expansion medium.

**Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis**

Total RNA was extracted from astrocyte cultures using the Trizol (Invitrogen, Carlsbad, CA, www.thermofisher.com) method. RNA concentration was determined by a spectrophotometric reading at 260 nm. For mRNA expression analysis, 1–3 μg of RNA were used to synthesize cDNA, and the SYBR green (Roche, Mannheim, Baden-Württemberg, Germany, www.roche-applied-science.com) method was performed using the StepOnePlusTM real-time PCR system (Applied Biosystems, Foster City, CA, www.appliedbiosystems.com), according to the manufacturer’s instructions. Primers used for real-time PCR were as follows:

- Tenascin-C (TNC)-Forward: 5'-CCTATTCGTCGGCCTGGTGT A3'; TNC-Reverse: 5'-TCCGGTCACTTGCCTGGT3';
- Epidermal growth factor receptor (EGFR)-Forward: 5'-GGACGCCACCAAGACA-3'; EGFR-Reverse: 5'-GTCAAGCTGACTCCACGGTTG-3';
- Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-Forward: 5'-CACGGCAATTTCAACGACAG-3'; GAPDH-Reverse: 5'-GA CGCCATGACTCCACGACAT-3'.

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Immunocytochemistry and Immunohistochemistry Assays

For immunocytochemistry, induced cells were fixed with 4% paraformaldehyde (PFA), after three washes in PBS. Cells were then permeabilized with 0.3% Triton X-100, and blocked with 5% normal goat serum for 2 hours. Cells were then incubated with the following primary antibodies: anti-GFAP (EMD Millipore, Billerica, MA, www.emdmillipore.com), anti-Nestin (Abcam, Cambridge, Cambridgeshire, U.K., www.abcam.com), anti-TNC (Abcam), anti-Tuj1 (Abcam), anti-β1a1 (Abcam), or anti-EGFR (Abcam), overnight at 4°C. On the following day, cells were incubated with secondary antibodies, and the nuclei were stained with Hoechst 33342 (1:1,000, Pierce, IL, www.pierce.com). For immunohistochemistry, 1, 3, 5, or 7 days after TBI, rats were anesthetized by intraperitoneal injection of chloral hydrate. Brains were cryoprotected in 30% sucrose in PBS, and frozen serial coronal brain sections (15 μm thickness) were cut on a cryostat. Sections were permeabilized with 0.3% Triton X-100, and blocked with normal goat serum in PBS for 2 hours. Next, sections were incubated overnight at 4°C with the following primary antibodies: anti-GFAP (EMD Millipore), anti-Nestin (Abcam), anti-Ki67 (Abcam), anti-DCX (Abcam), anti-TNC (Abcam), anti-Tuj1 (Abcam), or anti-EGFR (Abcam). After washing, sections were incubated for 2 hours at 37°C with the iFluor 594 goat anti-mouse IgG (16468, AAT Bioquest, Sunnyvale, CA, www.aatbio.com) and Alexa Fluor 488 goat anti-rabbit IgG (ab150077, Abcam). Nuclei were counterstained with Hoechst 33342 (Sigma Aldrich, ).

Cell Viability and Proliferation Assays

The proliferation of NSCs was assessed with the CCK-8 Kit (Dojindo, Kumamoto, Japan, www.dojindo.com) according to manufacturer’s instructions. Briefly, each well was diluted in 1:10 ratios using DMEM-F12. After 2 hours incubation at 37°C, the absorbance was measured at 450 nm on a microplate reader.

Co-Immunoprecipitation

NSC suspension of passage one was centrifuged at 1,000 g for 5 minutes and the supernatant was discarded. Cells were then washed once with PBS and incubated with ligands TNC (10 μg/ml) for 10 minutes at 37°C. After incubation, carefully wash cells with PBS twice. Co-immunoprecipitation was performed using the Pierce™ Classic Magnetic IP/Co-IP Kit (Thermo Scientific, Rockford, IL, www.thermoscientific.com) according to manufacturer’s instructions. Briefly cell lysates were immunoprecipitated using anti-EGFR (Abcam) and analyzed by Western blot with anti-TNC (Abcam).

