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Acute Intermittent Hypoxia Combined with Motor Training following Cervical Spinal Injury Alters the Glial Fibrillary Acidic Protein (GFAP) expression

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Abstract

Spinal cord injury (SCI) is a global neurological disorder that can result in significant functional impairment. The response of astrocytes to SCI, is complex and multifaceted. Astrocytes can play beneficial and detrimental roles in recovery, and the balance between these effects is likely important for functional outcomes. Astrocytes are versatile cells active when the brain and spinal cord sustains injury or damage. Astrocyte activation, or astrogliosis, plays a crucial role in neurological problems, including trauma, infections, stroke, and neurodegeneration. Activated astrocytes demonstrate elevated expression of the intermediate filament GFAP. Recently, acute intermittent hypoxia (AIH, brief exposures to low oxygen (O2) levels alternating with normal O2 levels) presents a fresh approach to non-invasive therapy. AIH has shown tremendous potential to induce spinal plasticity in respiratory and non-respiratory motor neurons and ultimately enhance locomotor function in animals and human subjects with an SCI. The effect of AIH treatment on astrocytes in the spinal cord following SCI, has

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not been explored. Therefore, this study aims to investigate the impact of AIH treatment and motor training on the expression of GFAP protein in the spinal cord of the SCI rats treated with AIH combined with motor training using immunofluorescence. In this study, we have used the AIH treatment protocol, which has been shown to improve forelimb functional recovery in this lesion model in rats. Specifically, the study assesses GFAP protein expression in the spinal segments of animals with incomplete cervical SCI exposed to AIH treatment + motor training for 1 or 7 days. The protocol of AIH treatment consisted of ten episodes, 5 min 11% O2: 5 min 21% O2 for seven days beginning at four weeks following SCI. AIH treatment and motor training reduced the expression of GFAP in the ventral grey matter at spinal segments C6-7. This effect was evident after seven days of treatment and training but not after one day of therapy and training.

Interestingly, AIH treatment and motor training for one day or seven days did not alter the expression of GFAP protein in the ventral grey matter at L4-5. The current research proposes that the combination of AIH treatment and motor training may mitigate the inflammatory response and decrease the activation of astrocytes in the L4-5 spinal segments of the cord cord after spinal cord injury (SCI). The findings of this study offer valuable perspectives on the therapeutic possibilities of AIH treatment for promoting functional recovery after SCI by modulating astrocyte reactivity. Further investigation is necessary to validate these findings and ascertain the most effective dosage and duration of AIH treatment for maximizing therapeutic benefits in the treatment of SCIs.

Key Words: Acute Intermittent Hypoxia, Inflammation, Glial cells, Astrocytes, GFAP, Spinal Cord Injury, Therapeutic intervention.

Introduction

Spinal cord injury (SCI) ia widespread neurological disorder, compromises the synaptic conections between brain and spinal cord, resulting in substantial alterations in motor, sensory, and autonomic functions below the injury(1). These alterations rapidly impact bodily functions, such as breathing, movement, muscle control, sexual activity, and elimination. (2). Most spinal cord injuries (SCIs) are categorized as incomplete, preserving some functional synaptic axonal pathways. These undamaged pathways facilitate the spontaneous restoration of specific limb and respiratory functions following SCI. Current knowledge suggests that spontaneous recovery mediated by spared pathways following incomplete spinal cord injuries demonstrates inherent limitations in achieving complete functional restoration. Ongoing research seeks to elucidate these limitations and identify potential therapeutic strategies to optimize recovery outcomes.

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Injuries to the central nervous system, encompassing the brain and spinal cord, often trigger inflammatory responses as a hallmark feature, particularly following traumatic events (3, 4). The way that brain cells that support neurons, such as astroglia and microglia, respond to injury depends on several things, such as the type of injury, the environment around the injured area, and how far away it is from the injury (5). These brain cells become active in response to injury (6). When these cells are active, it can have both good and bad effects on how well someone recovers. Glial activation, also known as gliosis, is necessary to limit inflammation by forming scar tissue, but this scar tissue can also make it harder for nerve fibers to regrow (7). Brain cells can release chemicals that either promote inflammation or suppress it. Chemicals that promote inflammation make inflammation worse and can slow down recovery; they also cause more gliosis (7, 8). On the other hand, chemicals that suppress inflammation reduce inflammation and make these brain cells less active, and this helps with recovery after injury (3, 7).

The response of astrocytes to SCI is complex and multifaceted. Astrocytes can play beneficial and detrimental roles in recovery, and the balance between these effects is likely important for functional outcomes. Brain and spinal cord injuries trigger the activation of astrocytes, which can have both beneficial and detrimental effects on the recovery process. (7, 9). Neurological disorders ranging from trauma and infections to stroke and neurodegeneration all involve astrocyte activation, known as astrogliosis, but the consequences and significance of this process remain under investigation. Activation of astrocytes is associated with increased expression of GFAP, an intermediate filament protein (10). Sustained or excessive astrocyte reactivity can aggravate chronic inflammatory processes and negatively influence neural function, ultimately hindering the return of normal function after neurological damage. Astrocytes respond to brain and spinal cord injuries through activation or astrogliosis. While appropriate astrocyte activation is vital for recovery, excessive or sustained activation can contribute to chronic inflammation and hinder functional recovery.

Researchers have explored numerous therapeutic strategies to enhance recovery after SCI, focusing on promoting plasticity in the remaining synaptic neural pathways (11-13). Acute intermittent hypoxia (AIH), characterized by brief excursions into hypoxia interspersed with normoxic periods, emerges as a potentially valuable non-invasive therapeutic strategy in the field of spinal cord injury (13-18). Studies show that AIH has remarkable potential to enhance spinal plasticity and improve locomotor function in animal models and individuals with SCI, impacting both respiratory and non-respiratory motor neurons(18-20). Moreover, AIH protocols offer these benefits without the adverse side effects commonly associated with conventional treatment options (12, 13, 21). The combination of AIH treatment with task-specific training has effectively enhanced forelimb function in both rats and humans with spinal cord injuries. (17, 19, 20, 22).

