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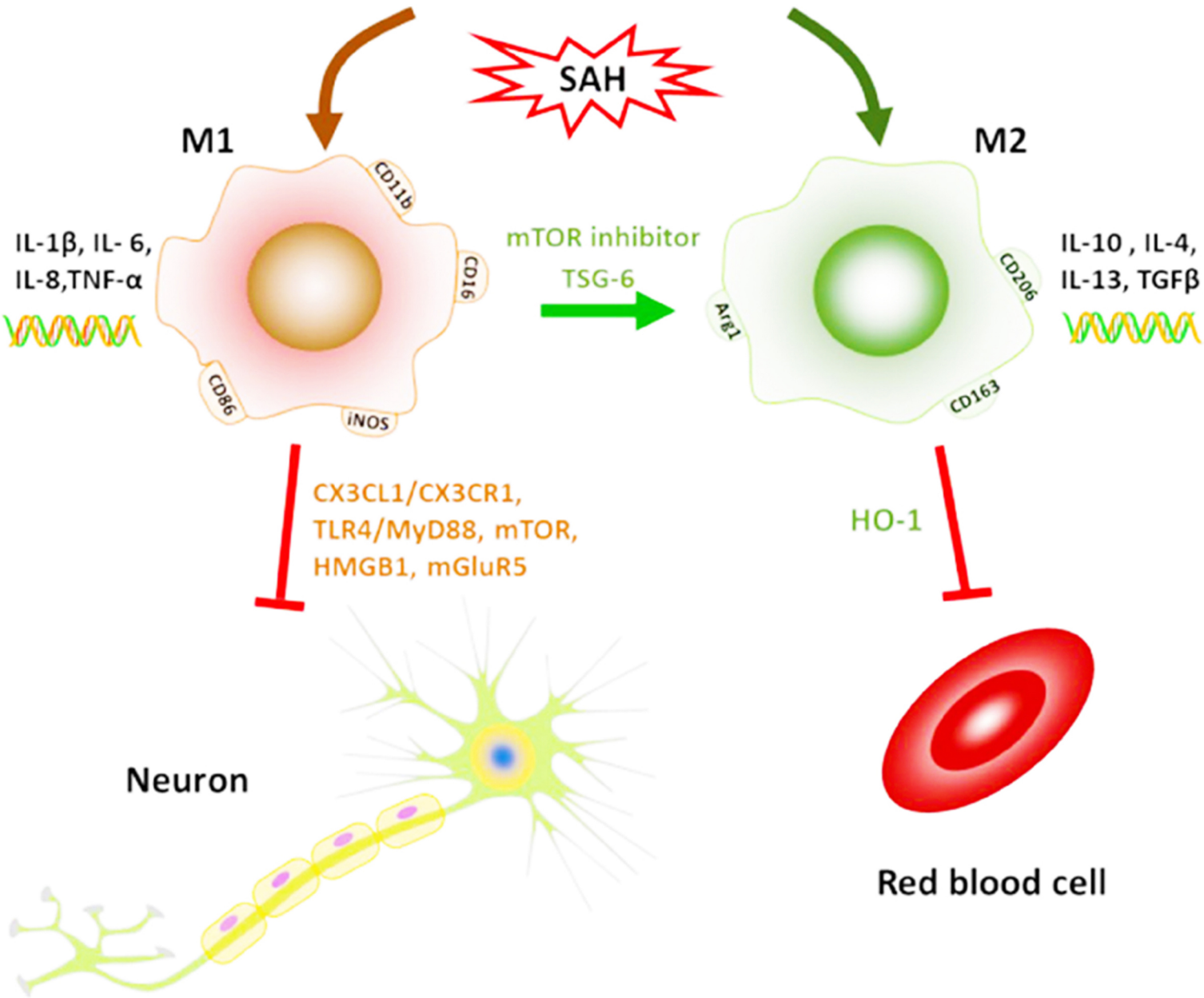

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Neuroimmunology and Neuroinflammation

Review

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Microglial activation and polarization after subarachnoid hemorrhage

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Abstract

Subarachnoid hemorrhage (SAH) is a devastating stroke type, with high mortality and morbidity. The neuroinflammatory response evolves over time from early brain injury to delayed cerebral deterioration. Microglia, the resident immune cells of the central nervous system, respond to the acute brain injury through activation and polarization. Microglia are able to polarize along two pathways, classic M1 and alternative M2, towards tissue injury and tissue repair respectively. The modulation of microglial activation has gained appreciation as a means to prevent the detrimental effects. In this review, we describe the progression of microglial polarization after SAH and summarize the key studies on mediators of microglial activation, including M1 and M2 specific microglial markers, transcription factors and key signaling pathways. Interactions between microglia and other cells are critical in modulating microglial activation and function, which are discussed as well. The preclinical application of microglia-dependent treatments is presented, aiming for a better understanding of modulating microglial function and suggesting future investigation for therapeutic approaches.

Keywords: Microglia, polarization, inflammation, mediator

INTRODUCTION

Subarachnoid hemorrhage (SAH) is a devastating condition accounting for 5% of the stroke population[1]. The mortality rate is as high as 30%-40% and approximately 50% of the survivors remain permanently disabled[2,3]. Primary brain damage develops within the first few hours to days after SAH as a result of extravasate blood or intracranial circulatory arrest. The increased intracerebral pressure and mass effect result in the sudden herniation and death. Recently, more attention has been focused on early brain injury (EBI) which appear to contribute to subsequent adverse cerebral events[4]. Current treatment for SAH mainly

concentrates on aneurysm repair to avoid further bleeding. Also, cerebral blood flow augmentation is needed to reduce vasospasm. Identification of specific events enriches our understanding of the pathophysiological processes of SAH and enhances the investigation of potential therapeutic strategies. The recent prospective studies failing to confirm the association between vasospasm and outcomes in SAH patients attests to the need to develop new therapeutic directions[5,6].

Microglia, the resident immune cells of the central nervous system (CNS), are a pivotal component of neuroinflammation after SAH[7,8]. Microglia respond to brain injury through altering their morphology and polarization to activate in response to pathophysiological brain insults. Microglia are activated and M1-dominated polarization is demonstrated in the early phase of SAH. Microglial activation contributes to the SAH pathogenesis including brain edema, blood-brain barrier permeability, and neuronal apoptosis. The depletion of microglia leads to a significant decrease in neuronal apoptosis in the early phase[9-11]. Microglial activation involves numerous mediators and signaling pathways, especially the toll-like receptor 4 (TLR4). TLR4 plays an important role in inflammatory response and neuronal death after SAH and the majority of TLR4 is expressed by microglia. TLR4 activation in microglia results in secretion of inflammatory factors such as tumor necrosis factor-α (TNF-α) and deletion of TLR4 significantly reduces the neuronal apoptosis in SAH[10,12]. Increasing experimental evidence supports manipulation of microglial polarization as a strategy for preventing disease progression and improving outcomes[13-15].

Microglia are the primary source of cytokines and chemokines, which contribute to the immunomodulatory signaling after SAH[11,16,17]. These molecules initiate secondary brain injury but can also participate in the subsequent brain repair processes. Elevations in neuroinflammatory factors including monocyte chemoattractant protein-1 and TNF-α are regarded as predictors of overall negative outcome, but not necessarily useful predictors of vasospasm[18,19]. The anti-inflammatory factors such as interleukin-4 (IL-4), IL-10 and transforming growth factor-β (TGF-β) are released following the acute pro-inflammatory response in SAH[11].

In this review, we summarize microglial function after SAH, with a specific focus on microglial activation and polarization. Furthermore, we discuss the potential modulators of microglial polarization and function. The interactions of microglia with other cells are discussed as well.

MICROGLIA

Microglia derive from yolk sac primitive progenitors and migrate into the CNS in early embryogenesis. Microglia have the capabilities of proliferation and differentiation, which are basically associated with disease states[8]. Microglia are highly dynamic in the resting state and activate rapidly in response to a set of transcription factors and growth factor receptors in brain injury or degeneration conditions[20]. Microglial cells activate through modulating the phenotype and generating a large number of cytokines and chemokines. Recent studies indicate that microglia have highly motile processes and continually survey the microenvironment, even in the normal brain[21]. Therefore, microglia represent the guardians of the brain in various injuries and diseases.

Microglia are classically considered as CNS-resident macrophage, as they share many macrophage-associated markers, such as CD11b[22]. However, the lineage relationship between microglia and macrophages clearly indicates that they are separate cell types. Microglia proliferate locally and tend to remain viable longer than macrophage. In addition, microglia are not normally supplied by bone marrow-derived cells[23].

The microglial phenotype is modified in response to the brain injury. Experimental evidence indicates that microglia dynamically and temporally polarize into a classically activated state and alternatively activated state, which contributes to tissue damage or repair respectively[24-26]. Microglial polarization is considered

a functional means by which they release inflammatory factors that contribute to the neuroinflammation responses in CNS disease.

MICROGLIAL POLARIZATION

Microglia generally polarize in two directions from a resting state. The classical activation is known as M1, which is the mediator of pro-inflammatory responses. The alternative activation, known as M2, is responsible for resolution and repair. The polarization of microglia has been clarified by measuring the markers both *in vitro* and *in vivo*. *In vitro* treatment mouse microglia with lipopolysaccharide (LPS) and IL-4 results in M1 and M2 phenotypes respectively[27]. The M1 microglial polarization is characterized by an increase in the expression of pro-inflammatory molecules. Alternatively, activated M2 microglia are categorized into four subtypes, M2a, M2b, M2c, and M2d. The subtypes of the M2 phenotype are firstly defined in the macrophage activation. Analogous to macrophage, we hypothesize the similar subtypes of M2 microglia[28-30].

Recently, the mixture of M1 and M2-phenotyped response are reported in experimental studies, which raise a controversy to the traditional concept of M1 *vs.* M2 microglial polarization. In addition, the classic M1 marker IL-6, which is induced by IL-4, functions as an anti-inflammatory mediator in a mouse model of experimental autoimmune encephalomyelitis[31].

Although the classification of M1/M2 phenotypes is now recognized as an oversimplification, as the pure M1 or M2 polarization is executively observed in *in vitro* studies, this conception remains useful for understanding the functional role of microglia in CNS diseases. The recent studies on SAH address the functional role of microglia by investigating the M1-like and M2-like markers, which are discussed below.

MICROGLIAL M1 POLARIZATION

Microglial polarization can be assessed by immunohistochemical analysis of the specific markers. Pro-inflammatory cytokines are considered to be produced predominantly by classically activated M1 microglia, and these pro-inflammatory factors are integral in the activation of downstream pathways. Therefore, the changes in pro-inflammatory cytokine profiles and pathways can be indicators of microglial polarization after SAH.

An intraparenchymal accumulation of KiM1P-positive microglia/macrophage cells is documented in the SAH patients. The microglia accumulation is evident between day 5 and 15[32]. On experimental SAH, the increase of Iba-1-positive microglial cells is observed around day 4 and 28 within the brain parenchyma. The peak occurs on day 14 after SAH induction. The microglia accumulation presents a centrifugal pattern, starting at the base to the cortex of both hemispheres and spreading globally to other regions of the brain[32].

Cell surface markers including CD11b[9], CD68[33], and ED-1[17] are used to distinguish the activated from resting microglia, both of which can be defined by immunostaining of iba1. In culture, microglia treated with oxyhemoglobin leads to M1 polarization, indicated by CD16+/CD11b+ cells [Figure 1][9]. Other M1-associated markers CD86 and inducible nitric oxide synthase (iNOS) increase immediately after SAH, peak at 24-48 h, and remain highly expressed for 72 h[11].

TNF-α and IL-1β are known to be the most important pro-inflammatory cytokines in human SAH pathology, as well as in other experimental models of SAH. M1-signature pro-inflammatory cytokines are upregulated prominently after SAH. IL-1β, IL- 6, TNF-α increase rapidly within 24 h and last for 48 h on experimental SAH model[9,17,33-35]. The increased pro-inflammatory cytokine expression is associated with poor outcomes in SAH[36].

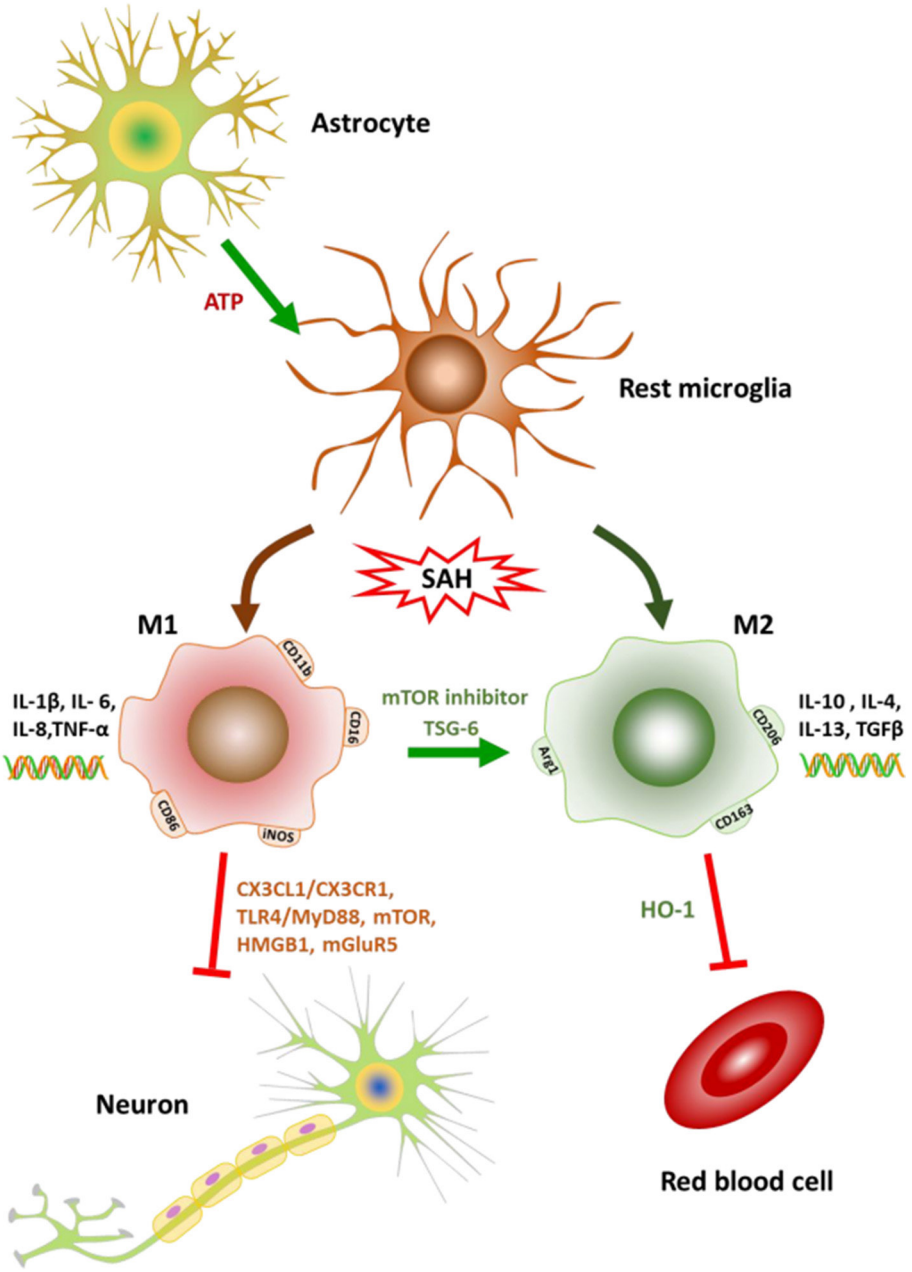

Figure 1. Schematic description of microglial polarization after SAH and the interaction of microglia with other cell types in central nervous system. Microglia are activated and polarized to M1 or M2 direction after SAH. M1 phenotype is characterized by the cell surface marker CD11b, CD16, CD32, CD86. M2 phenotype is characterized by the cell surface markers CD206, CD163, Arg1. M1 microglia exhibit the pro-inflammatory responses with the expression of IL-1β, IL-6, IL-8 and TNF-α. M2 exhibit the pro-inflammatory responses with the expression of IL-10, IL-4, IL-13 and TGF-β. The M1 1 microglial activation has an unfavorable effect on the neurons through the interaction of CX3CL1 and CX3CR1. The neuronal apoptosis is modulated by microglia-dependent TLR4-MyD88, mTOR, HMGB1 and mGluR5. The extracellular ATP released from the surrounding astrocytes trigger the rapid chemotactic response of microglia towards injury. This response can be inhibited by blocking G protein-coupled purinergic receptors and connexin channels, which are highly expressed in astrocytes. The expression of HO-1 in microglia helps to clear the extravasated red blood cells and attenuated neuronal apoptosis. SAH: subarachnoid hemorrhage; IL: interleukin; TNF-α: tumor necrosis factor-α; TGF-β: transforming growth factor-β; CX3CL1: CX3C motif chemokine ligand 1; CX3CR1: CX3C chemokine receptor 1; TLR4: toll-like receptor 4; MyD88: myeloid differentiation factor 88; mTOR: mammalian target of rapamycin; HMGB1: high-mobility group box 1 protein; mGluR5: metabotropic glutamate receptor 5; HO-1: heme oxygenase-1; TSG-6: tumor-specific glycoprotein-6

TLR4 plays an important role in mediating the microglia-dependent neuroinflammation. The activation of TLR4 towards the M1 microglial polarization and increased expression of TLR4 are correlated with the poor outcomes in SAH[37-39]. TLR4 expression in microglia increases 2-6 h after SAH and remains elevated

for 12-48 h[40]. High-mobility group box 1 protein (HMGB1) is a nuclear factor and potent pro-inflammatory mediator, and expressed by Iba1-positive microglia[41]. HMGB1 increases in the cerebrospinal fluid (CSF) of SAH patients with a poor functional outcome. The upregulation of HMGB1 correlates significantly with IL-6, IL-8, and TNF-α expression in CSF, developing towards a pro-inflammatory response after SAH[42-44]. While another study suggests no correlation between HMGB1 and IL-6 concentrations in plasma, which may indicate the acute activation of HMGB1 occurs only in the CNS[45].

MICROGLIAL M2 POLARIZATION

Although M2 microglia play a critical role in tissue repair and toxicity clearance in the other CNS diseases[46,47], M2-directed polarization in SAH has been less studied than M1 phenotype.

All M2a, b and c phenotypes are considered as anti-inflammatory repair M2 microglial cells. The subtypes are generally classified by the different profiles of pro-inflammatory cytokines. M2a activation is considered to be IL4Rα-dependent and follows exposure to IL-4 or IL-13. M2a microglia play a role in the cell repair and regeneration by expressing anti-inflammatory and immune-regulatory molecules. M2b and M2c microglia are largely phagocytic. M2b microglia, in particular, express high levels of IL-10 and low levels of IL-12, whereas, M2c is characterized by high TGF-β expression[28-30,48]. M2d, distinguished from the above subtypes of M2 polarization, results from the classically activated status through the activation of adenosine A_{2A} receptors in activated M1 pro-inflammatory cells[48].

Upregulation of the M2-associated markers Arg1, CD163 and CD206 are observed in experimental SAH. The expression of the markers increases slowly and peaks at 48-72 h[11]. The mRNAs encoding IL-4, IL-10, and TGF-β, show corresponding increments along with M2 polarization in SAH[33]. IL-4 is an important anti-inflammatory cytokine and instrumented in M2-like microglial responses leading to improved functional recovery in ischemic stroke and in intracerebral hemorrhage (ICH) as well[15,30,37,49].

Peroxisome proliferator-activated receptor-γ (PPARγ) is a superfamily of nuclear receptors with the antioxidant and anti-inflammatory properties. Treatment with PPARγ agonists has beneficial effects on SAH, reportedly due in part to reduced microglial activation and reduced pro-inflammatory cytokine expression[50,51]. PPARγ is considered as an important mediator in pro-inflammatory reaction toward M2-like microglial phenotype.

TNF stimulated gene-6 (TSG-6), a multifunctional glycoprotein, acts as a protective regulator against inflammation. The endogenous TSG-6 is mainly expressed in microglia with the peak release in 12-24 h after SAH injury. TSG-6 is considered as an endogenous inhibitor in pro-inflammation progression and induced M2-like polarization. The rh-TSG-6 treated SAH rats show improved neurobehavioral outcomes and reduced brain edema. The decreased M1 polarization and elevated M2 phenotype are observed with the remarkable downregulation of TNF-α and upregulation of IL-10 in SAH rats[11].

The activation of microglia to an M1 phenotype appears mainly in the acute phase after SAH and M2 microglial activation occurs in the subacute and delayed phase. The similar theme of microglial activation is observed in ICH[52]. However, microglia demonstrate the M2-dominated activation in early phase in ischemic stroke and gradually transforms to the M1 phenotype in peri-infarct regions. The ischemic neurons lead microglial polarization toward M1 phenotype[25]. The discrepant responses of microglia to the brain injuries largely depend on the different pathophysiological processes. Hemorrhagic stroke triggers an acute pro-inflammatory response mainly in proximity to the bleeding. The delayed ischemia occurred in one-third of SAH patients[53]. The ischemic neurons may critically contribute to the delayed M2 polarization and the protective M2 phenotype may involve blood clearance and tissue repair in reaction.

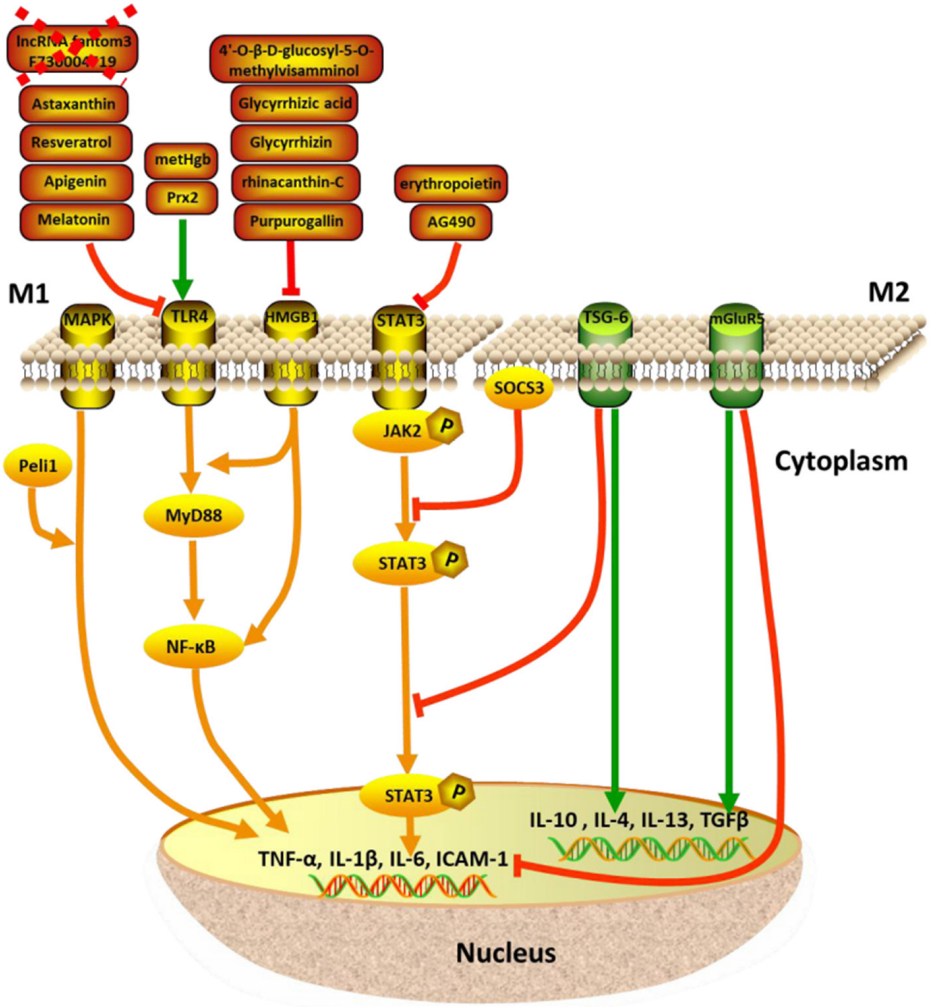

Figure 2. Mediators of microglial polarization after SAH. Activation of TLR4, HMGB1, STAT3 and MAPK signaling pathways promotes microglial M1 polarization with the expression of TNF-α, IL-1β, IL-6 and ICAM-1. The downstream mediators are shown in the figure. Activation of TSG-6 and mGluR5 promotes microglial M2 polarization, therefore reverses the pro-inflammatory responses. SAH: subarachnoid hemorrhage; TLR4: toll-like receptor 4; HMGB1: high-mobility group box 1 protein; STAT3: signal transducer and activator of transcription 3; MAPK: mitogen-activated protein kinase; TNF-α: tumor necrosis factor-α; IL: interleukin; ICAM-1: intercellular adhesion molecule 1; TSG-6: tumor-specific glycoprotein-6; mGluR5: metabotropic glutamate receptor 5; JAK2: Janus kinases 2; MyD88: myeloid differentiation factor 88; SOCS3: suppressor of cytokine signaling 3; TGF-β: transforming growth factor-β; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; Prx2: peroxiredoxin 2; metHgb: methemoglobin

MEDIATOR OF MICROGLIAL POLARIZATION

Microglia dynamically polarize in response to brain damage or pathogens. The mechanism mediating the microglial polarization remains to be fully elucidated. Recently, several mediators are characterized to help in understanding the underlying process and provide potential targets to suppress the M1-like pro-inflammation [Figure 2].

TLR4 is thought the most studied signaling pathway in regulating the microglial activation after SAH. In both SAH patients and animal models, TLR4 is upregulated and associated with cerebral vasospasm, delayed brain ischemia, and neuronal apoptosis in aneurysmal SAH[10,40,54,55]. The majority of TLR4 is expressed in microglia rather than astrocytes or neurons[10]. TLR4 is markedly increased in microglia in a neuron-microglia co-culture system *in vitro*, with consequent increases in pro-inflammatory cytokines and neuronal apoptosis. The downstream molecules, such as myeloid differentiation factor 88 (MyD88) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) are upregulated as well[37,38]. TLR4/MyD88/NF-

κB signaling pathway is more related to the M1 polarization. Based on the high-throughput sequencing and co-expression network analysis of long non-coding RNAs (lncRNAs) and mRNAs, knockdown of lncRNA fantom3_F730004F19 attenuates inflammation in LPS-treated BV-2 microglial cells through the downregulation of TLR4 and CD14, with decreased TNF-α, IL-1β and IL-6 expression[39]. Methemoglobin (metHgb) is considered as an endogenous TLR4 ligand. The application of metHgb into the rat subarachnoid space induces the widespread TLR4-mediated neuroinflammation resulting in the microglial activation and TNF-α upregulation[56].

Substantial treatment strategies have been investigated based on targeting TLR4. Treatment with astaxanthin significantly reduced the TLR4 activation in SAH rats. The subsequent pro-inflammatory releases of IL-1β, TNF-α, and intercellular adhesion molecule 1 are downregulated accordingly at both the protein and mRNA levels *in vivo* and *in vitro*[38]. Peroxiredoxin 2 (Prx2) is a member of Prx protein family. Prx2 activates microglia through TLR4/MyD88/NF-κB signaling pathway and the TLR4 knock-out mitigates the Prx2-induced neuronal cytotoxicity after SAH, which suggests the critical role of Prx2 in the inflammatory modulation responding to hemorrhage attack[37]. Post-SAH treatment with resveratrol, a naturally occurring polyphenolic compound with the anti-inflammatory activity, apigenin or melatonin ameliorates EBI after SAH by suppressing the activation of the TLR4 pathway and expression of MyD88 and NF-κB. The reduced microglia activation and inflammatory cytokines results in the mitigation of neural apoptosis, brain edema, and neurological deficits[12,57,58].

HMGB1 has endogenous cytokine-like activity and involves both in the EBI and delayed cerebral ischemia after SAH by modulating the microglia-dependent pro-inflammation[59]. Treatment with anti-HMGB1 antibody significantly reduces the expression of TLR4, IL-6, TNF-α, and iNOS and reverses the basilar artery vasospasm in the SAH model[35]. The application of glycyrrhizin and glycyrrhizin acid, rhinacanthin-C, purpurogallin, and 4'-O-β-D-glucosyl-5-O-methylvisamminol attenuating the expression of HMGB1, ameliorate inflammatory effect by downregulating M1-related cytokines[60-65]. The molecules targeting HMGB1 may be potential candidates for the treatment of inflammatory brain injury after SAH.

Signal transducer and activator of transcription 3 (STAT3) inflammatory signaling mediates microglial activation both in primary microglia and microglial cell lines. Increased STAT3 expression is accompanied by the elevated expression of IL-6 rather than IL-10. The STAT3/Janus kinases (JAK) cascade is a pivotal inflammatory signaling pathway and widely expressed in the brain maintaining[66,67]. Based on our studies and published data, STAT3 responses to the hemorrhage attack immediately through phosphorylation and translocation into the nuclei. STAT3/JAK pathway is activated and upregulated within 24 h after SAH with the increased pro-inflammatory cytokines release. The mediators and inhibitors, such as erythropoietin and AG490, suppress the STAT3/JAK pathway activation and reduce the M1-like inflammatory response and ameliorate brain injury after SAH[68,69]. The neuroprotective effect of TSG-6 on modulating microglial phenotype involves suppression of the STAT3/suppressor of cytokine signaling 3 pathway activation through impeding the translocation of phosphorylated STAT3[11].

Other mediators trigger the M1 classical activated status in brain in response to pro-inflammatory signals. Activation of metabotropic glutamate receptor 5 (mGluR5) attenuates the M1 microglial activation by downregulating both mRNA and protein expression of pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α, at 24 h after SAH[17]. Albumin suppresses microglial activation resulting in reduced Iba-1 and CD68 staining in the cortex on 1 day after SAH. Expression of M1 microglial markers including iNOS, IL-1b, CD16, and CD32 are remarkably suppressed as well. The albumin induced microglial modulation is associated with binding of albumin to a C-type lectin microglial receptor (mincle), followed by the reduction of mincle/spleen tyrosine kinase/IL-1b signaling in ipsilateral hemisphere subjected to SAH[33]. Peli1, an E3 ubiquitin ligase, mediates the induction of pro-inflammatory cytokines in microglia via mitogen-activated protein kinase (MAPK) signaling pathway. Peli1 promotes the expression of M1 microglia polarization biomarker CD16/32 and iNOS after SAH. Knockdown of Peli1 suppresses microglial activation by inhibiting

MAPK signaling and improves neurological outcomes and reduces cerebral edema after SAH[16].

TRANSITION BETWEEN M1 AND M2

The M2 to M1 shift is observed in models of traumatic brain injury[70] and ischemic stroke[14]. However, whether this transition is a result of phenotypic transformation of a single microglial population, or of the M2 microglial migration and infiltration remains to be determined.

In the SAH model, inhibition of mammalian target of rapamycin (mTOR) can induce a shift of microglia polarizing from M1 to M2 phenotype, as indicated by the reduction of the CD16: CD206 ratio. The shift is along with the decrease in the levels of TNF-α and IL-1β, apoptosis and neuronal degeneration index, brain water content and albumin extravasation in the cerebral cortex after SAH[9].

As described above, in the endovascular punctured SAH model, endogenous TSG-6 transforms the SAH-driven M1 polarization to a skewed M2 polarization and balances the M1/M2 ratio to a beneficial phenotype in the group of $CD11b^{+}$ $CD45^{low}$ labeled microglia. Deficiency of endogenous TSG-6 results in a conversion to pro-inflammatory microglial activation. TSG-6 is a promising candidate to modulate microglial polarization for the neuroprotective effects[11].

INTERACTION WITH OTHER CELLS

CX3C motif chemokine ligand 1 is expressed on neurons and its receptor CX3C chemokine receptor 1, is highly expressed on microglia[71]. Neuronal apoptosis is mediated in a TLR4-MyD88, mTOR and HMGB1 related microglial manner[9,10,62]. Activation of mGluR5 reduces the terminal deoxynucleotidyl transferase dUTP nick end labeling-positive cells and active caspase-3/NeuN-positive neurons in the cortex at 24 h after SAH[17]. The neuronal apoptosis is initiated from 24 h and increases up to two weeks after SAH[32,72,73].

Adenosine triphosphate (ATP) is a pivotal signaling molecule regulating the interactions among different cell types in the CNS and a high concentration of ATP induces microglial activation. Microglia extend the processes toward the site of injection of ATP without cell body movement. This response can be inhibited by blocking G protein-coupled purinergic receptors and connexin channels, which are highly expressed in astrocytes. The extracellular ATP released from the damaged tissue and surrounding astrocytes trigger the rapid chemotactic response of microglia towards injury. This provides evidence of the interaction between microglia and astrocyte in CNS injury[18].

Erythrocyte extravasation is a potential danger factor to the EBI, as heme, released from injured red blood cells, contributes to the pathogenesis of SAH. Heme is metabolized by heme oxygenase-1 (HO-1), which is minimally expressed in the uncompromised brain, but largely upregulated in microglia following injury. Expression of HO-1 in microglia is necessary to attenuate neuronal cell death, vasospasm, impaired cognitive function, and clearance of cerebral blood burden[74].

CLINICAL IMPLICATION

Microglial responses affect neuronal survival and contribute to poor outcome after SAH. Therapeutic intervention or suppression of unfavorable microglial response may accelerate the recovery. Several therapeutic strategies have been implicated to prevent the detrimental effects of microglia and attenuate the neurological impairments in preclinical studies. The application of rosuvastatin markedly inhibits microglia activation and therefore reduces cortical apoptosis, brain edema, and improve the neuronal function after SAH. Heparin reduces the microglial activation and reduces its production of pro-inflammation cytokines[75,76]. As discussed above, administration of resveratrol, apigenin, melatonin, and rh-TSG-6 may

reverse the M1-like pro-inflammatory effect after SAH. Clinical trials are essential to provide the advanced evidence for their therapeutic effect. With the expanding investigation of microglial polarization and function, the treatment that targets microglial phenotype switching may be an efficient approach for SAH therapy.

CONCLUSION

Microglial activation is an important pathological mechanism in the progression of SAH. Microglia undergo polarization into mainly M1 and M2 phenotypes contributing differently to neuroinflammation after SAH. These results indicate the presence of M1-related pro-inflammatory state early after SAH. While microglia polarize to M2 phenotype gradually on delayed phase. Although the dynamics of microglial polarization specifically after SAH remain to be defined, modulation of microglial activation is expected to enhance the tissue repair and functional recovery. The transition from M1 to M2 polarization is thought to be a target concerning the amelioration of the pro-inflammatory response. The investigation on the applications of microglia-targeted treatments is expected to improve our understanding of the pathogenesis of SAH and lead to potential therapeutic strategies for affected patients.

DECLARATIONS

Authors' contributions

Formulation of the key concepts and manuscript framework, literature research, manuscript draft and editing: Zheng ZV

Formulation of the key concepts and manuscript framework, manuscript revision: Wong GKC

Availability of data and materials

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

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Consent for publication

Not applicable.

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Neuroimmunology and Neuroinflammation

Editorial

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Immunotherapeutic approaches for treatment of brain tumors

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Antigenic differences between normal and malignant cells of the cancer patient form the rationale for clinical immunotherapeutic strategies. One emerging strategy in the treatment of tumors involves stimulation of an immunologic response against the neoplastic cells. The hope is that the immune system can be called into play to destroy malignant cells. However, in most instances, proliferating tumors do not provoke anti-tumor cellular immune responses. The precise mechanisms that enable antigenic neoplasms to escape host immunity are incompletely understood. The cells appear to escape recognition by the immune system in spite of the fact that neoplastic cells form weakly immunogenic tumor associated antigens.

The ultimate goal of cancer therapy is the elimination of every remaining tumor cell from the patient. It is unlikely that a single form of therapy is capable of achieving this goal. A number of papers in this special issue are presented which explore the various issues encountered with immunotherapeutic approaches to brain tumors. In particular one paper outlines the various clinical trials that have been attempted for treatment of brain tumors using various immunotherapeutic approaches. Although the results have been relatively modest, there is still enthusiasm for developing new and improved approaches with the speculation that immunotherapy will eventually play an important role in the treatment of brain tumors. Monitoring of the efficacy of immunotherapy remains an issue, and some important points regarding MRI techniques in monitoring these patients are outlined in another paper. The role of photodynamic therapy in the generation of specific anti-tumor immunity and vaccines for the treatment of brain tumors is outlined in one of the manuscripts. The specific problems encountered in developing immunotherapy for pediatric patients with brain tumors is a topic of another paper.

A number of exciting results have been found in patients with non-small cell lung cancer treated with immunotherapeutic approaches, and the issues involved in the treatment of these patients with tumors

metastatic to the brain is the topic of one manuscript. Some issues regarding the interaction of hematopoietic stem cells in the immune response is a topic of another paper. A discussion of pharmacological strategies that interfere with glioma associated microglia/macrophage function which might be an alternative/additional option to current approved cytotoxic regimens is reviewed in several manuscripts. Chimeric antigen receptor T (CAR-T) cells are being studied, both with systemic infusion and direct administration to the tumor and into the cerebrospinal fluid, with promising early results. A review aimed to discuss adoptive cell therapies with a focus on CAR-T treatment in patient with brain tumors is the topic of another manuscript. The therapeutic approach of adoptive lymphocyte transfer using lymphocytes primed and expanded *ex vivo* by exposure to total tumor RNA containing dendritic cells in certain pediatric patients with brain tumors is explored in another paper. A review of the current state of use of histone deacetylase (HDAC) inhibitors in gliomas, the mechanistic rationale for use of HDAC inhibitors in gliomas, and revelation of how certain HDAC inhibitors promote antitumor immunity in glioma patients is the focus of another manuscript. Finally a review of the immunosuppressive microenvironment generated by tumors along with various inhibitors that can impair these tumor immunosuppressive capabilities is outlined in another manuscript.

It is clear from the papers in this special issue that immunotherapy for brain tumors is being investigated from a number of different approaches, but significant enthusiasm remains that immunotherapy will be an important adjunct to the treatment of patients with brain tumors.

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Neuroimmunology and Neuroinflammation

Editorial

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Pulsed intravenous corticosteroids in chronic inflammatory demyelinating polyneuropathy: why not?

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Chronic inflammatory demyelinating polyneuropathy (CIDP) is a remitting/relapsing and/or chronic autoimmune disorder, characterized by symmetrical, sensorimotor neuropathic involvement and a slowly progressive onset. There are many clinical variants, suggesting that this disorder may not be an unique entity but rather a spectrum[1]. CIDP diagnostic criteria combine clinical and electrophysiological features. Supportive data include increased cerebrospinal fluid (CSF) protein levels[1].

Many, but not all, patients may be successfully treated with therapies aimed at arresting immunological mechanisms, such as corticosteroids[2] and intravenous immunoglobulins (IVIg)[3]. A systematic review concluded that there was no clear short-term difference with IVIg when compared with intravenous methylprednisolone and likely no improvement when compared with either oral prednisolone or plasma exchange[4]. More randomised trials are strongly needed[4].

Recently, a multicentre retrospective study compared safety and efficacy of daily prednisolone, pulsed dexamethasone, and pulsed intravenous methylprednisolone. Interestingly, corticosteroids led to improvement in 60% of subjects and to clinical remission in 61% of responders[2]. There were no significant differences in terms of safety and efficacy[2]. A therapeutic protocol with corticosteroids, with IVIg as an adjunctive treatment in case corticosteroid treatment was insufficient, could lead to improvement in 90% of CIDP patients[2].

Some patients may not respond to IVIg. For instance, we follow a 37-year-old male patient diagnosed with CIDP at age 7, based on a sensory ataxic phenotype. The diagnosis was supported by typical

electrophysiological features, CSF analysis and sural nerve biopsy. Molecular studies for hereditary neuropathies were unremarkable. At age 33 he had a severe relapse leading to a subacute flaccid, areflexic tetraparesis unresponsive to IVIg. He was then successfully treated with plasma exchange and intravenous corticosteroids. Subsequently he became corticosteroid-dependent needing chronic treatment with oral prednisone (25 mg every other day). Unfortunately, he developed bilateral cataract, right hip osteonecrosis and cushingoid appearance. Therefore, we switched the treatment to pulsed intravenous methylprednisolone (1 g daily for three consecutive days every two months). After one year of this schedule, the neuropathy is excellently controlled (apart from mild distal leg weakness) and corticosteroid toxicity is minimized, with improvement of hip osteonecrosis and regression of the cushingoid features.

In conclusion, corticosteroids are cheaper, easier to use, and much more widely available than IVIg. They have been suggested to lead to long-term remission more often than IVIg[2]. Furthermore, even if there are no significant differences in response and remission rate between these two regimens, pulsed intravenous corticosteroids have lower rates of serious adverse effects than long-term daily use[2]. Therefore, in our opinion pulsed intravenous methylprednisolone should be considered in CIDP patients, especially in non-responders to IVIg. In fact, it may represent the therapy of choice in these patients.

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Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Orsucci D

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Neuroimmunology and Neuroinflammation

Editorial

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Adiponectin: a pivotal role in the protection against cerebral ischemic injury

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Adiponectin (APN), an adipokine which weights 30 kDa, is first identified in 1995 and almost exclusively secreted by adipocytes[1]. Its physiological and clinical significance has been extensively explored in these years. A comprehensive review of adiponectin and its relating significance is beyond the scope of this article. Although its relationship with cerebral ischemia and ischemic stroke has been reviewed previously[2,3], I try to briefly address the pivotal role of adiponectin in the protection against cerebral ischemic injury in the current article.

STRUCTURE AND BIOSYNTHESIS

There are 3 major oliogmeric multimers of APN: the low-molecular-weight trimer, the middle-molecular-weight hexamer, and the high-molecular-weight (HMW) 12-18 multimers[4,5]. HMW adiponectin has been proposed to be the most potent form and drives the physiological role of adiponectin, as evidenced by some *in vitro* and human studies[1,6]. Also, a globular fragment of adiponectin (gAd) exists. It is generated as the full-length adiponectin is cleaved by leukocyte elastase which are secreted from activated monocytes and/or neutrophils. As compared with other isoforms, it remains at low circulating levels, only accounting for about 1% of total adiponectin[7]. The expression of adiponectin are under the regulation of several transcriptional factors, including CCAAT-enhancer-binding proteins, peroxisome proliferator-activated receptor γ, and sterol regulatory element binding protein 1c[6].

PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL ROLES IN HUMAN DISEASES

The physiological role of adiponectin is mainly involved in insulin sensitivity and regulation of metabolism of glucose and lipids[8]. Furthermore, it has pleiotropic effects including anti-inflammation, anti-

atherosclerosis, anti-thrombosis, and promotion of endothelial repair and angiogenesis which are protective in endothelial injury, atherosclerosis, and cardiovascular diseases[9-11].