In Vitro siRNA Transfection

Cholesterol-modified small-interfering RNA for TNC (si-TNC) and control siRNA (si-Con) were obtained commercially (RiboBio, Guangzhou, China, www.ribobio.com). The second-generation astrocytes were removed, digested with trypsin, and seeded into 6-well plates at a density of 5 x 10^4 cells to achieve a transfection density of 30%-50%. Next, 5 μl of 20 μM siRNA stock solution (RiboBio) was diluted with 120 μl of 1X riboFECTTMCP Buffer (RiboBio), and mixed gently. Next, 12 μl of riboFECTTM CP Reagent (RiboBio) was added and mixed by gentle pipetting, followed by incubation at room temperature for 0–15 minutes. Finally, riboFECTTMCP Transfection Complex was added to the appropriate amount of medium, and the culture plate was incubated at 37°C for 24–96 hours. After 24–72 hours of transfection, qRT-PCR was performed to detect the transfection efficiency of the target gene.

In Vitro Lentivirus Transfection

NSCs were inoculated into 6-well plates at a density of 1 x 10^5 cells/well. A constructed lentivirus plasmid (LV-shEGFR, from GeneChem, Shanghai, China, www.genechem.com) was used to knockdown EGFR expression in NSCs. A blank vector (LV-shVector) was also transfected into NSCs to establish the control groups. The MOI was set to 5, 10, and 20. The green fluorescence expression intensity was observed to determine the best MOI value of lentivirus infection. After the lentivirus was cultured for 20 hours to the best infection complex value, the lentivirus was removed and replaced with fresh NSC medium. After 3 days, subsequent detection was performed. The shRNA sequence targeting EGFR was 5′-GCCTCCAGAGGATGGTTCAA-3′.

Proteomic Sample Preparation

The conditioned medium from each group of samples was collected into a 50 ml centrifuge tube. CM was then centrifuged at 2,000g for 10 minutes at 4°C. The supernatant was pipetted into a centrifuge tube and stored at −80°C. The samples were then transferred to a 10 kDa ultrafiltration centrifuge tube, centrifuged at 14,000g, and the protein quantified using the BCA method. An aliquot (15 μg) of the sample from each group was used for enzymolysis. An aliquot (100 μl) of UA buffer (containing 8 M Urea, 150 mM TrisHCl, pH 8.0) was then mixed into the sample, followed by 5 mM DTT and the solution was transferred to a 10 kDa ultrafiltration centrifuge tube, followed by centrifugation at 14,000g for 15 minutes. An aliquot (200 μl) UA buffer was added and centrifuged at 14,000g for 15 minutes. The supernatant was then discarded and 100 μl IAA (50 mM IAA in UA) was added to the pellet and shaken at 600 rpm for 1 minute. The mixture was then incubated at room temperature for 30 minutes protected from light, followed by centrifugation at 14,000g for 10 minutes. An aliquot (100 μl) UA buffer was added and centrifuged at 14,000g for 10 minutes. An aliquot (100 μl) of 20 mM ammonium bicarbonate was added to the mixture and centrifuged at 14,000g for 10 minutes, and this was repeated twice. An aliquot (8 μl) of Trypsin buffer (containing 4 μg Trypsin) was added to the pellet and shaken at 600 rpm for 1 minute, followed by incubation at 37°C for 16–18 hours; this step was repeated twice. The collection tube was changed and centrifuged at 14,000g for 10 minutes. The supernatant was collected and subjected to OD280 peptide quantification.