Recent research findings provide growing evidence that this intervention may be effective in improving both hand and leg function in individuals with chronic, incomplete SCI. (15, 19). Emerging evidence from clinical trials suggests the efficacy of this therapy in promoting functional recovery in both the upper and lower extremities, improving hand

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dexterity and leg movement in individuals with chronic, incomplete SCI. (15, 19). The combination of AIH and overground walking training for five days offers a promising intervention for individuals with chronic, incomplete SCI, leading to sustained improvements in walking speed and endurance that remain evident at least one week post-treatment (23).

Extensive investigations have explored the intricate cellular and molecular pathways involved in AIH-mediated plasticity, enriching our understanding of its therapeutic potential (21, 24). Recent preclinical research in SCI animal models has demonstrated that AIH treatment combined with motor training upregulation of hypoxia-associated proteins, including brain-derived neurotrophic factor (BDNF) and its high-affinity receptors TrkB, in spinal motor neurons (22, 25, 26). However, the impact of AIH treatment on astrocytes in the spinal cord following SCI remains unexplored. This research explores the collective impacts of acute intermittent hypoxia (AIH) treatment and motor training on the expression of GFAP in the spinal cords of injured rats. Utilizing immunofluorescence techniques, we aim to elucidate the impact of this combined intervention on astrocyte activation within the injured spinal cord. The present investigation employs a well-established AIH treatment protocol consistently demonstrated to enhance forelimb functional recovery in preclinical spinal cord injury (SCI) models. This AIH protocol entails the exposure of 10 cycles of alternating hypoxia (5 minutes at 11% O_2) and normoxia (5 minutes at 21% O_2), culminating in a total of 70 hypoxic episodes delivered over one week. Following observations of motor recovery in a rat model of SCI induced by a previously established training protocol and AIH treatment, the present study sought to elucidate the influence of this combined intervention on astrocyte activity within the injured spinal cord (25, 27). To achieve this, we employed glial fibrillary acidic protein (GFAP), a specific astrocyte marker, to quantitatively assess changes in their expression, thereby evaluating the response of these non-neuronal cells to the combined training and AIH treatment in the context of SCI. In this study, we assess the expression of GFAP protein in the spinal segments of spinally injured animals exposed to AIH combined with motor training for one or seven days. The treatment protocol of AIH consists of ten hypoxic episodes of AIH (5 min at 11% O_2 followed by 5 min at 21% O_2) daily for seven days, commencing four weeks post-SCI. Our findings demonstrate that the combination of AIH treatment and motor training significantly reduces GFAP protein expression within the ventral grey matter of spinal segments C6-7. This reduction becomes noticeable after seven days of treatment and training, whereas no significant effect is observed after just one day. Notably, neither one-day nor seven-day AIH treatment combined with motor training affects the expression of GFAP in the ventral grey matter at L4-5 segment of the spinal cord. The findings of this study hold considerable significance in developing novel therapeutic interventions for spinal cord injury. This study's findings suggest a potential therapeutic role for acute intermittent hypoxia (AIH) in reducing glial fibrillary acidic protein (GFAP) expression, a marker of astrocyte activation. This decrease in GFAP may be associated with enhancement of functional recovery following spinal cord injury (SCI). However, these results are preliminary; further research is necessary to

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confirm these findings, elucidate the underlying mechanisms, and assess AIH's long-term efficacy and safety as a potential therapeutic intervention for SCI. Although this study shows potential, it is subject to certain limitations that warrant further investigation. Specifically, the small sample size and relatively short duration of treatment in the current study call for additional research to delve deeper into the findings.

In summary, the research presented in this study offers convincing evidence that AIH treatment holds potential as an encouraging therapeutic strategy for spinal cord injury. Nevertheless, further investigation is required to validate these results and determine the most influential parameters for AIH treatment in facilitating functional recovery post-SCI. The potential advantages of AIH in regulating GFAP expression and its impact on microglia activation are especially intriguing, offering avenues for developing novel therapeutic strategies for SCI.

Materials and Methods

Animals and Housing

Male Lewis rats weighing between 225-250g were obtained from Charles River Laboratories in Quebec and were group-housed, with three rats per cage, upon their arrival at the research facility Western College of Veterinary Medicine, University of Saskatchewan. They were given a five-day acclimation period in the colony room, which was maintained at 20°C and followed a 12-hour light-dark cycle. This facility is located The housing environment consisted of cages dimension 51 x 28 cm, furnished with wood chip bedding, PVC tubes for shelter and sleep, and wood blocks for gnawing. The rats had *ad libitum* access to rodent chow until reaching ~320g of body weight, after which their food intake was restricted to 4 pellets/rat/day throughout the study. Water was available to the rats without restriction. Handling procedures, conducted by the same individual, involved gentle handling of the rats for 10 minutes daily over three days or until the rats were comfortable with the handler. The research adhered to the regulatory standards stipulated from the Canadian Council on Animal Care and aapproved by the University of Saskatchewan Committee on Animal Care and Supply (UCACS).

Experimental Design:

In this study a total of twenty-four male Lewis rats were utilized. Following comprehensive ladder training and thorough motor function testing, the animals underwent a standardized SCI surgical procedure. Subsequently, a randomized block design was employed to allocate them into either the acute intermittent hypoxia (AIH) or normoxia control treatment group (n = 12 per group). Within each treatment group, a further randomized block design stratified the animals into two equal-sized subgroups (n = 6/group) to assess the potential influence of treatment duration. One subgroup received the assigned treatment (AIH or normoxia) for one day, followed by ladder training. The other subgroup received the same treatment for seven days, followed by ladder training. This resulted in four distinct

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experimental groups: 1-day normoxia, 7-day normoxia, 1-day AIH, and 7-day AIH, each composed of six animals.

Ladder-Walking Task

The rats were subjected to training for traversing a horizontal runway, equipped with an integrated ladder apparatus for detailed gait analysis.. The runway included opaque plexiglass platforms measuring 20 cm at each end to assist in rat maneuvering. The central ladder segment, spanning 80 cm, was comprised of wire rungs with a diameter of 2 mm, spaced 2 cm apart. Positioned above a 45° angled mirror, the ladder allowed observation of both lateral and ventral aspects of rat movements using a digital video camera (EOS Rebel, T2i EOS 550D Canon), which was oriented perpendicularly to the runway.

