The Effect of Neural Stem Cell Treatment on Astrocyte Phenotypes morphology and Galectin Expression in Following Rat Spinal Cord Injury

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Abstract

Traumatic spinal cord injury (SCI) results in complex neurological dysfunction. Astrocytes react in response to SCI by undergoing proliferation and migrating to the injury site, resulting in astrogliosis. Neural stem cells (NSCs) have been proposed as a promising therapeutic strategy to promote neurogenesis following transplantation post SCI. In this study the antigenic and morphological phenotype of astrocytes, and the pattern of galectin expression was examined following SCI. Female Sprague Dawley rats were contused at thoracic (T) level T9 and treated with Cyclosporin-A (CsA) and/or NSCs 1 week post-injury. CsA and NSC treatment led to increased glial fibrillary acidic protein (GFAP), brain lipid binding protein (BLBP) and vimentin expression. Quiescent and reactive astrocytes were the most abundant cell morphology observed. Astrocytes co-localised with galectin-1 (Gal-1) and galectin-3 (Gal-3). Gal-1 expression was increased in both CsA and CSA + NSC treated animals 2 weeks post-treatment. Treatment with CsA and/or NSCs offers many prospective beneficial effects. A detailed revelation of how astrocytes are heterogeneously responsive to SCI, in addition to their affinity to galectins and response to CsA and NSCs is shown in this study. The current study's findings support the potential application of NSCs as a novel therapeutic tool in the treatment of SCI.

Introduction

Spinal cord injury (SCI) is a worldwide, life threatening condition that still has no cure. The physical force which causes the primary SCI is followed by a secondary injury cascade comprised of inflammatory cells such as macrophages, microglia, T-cells and neutrophils which infiltrate the injury site. Apoptosis and necrosis is observed at the lesion epicenter followed by axonal collapse, demyelination, glial scar formation. Glial cells proliferate in mammals and contribute to the glial scar. After SCI, the introduction of neural stem cells (NSCs) to the damaged spinal cord boosts neural repair by encouraging host cell regeneration and replacing host cells that have died at the site of injury. NSCs can differentiate into neurons or glial cells, in particular astrocytes, and can influence the mobilisation of endogenous stem cells by providing cellular scaffolding during cell migration. Cell transplantation has been proposed to promote axon growth by modifying the astrocyte response to injury. In addition to cell replacement, NSCs may be suitable as a means of delivering therapeutic proteins, including various neurotrophic factors, as they migrate towards the site of an injury, reducing inflammation and inhibiting reactive astrogliosis. CsA is an immunosuppressive drug which is neuroprotective and improves functional recovery in SCI rats, and it is often used in neural regeneration strategies involving transplantation to avoid tissue rejection.

Astrocyte morphology and associated function is quite diverse and understanding astrocytic structural diversity provides a fundamental basis for insight into the consequential effects on function, especially for cells involved in astrogliosis. Astrocytes are the most abundant glial cell type in the mammalian central nervous system (CNS) and arise from radial glial cells. After neurogenesis, those radial glial cells that differentiate to become astrocytes begin to express brain lipid binding protein (BLBP) and vimentin. Transcription pathways are activated which affect radial glia expressing BLBP, causing them to...
lose BLBP expression and differentiate into astrocytes [19] by extending additional cell processes and expressing glial fibrillary acidic protein (GFAP) [20]. GFAP is therefore commonly used as an antigenic marker for astrocytes. Once development is complete, mature astrocytes can be distinguished based on their morphology and can be classified based on their location within the cord. Astrocytes located in the white matter (WM), are long and thin with few processes (fibrous astrocytes). Astrocytes located in the gray matter (GM) contain multiple processes (protoplasmic astrocytes). Fibrous astrocytes continue to express vimentin, whereas protoplasmic astrocytes have reduced expression of vimentin [21, 22]. In normal, uninjured spinal cord naive astrocytes are also known as quiescent cells [23]. Non-process bearing astrocytes relate to cells in an immature proliferative state [24].

Following SCI, quiescent/naïve astrocytes sequentially exhibit a reactive astrocyte phenotype involving cellular hypertrophy as well as an augmented production of the intermediate filament protein GFAP [13, 25, 26, 27]. Astrocytes also overexpress vimentin following SCI, highlighting a close relationship between GFAP and vimentin in reactive astrocytes [27, 28]. In injured spinal cord BLBP positive subpial radial glial processes are reportedly upregulated [29]. Reactive astrocytes migrate to the area of injury and secrete chondroitin sulphate proteoglycans (CSPGs) and other inhibitory molecules to form the glial scar. The presence of CSPGs and other inhibitory molecules within the glial scar that inhibit axonal regeneration indicates that reactive astrocytes exert a detrimental effect following SCI [30]. Despite these negative outcomes, the astrocytic response to SCI has many additional aspects, some astrocytes contribute to neuroprotection and display repair mechanisms [31]. However detailed studies on astrocyte morphology in NSC-treated SCI tissue which may help clarify beneficial effects associated with particular morphologies have not been carried out.