Adiponectin is abundant in peripheral circulation, representing about 0.01% total plasma proteins[12], and has a rapid turnover rate[10]. Decreased circulating adiponectin level is noted in obesity for increased oxidative stress in accumulated fat[13,14]. Clinically, hypoadiponectinemia has been noted in various diseases, such as ischemic stroke[15,16], coronary artery disease, insulin resistance and diabetes, hypertension, dyslipidemia, metabolic syndrome, hepatic steatosis and fibrosis, and cancer[17]. On the contrary, hyperadiponectinemia has been noted in congestive heart failure and chronic kidney disease[18,19].

ADIPONECTIN IS PROTECTIVE AGAINST CEREBRAL ISCHEMIC INJURY

Exogenous accumulation from the circulation but not endogenous production in damaged brain tissues after cerebral ischemic injury

In many pre-clinical studies, adiponectin has been consistently shown to be protective against cerebral ischemic injury. However, the expression of adiponectin after cerebral ischemic injury is not endogenous in ischemic cerebral tissues but exogenous from the peripheral circulation.

There are studies exploring the expression profiles of adiponectin after middle cerebral artery occlusion (MCAO) in mice and rats. The study by Yatomi *et al.*[20] showed that, plasma adiponectin levels peaked soon at 1-3 h, decreased later, reached the nadir in 48 h, and then returned to baseline gradually in the rat after MCAO. However, the expression pattern of adiponectin in ischemic cerebral hemisphere differed. Adiponectin showed higher levels in ischemic cerebral hemisphere than non-ischemic one during 72 h to 7 days after ischemia/reperfusion injury. Moreover, its expression was evident in endothelium only, not in neurons, glia, or macrophages. Finally, its expression in the endothelium of ischemic hemisphere seemed to be exogenous from the circulation but not endogenous from damaged cerebral hemispheres since there was no mRNA expression of adiponectin detected by reverse transcription polymerase chain reaction in these area. Another study by Shen *et al.*[21] showed similar findings. They found that adiponectin started to rise 1 h in ischemic hemisphere after cerebral ischemia/reperfusion injury in mice, peaked in 3 days and lasted till 7 days. They found the expression of adiponectin occurred only in vascular endothelial cells but not in neurons or glial cells. Furthermore, they could not find the mRNA expression of adiponectin in ischemic cerebral hemisphere. Taken together, adiponectin accumulates in vascular endothelial cells instead of *de novo* generation in ischemic brain after cerebral ischemic injury.

Adiponectin alleviates cerebral ischemic injury through multi-mechanisms

Adiponectin is protective against cerebral ischemic injury and the mechanisms accounting for this are diverse. The study by Nishimura *et al.*[22] reported that adiponectin exerted a cerebroprotective action through an endothelial nitric oxide synthase (eNOS)-dependent mechanism in cerebral ischemic injury. They showed that adenovirus-mediated delivery of adiponectin augmented the status of phosphorylation of endothelial nitric oxide synthase and reduced the infarction volume in adiponectin knockout (APN-KO) mice.

Another important mechanism of adipoenctin being protective against cerebral ischemic injury is anti-inflammation. The study by Chen *et al.*[23] showed that exogenous supplement of gAd via jugular vein reduced cerebral infarct size, neurological deficits, and expression of endogenous matrix metalloproteinase 9, interleukin (IL)-1β, tumor necrosis factor-α and IL-8, and inhibited the translocation of nuclear factor (NF)-κB from cytoplasm into the nucleus in the rat after MCAO. The indirect evidence of its anti-inflammatory mechanism comes from another study by Jung *et al.*[24]. They found more rolling leukocyte and leukocyte adhesion were observed in the APN-KO mice than in the wide type mice after cerebral ischemia/reperfusion injury. They proposed that adiponectin inhibits the interaction between the endothelium and leukocytes and hence alleviates the inflammatory insult in cerebral ischemic injury.

Adiponectin exerts anti-oxidation against cerebral ischemic injury as well[25,26]. The study by Song *et al.*[25] showed that intracerebral injection of gAd attenuated infarct size and neurological deficits aggravated by NADPH oxidase activator in mice after MCAO along with increased activities of superoxide dismutase (SOD) and catalase, and reduced content of malondialdehyde (MDA). The study by Li *et al.*[26] reported similar findings. They showed intraperitoneal supplement of adiponectin improved neurological deficits, decreased infarct size, and attenuated neuronal injury along with decreased MDA levels and increased SOD activity levels in mice after MCAO. The study by Wang *et al.*[27] reported adiponectin attenuated oxygen and glucose deprivation-induced neuronal injury and mitochondrial oxidative stress in hippocampal neuronal HT22 cells as evidenced by attenuated reactive oxygen species and malondialdehyde, and increased superoxide dismutase and glutathione peroxidase activity.

Also, adiponectin has been related to PKA, CREB, and BDNF in the protection against cerebral ischemic injury. The study by Bai *et al.*[28] reported activation of cAMP/PKA-CREB-BDNF signaling pathway by adiponectin was protective against ischemia/reperfusion injury with reduced infarct volume, neurological deficits and brain water content.

Finally, anti-apoptosis after cerebral ischemic injury by adiponectin has been found in *in vivo* and *in vitro* studies[25-27,29], including that by our group[30].

APN-gene modified cell therapy alleviates cerebral ischemic injury

The pre-clinical studies of APN-gene modified cell therapies in the treatment of cerebral ischemic injury are growing recently. The study by Nishimura *et al.*[22] showed that adenovirus-mediated expression of adiponectin reduced brain infarction volume, increased cerebral blood flow, and improved neurological deficits, through an eNOS-dependent mechanism in cerebral ischemic injury. The study by Shen *et al.*[31] reported that adiponectin could promote focal angiogenesis in cerebral ischemic injury. They showed that after MCAO mice receiving intracerebral injection of adeno-associated viral vector carrying the APN gene had reduced ischemia-induced brain atrophy, improved neurological function and increased number of microvessels along with increased AMPK phosphorylation and vascular endothelial growth factor expression. Furthermore, the study by Miao *et al.*[32] showed this angiogenetic effect was more significant in aged mice than young mice.

Our group[30] showed that pre-treatment of baculovirus-mediated expression of APN through intra-cerebral injection was protective against cerebral ischemic injury in both normal weight and obese rats through reducing brain infarct and edema, neurologic deficits, and p38-mediated neuronal apoptosis.

Recently, it has been shown that genetically-transplanted endothelial progenitor cells with adiponectin by lentivirus could reduce cerebral infarction volume, improve behavior outcome, increase microvessel density, and reduce cell apoptosis[33].

gAd alleviates cerebral ischemic injury

Among adiponectin isoforms, the globular adiponectin has been mostly studied and consistently shown to be protective against cerebral ischemic injury[23,25,29]. The data regarding whether other isoforms of adiponectin could exert protection against cerebral ischemic injury are currently lacking and warrant further studies.

In summary, adiponectin, an adipokine secreted by adipocytes, exists in a relatively large amount in the peripheral circulation. During the cerebral ischemic injury, it accumulates in the damaged cerebral vasculature instead of *de novo* generation by damaged cerebral tissues. It exerts multiple protective mechanisms against cerebral ischemic injury including eNOS-dependent mechanism, anti-inflammation, anti-oxidation, anti-apoptosis, and promotion of angiogenesis. Since adiponectin is an adipokine naturally

secreted by human adipose tissues, and has multi-mechanisms protective against cerebral ischemic injury, it is of great potential in the application of clinical treatment of ischemic stroke in the future.

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Authors' contributions

Wu MH is responsible for design and conceptualization of the study, drafting and revising the manuscript, and obtaining funding.

Availability of data and materials

Not applicable.

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Conflicts of interest

The author declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Neuroimmunology and Neuroinflammation

Review

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Speedy/RINGO: a molecular savior in spinal cord injury-based neurodegeneration?

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Abstract

Endogenous or exogenous insults can cause spinal cord injury (SCI), often resulting in the loss of motor, autonomic, sensory and reflex functions. The pathogenesis of SCI comprises two stages. The primary injury stage occurs at the moment of trauma and is characterized by hemorrhage and rapid cell death. The secondary injury stage occurs due to progression of primary damage and is characterized by tissue loss and functional disorder. One of the most important cellular mechanisms underlying secondary injury is glutamate excitotoxicity, which overactivates the calpain protease via excessive Ca^{2+} influx and induces neuronal apoptosis via p53 induction. Furthermore, Ca^{2+} influx elicits apoptosis by inducing p53, thus negatively affecting two pathways: the mitogenic extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway and the survival phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway. Speedy/rapid inducer of G2/M progression in oocytes (Speedy/RINGO) is a cell cycle regulatory protein that increases survival of p53-positive mitotic cells by inhibiting the apoptotic machinery. Moreover, this protein elicits p53-dependent anti-apoptotic effects on calpain-induced degeneration of primary hippocampal neurons, amyotrophic lateral sclerosis motor neurons, and astrocytes and microglia in spinal cord lesions. The pathophysiology of SCI has not been fully elucidated and this hinders the development of powerful therapeutic strategies. This review focuses on the cellular mechanisms underlying the anti-apoptotic effects of Speedy/RINGO and discusses how this protective function can possibly be exploited to facilitate recovery from SCI. Particular attention is paid to reversal of the negative effects on the ERK/MAPK and PI3K/AKT pathways via induction of p53.

Keywords: Speedy/RINGO, calpain, p53, extracellular signal-regulated kinase/mitogen-activated protein kinase, phosphoinositide 3-kinase/protein kinase B, spinal cord injury, glutamate excitotoxicity, calcium influx

INTRODUCTION

Spinal cord injury (SCI) can be defined as an endogenous or exogenous trauma resulting in the loss of motor, autonomic, sensory and/or reflex functions. SCI is a major cause of permanent disability. Researchers estimate that 230,000 people in the United States are living with an SCI, and that 10,000 new patients are diagnosed each year[1-7].

The pathology of human spinal cord injury is the result of two main mechanisms known as "primary" and "secondary" injury. Primary injury begins at the moment of trauma and is characterized by hemorrhage and rapid cell death. Secondary injury is an extension of the original injury and occurs when vascular and biochemical effects cause tissue loss and functional disorders[8,9]. It is important to state that primary injury always serves as the nidus of secondary injury. Secondary injury mechanisms primarily involve neurogenic shock, vascular damage, ischemia and hemorrhage, immunologic secondary injury, glutamate excitotoxicity and subsequent apoptosis.

Among all, the most destructive cellular mechanism underlying secondary injury is glutamate excitotoxicity, which overactivates calpain protease via excessive Ca^{2+} influx and induces neuronal apoptosis via p53 induction[10]. Furthermore, intracellular Ca^{2+} influx has an apoptotic effect, particularly through p53 induction on mitogenic extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and survival phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathways[11]. Therefore, a primary goal of SCI treatment could involve the prevention p53-induced apoptosis caused by glutamate excitotoxicity.

Speedy/rapid inducer of G2/M progression in oocytes (Speedy/RINGO) is a cell cycle regulatory protein that increases survival of p53-positive mitotic cells by inhibiting the apoptotic machinery[12]. Moreover, this protein elicits p53-dependent anti-apoptotic effects on calpain-induced degenerating primary hippocampal neurons[13], amyotrophic lateral sclerosis (ALS) motor neurons[14] and in astrocytes and microglia in spinal cord lesions[15]. In addition, evidence from breast and testis cancer studies strongly implicates the direct or indirect interaction of Speedy/RINGO as a p53-dependent anti-apoptotic factor for ERK/MAPK and PI3K/AKT pathways[16-18]. These findings strongly suggest a role for Speedy/RINGO as a shield against p53-mediated apoptotic death in SCI.

As yet, there is not any proven treatment regimen for SCI probably due to its lesser known pathophysiology. Revealing cellular mechanisms of SCI and correlating them with the clinical symptoms are of primary importance for developing effective SCI recovery treatments. In this regard, this review focuses on the underlying molecular mechanisms of Speedy/RINGO's anti-apoptotic function by correlating these mechanisms with the complex pathophysiology of SCI. Furthermore, this review discusses how this protective function could possibly be exploited to facilitate recovery from SCI. Particular attention is paid to reversal of the negative effects on the ERK/MAPK and PI3K/AKT pathways via induction of p53. This new approach may assist in identifying the most promising molecular targets for effective treatment modalities and may also uncover the molecular basis of SCI.

GLUTAMATE EXCITOTOXICITY AND DOWNSTREAM APOPTOTIC EVENTS IN SCI

Excitotoxicity is defined as cell damage or cell death resulting from exposure to excitatory amino acids such as glutamate. Glutamate is a major neurotransmitter that plays an important role in the central nervous system[19]. After spinal cord injury, glutamate levels increase in and around the trauma site. As a result of glutamate release, glutamatergic activity contributes to induction and progression of secondary injury in SCI[10]. When an excessive amount of glutamate is released from presynaptic nerve terminals and from astrocytes into the extracellular space, glutamate receptors (*N*-methyl-d-aspartate receptor, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor) are over stimulated[20]. Glutamate excitotoxicity leads to Ca^{2+} imbalance, free radical formation, and apoptosis. An excessive amount of Ca^{2+} influx into the cell is

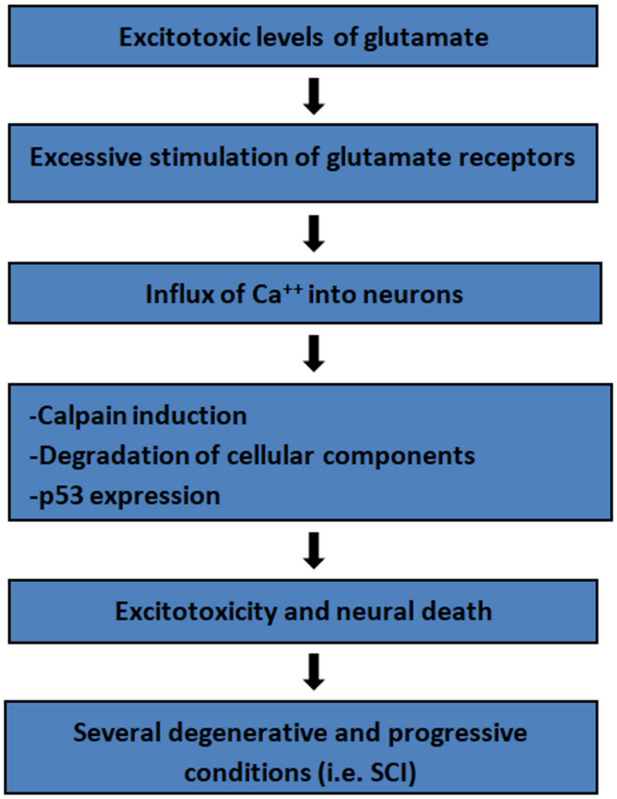

Figure 1. A diagram depicting the mechanism of glutamate excitotoxicity. SCI: spinal cord injury

neurotoxic because it causes the activation of certain enzymes such as calpain protease that degrade cellular proteins and membranes[21,22], leading to p53-mediated apoptosis[10,23] [Figure 1].

High level of intracellular calcium

Ionic balance is essential to protecting the functional integrity of neural cells. Therefore, Ca^{2+} imbalance provides a mechanism for a severe secondary injury. The influx of Ca^{2+} is triggered by trauma through glutamate toxicity and continues for some time once it has been triggered. Ca^{2+} influx has serious negative effects in neural cells, including mitochondrial damage that further destabilizes Ca^{2+} balance, generation of free radicals, and activation of many enzymes, including calpain. This ultimately triggers degradation of cellular components, leading to p53 induction and subsequent caspase-dependent apoptosis[24,25] and the spread of the axoplasmic damage into adjacent cells[26,27]. One therapeutic strategy to reduce the degree of secondary damage in the neural pathways of SCI patients would be the inhibition of the apoptotic downstream effects of Ca^{2+} influx.

Apoptosis after SCI

Programmed cell signaling pathways play an important role in the pathobiology of neurological diseases such as SCI. After spinal cord trauma, a number of cells at the lesion site die via apoptosis or necrosis. Apoptosis is a programmed cell suicide mechanism which can be triggered by cytokines, post traumatic inflammation, free radicals and excitotoxicity. Recent studies confirm that cells of injured spinal cord tissue primarily die due to apoptosis[28].

Apoptosis is commonly observed in both neurons and oligodendrocytes, increasing the possibility of paralysis in patients with SCI. An experimental study in rats showed that apoptosis occurred 4 h after trauma, and that the effect of the injury could be decreased as late as 3 weeks after SCI[29]. A caspase

mechanism is activated in neurons at the injury site and spreads to adjacent and distant oligodendrocytes. Thus, the primary injury caused by spinal cord trauma progresses into nearby tissue cells, leading to secondary injury.

In SCI, increased intracellular Ca^{2+} influx as a result of glutamate induction is one of the major apoptotic insults leading to overactivation of certain proteases which subsequently cause proteolytic degradation of myelin and cytoskeletal proteins and degeneration of axons. These are all hallmarks of secondary injury and contribute to the progression of SCI[30-34]. One of these proteases - calpain - is known to be a highly effective neurodegenerator. Upon its overactivation, calpain increases p53 and caspase-3 activation, causing neurons to degenerate through apoptosis[13]. In addition, calpain overactivity has been shown to disrupt the regulation of mitogenic ERK/MAPK and survival PI3K/AKT signaling cascades in a p53-dependent manner[11].

Based upon these findings, it is evident that glutamate-mediated p53 induction is the prominent reason for apoptosis in SCI. The p53-dependent anti-apoptotic function of Speedy/RINGO makes it an excellent therapeutic candidate for treatment of SCI.

CURRENT STUDIES FOR THE RECOVERY OF SCI

Current research approaches for developing novel therapeutic regimens target both primary and secondary injuries, which are the hallmarks of SCI. Since more complex, multifaceted neurodegenerative progression occurs in secondary injury, the main aim of such investigations is to understand the underlying molecular mechanisms and find the potential key molecules to target for effective SCI treatment. Since the complex molecular mechanisms of SCI have only been partially elucidated, most efforts so far have had limited efficacy. These efforts mainly involve providing anti-neuro-inflammatory conditions[30,35-37] preventing excitotoxicity in neurons[38,39], reducing oxidative damage[31,40,41] and regulating the effects of intracellular ionic changes, such as altered Ca^{2+} homeostasis.

Spinal cord injury results in loss of oligodendrocytes which, in turn, causes demyelination of axons. Since demyelination largely impedes functional recovery from SCI, an important treatment modality involves preventing oligodendrocytic death[42] and/or enhancing myelin formation by regulating myelin-related factors such as Nogo, ephrins, semaphorins, oligodendrocyte-myelin glycoprotein, and/or myelin-associated glycoprotein, all of which have been shown to increase neuroregeneration after spinal cord injury[43-49]. Neurotrophins and neurotrophin receptors are reported to provide neuronal survival[43,50-55] and enhance behavioral recovery in SCI[56-58], so another potential treatment strategy would be to enhance the expression of regeneration-associated genes such as neurotrophins, integrins, GAP-43 and CAP-23[50].

In addition to genetic and molecular-based studies, some researchers are studying the efficacy of transplanting stem cells, Schwann cells, peripheral blood stem cells and bone marrow to replace lost tissue[57,59,60].

Numerous studies on spinal cord injury in rodents, primates and humans have indicated that the level of inflammation increases as a result of glial cell activation and filtration of somatic immune cells through mechanically disrupted spinal cord tissue[61]. The effect of inflammation in the secondary mechanism of SCI has not yet been clarified. However, it is known that inflammation induces astrocytic gliosis. This, in turn, results in glial scar formation, spread of the inflammatory response and damage to the surrounding healthy neurons, leading to their apoptotic deaths[62]. In a study aiming to prevent astrocytic gliosis, researchers focused on the role of the mitogenic ERK/MAPK signaling cascade in astrocytic proliferation, since mitosis is the most important feature of reactive astrocytes[63]. In their experiments, an increase in expression and phosphorylation of ERK/MAPK members was observed in reactive proliferating astrocytes of SCI lesions. In order to downregulate ERK/MAPK signaling, liposomes containing the interferon-β (*IFN-β*) gene were

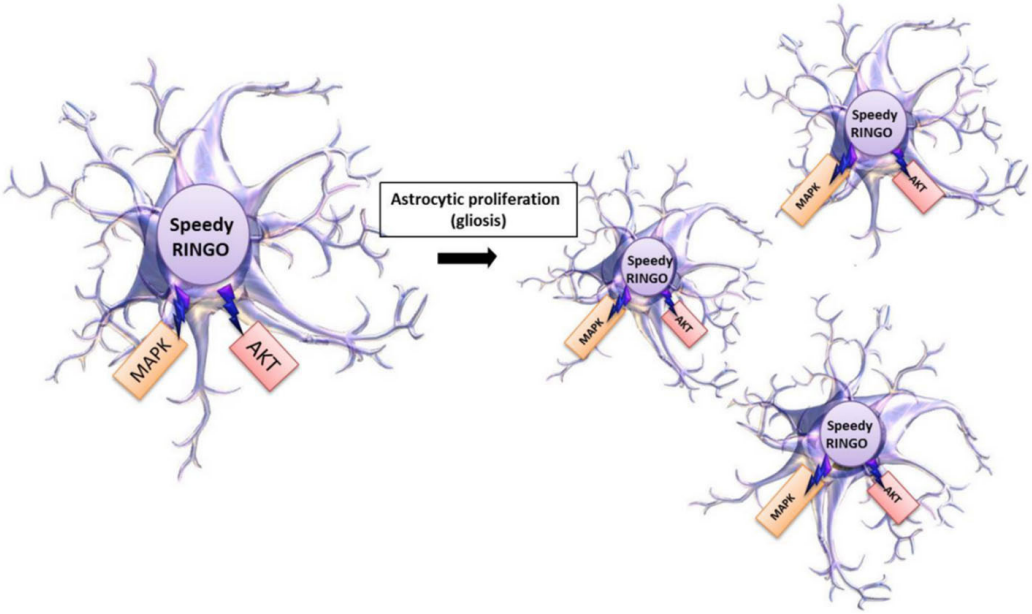

Figure 2. A schematic diagram for proposed proliferative regulation of Speedy/rapid inducer of G2/M progression in oocytes (Speedy/RINGO) on extracellular signal-regulated kinase/mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase/protein kinase B (AKT) signaling cascades in astrocytic gliosis in spinal cord injury

administered 30 min after injury. After 14 days, the ERK/MAPK phosphorylation and proliferation rates were significantly reduced. The animals that received the *IFN-β* gene exhibited neurobehavioral recovery, indicating the importance of regulating mitogenic ERK/MAPK signaling in SCI.

In addition to ERK/MAPK signaling, the other component responsible for this astrocytic proliferation was shown to be the mitotic regulator Speedy/RINGO[15]. Researchers indicated that 2 days after SCI, Speedy/RINGO expression peaked specifically in astrocytes and microglia cells in concordance with the increase in their proliferation rate. This finding points to Speedy/RINGO as another strong candidate to prevent astrocytic proliferation.

Findings of these studies on astrocytic gliosis indicate that the interaction between Speedy/RINGO upregulation and ERK/MAPK hyper-phosphorylation leads to glial scar formation [Figure 2]. Glial scar formation is one of the primary obstacles for axon regeneration after injury[63]. Therefore, an effective treatment strategy for SCI may involve preventing expansion of these scars by targeting Speedy/RINGO, and thereby affecting ERK/MAPK signaling and allowing axonal regeneration.

Apart from the research studies on astrocytic gliosis, other experiments showed that, in SCI, the intraneuronal Ca^{2+} level increases as a result of glutamate induction. Consequent deregulation of Ca^{2+} homeostasis leads to abnormal activation of proteases, which subsequently cause proteolytic cleavage and degradation of myelin and cytoskeletal proteins, along with degeneration of axons. These are all hallmarks of secondary injury that contribute greatly to the progression of SCI[30-34].

As previously mentioned, one of these proteases, calpain, experimentally induces apoptosis in degenerating neurons through increasing p53 and caspase-3 activation[13].

Furthermore, there is strong evidence indicating that intracellular Ca^{2+} influx gives rise to apoptotic deregulation of mitogenic ERK/MAPK and survival PI3K/AKT pathways through p53 induction[11]. In

certain neurodegenerative conditions, MAPK and PI3K/AKT signaling pathways provide a crucial function in neuronal survival[10,23,64]. In one *in vivo* study, researchers performed facial nerve axotomy on rats to create a peripheral nerve injury model. They then analyzed the ERK/MAPK and PI3K/AKT pathways by evaluating the phopshorylation levels of ERK and AKT in axotomized neurons[10]. Seven days after the nerve axotomy, ERK and AKT phosphorylation levels were shown to have increased, while the rate of apoptosis was shown to have decreased. These researchers also used the MAPK inhibitor or PI3K/AKT phosphorylation inhibitor to determine survival rate of facial neurons, regenerated axon number and length of regenerated axons, in the event that ERK/MAPK and PI3K/AKT signaling cascades were silenced. When ERK phosphorylation was inhibited, only the regenerated axon length was obviously decreased. However, inhibition of AKT phosphorylation significantly reduced not only the length of regenerated axons but also the number of new axons and the survival rate of neurons. Results of this study clearly indicate that nerve injury through axotomy activated both PI3K/AKT and ERK/MAPK signaling in neurons, implying an effort to survive[10].

In another *in vitro* study investigating the function of ERK/MAPK and PI3K/AKT pathways in neuronal survival after injury, researchers demonstrated that ciliary neurotrophic factor (CNTF) promotes survival and process outgrowth via ERK/MAPK and PI3K/AKT pathways in oxytocinergic neurons of hypothalamic organotypic cultures[64].

Finally, a research group performed an *in vitro* study to analyze the role of MAPK signaling in preventing cytosine arabinoside (araC)-induced apoptosis in sympathetic neurons[23]. They induced apoptosis with araC and used the selective MAPK inhibitor PD98059 to test whether MAPK inhibition affected the rate of apoptosis. Their results showed that MAPK inhibition increased the rate of araC-induced apoptosis in the presence of nerve growth factor (NGF) in a p53-dependent manner. This finding indicated that the MAPK signaling pathway plays a critical role in protecting primary neurons against apoptosis under certain pathological conditions[23].

The aforementioned studies dealing with neurons and astrocytes demonstrate the controllable nature of ERK/MAPK and PI3K/AKT pathways through different effector molecules, including p53, NGF, CNTF and, most probably, Speedy/RINGO. This implies that properly balancing the activity of these pathways with respect to different neuropathological conditions and different cell types may help prevent neurodegeneration and apoptosis.

To recapitulate, deregulation of intraneuronal Ca^{2+} influx - which is one of the well-known triggering events of secondary injury in SCI - results in activation of calpain. This activation subsequently increases p53 levels, which abnormally regulate MAPK and PI3K/AKT pathways and lead to severe neurodegeneration and apoptotic death. A number of investigations have studied whether the reduction of apoptotic effects of Ca^{2+} influx can prevent or minimize secondary injury in SCI.

Estrogen is one inhibitor that has been used to protect cells in culture and in rat models against apoptosis. Researchers showed that estrogen and its analogs decreased the activity of calpain protease[65] and inhibited apoptosis in microglia, neurons[66] and oligodendrocytes[67].

Melatonin, known for its antioxidant and anti-inflammatory properties, is another anti-apoptotic agent in SCI. It has been shown that melatonin promotes neuronal survival by preventing secondary injury through free oxygen radical scavenging[57,58]. Melatonin also works to alleviate intracellular Ca^{2+} influx and subsequent calpain activation[65-70].

Abnormal intracellular Ca^{2+} influx, as an integral part of SCI, has apoptotic effects such as aberrant regulation of MAPK and AKT signaling pathways via p53 induction. Because of this, it is vital to overcome, or at least minimize, this apoptotic effect of Ca^{2+} and provide neuroprotection to neurons at the injury

site. In order to achieve this goal, the first challenge is to thoroughly understand the exact pro-apoptotic mechanisms driven by Ca^{2+} influx and the key factors involved in these mechanisms. From this point of view, superior neuronal protection in SCI involves a two-pronged approach: (1) reversal of the apoptotic effect on injured neurons caused by the apoptotic deregulation of the ERK/MAPK and PI3K/AKT pathways; and (2) inhibition of pathological calpain protease activation.

The goal of our laboratory is to understand the pro-apoptotic mechanism of Ca^{2+} deregulation and to prevent apoptosis by inhibiting the downstream effects of lethal Ca^{2+} influx in neurons. To this effect, we are studying the novel cell cycle regulatory protein Speedy/RINGO due to its p53-dependent anti-apoptotic function which has previously been observed in U2OS osteosarcoma cells[12], calpain-induced degenerating primary neurons[13], ALS motor neurons[14] and astrocytes and microglia of spinal cord lesions[15].

A NOVEL CELL CYCLE REGULATOR, SPEEDY/RINGO, AS A STRONG CANDIDATE PROTEIN FOR NEUROPROTECTION IN SCI

The main function of Speedy/RINGO is the regulation of the cell cycle in mitotic cells. However, recent studies show that Speedy/RINGO also has an anti-apoptotic effect in DNA-damaged mitotic cells, allowing for their survival[12]. Speedy/RINGO has been shown to have a strong protective effect for mitotic cells exposed to extrinsic or intrinsic apoptotic factors such as UV irradiation. This anti-apoptotic function of Speedy/RINGO has also been utilized and confirmed in post-mitotic degenerating primary neurons[13] and in ALS motor neurons[14]. Speedy/RINGO performs this function by inhibiting caspase-3 activation and apoptosis in the presence of the gene regulatory protein p53[12,13]. Since p53-mediated apoptosis is inevitable for SCI patients, utilizing Speedy/RINGO's anti-apoptotic feature may turn the tide in the battle against SCI.

Speedy/RINGO protein structure and function

In eukaryotic cells, cell cycle progress is strictly controlled by cyclin-dependent kinases (CDKs) which are regulated by cyclins. Cyclins regulate CDK activity during different phases of the cell cycle by binding and phosphorylating them. Although cyclins are the key regulators of CDK activity, Speedy/RINGO, a novel cell cycle regulator, is shown to bind and regulate CDK activity in many eukaryotic cell types[71].

Speedy/RINGO was first identified in *Xenopus* oocytes as a meiotic cell cycle regulator accelerating G2/M progression during oocyte maturation[72].

Unlike cyclins, Speedy/RINGO binds and activates CDKs by a yet unelucidated phosphorylation-free mechanism[71].

There are at least three major branches in the Speedy/RINGO family (A, B and C), with a fourth branch (D) suspected. Speedy/RINGO A, the human homologue Spy1, is the most conserved and the most slowly evolving branch of Speedy/RINGO family. This is the branch used in our laboratory. Branch A is found in nearly all types of cells in fish, chickens, sea urchins and mammals. Expression levels are higher in testis tissue than in tissues such as brain, heart, lung, placenta, prostate, small intestine, *etc.*[71]. Since Speedy/RINGO is primarily a mitosis regulatory protein, it is not widely expressed in post-mitotic neurons.

Although the main function of Speedy/RINGO is cell cycle regulation, studies have attributed a p53-dependent anti-apoptotic function to Speedy/RINGO in DNA-damaged mitotic cells, resulting in those cells evading apoptosis and, thus, surviving[12].

Speedy/RINGO protein inhibits apoptosis and leads mitotic cells to become cancerous

When apoptotic insult occurs during a cell cycle, the resulting DNA damage triggers cell cycle arrest. This arrest, in turn, activates checkpoint responses to allow cells to repair the DNA damage[73]. Increased

expression and activation of p53 is the key event in these responses[74]. In the event that irreparable DNA damage occurs, cells activate their apoptotic machinery, ultimately leading to cell death.

Speedy/RINGO has been shown to prevent p53-dependent apoptosis which is normally induced in response to DNA damage in a mitotic human osteosarcoma cell line, U2OS[12]. The survival effect of Speedy/RINGO on mitotic cells was very strong and significant against a number of extrinsic or intrinsic apoptotic factors (e.g., carcinogenic-level UV irradiation). Speedy/RINGO helps cells evade apoptosis by inhibiting caspase-3 activation only in the presence of the gene regulatory protein p53[12,75].

Speedy/RINGO protein functions as an anti-apoptotic factor in degenerating post-mitotic neurons

Although there are intrinsic regulatory mechanisms for calcium influx into neurons, a number of insults such as glutamate neurotoxicity cause deregulation of calcium homeostasis by increasing intraneuronal calcium influx, as in SCI. This increase in calcium influx induces cystein proteases, including calpain, and results in pathologic calpain activation. Pathological activation of calpain is known to be one of the most important neurodegenerative factors triggering apoptosis, which it does by inducing p53 and activating caspase-3.

Calpain overactivation directly or indirectly induces p53 expression and drives neurons into apoptosis. Indirectly, overactivated calpain cleaves p35 protein into p25 and p10 fractions. Under normal conditions, p35 is the partner for non-mitotic neuron-specific kinase cdk5, forming a cdk5/p35 complex. This complex functions in important cellular events such as neuronal development and maturation[76-78]. When neuronal calpain is overactivated as a result of increased Ca^{2+} influx, however, p35 is cleaved by calpain into p25 and p10 fractions. Like p35, p25 can bind to cdk5, forming a cdk5/p25 complex. However, cdk5/p35 and cdk5/p25 complexes differ in both localization and function[79]. The cdk5/p25 complex has been shown to directly activate p53, with p53 acting as a substrate for cdk5[80]. Data indicate that the calcium-mediated calpain activation observed in SCI results in an increase in p53 expression and activation, leading to caspase-dependent apoptosis and the resulting degeneration of neurons.

Calpain overactivation leads to increased p53 expression and activation which, in turn, triggers caspase-mediated apoptosis of neurons[81]. Apparently, both direct and indirect calpain-induced apoptosis occur in a p53-dependent fashion.

In addition to its cell cycle regulatory function, Speedy/RINGO has also been shown to function in preventing apoptosis by inhibiting caspase-3 activation in a p53-dependent manner in mitotic U2OS cells.

Since Speedy/RINGO is primarily a cell cycle regulatory protein, it is highly expressed in mitotic cells compared to post-mitotic cells such as neurons[13]. One possible way to prevent p53 mediated apoptosis in neurons would be to transfect non-mitotic neurons with Speedy/RINGO.

With this in mind, our laboratory designed an *in vitro* experiment utilizing primary hippocampal neurons from post-natal (PN0) Sprague-Dawley rats. These neurons were transfected with Speedy/RINGO. After transfection, calpain was induced using calcium ionophore A23187, facilitating extracellular Ca^{2+} transport into the neurons[13]. Results of our study showed for the first time that Speedy/RINGO, a mitotic cell-specific protein, is protective against p53-mediated apoptosis in non-mitotic neurons. Calpain induction by A23187 was shown to drive neurons into apoptosis by increasing p53 expression and activating caspase-3, which is a typical characteristic of caspase-dependent apoptosis. However, overexpression of Speedy/RINGO in calpain-induced neurons prevented caspase-3 activation in a p53-dependent manner[13].

In another recent study, Speedy/RINGO expression levels were shown to substantially decreased in ALS motor neurons compared with wild-type controls[14]. As a result of decreased Speedy/RINGO expression,

reduction in cell viability and activation of the DNA damage response in SOD1 mutated cells was established. Conversely, increased Speedy/RINGO expression enhanced cell viability and prevented the DNA damage response in SOD1 mutated cells. These findings indicate that Speedy/RINGO confers a similar protective benefit for both ALS motor neurons and degenerating primary hippocampal neurons. It is therefore possible that Speedy/RINGO may someday play a role in treating neurodegenerative conditions such as SCI.

Even though the exact mechanism for the protective role of Speedy/RINGO in p53-mediated apoptosis requires further analysis, the effects are not due to the direct inhibition of calpain activity or p53 induction, as calpain-mediated p53 induction was maintained even in the presence of Speedy/RINGO[13]. The mechanism may instead occur downstream from the p53 activation.

In addition to the aforementioned studies on degenerating primary neurons and ALS motor neurons, results of carcinogenic[16-18] and astrocytic[15,63] proliferation studies point out that, among the downstream targets of p53, ERK/MAPK and PI3K/AKT, signaling cascades are the most potent in terms of clarifying the p53-dependent anti-apoptotic mechanism of Speedy/RINGO.

p53 regulation of ERK/MAPK and PI3K/AKT pathways in apoptosis

p53 is a tumor suppressive transcription factor that inhibits tumorigenesis under genotoxic conditions by regulating gene expression. p53 induces anti-tumorigenesis mechanisms - including cell cycle arrest, senescence and apoptosis - according to cellular conditions, type and intensity of stress signals[82-84]. Similarly, the regulation of apoptosis, cell cycle arrest and proliferation by the ERK/MAPK pathway differs depending on the type of stress signals received and the context of the cell[85,86].

Under stress conditions, different signaling pathways can be triggered. For example, p38[87-89] and ERK/MAPK[90-93] activate p53 and induce its transcriptional activity. The reverse also occurs: p53 may activate ERK/MAPK signaling[85,94,95] by inducing tyrosine kinase receptor DDR1[96]. In addition, p53 is also capable of suppressing the ERK/MAPK pathway via activation of various phosphatases. These phosphatases then dephosphorylate ERK and inhibit its anti-apoptotic function, leading to p53-dependent apoptosis[97,98]. It is evident that interaction between p53 and ERK/MAPK signaling differs depending on cellular context and the type of stress stimulus.

Another p53-related signaling pathway is PI3K/AKT. P13K/AKT has primarily been implicated in promoting cell survival in response to extracellular signals[99-101] that regulate intracellular signaling cascade by activating transmembrane receptors. This activation recruits PI3K isoforms to the plasma membrane which, in turn, results in phosphorylation and activation of AKT. Activated AKT has a number of cell survival stimulating effects through phosphorylation and inhibition of pro-apoptotic genes.

There is growing body of evidence indicating a negative regulatory function for p53 on cell survival in healthy cells. In this mechanism, p53 binds to the promoter site of PTEN (a phosphatase and tensin homolog deleted on chromosome ten)[102,103]. Active PTEN dephosphorylates the 3' phosphate of phosphoinositol triphosphate which results in the inhibition of the PI3K/AKT pathway. This inhibition subsequently causes a reduction in phospho-AKT levels, which has been shown to cause G1 arrest in glioblastoma cells, but trigger apoptosis in carcinomas[104,105]. In addition, p53 induction causes significant inhibition of PI3K/AKT in EB1 colon cancer cells which strongly implies that inhibition of PI3K/AKT is essential for p53-dependent apoptosis[94].

These two pathways have been shown to be equally important for neuronal survival and regeneration after nerve injury. Researchers found that 7 days after axotomy, ERK/MAPK and PI3K/AKT signaling activity was increased, causing reduced apoptosis[10]. Thus, it is reasonable to assume that the inhibitory effect of increased p53 levels on the ERK/MAPK and PI3K/AKT pathways may be the major death signal for neurons in SCI.

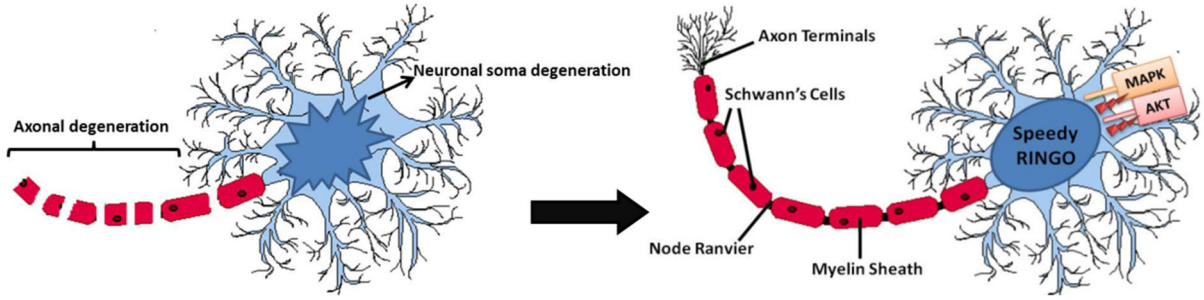

Figure 3. A schematic diagram for the proposed mechanism of anti-apoptotic action of Speedy/rapid inducer of G2/M progression in oocytes (Speedy/RINGO) on mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase and phosphoinositide 3-kinase/protein kinase B (AKT) signaling cascades in degenerating neurons

Evidently, ERK/MAPK and PI3K/AKT signaling is very important for cell survival. Depending on the cellular context, cell type, and internal/external stimuli, however, p53 may act as a strong anti-apoptotic or pro-apoptotic regulator of both pathways.

Potential regulatory function of Speedy/RINGO protein on ERK/MAPK and PI3K/AKT pathways as an inhibitor of apoptosis

Previous investigations by our lab indicate that Speedy/RINGO protects neurons against calpain-mediated p53-dependent apoptosis without decreasing p53 levels. This finding strongly implies that the anti-apoptotic effect of Speedy/RINGO is downstream of p53 activation, not directly on calpain or p53 itself. As explained above, the most remarkable downstream targets of p53, in terms of generating an apoptotic effect on neurons, are ERK/MAPK and PI3K/AKT pathways. Therefore, in degenerating neurons, Speedy/RINGO may use its ability to regulate ERK/MAPK and PI3K/AKT pathways to reverse the apoptosis-triggering effect of p53 induction on these pathways [Figure 3].

Furthermore, cancer studies show promising evidence of direct or indirect interaction of Speedy/RINGO with ERK/MAP and PI3K/AKT pathways. Several studies on tumorigenesis in breast tissue show that ERK/MAPK pathway overactivation results in Speedy/RINGO overexpression. As a result of inhibition of enzyme MEK1, a member of the MAPK pathway, Speedy/RINGO expression is shown to decrease[16].

In addition, studies using testis tissue revealed that Speedy/RINGO overexpression causes an increase in Cyclin A2-cdk2 expression[17]. Mouse embryonic stem cell studies indicated that Cyclin A2-cdk2 complex has an important role in AKT hyperphosphorylation, which is a highly effective apoptosis-prevention factor in many types of cancer[18].

On the other hand, studies on glial scar formation through astrocytic gliosis, a hallmark of secondary injury that inhibits axonal regeneration[62], show that increased Speedy/RINGO expression is one of the major events responsible for astrocytic proliferation[15]. Since glial scar formation is a very potent inhibitor of SCI recovery, researchers are studying how to therapeutically prevent astrocytic gliosis. In one of these studies, researchers focused on the mitogenic ERK/MAPK signaling cascade in astrocytic proliferation[63]. Their experimental results showed that ERK/MAPK phosphorylation/activation was increased in proliferating astrocytes of SCI lesions. Treating these cells with the *IFN-β* gene significantly reduced ERK/MAPK activity and the astrocytic proliferation rate.

Findings from these studies on astrocytic gliosis imply that there may be an interaction between upregulation of Speedy/RINGO and ERK/MAPK hyper-phosphorylation leading to glial scar formation. Taking these data into consideration, it is reasonable to think that Speedy/RINGO may have reversed the inhibitory effect

of p53 on ERK/MAPK and PI3K/AKT pathways, and thus prevented apoptosis in degenerating hippocampal and ALS motor neurons. Hence, it is worthwhile to further explore p53-dependent anti-apoptotic regulatory function of Speedy/RINGO on these pathways.