To objectively assess changes in locomotor performance across all animal groups, a standardized ladder task was employed. Following a two-week training period, animals achieved a criterion of consistent and rapid ladder traversal with minimal hesitation or pausing. To track potential treatment-related effects, we performed ladder task evaluations at specific time points: pre-surgery baseline, pre-treatment baseline, and daily throughout the seven-day treatment regimen. On treatment days, assessments occurred at least one hour post-completion of either acute intermittent hypoxia (AIH) or normoxia treatment. To achieve robust data acquisition, each recording session captured 12 complete ladder crossings at a frame rate of 60 frames per second.

Surgery

Prior surgery rats were injected glycopyrrolate 0.03mg/kg, to minimize the salivation (Sabex Inc., Boucherville, QC, Canada) then expose to isoflurane anaesthesia inclosed plexy glass chamber. Following surgery an antibiotic trimethoprim and sulfadoxine (TMS: 30mg/kg SC), and pre-emptive analgesia buprenorphine (0.05mg/kg SC) were admininstered in the animals (drugs were obtained from Trivetrin, Schering Canada Inc., QC, Canada, and Buprenex; Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA, USA). Adhering to aseptic principles, the surgical site on the dorsum of the neck was prepared. Under an operating microscope, a laminectomy and durotomy were performed, exposing the second cervical spinal segment (C2). Using a modified 25-gauge bevel-tipped needle, a unilateral transection of the left dorsolateral funiculus was conducted. Musculature and skin were closed utilizing a subcuticular suture technique. The complete duration of anesthesia lasted around 20 minutes. Post-operatively, animals received analgesia (buprenorphine, 0.05 mg/kg) and prophylactic antibiotics (trimethoprim-sulfadoxine, 30 mg/kg) for 48 hours, with extension if clinically indicated. Close monitoring for five days post-surgery included evaluation of weight changes, presence and severity of porphyrin staining, hydration status, incision site healing, mobility, and general behavior. On the third day post-surgery, rats were reintroduced to their original cage mates, following the described procedure.

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Acute Intermittent Hypoxia and Normoxia Treatment

Prior to commencing the 7-day AIH intervention outlined previously, individual rats were habituated to the treatment apparatus. Each animal was housed within a custom-built Plexiglas chamber (30cm x 17cm x 12cm) under normoxic conditions (21% inspired O2) for a duration of 30 minutes. This acclimatization procedure ensured familiarization with the environment before treatment initiation (25, 27). Throughout the 7-day treatment period, animals were individually housed within the same Plexiglas chambers and subjected to daily AIH exposure. The AIH protocol consisted of ten cycles of alternating 5-minute hypoxic episodes (11% inspired O_2) and 5-minute normoxic intervals (21% inspired O_2). This cyclical exposure was achieved by automated switching of incoming air between premixed O_2/N_2 gas (FlO_2 = 0.11) and medical air (FlO_2 = 0.21). For comparison, normoxic control animals were housed in adjacent chambers and exposed to uninterrupted normoxic conditions (FlO_2 = 0.21; 21% inspired O_2) for the same total duration. Oxygen levels within all chambers were continuously monitored using portable oxygen analyzers (AX300-1; Teledyne Analytical Instruments).

Preparation of Tissue

On the first and seventh days of the AIH treatment, animals within each experimental group were deeply anesthetized with isoflurane. Subsequently, they underwent trans-cardiac perfusion with heparinized phosphate-buffered saline (PBS) followed by 4% paraformaldehyde for tissue fixation. The spinal cord was meticulously dissected from the vertebral column, with specific segments C6-C7 (housing forelimb motoneurons) and L4-L5 (containing hindlimb motoneurons) further postfixed for 1-1.5 hours in 4% paraformaldehyde. Finally, these segments were cryoprotected in 10% and then 20% sucrose solutions overnight at 4°C for subsequent analysis.

Following dissection, spinal cord sections from both AIH-treated and normoxia control groups were prepared for further analysis. Each specimen was embedded within a cryomold (Tissue Tek, Miles Laboratories) using OCT compound (Tissue Tek, Miles Laboratories) and rapidly frozen using dry ice-cooled isopentane in an additional OCT-filled mold. To streamline processing, individual spinal cord segments from different experimental groups (1-day normoxia, 7-day normoxia, 1-day AIH, 7-day AIH) and spinal regions (C6-C7 and L4-L5) were strategically combined into larger blocks. Each block comprised eight individual pieces, meticulously arranged to represent all four experimental conditions from both spinal regions. This consolidated approach resulted in a total of three tissue blocks generated from the spinal cords of all 12 animals.

Tissue blocks were initially stored at -80°C for subsequent sectioning. Using a cryostat, the frozen tissue was sectioned at a consistent thickness of 10 μm. These thaw-mounted sections were then adhered to Superfrost Plus slides (VWR) and maintained at -80°C until further immunofluorescence processing. Notably, each slide accommodated eight

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distinct sections, each representing a unique experimental group. Notably, for enhanced consistency and reduced processing variations, sections from both forelimb and hindlimb regions obtained from the same animal (normoxia and AIH-treated; days one and seven) were strategically positioned on the same slide and subjected to identical processing conditions.

Immunofluorescence

Prior to immunofluorescence labeling, tissue slides were retrieved from the -80°C freezer and brought to room temperature over 30 minutes within a desiccator to avoid condensation. Subsequently, thorough washing was performed with 0.1M PBS (pH 7.4) for 30 minutes, changing the PBS solution every 10 minutes. This facilitated the removal of excess media and debris from the sections. Following washing, all slides were incubated with a blocking solution containing sea block in primary diluent (0.1% Triton X-100 in 0.1M PBS) for 1 hour at room temperature. This step aimed to minimize non-specific antibody binding during subsequent immunolabeling. Specific primary antibody was then diluted in 10% sea block within the same diluent at predetermined concentrations: rabbit anti-GFAP (Dako Canada, Inc) at 1:800.

