Adhesion molecules close homolog of L1 and tenascin-C affect blood–spinal cord barrier repair

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Mice deficient in the recognition molecules, close homolog of L1 (CHL1) and tenascin-C, show improved and reduced functional recovery, respectively, after spinal cord injury compared with wild-type littermates. In this study, we addressed the question whether the differential functional outcome was paralleled by differences in blood–spinal cord barrier (BSCB) repair in the two mouse strains. We conducted spinal cord compression injuries in knock-out and wild-type mice. BSCB permeability was assessed by measuring the Evans blue spread within the spinal cord tissue at 14–21 days after injury. Results show that CHL1 reduces and tenascin-C enhances BSCB permeability, suggesting a correlation between functional outcome and BSCB repair. ***NeuroReport*** **23:479–482 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

The blood–spinal cord barrier (BSCB) is a diffusion barrier that is integral in the central nervous system (CNS) as it regulates and restricts the transport of molecules and cells into and out of the spinal cord. Spinal cord injury (SCI) results in primary and secondary tissue damage and cellular responses after injuries. The primary responses refer to the mechanical damage that directly leads to BSCB breakdown, bleeding, cell death, destruction of essential neural structures, such as axons and myelin, as well as activation of central and peripheral glia and of the immune system, including the infiltration of neutrophils and macrophages [1]. Secondary responses include continued disruption of the BSCB and sustained inflammatory responses and astrogliosis. These responses greatly affect the degree of functional recovery [2].

The activation of astrocytes after SCI is of particular importance for repair of the BSCB [1]. Activated astrocytes upregulate the expression of various cell surface molecules including cell adhesion molecules (CAMs) and extracellular matrix (ECM) glycoproteins. Among these astrocyte-associated molecules are the CAM close homolog of L1 (CHL1) [3,4] and the ECM glycoprotein tenascin-C (TNC) [5,6]. As the two molecules have been implicated in evoking both conducive and repellent actions on neuronal cell bodies and neurites *in vitro* [7,8], we previously analyzed their functional roles in repair of the injured spinal cord using mice constitutively deficient in these molecules through genetic knock out. CHL1-deficient ($CHL1^{-/-}$) mice showed better locomotor recovery and axonal regeneration after SCI due to the lack of CHL1 expression in astrocytes and the resulting block of CHL1 homophilic binding mechanisms inhibiting axonal growth [4]. In contrast to $CHL1^{-/-}$ mice, mice constitutively ablated in TNC ($TNC^{-/-}$ mice) expression recover less well than their wild-type ($TNC^{+/+}$) littermates after SCI [9]. On the basis of these observations we were interested to analyze whether we could correlate an abnormal BSCB repair, different between the two mutants, with the distinct and opposite outcomes in recovery from SCI. Here we show that, compared with wild-type littermates, BSCB permeability is decreased in $CHL1^{-/-}$ mice and increased in $TNC^{-/-}$ mice with SCI. These findings suggest that CHL1 and TNC have differential impacts on BSCB repair correlating with different outcomes after SCI.

Materials and methods

Animals

Ten $CHL1^{-/-}$ and nine $CHL1^{+/+}$ mice, and 10 $TNC^{-/-}$ and 10 $TNC^{+/+}$ mice were obtained from heterozygous breeding pairs with a mixed C57BL/6J-129Ola genetic background and five backcrosses into C57BL/6J [10,11]. Mice at the age of 77–130 days were used. Genotypes were determined by PCR analysis. For the experiments with $CHL1^{-/-}$ and $CHL1^{+/+}$ mice, only females were used. For the experiments with $TNC^{-/-}$ and $TNC^{+/+}$ mice, both males and females were used after assuring that there was no statistical difference in results between sexes. Mice were kept under standard laboratory conditions and taken care of by the animal services staff of the

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Keck Center, Nelson Laboratories, Rutgers University, in accordance with the guidelines of Rutgers University and the National Institutes of Health. All experiments as well as data acquisition and analyses were performed in a blinded manner.

Spinal cord injury

Mice were deeply anesthetized by intraperitoneal injections of ketamine and xylazine (100 and 5 mg/kg body weight, respectively, both from Sigma-Aldrich, St Louis, Missouri, USA). The mice were then left on a 35°C heating pad for 20 min before surgery. Laminectomy was carried out at the T8–T10 level to expose the spinal cord using mouse laminectomy forceps (Fine Science Tools, Foster City, California, USA). The spinal cord was compressed for 1 s using a watchmaker forceps (Fine Scientific Tools) driven by an electromagnetic device to produce severe SCI [12,13]. The skin was closed using a 3-0 silk suture (Ethicon, Somerville, New Jersey, USA). All surgeries were performed using sterile technique. The mice were kept on heating pads (35°C) throughout the surgery and the following 5–6 h or until they awoke. During the postoperative period (14–21 days), mice were singly caged in a temperature-controlled room (22°C) with water and food within reach [4]. Bladders were manually voided twice daily.