Our laboratory is currently investigating the function of Speedy/RINGO on the ERK/MAPK and PI3K/AKT pathways using undifferentiated p53- and Speedy/RINGO-expressing neuronal-like neuroblastoma cells. Preliminary data give remarkable clues indicating that Speedy/RINGO plays an essential role on the regulation of ERK/MAPK and PI3K/AKT signaling pathways that directly affect the apoptotic state and survival rate of neuroblastoma cells. More precisely, silencing of the Speedy/RINGO gene significantly alters expression levels and phosphorylation states of certain members of the ERK/MAPK and PI3K/AKT pathways. This, in turn, leads to apoptotic death of neuroblastoma cells, likely due to the absence of Speedy/RINGO's regulatory function on these two pathways.

CONCLUSION

SCI is a critical clinical issue whose ongoing destructive path affects patients for life. It is one of the most important causes of disability and mortality around the world[106,107].

It has long been known that glutamate-induced Ca^{2+} influx through glutamate receptors, known as glutamate excitotoxicity, is indispensable for SCI. This influx ultimately causes the p53-mediated apoptotic death of neurons. It is most likely that p53 exerts its apoptotic function on the members of ERK/MAPK and PI3K/AKT signaling cascades[11].

The goal of our laboratory is to elucidate and prevent the pro-apoptotic intracellular Ca^{2+} deregulation in neurons. We are therefore optimistic about Speedy/RINGO, a novel cell cycle regulatory protein proven to have a p53-dependent anti-apoptotic function in different cell types, including U2OS osteosarcoma cells[12] as well as calpain-induced degenerating primary hippocampal neurons[13]. In addition, Speedy/RINGO expression levels were shown to be substantially decreased in ALS motor neurons compared with wild-type controls[14]. By contrast, increased Speedy/RINGO expression enhanced cell viability and prevented the DNA damage response in ALS motor neurons. These findings indicate that Speedy/RINGO plays a protective role in both ALS motor neurons and in degenerating primary hippocampal neurons. This implies a potential therapeutic role for oncogenic proteins in neurodegenerative conditions such as SCI.

Although the mechanism of Speedy/RINGO's anti-apoptotic function in degenerating neurons is not yet known, Speedy/RINGO most probably exhibits its protective function on downstream targets of p53, rather than on p53 levels directly[13]. ERK/MAPK and PI3K/AKT survival pathways are the most important downstream targets of p53 in cases of neurodegeneration, and Speedy/RINGO has been shown to act on both pathways. This evidence has come from both cancer studies[16-18] and studies on glial scar formation in SCI[63]. Exploring this interaction and revealing the possible regulatory function of Speedy/RINGO on these pathways may help to someday reverse the p53-induced apoptotic effect observed in SCI.

Overexpressing Speedy/RINGO in *in vitro* and *in vivo* SCI models and exploring its effects will provide important insights about underlying molecular mechanisms of secondary injury. One goal is to study the abnormal regulation of ERK/MAPK and PI3K/AKT pathways by transcription factor p53, one of the primary initiators of secondary injury.

As described elsewhere in this paper, we believe that Speedy/RINGO is likely to exhibit anti-apoptotic activity in the neuron and glia cells of areas affected by SCI, making this protein a strong potential candidate for therapeutic treatment of SCI patients. In order to confirm the presumed anti-apoptotic function of Speedy/RINGO in SCI, further studies should be performed with both *in vivo* and *in vitro* SCI models.

It is important to remember that Speedy/RINGO's anti-apoptotic function in neurons and astrocytes may be advantageous or disadvantageous depending on the cell type and function in SCI. Speedy/RINGO's anti-apoptotic function is desirable in neurons, but undesirable for astrocytes, since it causes glial scar formation and thereby prevents axonal regeneration. In developing an effective SCI recovery regimen, Speedy/RINGO will need to be regulated differentially depending on the therapeutic target.

The pathophysiology of SCI has not yet been fully elucidated, making it difficult to develop effective treatment methods. Overcoming this problem will require collaboration between basic and clinical researchers. Basic research must take place to gain a clear understanding of the basic neuronal and glial mechanisms seen in SCI before these mechanisms can be linked to clinical SCI symptoms and recovery. Versatile molecules like Speedy/RINGO are an excellent tool for increasing our understanding of the molecular mechanisms of SCI with the goal of developing effective treatment strategies.

DECLARATIONS

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Authors' contributions

Made substantial contributions to the conception and design of the study, performed data analysis and interpretation, and wrote the paper: Yildiz A

Performed data acquisition and provided technical support: Kaya Y

Availability of data and materials

Not applicable.

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Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Neuroimmunology and Neuroinflammation

Letter to Editor

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The involvement of anti-neurofascin 155 antibodies in central and peripheral demyelinating diseases

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The immunological aspects of autoantibodies directed against paranodal and nodal proteins form a prominent field of research. Contactin-1 and contactin-1 associated protein, gliomedin and neurofascin (NF) are Ranvier node-related proteins[1,2]. Antibodies against these proteins have been identified in chronic demyelinating conditions, such as multiple sclerosis (MS) and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP); and in acute demyelinating diseases such as Guillain-Barre syndrome[3,4]. Myelin and the paranodal axoglial junctions flanking the nodes of Ranvier are not merely passive transmitters of electric signals: instead, they have essential roles in maintaining the structural integrity of myelin-axolemmal interactions, bidirectional signaling and regulation of ion channels[5].

Here, we will focus on NF155, a major constituent of the Ranvier paranodal junction[6]. NF155 belongs to the L1 family of transmembrane cell adhesion molecules [Figure 1], typically presenting an evolutionary well-conserved protein domain structure with six immunoglobulin and five fibronectin type III domains, a transmembrane domain and a cytoplasmic domain with 113 amino acids[7,8]. It is expressed at paranodes by terminal loops of myelin[9] and its main function is to stabilize these paranodes. Other isoforms of NFs (NF166, NF180 and NF186) are equally involved in dynamic mechanisms of synaptic stabilization, neural outgrowth and clustering of sodium channels[7].

Patients with CIDP who present antibodies against NF155 have a particular disease course that involves onset at younger age, tremors, ataxia, dysarthria, nystagmus, extremely high protein levels in spinal fluid, symmetrical spinal root and plexus hypertrophy[10,11]. In addition, over 70% of these patients can have abnormal visual-evoked potentials during the course of CIDP[10]. CIDP patients with anti-NF155 antibodies

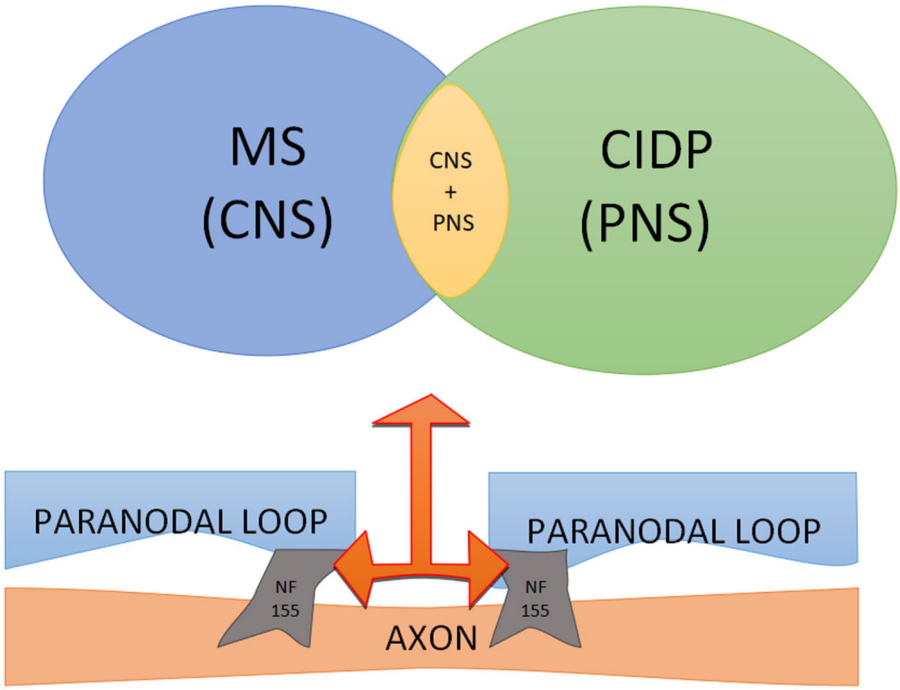

Figure 1. Neurofascin 155 (NF155) is a transmembrane adhesion molecule located in the paranodal region in the central (CNS) and peripheral nervous system (PNS), justifying the various clinical presentations. CIDP: chronic inflammatory demyelinating polyradiculoneuropathy; MS: multiple sclerosis

may present demyelinating lesions in the central nervous system, along with a remarkably poor response to intravenous courses of immunoglobulin[12]. Overall, patients with CIDP with overlapping central and peripheral demyelination associated with presence of anti-NF155 antibodies exhibit greater disability.

In addition to the peculiarities of peripheral demyelination associated with presence of NF155 antibodies, diseases of the central nervous system also show particular features when anti-NF155 antibodies are identified. Ten percent of patients fulfilling the diagnostic criteria for MS[13], may have antibodies against NF155[14]. Cases of neurological disease with onset typical of MS that were subsequently diagnosed as CIDP have been described[15-17]. Interestingly, presence of antibodies against NF155 is significantly more frequent in patients with primary progressive forms of MS than in those with relapsing disease[18].

Patients who develop antibodies against NF155 may ultimately present a different form of demyelinating disease with both central and peripheral involvement. There are often signs of cerebellar, spinal root and plexus involvement, optic nerve demyelination and high levels of protein in the spinal fluid in these cases. Anti-NF155 antibodies may form a biomarker for a particular form of demyelinating neurological disease. NF155 does not activate a complement[9]. Therefore, at least in theory, patients with anti-NF155 antibodies might respond well to therapies using ocrelizumab and rituximab. "Anti-NF155 demyelination" may be a different disease with peculiar presentation and therapeutic management.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception of the study, as well as provided administrative, technical, and material support: Goncalves MVM, Fragoso YD

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Both authors declared that there are no conflicts of interest.

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Neuroimmunology and Neuroinflammation

Case Report

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Bartonella henselae neuroretinitis in a patient without cat scratch

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Abstract

Neuroretinitis is characterized by optic disc swelling with macular star, and affects 1%-2% of the patients with Bartonella henselae infection. This condition needs to be considered in the differential diagnosis of unilateral optic neuropathy in young adults. A 44-year-old woman presented with a progressive visual loss in right eye that was described as a central scotoma with altered color perception. Medical history was negative except for Hashimoto's thyroiditis. The examination evidenced a relative afferent pupillary defect in right eye and marked papillary oedema involving the macular region. Brain and orbits magnetic resonance imaging were normal, and fundoscopy showed star-shaped hard exudates. Autoimmune and infective screening revealed IgM and IgG antibodies against Bartonella henselae, suggesting for recent cat-scratch disease. She was treated with high-dose intravenous steroids and doxycycline. One month later she fully recovered, and she had no relapses. We diagnosed a Bartonella henselae neuroretinitis (finally the patient recalled she had stroked stray cats, not being scratched). In conclusion time course and absence of pain differentiate neuroretinitis from other optic neuropathies. Fundoscopic image of macular star is a clue for diagnosis, and visual recovery is usually excellent.

Keywords: Neuroretinitis, optic neuropathy, bartonella henselae, cat-scratch disease

INTRODUCTION

Neuroretinitis is a rare inflammatory optic neuropathy with direct involvement or autoimmune activation against the optic nerve. The inflammation of the optic disc vasculature causes exudation of fluid into the

peripapillary retina, taking a characteristic macular star shape. Usual presentation is monocular central visual loss with discromatopsia and papillitis on funduscopic evaluation[1].

The differential diagnosis includes inflammatory optic neuropathies such as sarcoidosis, systemic lupus erythematosus, Behçet syndrome, inflammatory bowel disease, and paraneoplastic syndromes[2]. Other causes are infections: herpetic viruses, HIV, West Nile virus and rarely flu or mumps, toxoplasmosis and toxocariasis, syphilis, Lyme disease, tuberculosis, rickettsiosis, and cat-scratch disease (CSD)[3]. CSD is related to Bartonella species and transmitted through skin lesions after contact with infected animals[4]. A contact with cats is reported in 90% of CSD, and scratches or bites in 60% of the patients[5].

Typical CSD presentation is a small papule at the inoculation site, followed by regional and systemic lymphadenopathy and fever. Ocular involvement occurs in 5%-10% of the patients, mostly presenting Parinaud syndrome (fever, regional lymphadenopathy, and follicular conjunctivitis). Neuroretinitis occurs in 1%-2% of the patients[6], and other neurological manifestations are meningoencephalitis, myelitis, and acute polyneuropathies[7,8].

Neuroretinitis is characterized by subretinal fluid, retinal thickening, and hyper-reflective exudates in the outer plexiform layer[9] at optical coherence tomography. Fluorescein angiography shows fluid leakage, due to increased vessel permeability.

Currently, antibiotic therapy for Bartonella complicated infection with doxycycline and rifampin is recommended, either alone or in combination with corticosteroids. Habot-Wilner *et al.*[10] showed a significant visual acuity improvement in those patients treated with antibiotics and corticosteroids if compared to those receiving only antibiotics. In the majority of cases no further progression of ocular inflammation was observed after therapy, and visual acuity recovered after 1-4 weeks[11].

CASE REPORT

A 44-year-old woman presented to the Emergency Department with a progressive visual loss and altered color perception in the right eye, started one week before with no ocular pain, headache, dizziness, nausea or vomiting.

Her past medical history included Hashimoto's disease, treated with hormonal replacement therapy in the last 10 years. Particularly, the patient denied previous neurological or visual deficit. Her familial history was negative for neurological, vascular or autoimmune disease, and both her parents suffered from colorectal cancer. About two weeks before, she had fever and was treated with amoxicillin and clavulanic acid (875/125 mg b.i.d) for otitis. She reported a mild indolent cervical lymphadenopathy occurred 5-7 days after fever, and denied any cutaneous manifestation. Routine blood examinations were normal, including reactive C protein (0.05 mg/dL, N.V. < 1.00 mg/dL). She had neither fever, night sweats, weight loss or other constitutional symptoms nor lymphnod involvement at the examination.

Ophthalmological findings

Ophthalmological examination revealed severely compromised visual acuity (1/10 in right eye *vs.* 8/10 in left eye), and computerized visual field testing evidenced a temporal loss in right eye. Ocular tonometry was normal. At the slit lamp the anterior segment was regular with normal pupillary reflexes, but there was a right relative afferent pupillary defect. On that side, fundoscopy showed marked papillary oedema involving macular region, hard retinal exudates with appearance of macular star, increased retinal vessel tortuosity and arteriovenous nicking. Left eye fundoscopy showed milder signs of inflammation with moderate edema. A fluorescein angiography confirmed the presence of retinal edema with leakage and optic coherence tomography (OCT) showed retinal thickening with initial exudates within the outer plexiform layer [Figure 1].

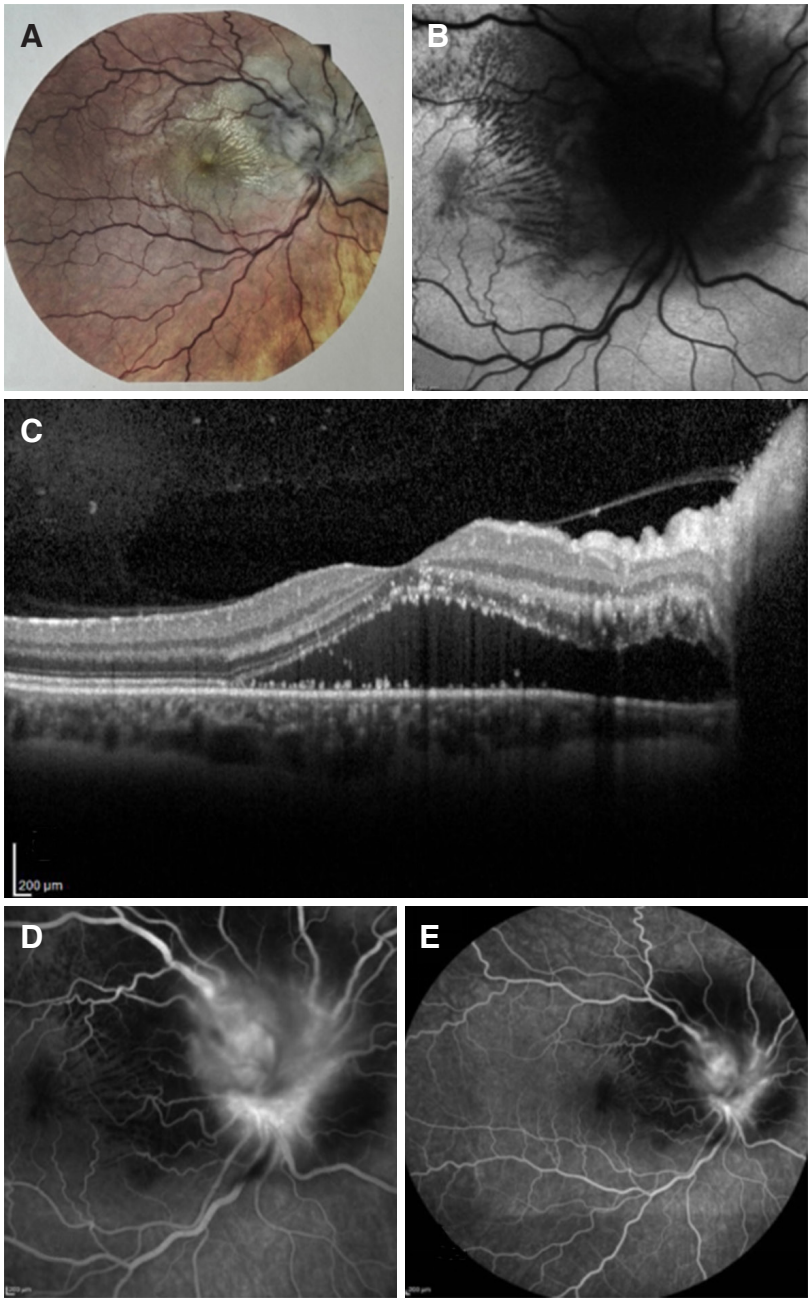

Figure 1. Instrumental imaging of right eye at symptoms presentation. A: colorful fundus photography; B: funduscopic scan with retinal exudates organizing in macular star appearance; C: OCT of right eye showing retinoic edema with peripapillary fluid; D: fluorescein angiography showing leakage of vessels from inflamed right optic disc; E: indocyanine green angiography

Neurological findings

A brain tomography was immediately performed to exclude an intracranial mass and one week later she had a magnetic resonance imaging (MRI) of brain and optic nerves, that resulted normal. Visual evoked potentials (VEP) showed a severe right delay of conduction (P100 latency was 123 ms) [Figure 2].

Since the ophthalmological pattern was highly suggestive for an inflammatory neuroretinitis, a full blood analysis for autoimmune and infective causes was performed. Erythrocyte sedimentation rate was mildly elevated (38 mm/h N.V. 0-15), and serologic test for herpetic viruses including cytomegalovirus and Epstein Barr showed immunization from previous infections. Serology for toxoplasmosis, syphilis, Lyme disease and

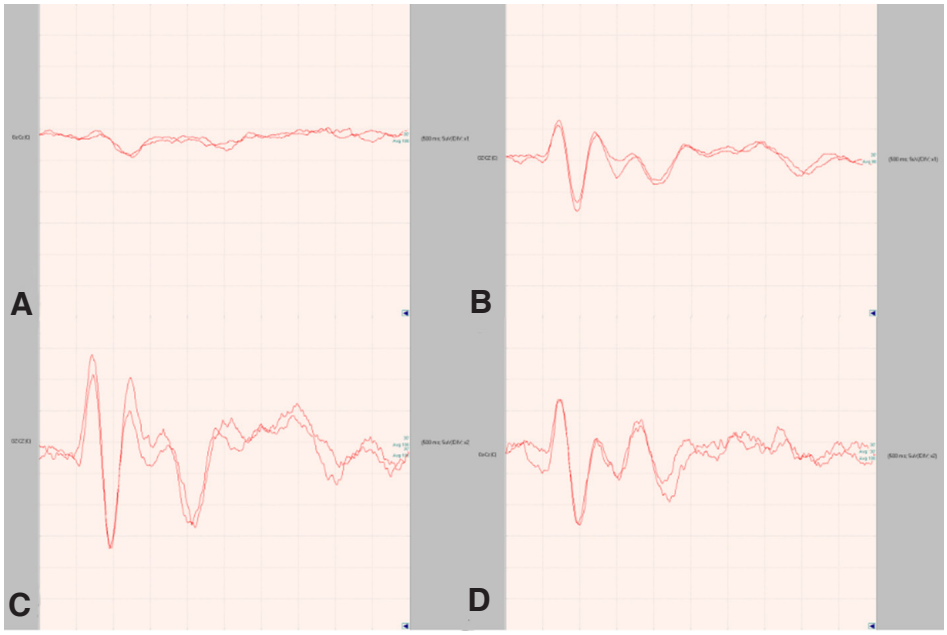

Figure 2. Visual evoked potential. A: right eye VEP at symptoms presentation (P100 latency 123 ms); B: left eye (P100 latency 86 ms); C: right eye VEP after one month; D: left eye VEP after one month

Toxocara canis were negative. Serology for Bartonella infections showed previous infection from Bartonella quintana (IgM antibody titer assay was not performed because of negative immunofluorescence, IgG titer was 1:128 that was at upper limit for positivity in our laboratory) and recent infection from Bartonella henselae with both IgM and IgG (IgM titer was 1:40 N.V. < 20, IgG was 1: 512 N.V. < 64).

Treatment and follow up

A high dose intravenous steroid regimen (methylprednisolone 1000 mg/die for 3 days) was started, followed by oral steroid tapering for one month. According to an infectiology consultation, antibiotic therapy with doxycycline 100 mg b.i.d for two weeks was started.

One month later the patient fully recovered the visual acuity (10/10 in both eyes). OCT and fluorescein angiography showed marked reduction of optic disk swelling and disappearance of retinal exudates. VEP returned to normal [Figure 3]. Six months later, the patient doesn't complain any visual deficit, as confirmed at ophthal mologic instrumental evaluation. Neurologic examination is normal.

At that time, the patient was asked about previous contact with potentially infected animals, as she didn't own domestic animals. She recalled having handled stray cats during previous summer holidays (about one month before visual symptoms started), but she denied having been scratched.

DISCUSSION

We presented a young woman with a unilateral loss of vision caused by a CSD isolated neuroretinitis with mild constitutional symptoms and no history of scratch. Neurological examination, brain MRI and VEP did not give a clue for this diagnosis, whereas ophthalmological results suggested the possibility of an infection. The prompt treatment with antibiotics and steroids brought a complete resolution of the vision deficit.

This case represents a clinical rarity, first because of the diagnosis of CSD without cat scratch. Notably, CSD diagnosis requires the presence of at least 3 of 4 criteria including: a history of traumatic cat exposure, a positive test for CSD antibody, regional lymphadenopathies, and serum analysis excluding concurrent

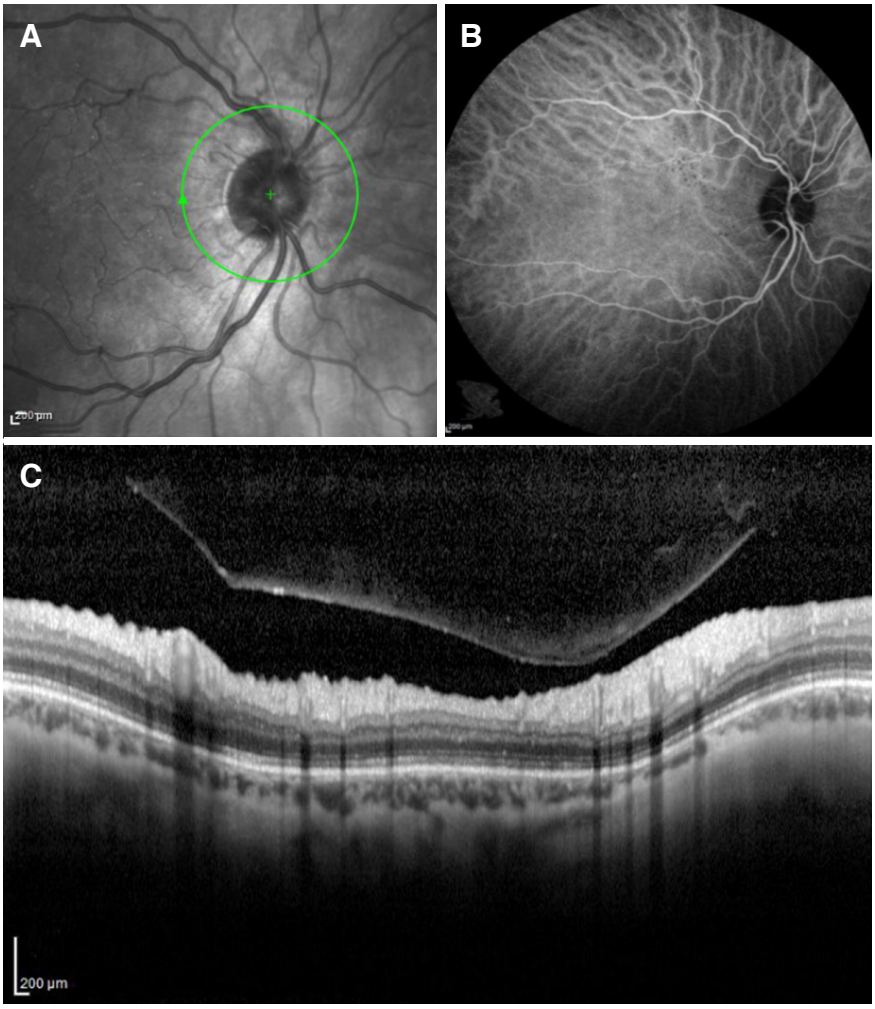

Figure 3. Instrumental imaging of right eye at one month, after treatment. A: funduscopic scan no retinal exudates; B: fluorescein angiography showing resolution of leakage; C: OCT of right eye showing resolution of edema

causes[12,13]. Only 2 criteria were fulfilled in our case. Celiker *et al.*[14] reported three cases of isolated CSD neuroretinitis without contact with cats, and only 26% of patients recalled a history of cat scratch or bite in a case series of 19 patients with ocular bartonellosis[15]. This could be related to animal transmission by feces, that host Bartonella for several days and could infect man through an open wound or mucosae. Moreover, direct ocular infection is possible through the conjunctiva by eye rubbing, contaminated cosmetics or contact lenses[16].

Our diagnosis of CSD neuroretinitis relied on the typical hallmarks for this condition: absence of pain, progressive course of visual loss in few days, initial bilateral involvement, and no altitudinal visual deficit. Time interval of 6 weeks between presumed exposition to infected animal and initial symptoms is highly suggestive[17]. Instrumental findings were also indicative for neuroretinitis: i.e., OCT scan with subretinal fluid (as happens at very early stage with direct ophthalmoscopy or fluorescein angiography still normal)[18], and the presence of macular star (pathognomonic of neuroretinitis). We also performed fluorescein angiography to exclude retinal arteriolar occlusions, a complication of ocular bartonellosis associated with very poor visual recovery[19].

Serological analysis confirmed our suspicion with both IgM and IgG for Bartonella henselae. This immunofluorescence assay has good specificity for IgM (87%-96%) and IgG (69%) although sensitivity is

greater for IgG (88%-98%) compared to IgM (50%-62%)[20]. False positives due to cross-reaction are highly improbable being about 2%-5% for Epstein Barr/Cytomegalovirus, and 20%-50% for Coxiella burnetii/non-henselae bartonellosis (very rare in our country)[21]. IgM presence allows to date back infection to the previous 3 months, whereas IgG remains detectable in the 25% of patients one year later.

Finally, we observed full clinical and instrumental recovery after specific antibiotic and steroid therapy. In a retrospective study on 74 CSD patients with ocular disease, 44 were treated with antibiotics alone, 17 with antibiotics and steroids (both intravenous high dose metil-prednisolone or oral prednisone), 2 with steroids alone and 11 patients didn't receive any treatment. Visual acuity improvement occurred in 50% of the patients treated with antibiotics alone, and in 87.5% treated with both antibiotics and corticosteroids[10].

The combination of doxycycline and steroids shorten the course of disease and accelerate visual recovery compared to natural history of illness in our case[22]. No side effects were reported.

CSD neuroretinitis is a rare clinical condition that must be considered in young patient with rapidly progressive unilateral visual loss. It requires a prompt neuro-ophthalmological evaluation in order to establish the correct diagnosis and start an early specific treatment.

DECLARATIONS

Authors' contributions

Made substantial contributions to realization of this case report: Montabone C, Vecchio D, Vujosevic S
Reviewed the case as well as the manuscript, providing substantial contribution to discussion and conclusion: De Cillà S, Cantello R

Availability of data and materials

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The patient gave written informed consent.

Consent for publication

The patient gave written informed consent for publication. All clinical information submitted is anonymous and the patient is not recognizable from the iconographic material.

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Review

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Current immunotherapies for multiple sclerosis and neuromyelitis optica spectrum disorders: the similarities and differences

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Abstract

Multiple sclerosis (MS) and neuromyelitis optica spectrum disorders (NMOSD) are autoimmune demyelinating diseases of the central nervous system. Neuromyelitis optica was considered a variant of MS until the discovery of NMO-IgG in 2004, which changed our understanding of the pathophysiology of NMOSD. This review focuses on the similarities and differences in the immune treatments of MS and NMOSD.

Keywords: Multiple sclerosis, neuromyelitis optica spectrum disorders, pathophysiology, treatment, disease-modifying drugs

INTRODUCTION

Multiple sclerosis (MS) and neuromyelitis optica spectrum disorders (NMOSD) are chronic immune-mediated demyelinating diseases of the central nervous system (CNS) with distinct immunological and pathological features[1-3]. MS is common in Western countries (incidence, > 100 per 100,000 in the European and North American populations), where it is the most common non-traumatic disabling disease among young people. However, MS is not common in Asia (incidence, 0-20 per 100,000 in Asian populations)[4]. Interestingly, the farther away one goes from the equator, the higher is the prevalence of MS[5]. MS generally progresses from a period of relapses and remissions to progressive disability. The pathogenetic mechanism

underlying MS is an autoimmune reaction to myelin or oligodendrocytes, but no MS-specific autoantigen has been identified.

NMO/NMOSD typically manifest as optic neuritis and longitudinally extensive transverse myelitis, and can lead to severe disability. The prevalence of NMOSD rarely exceeds 5/100,000, and is comparatively similar globally[4]. In 2004, Lennon *et al.*[6] discovered NMO autoantibodies, which clearly differentiated NMO from MS. Up to 80% of NMO patients test positive for antibodies against aquaporin4 (AQP4), which is a water channel protein found in many organs of the body[7]. In the CNS, AQP4 is expressed in a perivascular distribution on astrocytic foot processes[6]. The distinctive immunopathology of NMO lesions supports a central role for AQP4-IgG in the pathogenesis of this disease. AQP4-IgG damages the blood-brain barrier (BBB) through complement-dependent astrocytic damage. AQP4-IgG-positive NMOSD is not a classic demyelinating disease as MS, marked by secondary demyelination due to astrocyte loss[8,9]. In addition to the optic nerve and spinal cord, areas of high AQP4 expression around the ventricles are often involved, such as the area postrema of the medulla oblongata, thalamus, peripheral area of the third and fourth ventricles, corpus callosum, and white matter of the cerebral hemisphere. The high specificity of AQP4-IgG extends the study of NMO and its related diseases. Previously, the diagnostic criteria for NMO required optic nerve and spinal cord involvement. In 2007, Wingerchuk proposed the concept of NMOSD[10]. In 2015, the International Panel for NMO Diagnosis removed the separate definition of NMO and integrated NMO into the broader term of NMOSD[11]. NMOSD are a class of antigen-antibody-mediated CNS inflammatory demyelinating diseases that are primarily mediated by humoral immunity, with or without AQP4 positivity[12].

PATHOGENESIS OF MS AND NMOSD

MS is considered a classic autoimmune disease mediated by autoreactive T-lymphocytes, specifically CD4+ T-helper (Th)1 cells and Th17 cells. Th1 cells produce interferon (IFN)-γ, while Th17 cells are a T-cell subgroup producing IFN-γ and interleukin (IL)-17[13,14]. Activated T-cells can express a variety of adhesion molecules that combine with receptors on the vessel wall. Furthermore, vascular endothelial cells express selectins that bind to T-cells, and chemokines can induce T-cells to enter the CNS. Additionally, T-cells secrete matrix metalloproteinases that degrade the collagen component of blood-vessel walls, destroying the BBB and facilitating the entry of T- and B-lymphocytes and monocytes into the brain. In the CNS, T-cells secrete inflammatory cytokines and chemokines, which cause the activation of other inflammatory cells, resulting in a series of complex cascades of immune responses that finally lead to damage to the myelin sheath and even axons[13-16]. IL-4 stimulates the differentiation of CD4+ T-cells into anti-inflammatory Th2 cells and inhibits Th1 and Th17 cells. IL-2 and transforming growth factor (TGF)-β stimulate the production of regulatory T cells, which can inhibit Th17, Th1, and CD8+ T-cells in the CNS[17]. Th1 cells can recognize major histocompatibility complex (MHC) class II molecules, cross the BBB, and induce CNS autoimmunity. And Th17 lymphocytes are capable of crossing the BBB, and their secretion damages the BBB and promotes the entry of other inflammatory cells into the CNS[18]. In recent years, it has been found that B-cells also play an important role in the pathogenesis of MS. In most MS patients, oligoclonal bands and B-cell clonal proliferation occur in the CSF, and B-cell proliferation and germinal-center formation may occur in the meningeal follicles[19].

NMOSD are humoral immune-mediated autoimmune diseases. B-lymphocytes secrete specific antibodies that bind to complement, then deposit and destroy AQP4, which is expressed on the surface of astrocytes. However, the role of B-cells in the pathogenesis of NMOSD may not be limited to the production of AQP4-IgG, and an imbalance between pro-inflammatory and anti-inflammatory B-cell functions may also be involved[20]. Other inflammatory cells such as macrophages, eosinophils, and neutrophils are then attracted towards the injured tissue and secrete inflammatory factors that cause myelin loss and axonal damage[21]. A study has shown that peripheral blood neutrophils show a primed phenotype in NMOSD[22]. In some

NMOSD patients, antibodies against myelin-oligodendrocyte-glycoprotein (MOG) rather than AQP4 antibodies are detectable. The clinical features of MOG-IgG-positive NMOSD are different from those of the classic AQP4-IgG-positive NMOSD, and the underlying pathogenesis of the two conditions may also be different. MOG is a glycoprotein localized on the myelin surface as well as in the cell bodies and processes of oligodendrocytes. The MOG antibody is a subtype of IgG1, which is effective in regulating complement-dependent cytotoxicity. MOG antibodies target myelin-forming oligodendrocytes, whereas AQP4 antibodies damage astrocytes leading to secondary demyelination[23,24]. In terms of clinical features, MOG-IgG-positive NMOSD tends to be monophasic, more common among men, and have a younger onset age and a better prognosis[25]. At present, it is unclear whether CNS demyelinating diseases mediated by MOG antibodies should be independent of MS and NMOSD[26,27]. However, according to the revised NMOSD diagnostic criteria in 2015, AQP4-IgG positive or negative diseases and MOG-IgG positive diseases can be classified as NMOSD[11]. AQP4 antibodies and MOG antibodies are mainly produced extrathecally and are therefore less frequently found in the CSF than in the serum. AQP4 antibodies can be detected in the CSF in only 70% of patients who are seropositive for AQP4 antibodies and in none of the patients who are seronegative for AQP4 antibodies[28,29]. Furthermore, the levels of AQP4 and MOG antibodies may vary during the course of the disease. However, AQP4 antibody titers do not seem to predict long-term disease duration, and the serum AQP4 antibody status does not predict immunotherapy response[30,31].

TREATMENT OF ACUTE ATTACKS

At present, there is no cure for MS and NMOSD. The primary goal of therapy in the acute phase is to alleviate symptoms, shorten the disease course, and prevent complications. Currently available treatments only act on the inflammatory components of the disease process, and no therapy that can directly reverse myelin loss or neuronal damage exists. As in other autoimmune diseases, the recommended management strategy for patients with MS or NMOSD during the acute phase includes intravenous methylprednisolone (IVMP) pulse therapy, plasma exchange (PE), and intravenous immunoglobulin (IVIG)[32,33]. The treatment of acute attacks shortens the duration of relapses and reduces symptoms, but does not have long-term neuroprotective effects[34,35]. IVMP is considered the standard treatment for acute attacks[36,37]. Mainly in patients with contraindications to IVMP or disease that is refractory to IVMP, PE and IVIG are alternative therapies. NMOSD lesions are associated with IgG, IgM, and complement deposition; all of these are targeted by PE, which has a good therapeutic effect in NMOSD[38]. Immunoadsorption (IA) can remove immunoglobulins from the circulation, and is an alternative treatment for acute attacks[39,40]. For severe attacks, PE and IA can be used as initial therapies[41]. IVIG is a safe and well-tolerated immunotherapy that could also be used as a treatment alternative for MS and NMOSD[42,43]. However, this recommendation is based mostly on clinical experience, because of a lack of trials on IVIG monotherapy in the treatment of acute attacks. Furthermore, IVIG probably confers no advantage over IVMP and PE[44]. For MS, IVIG is usually only used for patients with contraindications to IVMP and PE, as the efficacy of IVIG is uncertain. NMOSD are humoral-mediated diseases, and therefore, therapeutic agents that inhibit B-cells or antibody production may be effective[45]. IVIG can reduce anti-AQP4 levels[46]; however, the efficacy of IVIG for acute attacks still needs to be proven.

Corticosteroids are an important therapy in the acute phase of relapse. High-dose methylprednisolone (0.5-1.0 g intravenously for 3-5 days) is recommended in the acute phase. The mechanisms of action of IVMP include dampening the inflammatory cytokine cascade, inhibiting the activation of T-cells, decreasing the expression of MHC-II molecules on antigen-presenting cells and the entry of immune cells into the CNS, and facilitating the apoptosis of activated immune cells[47]. Some studies have shown that oral methylprednisolone is no worse than IVMP in terms of the clinical and radiological outcomes of MS relapses[48-51]. The European Federation of Neurological Societies guidelines recommend an oral methylprednisolone dose of at least 0.5 g/day for 5 days (cumulative dose, 2.5 g)[52]. Several studies have

shown the safety of stopping a short course of high-dose corticosteroids without a tapering regimen[53,54]. In addition, one study showed that in MS, IVMP combined with low-dose oral hormones did not improve disability progression compared with IVMP alone[55]. However, low-dose oral corticosteroids may help prevent relapses in NMOSD[56]. In some patients with NMOSD, a rebound effect may occur if corticosteroids are stopped quickly. A study of 59 patients with relapsing MOG antibody-associated demyelination showed that most cases of relapse occurred within 2 months of prednisolone cessation and in patients who had been administered daily doses of < 10 mg[57]. Therefore, an oral weaning course of prednisolone over 2-6 months and long-term maintenance with low-dose oral prednisolone is recommended[58,59]. Compared with MS, NMOSD relapse is often more severe and less responsive to IVMP[60,61]. A retrospective study showed that IVMP has a significant effect on acute relapses in both MS and NMOSD patients, but the effects in MS were slightly better than those in NMOSD based on the changes in the Expanded Disability Status Scale score before and after IVMP[62].

PE can remove circulating autoantibodies, macromolecular immune complexes, inflammatory cytokines, and other mediators. It can also affect lymphocyte proliferation and activation[63]. Common side effects of PE include hypocalcemia, bleeding, and infections. When remission is absent or insufficient, PE every other day is recommended, with removal of 1-1.5 plasma volumes each time (30-40 mL/kg). A total of 5-7 treatments are recommended. Studies have found that the exchanged molecules will drop to less than 20% of their initial level after 5 exchanges[64,65]. In addition, IA is a more selective method that eliminates certain proteins, such as antibodies, while retaining other plasma proteins. The effects of IA and PE are comparable in the treatment of MS- or NMOSD-relapses[66]. Patients with a suboptimal response to methylprednisolone and those who present with severe symptoms should be treated with PE/IA. Some results support the use of PE in severe relapses of MS unresponsive to corticosteroids[67]. PE and IA can clear AQP4-IgG and are effective therapies for NMOSD. The results of a retrospective cohort study suggest early use of PE/IA in NMOSD attacks[68]. And no superiority was shown for one of the 2 apheresis techniques[68]. PE/IA combined with IVMP is more effective than IVMP alone in NMOSD[69,70]. In addition to steroids, it is recommended that PE/IA be started as soon as possible[71,72]. A study showed that the early initiation of PE (≤ 5 days) is more beneficial than delayed PE for cases that are refractory to IVMP[71].

IVIG is another important therapy that can affect a variety of immunomodulatory and antigenic-recognition pathways, including humoral and cellular immunity. It interacts with various subsets of B- and T-cells, modulates cytokines, scavenges complement, and blocks idiotypic antibodies[73]. Patients should be given IVIG at a dose of 0.4 g/kg/day for 5 days[74]. In MS, studies have shown that IVIG combined with IVMP is not superior to IVMP alone[75,76]. Few studies have assessed the efficacy of IVIG monotherapy for MS relapses. IVIG has a good effect in other humoral immune-mediated neuroimmunological diseases. IVIG may affect certain steps of pathological processes in NMOSD. Clinical experience suggests that this therapy may be of benefit in NMOSD patients[44]. A retrospective study with a small sample size has shown the efficacy of IVIG treatment for NMOSD relapses[43]. Furthermore, it has been shown that regular IVIG could prevent relapses in both MS and NMOSD[77-80].

TREATMENTS IN THE REMISSION PERIOD

In most instances, the initial course of MS consists of relapses and remissions, known as relapsing-remitting MS (RRMS), with disability progression over the course of the disease. Most patients eventually enter a secondary phase of progressive disease, i.e., secondary progressive MS (SPMS). In a few patients, the initial course is progressive with no relapsing-remitting phase; that is known as primary progressive MS (PPMS)[81]. The relationship between disability progression and relapses in MS is not yet clear. Unlike MS, in NMOSD, disability is the result of cumulative inflammatory damage caused by acute attacks[58]. The purpose of treatment during the remission period is reducing the risk of relapse and disability progression. In both MS and NMOSD, treatment during remission should be started as soon as possible[58,59,82,83]. Therapeutic drugs

in the remission period include conventional immunosuppressants and some new immunomodulators as well as biological agents. The latest guidelines in the United States and Europe recommend disease-modifying drugs (DMDs) to regulate MS[82,83]. Most recommendations for NMOSD are still based on expert advice because of the lack of clinical evidence, as until recently, most studies reporting treatment outcomes were conducted in a non-random and often retrospective environment[84-86]. There exist differences in the mechanisms of action, routes of administration, and approved indications of different drugs. The various medications are presented in Table 1. Table 2 lists the results of some important trials.