Following a 24-hour incubation at 4°C with their respective primary antibodies, the slides were subjected to three 10-minute washes with 0.1M PBS to remove unbound primary antibodies. Subsequently, fluorescent labeling was achieved by incubating the slides with an appropriate secondary antibody. This study employed Cy3 Donkey-anti-rabbit (Jackson Immuno Research Laboratories, Inc) diluted to 1:4000 in 0.1M PBS. This incubation took place in the dark at room temperature for 1 hour. To remove unbound secondary antibodies, three further 10-minute washes with 0.1M PBS were performed. Finally, subsequently slides were mounted with Antifade Reagent ProLong Gold (P36931, Molecular Probes, Invitrogen) for preservation and fluorescence stabilization.

Image Analysis / Quantification

Immunofluorescence imaging of the spinal cord sections was performed using a Zeiss Axioskop microscope equipped for the technique. Appropriate filters were employed to optimize visualization of the fluorescent labels. To ensure standardized image acquisition, all sections of interest within a single slide were imaged at 20x magnification under identical settings. Subsequently, two slides representing distinct animals from each experimental group were chosen for further quantitative analysis.

Image analysis employed a blinded approach within a predefined region of interest: the ventral grey matter. This analysis encompassed photographs from both C6-C7 and L4-L5 spinal cord segments across all experimental groups. For GFAP staining, Image J software was used to place four equally spaced 100µm x 100µm boxes within the ventral

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horn of each section, on both sides. Background-subtracted mean grey values were calculated for each box, subsequently averaged across both sides of the spinal cord. To derive representative data, these net mean grey values were further averaged across the three animals within each experimental group (1-day normoxia, 7-day normoxia, 1-day AIH, 7-day AIH). This resulted in a single mean grey value per group for subsequent analysis and interpretation.

Statistical Analysis

IBM SPSS Statistics v20 for Windows software were used for all statistical analysis. Four experimental groups were defined: 1-day normoxia, 7-day normoxia, 1-day AIH, and 7-day AIH. Mean grey values of GFAP expression were compared across these groups for both C6-7 and L4-5 spinal cord segments using a one-way analysis of variance (ANOVA). Post hoc analysis employed Tukey's HSD test to identify significant pairwise comparisons. Statistical significance was defined as $p < 0.05$.

Results

Specificity controls devoid of primary antibodies were implemented for all investigated markers to confirm the authenticity of positive immunofluorescence signals and preclude the possibility of nonspecific secondary antibody binding. Notably, no discernable positive immunofluorescence was observed for any marker in the absence of the primary antibody.

AIH treatment combined with motor training over seven days effectively reduces the expression of GFAP protein within the ventral horn of the C6-C7 spinal cord segment.

In spinal cord injury (SCI), astrocytes are key players in the inflammatory response. Following SCI , astrocytes undergo morphological and functional changes, characterized by glial fibrillary acidic protein (GFAP) expression, a marker of their "reactive" state.Treatments or factors that mitigate their activation demonstrably enhance functional recovery post-injury (7). Daily co-administration of acute intermittent hypoxia (AIH) and motor training for seven days resulted in a marked reduction of GFAP expression within the ventral gray matter of C6-C7 spinal cord segments in a rat model of SCI. GFAP, a well-established marker of astrocyte activation, exhibited significantly decreased immunoreactivity as visualized by immunofluorescence microscopy (Figures 1-3) compared to the control group. Interestingly, this effect was not observed after a single day of the combined intervention. Negative controls processed without the primary antibody displayed no GFAP staining (Figure 2), confirming the specificity of the observed downregulation.

Quantitative analysis, utilizing one-way ANOVA revealed a statistically significant ($p < 0.05$) decrease in glial fibrillary acidic protein (GFAP) protein expression within the ventral gray matter of C6-C7 spinal segments in rats subjected to daily dAIH and motor training for seven days compared to normoxic controls (Figure 3). This finding suggests a potential for this intervention to modulate astrocyte activation in the context of spinal cord injury (SCI).

Interestingly, both one-day and seven-day co-administration of AIH and motor training did not lead to any noticeable changes in GFAP expression within the ventral gray matter of the L4-5 spinal cord segments in a rat model of SCI.

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Immunofluorescence microscopy revealed no observable differences in GFAP immunoreactivity between AIH-treated and normoxic control groups at either time point (Figure 4). This lack of effect was further confirmed by the absence of any GFAP staining in negative controls processed without the primary antibody (Figure 5). These findings suggest that the observed downregulation of GFAP in C6-C7 segments (reported earlier) might be specific to those particular spinal segments and may not generalize to other regions like L4-5. Quantitative analysis, utilizing one-way ANOVA, revealed no statistically significant alterations in GFAP protein expression within the ventral gray matter of L4-L5 spinal segments in rats subjected to AIH treatment and motor training for either one or seven days compared to normoxic controls (Figure 6). This finding indicates a segment-specific effect of this intervention on astrocyte activation, suggesting differential responses across the spinal cord and prompting further investigation into underlying mechanisms and potential therapeutic implications tailored to distinct spinal regions.