Cell and tissue glycosylation is altered during injury and repair, and glycosylation plays an important role in many biological processes including cell differentiation, maturation, inflammation and tissue repair [32, 33, 34]. Carbohydrates exert their biological effects by interacting with lectins, carbohydrate-binding receptors, and galectins, a lectin family with binding affinity for β-linked galactosides, play important roles in a variety of physiological and pathological processes including immune responses and neuronal regeneration [35, 36]. Galectin-1 (Gal-1) expression is upregulated after SCI in many cells types including reactive astrocytes around the lesion site post-injury [37, 38]. Galectin-3 (Gal-3) expression is up-regulated by astrocytes and microglia at sites of pathology in SCI and is expressed by macrophages and microglia that phagocytose degenerated myelin [39]. In brain injury, Gal-3 has been shown to be expressed by reactive astrocytes associated with phagocytosis [40, 41]. In addition, Gal-3 plays a role in regulating the severity of neuroinflammation following SCI [42]. Given the importance of Gal-1 and Gal-3 in modulating the neural inflammatory response and promoting neuronal regeneration, these galectins have been proposed as important targets of therapeutic potential [43].

In this work, we carried out a detailed study of the phenotype of astrocytes in rat spinal cord tissues at 2 and 6 weeks following contusion SCI. We hypothesised that transplantation of NSCs alters the endogenous cellular environment with regard to the antigenic and morphological phenotype of astrocytes following SCI and that NSCs may alter expression levels of Gal-1 and Gal-3 following SCI.
Results

Four animal groups were examined in this study: uninjured, spinal cord injured, spinal cord injured treated with CsA, and spinal cord injured treated with a combination CsA and NSCs (CsA+NSC). While CsA has neuroprotective properties and can lead to functional recovery from SCI [8,15,16], in this instance it was also administered to prevent rejection of mouse NSCs by the host rat spinal cord tissue. The spinal cord WM and GM was examined and the lesion site was examined in all injured animal groups. Two time points post-treatment, 2 and 6 weeks, were examined for astrocyte density and morphology while Gal-1 and Gal-3 expression was examined at 2 weeks post-treatment.

Astrocyte density alters over time in spinal cord injured tissue

The percentage density of GFAP-positive cells at 2 weeks post-treatment was higher in CsA+NSC-treated animals at approximately 16% compared to injured and CsA-treated animals (approximately 8.5% and 9.5%, respectively, Figure 1A). At 6 weeks, the percentage density of GFAP-positive cells had decreased to approximately 12% in the CsA+NSC-treated group, which was still higher than the injured group, which had decreased to approximately 6% (Figure 1A). However the CsA-treated animal group had the highest overall percentage density of GFAP-positive cells at 6 weeks post-treatment with approximately 15% (Figure 1A). Vimentin-positive cells showed a similar response pattern to that observed with GFAP-positive cells at 2 weeks post-treatment, with approximate percentage densities of 16.5% for the CsA+NSC-treated group, 11% for the CsA-treated group, and 7% for the injured group (Figure 1B). There were no significant differences in percentage density of vimentin-positive cells between groups at 6 weeks and compared to 2 weeks post-treatment percentage density was the same for the injured animal group, decreased for the CsA+NSC-treated group and increased for the CsA-treated group (Figure 1B). At 6 weeks, the vimentin-positive cells percentage density was similar between the CsA-treated and CsA+NSC-treated groups (approximately 11% and 10.5%, respectively) and both groups had greater percentage densities than the injured group (approximately 7%, Figure 1B). No significant differences in radial glial cells labelled with BLBP were observed between groups at either time point, but both treatment groups exhibited an overall increase in percentage density (approximately 7.5% to 10% from 2 weeks to 6 weeks post-treatment for CsA-treated animals, and approximately 10% to 12.5% for CsA+NSC-treated animals) (Figure 1C). In contrast, the injured group displayed a small overall reduction in BLBP-positive cells percentage density (approximately 7% to 6% from 2 weeks to 6 weeks post-treatment) (Figure 1C).

In the uninjured spinal cord tissue, the distribution of immunohistochemical staining in the WM was measured (Supplementary Figure 1C). There were significantly more GFAP and vimentin-positive cells in the WM in comparison to BLBP-positive cells in unjured WM.

Astrocyte morphology in injured spinal cord

In uninjured WM, fibrous morphologies were the most abundant morphological phenotype observed (Supplementary Figure 2A-2C). For all antibody stains for the injured, CsA-treated, and CsA+NSC-treated
groups, there were markedly more quiescent cells within each animal group at both 2 and 6 weeks post-treatment compared to any other cell morphology (Figures 2A, 3A, 4A).