Attack prevention in MS

The pathogenesis of MS includes focal inflammatory demyelination and axonal loss. The available DMDs are mainly beneficial for controlling inflammation and have a poor effect on the degenerative component of the disease[173]. Since the first DMD, IFN-β1b became available in 1993, the US Food and Drug Administration has approved more than a dozen DMDs for MS: IFN-β1b, IFN-β1a, glatiramer acetate (GA), mitoxantrone, natalizumab, fingolimod, teriflunomide, dimethyl fumarate (DMF), alemtuzumab, pegylated IFN-β1a, daclizumab, ocrelizumab, cladribine and siponimod. The mechanisms of action of these DMDs have been depicted in [Figure 1].

Currently, three types of IFNs have been approved for RRMS: IFN-β1b, IFN-β1a, and pegylated IFN-β1a. The biological activity of IFN-β1a is 10 times higher than that of IFN-β1b. However, pegylated IFN-β1a, which consists of covalently linked IFN and polyethylene glycol, has a long half-life, which decreases the required frequency of administration[99,100]. IFN-β and GA, which were approved more than 20 years ago, are safe and effective. Both drugs are often considered as standard therapies in clinical trials of new DMDs. Among the DMDs for MS, mitoxantrone is not recommended firstly because of its cardiac toxicity. The cardiotoxicity of anthracyclines is thought to be dose dependent and irreversible, leading to a reduction in left ventricular ejection fraction and congestive heart failure. Regular and frequent cardiac monitoring is required during mitoxantrone therapy[174]. Daclizumab was delisted because of its high risk of serious inflammatory brain disorders, including encephalitis and meningoencephalitis[175,176]. Ocrelizumab, which has an anti-CD20 action, is the only drug approved for PPMS. Last month, siponimod has been approved by FDA. It may reduce the activity of the disease and has a modest effect on the gradual disability accrual in SPMS[156].

Monoclonal antibodies are more effective than other immunomodulators and can reduce the annual relapse rate by almost 50%[82]. Alemtuzumab (anti-CD52), fingolimod, or natalizumab (α4-integrin inhibitor) are recommended for patients with highly active MS[83]. Patients who use fingolimod, DMF, natalizumab, ocrelizumab, or rituximab should be evaluated for their risk of progressive multifocal leukoencephalopathy (PML). Cases of PML due to the use of fingolimod or DMF are fortunately rare[177,178]. However, the overall risk of PML with natalizumab use is high (4 per 1000)[179-181]. Patients with MS taking natalizumab should be switched to another DMD with a lower PML risk, if the anti-JC virus antibody index exceeds 0.9 during treatment. High-dose steroid and maraviroc (1000-3000 mg/day, po) may be beneficial for natalizumab-associated PML, and are lacking in experience[182,183]. The advent of oral DMDs has greatly facilitated the daily management of MS patients and improved compliance to treatment. Rituximab, which is usually used to treat NMOSD, has also been used for MS since the discovery of the role of B-cells in the pathogenesis of MS[160-162].

Attack prevention in NMOSD

Since the cumulative inflammatory damage caused by acute attacks leads to disability in NMOSD, attack prevention is crucial for long-term efficacy. It is accepted that first-line immunotherapies for the prevention of relapses in NMOSD include azathioprine, mycophenolate mofetil, and rituximab[41,84-86]. It should be noted that most studies on this topic were not well-controlled or randomized, and may have some bias in their results. Azathioprine antagonizes purine metabolism, and was the first immunosuppressant drug that was found to be effective in preventing NMOSD relapses[165]. Mycophenolate mofetil blocks lymphocyte

Table 1. Disease-modifying drugs for multiple sclerosis and neuromyelitis optica spectrum disorders

DMD	Trade name, available since	Dosage	Mechanism of action	Clinical trials
IFN-β1b	Betaseron, 1993 Extavia, 2009	250 μg, every other day, sc	Reduces Th1 and Th17 production; promotes Th2 proliferation; regulates T-, B-, natural killer, and dendritic cells; blocks leukocyte migration to the central nervous system[99-101]	RRMS[87-93], SPMS[94-96], PPMS[97,98]
IFN-β1a	Avonex, 1996 Rebif, 2002	30 μg, once a week, im 22 or 44 μg, three times a week, sc		
Pegylated IFN-β1a	Plegridy, 2014	125 μg, once 2 weeks, sc		
GA	Copaxone, 1996	20 mg, once a day, sc 40 mg, 3 times a week, sc	Binds MHC class II; interferes with development of self-reactive proinflammatory T-cells; promotes Th2 proliferation; regulates various immune cells[102-104]	RRMS[105-109], PPMS[110]
Mitoxantrone	Novantrone, 2000	12 mg/m^2, once every 3 months, iv	Inhibits type-II topoisomerase; disrupts DNA synthesis	RRMS[111,112], SPMS[113-115], PPMS[114]
Fingolimod	Gilenya, 2010	0.5 mg, once a day, po	Sphingosin-1 phosphate receptor agonist; induces lymphocytes to enter secondary lymphoid organs[116-118]	RRMS[119-124], PPMS[125]
Teriflunomide	Aubagio, 2012	7 or 14 mg, once a day, po	Prevents dihydroorotate dehydrogenase activation; suppresses activated T-lymphocyte proliferation[126,127]	RRMS[128-131]
Dimethyl fumarate	Tecfidera, 2013	240 mg, twice a day, po	Th1-Th2 shift, lymphocyte apoptosis[132,133]	RRMS[134-136]
Natalizumab	Tysabri, 2006	300 mg, once every 4 weeks, iv	Inhibits α4-integrin; prevents activated CD4+ T-cells from crossing the blood-brain barrier[137-139]	RRMS[140-145], SPMS[146]
Alemtuzumab	Lemtrada, 2013	12 mg, once a day for 5 days, then for 3 days one year later, iv	Anti-CD52; depletes CD52-positive lymphocytes[147]	RRMS[148-150]
Ocrelizumab	Ocrevus, 2017	600 mg, every 6 months, iv	Anti-CD20, depletes a large part of the B-cell lineage	PPMS[151], RRMS[152,153]
Cladribine	Mavenclad, 2017	Cumulative doses: 3.5 mg/kg or 5.25 mg/kg, po	Synthetic purine nucleoside analogue, disrupts DNA repair and synthesis, achieves therapeutic depletion of lymphocytes	RRMS[154,155]
Siponimod	Mayzent, 2019	2 mg, once a day, po	A new sphingosine 1-phosphate receptor modulator, depletes circulating lymphocytes, promotes CNS repair by modulating S1P1 on astrocytes and S1P5 on oligodentrocytes[157]	SPMS[156] RRMS[157,158]
Rituximab	Mabthera 1997	Two sessions of slow iv infusion of 1 g rituximab 14 days apart or 375 mg/m^2 each week for 4 weeks	Anti-CD20, attacks B-cells and plasmoblasts	NMOSD[159], RRMS[160,161], PPMS[162]
Azathioprine		2-3 mg/(kg·day) , po	Inhibits purine nucleotide synthesis; activates mitochondrial apoptotic pathway; activated T-cell apoptosis[163,164]	NMOSD[159,165,166], RRMS[167]
Mycophenolate mofetil	CellCept	1000-3000 mg/day, po	Blocks guanine nucleotide production; inhibits lymphocyte proliferation[168,169]	NMOSD[170-172]

DMD: disease-modifying drug; IFN: interferon; sc: subcutaneous; iv: intravenous; im: intramuscular; po: per os; RRMS: relapsing-remitting multiple sclerosis; SPMS: secondary progressive multiple sclerosis; PPMS: primary progressive multiple sclerosis; MHC: major histocompatibility complex; NMOSD: neuromyelitis optica spectrum disorders

proliferation by inhibiting the synthesis of guanine. It causes fewer adverse reactions, so it is a safe and generally well tolerated drug for NMOSD[169]. The efficacy of rituximab is better than that of azathioprine

Table 2. Clinical trials of multiple sclerosis and neuromyelitis optica spectrum disorders

Drug	Comparator	Trial	Disease	Duration	Sample size	Findings
IFN-β1b[87]	Placebo	Randomized, double-blind	RRMS	2 years	*n* = 372, 1:1:1 ratio of placebo, 1.6 million IU, and 8 million IU	Annual exacerbation rate: Placebo, 1.27; 1.6 million IU, 1.17; 8 million IU, 0.84
IFN-β1a[89]	Placebo	Randomized, phase III, double-blind	RRMS	104 weeks	*n* = 301, 1:1 ratio of placebo and 30 μg IFN-β1a	Annual exacerbation rate: Placebo, 0.9; interferon β-1a 0.61; Patients with disability progression: Placebo, 34.9%; IFN-β1a, 21.9%
PegIFN-β1a, ADVANCE trial[93]	Placebo	Randomized, phase III, double-blind	RRMS	2 years	Placebo (*n* = 500), PegIFN every 2 weeks (*n* = 512), PegIFN every 4 weeks (*n* = 500)	Annual relapse rate: Placebo, 0.397 (95%CI: 0.328-0.481); PegIFN every 2 weeks, 0.256 (95%CI: 0.206-0.318); PegIFN every 4 weeks, 0.288 (95%CI: 0.234-0.355)
GA[105]	Placebo	Randomized, phase III, double-blind	RRMS	2 years	GA (*n* = 125), placebo (*n* = 126)	Annual relapse rate: Placebo, 0.84; GA, 0.59
GA[109]	Placebo	Randomized, double-blind	RRMS	1 year	GA (*n* = 943), placebo (*n* = 461)	Annual relapse rate: Placebo, 0.505; GA, 0.331
Teriflunomide, TOWER trial[128]	Placebo	Randomized, phase III, double-blind	RRMS	48 weeks	Placebo (*n* = 388), 7 mg (*n* = 407), 14 mg (*n* = 370)	Annual relapse rate: Placebo, 0.50 (95%CI: 0.43-0.58); 7 mg, 0.39 (95%CI: 0.33-0.46); 14 mg, 0.32 (95%CI: 0.27-0.38) No effect on sustained accumulation of disability (7 mg) (HR: 0.95, 95%CI: 0.68-1.35)
Teriflunomide, TEMSO[129]	Placebo	Randomized trial	RRMS	108 weeks	*n* = 1088 1:1:1 ratio of placebo, 7 mg, and 14 mg	Annual relapse rate: Placebo, 0.54; 7 mg, 0.37; 14 mg, 0.37 Patients with confirmed disability progression: Placebo, 27.3%; 7 mg, 21.7%; 14 mg, 20.2%
DMF[135]	Placebo, GA	Randomized, phase III, double-blind	RRMS	96 weeks	Placebo (*n* = 363) Twice-daily DMF (*n* = 359), Thrice-daily DMF (*n* = 345), GA (*n* = 350)	Annual relapse rate: Placebo, 0.40; Twice-daily DMF, 0.22; Thrice-daily DMF, 0.20; GA, 0.29 Fewer new or enlarging hyperintense lesions on T2-weighted images ($P < 0.001$)
DMF[134]	Placebo	Randomized, phase III, double-blind	RRMS	2 years	Placebo (*n* = 408), Twice-daily DMF (*n* = 410), Thrice-daily DMF (*n* = 416)	Annual relapse rate: Placebo, 0.36; Twice-daily DMF, 0.17; Thrice-daily DMF, 0.19 Confirmed disability progression: Placebo, 27%; Twice-daily DMF, 16%; Thrice-daily DMF, 18%
Fingolimod, FREEDOMS II trial[120]	Placebo	Randomized, phase III, double-blind	RRMS	24 months	Placebo (*n* = 355), 0.5 mg (*n* = 358),	Annual relapse rate: Placebo, 0.40 (95%CI: 0.34-0.48); 0.5 mg, 0.21 (95%CI: 0.17-0.25); Percentage brain volume change: Placebo, -1.28 (SD, 1.50); 0.5 mg, -0.86 (SD, 1.22)

Fingolimod[119]	Placebo	Randomized, phase III, double-blind	RRMS	24 months	Placebo (*n* = 355), 0.5 mg (*n* = 358), 1.25 mg (*n* = 370)	Annual relapse rate: Placebo, 0.40; 0.5 mg, 0.18; 1.25 mg 0.16 Cumulative probability of disability progression (confirmed after 3 months): Placebo, 24.1%; 0.5 mg, 17.7%; 1.25 mg, 16.6%
Cladribine, CLARITY study[154]	Placebo	Randomized, phase III, double-blind	RRMS	96 weeks	Placebo (*n* = 437), 3.5 mg/kg (*n* = 433), 5.25 mg/kg (*n* = 456)	Annual relapse rate: Placebo, 0.33; 3.5 mg/kg, 0.14; 5.25 mg/kg, 0.13
Natalizumab, AFFIRM trial[140]	Placebo	Randomized, phase III, double-blind	RRMS	2 years	Placebo (*n* = 627), Natalizumab (*n* = 315)	Cumulative probability of progression: Placebo, 29%; Natalizumab, 17% Rate of relapse at 1 year reduced by 68%
Alemtuzumab[148]	IFN-β1a	Randomized, phase III, double-blind	RRMS	2 years	IFN-β1a (*n* = 231) Alemtuzumab (12mg) (*n* = 436)	Patients with relapse: IFN-β1a, 51%; Alemtuzumab, 35% Cumulative disability: IFN-β1a, 20%; Alemtuzumab, 13%
Ocrelizumab[151]	Placebo	Randomized, phase III, double-blind	PPMS	120 weeks	Placebo (*n* = 244), Ocrelizumab (*n* = 488)	Worse performance on timed 25-foot walk: Placebo, 55.1%; Ocrelizumab, 38.9%
Siponimod[156]	Placebo	Randomized, phase III, double-blind	SPMS	3 years	Placebo (*n* = 546), Siponimod (*n* = 1099)	Patients with 3-month confirmed disability progression: Placebo, 32%; Siponimod, 26%
Rituximab[161]	Self	Phase II	RRMS	52 weeks	*n* = 30	Median GdE lesions reduced from 1.0 to 0; MSFC improved (*P* = 0.02)
Rituximab[162]	Placebo	Randomized, double-blind	PPMS	96 weeks	Placebo (*n* = 147), Rituximab (*n* = 292)	Patients with CDP: Placebo, 38.5%; Rituximab, 30.2% (*P* = 0.14) Mean (SD) T2 volume change: Placebo, 2,205 (4306); Rituximab, 1,507 (3739)
Rituximab[159]	AZA	Randomized clinical trial	NMOSD	12 months	Rituximab (*n* = 33), AZA (*n* = 35)	Decreased annual relapse rate: Rituximab, 1.09; AZA, 0.49 Relapse-free disease: Rituximab, 78.8%; AZA, 54.3%
AZA[167]	IFN-β	Randomized, phase III, single-blind	RRMS	2 years	AZA (*n* = 77), IFN-β (*n* = 73)	Annual relapse rate: AZA, 0.26; IFN-β, 0.39 Annualized new T2 lesion rate: AZA, 0.76; IFN-β, 0.69

IFN: interferon; PegIFN: pegylated interferon; RRMS: relapsing-remitting multiple sclerosis; SPMS: secondary progressive multiple sclerosis; PPMS: primary progressive multiple sclerosis; AZA: azathioprine; DMF: dimethyl fumarate; GA: glatiramer acetate; MSFC: multiple sclerosis functional composite; CDP: confirmed disease progression; GdE: gadolinium enhanced; CI: confidence interval; HR: hazard ratio; SD: standard deviation

and mycophenolate mofetil, and is probably the best choice at present[184-187]. Rituximab is a human-mouse chimeric monoclonal antibody against CD20, which is a regulatory factor for the early activation and differentiation of B-cells. It acts on B-cells and plasmablasts. After a single dose of rituximab, the number of B-cells typically decreases to their minimum value by 2 weeks, and this effect is generally maintained for 6 months. Studies have found that long-term rituximab treatment often leads to significant reduction in immunoglobulins[188]. There have been reports of infections with long-term rituximab treatment. It is important to monitor CD19+ B-cell counts, the total and specific Ig levels before and during treatment with

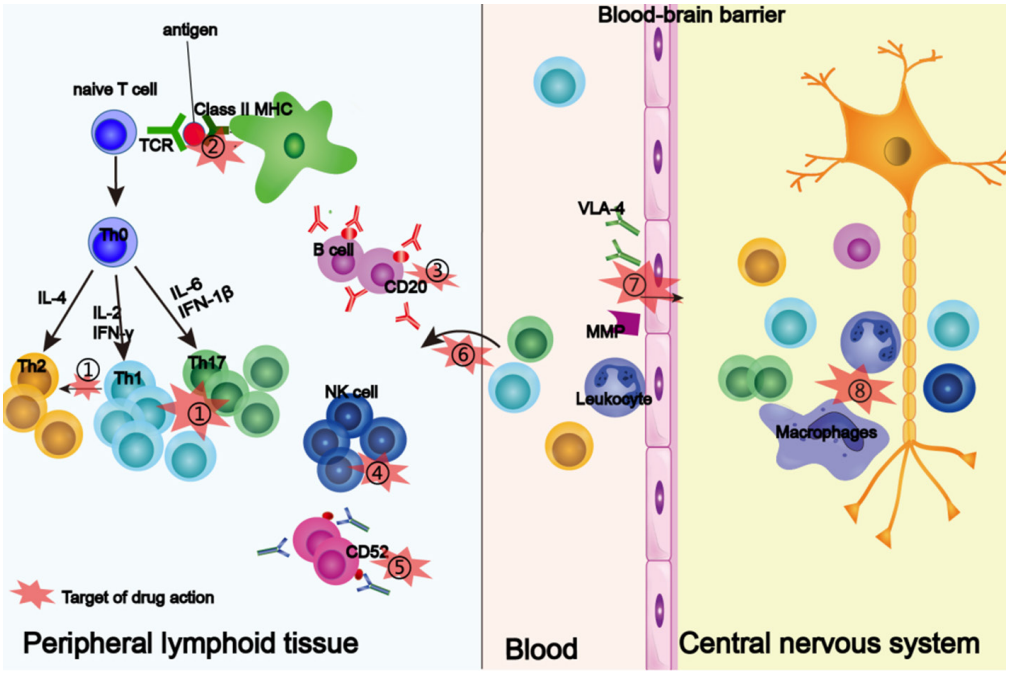

Figure 1. Pathogenesis of multiple sclerosis and targets of drug action. ① Reduced production of Th1 and Th17 cells and Th1-Th2 shift (interferon-β, teriflunomide, dimethyl fumarate); ② Competitive binding of MHC class II molecules (glatiramer acetate); ③ Depletion of CD20-positive lymphocytes (ocrelizumab, rituximab); ④ Regulation of T-cells, B-cells, NK cells, and dendritic cells (interferon-b, glatiramer acetate); ⑤ Depletion of CD52-positive lymphocytes (alemtuzumab); ⑥ Alteration of lymphocyte distribution (fingolimod); ⑦ Preventing activated CD4+ T-cells from crossing the blood-brain barrier (natalizumab, interferon-β); ⑧ Promoting leukocyte migration to the central nervous system (glatiramer acetate). VLA-4: very late antigen-4; MMP: matrix metalloproteinase; MHC: major histocompatibility complex; IFN: interferon; IL: interleukin; NK: natural killer; TCR: T-cell receptor; Th: T helper

rituximab to prevent complications[188,189]. Other immunosuppressants that have been used to treat NMOSD include tacrolimus, cyclophosphamide, methotrexate, and cyclosporin A. Tacrolimus and cyclosporin A produce good selective inhibition of Th cells, and methotrexate inhibits folate metabolism. However, these drugs have not been used frequently because of their uncertain effects[84-86]. Some studies have found that some new DMDs for MS, such as fingolimod, DMF, alemtuzumab, and natalizumab, may cause the disease to worsen, mainly in patients with AQP4-IgG-positive NMOSD[190-194]. There are insufficient data to support or discourage the use of GA and IFN-β in NMOSD[195,196]. Currently, experience in the treatment of MOG-IgG-positive NMOSD is still lacking, and long-term immunosuppression may be effective[197,198].

CONCLUSION

Currently, MS and NMOSD are incurable diseases. There is no consensus on the best treatment strategy or treatment target. Early, conventional immunosuppressive agents, such as azathioprine and cyclophosphamide, have been used for the treatment of MS and NMOSD. Various immunosuppressive agents have different degrees of efficacy in MS or NMOSD. Among them, only azathioprine and mycophenolate mofetil are currently recommended for the treatment of NMOSD, but no credible randomized controlled trial has yet proved their effects. Now, more than a dozen DMDs are available for MS, with varying levels of efficacy and safety. Immunomodulators against MS have been marketed since 1993, and conventional immunosuppressive agents have rarely been used in this condition. Compared with new immunomodulators, conventional immunosuppressants have more side effects and worse drug targeting. However, in some countries and regions, due to economic reasons or a lack of DMDs, cyclophosphamide, tacrolimus, and other drugs are still used to treat MS and have some therapeutic effect[199-201]. Despite the use of DMDs, some patients still have exacerbations and develop progressive disease. Few DMDs are available for NMOSD, and there is a lack of

large-scale clinical trials.Several new drugs are currently undergoing clinical trials, including tocilizumab (IL-6 receptor blocker), eculizumab (C5 complement inhibitor), and inebilizumab (CD19 B-cell depletion)[202].

More efficacious therapies that alter the disease course are therefore required. Additional research on neuroprotection and repair is urgently needed. Many therapies are currently under study, including hematopoietic stem cell transplantation, neural stem cell-based regenerative approaches, and exosomes derived from bone marrow mesenchymal stem cells. The future of MS and NMOSD treatment is extremely promising as more effective treatments are being developed.

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Authors' contributions

Summarized the references and wrote the manuscript: Zhang L
Discussed paper writing and revised the manuscript: Zhang L, Tian JY, Li B
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Review **Open Access**

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Economic impact of traumatic spinal cord injuries in the United States

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Abstract

Individuals having sustained traumatic spinal cord injury (TSCI) in the United States are living longer as compared to historical trends, thanks to an ever-evolving understanding of the nature of this injury. Despite this, multiple barriers to care for TSCI patients remain including variations in government-issued veteran insurance, privatized insurance, and among uninsured individuals. The United States alone experiences 12,000 new TSCI cases every year, many of these are found to occur in a growing proportion of elderly individuals. It is crucial to understand both the short-term direct costs as wells as the long-term rehabilitation costs required by these TSCI patients. The lifetime financial burden for those having sustained a TSCI can be immense for patients, insurance companies, and hospital systems alike. Among those with TSCI, re-hospitalization rates are high, leading to increased healthcare resource utilization within this specific patient population. Costs can quickly balloon into hundreds of thousands of dollars and cause a profound financial burden for these patients. This review article seeks to communicate an understanding of the current financial landscape surrounding TSCI patients. The authors will also examine the costs of acute emergency room surgical care such as American spinal injury association grade, hospital length of stay, as well as the timing delay between injury and surgical decompression. Long-term costs associated with TSCI such as rehabilitation, care of secondary comorbidities, and post-injury employment prospects will be examined as well. These costs will be framed from the patient's perspective as well as from both the hospital and insurance company's perspectives. It is hoped a complete understanding as to what makes TSCI such a medically and financially burdensome injury will allow for improved healthcare resource utilization in this population.

Keywords: Traumatic spinal cord injury, healthcare resource, American spinal injury association grade, hospital length of stay, rehabilitation, post-injury employment

INTRODUCTION

To begin a discussion of the economic impact of traumatic spinal cord injury (TSCI), it becomes necessary to first have an understanding of the epidemiology and disease burden of TSCI. TSCIs are sustained following major traumatic events, such as falls, motor vehicle accidents, or acts of violence. TSCIs are life-changing, economically impactful traumas that are estimated globally to affect 13 new individuals per 100,000 per year. But this incidence was found to double in North America, affecting nearly 26 new individuals per 100,000 per year[1]. The National Spinal Cord Injury Statistical Center, which is both the longest running and largest database containing the specifics of TSCIs in the United States, estimates the incidence of new TSCIs to be roughly 40 cases per one million in the United States, or roughly about 12,000 new cases per year[2]. The incidence of TSCI in the United States has historically been held constant, with the largest increases in incidence being observed in the elderly population in the specific context of an increase in the number of falls as an individual ages[3,4]. The prevalence of TSCI in the United States is estimated to be approximately 273,000, within a range of 238,000 to 332,000[2]. Within the prevalent population as a whole, more severe injuries were observed in younger individuals as compared to those living to older age with incomplete and/or lower level injuries with resulting high degrees of independence[5]. The average age at the time of spinal cord injury is estimated to be 42.6 years of age with males accounting for 80.7% of new cases, vastly outnumbering their female counterparts.

Those with TSCIs have recently been found to be living longer, when compared to historical trends[6]. Vehicle crashes remain the leading cause of injury, followed by falls, and then acts of violence (i.e., gunshot wounds) [Figure 1][2]. The neurological deficits sustained following a TSCI are categorized by its corresponding American Spinal Injury Association (ASIA) score, ranging from A to E with A indicating profound deficit and E indicating normal function [Table 1][7]. The extent of injuries varies as well, with incomplete tetraplegia being the leading extent of injury, followed by incomplete paraplegia, complete paraplegia, and finally complete tetraplegia [Figure 2][2]. The limitations on an individual's activities of daily living were found to be largely determined by the location and completeness of the injury sustained, where total hours of care were dependent upon injury level and severity[8]. TSCIs exact a heavy financial burden both in the acute care setting as well as within the context of longer-term rehabilitation that often follows the initial injury[9]. The costs associated with TSCIs are greatly affected by both the patient's extent of injury and subsequent degree of disability. Unsurprisingly, the overall life expectancy for those individuals sustaining a TSCI remain significantly below the average life expectancy in the United States[10]. An understanding of the epidemiological burden of TSCI in the United States warrants a further discussion on the cost, reimbursement, and subsequent disability associated with such an economically, medically, and psychologically impactful event.

ACUTE CARE COSTS FOLLOWING TRAUMATIC SPINAL CORD INJURY

Immediately following a TSCI, the vast majority of patients will promptly seek medical care consisting of both surgical stabilization and vertebral decompression[11-14]. The high acuity of TSCIs often exacts a heavy financial burden in addition to a life-altering disability for these patients. In the United States, approximately 50% of TSCI patients have their medical costs covered through a private insurer. Medicaid, a state-run medical insurance provider for financially disadvantaged patients, covers 28% of those having sustained a TSCI. The remaining population has their medical costs covered through Medicare or the Veterans Health Administration (VHA)[13]. The average cost for the initial injury and recovery phase, termed the *acute phase*, can run $142,366[12]. The majority of these charges will be covered through a patient's primary medical insurance. Most patients, with the exception of eligible military veterans through the VHA, are often left with high co-pays that place an additional undue financial burden on the recovery process.

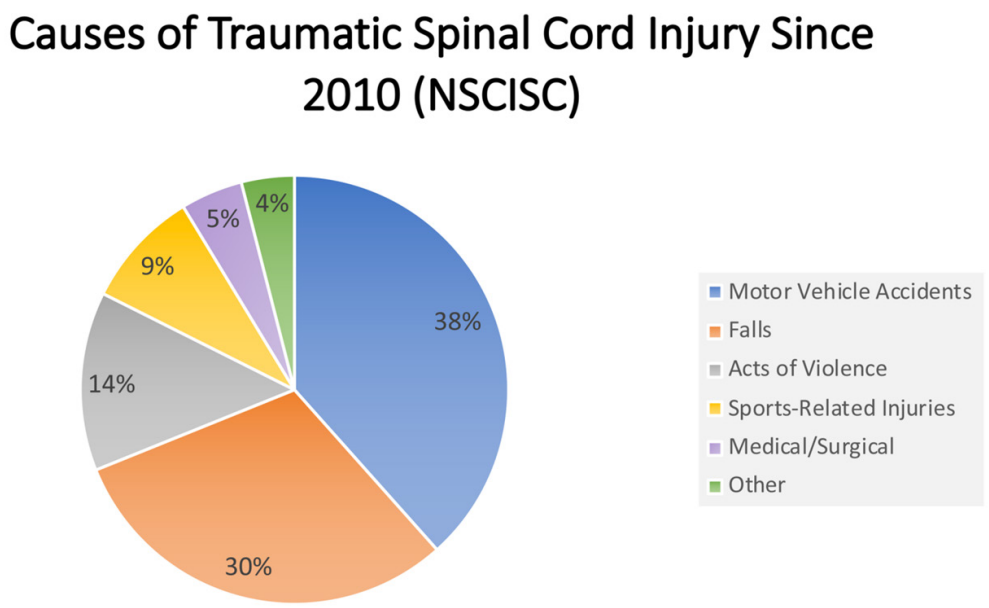

Figure 1. A pie chart illustrating the major causes of TSCI since 2010[2] according to the NSCISC. The NSCISC estimates that the most common causes of TSCI include motor vehicle accidents (blue), mechanical falls (orange), and acts of violence (gray). Less commonly TSCI is caused by sports-related injuries (yellow), medical/surgical causes (pink), and other miscellaneous causes not previously listed (green). TSCI: traumatic spinal cord injury; NSCISC: National Spinal Cord Injury Statistical Center

Table 1. Percentage of patients with ASIA grade at ER discharge and resultant one year ASIA improvements

ASIA Grade	Injury type	Definition Of ASIA Grade	TSCI patients with ASIA Grade at time of Discharge*	ASIA Grade one year improvement rates (≥ 1 Grade level)**
Grade A	Complete	Complete sensorimotor loss	36.4%	25.1%
Grade B	Incomplete	Complete motor loss with incomplete sensory loss	13.8%	71.1%
Grade C	Incomplete	Motor function is preserved, but more than 50% of key muscles below the neurological level have a muscle grade < 3	11.9%	78.8%
Grade D	Incomplete	Motor function is preserved but the at least 50% of key muscles below the neurological level have a muscle grade ≥ 3	37.6%	14.1%
Grade E	Normal	Motor and sensory functions are normal	0.3%	N/A

ASIA: American spinal injury association; *: within each of the ASIA grade rows, there is the percentage of total TSCI patients at the time of hospital discharge with that specific ASIA grade injury out of all TSCI patients; ER: emergency room; TSCI: traumatic spinal cord injury; **: percentage of patients who have improved ≥ 1 ASIA grades from their original ASIA grade assignment (column 1) at one year post-discharge. Grade B and C injuries have the highest chance of improvements at 71.1% and 78.8%, respectively[26,27]

Evidence demonstrates that surgical intervention within the first 72 h post-injury is both a key prognostic and cost-determining factor in the context of TSCI. Surgical intervention within this crucial window has been shown to directly correlate with a decreased hospital length of stay (LOS) and subsequent decreased medical costs[15,16]. If surgical intervention is received within 72 h following the initial injury, hospitals were found to save an average of $14,000 on resource utilization. Additionally, patients were found to have a greater chance of neurological recovery and were spared approximately $45,000 in medical costs. A prospective cohort study investigating the relationship between the delay of surgical decompression following TSCI and neurological recovery found that decompression within the first 24 h more than doubled the chance of recovery of a 2 ASIA grade TSCI as compared to those who received spinal cord decompression outside of this 24 h window[15,17]. This rapid surgical turnaround within 24 h was found to be just as safe[18]. However, this crucial window presents an access to care issue for those living in rural areas in which there is a high prevalence of TSCI, but low rate of hospitalization with subsequent inflated

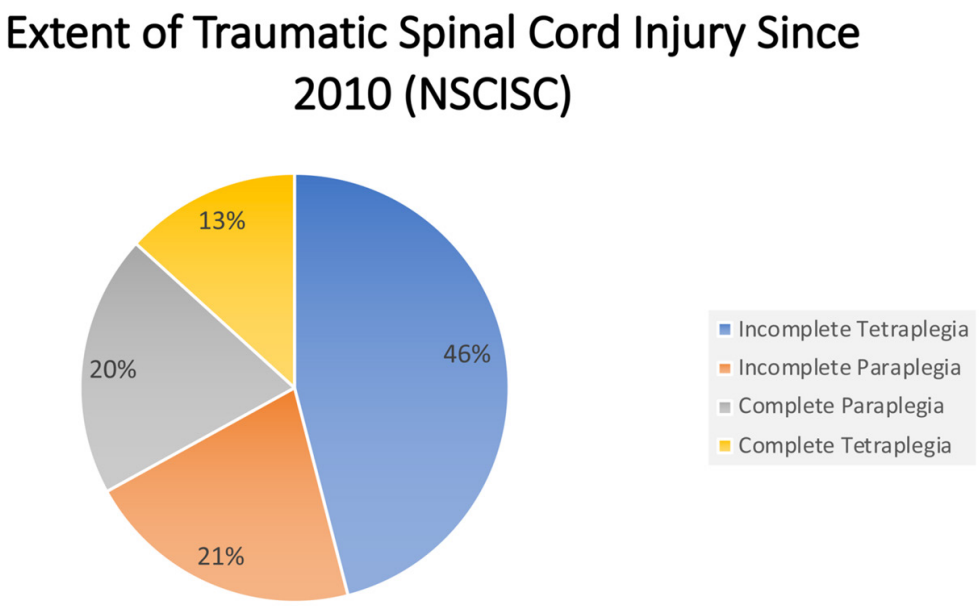

Figure 2. A pie chart illustrating the extent of injury following TSCI since 2010[2] according to the NSCISC. The NSCISC estimated that nearly half of all TSCI resulted in the extent of injury known as incomplete tetraplegia (blue). Incomplete and complete paraplegia were similar in prevalence following TSCI (represented by orange and gray, respectively) while complete tetraplegia (yellow) was the least common extent of injury following TSCI as compared to the other major extent of injury categories. TSCI: traumatic spinal cord injury; NSCISC: National Spinal Cord Injury Statistical Center

healthcare costs[12]. Sparsely located hospitals in rural areas ill-equipped to manage complex TSCIs may underlie the delayed care observed in rural areas[12,19].

HOSPITAL LENGTH OF STAY FOLLOWING TRAUMATIC SPINAL CORD INJURY

The average hospital LOS following a TSCI was found to be approximately 12 days, twice as long as patients without TSCI. Interestingly, patients between the ages of 18-29 averaged 13.5 days in the hospital, while elderly patients (over 60 years old) averaged only 10 days. This is a surprising observation that can be attributed to younger age being a major risk factor for more severe forms of TSCI[12]. Surgical intervention is often necessary for severe TSCIs and is significantly more expensive than conservative medical management. In a study conducting a cost/benefit analysis in elderly patients with odontoid (C1-C2) fractures, it was found that the cost of surgical intervention was approximately $50,000 per patient, while the cost of medical management alone was more akin to $30,000 per patient. When considering the options between surgical and medical management, it is important to note that patients between the ages of 65-85 had a favorable increase in quality adjusted life years (QALY) following surgical management. These patients' qualities of life improved following surgical management to offset the high costs of care. Patients over the age of 85 did not see the favorable QALY improvement from surgical intervention, suggesting this population would have the greatest cost-benefit from conservative medical management as compared to surgical intervention[20]. The ASIA score can be utilized as a determinant of emergency room (ER) cost as well[15,21]. Using this information, TSCI surgical hospital costs can be lowered by trying to target certain age groups (under 85) and by attempting surgical intervention sooner[22].

RECOVERY AND LONG-TERM DISABILITY FOLLOWING TRAUMATIC SPINAL CORD INJURY

Post-injury rehabilitation

TSCI recovery is divided into three major phases: acute, post-acute, and chronic[23,24]. The acute phase is marked by post-injury care received in the hospital, while post-acute and chronic phases are distinguished

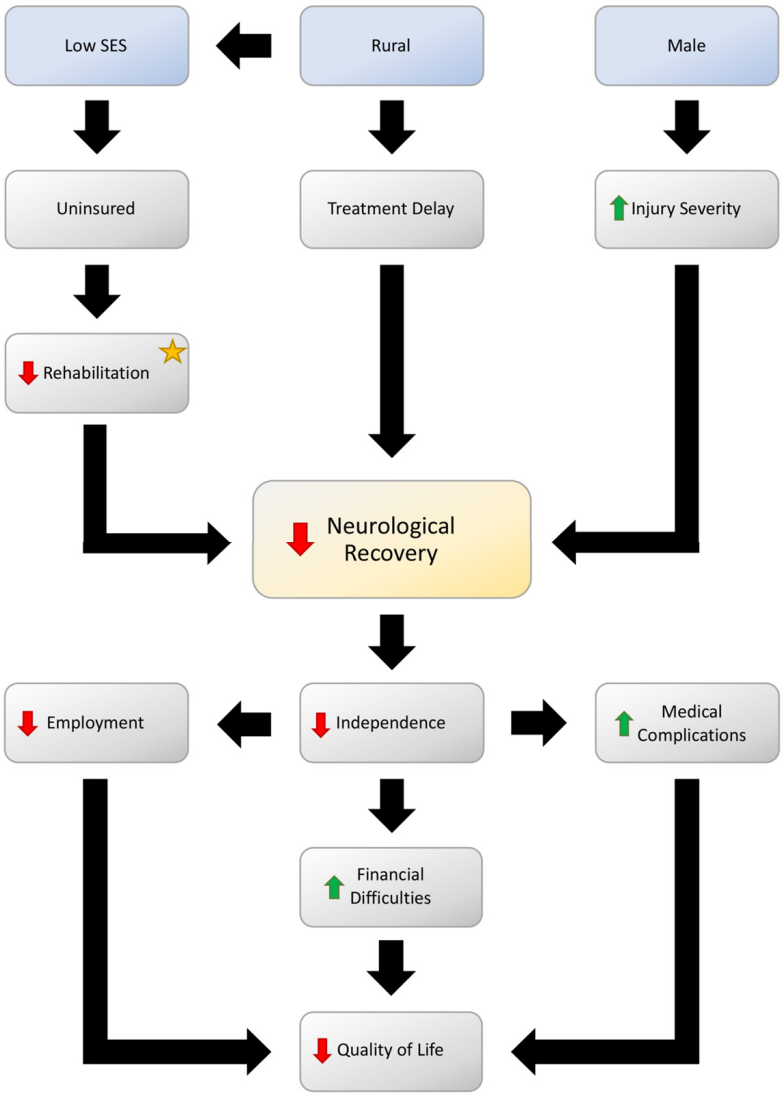

Figure 3. A flowchart illustrates the primary risk factors for a TSCI (blue) and the obstacles TSCI patients may face throughout their lives. Decreased neurological recovery (yellow) is the factor that has the greatest negative impact on a patient. Access to rehabilitation (star) is the only modifiable attribute shown that can reduce the cascade of negative events leading to a decreased patient quality of life. SES: socioeconomic status; TSCI: traumatic spinal cord injury

through post-injury care delivered in an outpatient setting[24]. While the timeframe of each of these phases varies, neurological recovery has been found to occur during the acute and post-acute phases. This crucial recovery period has been found to last between 12-18 months, with the majority of improvement observed in the first 3 months post-injury[25]. During the acute and post-acute phases, rehabilitation seeks maximize neurological recovery as measured by the ASIA grade[26,27]. A patient will enter the chronic phase when they have reached their maximum neurological recovery; therefore, priorities in the chronic phase shift to minimizing common long-term TSCI co-morbidities and normalizing a patient's new post-injury standard of living[28]. The neurological recovery and the quality of life of the TSCI patients are dependent on various primary risk factors and the obstacles that they may face throughout their lives [Figure 3].

To date, few studies have examined the recovery rates corresponding with the time between TSCI and initiation of rehabilitation[23]. Regardless, studies have shown TSCI patients having access to rehabilitation corresponds to better outcomes and a greater chance for patients to reclaim their roles as active members of

the society[29]. Despite its importance, discrepancies of who should receive rehabilitation continue to exist. A study investigating rehabilitation rates in patients with TSCI examined patients with private insurance, government insurance (Medicare/Medicaid), and the uninsured. Patients with private insurance were referred to rehabilitation services 84.6% of the time, while government and the uninsured were referred rehabilitation 55.5% and 55.2% of the time, respectively, despite both populations having similar injury severities. This study also found that patients with government insurance had an average LOS of 12 days longer than both privatized insurance and those who remain uninsured. However, the explanations are varied. Claridge *et al*.[28] hypothesizes that uninsured patients are simply rejected from most rehabilitation facilities and are inevitably sent home, while privately insured patients are transferred to rehabilitation facilities as soon as possible. Patients with government insurance are kept in the hospital while case management explores potential options, explaining their increased LOS[28]. Although not surprising, these results give rise for concern. Increased time between injury and rehabilitation has been associated with decreased long-term quality of life and a decreased ability to live independently; thus, raising the long-term cost of care for these individuals. Rehabilitation teaches patients to prevent secondary health complications, maximizing function and work towards long term healthy lifestyles[23].

Long-term complications of a traumatic spinal cord injury

In the years following a TSCI, patients face a risk of several severe co-morbidities. Most fatal complications are due to urinary tract infections (UTIs), sepsis due to pneumonia, and pressure ulcers (in those with T1-S5 injuries)[30,31]. A medium-sized cohort study found that 47.6% of TSCI participants were treated for a UTI, 33.8% were treated for pneumonias, 27.5% for depression, and 19.7% for a decubitus ulcers[32]. Characterized as a "never event", almost one third of all pressure ulcers are seen in paralyzed patients. The estimated cost for treating a stage IV pressure ulcer (an ulcer that extends into the underlying bone and muscle[33]) is approximately $124,000-$129,000 per instance[34,35]. Sepsis, the second most expensive of the above listed comorbidities in TSCI patients, was found to cost around $27,000 per stay in the intensive care unit (ICU). When broken down to the cost by day, the cost of sepsis in the ICU per day in the United States was just over $4,500[36]. As suggested in the data from the medium sized cohort, TSCI paralysis is a risk factor for increased UTI rates[32,37]. The most common of the comorbidities and the least expensive, it cost around $8,300 per hospital treatment[37].

Post-injury re-hospitalization rates

Patients within the first year following a TSCI are at a significant risk for re-hospitalization. One study estimates a re-hospitalization rate between 36%-45% in the first year post-injury, decreasing to a 30% re-hospitalization risk in subsequent post-injury years[38]. The authors of a 2015 study investigating emergency room visits (ERV) and emergency re-hospitalizations (ERH) in chronic TSCI patients found that 37% of participants had at least 1 ERV in the last year, with half of those visits progressing to an ERH[39]. The average hospital LOS for these patients was found to be 21 days[40]. An additional study found that the only modifiable risk factor for a TSCI patient ERH is lower functional independence following initial rehabilitation[41]. Lack of independence is an important issue for uninsured TSCI patients, who encompass 12% of the TSCI population[11]. As stated previously, most uninsured TSCI patients forego rehabilitation, causing decreased functional independence and a subsequent increased risk of medical emergencies[42].