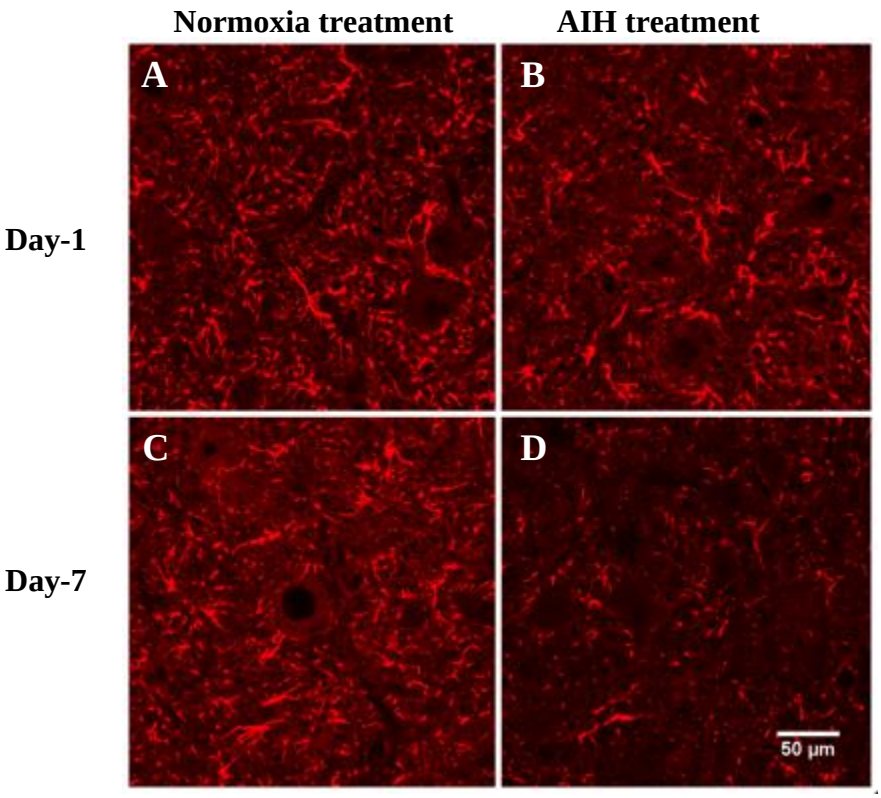

Figure 1: Exposure to AIH combined with motor training treatment for seven days significantly downregulated GFAP expression in the ventral grey matter of C6-C7 spinal segments. Photomicrographs of the C6-7 ventral grey matter immunostained for GFAP at Day 1 (B) and Day 7 (D) in AIH-treated spinal-injured rats compared to normoxia-treated spinal-injured rats (A and C). The scale bar measures 50 µm.

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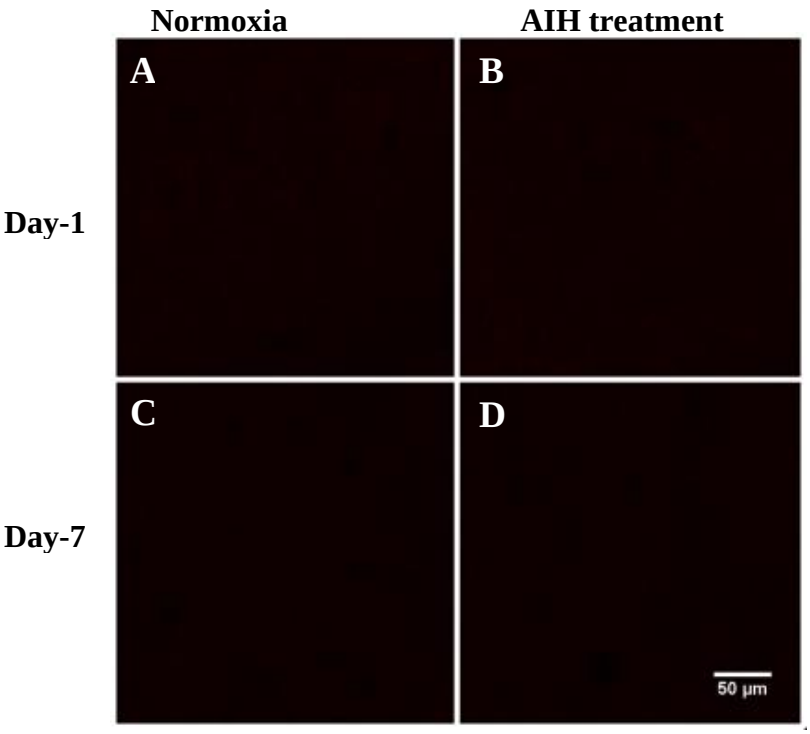

Figure 2: Photomicrograph of the C6-7 spinal cord segment ventral horn stained for GFAP protein expression without a primary antibody, showing background fluorescence (control). Photomicrographs of the C6-7 ventral grey matter immunostained for GFAP at Day 1 (B) and Day 7 (D) in AIH-treated spinal-injured rats compared to normoxia-treated spinal-injured rats (A and C). The scale bar measures 50 µm.

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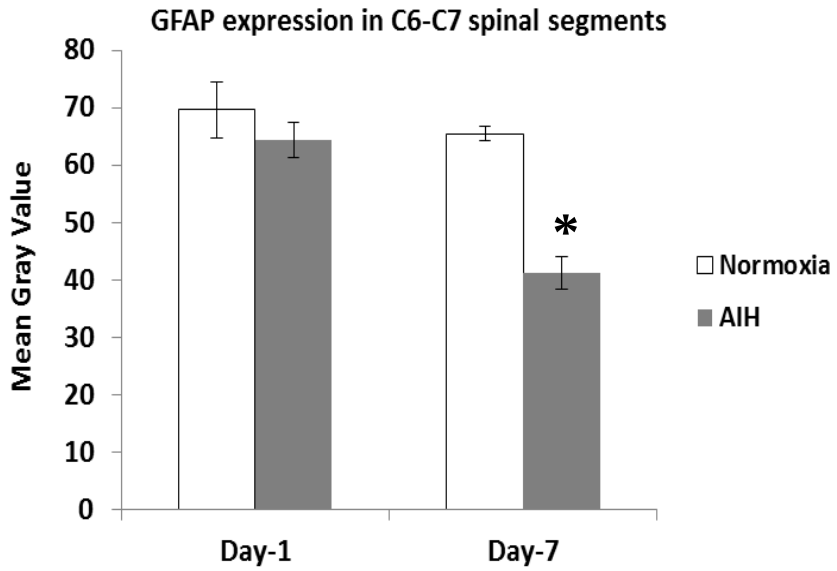

Figure 3: AIH tically significant downregulation of GFAP protein levels within the C6-C7 ventral grey matter. Data represent mean ± SEM from normoxia (n = 3, for each time point i.e. Day-1 and Day-7) and AIH (n = 3, for each time point i.e. Day-1 and Day-7). * $p < 0.05$ difference between normoxia and AIH treated groups.