In GFAP-reactive tissue for all groups, there was a greater overall number of astrocytes present at 2 weeks compared to 6 weeks post-treatment and there were significantly more quiescent cells present compared to all other cell morphologies at both time-points (Figure 2A, 2C). The CsA treated group had the greatest number of GFAP-positive cells compared to the injured and CsA+NSC-treated groups at both time-points and the injured group had the lowest number of GFAP-positive cells. Injured tissue at 2 weeks post-treatment also had a reactive astrocyte morphology, which was approximately 23% of the number of quiescent cells, with very low numbers of fibrous and non-process bearing morphologies (Figure 2A, 2B). At 6 weeks post-treatment, injured tissue had no non-process bearing cells, there was a slight increase in fibrous cell number compared to 6 weeks and, while the overall number of reactive cells was lower than at 2 weeks, the proportion of reactive cells was greater (approximately 36% of the number of quiescent cells) (Figure 2A). GFAP-positive cells with a reactive astrocyte morphology were also overall decreased in number at 6 weeks compared to 2 weeks post-treatment for the CsA- and CsA+NSC-treated groups, but the proportion relative to quiescent cells remained similar for the CsA-treated group (reactive cell number approximately 40% of number of quiescent cells at 2 weeks and 37% at 6 weeks) while it decreased dramatically by 6 weeks for the CsA+NSC-treated group (reactive cell number approximately 45% of the number of quiescent cells at 2 weeks compared to approximately 14% at 6 weeks post-treatment) (Figure 2A). The CsA+NSC-treated group also had a similar low number of non-process bearing cells at both time points while the number of cells with fibrous morphology decreased from 2 weeks to 6 weeks post-treatment which was a marked decrease in relative proportion of GFAP-positive cells (fibrous cell number 25% of the number of quiescent cells at 2 weeks compared to 7% at 6 weeks post-treatment) (Figure 2A). Similarly, fibrous morphology cell number decreased over time for the CsA-treated group, but the relative proportion of GFAP-positive fibrous cells remained the same (fibrous cell number approximately 14% of the number of quiescent cells at 2 weeks compared to approximately 16% at 6 weeks post-treatment) (Figure 2A). Finally, CsA-treated tissues at 2 weeks post-treatment had the highest number of GFAP-positive non-process bearing cells of all groups and time-points (17% of the number of quiescent cells at 2 weeks) and cell number decreased to very few cells (<10 cells/mm²) by 6 weeks post-treatment (Figure 2A).

Spinal cord tissue stained with vimentin showed significantly more vimentin-positive cells with a quiescent cell morphology in all animal groups at both 2 and 6 weeks post-treatment, and there was an overall higher vimentin-positive cell number at 2 weeks compared to 6 weeks post-treatment (Figure 3A, 3D). The CsA+NSC-treated group had the lowest number of vimentin-positive cells at 2 weeks (Figure 3A). The overall number and relative proportion of reactive vimentin-positive cells decreased for the injured group by 6 weeks post-treatment (to approximately 8% of the number of quiescent cells), but both cell number and relative proportion increased for reactive cells for both the CsA-treated and CsA+NSC-treated groups (to approximately 61% and 13% of the number of quiescent cells, respectively) (Figure 3A, 3B, 3C). Vimentin-positive cell numbers for the non-process bearing morphology were low to none for all
groups at both time points (Figure 3A). Fibrous cell number increased from none at 2 weeks to low numbers at 6 weeks for the injured group, remained similar for the CsA-treated group at both time points, and decreased from 2 weeks to 6 weeks post-treatment for the CsA+NSC-treated group (Figure 3A).

In the injured spinal cords, there were higher numbers of BLBP-positive cells in all tissues at 2 weeks compared to 6 weeks post-treatment, and there were statistically more BLBP-positive cells with quiescent cell morphology in injured and CsA treated animals at 2 weeks compared to 6 weeks post-treatment (Figure 4A). The number of BLBP-positive cells with reactive morphology were higher overall at 2 weeks compared to 6 weeks for all animal groups, and the CsA-treated group at 2 weeks had the highest number of reactive cells (Figure 4A-4D). Similarly the relative proportion of reactive BLBP-positive cells decreased for each group from 2 weeks to 6 weeks post-treatment, with the injured group decreasing from a reactive cell number of approximately 25% of the number of quiescent cells to essentially none, the CsA-treated group from approximately 85% of the number of quiescent cells to 41%, and the CsA+NSC-treated group from approximately 54% of the number of quiescent cells to 22% (Figure 4A-4D). At 6 weeks post-treatment there was significantly more fibrous BLBP-positive cells in the CsA+NSC-treated group compared to the injured group, and this number was increased compared to the CsA+NSC-treated group at 2 weeks (Figure 4A). There were no BLBP-positive cells with non-process bearing morphology in any group at any time point except for the CsA-treated group at 2 weeks post-treatment, when the reactive cell number was approximately 9% of the number of quiescent cells (Figure 4A).