Mass Spectrometry Data Acquisition and Analysis

The mass-to-charge ratios of polypeptide fragments and peptides were collected in the following manner: 12 MS fragments (MS2 scan) were acquired after each full scan (MS1 scan 300–1,800 m/z), and the MS1 scan was performed in profile mode at a resolution of 70,000. The MS2 scan was performed in a profile mode at a resolution of 17,500, and the fragmentation mode used HCD (high energy collision dissociation) with a normalized energy of 30%, and a dynamic exclusion of 30 seconds. Additional parameters were as follows: detection
instrument temperature: 270°C; instrument company: Thermo-Fisher Scientific; detection: positive ion; column size: 0.075 mm × 150 mm (RP-C18; 3 μm, self-packing). The raw file was analyzed using MaxQuant 1.5.2.8, and MaxQuant’s own algorithm was used to identify polypeptides and proteins. The filtering parameters were as follows: peptide FDR ≤ 0.01; protein FDR ≤ 0.01; phosphosite FDR ≤ 0.01.

**Traumatic Brain Injury and Injection**

For the adult TBI model, 12-week-old SD rats were anesthetized and placed in a stereotaxic frame. The head was incised using a scalpel blade. One hole was drilled over the right hemisphere at approximately 1.5 mm right of the midline and 0.8 mm posterior to the bregma using a dental drill. Stab wound injury was caused by inserting a 26-gauge needle. The coordinates of stab lesion to the right cortex were as follows: anterior–posterior (AP): 0.8; lateral (L): 1.5; dorsal/ventral (D/V): 1.0; all relative to Bregma = 0. Immediately after injury, 10 or 20 μl of TNC recombinant protein (0.1 μg/μl) or TNC-overexpressing lentivirus (LV-TNC, from GeneChem) was injected (AP: 0.8; L: 1.2–1.8; D/V: 1.0; all relative to Bregma = 0). For control rats, PBS or blank vector (LV-Vector) only was injected. This study used a CRISPR/Cas9-based synergistic activation mediator (SAM) system to amplify the expression of TNC. Similarly, for the neonatal TBI model, 1 to 2 day-old postnatal SD rats were anesthetized on ice and injured in the right cortex (AP: 0.2; L: 0.4; D/V: 0.5; all relative to Bregma = 0). Then, 1 μl of previously diluted TNC/control siRNA (1 nmol/μl) was slowly injected (AP: 0.2; L: 0.3–0.5; D/V: 0.5; all relative to Bregma = 0). After the surgery, the rats were kept warm and left to recover.

**Statistical Analysis**

All data are presented as the mean ± SD of at least three independent experiments. Most statistical analyses were made using a one-way analysis of variance, followed by a Dunnett’s multiple comparison test. p < .05 was considered to be statistically significant.

**RESULTS**

**Effects of Neonatal and Adult Astrocytes on NSC Proliferation**

To investigate the effect of normal or activated neonatal and adult astrocytes on NSC proliferation, we first extracted primary astrocytes from SD rats 1 day or 8 weeks after birth. Cells were observed by light microscopy and identified by immunofluorescence (supplemental online Fig. S1A, S1B). Next, we extracted CM from neonatal and adult rat astrocytes under LPS-stimulated and unstimulated conditions. NSCs were treated with the different types of CM listed above, and the CCK-8 assay and Ki67 immunofluorescence were performed to detect the proliferation efficiency of NSCs. Results showed that regardless of whether the astrocytes were stimulated by LPS or not, the neonatal astrocyte CM groups (Neonatal and LPS-Neonatal) exhibited significantly greater proliferation of NSCs, compared with the adult astrocyte CM groups (Adult and LPS-Adult) (Fig. 1A–1C). Furthermore, the CCK-8 assay revealed a statistically significant difference in the cell viability of NSCs at the 48 hours CM-treated time-point (Fig. 1A). Similarly, after being treated with CM for 48 hours, the number of Ki67-expressing in NSCs was increased in the neonatal groups compared with adult groups, regardless of LPS stimulation state (Fig. 1B, 1C). In addition to the CM from astrocytes, we further explored the effect of exosomes (supplemental online Fig. S2A, S2B) secreted by neonatal rat astrocytes. Results showed that these exosomes did not significantly increase NSC proliferation (Fig. 1D). These findings suggest that astrocytes in neonatal rats may regulate NSC proliferation through a non-exosomal pathway.