TSCI patients re-admitted to a hospital post-injury experience a wide range of costs that are dependent on their co-morbidities. A 2018 study followed a cohort of TSCI patients over a decade while analyzing their use of health care services over that period. This study found that a combined $49.4 million was spent on health care services over this 10-year span for all 303 participants. Interestingly, two-thirds of those costs were utilized by only 16.5% of the study population (termed High Utilizers), with each individual charging $51,860 per year. High Utilizers had an ERH 2.6 times per year with an average LOS of 9.6 days, often being treated for multiple co-morbidities. High Utilizers were commonly male, of a racial minority, of low

socio-economic status, with high-grade TSCI, and experienced frequent pressure ulcers. In contrast, 53% of chronic TSCI patients were considered Low Utilizers. These patients on average visited the ED 0.1 times per year and only stayed in the hospital 0.3 days per year[38].

MILITARY VETERANS SUSTAINING TRAUMATIC SPINAL CORD INJURY

According to the department of veterans affairs, the VHA is the largest network of TSCI care in the country, with over 1,200 integrated healthcare facilities distributed throughout the country. As of 2018, there are over 19 million United States military veterans, and approximately 9.15 million of those veterans are enrolled in the veterans affairs (VA) health care system; making the VHA a healthcare provider for approximately 2.8% of the American population[43,44]. In order for a military veteran to qualify for VA-sponsored healthcare, they must have served under active duty and have been honorably discharged. Veterans sustaining a TSCI while in active military service are eligible for monthly disability compensation in addition to the healthcare coverage that all VHA-eligible veterans receive[45]. Veterans who are injured in connection to their military service are entitled to comprehensive healthcare coverage with zero monetary responsibility falling onto the patient[46]. The VHA provides an interesting perspective on health care resource allocation due to eligible veterans being the sole TSCI population in the United States with no financial responsibility for their post-injury TSCI care.

Traumatic spinal cord injury costs in the veterans affairs health care system

According to the VHA, there are approximately 26,000 TSCI patients who are eligible to receive VHA-sponsored treatment, half of which chose to undergo specialty treatment within the VA health care system[45]. The first 12 months post-injury were found to be the costliest, with the average patient being charged $606,349 within the first year. Patients were then charged an average of $92,454 annually for long-term care[47]. However, these charges can vary greatly depending on the severity and extent of the injury. Veterans with C1-C4 tetraplegia accrue an average of $1,064,716 in costs within the first year with $184,891 annually, while veterans who still retain some motor function at all levels average $347,484 in costs within the first year and $42,206 annually[48].

Prescription medication coverage for those with TSCI

Considering the high cost of many prescription medications, 88% of veterans with TSCI obtain prescription medication coverage through the VHA. The remaining 12% utilize either a combination Medicare Part D & VHA (9.5%) or Part D alone (2.8%). This trend is likely to continue as most veterans with TSCI are exempt from medication co-payments through the VHA. Patients sustaining a TSCI or secondary co-morbidity (i.e., pressure ulcer, UTI, diabetes) were found to rely less on Medicare Part D and more on the VHA for their prescription medication needs[49].

UNEMPLOYMENT AND BANKRUPTCY FOLLOWING TRAUMATIC SPINAL CORD INJURY

It is unsurprising that TSCIs of all severities are one of the most debilitating injuries a person can experience, often causing significant undue financial strain[50]. Despite many TSCI patients having a desire and capability to work, data show that only 35% of those having sustained TSCI eventually return to active employment[51-53]. Five years post-injury, 25% of these patients were found to file for bankruptcy[54]. TSCIs decrease the quality of life in patients due to their consequent inability to work and increased healthcare costs[55]. Following a TSCI, mobility/physical impairments and incontinence issues may limit the type of work available to TSCI patients[56,57]. Following a TSCI, skilled labor jobs may no longer be an option and many patients unable to return to their old jobs are forced to find new avenues of employment[58,59].Realizing this difficulty, the Rehabilitation Act of 1973 was amended in 1992 to include supported employment (SE), which promotes disabled persons to return to the workforce. SE encourages those with significant disability to find jobs with competitive pay and have supportive services provided to those that in need[60].

Effectiveness of the supported employment initiative in veterans with TSCI

In 2012, a randomized multisite study investigated the effectiveness of a SE rehabilitation program in aiding military veterans with TSCI find post-injury employment. The initial results showed that veteran participants were 11.4 × more likely to find employment as compared to veterans without any form of rehabilitation program[60]. Two years later, a follow-up study was performed by the same investigators assessing the long-term performance of the previously studied SE rehabilitation program. The results showed that veterans were 30.8% more likely to achieve employment; however, veterans were significantly more likely to achieve employment within the first 12 months after their TSCI compared to those who waited longer than a year[61].

In the same year as the 2-year follow-up study, a cost-effectiveness analysis was performed on the SE rehabilitation program for veterans with TSCI. Each participant received approximately 35 h of rehabilitation services costing $1,821 on average. The costs associated with the program were then compared to the quality of life improvement self-reported by each of the participants. The results showed that participants in the SE rehabilitation program had marginally reduced societal costs compared to the control group. But these results, coupled with an insignificant difference in quality of life improvement, led to the determination that the SE rehabilitation program was not cost-effective as compared to standard care[62].

Bankruptcy prevalence in those having sustained a TSCI

In the United States, the leading cause of bankruptcy is the inability to pay medical bills[63]. A study comparing the risk of bankruptcy before and after TSCI found that patients sustaining a TSCI have a 3.5% chance of bankruptcy in the first five years post-injury. Interestingly, those with private insurance were twice as likely to file for bankruptcy as compared to those with Medicaid. The authors attributed this finding to private insurance patients accruing additional debts pre-injury that they can no longer be paid back (i.e., car, mortgage, etc.)[64]. Race and income were also found play an important role for those returning to work post-injury. For caucasian patients it took a median of 566 days to return to work. However, their non-Caucasian counterparts took 1382 days to return to work, almost 2.5 times slower. Considering income, higher income patients in the upper 75th percentile returned to work in 557 days. In contrast, TSCI patients in the lower 25th percentile of income returned to work over 200 days later than their higher income counterparts. Phillips *et al.*[58] attributed this delay to lower paying jobs often requiring skilled physical labor, causing an obvious barrier to TSCI patients.

Following a TSCI, patients report unemployment and financial difficulties as primary factors contributing to unhappiness, to a greater extent than the extent of their disability. Employment gives these patients a sense of both purpose and financial independence[57]. Patients with greater levels of social support, community integration and higher levels of education were more likely to gain steady employment[65,66]. TSCI may leave patients emotionally drained and separated from their social lives[67]. Prolonged unhappiness can exacerbate a variety of mental illnesses, with studies showing that 18%-37% of TSCI patients presented with signs of major depressive disorder (MDD)[56]. Financial stressors such as job loss, financial crisis, and inability to pay bills are found in 31.2% of those with MDD[68,69]. Patients following a TSCI may additionally have altered decision making capabilities due to the increased incidence of MDD[70].

CONCLUSION

TSCI is a lifelong costly injury for both hospital systems and the patient. Fast access to decompression surgery and early rehabilitation has been shown to improve injury outcomes. Although access to rehabilitation can be difficult through certain forms of insurance, it is critical in TSCI care. Lack of rehabilitation services has been associated with greater levels of comorbid secondary health conditions.

Condition's such as UTI, sepsis, and pressure ulcers account for higher health care cost utilization by TSCI patients. Reduction of secondary heath conditions is one of the few areas that can be modified in TSCI patients. Implementing rehabilitation and education about secondary health conditions for all TSCI patients would save both hospitals and patients money. These savings would allow for allocation of healthcare resources to other areas.

DECLARATIONS

Authors' contributions

Conceptualized the theme and conducted the literature review process: Merritt CH, Taylor MA, Yelton CJ
Contributed to preparation of the manuscript, interpretation of subtopics, preparation figures, and revision of the manuscript; and approved the final version to be published: Merritt CH, Taylor MA, Yelton CJ, Ray SK

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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LFA-1 antagonist (BIRT377) similarly reverses peripheral neuropathic pain in male and female mice with underlying sex divergent peripheral immune proinflammatory phenotypes

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Abstract

Aim: The majority of preclinical studies investigating aberrant glial-neuroimmune actions underlying neuropathic pain have focused on male rodent models. Recently, studies have shown peripheral immune cells play a more prominent role than glial cells in mediating pathological pain in females. Here, we compared the onset and duration of allodynia in males and females, and the anti-allodynic action of a potentially novel therapeutic drug (BIRT377) that not only antagonizes the action of lymphocyte function-associated antigen-1 (LFA-1) to reduce cell migration in the periphery, but may also directly alter the cellular inflammatory bias.

Methods: Male and female mice were subjected to peripheral nerve injury chronic constriction injury (CCI) applying two methods, using either 4-0 or 5-0 chromic gut suture material, to examine potential sex differences in the

onset, magnitude and duration of allodynia. Hindpaw sensitivity before and after CCI and application of intravenous BIRT377 was assessed. Peripheral and spinal tissues were analyzed for protein (multiplex electrochemiluminescence technology) and mRNA expression (quantitative real-time PCR). The phenotype of peripheral T cells was determined using flow cytometry.

Results: Sex differences in proinflammatory CCL2 and IL-1β and the anti-inflammatory IL-10 were observed from a set of cytokines analyzed. A profound proinflammatory T cell (Th17) response in the periphery and spinal cord was also observed in neuropathic females. BIRT377 reversed pain, reduced IL-1β and TNF, and increased IL-10 and transforming growth factor (TGF)-β1, also an anti-inflammatory cytokine, in both sexes. However, female-derived T cell cytokines are transcriptionally regulated by BIRT377, as demonstrated by reducing proinflammatory IL-17A production with concurrent increases in IL-10, TGF-β1 and the anti-inflammatory regulatory T cell-related factor, FOXP3.

Conclusion: This study supports that divergent peripheral immune and neuroimmune responses during neuropathy exists between males and females. Moreover, the modulatory actions of BIRT377 on T cells during neuropathy are predominantly specific to females. These data highlight the necessity of including both sexes for studying drug efficacy and mechanisms of action in preclinical studies and clinical trials.

Keywords: Neuropathic pain, glia, neuroimmune, peripheral immune, T cells

INTRODUCTION

While male and female rodent models of peripheral neuropathic pain generate similar clinical features such as pathological sensitivity to light touch referred to as allodynia, emerging evidence suggests that the biochemical and cellular aspects underlying allodynia are different between the sexes. Clinical evidence strongly implicates sex differences in pain sensitivity[1,2], and preclinical data supports these clinical findings by demonstrating that peripheral immune and glial cells exhibit sex differences in response to peripheral nerve injury leading to neuropathy[3-6]. Understanding sex divergent components of pain pathophysiology has drawn significant attention and is of paramount importance for identifying effective pain therapeutics in males and females.

Chronic neuropathic pain following peripheral nerve injury involves dynamic neuroimmune interactions between peripheral immune cells that traffic to the injured nerve, the dorsal root ganglia (DRG), and the spinal cord, and the actions of glial cells within the spinal cord[7-9]. Peripheral nerve injury leads to alterations in proinflammatory cytokines including interleukin (IL)-1β, IL-6, and TNF, and anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β1) at anatomical sites of the pain pathway[10-16]. Spinal glia and DRG satellite glia contribute to persistent allodynia by responding to and releasing these proinflammatory cytokines and by reducing IL-10 expression[12,14,17-19]. Additionally, the chemokine CCL2 is elevated in DRG of injured nerves and facilitates leukocyte migration to DRG and spinal cord[19,20]. However, most of these reports were studied male models of neuropathy or the sex was unspecified, with few studies utilizing females[21-24], or compared sex differences underlying pathological pain[3-5,25-28].

A substantial role of the adaptive immune response is now recognized as underlying aberrant neuroimmune actions following nerve injury. Activated CD4 T cells, specifically proinflammatory Th1 and Th17 cells, infiltrate the injured peripheral nerve and the lumbar spinal cord (LSC)[29-33], and are thought to contribute to glial activation[34]. Conversely, anti-inflammatory T regulatory (Treg) cells control spinal glial-immune proinflammatory activation and are protective against neuropathy[35]. A few studies have

implicated a T cell role by examining cell migration to the DRGs[27,36] or the spinal cord[30] in neuropathic females. However, it is critical to identify T cell subtypes present in these key anatomical regions because discrete subtypes exert a distinctly different impact on surrounding tissue during chronic pain. The critical roles of subtypes of T cells within discrete anatomical pain-related regions (peripheral or central) remain unclear.

We hypothesized that peripheral immune and glial responses following peripheral nerve damage are quantitatively and qualitatively (specific immune cells) different between sexes. If true, pain therapy that targets specific immune actions may require distinctly different mechanisms to exert efficacy. Lymphocyte function-associated antigen-1 (LFA-1) is an adhesion molecule expressed on myeloid and T cells and possibly spinal microglia and is critical for immune cell adhesion and migration[37]. In addition to the widely characterized role of LFA-1, emerging evidence suggests that LFA-1 regulates various macrophage proinflammatory functions as well as T cell activation and differentiation[38-43]. Based on existing gaps in understanding sex differences in glial, innate or adaptive immune cell function driving neuropathic pain and the related cytokine/chemokine repertoire, the current study examined whether: (1) the development, magnitude, and duration of mechanical allodynia were different between male and female mice subjected to a well-established peripheral nerve injury (CCI) model; (2) blocking immune cell migration and/or altering the proinflammatory phenotype by preventing peripheral LFA-1 actions using a blood-spinal barrier impermeable small molecule antagonist, BIRT377[42,44,45], reduces allodynia in males and females; and (3) BIRT377 directly modulates myeloid/glial-derived and T cell-related pro- and anti-inflammatory cytokine expression. The results identified differences in the magnitude of immune factor expression contributing to neuropathy between males and females, and that BIRT337 reversed allodynia similarly between males and females, and altered the corresponding expression of sex-specific immune factors.

METHODS

Animals

Experiments were performed using 10-14 week-old C57BL/6 mice (wildtype; FFID: IMSR JAX:000664) purchased from Jackson Laboratories or were bred in-house with parent mice purchased from Jackson Laboratories (Bar Harbor, ME, USA). Age at the time of surgery ranged from 11-12 weeks for males and 10-12 weeks for females. Mice were housed with their cage-mates in groups of 2-5, in temperature (23 ℃ ± 2 ℃) and light (12:12 light:dark; lights on at 6:00 am) controlled rooms, fed standard rodent chow and water *ad libitum*, and acclimated for 1-2 weeks prior to handling. All mice were routinely monitored by the animal care staff under the direction of the institutional veterinarian, with cages and bedding changed every 7 days. Mice were maintained in separate male or female mouse colonies and were behaviorally assessed in separate testing rooms at the University of New Mexico (UNM) Health Sciences Center (HSC) Animal Facility. Pilot studies of behavioral hindpaw threshold responses were conducted to determine whether different phases of the estrous cycle altered behavioral outcomes at baseline (BL) and after surgical manipulation. Despite female mice entering experiments at different phases of the estrous cycle, hindpaw responses remained stable and predictable. Consequently, the stage of the estrous cycle varied and was not considered a key factor influencing hindpaw sensory responses throughout the chronic neuropathy paradigm.

All procedures were approved by the Institutional Animal Care and Use Committee of the UNM HSC, conducted in accordance to the NIH Guidelines for the Care and Use of Laboratory Animals, and closely adhered to recommendations from the International Association for the Study of Pain for the use of animals in research (Foundation for Biomedical Research, The Biomedical Investigator's Handbook for Researchers Using Animal Models. Washington, D.C.: FBR, 1987. WWW: http://www.fbresearch.org/).

Chronic constriction injury

A modification of Bennett and Xie's[46] sciatic nerve (SCN) Chronic constriction injury (CCI) was used after BL hindpaw threshold assessment, as detailed previously[47]. Briefly, following isoflurane anesthesia (induction at 3.0 vol.% followed by 1.5 vol.%-2.0 vol.% in oxygen, 2.0 L/min), the dorsal left thigh was shaved and cleaned using 70% Ethanol (EtOH) that was air dried prior to surgery. Using aseptic procedures, the SCN was exposed using blunt dissection scissors through the muscular fascia. Sterile plastic probes were used to locate and lift the SCN from its position between the muscles. Three, 2 cm-length pieces of chromic gut suture (Ethicon: 4-0 or 5-0, Cat#635H and Cat#634G, respectively) were then snugly tied around the SCN proximal to the trifurcation with care to avoid pinching the nerve, with ~1.5 mm spacing between sutures. Throughout this process, the nerve was kept thoroughly irrigated using isotonic sterile saline (Hospira; Cat#NDC 0409-4888-03). Sham surgeries were performed identically, but without the chromic gut ligation. The nerve was then placed back into its position using the plastic probes, and the muscles were then closed using one 4-0 silk suture (Ethicon; Cat#83G). Skin was closed using two Reflex™ wound clips (Kent Scientific Corp; Cat#INS750344). Total time for the surgical procedure was ~20 min, followed by a ~10 min recovery from anesthesia. Body weight was monitored prior to and after surgery to confirm healthy recovery [Supplementary Figure]. Following surgery, wound condition, hindpaw autotomia, activity levels and grooming appearance were checked routinely (each 1-2 days). Less than 1.0% of animals revealed abnormal recovery and were immediately euthanized when identified.

Behavioral assessment of hindpaw mechanical allodynia

Mechanical allodynia was chosen for investigation because pathological pain intensity (touch sensitivity) occurs clinically at much lower ranges of stimulus intensity compared to that observed when examining mechanical or thermal hyperalgesia. Thus, the impact of clinical allodynia is thought to be much greater[48]. Mice were habituated to the testing environment for ~45 min within the first 4 h of the light cycle, for four periods over the course of one week prior to BL hindpaw assessment. Hindpaw threshold responses to light mechanical stimuli were assessed by adopting principles of the von Frey fiber test originally developed for the rat[49], and modified for the mouse, as recently described in detail[47]. Hindpaw assessment occurred within the first 2 h of the light cycle in testing groups of 4-6, with testers blind to experimental conditions. Each group comprised a minimum of 1-4 mouse/mice per condition to ultimately reach n = 6 mice/experimental condition. Time points for behavior assessments were chosen based on pilot studies and prior reports to capture potential subtle differences during the development of allodynia and BIRT377-mediated pain reversal.

The von Frey test was applied using nine calibrated monofilaments (touch-test sensory evaluator: North Coast Medical; Cat#NC12775) applied for a maximum of 3.0s to the plantar surface of both the left and right hindpaws, with laterality of hindpaw testing occurring randomly, with repeated stimulus presentations to a single animal using a minimum inter-trial stimulus period of 30s. The log intensity of the nine monofilaments used is defined as $\log_{10}$ (grams × 10,000) with the range of intensity being as follows, reported in log (grams): 2.36 (0.022 g), 2.44 (0.028 g), 2.83 (0.068 g), 3.22 (0.166 g), 3.61 (0.407 g), 3.84 (0.692 g), 4.08 (1.202 g), 4.17 (1.479 g), and 4.31 (2.042 g). Testing began using the fiber marked 3.22 with subsequent monofilaments used based on the response/non-response of the mouse to the previous monofilament tested: if no response was elicited by the monofilament stimulus presented (e.g., 3.22), the next "heavier" monofilament was tested (e.g., 3.61); if a response was elicited by the monofilament stimulus presented (e.g., 3.22), the next "lighter" monofilament was tested (e.g., 2.83). A maximum total of six stimulus presentations were applied to each paw. The total number of positive and negative responses were then entered into the computer software program, PsychoFit (http://psych.colorado.edu/~lharvey: RRID: SCR_015381) to determine the absolute withdrawal threshold (50% paw withdrawal threshold), as previously described[50]. The PsychoFit program fits a Gaussian integral psychometric function to the

observed withdrawal rates for each monofilament using a maximum-likelihood fitting method[47,51]. The interpolated 50% withdrawal thresholds were then used for statistical analysis.

BIRT377 preparation

(*R*)-5-(4-bromobenzyl)-3-(3,5-dichlorophenyl)-1,5-dimethylimidazolidine-2,4-dione (BIRT377) was first reported and characterized by Kelly *et al.*[45]. BIRT377 is a small molecule that blocks the active conformational change of the transmembrane β_2-integrin adhesion molecule, leukocyte function-associated antigen-1 (LFA-1), that is expressed on leukocytes (e.g., T cell and myeloid cells)[37]. Upon activation from chemotactic signaling, LFA-1 undergoes a series of conformational changes from a bent inactive position to a progressively straightened and active position, thus allowing binding of LFA-1 with the surface receptor, intercellular adhesion molecule-1 (ICAM-1) expressed on endothelial cells[52]. Upon LFA-1/ICAM-1 interaction, cells expressing LFA-1 (leukocytes) are capable of undergoing transendothelial migration, and subsequently traffic to regions where damage- or pathogen-associated tissue signals arise. Therefore, BIRT377 binding to LFA-1 inhibits LFA-1/ICAM-1 molecular interactions, and prevents circulating leukocyte cell adhesion and migration[44,45,53] to sites of inflammation. BIRT377 abolishes T cell and antigen presenting cell interactions (referred as immune synapse), which is crucial for T cell activation[54]. Moreover, BIRT377 is bioavailable and easier to formulate for oral administration than antibodies against LFA-1[44,45] and BIRT377 is impermeable to blood-spinal cord barrier[42]. Therefore, i.v. injection of BIFT377 is expected to impact: (1) leukocyte migration to peripheral sites and across spinal endothelial cells; (2) macrophage proinflammatory function and possibly T cell differentiation in the periphery; and (3) neuron-to-glial and immune communication in the LSC due to BIRT377-mediated modulation in the periphery during neuropathy.

In an initial experiment, BIRT377 was gifted by HTW and CRW (University of Minnesota, College of Pharmacy, MN, USA), with later experiments where BIRT377 was made commercially available (Tocris; Cat#4776). BIRT377 was initially reconstituted in 200 proof ethyl alcohol EtOH (Sigma-Aldrich; Cat#7023) as a stock solution (22.156 mg/mL), followed by creating aliquots (221.56 μg of BIRT377 in 10 μL), which were stored in a clean sealed container at 4 °C for later use. On the day of intravenous (i.v.) injection, one aliquot was diluted using sterile water (Hospira; Cat# NDC 0409-4887-10), such that each 50 μL i.v. injection contained 2.5 μg BIRT377 (113.089 μM), and vortexed for 2 min. This dose was chosen based on a pilot study of various doses (ranging from 100 ng to 5 μg) that 2.5 μg was the lowest reliably efficacious dose in rats (unpublished data and[42]). Vehicle contained 0.226% EtOH in sterile water. Animals were injected within the hour following BIRT377 dilution.

Intravenous BIRT377 injection

For all experiments characterizing BIRT377 efficacy, i.v. BIRT377 or equivolume vehicle injection into tail veins of unanesthetized mice occurred on Day 10 post-surgery within 2.5 h of the initiation of the light cycle. Using aseptic procedures, 50 μL of BIRT377 or vehicle was collected into individual 1 cc, 27-G 5/8 insulin syringes (Becton Dickinson; Cat#329412). The weight of each mouse was recorded followed by placement for 30 s under a heat lamp with the mouse held in place by the tail and a soft, clean cloth placed over the body to avoid excessive heat to the body while leaving the tail exposed. Heating the tail facilitates tail vein dilation for ease of injection. Each mouse was moved immediately into a plastic restraint with a slit through the top and back so as to allow easy placement of the mouse into the restraint, and proper positioning of the tail. Held firmly in place, a 27-G sterile needle attached to a sterile 1 cc syringe was inserted into the lateral tail vein, followed by a small amount of blood efflux into the syringe hub, with a subsequent 5 s injection. Success of achieving accurate needle placement upon the first attempt was greater than 99%. Following injection, a small piece of sterile gauze was placed over the injection site to stem bleeding, and the mouse was removed from the restraint and placed back into its home cage. All mice appeared normal (e.g., moving, grooming, interacting) following injections. The total time required for handling and injection was less than 2 min without anesthesia.

Sciatic nerve biopsy

Following characterization of the timecourse of hindpaw sensory thresholds from sham or CCI treated mice using either 4-0 or 5-0 chromic gut, the presence of suture material and the condition of the SCN were carefully examined. Following complete return of sensory thresholds similar to BL levels, the ipsilateral SCN was dissected, and the degree of both suture absorption and nerve perturbation was noted. The appearance of each SCN at the time of dissection was documented by photograph.

To accomplish biopsies, animals were euthanized just prior to biopsy using 2% CO_2 in a closed container followed by cervical dislocation. An incision was then made on the ipsilateral skin overlaying the CCI manipulation, and the skin was retracted to expose the underlying muscle and surrounding area, which appeared to be healthy. Blunt dissection scissors were used to re-expose the underlying SCN. The surrounding muscle and remaining sutures and encapsulating sheath were removed, followed by dissection of an approximately 1 cm length of SCN. The nerve segment was then placed next to a ruler (in centimeters), on a clean black surface for documentation.

Tissue collection for RNA and protein analysis

Tissue collection was conducted in six cohorts of mice (8 mice in each cohort, N of 1 from each experimental condition) as previously described[47,51] and modified as described here. Immediately following behavioral analysis on Day 13 post-surgery (Day 3 post-injection), mice were deeply anesthetized under isoflurane (10 min in 5% isoflurane and in oxygen at 2 L/min), followed by rapid transcardial perfusion with ice cold 0.1 M phosphate buffered saline (PBS; pH = 7.4; flow rate 10 mL/min). Following collection of the spleen, mice were placed on a frozen gel refrigerant pack (Glacier Ice, Pelton Shepherd Industries), and the LSC (L3-L6) was dissected, with the dorsal spinal cord ipsilateral and contralateral to the sciatic ligation stored separately. Additionally, lumbar DRG (L4-L6) ipsilateral to the sciatic ligation, and the SCN were dissected. All tissues were immediately placed in DNase/RNase/Protease-free 1.5 mL disposable pellet mixer microtubes (VWR International; Cat#47747-358), briefly spun down, frozen on dry ice, and stored at -80 °C for future analysis.

Total RNA isolation

Total RNA was extracted as described previously[47], with minor modifications as briefly described here. Extraction was performed using the miRNeasy Micro Kit (Qiagen; Cat#217084) per manufacturer's instructions except where noted. Homogenization was performed using a motormlized VWR disposable pellet mixer and cordless motor pestle system (VWR; cordless pestle motor: Cat#47747-370; 1.5 mL microtubes: Cat#47747-362; 1.5 mL pestle: Cat#47747-358; and 1.5 pestle and microtube combo: Cat#47747-366: DRGs only) followed by addition of Qiazol Lysis Reagent (Qiazol; Qiagen; Cat#79306). DRGs were then transferred into microtubes prior to homogenization. Samples were homogenized in Qiazol with the motorized pestle for 60 s, and used for RNA extraction (Qiagen; miRNeasy Micro Kit). SCN and LSC were homogenized prior to aliquoting the tissue into two microtubes for protein or RNA extraction. 100 µL of chilled 1 × phosphate buffered saline (PBS; 10 × PBS diluted to 1 × with DNase/RNase free water; Sigma-Aldrich; Cat#P7059; Cat#W4502 respectively) was added to the tube containing the tissue. The SCN was chopped quickly using scissors for 30 s. Both SCN and LSC were then homogenized with the motorized pestle for 15 s. After initial homogenization, 40 µL of the homogenized solution was removed and placed into a separate 1.5 microtube containing 150 µL of chilled Qiazol and homogenized for an additional 15 s for LSC, or 30 s for SCN, prior to using the miRNeasy Micro Kit for RNA extraction.

Minor changes were incorporated for mRNA extraction using the miRNeasy kits as follows. An initial homogenization in 150-200 µL of Qiazol occurred, with the final volume increased to 700 µL following homogenization. Samples were vortexed and incubated at room temperature (RT) for 7 min, followed by the addition of 140 µL of chloroform (Sigma-Aldrich; Cat#C2432). The samples were then hand-shaken

vigorously for 15 s, incubated for 4 min at RT, hand-shaken vigorously for 10 s, and then centrifuged in 4 °C at 12,000 × *g* for 15 min. A portion, 300 µL, of the aqueous layer was extracted and placed into a clean RNase/DNase/Protease free 1.5 mL tube and 1.5 × aqueous layer (450 µL) of 200 proof EtOH (Sigma-Aldrich; Cat#E7023) was added to tube, pipetted 4-6 × to mix, moved to collection columns, and centrifuged in ~20 °C at 9,000 × *g* for 30 s. This was followed by a wash of 700 µL of RWT (provided with Qiagen kit) and centrifuged (~20 °C at 9,000 × *g*, 30 s), washed 2 × with 500 µL RPE (provided with Qiagen kit) and centrifuged (~20 °C at 9,000 × *g*, 30 s) after each, and washed 2 × with 500 µL 80% EtOH (100% EtOH diluted with sterile RNase/DNase/Protease free water; Sigma-Aldrich; Cat#W4502), and centrifuged (~20 °C at 9,000 × *g*, 2 min) after each. Caps were cut from columns and samples were dried by centrifugation (~20 °C at 20,627 × *g*, 12 min), and placed into RNA collection tubes with 14 µL sterile water (provided with Qiagen kit) added directly to the column filter, and centrifuged (~20 °C at 20,627 × *g*, 1 min). The concentration and quality of the total RNA was assessed by NanoDrop (Thermo Scientific, MA, USA).

mRNA Analysis by Quantitative Real-Time PCR

Total RNA samples were diluted to a standardized RNA concentration: 90 ng/µL for SCN, 70 ng/µL for lumbar dorsal horn, and 100 ng/µL for DRG. Total RNA (0.9-1.2 µg) was used to synthesize cDNA. For reverse transcription (cDNA), SuperScript™ IV VILO™ cDNA Synthesis Kit (Invitrogen) was used per manufacturer's instructions. Levels of mRNA transcripts were measured and analyzed, as previously described[47,55]. The following dilution factors (indicated in parentheses) were applied to cDNA samples for assessment of transcripts of interest in given tissues: ipsilateral and contralateral LSC (1:2.2), ipsilateral SCN (1:2.5), and ipsilateral DRG (1:3). The 1:200 dilutions of cDNA were used for assessment of the normalizer transcripts (18s RNA) for each of the tissue samples. Levels of mRNAs as well as 18s rRNA (*Rn18s*) were assayed in triplicate via quantitative real-time PCR (qRT-PCR) with Taqman Gene Expression Assays (cat# 4351370, ThermoFisher Scientific). In cases of triplicates with standard deviation of more than 0.1, the average value of the two closest replicates were included. All selected gene expression assays were identified by the manufacturer to be the "best coverage" assays, unless otherwise noted, and designed to exclude detection of genomic DNA. mRNA levels were analyzed with the formula: $C = 2^{CT^{Normalizer}}/2^{CT^{Target}}$, as previously described[55,56].

To test whether BIRT377 treatment influenced the inflammatory milieu in collected tissues, the following pain-relevant proinflammatory and anti-inflammatory factors were assessed: C-C motif chemokine ligand 2 (CCL2, *Ccl2*), interleukin-1β (IL-1β, *il1b*), (TNF, TNFα, *Tnf*), interleukin-10 (IL-10, *Il-10*), TGF-β1, *Tgfb1*. Monocyte and T cell-specific cytokines and cellular markers were analyzed: integrin alpha M (CD11b, *Itgam*, a common monocyte/macrophage marker), cluster of differentiation 3 (CD3; expressed on all T cells), forkhead box P3 (FOXP3, *Foxp3*) which is expressed by Treg cells, and Interleukin-17a (IL-17A, *Il-17a*, expressed by proinflammatory Th17 cells)[57-60]. To assess whether BIRT377 treatment may lead to allodynia reversal by modulating glial activation in the LSC, the transcript levels of the microglial specific marker, transmembrane protein 119 (TMEM119, *Tmem119*)[47,61], and the astrocyte activation marker, glial fibrillary acidic protein (GFAP) were evaluated. All 48 samples (96 for ipsilateral and contralateral LSC) and a "no template control" sample for each tissue type was processed for the cDNA preparation or real-time PCRs simultaneously.

Multiplex determination of splenic cytokine and chemokine expression

Frozen spleens were homogenized for 60 s in a buffer with protease inhibitors (MesoScale Discovery) while kept on ice, and subsequently sonicated (settings: 5 pulses, at 50%, Fisher Scientific). Tissue samples were then centrifuged at 4200 × *g* at 4 °C for 10 min to pellet cellular debris. Cellular lysates (supernatant) were collected in a new set of tubes, and protein concentrations were measured by Quickstart™ Bradford Protein Assay Kit (Biorad, CA, USA). Splenic cytokine and chemokine levels were determined using V-Plex™ multiplex immunoassays (MesoScale Discovery), as described previously[47,51,62,63]. Briefly,

calibrators (provided by the kit) or samples (100 μg protein from each experimental sample per well) were loaded onto a "multi-spot" plate in duplicates. Each plate-well is pre-coated with antigen-specific "capture" antibodies on independent, spatially well-defined "spots" that are in turn connected to a working electrode surface. Following incubation with protein lysates, immobilized proteins were recognized by SULFO-TAG™-conjugated antigen-specific "detection" antibodies. Samples were read using a Quickplex SQ120 Imager (MesoScale Discovery).

Preparation of Naïve CD4 T cell suspension

To further investigate whether LFA-1 contributes to T cell differentiation and their functional responses, CD4 T cells were cultured with or without BIRT377 (500 ng/mL). A total of 20 mice (wildtype, FFID: IMSR_JAX:000664; 10 females and 10 males, 8-10 week-old) were compared in this study. In each experiment, 5 male and 5 female mice were used, with two repeat experiments (total of 10 male and 10 female mice). No handling occurred with these mice. Mice were sacrificed with CO_2 asphyxiation, followed by cervical dislocation. Under sterile conditions, spleens and lymph nodes (cervical, inguinal and brachial) were collected in tubes containing ice-cold PBS with 2% fetal bovine serum (FBS; Gibco, Thermofisher Scientific, MA, USA). Spleens and lymph nodes were disrupted using a micro-plunger to press the tissues through a 70 μm nylon mesh. Cells were centrifuged at 300 × *g* for 10 min at 4 °C and resuspended at 1×10^8 nucleated cells/mL in PBS with 2% FBS and 1mM EDTA (ethylene diaminetetraacetic acid). Naïve CD4 T cells ($CD4^+CD44^{low}CD62L^{high}$) were isolated using EasySep™ Naïve CD4 T Cell Isolation Kit, per manufacturer's instructions (Stemcell Technologies, BC, Canada). In this technique, non-naïve T cells were labeled with biotinylated antibodies and magnetic particles, allowing for the collection of desired naïve T cells using an EasySep™ magnet (Stemcell Technologies, BC, Canada). Live cells were counted on a hemocytometer.

CD4 T cell culture with BIRT377

Our prior data demonstrated that BIRT377 (500 ng/mL) induces a switch of stimulated macrophage (RAW264.7) from a proinflammatory bias to an anti-inflammatory state[42]. To examine effects of blocking LFA-1 actions on T cells *in vitro*, isolated male or female derived CD4 T cells were resuspended with complete RPMI media[51] and treated with BIRT377 (500 ng/mL). A 24-well tissue culture plate was pre-coated with anti-CD3 antibody (10 μg/mL in sterile PBS; R&D systems), and stored overnight at 4 °C. The tissue culture plate was pre-warmed and washed 3 × with sterile PBS before use. One million CD4 T cells were plated per well, with 2-3 well-replicates per experimental condition. Cells were cultured with either Th17 or Treg differentiation conditions, as described previously[64,65], with minor modifications. Briefly, in the presence of TCR (T cell receptor) stimulation by anti-CD3 antibody, a cocktail of Th17 polarizing cytokines was applied as follows: anti-mouse CD28 (5 μg/mL), TGF-β1 (2.25 ng/mL), IL-1β (20 ng/mL), IL-6 (30 ng/mL), IL-23 (30 ng/mL), anti- IFNγ (10 μg/mL). For Treg differentiation, a cocktail containing CD28 (2 μg/mL), IL-2 (20 ng/mL), and TGF-β1 (5 ng/mL) was added to the cells. BIRT377 treatment (500 ng/mL) was applied simultaneously with the Th17 or Treg cytokine T cell stimulation mixtures throughout the culture timecourse (4 days). Anti-CD3, anti-CD28, and anti-IFNγ were purchased from eBioScience (Thermofisher Scientific, MA, USA), and TGF-β1, IL-1β, IL-6, IL-23 and IL-2 were purchased form PeproTech (NJ, USA). Pooled CD4 T cells from naïve mice (5 male or 5 female mice maintaining sex-separate tubes) were used for two independent experiments, followed by flow cytometry procedures similar to that detailed in prior reports[66-69].

Intracellular staining and flow cytometry

On Day 4 of T cell differentiation culture, cells were collected, washed with 1 × PBS (300 × *g*, 5 min, 4 °C), kept on ice to prevent cytokine secretion, and stained for intracellular levels of pro- or anti-inflammatory cytokine or transcription factor (protein) production. Proinflammatory markers included retinoid-related orphan receptor-γt (RORγt), the Th17-associated transcription factor, and the cytokines, IL-17A and TNF. Anti-inflammatory markers included the cytokines IL-10 and TGF-β1. Cells were first stained for viability,

surface antigens, and intracellular proteins, as described previously, with minor modifications[51]. Briefly, cells were pelleted into FACS staining tubes (BD Falcon) and incubated with Fc-block (blocking buffer) for 10 min on ice, stained with viability dye (25 min, on ice, dark) and then incubated with CD4 antibody (25 min, on ice, dark). To stain for RORγt and intracellular cytokines (IL-17, TNF, IL-10 and TGF-β1), an intracellular cytokine staining kit (Cat#00-5523-00, eBioScience, Thermofisher Scientific, MA, USA) was used. With this protocol, cells were fixed and permeabilized for 60 min at RT and protected from light. Then cells were washed 2× with permeabilization buffer (2 mL/tube) for 5 min at RT. To prevent non-specific binding of the antibodies, cells were incubated with blocking buffer (containing 2.5 μg anti-mouse CD32 purified antibody) for 15 min on ice. Without washing, fluorochrome conjugated antibodies against RORγt, IL-17A, and TNF (cells from Th17 differentiation wells), or against IL-10 and TGF-β1 (cells from Treg differentiation wells), were added, and cells were incubated for 45 min at RT in the dark. Cells were then washed 2 × again with 2mL of permeabilization buffer at 300 × *g* for 5 min, at 4 °C, resuspended in 300 μL FACs buffer (1 × PBS containing 1% bovine serum albumin and 1mM EDTA), and kept on ice protected from light until data acquisition. Blocking buffer was purchased from BD Biosciences (Fc block, Cat#553141). All the fluorochrome conjugated antibodies were purchased from eBioscience (Thermofisher Scientific, MA, USA) and used at 0.125-1 μg/per 10^6 cells per tube, as recommended by the manufacturer. T cell events (50,000) were collected using a BD LSR Fortessa Cell Analyzer, and later analyzed via FlowJo software v.8.7.4. Only viable (based on light scatter properties and viability dye) CD4 T cells (based on positive staining of surface CD4 antigen) were included for further analysis. Positive staining for transcription factor and/or cytokines were determined based on staining with fluorochrome conjugated isotype controls (IgG2b and IgGa).

Experimental design and statistical analysis

Three independent behavioral experiments were conducted with an $n = 6$ mice in each group in each experiment. Prior work demonstrates $n = 4$-6 mice/experimental condition is sufficient to yield reliable group differences when examining similar endpoints[3,4,13,33-35,47]. The initial experiment was an examination of differences in hindpaw sensitivity between sexes following peri-sciatic manipulations (sham *vs.* 4-0 *vs.* 5-0 CCI) during a 56-days timecourse. Thus, a 2 × 3 repeated measures analysis of variance (ANOVA) was conducted, with hindpaw thresholds assessed at BL and re-assessed every 1, 2, 3, or 5 days after surgical manipulation until complete resolution of allodynia was observed. The behavioral profile of the mice presented in Figure 1 was predicted to inform parameters of Experiment 2. That is, to characterize the earliest maximal onset, stable maintenance and duration of allodynia in both male and female mice. For experiment 2, injection of BIRT377 was administered on Day 10 after surgery, and the efficacy and duration of reversal from allodynia by BIRT377 was assessed. Experiment 2 design was a 2 (male *vs.* female) × 2 (vehicle *vs.* BIRT377) and analyzed by a 2-way repeated measures ANOVA where hindpaw assessment occurred prior to and following BIRT377 treatment. The goal of Experiment 3 was to examine the biochemical profile (protein and mRNA) in discrete tissue systems at a time when BIRT377 exerts maximal efficacy on stable allodynia as determined by Experiment 2. Therefore, Experiment 3 design was a 2 (male *vs.* female) × 2 (sham *vs.* CCI) × 2 (vehicle *vs.* BIRT377) and analyzed by a 3-way repeated measures ANOVA, with re-assessment of hindpaw thresholds terminating at peak BIRT377 efficacy. At this time, spleen, SCN, DRG, ipsilateral and contralateral LSC tissues were dissected and processed.