Skeletal length was examined in vimentin-positive cells in uninjured WM and at the lesion site of injured tissue at both 2 and 6 weeks post-treatment. There was a significant difference in skeletal lengths of vimentin-positive fibres in the uninjured WM compared with the injured animal group 2 weeks and 6 weeks post-treatment (Supplementary Figure 3A). No difference in skeletal diameter of vimentin-positive cells was observed between uninjured and injured tissue (Supplementary Figure 3B).

**Gal-1 and Gal-3 expression in injured spinal cord**

Gal-1 and Gal-3 expression and localisation was examined at the injury site, WM and GM of spinal cord tissue at 2 weeks post-treatment (Figure 5A).

There was no statistical difference in expression of Gal-1 in WM and GM of uninjured (control) and injured groups (Figure 5A). However, Gal-1 expression was significantly higher in the CsA- and CsA+NSC-treated groups compared to both the injured animals without treatment and uninjured animals for each region of interest examined (Figure 5A). In addition, overall Gal-1 expression was significantly higher in the CsA+NSC-treated group compared to the CsA-treated group (Figure 5A). Immunostaining showed Gal-1-positive staining in each region examined (Figure 5B-L), with colocalisation of Gal-1-positive staining with the GFP-positive NSCs at the lesion site in the CsA+NSC-treated group (Figure 5L), slight colocalisation in the WM (Figure 5J) but none in GM (Figure 5K). Dual staining to identify the Gal-1-positive cells with the astrocytic marker GFAP in injured tissue showed that Gal-1-positive cells in the GM and WM appeared to be astrocytes (Figure 6A, 6B), but the Gal-1 stained cells at the injury site were not positive for GFAP (Figure 6C). The Gal-1-positive astrocytes in GM and WM had longer processes,
identifying them as either fibrous or quiescent (Figure 6A, 6B). Some of the dual stained processes appear to enwrap larger cells, in particular in the WM (Figure 6A, 6B). The βIII integrin was previously identified as mediating neuron to astrocyte communication with the Thy-1 glycoprotein [44], and integrin glycosylation was previously shown to alter binding to galectins and change signalling responses [45,46]. Thus βIII integrin immunohistochemical staining was employed in combination with Gal-1 staining to ascertain any co-localisation (Figure 6D-F). As noted for the GFAP staining (although with a different pattern), there was some dual staining in both WM (Figure 6D) and GM (Figure 6E) but none at the injury site (Figure 6F). Dual staining appeared most intense at foci on processes near or surrounding larger cells (Figure 6D, 6E).

Gal-3-positive staining was observed in all regions of interest examined (Figure 7B-7L). There was a slight increase in Gal-3 expression at the injury site in injured and CsA-treated tissue, and a slight decrease compared to these tissues in the CsA+NSC-treated group, but there were no significant quantification differences between areas of the staining when regions of interest or animal groups were compared (Figure 7A). Gal-3-positive cells co-localised with GFP-positive NSCs in the CsA+NSC-treated tissues (Figure 7J-JL) but most markedly within the lesion site (Figure 7L). Again dual staining was performed with GFAP and Gal-3 to observe and dual staining with astrocytes in the injured tissue. Some co-localisation of Gal-3 staining with GFAP-positive astrocytes was also evident at the lesion site and the WM of the injured tissue (Figure 8A, 8C), but not in the injured GM (Figure 8B).

Discussion

GFAP expression increases throughout the lesion border in response to SCI, indicating the recruitment of astrocytes to the damaged tissue [25, 47], but astrocyte morphology in SCI tissue is not well investigated. In this work, the antigenic markers GFAP, BLBP and vimentin were used to identify the expression patterns and morphological phenotypes of spinal cord cells. CsA has been previously shown to offer protective effects to astrocytes [48] and in this case CsA also spared astrocytes from damage. Additionally we have demonstrated that CsA induces an up-regulation of GFAP-positive astrocytes at the glial scar at 6 weeks (Fig. 1A). Previously a notable increase in GFAP-immunoreactive astrocytes following CsA treatment was reported [49]. Here we show there was also an increase of GFAP-positive cells in CsA+NSC-treated animals compared to injured animals, both 2 and 6 weeks post treatment (Fig. 1A). Previous reports suggest that administration of NSCs reduce the amount of glial scarring following injury [8].