**Mass Spectrometry Detection and Analysis**

In order to investigate any difference in protein composition that may mediate the effect of CM on NSC proliferation, we extracted CM from neonatal and adult rat astrocytes stimulated by inflammatory and noninflammatory factors, and performed proteomic analysis with liquid chromatography–tandem mass spectrometry (LC–MS/MS). The heat map (Fig. 2A) showed a large number of differentially expressed proteins between the adult and neonatal groups. In each group, many low-abundance proteins were measured (shown in green), in addition to a smaller number of high-abundance proteins (red). Then, we selected a group of differential proteins related to cell proliferation, nerve regeneration, and inflammation, such as PTN, TNC, ATRN, GRN, etc. The final validation via RT-PCR (data not shown) found that TNC showed the most significant difference in expression between the neonatal and adult groups. Moreover, the TNC protein itself has exocrine properties and can participate in intercellular communication. Likewise, further analysis of the subcategories of the matrisome showed that the LPS-stimulated and LPS-unstimulated neonatal groups contained a greater abundance of TNC, whereas the LPS-stimulated and LPS-unstimulated adult groups were virtually TNC-free. In order to further compare the degree of difference in TNC abundance between the adult versus the neonatal group, a volcano plot was generated to demonstrate the fold change in protein abundance (neonatal versus adult) based on statistical p-values. In the volcano plot, it can be seen that TNC is enriched in the neonatal groups compared with the adult groups, regardless of whether or not they are stimulated by LPS (Fig. 2B, 2C).

TNC was found to be abundantly enriched in the CM of neonatal rat astrocytes, but it is unknown whether TNC specifically regulates NSCs. We predicted through the String database that TNC may be able to promote proliferation through multiple pathways by binding to EGFRs, which are present on NSCs (Fig. 2D).

**The Expression of TNC in Astrocytes In Vitro and In Vivo**

In vitro, we performed immunofluorescence and Western blot analyses to verify the expression of TNC in astrocytes. Results showed that TNC expression was present in GFAP-labeled neonatal rat astrocytes, but not in adult rat astrocytes (Fig. 3A, 3J). Similarly, Western blotting confirmed the presence of TNC protein in neonatal rat astrocytes, but its near-absence in adult rat astrocytes (Fig. 3B, 3K). Furthermore, we determined its secretion level by enzyme-linked immunosorbent assay. The result demonstrated that neonatal rat astrocytes secrete large amounts of TNC into the CM, compared with adult rat astrocytes which secreted no detectable TNC (Fig. 3C). However, no
TNC was detected in exosomes from neonatal rat astrocytes (Fig. 3D).

In vivo, we used an SW lesion model in both neonatal and adult rats. Results showed that on the 1st and 5th days post-injury, a large number of GFAP and TNC double-labeled cells could be found around the cortical lesion site in neonatal rats, whereas in the adult rat cortical lesion area the number of GFAP+ and TNC+ cells was significantly lower (Fig. 3E, 3L). Moreover, we noticed that although fewer than neonatal astrocytes, the adult astrocytes expressed TNC slightly on the 5th day post-injury in vivo compared with in vitro, which may be caused by the complicated neuro-microenvironment composed of many different types of nerve cells. In addition to astrocytes, we have also found some Tuj1+ or NG2+ (specific markers of neurons and oligodendrocyte progenitor cells respectively) cells which can also express TNC (supplemental online Fig. S3A, S3B).

The above studies confirm that TNC can be expressed and secreted abundantly by astrocytes of neonatal rats, but much fewer by astrocytes of adult rats.

The Expression of EGFR in NSCs In Vitro and In Vivo

Using String database analysis, we had previously predicted that TNC may bind to the EGFR. We next sought to detect the expression of EGFR on NSCs. Immunofluorescence results showed EGFR expression on Nestin+ NSCs (Fig. 3F), and Western blot analysis also revealed the expression of EGFR in NSCs (Fig. 3G). In addition, co-immunoprecipitation was conducted to confirm the relationship between TNC and EGFR in NSCs (Fig. 3I). Furthermore, we found that EGFR;Nestin double-labeled cells could easily be detected around the border of the injured cortex in neonatal rats in vivo, whereas fewer were found in the cortex of injured adult rats (Fig. 3H, 3M).