All behavioral data was graphed in GraphPad Prism version 7.02 (GraphPad Software Inc.; RRID:SCR_002798). All statistics were run using IBM SPSS Statistics version 24 (IBM; RRID:SCR_002865). ANOVAs were performed for data collected at BL and on injection day. For all other behavioral timepoints, repeated measures ANOVA were performed to assess differences of group and timecourse between treatments. The assumption of sphericity was assessed using Mauchly's Test of Spericity ($\alpha = 0.05$) and, if the assumption of sphericity was violated ($P > 0.05$), the reported degrees of freedom and p-values were adjusted using the Greenhouse-Geisser correction to protect against Type I errors[47,51]. Fisher's LSD test was

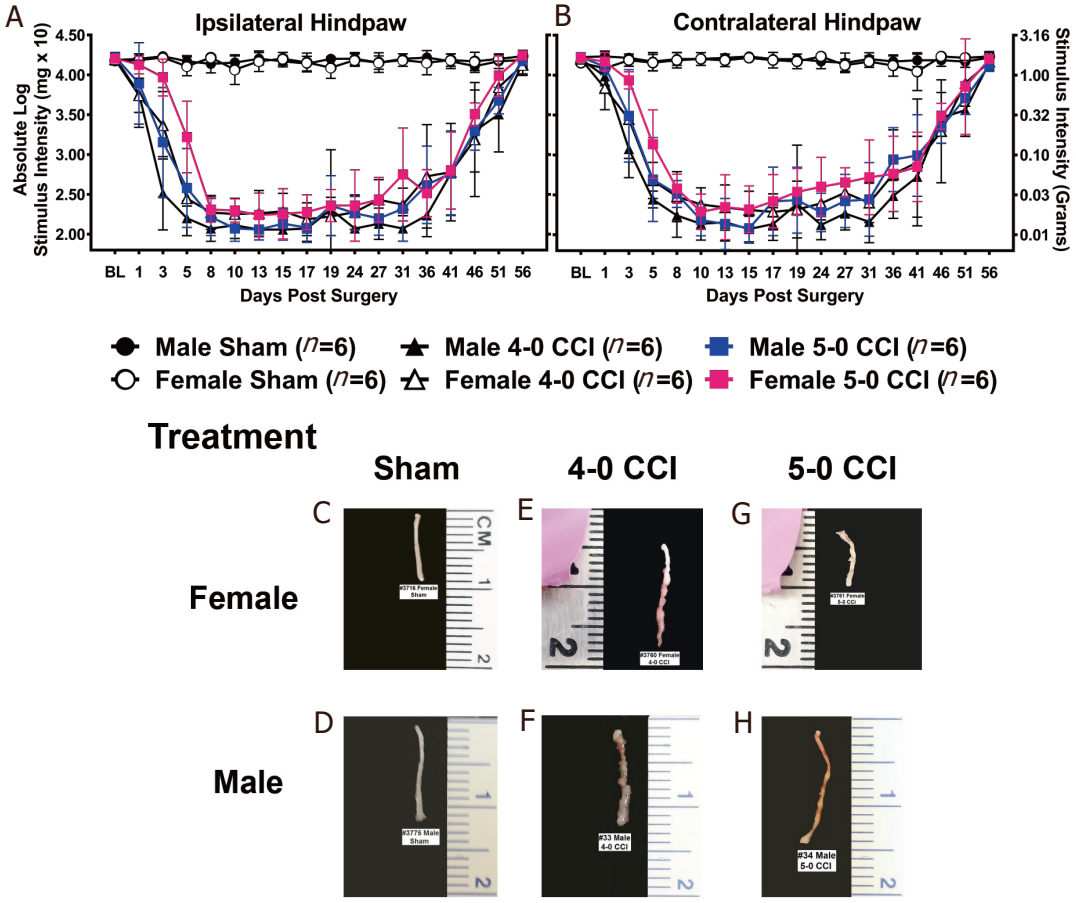

Figure 1. The timecourse of CCI-induced allodynia comparing 4-0 or 5-0 suture material is similar in both males and females. No significant differences were observed between groups at baseline (BL) assessment for hindpaw threshold responses either (A) ipsilateral or (B) contralateral to the sciatic nerve injury. Compared to mice that underwent sham manipulations, all mice with either 4-0 or 5-0 CCI reached maximal bilateral allodynia by Day 8-10 post-surgery and remained stably allodynic through Day 36 post-surgery. Main effects of time on hindpaw responses was observed from BL to Day 8 in the ipsilateral side ($F_{2.4,74.7}$ = 207.56, P < 0.001) or Day 10 in the contralateral side ($F_{2.7,81.5}$ = 212.64, P < 0.001). A main effect of surgery (ipsilateral: Day 8 - 27: $F_{2,30}$ = 591.25, P < 0.001; contralateral: Day 10 - 19: $F_{2,30}$ = 352.59, P < 0.001) was observed from hindpaw responses showing stable allodynia. A gradual spontaneous reversal of hindpaw responses similar to BL values was evident by Day 56 post-surgery, as supported by a main effect of time (ipsilateral: Day 27 - 56: $F_{3.5,105.0}$ = 113.37, P < 0.001; contralateral: Day 19 - 56: $F_{4.3,131.6}$ = 91.55, P < 0.001). Interestingly, sex and suture size had an effect on hindpaw responses only during the onset of allodynia, as shown by the main effect of sex (ipsilateral: BL - Day 8: $F_{1,30}$ = 13.05, P = 0.001; contralateral: BL - Day 10: $F_{1,30}$ = 9.03, P = 0.005). (C-H) Sciatic nerves were biopsied on Day 56 post-surgery. Compared to Sham (C) female and (D) male sciatic nerves, (E) female and (F) male nerves with peri-sciatic 4-0 CCI revealed a translucent sheath and remaining suture material surrounding the injury site, combined with significant discoloration and indentation of the nerves. (G) Female and (H) male mice with peri-sciatic 5-0 CCI revealed diminished or lack of sheath, and minimal suture material surrounding the injury site, combined with far less discoloration and indentation of the nerves. n = 6 for all groups

applied for *post hoc* analysis. Relative mRNA transcript levels from qRT-PCR were analyzed using 3-way ANOVA on GraphPad PRISM version 7.02 or SPSS. To control the type I error rate during all multiple comparisons, Fisher's LSD test (reported with adjusted P values) was applied for *post hoc* examination of possible group differences selected *a priori*. Within-group outliers were detected by Grubbs' Test using the GraphPad QuickCalc Outlier Calculator (https://graphpad.com/quickcalcs/grubbs1/) with α = 0.05.

An *in vitro* tissue culture experiment (Experiment 4) was conducted using CD4 T cells isolated from naïve male and female mice to investigate the effects of BIRT377 on T cell activation and differentiation. No behavioral assessment was performed on these mice. Flow cytometry data from Experiment 4 were analyzed by 2-way ANOVA using Graphpad Prism and Fisher's LSD test for *post hoc* comparisons. The

threshold for statistical significance for all sets of multiple comparisons was set *a priori* to $\alpha = 0.05$. All data are presented as the mean ± Standard Error of the Mean.

RESULTS

Male and female mice with CCI of the sciatic nerve using either 4-0 or 5-0 chromic gut suture material develop allodynia with similar onset, duration and spontaneous recovery

The mouse CCI model has been performed using a range of suture types and sizes[4,47,70,71]. Here, we examined the profile of allodynia using chromic gut suture material of two thickness characteristics (4-0 *vs.* 5-0), whereby the 4-0 suture material is thicker than the 5-0 suture material. Assessment for hindpaw light mechanical touch responses at BL revealed no difference between male or female mice (ipsilateral: $F_{5,30} = 0.78$, $P = 0.576$; contralateral: $F_{5,30} = 2.37$, $P = 0.063$) [Figure 1A and B].

Compared to mice that underwent sham manipulations, mice that underwent CCI surgery developed bilateral allodynia, which replicated similar experiments reported previously[47,51,72-75]. All mice with either 4-0 or 5-0 CCI reached maximal bilateral allodynia by Day 8 (ipsilateral) or Day 10 (contralateral) post-surgery, with main effects of time (ipsilateral: $F_{2.4,74.7} = 207.56$, $P < 0.001$; contralateral: $F_{2.7,81.5} = 212.64$, $P < 0.001$) and surgery (ipsilateral: $F_{2,30} = 141.3$, $P < 0.001$; contralateral: $F_{2,30} = 420.18$, $P < 0.001$), and an interaction between time and surgery (ipsilateral: $F_{4.9,74.7} = 53.99$, $P < 0.001$; contralateral: $F_{5.4,81.5} = 56.28$, $P < 0.001$). Compared to sham-treated mice, stable bilateral hindpaw sensitivity (allodynia) persisted in CCI-treated mice ipsilaterally (Day 8-27) and contralaterally (Day 10-19), as supported by a main effect of surgery (ipsilateral: $F_{2,30} = 591.25$, $P < 0.001$; contralateral: $F_{2,30} = 352.59$, $P < 0.001$). A gradual and spontaneous return to levels similar to BL was observed bilaterally with complete reversal occurring by Day 56, as supported by a main effect of time (ipsilateral: Day 27-56 post-surgery: $F_{3.5,105.0} = 113.37$, $P < 0.001$; contralateral: Day 19-56 post-surgery: $F_{4.3,131.6} = 91.55$, $P < 0.001$) and surgery (ipsilateral: $F_{2,30} = 151.37$, $P < 0.001$; contralateral: $F_{2,30} = 192.63$, $P < 0.001$), and the interaction between time and surgery (ipsilateral: $F_{7.0,105.0} = 27.86$, $P < 0.001$; contralateral: $F_{8.7,131.6} = 21.52$, $P < 0.001$) [Figure 1A and B]. While hindpaw responses between males and females were similar during most of the timecourse following surgery, differences during the initial phase of allodynia were observed. Statistical differences in the onset of allodynia were revealed between males and females, as supported by a main effect of sex (ipsilateral: BL-Day 10 post-surgery: $F_{1,30} = 13.05$, $P = 0.001$; contralateral: BL-Day 10 post-surgery: $F_{1,30} = 9.03$, $P = 0.005$), and between 4-0 and 5-0 chromic gut suture. That is, in comparison with other groups, males with 4-0 chromic gut suture material (thicker than 5-0) developed robust allodynia by Day 3 post-surgery, while females with 5-0 chromic gut suture material did not develop clear maximal allodynia until Day 8 post-surgery.

Reversal from allodynia prior to full reabsorption of the chromic gut suture material was observed in virtually all of the mice treated with CCI, regardless of the chromic gut suture thickness. Representative photographs of each treatment condition (with suture removed) are presented [Figure 1C-H]. Unpublished reports that examined reabsorption of 4-0 chromic gut from the SCN at Day 72 post-surgery in a rat model of CCI revealed variable degrees of reabsorption, and often observed a complete absence of chromic gut material, despite the presence of allodynia[75]. This further supports a published report that by Day 60 and 120 post-CCI in rats, the connective tissue capsule has been resorbed[46]. The current report sought to conduct gross morphological examination of the SCN in the mouse model of 4-0 and 5-0 chromic gut CCI following resolution of allodynia.

The data suggest that, while SCNs from sham-operated mice appear translucent with little discoloration [Figure 1C and D], the nerves treated with 4-0 chromic gut suture were found to possess a sciatic sheath/capsule surrounding the sutures, which was similar between male and female mice [Figure 1E and F]. However, SCNs from mice treated with a 5-0 chromic gut CCI revealed visibly less remaining suture

material and less encapsulating sheath compared to SCNs treated with 4-0 CCI [Figure 1G and H]. Upon further dissection of the sheath and sutures away from the nerve in 4-0 and 5-0 CCI, marked indentations beneath the ligature in both conditions were observed. These observations suggest that reversal of allodynia from CCI in mice involves processes that are independent of the presence of the sutures. That is, the physiological response to peri-sciatic CCI is critical in the resolution of allodynia, and is not dependent on the presence of factors from the suture material itself.

The LFA-1 antagonist BIRT377 reverses allodynia in male and female mice

Given the onset, intensity, and duration of allodynia was similar following either 4-0 or 5-0 peri-sciatic CCI in both males and females, subsequent experiments applied 5-0 chromic gut suture for CCI. Mice were assessed using the von Frey fiber test at BL, and no significant differences were observed. Mice with CCI developed maximal bilateral allodynia by Day 8 post-CCI [Figure 2A]. On Day 10 post-CCI, when all animals revealed stable and maximal allodynia, an i.v. injection of BIRT377 or vehicle was given followed by hindpaw re-assessment. Compared to mice given vehicle, complete reversal from allodynia was observed in both male and female animals following BIRT377 injection. Interestingly, a slight delay and duration of reversal of ipsilateral hindpaw sensitivity was observed in females compared to males. Specifically, BIRT377-mediated reversal of allodynia was delayed by 24 h in female mice, with allodynia returning 24 h earlier than their male counterparts. Contralateral hindpaw sensitivity was reduced by BIRT377 treatment to a similar degree and magnitude between males and females, as no statistical differences were observed. While it is clear that both male and female mice develop allodynia to the same degree with a similar duration, the difference in their response to i.v. BIRT377 suggests that the underlying processes leading to allodynia may not simply overlap, but instead may include distinct mechanisms between male and female mice.

In an effort to expand on characterizing potential sex-dependent differences in expression levels of peripheral immune signaling molecules (pro- and anti-inflammatory cytokines) during established peripheral neuropathy or BIRT377-induced reversal from neuropathy, a separate experiment was conducted to replicate the effect of BIRT377 on allodynia which was terminated at the peak of BIRT377 mediated pain reversal [Figure 2B] and tissues were collected for protein or mRNA (represented in subsequent figures) analysis. In this replication study, while differences in ipsilateral, but not the contralateral hindpaw threshold responses were observed at BL ($F_{7,40}$ = 2.27, P = 0.048), these differences may simply be due to an exceptionally small variance in the threshold responses of female mice compared to males. However, these differences are not considered physiologically meaningful, as such variance was not observed previously or routinely in either the ipsilateral or contralateral hindpaws. Compared to sham-operated animals, male and female mice with CCI developed clear allodynia through Day 10 [Figure 2B]. A main effect of time (ipsilateral: $F_{2.22,88.85}$ = 265.36, P < 0.001; contralateral: $F_{2.11,84.20}$ = 213.38, P < 0.001) and surgery (ipsilateral: $F_{1,40}$ = 1612.46, P < 0.001; contralateral: $F_{1,40}$ = 978.01, P < 0.001), and an interaction between time and surgery (ipsilateral: $F_{2.22,88.85}$ = 240.45, P < 0.001; contralateral: $F_{2.11,84.20}$ = 200.82, P < 0.001) was observed. Following BIRT377 treatment, while sham animals remained stably responsive and close to BL thresholds throughout the timecourse, partial bilateral reversal from allodynia was observed by 24 h in males, but not females. Additionally, maximal effects of BIRT377 on allodynia were observed a full day sooner in males than in females [Figure 2B]. Main effects of time (ipsilateral: $F_{3,120}$ = 65.14, P < 0.001; contralateral: $F_{3,120}$ = 71.58, P < 0.001), injection (ipsilateral: $F_{1,40}$ = 218.80, P < 0.001; contralateral: $F_{1,40}$ = 306.81, P < 0.001), and surgery (ipsilateral: $F_{1,40}$ = 1818.98, P < 0.001; contralateral: $F_{1,40}$ = 1816.36, P < 0.001), and interactions between time and sex (ipsilateral: $F_{3,120}$ = 5.31, P = 0.002; contralateral: $F_{3,120}$ = 2.86, P = 0.040), time and injection (ipsilateral: $F_{3,120}$ = 62.77, P < 0.001; contralateral: $F_{3,120}$ = 69.52, P < 0.001), and sex and injection (ipsilateral: $F_{1,40}$ = 16.08, P < 0.001; contralateral: $F_{1,40}$ = 10.70, P = 0.002) were observed. However, by Day 3 post-injection, both males and females achieved similar levels of reversal from allodynia.

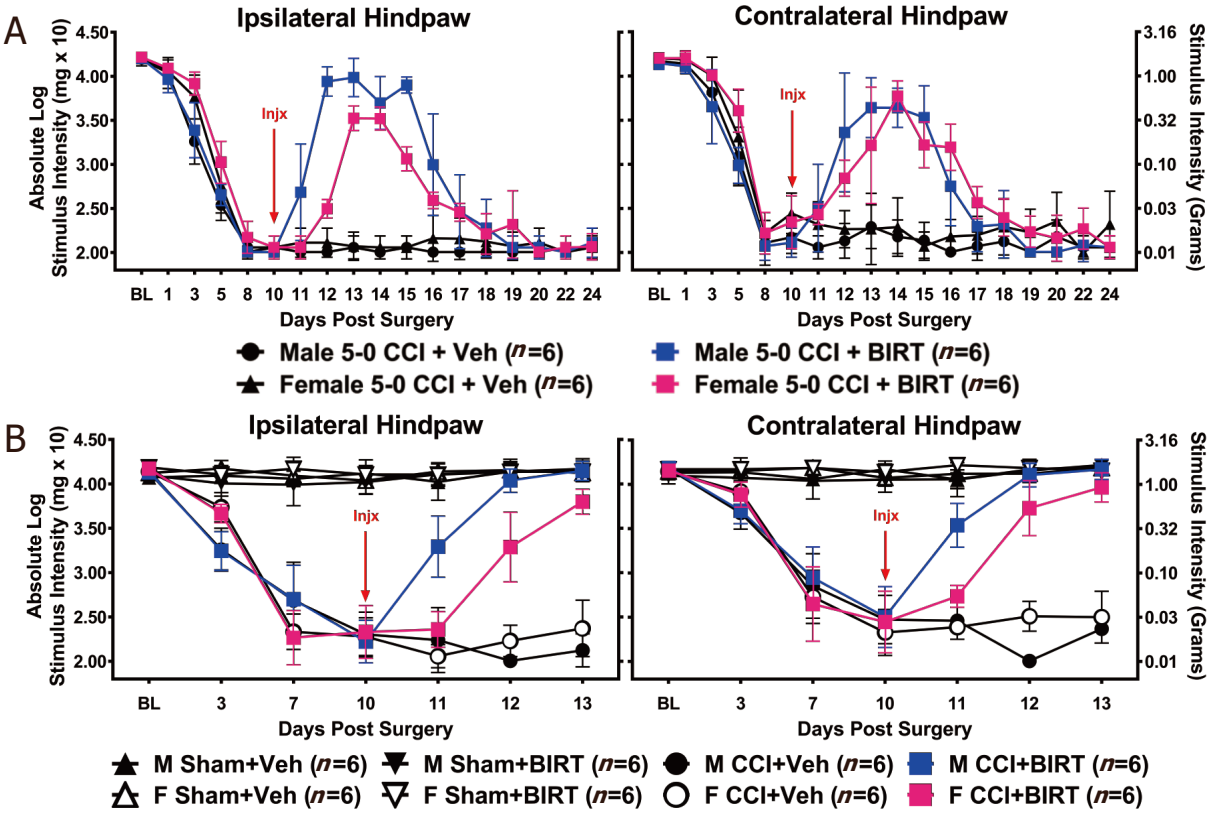

Figure 2. The LFA-1 antagonist, BIRT377, similarly reverses allodynia in males and females. Mice were either sham or treated with perisciatic 5-0 suture. (A) All groups of mice show similar BL threshold hindpaw sensitivity. Following 5-0 CCI, all animals develop clear allodynia during an 8-day timecourse, showing stable allodynia on Day 10, with a main effect of time (ipsilateral: $F_{3.02,60.48}$ = 1003.02, $P < 0.001$; contralateral: $F_{3.56,71.15}$ = 528.60, $P < 0.001$). Additionally, a main effect of sex during the development of allodynia is observed (ipsilateral: $F_{1,20}$ = 21.27, $P < 0.001$; contralateral: $F_{1,20}$ = 13.06, $P = 0.002$), with a significant interaction between time and sex (ipsilateral: $F_{3.02,60.48}$ = 10.899, $P < 0.001$; contralateral: $F_{3.56,71.15}$ = 3.23, $P = 0.021$). Following injections on Day 10 post-surgery, clear reversal from allodynia resulting from BIRT377 injection is observed compared to vehicle treated mice, supported by a main effect of injection (ipsilateral: $F_{1,20}$ = 328.97, $P < 0.001$; contralateral: $F_{1,20}$ = 74.47, $P < 0.001$). In addition, male mice treated with BIRT377 appeared to reverse from allodynia 1 day sooner than female BIRT377-treated mice, as observed in hindpaw responses ipsilateral ($F_{1,20}$ = 12.12, $P = 0.002$) but not contralateral to the CCI, with an interaction between time and sex (ipsilateral: $F_{4.33,86.61}$ = 9.33, $P < 0.001$; contralateral: $F_{4.92,98.34}$ = 3.15, $P = 0.012$), and time and injection (ipsilateral: $F_{4.33,86.61}$ = 70.29, $P < 0.001$; contralateral: $F_{4.92,98.34}$ = 32.77, $P < 0.001$). (B) Experimental replication of BIRT377 reversal in males and females following CCI, with the onset and full development of bilateral allodynia occuring during a 10-day timecourse. Female mice reveal delayed onset of allodynia but no sex differences are observed by Day 10 post-surgery, when maximal allodynia is observed in both males and females. As previously observed, female 5-0 CCI mice treated with BIRT377 displayed slightly slower reversal from allodynia compared to males, with maximal bilateral reversal observed by Day 3 post-injection. n = 6 for all groups

Characterization of sciatic nerve anti- and proinflammatory cytokine/chemokine mRNA levels in males and females following BIRT377 treatment

Prior studies suggest contralateral allodynia referred to as "mirror pain" corresponds to pathological events at the spinal cord[22,72,73,76-81]. In the current study, inflammatory cytokine changes were examined in the ipsilateral SCN and DRGs, as well as in both the ipsilateral and contralateral LSC dorsal horn to complement prior reports. In the ipsilateral SCN, mRNA levels of the proinflammatory cytokines, CCL2, IL-1β and TNF, were robustly elevated in both males (CCL2, IL-1β and TNF: $P < 0.0001$) and females (CCL2: $P = 0.039$; IL-1β: $P = 0.0007$; TNF: $P = 0.0006$), compared to the corresponding sham-treated controls [Figure 3A-C]. A greater magnitude of CCL2 ($P = 0.015$) and IL-1β ($P < 0.0002$) increase was observed in CCI + Veh males when compared to CCI + Veh females. Treatment with BIRT377 in CCI-operated mice (CCI + BIRT) revealed a reduction in CCL2 in males ($P = 0.006$), and in both males and females, a reduction in IL-1β (male: $P < 0.0001$; female: $P = 0.049$) and TNF (male: $P = 0.0001$; female: $P = 0.022$) mRNA levels, with the largest magnitude

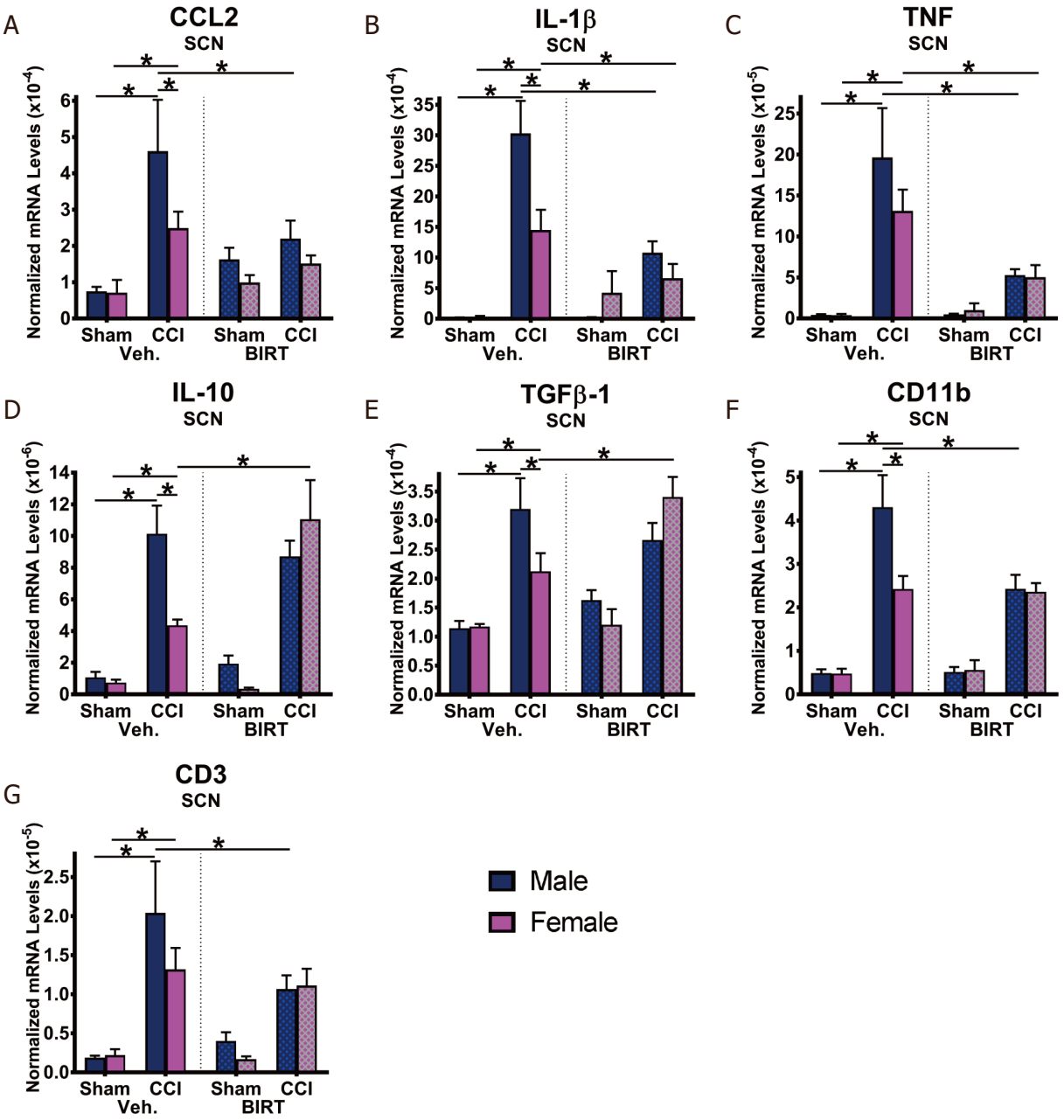

Figure 3. BIRT377 treatment reduced pro-inflammatory cytokine/chemokine in males and females and increased anti-inflammatory cytokines only in females around the injured sciatic nerve. Ipsilateral sciatic nerves were collected from behaviorally verified mice as represented in Figure 2B. (A) Sciatic nerve damage (CCI) induced a significant increase in CCL2 mRNA expression ($F_{1,1}$ = 16.33, P = 0.0002), which was greater males than females ($F_{1,1}$ = 4.36, P = 0.043). BIRT377 treatment reduced CCL2 mRNA expression in male mice with CCI ($F_{1,1}$ = 7.43, P = 0.009). (B) CCI increased IL-1β mRNA expression ($F_{1,1}$ = 54.46, P < 0.0001), which was greater in males than females ($F_{1,1}$ = 9.68, P = 0.003). BIRT377 treatment reduced IL-1β mRNA levels in mice with CCI ($F_{1,1}$ = 16.3, P = 0.0002). (C) Similarly, after CCI, TNF mRNA expression was elevated ($F_{1,1}$ = 35.85, P < 0.0001). BIRT377 treatment reduced TNF mRNA expression in mice with CCI ($F_{1,1}$ = 11.53, P = 0.001). (D) Following CCI, IL-10 mRNA was dramatically increased ($F_{1,1}$ = 83.79, P < 0.0001). BIRT377 treatment further induced IL-10 mRNA expression in females, as a significant interaction between BIRT377 treatment and sex ($F_{1,1}$ = 4.35, P = 0.04) was observed. (E) CCI induced an increase in TGF-β1 mRNA expression ($F_{1,1}$ = 52.7, P < 0.0001). BIRT377 treatment further increased TGF-β1 in female s with CCI ($F_{1,1}$ = 6.98, P = 0.012). (F) After CCI, CD11b mRNA levels were increased ($F_{1,1}$ = 104.2, P < 0.0001). Following CCI, males displayed greater levels of CD11b mRNA than females ($F_{1,1}$ = 4.58, P = 0.038). BIRT377 treatment reduced CD11b mRNA levels in male mice with CCI ($F_{1,1}$ = 4.884, P = 0.032) but not females, as a main effect of sex was observed for CD11b mRNA levels ($F_{1,1}$ = 4.22, P = 0.046). (G) CD3 mRNA levels were elevated in mice with CCI ($F_{1,1}$ = 34.3, P < 0.0001). Post hoc comparisons revealed that BIRT377 treatment reduced CD3 levels in males with CCI (P = 0.016). *p values from post hoc comparisons ranges from P = 0.039 to P < 0.0001. n = 5 in female Sham + Veh and CCI + Veh for TGF-β1 data. n = 6 per group unless otherwise indicated

of changes observed in males. mRNA levels of the anti-inflammatory cytokines, IL-10 (male: $P < 0.0001$; female: $P = 0.034$) and TGF-β1 (male: $P = 0.0001$; female: $P = 0.046$) were increased in CCI + Veh mice compared to sham-operated conditions (Sham + Veh) [Figure 3D and E]. These data reflect the predicted peri-sciatic anti-inflammatory compensatory response to control ongoing inflammation at the injured SCN [47,51]. Neuropathic females had lower levels of IL-10 mRNA than their male counterparts (CCI + Veh: $P = 0.001$). Interestingly, while BIRT377 treatment in neuropathic males did not induce further increases in these anti-inflammatory cytokines, notable mRNA increases in both IL-10 ($P = 0.002$) and TGF-β1 ($P = 0.006$) were measured in females [Figure 3D and E]. Additionally, because low-level cytokine/chemokine expression remains unaltered in sham-surgery animals given BIRT377 (Sham + BIRT), these data suggest a permissive effect of BIRT377's action in and around activated peripheral immune cells that have already migrated to the local site of injury in the female SCN microenvironment.

To confirm the presence of the monocytes/macrophages and T cells, which are well-characterized to produce CCL2, IL-1β, TNF, IL-10 and TGF-β1, mRNA levels for CD11b (pan myeloid cell marker) and CD3 (pan T cell marker) were evaluated [Figure 3F and G]. While all neuropathic mice (CCI + Veh) reveal significant increases in peri-sciatic CD11b (male: $P < 0.0001$; female: $P = 0.0001$) and CD3 (male: $P < 0.0001$; female: $P = 0.005$) mRNA levels, reflecting that these peripheral immune cells have migrated to the damaged SCN, the magnitude of increase was greater in males than females for CD11b ($P = 0.0002$). Sham animals treated with BIRT377 did not result in alterations of CD11b and CD3 mRNA levels. However, BIRT377 did significantly reduced mRNA levels of CD11b ($P = 0.0002$) and CD3 ($P = 0.016$) in neuropathic males. These data indicate that by 4 days following i.v. BIRT377 injection, a reduction in both monocyte/macrophage and T cell recruitment around the injured nerve occurred in males. Importantly, these data demonstrate that despite similar levels of CD11b and CD3 mRNA in CCI + Veh and CCI + BIRT females, TNF and IL-1β are reduced, indicating that BIRT377 alters functional responses of immune cells previously recruited around the SCN in females. That is, BIRT377 may be exerting actions on immune cells beyond simply preventing leukocyte trafficking. Given the observed elevation in anti-inflammatory IL-10 and TGF-β1 mRNA levels and reduction in proinflammatory cytokines discussed above, BIRT377 may be dampening the degree of peripheral "damage" signals relayed from the peripheral nervous system to the central nervous system (CNS).

Sex differences observed in the effects of BIRT377 on reduced mRNA levels of T cell-specific pro- and anti-inflammatory responses

Previous reports demonstrate potential differential contribution of T cell-mediated responses in males and females[3]. In the current report, the T cell specific factors, FOXP3 (anti-inflammatory-like T cells) and IL-17A (proinflammatory-like T cells) were analyzed in key anatomical regions of the pain pathway following CCI [Figure 4]. A potential cellular source of anti-inflammatory cytokines is from a subset of T cells referred to as Tregs cells[57,82]. The transcription factor responsible for generating Treg cells is FOXP3[57,59]. Therefore, to identify a possible source of IL-10 and TGF-β1 (demonstrated in Figure 3D and E), the contribution of Treg cells was indirectly explored by examining FOXP3 mRNA levels. Compared to sham treatment, elevated FOXP3 mRNA was observed in SCN of males ($P < 0.0001$) and females ($P = 0.003$) given vehicle. FOXP3 mRNA levels were further elevated from CCI + Veh group following BIRT377 treatment, only in females ($P < 0.0001$) [Figure 4A]. Similarly, DRGs revealed elevated FOXP3 mRNA levels in CCI + Veh females ($P = 0.034$), while no such increases were observed in males [Figure 4B]. However, BIRT377 did not alter basal FOXP3 mRNA levels in DRG in sham or CCI groups. These data indicate a modest recruitment of Tregs in female DRGs. In contrast to effects observed in females of peripheral tissues (SCN and DRG), only males revealed changes in FOXP3 mRNA levels in the ipsilateral spinal cord, with no FOXP3 mRNA changes observed in the contralateral spinal cord [Figure 4C and D]. Th17-specific proinflammatory cytokine, IL-17A, increases were detected in SCN of both neuropathic males ($P = 0.04$) and females ($P < 0.0001$), when compared to sham-surgery groups [Figure 4E]. Neuropathic (CCI + Veh) females displayed about twice as much upregulation

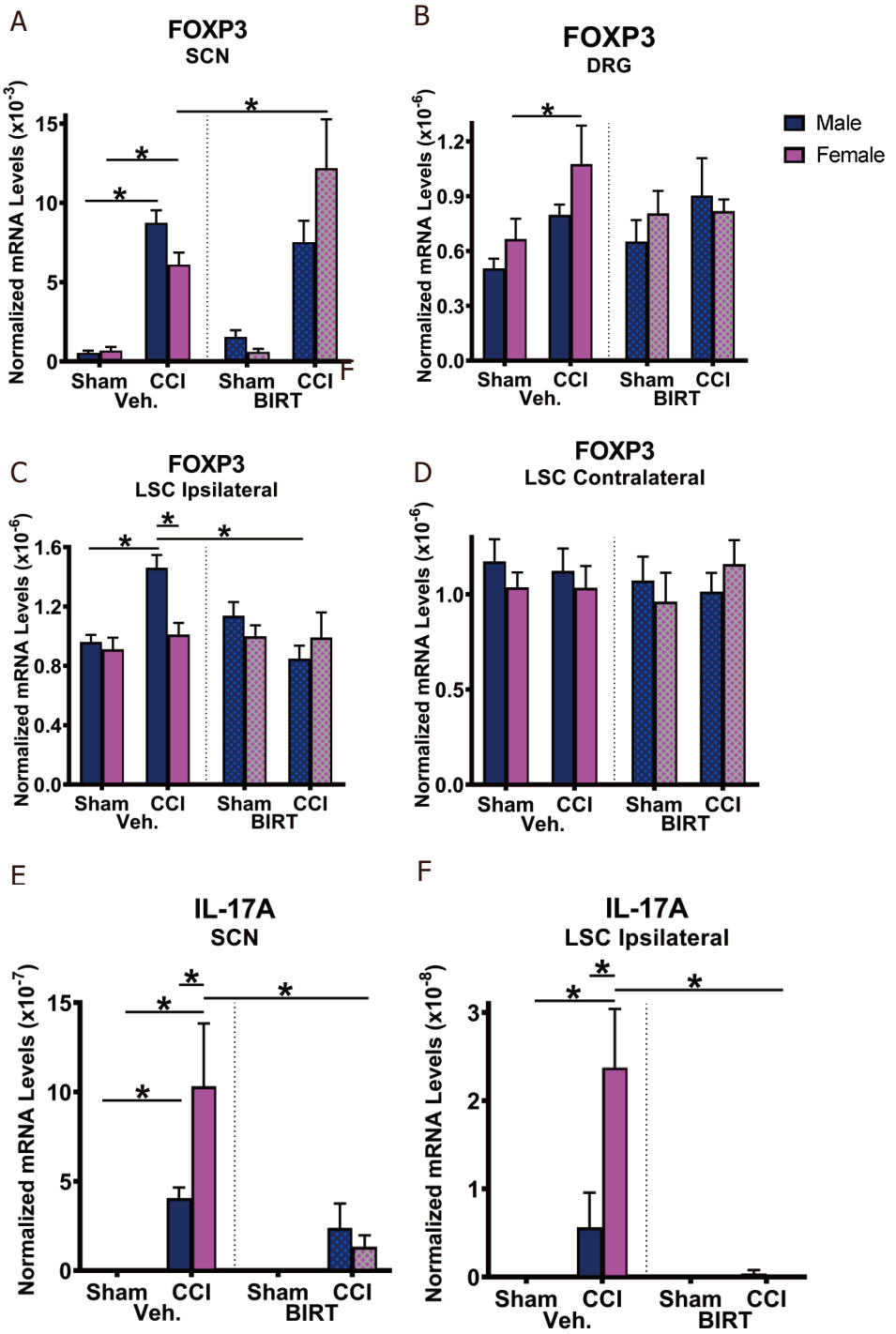

Figure 4. Sex differences in anti-inflammatory FOXP3 and proinflammatory IL-17A from damaged sciatic nerve and in spinal cord. Tissues were collected from behaviorally verified mice as represented in Figure 2B. (A) From ipsilateral sciatic nerve (SCN), CCI induced a significant increase in FOXP3 mRNA expression ($F_{1,1}$ = 76.18, P < 0.0001), with CCI-treated females responding to BIRT377 treatment to a greater degree than males ($F_{1,1}$ = 5.38, P = 0.025). (B) In the ipsilateral DRGs, FOXP3 mRNA levels were elevated following CCI ($F_{1,1}$ = 6.55, P = 0.014), post hoc comparisons revealed significant increases in FOXP3 mRNA levels in females with CCI compared to the sham controls. (C) In the ipsilateral lumbar spinal cord (LSC) dorsal horn, post-CCI induction in FOXP3 mRNA levels was observed in males (P = 0.0006). Following CCI, males displayed significantly greater FOXP3 mRNA levels than in females (P = 0.001). BIRT377 treatment reduced FOXP3 in males with CCI (P < 0.0001), a significant interaction between surgery, injection, and sex ($F_{1,1}$ = 6.5, P = 0.014) was observed. (D) In the contralateral dorsal horn, FOXP3 levels were comparable between groups. (E) Post-CCI IL-17A mRNA levels were significantly elevated at the ipsilateral sciatic nerve ($F_{1,1}$ = 21.93, P < 0.0001). BIRT377 treatment reduced post-CCI IL-17A mRNA levels ($F_{1,1}$ = 21.93, P < 0.0001), with post hoc comparisons revealing a significant reduction of IL-17A mRNA levels following BIRT377 treatment in females with CCI. (F) In the ipsilateral dorsal horn, post-CCI IL-17A mRNA levels were elevated ($F_{1,1}$ = 14.85, P = 0.0004). BIRT377 treatment reduced IL-17A mRNA levels in mice with CCI ($F_{1,1}$ = 14.07, P = 0.0006), which occurred in females to much a greater degree than males ($F_{1,1}$ = 5.71, P = 0.02). Post-CCI induction in IL-17A mRNA levels were much greater in females, than in males ($F_{1,1}$ = 5.23, P = 0.02). *p values from post hoc comparisons ranges from P = 0.04 to P < 0.0001. n = 5 in female CCI + BIRT data for DRG FOXP3. n = 6 per group unless otherwise indicated

of IL-17A than neuropathic males (P = 0.002). These data suggest that Th17 cells may play a more prominent role in females with peripheral neuropathy. This is further supported by BIRT377-mediated reduction of IL-17A mRNA levels in SCNs of pain-reversed females (P < 0.0001). Spinal IL-17A mRNA transcripts were absent under non-neuropathic sham-treated conditions in males and females [Figure 4F]. Compared to sham controls, large increases in LSC IL-17A mRNA levels were observed ipsilaterally in neuropathic females (P < 0.0001), with modest increases in IL-17A in neuropathic males [Figure 4F]. IL-17A was not reliably detected in the DRGs or contralateral spinal cord samples in any groups. BIRT377 treatment abolished ipsilateral spinal IL-17A mRNA levels in neuropathic females (P < 0.0001) and, to a lesser extent, in neuropathic males. These data, along with the data presented in Figure 3G suggest that though there was no difference in overall content of the T cell (CD3 mRNA) population, the quality and differentiation status of these T cells varied in neuropathic animals. These data also show that the actions of BIRT377 on these differentiated T cell subsets is most pronounced in females and may reflect a phenotypic change from proinflammatory to anti-inflammatory, rather than simply reflecting a suppression of T cell recruitment.

BIRT377 treatment exerts sex-dependent differential effects on T cell differentiation and functional responses

While BIRT377-mediated reduction of IL-17A is indicative of effects of BIRT377 on CD4 T cell differentiation and function, these effects may also be due to the indirect effects of a general reduction in proinflammatory cytokine production (such as TNF) from monocytes that promote Th17 differentiation[83]. Therefore, to examine the direct actions of BIRT377 on CD4 T cell differentiation and function, CD4 naïve T cells were given conditioned media to induce the generation of either a Treg or Th17 phenotype, in the presence of control (media only) or BIRT377. Subsequently, the proportion of T cells positive for RORγt$^+$ (transcription factor required for the generation of Th17 cells), was analyzed. BIRT377 only reduced the generation of RORγt$^+$ T cells in females (P = 0.0007), but not in males [Figure 5A]. Additionally, CD4 T cells that are IL-17A$^+$, and also produce TNF, were examined as an indication of their functional proinflammatory capacity. Compared to conditioned media alone, the population of IL-17A$^+$TNF$^+$ CD4$^+$ T cells was substantially reduced by BIRT377 exposure only in CD4$^+$ T cells derived from females (P = 0.001), but not males [Figure 5B]. Furthermore, Treg generation and function in the presence of BIRT377 was examined. Fully differentiated Tregs exert their immune suppressive actions by producing the characteristic anti-inflammatory cytokines, IL-10 and TGF-β1[57]. Therefore, the expressions of IL-10 and TGF-β1 proteins were examined as direct evidence of the fully differentiated functional Treg cells. Given that FOXP3 drives Treg generation concurrent with IL-10 and TGF-β1 production, FOXP3 expression was considered redundant. During Treg differentiation in the presence of BIRT377, a large increase in the production of the IL-10 (P = 0.0005) and TGF-β1 (P = 0.014) was observed [Figure 5C and D] in female-derived pooled T cells, while BIRT377-induced changes in these anti-inflammatory cytokines were absent in male derived T cells. While a trend of increased IL-10$^+$ CD4$^+$ T cells was also observed in males [Figure 5C], these data demonstrate that female T cells are much more responsive to BIRT377-mediated modulation of pro- and anti-inflammatory T cell-related cytokines. Therefore, BIRT377 regulates one aspect of T cell differentiation and function more readily in females under pathological conditions, which may provide a mechanism for the IL-17A reduction reliably detected only in females at the SCN [Figure 4].