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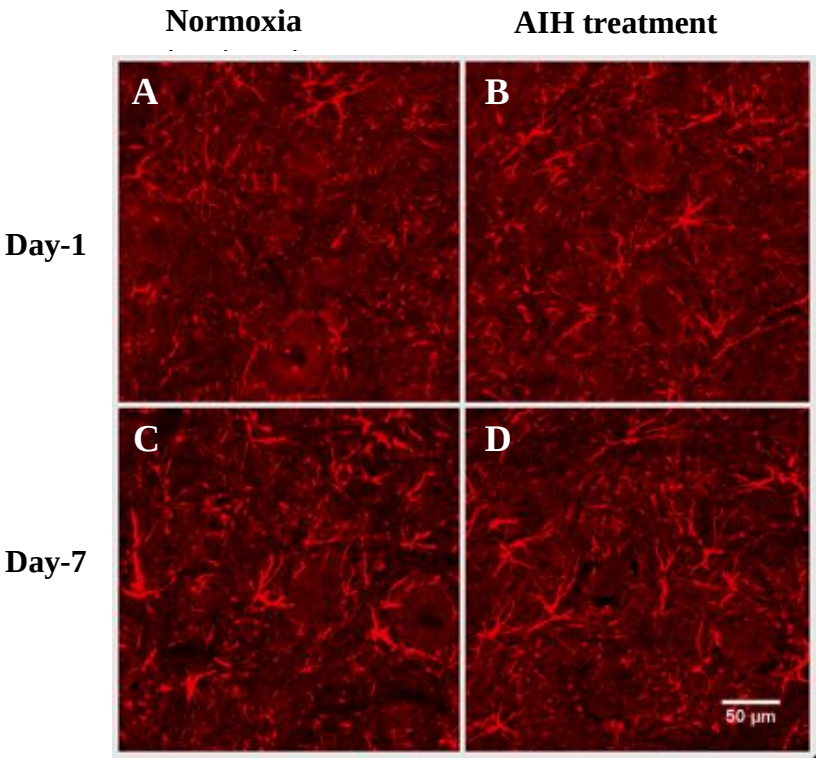

Figure 4: Combined exposure to AIH and motor training for one or seven days did not induce significant changes in GFAP levels within the L4-5 spinal segments. Photomicrographs depicting the L4-5 ventral grey matter from spinal-injured rats treated with either normoxia (A, C) or AIH (B, D) showcase GFAP immunofluorescence staining at Day 1 and Day 7. No significant differences in GFAP expression are observed between the groups. The scale bar measures 50 µm.

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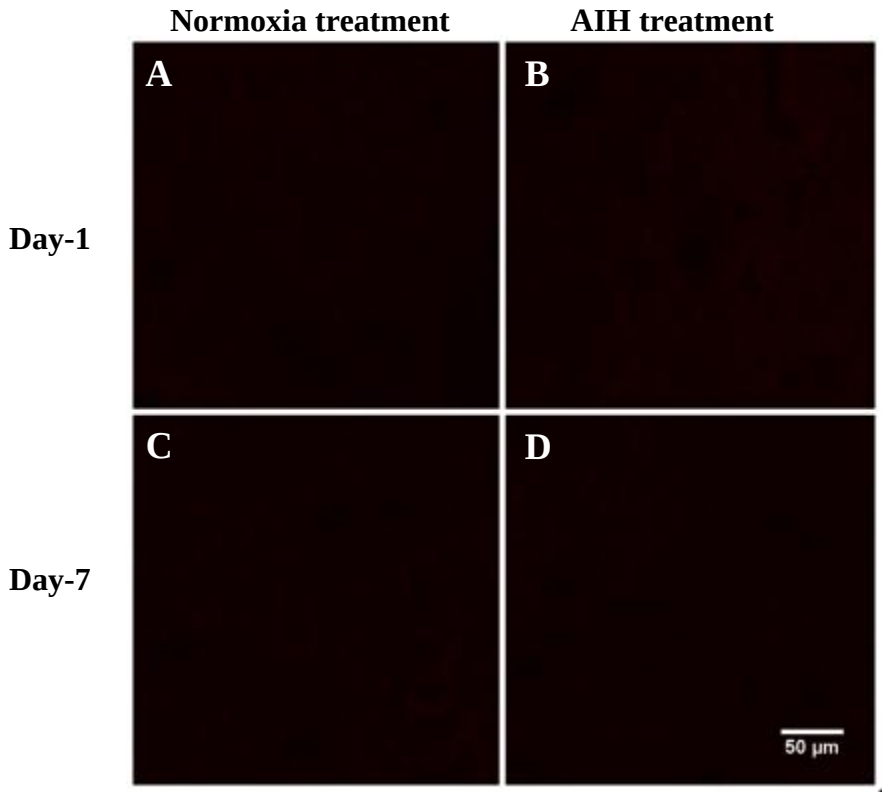

Figure 5: Photomicrograph of the L4-5 spinal cord ventral horn stained for GFAP protein expression without a primary antibody, showing background fluorescence (control). Photomicrographs of the L4-5 ventral grey matter immunostained for GFAP at Day 1 (B) and Day 7 (D) in AIH-treated spinal-injured rats compared to normoxia-treated spinal-injured rats (A and C). The scale bar measures 50 µm.