Figure 1. NSC proliferation affected by neonatal and adult astrocytes. (A): The CCK-8 assay showed that the neonatal astrocyte conditioned media (CM) significantly enhanced the proliferation of NSCs compared with the adult astrocyte CM groups, regardless of whether LPS was used to stimulate astrocytes or not (N = 3; **, p < .01; ***, p < .001). (B, C): Representative images and quantification showing significantly increased Ki67 (red) expression in NSCs treated with neonatal astrocyte CM for 48 hours compared with the adult astrocyte CM groups, regardless of LPS stimulation states (**, p < .01). Scale bar = 200 μm. (D): The CCK-8 assay showed no significant effect of exosomes derived from neonatal rat astrocytes on the proliferation of NSCs (ns = no statistical significance). Abbreviation: LPS, lipopolysaccharide.
TNC Combines with EGFR on the Surface of NSCs to Promote Proliferation

Although our experiments have confirmed the existence of TNC secretion by neonatal rat astrocytes, as well as EGFR expression in NSCs, the specific mode-of-action of TNC required further exploration. We first knocked-down TNC in neonatal rat astrocytes by the use of TNC siRNA (si-TNC), and extracted the corresponding CM (si-TNC CM). The efficiency of TNC knock-down was verified (supplemental online Fig. S4A, S4B). After treating NSCs with CM for 48 hours, results confirmed that NSCs grown with si-TNC CM were significantly less proliferative than cells grown in the si-Con and normal CM groups, as assessed by a CCK-8 assay and Ki67 expression (Fig. 4A–4C). Moreover, when different concentrations of TNC recombinant protein were added into the NSC culture medium for 3 days, the proliferation level of NSCs was increased, reaching a peak at a TNC concentration of 10 μg/ml (Fig. 4D–4F). Interestingly, the differentiation of NSCs into neurons could also been promoted by TNC recombinant protein in vitro (supplemental online Fig. S5).

Figure 2. Proteomic analysis of astrocyte conditioned media (CM) with liquid chromatography–tandem mass spectrometry. (A): The heat map showed differential proteins between the neonatal and adult astrocyte CM groups. (B, C): The volcano plot images showed that TNC was rich in both (C) LPS-stimulated and (B) LPS-unstimulated neonatal groups but not in adult groups. (D): String database analysis predicted TNC can promote proliferation through multiple pathways by binding to epidermal growth factor receptors on NSCs. Abbreviations: LPS, lipopolysaccharide; TNC, Tenascin-C.
Figure 3. (Legend appears on next page.)
Figure 3. Expression of TNC in astrocytes and EGFR in NSCs in vitro and in vivo. (A): Immunofluorescence images showed that TNC (red) expression was present in GFAP-labeled (green) neonatal rat astrocytes but not in adult rat astrocytes in vitro. Scale bar = 100 μm. (B): Western blot analysis showed that TNC protein was enriched in neonatal rat astrocytes, but almost absent in adult rat astrocytes. (C): ELISA results demonstrated that large amount of TNC could be secreted into the conditioned media by neonatal rat astrocytes compared with adult rat astrocytes. (D): Western blot showing no detectable expression of TNC in exosomes secreted from neonatal rat astrocytes. (E): In vivo, representative images showed a large number of GFAP (green) and TNC (red) double-positive cells were observed around the cortical lesion site in neonatal rats on 1st and 5th days after injury, whereas in the adult rats were obviously fewer. Scale bar = 200 μm. (F): Representative immunofluorescence images showing expression of EGFR (red) in Nestin“ (green) NSCs. Scale bar = 200 μm. (G): Western blot analysis showing expression of EGFR in NSCs. (H): In vivo, EGFR (red) and Nestin (green) double-positive cells were located at the border of the injured cortex in neonatal rats, whereas fewer in injured adult rats. Scale bar =200 μm. (I): Co-immunoprecipitation showing relationship between TNC and EGFR in NSCs. (J–M): Quantification of (A), (B), (E), and (H) respectively (*, p < .05; **, p < .01; *** p < .001). Scale bar = 200 μm. Abbreviations: EGFR, epidermal growth factor receptor; GFAP, glial fibrillary acidic protein; LPS, lipopolysaccharide; NSCs, neural stem cells; TNC, Tenasin-C.