BIRT377 modulates the proinflammatory cytokine milieu in the DRGs to favor pain reversal

The most widely examined proinflammatory cytokines known to be critical for pain processing were examined in the ipsilateral DRGs. As predicted, neuropathic male and female (CCI + Veh) mice revealed increases in CCL2 (male: P = 0.01; female: P = 0.016), IL-1β (males: P = 0.0005; female: P < 0.0001), TNF (P < 0.0001: both sexes). In support of prior reports, a compensatory elevation in anti-inflammatory IL-10 (P < 0.0001: both sexes) mRNA levels in the ipsilateral DRGs as compared to Sham + Veh was also measured [Figure 6]. While CCL2 mRNA levels were increased in males and females following CCI, BIRT377 did not alter CCL2 mRNA levels under either condition, and sex differences were not observed. However, a reduction in IL-1β

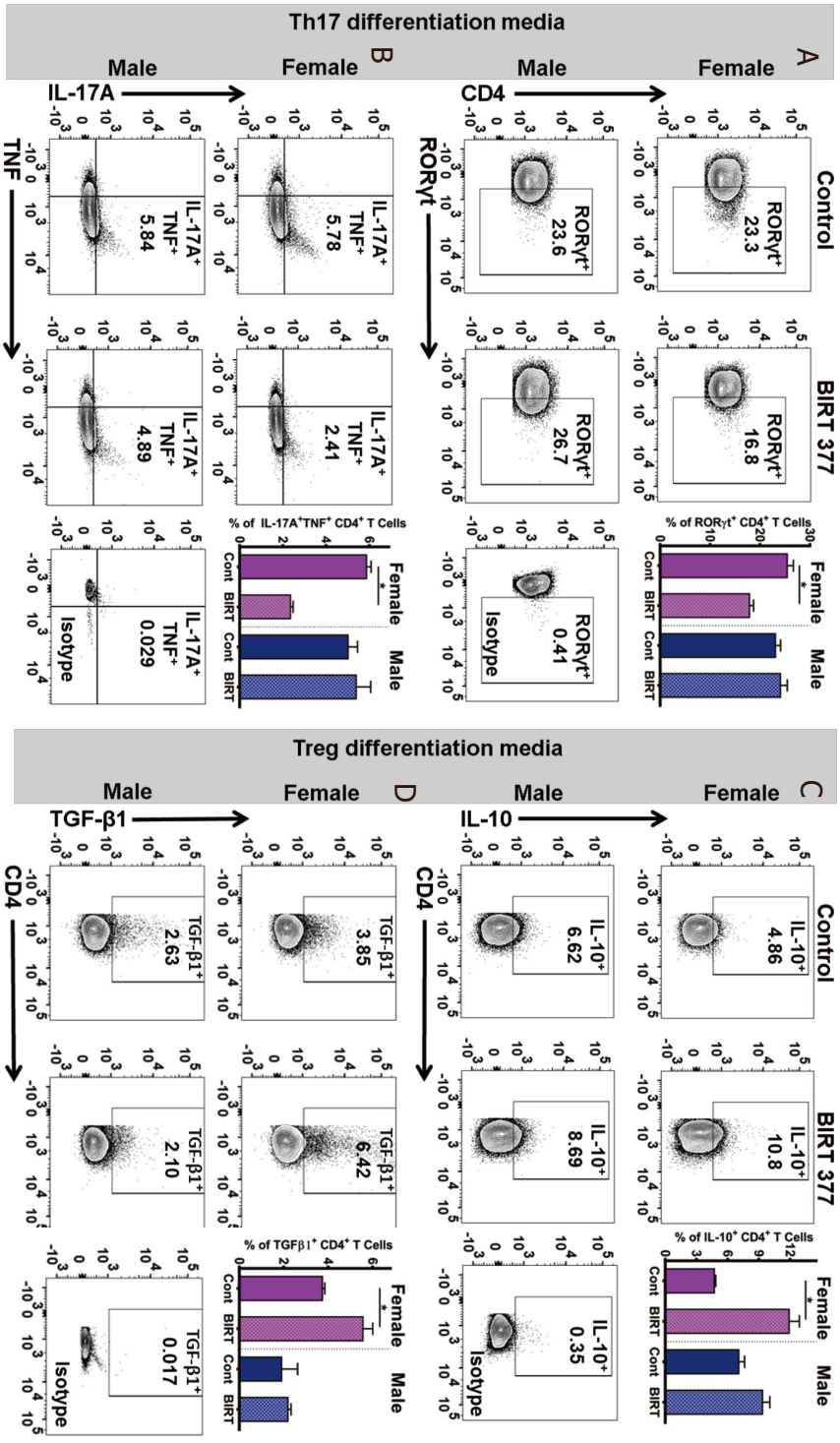

Figure 5. Flow cytometric characterization of *ex vivo* T cells: sex differences in the anti-inflammatory response to BIRT377. Naïve CD4 T cells were conditioned with (A-B) Th17 or (C-D) Treg inducing cytokines, with or without BIRT (500 ng/mL). After 4 days, all viable CD4 T cells were identified and analyzed for the expression of: (A) RORɣt (major transcription factor for Th17 cells) or (B) intracellular levels of pro-inflammatory cytokines IL-17A and TNF. BIRT377 treatment reduced RORɣt$^+$ CD4 T cells ($F_{1,8}$ = 17.99, P = 0.002) and IL-17A protein production ($F_{1,8}$ = 24.3, P = 0.001) in females. (C-D) From Treg inducing culture, all viable CD4 T cells were analyzed for intracellular levels of (C) TGF-β1 and (D) IL-10. BIRT377 treatment increased intracellular TGF-β1 ($F_{1,5}$ = 12.85, P = 0.015) and IL-10 ($F_{1,6}$ = 10.57, P = 0.017) protein levels in females. (A-D) Representative flow cytometry plots are shown. Numbers represent the percentages of the (A) RORɣt or (B-D) cytokine positive CD4 T cells, where total CD4 T cells are taken as 100%. Corresponding isotype controls (stained with IgG, IgG2a or IgG2b fluorochrome conjugated antibody) for the intracellular staining are shown. Each experimental condition was run in 2-3-well replicates. Error bars represent variations in the well replicates. Data are representative of two independent experiments where T cells were pooled from n = 5 males or n = 5 females in each experiment. Viable cells were identified based on their light scatter properties (forward and side scatter plot) and viability dye staining. Viable cells were then gated for CD4 cell surface expression; only CD4$^+$ T cells were included for further analysis. Positive staining for transcription factor and/or cytokines were determined based on staining with fluorochrome conjugated isotype controls (negative controls). *P values from post hoc comparisons ranges from P = 0.014 to P = 0.0005

(male: $P = 0.029$; female: $P < 0.0001$) and TNF (males: $P = 0.001$) mRNA levels were measured in allodynic-reversed mice given BIRT377. Unexpectedly, no further increases in IL-10 mRNA levels were observed in BIRT377-treated allodynic-reversed mice. It is notable that the magnitude of IL-1β increase was greater in female CCI + Veh mice ($P = 0.0002$) than males. Correspondingly, the magnitude of BIRT377-induced decreases in IL-1β was greatest in female CCI + BIRT mice [Figure 6B]. In general, the effects of BIRT377 on these pro- and anti-inflammatory cytokines revealed similar trends in both male and female DRGs. Together, these data support that BIRT377 not only affects immune cells at the nerve injury, but also is able to modulate immune cells locally in the DRGs thereby dampening the proinflammatory environment contributing to pain reversal.

BIRT377 predominantly restores IL-10 levels in the dorsal spinal cord

It is possible that BIRT377-mediated changes in cytokine mRNA levels at the damaged SCN and the DRG together influence the inflammatory signals ultimately relayed to the spinal cord dorsal horn where critical pain relays can be facilitated by spinal glial and resident immune cells. Moreover, it is reasonably possible that BIRT377 additionally modulates leukocyte adhesion and spinal trafficking, thereby controlling the peripheral leukocyte milieu recruited to the spinal cord as a consequence of nerve injury. Therefore, spinal mRNA levels of CCL2 was assessed, as CCL2 is a well-established chemokine released from damaged neurons that signals to circulating leukocytes (macrophages as well as subsets of T cells) facilitating immune cell migration to the spinal cord. As predicted, a significant induction of CCL2 mRNA was observed in the dorsal horn of the LSC ipsilateral to the SCN lesion both in males ($P = 0.014$) and females ($P < 0.0001$), with a trend toward increased CCL2 in LSC contralateral to the SCN lesion in CCI females compared to Sham conditions [Figure 7A and B]. Interestingly, CCL2 mRNA levels were similar in neuropathic males given vehicle or BIRT377, whereas a significant bilateral reduction of CCL2 was observed in females given BIRT377 (LSC ipsilateral: $P = 0.0006$; LSC contralateral: $P = 0.04$) [Figure 7A and B]. In support of prior reports documenting the crucial role of IL-1β actions in mediating allodynia, a small but significant increase in the levels of IL-1β mRNA were observed in female CCI + Veh ($P < 0.0001$), with a similar trend observed from the contralateral side. Unexpectedly, BIRT377 treatment did not change IL-1β mRNA levels in the spinal cord [Figure 7C and D].

Anti-inflammatory cytokines TGF-β1 and IL-10 were analyzed in the LSC both ipsilateral and contralateral to the SCN lesion [Figure 7E-H]. Compared to Sham + Veh, a significant induction in ipsilateral TGF-β1 (male: $P = 0.0004$; female: $P = 0.013$) and reduction of IL-10 (male: $P = 0.0001$; female: $P < 0.0001$) mRNA levels were measured in all CCI + Veh mice [Figure 7E and G]. However, following BIRT377 treatment, ipsilateral LSC IL-10 mRNA levels were elevated in both neuropathic males ($P = 0.005$) and females ($P = 0.02$), with similar observations made in female ipsilateral LSC TGF-β1 mRNA levels [Figure 7E and G]. However, the magnitude of IL-10 increases in ipsilateral LSC was greater in CCI + BIRT males than females, and CCI + BIRT females displayed lower IL-10 levels than males ($P = 0.019$). Contralateral IL-10 mRNA levels displayed the same pattern as ipsilateral dorsal horn following CCI: IL-10 mRNA levels were significantly decreased in neuropathic females ($P = 0.004$), along with a similar trend in males ($P = 0.07$), compared to their corresponding Sham + Veh groups. BIRT377 treatment increased contralateral IL-10 significantly from CCI + Veh in males ($P = 0.04$), with a similar trend observed in females ($P = 0.06$) [Figure 7H]. These data indicate that BIRT377-mediated pain reversal corresponds to increased bilateral spinal IL-10 mRNA levels in neuropathic animals of both sexes.

BIRT377 reduced astrocyte activation in the spinal cord

The data above show that i.v. BIRT377 corresponds to reduced proinflammatory cytokines in both the SCN and DRGs, and reduced CCL2 in the ipsilateral spinal cord, while elevating anti-inflammatory cytokines in the SCN, DRGs, and LSC. Persistent microglial and astrocyte activation in the spinal cord is critical for the chronicity of sciatic neuropathy. Reducing the pro-inflammatory cytokine milieu at peripheral anatomical

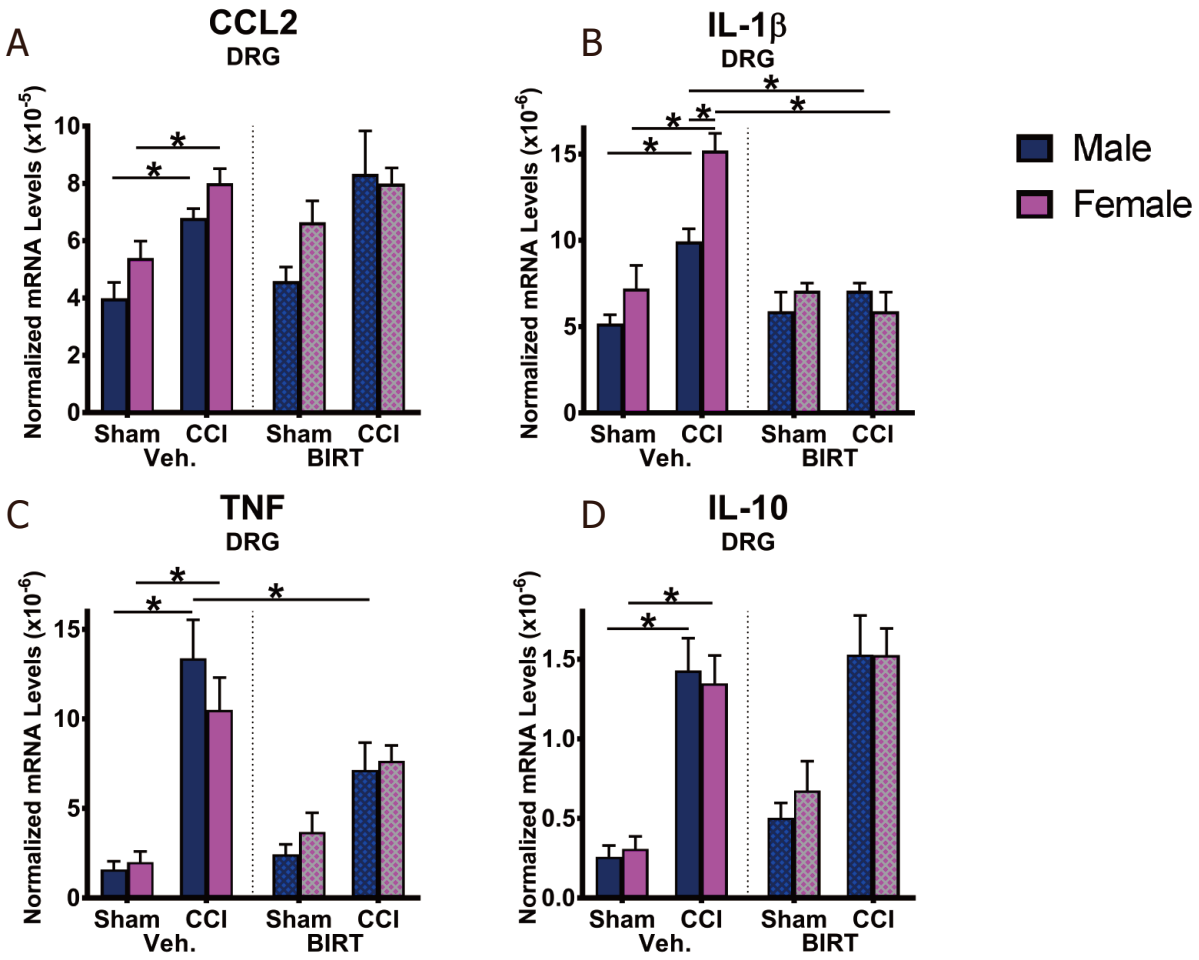

Figure 6. DRGs from males and females reveal similarly reduced IL-1β and TNF mRNA levels following BIRT377 treatment. Total RNA was isolated from ipsilateral lumbar DRGs from the same mice used in Figure 2B, and analyzed for inflammatory cytokines. (A) In the DRGs, CCI induced CCL2 mRNA levels ($F_{1,1}$ = 25.5, P < 0.0001) remained unchanged following BIRT377 treatment. (B) CCI increased IL-1β mRNA expression in females ($F_{1,1}$ = 25.4, P < 0.0001) to a much greater degree than in males ($F_{1,1}$ = 8.3, P = 0.006). BIRT377 treatment reduced IL-1β mRNA levels in mice with CCI ($F_{1,1}$ = 25.4, P < 0.0001), with a greater magnitude in females ($F_{1,1}$ = 4.95, P = 0.031). (C) Post-CCI TNF mRNA expression levels were increased ($F_{1,1}$ = 61.81, P < 0.0001). BIRT377 treatment reduced TNF mRNA expression levels in mice with CCI ($F_{1,1}$ = 9.92, P = 0.003). Post hoc comparisons revealed a significant reduction of TNF mRNA levels following BIRT377 treatment in CCI-treated males. (D) Following CCI, IL-10 mRNA expression levels were increased ($F_{1,1}$ = 77.99, P < 0.0001). BIRT377 treatment did not further elevate IL-10 mRNA levels during neuropathy. *P values from post hoc comparisons ranges from P = 0.029 to P < 0.0001, n = 6 per group

regions (SCN and DRG) of the pain pathway may likely reduce chronic pain relays to the spinal cord, and in doing so, may reduce spinal glial activation and ultimately, pathological pain processing. In support of this possibility, GFAP (marker of astrocyte activation) and TMEM119 (related to microglial activation) mRNA levels were examined in the ipsilateral and contralateral LSC [Figure 8]. Data revealed that GFAP mRNA levels were significantly increased in the ipsilateral dorsal horn of CCI + Veh animals compared to Sham + Veh animals (male: P = 0.0001; female: P < 0.0001), in support of prior reports[26,84]. Compared to CCI + Veh animals, GFAP mRNA levels of CCI + BIRT males were significantly decreased (P = 0.044), with a similar trend (P = 0.056) observed in females [Figure 8A]. Similar increases in GFAP mRNA levels were observed in contralateral LSC in CCI + Veh animals (male: P = 0.015; female: P < 0.0001). Similarly, contralateral GFAP levels returned to BL in all neuropathic mice following BIRT377 treatment (male: P = 0.04; female: P = 0.0003) [Figure 8B]. Of note, GFAP transcripts from the contralateral side of the spinal cord were significantly greater in neuropathic females than in males (P = 0.001). Ipsilateral TMEM119 mRNA levels were also increased, indicative of increased microglial activation following CCI [Figure 8C and D] in both

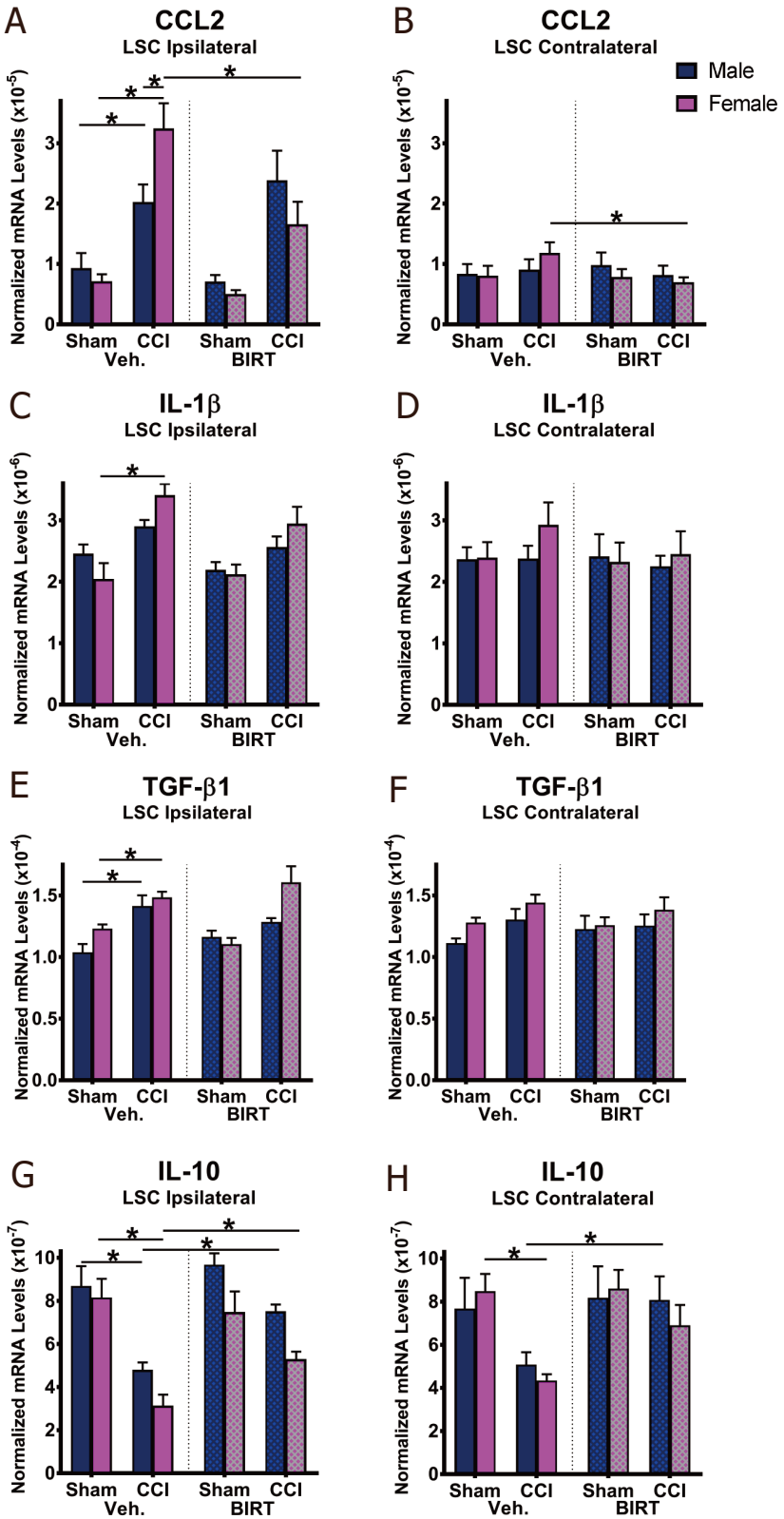

Figure 7. Ipsilateral dorsal spinal cord mRNA levels reveal BIRT377 treatment reduces CCL2 in sciatic damaged females only, while no sex differences occur in elevated IL-10. Lumbar spinal cord (LSC) tissues from behaviorally verified mice (Figure 2B), were collected and analyzed for pain-relevant cytokines. (A-B) Ipsilateral CCL2 mRNA levels were increased following CCI ($F_{1,1}$ = 57.38, P < 0.0001). BIRT377 treatment reduced CCL2 mRNA expression in females with CCI ($F_{1,1}$ = 5.28, P = 0.026). Neuropathic females displayed significantly more CCL2 mRNA levels than in neuropathic males (P = 0.006). Post hoc comparisons revealed significant reduction in contralateral CCL2 mRNA levels in CCI-treated females following BIRT377 treatment. (C-D) Ipsilateral IL-1β mRNA levels were increased following CCI ($F_{1,1}$ = 32.8, P < 0.0001) that occurred in females to much a greater degree than males ($F_{1,1}$ = 6.86, P = 0.012). (E-F) While sciatic nerve CCI induced a bilateral elevation in TGF-β1 mRNA levels in both males and females (ipsilateral: $F_{1,1}$ = 40.95, P < 0.0001; contralateral: $F_{1,1}$ = 5.23, P = 0.027), the magnitude of TGF-β1 mRNA increase following BIRT377 treatment was greater in ipsilateral LSC from females than males ($F_{1,1}$ = 6.56, P = 0.014). No differences in TGF-β1 mRNA levels from contralateral LSC were revealed following BIRT377 treatment. (G-H) IL-10 mRNA levels were decreased following CCI (ipsilateral: $F_{1,1}$ = 52.26, P < 0.0001; contralateral: $F_{1,1}$ = 9.45, P = 0.004). BIRT377 treatment increased IL-10 mRNA expression levels in mice with CCI (ipsilateral: $F_{1,1}$ = 6.19, P = 0.017; contralateral: $F_{1,1}$ = 4.94, P = 0.032). *P values from post hoc comparisons ranges from P = 0.04 to P < 0.0001. n = 5 in male Sham + Veh and CCI + BIRT for IL-10 contralateral data. n = 6 per group unless otherwise indicated

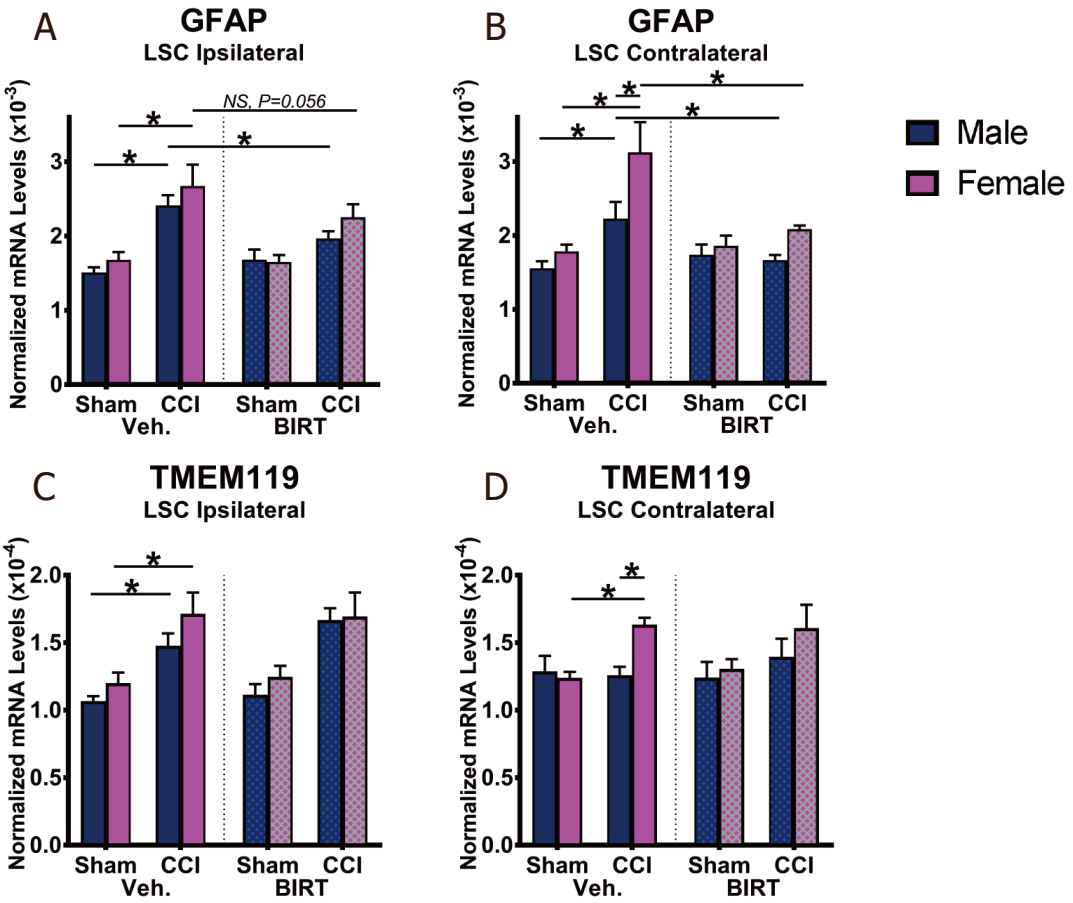

Figure 8. Spinal GFAP mRNA levels are reduced following BIRT377 in both males and females. mRNA was extracted from tissues behaviorally verified in Figure 2B. (A-B) Astrocyte activation marker, GFAP, mRNA levels were increased on both sides of the spinal cord, in all mice with CCI (ipsilateral: $F_{1,1}$ = 41.91, P < 0.0001; contralateral: $F_{1,1}$ = 16.68, P = 0.0002). BIRT377 treatment reduced spinal GFAP mRNA levels in mice with CCI (ipsilateral: $F_{1,1}$ = 5.533, P = 0.023; contralateral: $F_{1,1}$ = 12.28, P = 0.001). In the contralateral side, CCI-induced GFAP mRNA levels were greater in females than in males ($F_{1,1}$ = 9.9, P = 0.003). (C-D) Microglial proliferation marker, TMEM119, mRNA levels were increased following CCI (ipsilateral: $F_{1,1}$ = 40.49, P < 0.0001; contralateral: $F_{1,1}$ = 7.66, P = 0.008). Neuropathic females displayed significantly greater contralateral TMEM119 expression than neuropathic males (P = 0.015), as a main effect of sex ($F_{1,1}$ = 4.16, P = 0.048) was observed. TMEM119 mRNA levels were comparable between BIRT377 or vehicle treated neuropathic males or females bilaterally. No significant difference was detected between male or female Sham + Veh. and Sham + BIRT group. *P values from post hoc comparisons ranges from P = 0.04 to P < 0.0001. n = 6 per group

males (P = 0.009) and females (P = 0.001). An elevation in TMEM119 mRNA levels were also observed from the contralateral spinal cord, but only in females (P = 0.011). Surprisingly, BIRT377 treatment did not change TMEM119 mRNA levels from the ipsilateral or contralateral spinal cord in males or females.

BIRT377 treatment did not result in systemic immune changes

Spleens were collected to capture a broad population of peripheral circulating immune cells inclusive of monocytic macrophages, neutrophils, dendritic, and T and B cells. Importantly, all splenic protein data presented [Figure 9A-J] are from behaviorally characterized mice, as demonstrated in Figure 2B. Protein analysis of spleen revealed that, compared to sham treatment, a trend toward increased proinflammatory cytokine IL-1β and chemokine C-X-C motif ligand 1 (CXCL1) occurred in CCI male mice, which returned to basal levels following BIRT377 treatment [Figure 9A and E]. However, comparisons did not reveal statistically significant differences, suggesting that the reservoir of circulating leukocytes, such as monocytes and lymphocytes represented in the spleen cannot act as surrogate indicators of atypical neuroimmune events in key anatomical regions of the pain pathway. It is possible that the immune

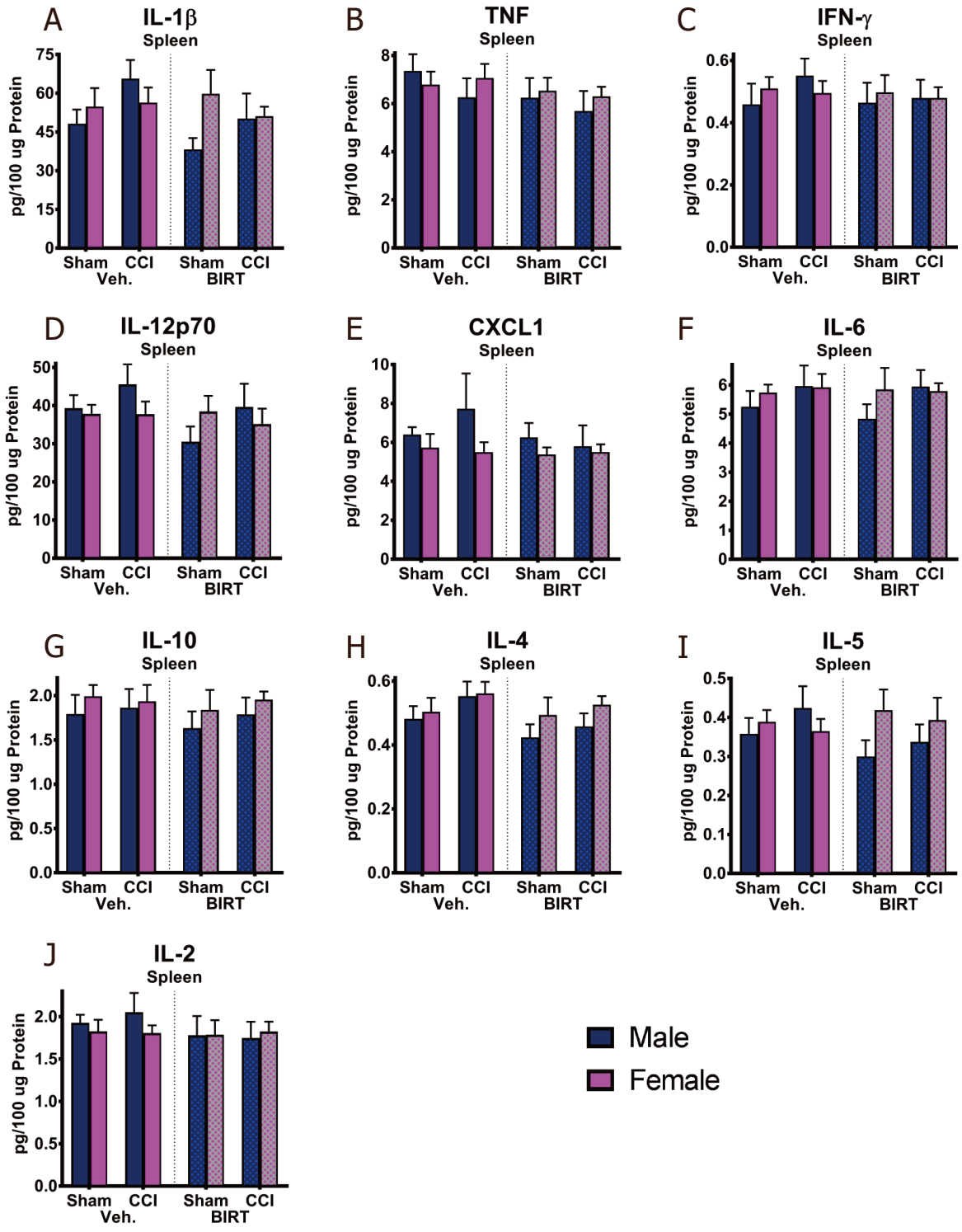

Figure 9. BIRT377 treatment did not result in systemic immune changes. (A-J) Spleens were collected from the same mice used in Figure 2B and analyzed for inflammatory cytokines. Splenic cytokine and chemokine protein levels were similar regardless of surgical manipulation, treatment, or sex. *n* = 6 in each group

changes observed in discrete regions involved in the pain pathway (SCN, DRGs and LSC) are exceedingly localized such that detection from the systemic pool of immune cells is not measurable. More likely, these

observations may indicate that further immune cell differentiation occurs after their migration to key pain-relevant nervous tissue regions in response to signals from local tissue-damage.

DISCUSSION

Reports focused on understanding neuroimmune changes when performing a comparative approach between sexes are rare with most studies applying male rodent models[4,25-27]. In recent years, published reports provide compelling evidence that activation of spinal microglia play a direct role in generating pathological pain in males, while in females, the actions of T cells are critically important[3,4]. Consistent with prior reports[4,26], we find that, while the onset, magnitude and spontaneous reversal of allodynia are similar in males and females [Figure 1], divergent peripheral immune and neuroimmune responses are present during neuropathy. We demonstrate for the first time that during neuropathy, T cell-associated pro- and anti-inflammatory responses in males and females are different at discrete anatomical regions critical in the pain pathway of sciatic neuropathy. During neuropathy, females displayed more profound Th17 specific responses (IL-17A) than males, both at the injured nerve and in the corresponding LSC [Figure 4]. While regulatory T cell (Tregs) recruitment (FOXP3 expression) was evident at the injured SCN in both males and females, only females displayed reliable increases of FOXP3 at the DRGs [Figure 4]. The beneficial role of blocking the active conformational state of LFA-1 was demonstrated in both sexes [Figure 2] by reducing immune cell accumulation in damaged SCN [Figure 3]. However, BIRT377 modulated T cell function in a sex-specific manner [Figure 5]. For example, T cells from females were significantly more responsive to the anti-inflammatory effects of BIRT377 [Figure 5]. Similarly, BIRT377 treatment elevated T cell-associated anti-inflammatory factors (FOXP3, IL-10 and TGF-β1) and reduced the proinflammatory T cell cytokine, IL-17A in the peri-sciatic milieu, predominantly in neuropathic females [Figures 3 and 4]. Interestingly, despite the fact that there was no additional change in FOXP3 expression in the LSC, the profound reduction of IL-17A in the LSC in i.v. BIRT377 treated females indicates a limited role exists for spinal Treg actions on pain reversal. Importantly, a reduction of peri-sciatic IL-17A co-occurs with profound spinal cord suppression of IL-17A, suggesting the excitatory input from centrally projecting nerve terminals into the lumbar spinal region ultimately leads to a reduction in proinflammatory factors that includes IL-17A. While these data demonstrate that a potential role for spinal IL-17A in pro-nociceptive signaling occurs, it remains unclear whether IL-17A acts in concert with other well-characterized spinal proinflammatory factors, or whether IL-17A is a necessary factor in pain signaling. Therefore, the results from the current data provide the rationale for performing future studies to examine whether specifically blocking the spinal actions of IL-17A also suppresses allodynia from peripheral neuropathy. While these additional studies would aid in understanding the role of IL-17A in chronic neuropathic pain, the current data are the first documented evidence that a reduction in lumbar spinal IL-17A expression co-occurs with a reduction in allodynia from CCI in both males and females [Figure 4]. Strikingly, peripheral BIRT377 reduced spinal astrocyte activation, but had little impact on microglial activation [Figure 8]. Overall, BIRT377 created an anti-inflammatory bias in discrete regions along the pain pathway of the CCI model in both sexes [Figures 3-7], thereby contributing to pain reversal. A brief summary of immune changes during CCI-induced neuropathy and BIRT377-mediated effects are listed in Table 1. This initial comparative analyses of glial/myeloid and T cell-related cytokines and their corresponding transcription factors that are altered by preventing β2-integrin (LFA-1) signaling, provides insight into possible mechanisms leading to peripheral sciatic neuropathy between males and females.

Sex differences in peripheral inflammatory reactions to nerve injury

Remarkable sex differences of immune system activity are observed in different disease models[85-89]. Sex differences in TLR4 responses to pathogen stimulation have been observed, whereby females produce similar or less IL-1β and TNF compared to males[85]. Female-derived immune cells are more efficient in antigen presentation and initiating adaptive immune responses[90]. In the CCI model, peripheral inflammatory reactions to nerve injury are mediated by endothelial cells of the blood-nerve barrier and

Table 1. A brief summary of changes in immune factors following CCI and BIRT377 treatment

Tissue Regions	Immune Parameters	Male		Female	
		CCI[#]	CCI+ BIRT377[##]	CCI[#]	CCI+ BIRT377[##]
Sciatic Nerve (Ipsilateral)	CCL2	Up	Down	Up*	
	IL-1β	Up	Down	Up*	Down
	TNF	Up	Down	Up	Down
	IL-10	Up		Up*	Up
	TGFβ-1	Up		Up*	Up
	CD11b	Up	Down	Up*	
	CD3	Up	Down	Up	
	FOXP3	Up		Up	Up
	IL-17A	Up		Up*	Down
DRGs (Ipsilateral)	CCL2	Up		Up	
	IL-1β	Up	Down	Up*	Down
	TNF	Up	Down	Up	
	IL-10	Up		Up	
	FOXP3			Up	
Lumbar Spinal Cord (Ipsilateral)	CCL2	Up		Up*	Down
	IL-1β			Up	
	TGFβ-1	Up		Up	
	IL-10	Down	Up	Down	Up
	FOXP3	Up*	Down		
	IL-17A			Up*	Down
	GFAP	Up	Down	Up	
	TMEM119	Up		Up	
Lumbar spinal Cord (Contralateral)	CCL2				Down
	IL-1β				
	TGFβ-1				
	IL-10		Up	Down	
	FOXP3				
	GFAP	Up	Down	Up*	Down
	TMEM119	Up		Up	

*Fold changes were significantly different in males versus females. [#]Comparison between Sham+Veh and CCI+Veh, [##]comparison between CCI+Veh and CCI+BIRT377

Schwann cells (e.g., undergoing myelin degeneration), followed by circulating leukocytes recruited in response to injury[7]. CCL2 signaling recruits monocytes, neutrophils and a subset of T cells[18,91,92]. We observed greater induction of SCN CCL2, along with greater SCN CD11b levels in males than females during neuropathy. These data, in combination with greater SCN IL-1β production in males suggest greater monocyte/macrophage-driven immune responses in males than females.

Though we detected T cell recruitment in both sexes, the critical finding was in detecting a T cell differentiation bias toward a proinflammatory status that was significantly greater in females than males. Moreover, responses to BIRT377 in females were robustly anti-inflammatory. For example, while an induction of SCN FOXP3 (transcription factor in Tregs for IL-10 and TGF-β1) was detected from both neuropathic males and females, BIRT377 induced additional increases only in SCNs of females with no change in FOXP3 levels in males. Even more striking were the robust levels of SCN IL-17A of neuropathic females compared to males, with profound blunting of IL-17A in pain reversed females relative to pain-reversed males [Figure 4A to F]. These data indicate that females mount stronger proinflammatory T cell responses following nerve injury compared to males despite an abundance of peri-sciatic T cells (as indicated by CD3, global T cell marker) present in both males and females. Moreover, the striking FOXP3 increase and simultaneous IL-17A decrease predominantly in female SCN suggests that BIRT377 favors targeting T cells derived from females than from males.

Interestingly in DRGs, IL-1β levels were greater in neuropathic females. It is possible that the combination of T cell-mediated responses, along with myeloid-driven proinflammatory actions culminate in greater nociceptive factors that induce further hyperexcitability relayed to the spinal cord in females. The fact that reliable induction of FOXP3 was observed only in female DRGs may reflect the anti-inflammatory rebound in response to inflammatory signals. Recruitment of Treg cells could function to control bystander injury-related proinflammatory cytokines.

Sex convergent and sex divergent aberrant spinal immune responses underlying chronic pain

Despite evidence of microglial activation in neuropathic females[26], microglial TLR4 signaling is only necessary for the development of neuropathy in males, whereas astrocytic signaling under neuropathic conditions is observed in both males and females[3,25,26]. Supporting prior observations, we detected astrocytic and microglial activity in both sexes during neuropathy [Figure 8][4]. However, we noticed that induction of ipsilateral CCL2 in conjunction with IL-1β was greater in females [Figure 7]. The reduction of basal IL-10 levels, a finding that our group has previously observed in chronic neuropathic male rats[93,94], appeared more pronounced in neuropathic female than male mice. Note that along with greater astrocyte (as assessed by increased GFAP) and microglial activation (TMEM119) and increased CCL2 in the contralateral side, a simultaneous decrease in IL-10 was measured, indicating that contralateral spinal cord IL-10 expression in females may reflect a greater impact of this cytokine in controlling proinflammatory contralateral glial activation. It is noteworthy that, other than CCL2 in females, changes in injury-related contralateral spinal IL-1β or TGF-β1 were not detectable in males or females, despite ongoing contralateral allodynia. Therefore, the reduction of the basal levels of spinal IL-10, rather than the presence of these specific proinflammatory cytokines, is likely a better indicator of ongoing allodynia.

We speculate that T cell-mediated proinflammatory cytokines (e.g., IL-17A) at the injury site may consequently drive sciatic "damage" signals, leading to the release of factors from nerve terminals in the spinal cord that communicate to pain projection neurons. Astrocytes and microglia local to the dorsal horn of the spinal cord respond to these damage signals from SCN terminals. Though activated astrocytes are capable of producing IL-17A[95,96], contralateral IL-17A was not detected despite astrocyte activation, suggesting that contralateral IL-17A is not a key factor in contralateral glial activation. In fact, the absence of contralateral IL-17 may reinforce the possibility that ipsilateral immune-related signaling may drive contralateral spinal cord pain neuron excitability via astrocyte-specific gap junctional communication[73,80]. Interestingly, supraspinal mechanisms such as activation of cortical areas important in pain processing, and descending facilitation from key brainstem areas may contribute in contralateral allodynia as well[97,98]. Proinflammatory cytokines in pain related brain regions are capable of impairing descending inhibitory pain pathways[99]. Whether, differential immune mechanisms following nerve injury influence the descending pathways involved in manifesting mirror image pain in different sexes would be an interesting avenue for future exploration.

Though astrocytic activation during neuropathy is common in both sexes, it is possible that microglia in males and infiltrating Th17 cells in females are the predominant cell types responsible for driving chronic excitation of astrocytic-neuronal interaction. In support of this possibility, the current report demonstrated a robust upregulation of IL-17A in the spinal cord of neuropathic females. Our prior data indicates the presence of activated T cells (T-bet and RORγt mRNA transcripts, which are critical transcription factors for Th1 and Th17 respectively) in the ipsilateral LSC in neuropathic female rats[100]. Therefore, Th17 cells likely infiltrate the ipsilateral spinal cord and interact with astrocytes where ongoing pathology is present, inducing a feed-forward astrocyte-proinflammatory chemokine (e.g., CCL2) and cytokine production[101,102], as observed in this study. However, re-programming of differentiated T cells and their functional responses can occur in response to the local cytokine milieu in the CNS[102-104]. Therefore, the absence of contralateral IL-17A does not prove a lack of T cell recruitment or their actions in contralateral neuropathy.

Sex-specific mechanistic differences of BIRT377 pain reversal

BIRT377-mediated effects on myeloid/glial activation

Numerous reports suggest that blocking LFA-1 actions restricts migration of monocytes and T cells to injured tissues[37,105]. Following BIRT377 treatment, both males and females display decreases in peri-sciatic IL-1β and TNF, which are generally myeloid-derived. Reduced CD11b levels around the injured nerve are found only in males. Together, these data suggest BIRT377 may reverse pain in males mainly by blocking myeloid cell migration and consequent exposure to proinflammatory cytokines. However, in females, the effect of blocking the active conformation of LFA-1 by BIRT377 appears to directly alter transcriptional regulation of pro- and anti-inflammatory cytokines of myeloid-derived cells. Previous studies suggest that a lack of LFA-1 interaction with leukocytes increases IL-10, switching macrophage activation from a proinflammatory bias to an anti-inflammatory state[38,42]. Therefore, BIRT377-mediated re-programing of myeloid cell function may also occur, and possible sex differences regarding these observations need to be further explored.