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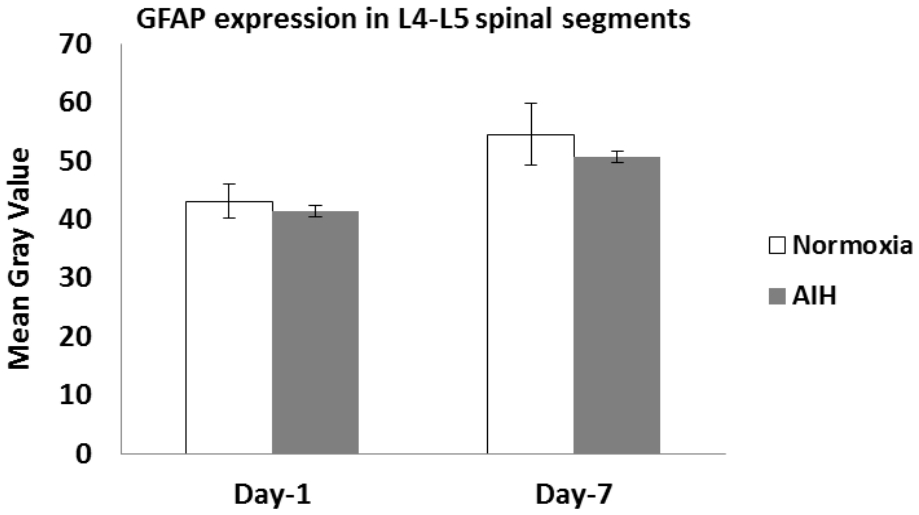

Figure 6: Exposure to AIH combined with motor training for one or seven days did not induce significant changes in GFAP levels within the ventral grey matter of L4-5 spinal segments. Data represent mean ± SEM from normoxia (n = 3, for each time point i.e. Day-1 and Day-7) and AIH (n = 3, for each time point i.e. Day-1 and Day-7).

Discussion

This study evaluated the combined effects of acute intermittent hypoxia (AIH) and motor training on GFAP expression in the ventral gray matter of spinal cord of rats with cervical spinal cord injury (cSCI). Rats received 10 daily AIH exposures (5 minutes each) alongside motor training for seven consecutive days. Results revealed a significant downregulation of GFAP in the C6-C7 spinal segments, suggesting potential neurorehabilitative implications. However, GFAP expression remained unchanged in the L4-L5 segments.

Effect of dAIH and motor training on Glial cells following SCI

Inflammation is a characteristic of CNS injuries, including traumatic brain and spinal cord injuries (28). Glial cell response, encompassing both astrocyte and microglial activation, following CNS injury exhibits intricate dependence on a constellation of factors including the type of injury, the local environment surrounding the lesion, and the distance from the injury site (5). Glial cells activate in response to injury (6). This activation can support recovery by promoting tissue repair, debris removal, and neuroprotection. However, excessive or prolonged activation can contribute to neuroinflammation, scar formation, and neuronal dysfunction, ultimately hindering recovery. While gliosis, characterized by activated glial cells and subsequent scar formation, serves to restrict inflammation and facilitate tissue repair in the acute phase, it ultimately creates an inhibitory microenvironment for axonal regeneration, thereby

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hindering neuronal repair and functional recovery in the long term. (7). Glial cells secret pro- and anti-inflammatory factors, pro-inflammatory factors promote inflammation and produce detrimental effects on recovery; these factors promote activation of gliosis (7, 8). On the other hand, anti-inflammatory factors suppress inflammation and reduce the activation of glial cells, and these anti-inflammatory factors promote recovery following injury (7, 28).

Astrocytes are versatile glial cells, undergo activation in response to central nervous system (CNS) injuries, including trauma, infections, stroke, and neurodegeneration (7, 9). This activation, termed astrogliosis, plays a complex role in these neuropathologies. While initially serving a neuroprotective function by mitigating inflammation and promoting tissue repair, excessive or prolonged reactive astrogliosis, marked by high levels of the intermediate filament glial fibrillary acidic protein (GFAP), can be detrimental. This chronic state contributes to neuroinflammation and neuronal dysfunction, hindering functional recovery(7-9).

AIH treatment, applied ten times daily, three times per week for four weeks beginning on the third day post-C2 spinal hemisection (C2HS), demonstrated potential in attenuating the inflammatory response. This intervention was associated with downregulated expression of both proximal and distal inflammatory genes to the injury site. (28). This study examined the combined effects of AIH treatment and motor training on GFAP expression in the ventral grey matter of the cervical (C6-7) and lumbar (L4-5) spinal cord segments in a rat model.
Following seven consecutive days of combined AIH and motor training, GFAP expression was significantly downregulated within the C6-7 segments of the spinal cord. This finding suggests potential therapeutic implications for modulating glial responses within the cervical spinal cord. Conversely, no significant alterations in GFAP expression were detected after just one day of intervention in the cervical or lumbar segments. Interestingly, AIH treatment, administered for one or seven days, did not elicit any changes in GFAP expression within the L4-5 segments. Previous research has shown that rAIH has differential effects on glial activities below and above the injury (28). Prior investigations employing a proteomic approach suggest that AIH treatment does not exert a significant modulatory effect on the expression of proteins specific to astrocytes and microglia within the brain; these findings tentatively support the hypothesis that AIH exerts a neutral modulatory effect on these glial populations, potentially mitigating its potential to induce or exacerbate inflammatory cascades. (26, 29).

Building upon existing literature and the findings of this study, a compelling argument emerges for the potential of AIH treatment in curtailing glial activation, thereby contributing to an attenuated inflammatory response. Therapies or factors demonstrably capable of downregulating pro-inflammatory mediators hold significant promise in achieving the deactivation or maintenance of the quiescent state of astrocytes and microglia. Nevertheless, further investigation

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remains imperative to delineate the precise mechanisms by which AIH modulates glial cell activation and the subsequent secretion of pro-inflammatory mediators.