Figure 4. TNC combined with EGFR on NSCs to promote NSC proliferation. (A–C): After treating NSCs with neonatal astrocyte CM for 48 hours, CCK-8 assay (C) and Ki67 (red) expression (A, B) showed that NSCs grown with si-TNC CM (si-TNC) were significantly less proliferative than cells grown with the si-Con CM (si-Con) and normal CM (**, p < .01; ***, p < .001). Scale bar = 200 μm. (D–F): The CCK-8 assay (F) and Ki67 (red) expression (D, E) showed an increased proliferation of NSCs after treatment with different concentrations of TNC recombinant protein for 3 days (*, p < .05; **, p < .01; ***, p < .001). Scale bar = 200 μm. (G–I): After TNC recombinant protein was re-added into the CM of neonatal rat astrocytes (si-TNC+ TNC) in which TNC expression had previously been knocked down (si-TNC), the CCK-8 assay (I) and Ki67 (red) expression (G, H) showed that the proliferation of NSCs was restored (**, p < .01; ***, p < .001). Scale bar = 200 μm. (J–L): The CCK-8 assay (L) and Ki67 (red) expression (J, K) showed that TNC did not significantly promote the proliferation of NSCs after knocking down EGFR (LV-shEGFR) (***, p < .001). Scale bar = 200 μm. Abbreviations: CM, conditioned media; EGFR, epidermal growth factor receptor; TNC, Tenasin-C.
Further validation revealed that, if TNC recombinant protein was re-added into the CM of neonatal rat astrocytes in which TNC expression had previously been knocked down, the proliferation of NSCs was restored (Fig. 4G-4I). These experiments are sufficient to demonstrate that TNC from neonatal rat astrocytes can act on NSCs and regulate their proliferation. To explore the relationship between TNC and the EGFR, we further knocked down EGFR expression in NSCs using an EGFR lentivirus (LV-shEGFR) (supplemental online Fig. S4C). TNC recombinant protein was added into the LV-shEGFR NSC medium, and NSC proliferation was tested using a CCK-8 assay and Ki67 and PCNA expression (Fig. 4J-4L and supplemental online Fig. S6). We found that TNC was unable to significantly stimulate the proliferation of NSCs following the administration of LV-shEGFR. These results indicate that TNC secreted from neonatal rat astrocytes can promote the proliferation of NSCs, via activation of the EGFR on NSCs.

To further explore the activation of particular downstream pathways in TNC-treated NSCs, we selected a classic proliferation-related pathway. Western blot analyses were performed and revealed that the expression of p-AKT and p-PI3K in NSCs was upregulated after the treatment with TNC (Fig. 5A, 5B). Furthermore, it was found that the addition of TNC to NSC medium produced continuous upregulation of IRS1, PDK1, p-GSK-3β, and p-p21 (Fig. 5C, 5D). However, all these upregulation and activation of PI3K-AKT pathway induced by TNC were blocked when EGFR in NSCs was knocked down. Thus, we believe that TNC may promote NSC proliferation through the PI3K-AKT pathway.

Figure 5. Activation of the PI3K-AKT pathway in TNC-treated NSCs. (A, B): Representative Western blot showing expression of p-PI3K and p-AKT in each group (*, p < .05; **, p < .01; ***, p < .001). (C, D): The changes in PI3K-AKT pathway related proteins were confirmed by Western blot (*, p < .05; **, p < .01; ***, p < .001). Abbreviations: EGFR, epidermal growth factor receptor; TNC, Tenascin-C.