Vimentin is expressed in fibrous astrocytes in the adult spinal cord [22]. Following injury, vimentin is upregulated and expressed by reactive astrocytes which surround the lesion site [8]. CsA+NSC treated animals showed a higher expression of vimentin-positive cells compared to injured and CsA treated animals at 2 weeks post-treatment (Fig. 1B). These findings suggest that NSCs caused stimulation of immature astrocytes, resulting in an augmented vimentin immunostaining.

Although there was no significant difference observed in radial glial cells labelled with BLBP after treatment at either the 2 or 6 weeks post-treatment (Fig. 1C), there was a trend toward increased BLBP
levels in CsA and CsA + NSC treated animal groups. Cells transplanted into the spinal cord should display long-term survival in patients and possess the ability to differentiate into cell types suitable for repair [50]. Administration of NSCs has been reported to cause an increase in the number of migratory cells at the lesion site [51]. This indicates a possible initiation of regeneration and hence explains the increase in BLBP expression observed.

The response to SCI initiates a series of events, conceptually similar to those occurring during development [52]. Astrocytes undergo rapid morphological changes in response to SCI forming reactive astrocytes, resulting in thick, highly branched cell processes accumulating around the lesion site [53]. These alterations in phenotype contribute to neuroprotection through formation of the glial scar, but prohibit axonal regeneration in the process [54]. Spinal cord tissue stained for GFAP, BLBP and vimentin, all displayed quiescent cells as the most prevalent morphological structure at both 2 and 6 weeks post-treatment (Figs. 2A, 3A, 4A). However, there was a decrease in the number of these inactive astroglial cells at 6 weeks across the three cellular markers which suggests a loss of quiescence over time. CsA treatment appeared to upregulate the number of quiescent and reactive cells at both 2 and 6 weeks post-treatment (Figs. 2A, 3A, 4A). Similar outcomes have been observed in other recent studies [49]. Recent reports have begun to look at reactive astrocytes and their role in the glial scar, in a positive light. Studies have concluded that loss of reactive cells exaggerates tissue degeneration and increases levels of immune cell penetration, thereby inhibiting functional recovery [31, 55, 56]. In this study, administration of NSCs had no significant effect on the number of quiescent or reactive astrocytes present. However, there did appear to be a trend toward decrease in the number of quiescent and reactive cells in certain cases. The observed trend, albeit non-significant, supports the proposal that administration of NSCs reduces glial scarring following injury [8]. Examination of the effect of transplanted cells on the environment of endogenous tissue is an important topic. Previous studies have also shown that environmental cues regulate astrocytic fate decisions [57].

GFAP expression has been observed in fibrous astrocytes [18]. A number of fibrous morphologies were also observed in low quantities in the area surrounding the lesion (Fig. 2A). The presence of fibrous astrocytes may be due to translocation of cells from rostral and caudal aspects of the WM, toward the injury site. Fibrous astrocytes have been reported to exist at a time corresponding with new axon remyelination [58]. There was also a significant increase in the number of fibrous cells identified with BLBP, post-CsA + NSC treatment (Fig. 4A). This result reiterates the above hypothesis and suggests an increase in the number of migratory fibrous cells in response to NSC treatment. These non-process bearing cells denote the presence of astrocytes undergoing developmental changes and may consist of cells that as of yet, have failed to express any structural processes.

Increased Gal-1 protein expression is at its peak in SCI tissue at 14 days post-injury and remains upregulated up to 28 days post-injury [38]. In brain injury, Gal-3 expression in reactive astrocytes was previously shown to have peaked at 7 days post-injury and lasted for at least 14 days post-injury [40]. In a previous study we showed NSCs co-localised with markers of astrocytes and neurons at 2 and 6 weeks post injury [8]. Here we observed NSCs at the injury site were Gal-1-positive (Fig. 5L). Gal-1 expression
promotes NSC proliferation, promotes axonal regeneration after SCI and stimulates neurotrophic factors in astrocytes [43, 59, 60]. Gal-1 secreting NSCs were previous shown to confer long term neuroprotection against cerebral ischemia [61] and may be exerting a similar effect here. However, NSCs outside the lesion region were not Gal-1-positive in the GM and had only slight colocalisation in the WM, but Gal-1 expression was associated with a subset of astrocytes. Indeed, Gal-1 expression was previously demonstrated in reactive astrocytes [62] which may be consistent with our findings and thus a subpopulation of reactive astrocytes may exert a neuroprotective effect surrounding injured tissue in vivo.

In this study the expression of Gal-1 was increased with the treatment of CsA and further increased with CsA + NSC treatment (Fig. 5A). A previous study demonstrated an injury-induced up-regulation of Gal-1 in the white matter, gray matter, and in the ependyma rostral and caudal to the lesion site following injury [63], which correlates with our findings. Elevating levels of Gal-1 in the rat brain has been shown to result in reduced astrocytosis associated injuries, reduced neuronal apoptosis and decreased volume of the infarct [64]. CsA has also been demonstrated to elicit neuroprotective effects [15, 49] and thus CsA may further promote Gal-1 expression in the CsA- and CsA + NSC-treated groups.