Evans blue injection and measurement of spread in the spinal cord

During the postoperative days, Evans blue (EB, E2129; Sigma-Aldrich) was intravenously injected into the tail vein at days 14, 18, and 21 after SCI. These time points were chosen during a preliminary evaluation of changes in BSCB permeability in our compression SCI model. EB has the property to bind quantitatively to albumin *in vivo* [14]. The EB/albumin complex leaks through the disrupted BSCB into the tissue after SCI and is thus an indicator of BSCB integrity [15]. The extent of extravasation of the systemically applied EB (1% in saline, 1 ml/100 g body weight) along the spinal cord axis served as an estimate of BSCB integrity [16]. Twenty-four hours after the injection, the mice were again deeply anesthetized and transcardially perfused with 25 ml of PBS, pH 7.3 for 10 min at room temperature. Twenty-four hours after injection was chosen as the time point for sacrifice as at this time the plasma levels of EB decrease to insignificant levels coinciding with the uptake of the EB/albumin complex by astrocytes and microglia/macrophages for degradation [17].

Presence of EB was quantitatively assessed by measuring the spread of EB along the spinal axis after exposure of the dorsal surface of the spinal cord. Images were taken with a dissection microscope (Zeiss SteREO V8, Jena, Germany), and the maximum spread of the dye along the longitudinal axis of the spinal cord was measured using the Image J software (NIH, Bethesda, Maryland, USA).

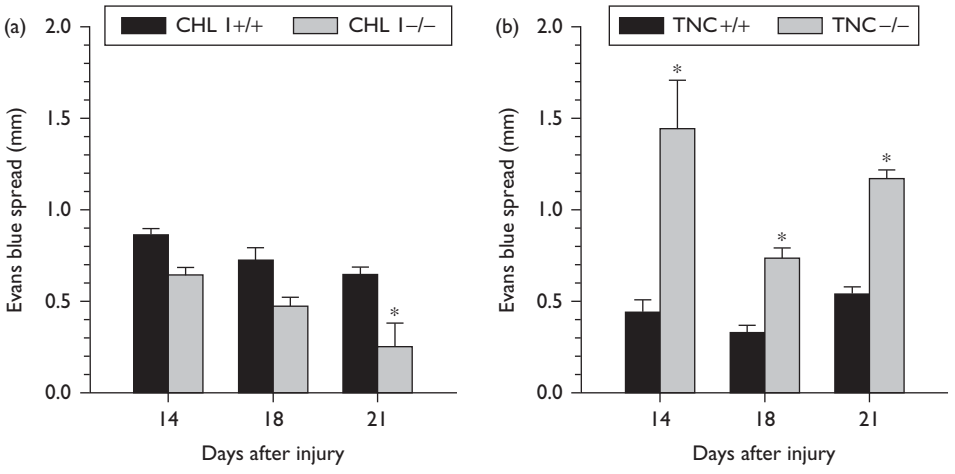
Statistical analysis

Data were collected in a blinded matter. Data were analyzed using two-way analysis of variance (ANOVA) with 'genotype' and 'time' as factors followed by the multiple comparison procedure of Holm–Sidak. P values of less than 0.05 were considered as significant.

Results

Measurements of the maximum rostrocaudal spread of EB were performed at 14, 18, and 21 days after compression SCI in *CHL1*$^{-/-}$ mice and their wild-type littermates and the

Fig. 1



Evans blue spread in the injured spinal cords. Mean Evans blue spread (± SEM) in spinal cords of *CHL1*$^{-/-}$ (a) and *TNC*$^{-/-}$ (b) mice and their wild-type littermates at 14, 18, and 21 days after compression SCI. Significant differences between mean values at a given time point (wild-type vs. knock-out mice) are indicated by asterisks ($P<0.05$, two-way analysis of variance with Holm–Sidak post-hoc test).

results showed overall higher values in $CHL1^{+/+}$ compared with $CHL1^{-/-}$ mice. Two-way ANOVA revealed significant effects of 'genotype' ($CHL1^{+/+}$ vs. $CHL1^{-/-}$, Fig. 1a, $F_{1,12} = 27$, $P < 0.001$, respectively) and 'time' ($F_{2,11} = 9.9$, $P = 0.003$). No interaction between the two factors ('genotype' × 'time') was found ($F_{2,12} = 0.9$, $P = 0.432$). Pairwise multiple comparisons using the Holm–Sidak method showed no changes over time (14–21 days) for wild-type mice ($CHL1^{+/+}$, $P > 0.05$) but differences for $CHL1^{-/-}$ mice (21 days < 18 and 14 days, $P < 0.05$). These results indicate lower BSCB permeability in $CHL1^{-/-}$ mice at 14–21 days after SCI and faster decline in this permeability over time in $CHL1^{-/-}$ compared with $CHL1^{+/+}$ mice.