TNC Can Promote the Proliferation of NSCs around the Damaged Area After TBI

To investigate the role of TNC in injury repair after TBI in vivo, we first established a model of TBI using an SW lesion in neonatal and adult rats. After administration of the TBI, 10 or 20 μl of TNC recombinant protein, or PBS, were injected near the injury site in adult rats. The expression of Ki67 in Nestin+ cells and colocalization of Ki67 and Nestin in total Hoechst+ cells both revealed a significant increase in the proliferation of NSCs around the injury site after injection of TNC protein (Fig. 6A–6C). In addition, we found that injection of TNC recombinant protein increased the number of newly born (DCX+) neuronal progenitors.

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differentiated from NSCs by increasing NSC proliferation, although it did not significantly promote NSC differentiation into neurons in vivo (supplemental online Fig. S7A–S7C).

To further confirm the function of TNC, we used Cas9 technology to overexpress TNC by lentivirus. In vitro, we used RT-PCR to verify the overexpression efficiency of the TNC lentivirus (supplemental online Fig. S4D). We then injected the constructed TNC lentivirus into the cortex in vivo around the lesion site in adult rats. Results showed that the expression of Ki67 in Nestin+ cells around the injury site was significantly increased after injection of 10 or 20 μl of TNC recombinant protein (0.1 μg/μl). Scale bar = 200 μm. (B) Quantification of Ki67 and Nestin double-positive cells in total Hoechst+ cells (**, p < .01; ***, p < .001). (C) Percentage of Ki67+ among Nestin+ cells (***, p < .001). Abbreviations: PBS, phosphate-buffered saline; TNC, Tenascin-C.

Together, these in vivo results demonstrate that TNC is highly expressed in neonatal rat astrocytes, and that it is capable of enhancing the proliferation of Nestin+ NSCs post-TBI, which promotes injury repair.

**DISCUSSION**

Previous studies have only assessed the effect of astrocytes on NSCs during the neonatal period. The effect of adult astrocytes on NSCs remains unknown. Therefore, in our experiments, to identify the reason for the differences in post-TBI regenerative ability between neonates and adults, the effect of astrocytes of different ages on NSC proliferation was explored. The results of our experiments confirmed that neonatal astrocytes...
have more potent NSC-stimulating effects than adult astrocytes in vitro and in vivo, regardless of whether the astrocytes were stimulated by LPS or not.

Elucidating the specific molecular mechanisms at play is crucial to understanding this phenomenon described above. It is worth noting that immature astrocytes can produce and secrete different types of proteins, enabling them to remodel the peripheral and synaptic extracellular matrix in the CNS. Therefore, we hypothesized that astrocytes of different ages can exert different regulatory effects on NSCs through their complement of exocrine proteins. We therefore used CM from neonatal or adult rat astrocyte cultures, in the presence or absence of an inflammatory stimulus, and detected and analyzed their composition by mass spectrometry. Through proteomic analysis with LC–MS/MS, we revealed a significant difference in the presence of TNC protein between neonatal and adult rat astrocytes, regardless of whether they were stimulated by LPS or not. Neonatal astrocytes are thus capable of secreting TNC protein, whereas in adult astrocytes, TNC secretion is minimal.

Furthermore, immunofluorescence analysis also confirmed that TNC is abundantly present in the cerebral cortex of neonatal rats, but much less in adult rats in vivo. Previous studies have demonstrated that TNC is present in the extracellular matrix, and is highly expressed during embryonic development,

Figure 7. Effect of TNC on NSC proliferation around the damaged area in vivo. (A, C): Images and quantification showing the increased expression of Ki67 (red) in Nestin+ (blue) cells around the injured site after injection of a lentivirus overexpressing TNC (green) into the cortex of adult rats (**, p < .01). Scale bar = 200 μm. (B, D): Images and quantification showing injection of siRNA against TNC (red) into the neonatal rat cortex near SW lesions induced a significant decrease in the number of Ki67+ (green) among Nestin+ (blue) cells (*, p < .05). Scale bar = 200 μm. Abbreviations: GFP, green fluorescent protein; RFP, red fluorescent protein; TNC, Tenascin-C.
as well as during tissue repair and in pathological conditions such as chronic inflammation [35,36]. TNC is also present in radial astrocytes in the cerebral cortex, as well as in optic nerve astrocytes. Additionally, TNC is released from radial astrocytes in the SVZ, where it functions to promote the growth and development of the neocortex [37]. These observations are consistent with our finding that TNC is abundant in the CM of neonatal rat astrocytes, but is rare in the CM of adult rat astrocytes.