Reactive astrocytes express increased β3 integrin which is involved in astrocyte migration, communication with neurons and regulation of astrocyte reactivity [44, 65, 66]. The GFAP and βIII integrin dual positivity observed in the WM and GM (Figs. 6D,6E) could indicate glycosylation-mediated interactions while the lack of dual GFAP and βIII integrin antibody staining that we observed in the injured tissue (Fig. 6F) may indicate a disruption in astrocyte-neuron communication.

In this work some astrocytes expressed Gal-3 in WM and the lesion of injured spinal cord tissue, but not in GM (Fig. 8). This finding is consistent with a similar study that found astrocytes, as well as microglia, express Gal-3 [39]. However, we found that only a subpopulation of astrocytes express Gal-3. In agreement with our data, a subset of Gal-3-positive reactive astrocytes were previously observed post-brain injury [40]. NSCs in the lesion site, WM and GM were Gal-3-positive in this work, but only astrocytes in the WM and the lesion site were Gal-3-positive. An absence of Gal-3 was previously shown to reduce tissue damage and improve functional outcome after SCI by modulating inflammation [67] but Gal-3-positive reactive astrocytes were associated with phagocytosis of damage tissue to help remodel tissue after brain injury [40]. Thus it is possible that this observed localisation of Gal-3-positive astrocytes reflects specialised functions associated with location, i.e. tissue remodelling in the WM and lesion and limiting inflammation within the GM.

A better understanding of astrocyte morphological and functional responses to various regenerative treatments would be useful in progression of novel therapeutic strategies. Astrocytes alter their morphology in response to different stimuli and changes in their cellular environment. Treatment for SCI is an area of intense investigation and astrocytes have fundamental roles in repair and axonal growth. Knowledge of the diverse and dynamic capabilities of astroglial cells is important for constructing regenerative approaches for current and future sufferers of SCI. Further, a better understanding of the expression of Gal-1 and Gal-3 in astrocyte subsets and their specialised roles could provide a key
breakthrough in augmenting therapeutic strategies for optimal neuronal repair and modulation of inflammation.

**Methods**

**Spinal cord injury**

This study was performed in accordance with the Health Products Regulatory Authority and the European Union Directive 2010/63/EU for the use of experimental animals. All animal experiments were reviewed and approved by the Animal Care and Research Ethics Committee of the National University of Ireland Galway, which were in compliance with the ARRIVE guidelines. The animals were housed in a controlled environment with a 12-h light–dark cycle at a temperature of 25°C and were allowed free access to food and water. Twenty-one female Sprague Dawley rats (Charles River U.K. Ltd, Margate, UK) weighing between 200–220 g were used in this study. Three animals were used as control (uninjured) rats and 18 rats received a SCI. The rats were anaesthetised by intraperitoneal injection of ketamine and xylazine (100 and 10 mg/kg, respectively) following which a laminectomy was performed at T8-T10. Injured animals received a 200 kilodyne moderate contusion injury at T9 using an Infinite Horizon Impactor Device (Precision Systems and Instrumentation, Lexington, KY, USA). The muscle and skin was sutured with absorbable suture material (Vicryl, 4 metric) and animals kept warm on a heated blanket until fully recovered. Each animal received a subcutaneous injection of 5–10 mg/kg enrooxacin (Baytril 5%, Bayer) antibiotic once daily for a minimum period of a week. Pain relief was provided by administering buprenorphine (Torbugesic, FortDodge Animal Health Ltd.) at 0.1–0.25 mg/kg twice daily for 7 days after surgery. Saline solution (3.5 ml) was administered subcutaneously for 3 days following surgery. Bladders were manually expressed twice daily from day of injury.

**CsA administration**

The 18 injured animals were randomly divided into groups – injured only (n = 6), CsA (n = 6), and CsA+NSC (details outlined below, n = 6). In animals that received CsA treatment, a subcutaneous injection of 5 mg/kg CsA was administered 4 days post-injury and every day thereafter for the duration of the experiment.

**NSC transplantation**

NSCs were isolated from E16.5 mouse fetal forebrain and transduced with lentiviral vector encoding eGFP to allow for cell tracking [11]. NSCs were cultured as previously described [8]. One week following injury, the 6 injured animals treated with CsA received an injection of NSCs. The remaining 12 injured rats (injured only and injured group treated with CsA groups) received an injection of vehicle (NSC media) at 1 week post-injury. On the day of cell or vehicle injection, animals were re-anaesthetised and spinal cords re-exposed. NSCs (50,000 cells/µl) or vehicle was injected into the injured spinal cords 2 mm rostral and 2 mm caudal to the site of injury (1 µl per injection site). Cells were delivered using a Hamilton microsyringe which was lowered 1.5 mm into the spinal cord. The needle was left in position for 2 min before removal
to prevent backflow of the solution. The muscle and skin was again sutured. Each injured animal group was subdivided into two groups and killed at 2 and 6 weeks post-injection of NSCs/vehicle (n = 3 per time point).