In contrast to $CHL1^{-/-}$ mice, the mean spread of EB was larger in $TNC^{-/-}$ than in $TNC^{+/+}$ mice at 14, 18, and 21 days after compression SCI (Fig. 1b). Statistically significant effects were found for 'genotype' ($TNC^{+/+}$ vs. $TNC^{-/-}$ mice, $F_{1,12} = 72$, $P < 0.001$) and 'time' ($F_{2,11} = 9.7$, $P = 0.004$) (two-way ANOVA). In addition, there was a significant interaction between these two factors ($F_{1,12} = 5.0$, $P < 0.001$). Pair-wise multiple comparisons showed no changes over time for $TNC^{+/+}$ mice ($P > 0.05$, Holm–Sidak test) but differences for $TNC^{-/-}$ mice (18 days < 14 and 21 days, $P < 0.05$). These results indicate higher BSCB permeability in $TNC^{-/-}$ than in $TNC^{+/+}$ mice at 14–21 days after SCI and no decline in this permeability over time.

We thus conclude that in the absence of the two molecules during ontogenetic development and in the adult, the BSCB is different from those of wild-type mice and in opposite manners: the CHL1-/- mouse repairs the broken BSCB more efficiently that its CHL1 + / + littermate and the TNC-/- mouse repairs the BSCB barrier less well than its TNC +/+ littermate. The results imply that CHL1 impairs, while TNC enhances the repair of the adult BSCB.

Discussion

In this study, we provide evidence that the CAM and ECM proteins, CHL1 and TNC respectively, affect the repair of the BSCB. Previous studies had demonstrated that $CHL1^{-/-}$ mice functionally recover more efficiently after SCI compared with $CHL1^{+/+}$ mice and $TNC^{-/-}$ mice functionally recover worse after SCI compared with $TNC^{+/+}$ mice.

Choosing the appropriate time points was an important decision as previous studies on blood–brain barrier repair in the rat suggested that protein leakage into and around the injured site was maximal between 3 h to 1 day [18] with the barrier being re-established by 14 days. A study by Popovich *et al.* [19] shows, however, that the time course for leaking of molecules circulating in the blood stream through the barrier varies from 4 to 28 days. It is noteworthy in this context that the inflammatory response to a traumatic injury, such as spinal cord compression, is not the same as a wound inflicted to the cerebral cortex, let alone comparing rats and mice. It has been reported that the area and duration of increased membrane permeability is much greater in the cord than in the brain [20]. In our preliminary studies, we looked at the wild-type mice at time points ranging from 1 to 28 days after injury. One to 10 days after the injury, high concentrations of the dye had leaked into the spinal cord tissue and could not be measured differentially and precisely, whereas at ~4 weeks, infiltration of dye was minimal and not measurable. Therefore, the time range of 2–3 weeks was selected as the appropriate times to study the mouse BSCB repair.

The primary and secondary responses to tissue injury are important for recovery after SCI. Of these responses, particularly the activation of microglia/macrophages and astrocytes affect the BSCB repair. TNC, a major ECM molecule, is abundantly expressed in astrocytes, which play important roles in BSCB functions [6]. Our previous study showed that genetic deficiency in TNC inhibited axonal outgrowth and locomotor recovery [9], whereas deficiency in CHL1 prevents fibroblast growth factor-2-mediated upregulation of the CHL1 expression in astrocytes and inhibitory homophilic binding mechanisms of CHL1 [4]. The present study shows a positive correlation between closure of the BSCB and success in regeneration, but it cannot deduce mechanisms by which TNC and CHL1 affect BSCB repair. However, our study draws attention to adhesion molecules in BSCB functions and to a plausible connection between adhesion molecules and tissue homeostasis affected by the BSCB.

It is important to note that inflammatory responses following SCI, such as infiltration of macrophages and other immune cells into the spinal cord, also affect BSCB repair. The functions of CHL1 and TNC in the immune system thus need to be studied, not only in CNS injury, but also in other acute and chronic inflammatory responses outside the CNS. Therefore, further studies investigating the infiltration of the immune system cells into the spinal cord of $TNC^{-/-}$and $CHL1^{-/-}$ mice after SCI will be necessary. These studies should lead to further insights into our understanding of the mechanisms by which TNC aids and CHL1 hinders the recovery of spinal cord injured mammals.

Conclusion

In this study, we show a positive correlation between improved locomotor recovery after SCI and improved repair of the BSCB in $CHL1^{-/-}$ and $TNC^{+/+}$ mice compared with $CHL1^{+/+}$ and $TNC^{-/-}$, respectively. This novel evidence draws attention to adhesion molecules as players in spinal cord regeneration in correlation with BSCB repair and may lead to targets for therapy both at the level of the spinal cord as well as of the BSCB.

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Conflicts of interest

There are no conflicts of interests.

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