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References

1. Astorino TA, Harness ET, White AC. Efficacy of acute intermittent hypoxia on physical function and health status in humans with spinal cord injury: a brief review. Neural Plasticity. 2015;2015.
2. Donovan J, Forrest G, Linsenmeyer T, Kirshblum S. Spinal Cord Stimulation After Spinal Cord Injury: Promising Multisystem Effects. Current Physical Medicine and Rehabilitation Reports. 2021;9(1):23-31.
3. Small A, Nikodemova M, Dougherty B, Mitchell G, Watters J. Repetitive acute intermittent hypoxia differentially affects CD11b+ cell phenotypes after C2 spinal hemisection (713.6). 2014.
4. Maheshwari S, Dwyer LJ, Sîrbulescu RF. Inflammation and immunomodulation in central nervous system injury–B cells as a novel therapeutic opportunity. Neurobiology of Disease. 2023;180:106077.
5. Davies SJ, Field PM, Raisman G. Regeneration of cut adult axons fails even in the presence of continuous aligned glial pathways. Exp Neurol. 1996;142(2):203-16.
6. Gensel JC, Zhang B. Macrophage activation and its role in repair and pathology after spinal cord injury. Brain Res. 2014.
7. Kang W, Balordi F, Su N, Chen L, Fishell G, Hebert JM. Astrocyte activation is suppressed in both normal and injured brain by FGF signaling. Proc Natl Acad Sci U S A. 2014;111(29):E2987-95.
8. Sofroniew MV, Vinters HV. Astrocytes: biology and pathology. Acta Neuropathol. 2010;119(1):7-35.
9. Lee KM, MacLean AG. New advances on glial activation in health and disease. World J Virol. 2015;4(2):42-55.
10. Zhang Y, Xu S, Liang KY, Li K, Zou ZP, Yang CL, et al. Neuronal mTORC1 Is Required for Maintaining the Non-reactive State of Astrocytes. J Biol Chem. 2017;292(1):100-11.

ISSN:2093-4777 | E-ISSN:2093-6931
Vol. 28 Iss. 1 (2024)
DOI: 10.5123/inj.2024.1.inj27

11. Dale EA, Ben Mabrouk F, Mitchell GS. Unexpected benefits of intermittent hypoxia: enhanced respiratory and nonrespiratory motor function. Physiology (Bethesda). 2014;29(1):39-48.
12. Hassan A, Nasir N, Muzammil K. Treatment Strategies to Promote Regeneration in Experimental Spinal Cord Injury Models. Neurochemical Journal. 2021;15(1):1-7.
13. Hassan A, Nasir N, Muzammil K, Khan MS, Asad MR, Wahab S. Beneficial Effects of Novel Non-invasive therapeutic Acute Intermittent Hypoxia (AIH) to treat spinal cord injury. Psychiatry. 2022;23:01-7.
14. Trumbower RD, Hayes HB, Mitchell GS, Wolf SL, Stahl VA. Effects of acute intermittent hypoxia on hand use after spinal cord trauma: a preliminary study. Neurology. 2017;89(18):1904-7.
15. Navarrete-Opazo A, Alcayaga J, Sepúlveda O, Rojas E, Astudillo C. Repetitive intermittent hypoxia and locomotor training enhances walking function in incomplete spinal cord injury subjects: a randomized, triple-blind, placebo-controlled clinical trial. Journal of neurotrauma. 2017;34(9):1803-12.
16. Welch JF, Sutor T, Vose AK, Perim RR, Fox EJ, Mitchell GS. Synergy between acute intermittent hypoxia and task-specific training. Exercise and sport sciences reviews. 2020;48(3):125.
17. Hayes HB, Jayaraman A, Herrmann M, Mitchell GS, Rymer WZ, Trumbower RD. Daily intermittent hypoxia enhances walking after chronic spinal cord injury: a randomized trial. Neurology. 2014;82(2):104-13.
18. Michel-Flutot P, Lane MA, Lepore AC, Vinit S. Therapeutic Strategies Targeting Respiratory Recovery after Spinal Cord Injury: From Preclinical Development to Clinical Translation. Cells. 2023;12(11):1519.
19. Trumbower RD, Hayes HB, Mitchell GS, Wolf SL, Stahl VA. Effects of acute intermittent hypoxia on hand use after spinal cord trauma A preliminary study. Neurology. 2017;89(18):1904-7.
20. Prosser-Loose EJ, Hassan A, Mitchell GS, Muir GD. Delayed Intervention with Intermittent Hypoxia and Task Training Improves Forelimb Function in a Rat Model of Cervical Spinal Injury. J Neurotrauma. 2015;32(18):1403-12.
21. Navarrete-Opazo A, Mitchell GS. Therapeutic potential of intermittent hypoxia: a matter of dose. Am J Physiol Regul Integr Comp Physiol. 2014;307(10):R1181-97.
22. Hassan A. Intermittent hypoxia induces spinal plasticity in rats with cervical spinal cord injury: University of Saskatchewan; 2015.
23. Navarrete-Opazo A, Vinit S, Dougherty B, Mitchell G. Daily acute intermittent hypoxia elicits functional recovery of diaphragm and inspiratory intercostal muscle activity after acute cervical spinal injury. Experimental neurology. 2015;266:1-10.
24. Sandhu MS, Rymer WZ. Brief exposure to systemic hypoxia enhances plasticity of the central nervous system in spinal cord injured animals and man. Curr Opin Neurol. 2021;34(6):819-24.

ISSN:2093-4777 | E-ISSN:2093-6931
Vol. 28 Iss. 1 (2024)
DOI: 10.5123/inj.2024.1.inj27

25. Hassan A, Arnold BM, Caine S, Toosi BM, Verge VM, Muir GD. Acute intermittent hypoxia and rehabilitative training following cervical spinal injury alters neuronal hypoxia-and plasticity-associated protein expression. PLoS One. 2018;13(5):e0197486.
26. Lovett-Barr MR, Satriotomo I, Muir GD, Wilkerson JE, Hoffman MS, Vinit S, et al. Repetitive intermittent hypoxia induces respiratory and somatic motor recovery after chronic cervical spinal injury. J Neurosci. 2012;32(11):3591-600.
27. Prosser-Loose EJ, Hassan A, Mitchell GS, Muir GD. Delayed intervention with intermittent hypoxia and task training improves forelimb function in a rat model of cervical spinal injury. Journal of neurotrauma. 2015;32(18):1403-12.
28. Small A, Nikodemova M, Dougherty B, Mitchell G, Watters J. Repetitive acute intermittent hypoxia differentially affects CD11b+ cell phenotypes after C2 spinal hemisection (713.6). 2014.
29. Satriotomo I, Dale EA, Dahlberg JM, Mitchell GS. Repetitive acute intermittent hypoxia increases expression of proteins associated with plasticity in the phrenic motor nucleus. Exp Neurol. 2012;237(1):103-15.

ISSN:2093-4777 | E-ISSN:2093-6931
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