**Tissue processing**

All 21 animals were deeply anaesthetised and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1M PBS. Spinal cords were dissected out and post-fixed overnight with 4% paraformaldehyde. Spinal cords were dissected out, post-fixed overnight with 4% paraformaldehyde, immersed in 30% sucrose cryoprotectant overnight and frozen in liquid nitrogen-chilled isopentane. Spinal cords were cryosectioned transversely at 20 µm thickness in a rostral to caudal direction. Sampling regions, located at 200-µm intervals, were examined within each spinal cord. Eight spinal cord sections were collected at each sampling region through the centre of the injury and two sections were placed on each slide.

**Tissue immunohistochemistry**

Tissue immunohistochemistry was carried out essentially as previously described [32]. In brief, frozen sections were rehydrated and incubated with 20% normal goat serum (NGS) in phosphate buffered saline, pH 7.2 (PBS) containing 0.2% Triton X-100 for 20 min. The primary antibodies BLBP (rabbit polyclonal, 1:100, Chemicon), vimentin (mouse IgG, 1:100, Sigma-Aldrich) and GFAP (rabbit polyclonal, 1:300, DakoCytomation) were diluted in PBS containing 2% NGS + 0.02% Triton X-100 and used to stain tissue at 2 and 6 weeks post treatment. The primary antibodies Gal-1 (mouse monoclonal IgG, 1:300, Abcam), Gal-3 (mouse monoclonal IgG, 1:200, Abcam) and βIII integrin (rabbit polyclonal IgG, 1:100, Chemicon) were diluted in PBS containing 2% NGS + 0.02% Triton X-100 and used to stain tissue at 2 weeks post-treatment. Tissue sections were incubated in primary antibody for 2 h at room temperature and washed three times in PBS. All of the following steps were carried out with limited light exposure. The corresponding secondary antibody labelled with either tetramethylrhodamine isothiocyanate (TRITC, Sigma-Aldrich) or fluorescein isothiocyanate (FITC, Sigma-Aldrich), was diluted 1:100 in PBS and incubated with the tissue for 1 h at room temperature. Tissue sections were then washed and incubated with 1 µg/mL DAPI (Sigma-Aldrich) in PBS for 5 min. A negative control was carried out for each antibody by omitting the primary antibody. All tissues were then washed three times in PBS, coverslipped with one drop of ProLong Gold antifade reagent (Life Technologies, Grand Island, NY) and left to cure for 1 day in the dark before imaging.

**Imaging and image analysis**

All images were captured on an Olympus IX81 fluorescent microscope using a Volocity grid pseudo-confocal acquisition system (PerkinElmer, Dublin, Ireland). A Z-stack of images was captured at a 40X magnification. Ten image stacks were captured from each tissue section. Each image was exported as a library clipping or bitmap file, depending on the type of image analysis and software compatibility. Astrocyte morphology was analysed using Image Pro Plus software to differentiate morphological
variations between astrocytes in both injured and uninjured samples. Morphology was identified and classified as appropriate [68] in uninjured tissue (fibrous, protoplasmic, immature or non-process bearing) and in injured tissue (fibrous, quiescent, reactive and non-process bearing) using the manual tagging tool. Average percentage density/area of GFAP, BLBP, vimentin, Gal-1 and Gal-3-positive staining (intensity ratio for each sampling region within each animal group) was analysed using ImageJ software. This was carried out by inverting the image and converting to 8 bit grayscale for easier identification of positively stained tissue (Supplementary Fig. 1A, 1B). Astrocyte skeletal length and diameter was analysed using PerkinElmer Volocity image acquisition software. The threshold tool was used on each image as desired, excluding all lengths less than 100 µm (Supplementary Fig. 3C and 3D). Each length was calculated based on the subsequent stain intensity and assigned a unique colour. Skeletal length results were in µm and exported to Microsoft Excel. Skeletal diameter was also calculated for the same astrocytes.

Statistical analysis

The average percentage density/area of immunohistochemical staining, average number of morphology types and average astrocyte skeletal length/diameter for uninjured and injured spinal cord tissue slices was calculated. This was carried out for all GFAP, BLBP, vimentin, Gal-1 and Gal-3-positive cells and where results were tabulated using Microsoft Excel and standard error of the mean (SEM) was calculated. Statistical calculations were performed using Minitab 16 software (Minitab Ltd., Coventry, U.K.). A one-way analysis of variance (ANOVA) was carried out. Post-hoc comparisons were undertaken by Tukey’s test. Differences were considered to be statistically significant at a probability value (P) < 0.05.