More crucially, we found that the addition of TNC recombinant protein to NSC medium was able to promote NSC proliferation, whereas the addition of si-TNC to neonatal rat astrocyte cultures was able to reverse the effect of astrocyte CM on NSC proliferation. Moreover, using a TBI-SW lesion model, we found an increase in TNC protein expression in neonatal rats compared with adult rats, as well as large number of Nestin+ cells around the cortex lesion area in neonatal rats. Immunofluorescence analysis also revealed an increase in Ki67 expression among Nestin+ cells around the injury area after injection of TNC recombinant protein or TNC-overexpressing lentivirus in adult rats. In subsequent experiments, after injection of siRNA against TNC into the cortex of neonatal rats with a cortical SW injury, the expression of Ki67 in Nestin+ cells surrounding the site of injury was significantly decreased, implying that TNC protein can promote the proliferation of NSCs. To further investigate cell fate of the proliferated Nestin+ cells, in vitro and in vivo experiments were conducted. Immunofluorescent images revealed that increasing concentrations of TNC induced neuronal differentiation of NSCs in a dose-dependent manner in vitro (supplemental online Fig. S5). More crucially, injection of TNC recombinant protein increased the number of newborn immature neurons differentiated from NSCs by enhancing NSC proliferation, although it did not significantly promote NSC differentiation into neurons in vivo (supplemental online Fig. S7), which was different from the result in vitro and may be due to the inhibition of neurogenesis by the inflammatory microenvironment in adult injured brain after TBI [38]. In addition, doublecortin (DCX) has been proved to be a specific marker of newborn neurons [39] and in this experiment above, Nestin and DCX double-positive cells indicated the direction of NSC differentiation into neurons.

To further investigate the mechanism by which TNC is able to promote NSC proliferation, we focused our attention on the EGFR, which is a surface receptor for TNC that plays an important role in NSC regulation [40]. Previous studies have confirmed that TNC is able to bind to the EGFR [41]. In our study, we knocked down EGFR expression in NSCs, using an EGFR lentivirus, and then treated these NSCs with TNC recombinant protein. Results showed that, following EGFR knockdown, the effect of TNC recombinant protein on NSCs was reversed; the TNC-mediated proliferation of NSCs was significantly reduced. This demonstrates that TNC is able to promote NSC proliferation via binding of the EGFR in NSCs. Moreover, after bioinformatic and Western blot analyses, we found that both p-AKT and p-PI3K expression in NSCs following treatment with TNC was significantly upregulated. Thus, we believe that TNC secreted from neonatal rat astrocytes may bind to the EGFR on NSCs, thereby promoting NSC proliferation via upregulation of the PI3K-AKT pathway.

**CONCLUSION**

We demonstrated that neonatal, but not adult, rat astrocytes can significantly stimulate NSC proliferation, regardless of their inflammatory status. Additionally, we have found a novel role for the protein TNC, which is secreted from astrocytes of the neonatal brain, demonstrating that it plays an important role in the process of NSC proliferation after TBI. We believe that TNC is an important potential future target for the promotion of NSC proliferation in adults after TBI. Further investigation into the clinical applications of TNC should be carried out in order to promote CNS regeneration after TBI.

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**AUTHOR CONTRIBUTION**

Y.D.: conception and design, collection and assembly of data, and manuscript writing; F.S.: conception and design, collection and assembly of data, data analysis and interpretation, and manuscript writing; H.Z. and Q.L.: conception and design, administrative support, provision of study material; X.X., P.G., and R.J.: conception and design; G.J., J.Q.: provision of study material; J.C., X.Z., W.S.: conception and design, financial support, and final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicated no potential conflicts of interest.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.
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