Declarations

Data availability

The data generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

MK contributed to concept/design, data analysis/interpretation, drafting of the manuscript, revision of the manuscript and approval of the article. DD contributed to concept/design, data analysis/interpretation, drafting of the manuscript. AP contributed to data analysis/interpretation. NB contributed to data analysis/interpretation. LD contributed to data analysis/interpretation. KT contributed to data analysis/interpretation and revision of the manuscript. TA contributed to data analysis/interpretation and revision of the manuscript. FB contributed to data analysis/interpretation. SMcM contributed to concept/design, drafting of the manuscript, critical revision of the manuscript and approval of the article.

Competing interests

The author(s) declare no competing interests.

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Figures

Figure 1
Graphs show percentage density of GFAP- (A), vimentin- (B) and BLBP-positive (C) spinal cord tissue at the lesion site 2 and 6 weeks post-treatment. Error bars represent one standard error of the mean. * = P < 0.05.

Figure 2

Graph shows number of morphologies per unit area in GFAP-positive cells at 2 and 6 weeks post-treatment in injured, CsA, and CsA + NSC animal groups (A). Error bars represent one standard error of the mean. * = P < 0.05 showing difference between quiescent and reactive cell morphology comparing 2 and 6 weeks post-treatment. ∞ = significant difference between quiescent morphology in CsA-treated and...
injured/CsA+NSC treated groups. † = significant difference between reactive morphology in CsA-treated and injured/CsA+NSC treated groups. Photomicrographs show GFAP-positive reactive astrocytes (arrow) around the lesion site at 2 weeks post-treatment (B) and quiescent GFAP-positive astrocytes in the CsA treatment group at 2 weeks post-treatment (C). Scale bar = 100 μm,

Figure 3
Graph shows number of morphologies per unit area in vimentin-positive cells at 2 and 6 weeks post-treatment in injured, CsA-treated, and CsA + NSC-treated animal groups (A). Error bars represent one standard error of the mean. * = P < 0.05 showing difference between quiescent cell morphology and reactive cell morphology comparing 2 and 6 weeks post-treatment. Photomicrographs show reactive vimentin-positive cells at the lesion site 6 weeks following CsA treatment (B, C). Region in yellow box in B is shown at higher magnification in C. Photomicrograph (D) shows vimentin-positive quiescent cells 6 weeks after treatment with CsA + NSCs. Scale bars in B, D = 100 μm, scale bar in C = 70 μm.
Figure 4

Graph shows number of morphologies per unit area in BLBP-positive cells at 2 and 6 weeks post-treatment in injured, CsA-treated, and CsA + NSC-treated animal groups (A). Error bars represent one standard error of the mean. * = P < 0.05 showing difference between reactive cell morphology and quiescent cell morphology comparing 2 and 6 weeks post-treatment. ∞ = significant difference between fibrous cell morphology in injured and CsA+NSC-treated groups. † = significant difference between reactive morphology in injured and CsA-treated groups. Photomicrographs show BLBP-positive cells with reactive cell morphology around the lesion site at 2 weeks post-treatment in the injured (B, C) and the CsA-treated animal groups (D). Arrows in C and D indicates reactive morphology. Scale bars in B, D = 100 μm, scale bar in C = 40 μm.
Figure 5

Graph shows area of Gal-1-positive cells in GM and WM of uninjured and injured animals and injury site of injured animals at 2 weeks post-treatment (A). Error bars represent one standard error of the mean. * = P ≤ 0.05. Photomicrographs show Gal-1 immunostaining in the WM and GM of uninjured animals (B, C) and in the WM, GM and injury site of animals from the injured (D-F), CsA-treated (G-I) and CsA+NSC-treated groups (J-L). Scale bars = 50 μm.
Figure 6

Photomicrographs show Gal-1 and GFAP-positive cells in the WM (A), GM (B) and injury site (C), and Gal-1 and β-III integrin-positive staining in the (D) WM, (E) GM and (F) injury site of animals from the injured group at 2 weeks post-treatment. Scale bars = 50 μm.
Figure 7

Graph shows area of Gal-3-positive cells in GM and WM of uninjured and injured animals and injury site of injured animals at 2 weeks post-treatment (A). Error bars represent one standard error of the mean. * = P ≤ 0.05. Photomicrographs show Gal-3-positive cells in the WM and GM of uninjured animals (B, C) and in the WM, GM and injury site of animals from the injured (D-F), CsA-treated (G-I) and CsA+NSC-treated groups (J-L). Scale bars = 50 μm.
Figure 8

Photomicrographs show Gal-3 and GFAP-positive staining in the WM (A), GM (B) and injury site (C) of animals from the injured group at 2 weeks post-treatment. Scale bar = 50 μm.

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