BIRT377-mediated effects on T cells

Though the exact mechanism(s) are unclear, LFA-1 signaling interacts with T cell activation, and therefore, modulates adaptive immune responses[44,54]. While sex was not specified, previous studies suggest that blocking LFA-1 actions decreases Th17 differentiation and increases $FOXP3^+$ Tregs[39,106]. Consistent with the *in vitro* T cell findings demonstrated in the current report, *in vivo* increases in IL-10 and TGF-β1 were observed along with increases in FOXP3 and reduced IL-17A levels at the SCN only in females following BIRT377 treatment. Therefore, BIRT377 treatment is beneficial for pain reversal by affecting both immune cell migration and modulation of their actions at local sites of inflammation, thereby, indirectly influencing the spinal-immune milieu during neuropathy. Interestingly, for both sexes, BIRT377 did not change microglial activation, suggesting that reducing astrocytic activation and increasing IL-10 levels at the spinal cord is sufficient to reverse allodynia.

We have recently reported that intrathecal (spinal) application of BIRT377 in a rat CCI model leading to chronic neuropathy, dorsal horn spinal astrocyte activation and IL-1β are reduced, as evidenced by immunohistochemical staining and image analysis measures[107]. Though BIRT377-mediated effects on mRNA levels of IL-17A, IL-10, and TGF-β1 are supportive of the protein levels of these cytokines [Figure 5], direct quantification of protein levels of all the diverse immune markers would further strengthen the findings in the current report[30,32], along with semi-quantitative analysis of expression markers using immunohistochemical and image analysis methods that can capture within-region specific changes in comparatively sparse T cell subtypes[33,108].

In conclusion, this study supports the presence of divergent proinflammatory cytokines in males and females following peripheral nerve injury, which has important implications when developing pain therapeutics. Despite the observed cytokine/chemokine and related transcription factor expression differences in SCN, DRG and LSC, systemic blockade of LFA-1 activation is beneficial for pain reversal in both sexes. Therefore, BIRT377 may serve as a novel therapeutic for chronic pain and other CNS diseases.

DECLARATIONS

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Authors' contributions

Performed behavioral assessments and extracted RNA from DRG tissues: Havard MA
Performed behavioral assessments: Sanchez JE, Harris NW
Experimental design, flow cytometry, real time PCR, data analysis and manuscript preparation: Noor S

Experimental design, performed behavioral assessment and data analysis for hindpaw responses, RNA extractions and aided with manuscript preparation: Sun MS
Tissue collection, aided with for behavioral assessment, small tissue RNA extraction, and generated PCR data: Vanderwall AG
Performed behavioral assessments and extracted RNA from DRG tissues: Havard MA, Sanchez JE, Harris NW
Provided resources and performed intravenous injection of BIRT377: Nysus MV, Norenberg JP
Gifted some batches of BIRT377 and provided guidance about BIRT377 usage: Wagner CR, West HT
Provided equipment, critical guidance and training for MSD immunoassays data analysis, and manuscript preparation: Jantzie LL
Provided equipment and critical guidance for quantitative real-time PCR with Taqman probes: Mellios N
Designed the studies, performed tissue dissection, reviewed data analysis, provided data interpretation and manuscript preparation: Milligan ED

Availability of data and materials

All data generated and analyzed for the current report is included within the article.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of The University of New Mexico Health Sciences Center, and closely adhered to guidelines from the International Association for the Study of Pain for the use of animals in research.

Consent for publication

Not applicable.

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Neuroimmunology and Neuroinflammation

Review

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Roles of miRNAs in spinal cord injury and potential therapeutic interventions

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Abstract

Spinal cord injury (SCI) affects approximately 200,000 individuals per year worldwide. There are more than 27 million people worldwide living with long-term disability due to SCI. Historically, it was thought that the central nervous system (CNS) had little ability for regeneration; however, more recent studies have demonstrated potential for repair within the CNS. Because of this, there exists a renewed interest in the discovery of novel approaches to promote regeneration in the CNS including the spinal cord. It is important to know the roles of the microRNAs (miRNAs) in modulation of pathogenesis in SCI and the potentials of the miRNA-based clinical interventions for controlling post-injury symptoms and improving functional recovery. The miRNAs, which are non-coding RNAs with an average of 22 nucleotides in length, are post-transcriptional gene regulators that cause degradation of the target mRNAs and thus negatively control their translation. This review article focuses on current research related to miRNAs and their roles in modulating SCI symptoms, asserting that miRNAs contribute to critical post-SCI molecular processes including neuroplasticity, functional recovery, astrogliosis, neuropathic pain, inflammation, and apoptosis. In particular, miR-96 provides a promising therapeutic opportunity to improve the outcomes of clinical interventions, including the way SCI injuries are evaluated and treated.

Keywords: Spinal cord injury, miRNAs, astrogliosis, neuropathic pain, inflammation, apoptosis, functional recovery

INTRODUCTION

Spinal cord injury (SCI) results from contusion/compression or transection of the spinal cord. SCI is a significant health issue with an estimate putting the number of people living with this neurological condition at more than 300,000 in the United States[1]. Because of the high number of casualties, SCI is also associated with various socio-economic challenges[2]. Hence, it is essential to understand the molecular mechanisms of pathogenesis in SCI in animal models and to elucidate novel therapeutic interventions for this devastating neurological condition[3]. The emergence of microRNAs (miRNAs) as potent regulators of gene expression at the post-transcriptional level has vast implications in many critical biological processes that include cell proliferation, differentiation, survival, and metabolism[4]. Studies indicate that miRNAs are currently attractive candidates as the upstream regulators of secondary injury progression in SCI because miRNAs are known to regulate entire sets of genes post-transcriptionally. Specific miRNAs (such as miR-96 and miR-544a) are potentially deregulated after SCI and the impact of this deregulation is an area of great interest[5].

Bioinformatic analysis indicates that the potential targets of miRNAs altered after SCI include genes encoding components that are involved in inflammation, oxidative stress, and apoptosis, all of which are known to be crucial for progressive pathogenesis in SCI, suggesting that abnormal expression of miRNAs may contribute to the pathogenesis in SCI. Levels of expression of miRNAs were identified to be deregulated (decreased or increased) in SCI animals[6]. A later investigation showed dramatic decreases in the expression of miRNAs including miR-96 in SCI[7]. Upregulation of miR-96 is likely to promote cell proliferation[8] and prevent neurodegeneration[9] for contribution to functional neuroprotection in SCI. Because miRNAs highly decrease specific gene expression and deregulation of miRNAs does occur in SCI, the potential of particular miRNAs as therapeutic agents should now be explored for functional neuroprotection in SCI[10]. This review article mainly focuses on recent research related to changes in expression of miRNAs following induction of SCI and effects of modulation of levels of miRNAs on critical molecular processes including neuroplasticity, astrogliosis, neuropathic pain, inflammation, apoptosis, and functional recovery in SCI. The last section of this review article focuses explicitly on miR-96 as an emerging post-transcriptional regulator that has the potential to revolutionize SCI clinical interventions.

SCI PATHOPHYSIOLOGY AND MIRNAS

The occurrence of SCI is classified into two different stages: primary stage (a few moments following the initial injury) and secondary stage (hours, days, or weeks after the initial injury). Research shows that the first phase of SCI is the best predictor of future prognosis[11,12]. During the first phase, SCI is manifested with immediate changes in pathophysiology such as hemorrhage, Ca^{2+} overload, and activation of the Ca^{2+}-dependent cysteine protease calpain causing necrotic and apoptotic neuronal death at the site of impact[13]. The secondary phase begins with molecular and physiological changes responsible for bleeding, loss of neurological functions, expansion of the lesion area, and overall amplification of the injury[14,15]. The secondary phase is also characterized by biochemical reactions, vascular alterations, inflammation, and edema[16]. The main pathological mechanisms responsible for the changes during the secondary phase are the depletion of energy, which is caused by ischemia and oxidative stress, neuroinflammation, and activation of calpains and caspases for cell death at the site of injury and the penumbra[17]. The combination of cellular and molecular modifications leads to various pathological events ranging from astrogliosis to apoptosis and tissue atrophy[18]. Understanding the pathological events is highly crucial for modulating progression of pathogenesis leading to activation of cysteine proteases in both acute and chronic SCI and for implementing therapeutic interventions[19]. It has been shown that attenuation of neuroinflammation and neurodegeneration with appropriate therapeutic interventions is essential for functional recovery in preclinical models of SCI[20,21]. A relatively novel therapeutic intervention evaluated for the treatment of SCI in preclinical models is the utilization of miRNAs[22]. The miRNAs are small non-coding RNA molecules

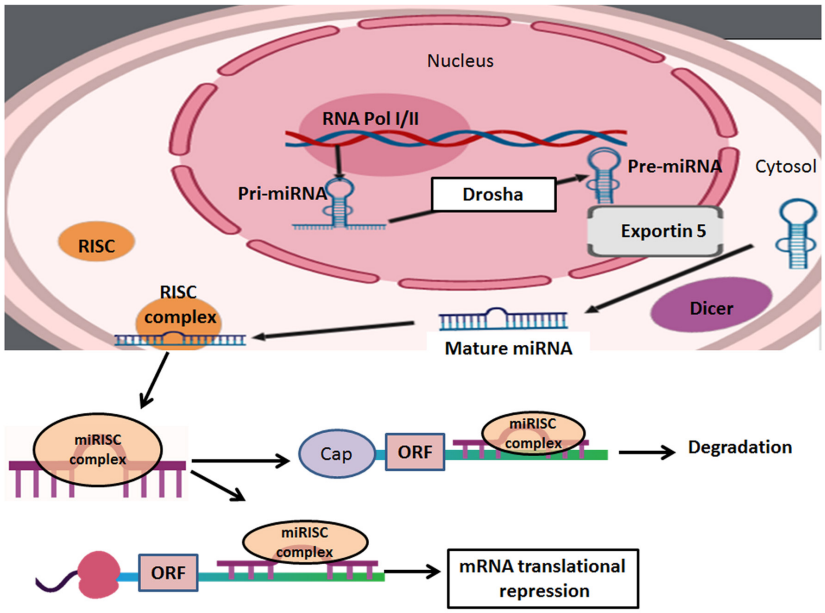

Figure 1. The biosynthesis of miRNAs begins in the nucleus. RNA polymerase (RNA pol) transcribes primary miRNAs, which consist of a poly-A tail and a 5′ cap. The multi-processor complex made up of double-stranded-RNA-binding protein and the RNase III enzyme Drosha to form pre-miRNAs. The Ran-GTP complex and karyopherin export pre-miRNAs into the cytoplasm. To finalize the process, the RNase III enzyme Dicer cleaves pre-miRNAs and triggers a further processing step by generating the miRNA. When miRNA-induced silencing complex (miRISC) is in the cytoplasm, miRNAs act on the target transcripts via complementary Watson-Crick base pairing to the corresponding miRNA response elements (MREs), which are usually present within the 3′-untranslated regions (3′UTRs) of target genes. Upon binding to MREs within 3′UTRs, miRNAs reduce protein outcome from the target transcripts due to translational repression and/or mRNA deadenylation and decay mechanisms

that post-transcriptionally regulate gene expression by degrading and inhibiting translation of target mRNAs [Figure 1].

The biogenesis of miRNAs occurs from pre-miRNA precursors and miRNAs are present not only in body fluid (sputum, serum, and blood) but also in body tissues where they are associated with the regulation of expression of target mRNAs[23]. Different miRNAs are also found in the central nervous system (CNS) where they act as essential mediators of neurodevelopment in the brain and spinal cord in mammals. For example, specific miRNA expression (e.g., miR-17∼92 clusters) controls neural growth as well as neurogenesis-gliogenesis switch in the developing CNS including spinal cord. Likewise, miRNAs target genes that are involved in the regulation of essential pathophysiological processes including apoptosis (miR-124 and miR-21), inflammation (miR-544a), and astrogliosis (miR-145 and miR-21) in the spinal cord[24]. The alterations in the expression of miRNAs following SCI can be categorized into three groups: (1) increased miRNAs; (2) decreased miRNAs; and (3) bidirectional (increased or decreased) miRNAs. The expression of specific miRNAs such as miR-146a and miR-129-2 is significantly influenced by the injury severity. The down regulation of specific miRNAs such as miR-219 and miR-124 is associated with the death of neural cells. On the other hand, the overexpression of specific miRNAs (e.g., miR-223) is due to infiltration of vascular and immune cells. Such infiltration of immune cells has been observed during the acute stage of SCI. Research has shown changes in miR-451 during the initial phase of SCI[25]. Other studies also indicate the important roles of miRNAs in regulating immune response and alleviation of inflammation following SCI. For example, miR-544a is down regulated after SCI while genes associated with inflammation (especially NEUROD4) are overexpressed[26]. These results suggest that miR-544a is essential in the repair process after SCI, although further research is required to confirm this assertion.

POTENTIAL ROLES OF MIRNAS IN SCI

miRNAs in astrogliosis

Astrogliosis or astrocytosis refers to the response of astrocytes in response to SCI. Astrogliosis occurs in the area close to the SCI and is a part of a complex multicellular response to SCI. Astrogliosis is characterized by functional, molecular, and morphological changes in astrocytes[27]. These changes usually occur within a few hours following the initial injury and evolve with time[28]. The process of astrogliosis is beneficial in the acute stages where it triggers the repair of the spine-blood barrier, cell regeneration, and prevention of inflammation. However, astrogliosis becomes detrimental in the later stages (4 to 6 weeks) following SCI when astrocytes change from hypertrophic to hyperplastic, producing glial scar with expression of chondroitin sulfate proteoglycans[29].

Under specific pathological conditions, molecular signaling mechanisms associated with miRNAs affects the process of astrocyte proliferation and astrogliosis. For example, the addition of anti-miR-125b is linked with reduced glial cell proliferation and overall regulation of cell growth[30,31]. Similarly, overexpression of miR-145 following SCI has been shown to increase astrogliosis in astrocytes close to the injured area[32]. There was focus of another study, which sought to determine the regulating mechanism of miR-21 in regard to glial scars and astrocytic hypertrophy[33]. More specifically, these investigators carried out tests involving overexpression of miR-21 in mouse astrocytes and concluded that overexpression of miR-21 in astrocytes attenuated hypertrophic response to SCI but expression of the miR-21 sponge augmented hypertrophic phenotype, even in chronic phase of SCI. Other researchers have studied the role of miR-21 in the hypertrophy-hyperplasia shift. According to recent research, miR-21 suppresses the expression of the glial fibrillary acidic protein (GFAP) and vimentin (VIM) under the influence of bone morphogenic protein (BMP) receptors[34]. Activation of different BMP receptors results in differential expression of miR-21 in astrocytes and controls astrogliosis[35,36]. BMP receptor type 1a (BMPR1a) and BMPR1b exert opposite effects on reactive astrocytic hypertrophy. BMPR1b plays a role in glial scar progression in the chronic stages following SCI. These receptors exert opposite effects on expression of miR-21 in astrocytes. Activation of BMPT1a causes overexpression of miR-21 with a dramatic reduction in GFAP levels, limiting the detrimental effects of BMPR1b signaling on glial scar formation following SCI.

miRNAs in apoptosis

Some researchers studied the action of miRNAs in the context of apoptosis. For example, overexpression of miR-96-5p inhibits apoptosis but promotes migration and proliferation in MDA-MB-231 and MCF-7 cells[37]. In this study, these investigators overexpressed miR-96-5p by transfecting MDA-MB-231 and MCF-7 cells with a miR-96-5p mimic. Treatment and incubation of these cells were done for two days after which cells were double stained with Annexin V/propidium iodide. Flow cytometry was then used to quantify the apoptotic cells. The conceivable molecular events leading to induction of apoptosis in SCI are shown [Figure 2]. Upregulation of miR-96 may suppress programmed cell death protein 4 (PDCD4) and subsequently decrease apoptosis. It is known that reduction of miR-21 promotes apoptosis by inhibiting the expression of phosphatase and tensin homolog (PTEN) and PDCD4[38]. Down regulation of the pro-apoptotic factors PTEN and PDCD4 can increase expression of the cell survival factor Akt or protein kinase B, leading to a reduction in apoptosis in SCI. Studies indicated that specific miRNAs (e.g., miR-21, miR-7-1) could significantly enhance efficacy of promising therapeutic agents (e.g., estrogen receptor agonists) for functional protection of spinal cord motoneurons and this combination therapeutic strategy could be used in the future to attenuate apoptosis of motoneurons in SCI[39,40].

miRNAs in axon regeneration and remodeling

It has been demonstrated that exercise, which promotes spinal cord plasticity, results in the down regulation of miR-199a-3p and upregulation of miR-21[41]. Alteration in expression of these miRNAs modulates the mechanistic target of rapamycin (mTOR) and PTEN, which are postulated to underlie

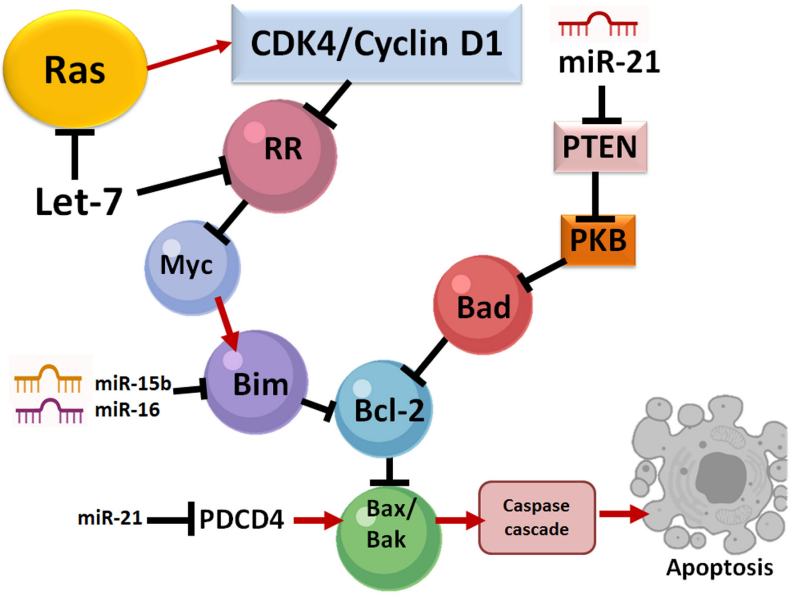

Figure 2. Regulation of apoptosis by miRNAs. All of miR-16, miR-15b, and miR-21 act on several molecular pathways, including Bak, Bcl-2, and Bax pathways during this process. miR-15b, miR-16, and Let-7 down regulate the expression of Bcl-2 (an anti-apoptotic protein) and trigger the release of cytochrome c (a pro-apoptotic factor). Down regulation of Bcl-2 reduces the mitochondrial membrane potential while cytosolic cytochrome c activates the caspase cascade, which subsequently leads to apoptosis. The role of miR-21 is two-fold: protection of neural cells and inhibition of apoptosis. Also, miR-21 down regulates PDCD4 to limit the activation of pro-apoptotic molecular pathways involving Bak and Bax. This, in turn, hinders apoptosis. Also, pro-apoptotic PTEN is down regulated by miR-21, resulting in an anti-apoptotic effect

exercise-induced enhancement of neuronal regeneration in SCI[42]. In another study, it has been shown that upregulation of mTOR occurs by deleting the *PTEN* gene in mice, resulting in axon regeneration in the injured optic nerves[43,44]. Attenuation of axonal damage and neuronal death is highly crucial for recovery of locomotor function in preclinical models of SCI[45]. Animal model research also suggests that miR-210 carries regenerative properties to cause axon growth in the context of SCI[46]. Administration of miR-210 to SCI mice decreased the expressions of protein-tyrosine phosphate 1B and ephrin-A3 to contribute to spinal cord repair by promoting angiogenesis.

miRNAs in neuronal cell cycle and functional recovery

There is evidence to show that miRNAs not only contribute to regeneration but also stimulate neuron growth and promote functional recovery[47]. Bioinformatic studies propose that miRNAs promote balance between the cell division cycle 42 gene and the brain-derived neurotrophic factor (*BDNF*) gene, both of which influence self-repair in SCI[48]. Studies show that miR-124 is associated with inhibition of neuronal apoptosis and improvement of motor scores in SCI, with the possibility of even restoring limb functionality after SCI[49]. Study suggests that miR-133b promotes neurite outgrowth via ERK1/2 and PI3K/Akt signaling pathway by RhoA suppression[50]. In terms of functional recovery, evidence shows that miR-133b has the ability to suppress the molecules that inhibit axon regrowth and thereby promote recovery of locomotor function after SCI[51].

miRNAs and neuropathic pain

A rich body of evidence has linked miRNAs to the regulation of SCI-related pain, both neuropathic and inflammatory[52]. According to researchers, the changes in miRNA expression induce increase in insulin-like growth factor-1 expression and down regulation of BDNF. This study concludes that the combination of these changes results in a decrease in inflammation and pain in SCI animals. Down regulation of miR-218 alleviates neuropathic pain by controlling the expression of cytokine signaling, which in turn

inhibits the JAK/STAT3 pathway in SCI animals[53]. The expression of miR-124 mainly in neurons is found throughout the brain and spinal cord. A recent study has corroborated the sensitivity of miRNAs to SCI by showing that expression of miR-124 in neurons is significantly decreased within 7 days after SCI to show the severity of injury[54]. The dynamic changes in expression of miRNAs play multiple regulatory mechanisms that may be leveraged to reduce neuropathic pain and potentially shed new light on the progression of maladaptive plasticity in SCI[55].

It has been demonstrated that miR-146a and miR-129-2 regulate pain during the early stages of SCI[56]. In this study, the investigators validated the expression of miRNAs by quantitative reverse transcription-polymerase chain reaction and *in situ* hybridization assays, revealing that SCI affected miRNA expression that persisted up to 14 days and expanded both anteriorly and caudally beyond the lesion site. It has been suggested that the effect of miRNAs on pain is not necessarily limited to the lesion site. It has been suggested that SCI induces changes in the expression of miRNAs in higher cortical structures, which control neuropathic and inflammatory pain, and miRNAs may serve as specific biomarkers for future targeted therapy of neuropathic and inflammatory pain conditions[57].

MOLECULAR INSIGHTS INTO ROLES OF MIR-96 IN SCI

miR-96 in neuroprotective therapy in SCI

It is essential to mention that miR-96 is one of three miRNAs that make up the miR-183 cluster (the other two miRNAs of this cluster are miR-183 and miR-182)[58]. Recent studies confirm that expression of miR-96 is dramatically decreased after SCI, favoring induction of apoptosis due to increase in expression of its targets, which are pro-apoptotic proteins[59]. Another recent research showed that increase in expression of miR-96 suppressed microglia activation marker proteins and inhibited inflammatory cytokines such as tumor necrosis factor-α and interleukin-1β to promote recovery in SCI[60]. Additionally, it has been reported that miR-96-5p regulates cysteine transporters such as the excitatory amino acid (EAA) transporter 3/EAA carrier 1 (one of the amino acid carriers involved in neuronal glutathione synthesis), which produces neuroprotective benefits against oxidative stress[61]. According to these researchers, rhythmic diurnal fluctuations of glutathione levels occur when miR-96-5p is blocked indirectly influencing the neuroprotective effect[62]. The subsequent section of this article will describe recent research related to roles of miR-96 in inhibition of apoptosis, promotion of cell proliferation, and alteration of other molecular pathways.

miR-96 in inhibition of apoptosis

Research shows that miR-96 may target and inhibit apoptotic factors at the protein and mRNA levels. For instance, miR-96 indirectly inhibits apoptosis through its effect on the forkhead transcription factor of the O class 1 (FOXO1) transcription factor[63]. Studies have shown that FOXO1 induces apoptosis via mitochondria-independent and mitochondria-dependent pathways. Also, miR-96-5p decreases the levels of caspase-9 (an important caspase in the mitochondrial pathway of apoptosis) by binding to the CASP9 3'-untranslated region (3'UTR)[64]. Overexpression of miR-96-5p is associated with inhibition of apoptosis[65].

miR-96 in cell proliferation

There is a growing interest in the study of miR-96, its effect on FOXO1 levels, and how this affects cell proliferation. For example, it has been shown that breast cancer causes upregulation of miR-96 by targeting protein tyrosine phosphatase, non-receptor type 9, this increases cell migration and proliferation and indirectly affects the pathophysiology mechanisms of breast carcinogenesis[66]. Studies on miR-96, specifically its role in hepatocarcinogenesis and treatment of hepatocellular carcinoma (HCC), have shown that miR-96 is significantly upregulated in HCC[67]. Studies also demonstrate that miR-96 targets the

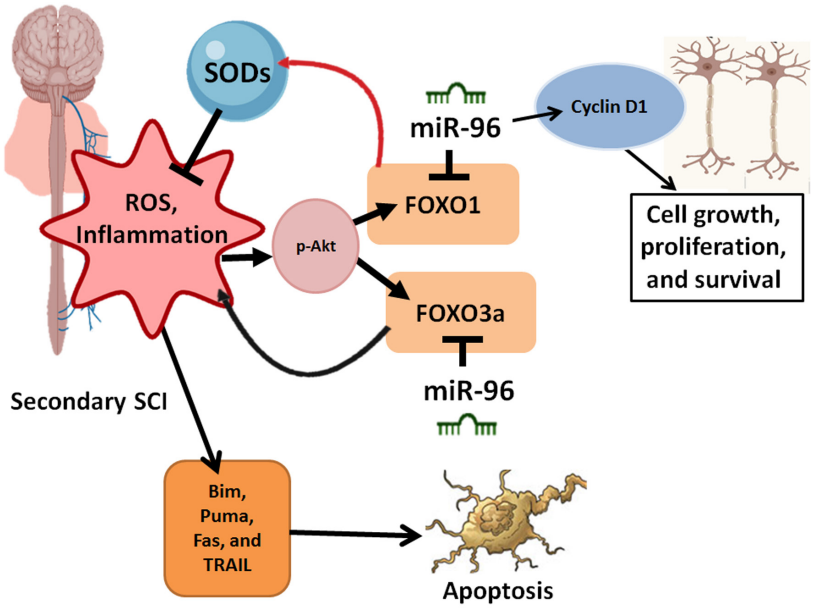

Figure 3. FOXOs facilitate cell cycle and metabolic regulation, which ultimately regulates apoptosis. At the beginning stage of spinal cord injury (SCI), FOXO1 activates the enzymes (e.g., superoxide dismutases or SODs) responsible for regulating oxidative stress from reactive oxygen species (ROS). With time FOXO1 induces autophagy, controls inflammation, and leads to cell cycle arrest. The role of miR-96 is most profound during the secondary stage of SCI, whereby it regulates FOXO proteins, including FOXO1 to enhances the survival of cells. FOXO3a is also down regulated by miR-96 affecting the expression of inflammatory pathways during SCI. This process also affects pro-apoptotic factors such as TRAIL, Fas, and Puma

FOXO subfamily, specifically FOXO3a and FOXO1. Inhibiting miR-96 upregulates FOXO1 and FOXO3a expression, suppressing colony formation and cell proliferation in HCC[68].

Studies have also shown that prostate cancer is associated with elevated expression of miR-96 and subsequent down regulation of FOXO1, a phenomenon that can be leveraged to control cell proliferation[69]. Other investigators studied the same phenomenon with a dataset containing non-malignant benign prostate tissue samples and prostate cancer tissue samples[70]. According to these researchers, overexpression of miR-96 decreases FOXO1 expression in both the non-malignant tissue samples and the prostate cancer tissue samples, even when the two samples are combined. Down regulation of FOXO3a and p27kip1 promotes axonal regeneration and proliferation of glial cells after SCI in rats[71]. An earlier investigation showed that down regulation of FOXO3a decreased p27kip1 at mRNA and protein levels after injury[72]. A recent study shows that miR-96 is highly essential for normal development of the auditory system, which is required for functional maturation in the peripheral and central auditory system[73].

miR-96 in regulation of FOXO pathway and other molecular pathways

FOXO pathways are of vital importance in regulating biological processes such as glucose metabolism, cellular proliferation, apoptosis, and repair of DNA damage[74]. FOXO transcription factors are also crucial in controlling neurodegenerative disorders through autophagy and apoptosis in the presence of oxidative stress[75]. Mounting evidence demonstrates that miR-96 plays a crucial role in regulating these FOXO pathways. For example, the expression of FOXO3a and FOXO1 is suppressed by miR-96, which controls the cell cycle, cell proliferation, and migration[76]. FOXO3a transcriptionally down regulates the level of p27kip1 (an important neurogenesis regulatory factor in mammals) positioning it as a candidate for controlling axonal regeneration after SCI. The role of miR-96 in regulating FOXO pathways has also been studied *in vitro*[77], demonstrating that miR-96 binds to the FOXO1 3'UTR sequence lowering the transcript levels of FOXO1 and elevating cell growth [Figure 3].

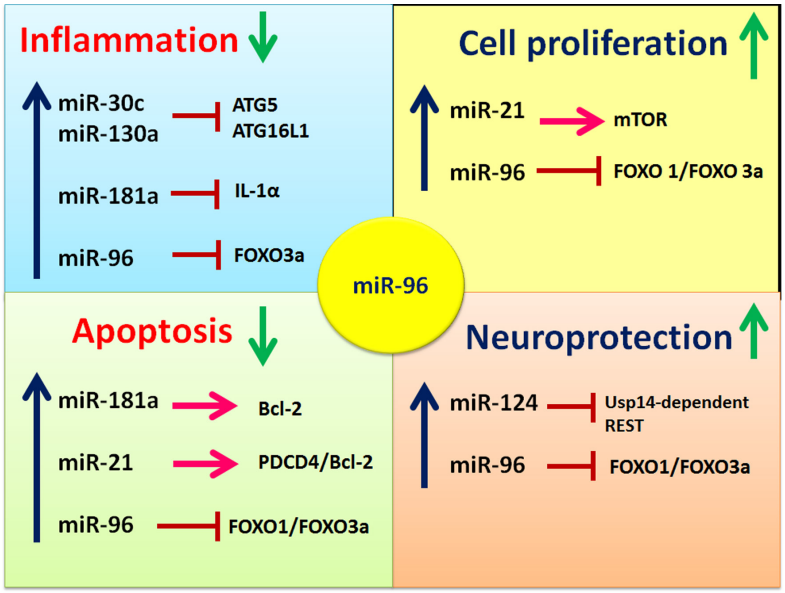

Figure 4. Summary of the roles of essential miRNAs including miR-96 in spinal cord injury (SCI). SCI affects the expression of miRNAs, which are known to regulate processes such as cell proliferation, inflammation, and apoptosis. Particularly, miR-96 is essential for neuroprotection as it induces multi-protective functions by targeting and down regulating FOXO pathways

Research shows that FOXO3a targets the cyclin-dependent kinase inhibitor p27kip1 and the pro-apoptotic molecule Bim, a phenomenon that triggers apoptosis[78]. Research has also shown that the effect of FOXO3a in regulating apoptosis is dependent on the expression of death receptor ligands such as the FasL[79]. There is evidence showing that miR-96 causes a reduction in the levels of both FOXO1 and FOXO3a for promoting cell proliferation[80], an effect that can be further investigated for neuroprotection and regeneration in SCI.

CONCLUSION

Recent preclinical evidence makes it clear that miRNAs are useful therapeutic tools that modulate critical molecular processes and enhance functional recovery after SCI. In this article, we have discussed some examples, highlighting how the expression of miRNAs triggers complex interactions and changes at the cellular and protein levels. These changes affect SCI pathophysiology and have been identified as regulators and contributors to secondary injury. The assertions made in recent studies indicate that manipulating the expression of miRNAs may provide an opportunity for developing the improved therapeutic and clinical interventions for dealing with the devastating consequences of SCI [Figure 4]. In fact, it is essential to note that not all miRNAs affect SCI positively. However, many of the miRNAs discussed in this review article have been shown to contribute positively to the management of SCI. For example, miR-96 promotes axonal growth, cell regeneration, neuroplasticity, and facilitates functional recovery, although more research is needed in this regard.

DECLARATIONS

Authors' contributions

Conceptualized the theme and conducted the literature review process: Almurshidi B
Contributed to preparation and revision of the manuscript, interpretation of subtopics, and preparation figures: Almurshidi B, Ray SK
Approved the final version to be published: Almurshidi B, Carver W, Scott G, Ray SK

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Bee venom acupuncture reduces neuroinflammation modulating microglia/macrophage phenotype polarization in spinal cord injury compression model

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Abstract

Aim: The present study aimed to examine whether apipuncture (stimulation of acupuncture points with bee venom) at ST36 and GV3 acupoints promotes neuroprotection and reduces neuroinflammation by modulating M1 and M2 phenotype polarization.

Methods: Wistar rats were treated with bee venom (BV) (0.08 mg/kg) injection at acupoints ST36 and GV3 [BV (ST36 + GV3)-spinal cord injury (SCI)] or BV injection at non-acupoints [BV (NP)-SCI] or no treatment (CTL-SCI) after SCI by compression. The spinal cord mRNA expression of iNOS, Arg-1 and TGF-β was measured by real time PCR and the levels of IBA-1; BCL-2; NeuN e CNPase was measured by western blotting. Locomotor performance was measured by Basso, Beattie, and Bresnahan (BBB) and grid-walking tests.

Results: Apipuncture treatment was able to (1) ameliorate locomotor performance; (2) reduce inflammatory markers (Cox-2 levels) and activation of microglia and macrophages; (3) reduce the polarization of the M1 phenotype marker

(iNOS) and increase M2 (Arg-1 and TGF-β) phenotypic markers; (4) promote neuroprotection by reducing the death of neurons and oligodendrocytes; and (5) increase the expression of the anti-apoptotic factor BCL-2.

Conclusion: Apipuncture treatment induces locomotor recovery and neuroprotection after the compression model of spinal cord injury. Further, it reduces neuroinflammation by decreasing M1 polarization and increasing M2 phenotype.

Keywords: Acupuncture, bee venom, spinal cord injury compression, microglia, macrophage, neuroinflammation

INTRODUCTION

Acupuncture, one of the therapies of Traditional Chinese Medicine based on the stimulation of specific body points[1], has shown positive effects in different experimental models of neurodegenerative diseases such as Parkinson's disease (PD)[2], amyotrophic lateral sclerosis (ALS)[3] and spinal cord injury (SCI)[4]. Different modalities of acupuncture points (acupoints) stimulation have been used to improve locomotor performance, reduce inflammation and promote neuroprotection in SCI models[4-7]. Bee venom (BV) acupuncture (apipuncture) is a therapeutic practice that BV (bee sting itself or diluted) is injected into acupuncture points[8,9].

BV is a complex substance with approximately 18 bioactive components, including phospholipase A2, histamine, norepinephrine, apamin and melittin (its principal component, representing 40%-60% of the BV total dry extract)[9,10]. The BV therapy (systemic administration of BV at doses generally between 0.1 and 0.5 mg/kg) has anti-nociceptive, anti-tumor, anti-inflammatory and anti-apoptotic effect[9-11]. Although several mechanisms of action have been implicated in the therapeutic effects of melittin or the other components of BV, e.g., the anti-arthritis effect of BV has been related to decrease in COX-2 and PLA2 expression and the decrease in the levels of TNF-α, IL-1, IL-6, NO, and ROS[9]. As BV is a complex substance, various mechanisms of it still need to be revealed.

When BV is applied in acupoints, it promotes intensification of its effects and more intense and lasting acupoint stimulation effects[12,13], which contribute to the clinical improvement of many diseases[9]. BV apipuncture has been used to treat inflammatory diseases such as rheumatoid arthritis[14] and to promote pain relief in patients with chronic low back pain[10,15]. Besides, it can also be applied as a complementary therapy to treat PD and ALS in humans[10,11,16]. Unfortunately, the mechanisms involved in the improvement of nervous system disorders are still poorly understood.

Previous results from our group, using a compression SCI model, showed that apipuncture in ST36 and GV3 acupoints was able to modulate the balance between pro-inflammatory (IL-6) and anti-inflammatory (IL-10) cytokines, promoting a reduction of spinal cord tissue loss and improvement of locomotor performance[17]. The neuroinflammation generated after a central nervous system (CNS) traumatic event is one of the most important factors that contribute to neurological losses leading to aggravation of sensory and motor neurological impairment[18-20]. However, the inflammatory response also promotes benefits, at certain stages of the inflammatory process, by phagocytizing cellular debris and stimulating tissue repair[18,21,22]. The duality of the inflammatory response is believed to be associated with the plasticity of microglia/macrophage polarization status[22-26].

The profile of microglia/macrophage polarization phenotypes in M1/M2 are initially simplified paradigms in an attempt to understand the complexity of the inflammatory response[22,25], in which M1 is described for presenting a pro-inflammatory profile, by stimulating and secreting inflammatory factors such as

iNOS, IL-1β, TNF-α; whereas M2 presents anti-inflammatory profile by promoting the release of anti-inflammatory cytokines such as IL-10, phagocyte myelin remnants and inhibiting factors to tissue regeneration[18,23,25,27,28]. After SCI, there seems to be a dominance of the M1 phenotype[25,27], and although more recent studies have shown that microglia can have multiple activation phenotypes (a "full spectrum of activation") and consider the model of two dualistic microglial state is too simplistic for revision[22]; therapeutic approaches that stimulate greater phenotypic polarization of microglia/macrophages in the M2 profile are likely to represent a promising tool[18,23,25].

Thus, in order to expand the previous results, using the same SCI model and followed the same therapeutic methodology, the present study aims to evaluate whether BV injection at ST36 and GV3 acupoints could reduce neuroinflammation by modulating the microglia/macrophages polarization in the M1 and M2 status, reduce apoptosis and promote the neuroprotection of neurons and oligodendrocytes in the SCI model by compression in rats.

METHODS

Spinal injury compression and groups

Adult male Wistar rats, weighing between 270 g and 300 g, were kept in 12/12-h light and dark cycles at a constant temperature, with food and water ad libitum. All procedures were approved by the Ethics Committee on Research of the Federal Rural University of Rio de Janeiro (23083.005880/2013).

Before the surgical procedure, the rats were anesthetized with a mixture of ketamine and xylazine (200 and 10 mg/kg, i.p.; respectively, FortDodge, São Paulo, Brazil). SCI induced by compression was similar to previously described by Vanický *et al.*[29]. As previously described[17], after exposure of the T10-L1 vertebra, a 2-French Fogarty catheter (Edwards Lifesciences, EUA, CA, USA) was inserted into the epidural space through a small hole in the vertebral arch (mini-laminectomy) of T10 and advanced cranially until the center of the balloon rested at the T8 and T9 level. The balloon catheter was inflated with 15 μL of saline for 5 min using a Hamilton syringe (type 1705). A sham-operated group was submitted to a mini-laminectomy without the insertion of the catheter[17].

At the end of the surgical procedure, the animals received injections of analgesic (fentanyl, 0.032 mg/kg, s.c.; Janssen Pharmaceutica, Beerse, Belgium) and prophylactic antibiotic (pentabiotic, 40,000 IU/kg, s.c.; FortDodge, São Paulo, Brazil). The rats with difficulty in spontaneous urination had their bladders emptied manually until they regained voiding function (generally in 12 h after surgery).

For this study, the animals were randomly divided into 4 groups: (1) Sham group, submitted to the mini-laminectomy without the insertion of the catheter; (2) CTL-SCI group which was only submitted to the spinal cord compression; (3) BV (NP)-SCI group received BV at non-acupuncture points at different time points after the SCI; (4) BV (ST36 + GV3)-SCI group received BV injection at ST36 and GV3 acupoints at different time points after the SCI[17].

BV solution preparation and treatments

BV (*Apis mellifera*, catalog #: V3375; Sigma, St. Louis, MO, USA) at a dose of 0.08 mg/kg was diluted in saline solution and the application of BV solution was performed according to the group as previously described[17]. BV (ST36 + GV3)-SCI group received a subcutaneous injection of BV solution at acupoints ST36 and GV3 (20 μL at each point). The acupoint ST36 is located approximately 5 mm below and lateral to the anterior tubercle of the tibia, and GV3 is located on the dorsal midline at the depression between the spinal processes of the last lumbar and the first sacral vertebrae[30]. The BV (NP)-SCI group was injected with the same dose and volume of BV at non-acupoints located in the same dermatome as the acupoints.

For ST36, the non-acupoint was located 5 mm lateral to the midline of the posterior face of the hindlimb, and for GV3, approximately 1 cm lateral to the GV3 on the crest of the ilium[17].

Animals subjected to behavioral analysis received BV immediately after the SCI, and then weekly until the fifth week; while rats subjected to methods of spinal tissue extraction (qPCR and Western blotting analyzes) received BV only immediately after SCI. Spinal cord samples were collected at different time points as described in the following topics.

Behavioral analyzes after SCI

For the evaluation of the locomotor capacity, the animals were submitted to the Basso, Beattie, and Bresnahan (BBB) test as described previously[31]. The BBB test, developed by Basso and colleagues, is a well-established test widely used for investigating the mechanisms involved with the pathophysiology of SCI and possible therapeutic targets. Each animal was placed individually in the center of the open field and was observed for 4 min. During walking in the open field, locomotor parameters are observed and compared with the BBB test scale which has scores ranging from 0 (no spontaneous movement in the hind paws) to 21 (normal locomotion). The evaluation was performed weekly and sometimes up to twice in the same week from the first to the thirty-fifth-day post-injury (1, 4, 7, 10, 14, 21, 28, 35 days after SCI) by two raters blinded to the experiment. The values were represented as mean ± standard error.

Based on prior publications[32], a grid walk was constructed for rats using two parallel pieces of acrylic plates (1 m in length) to hold metal bars (10 cm in length) with 1-4 cm apart[32]. Before the injury, rats were trained for 3 days on the apparatus. Each rat was allowed to cross the grid walk 3 consecutive times at 35th day after injury and the number of "footfalls", or the number of times that the animals' hind paws fall through the rungs were counted and represented as mean ± standard error. Animals unable to move the hind limbs were assigned a maximum of 20 footfalls[4]. The grid walk test evaluates the sensory-motor coordination between hindlimbs and forelimbs and examines the deficits in descending motor control[32]. Although the BBB is a reliable test, the combined use of other tests as the grid walk can facilitate the distinction of different motor and sensory impairments. Pajoohesh-Ganji *et al*.[33] have shown that combined scoring method can help the discrimination of different injury levels and produce less variability than the individual tests, which can help to follow motor recovery after SCI.

Western Blotting

The Western blotting technique was used to evaluate IBA-1; BCL-2; NeuN e CNPase, where approximately 1 cm of the spinal cord at the lesion site was collected at different time points (days 1, 3, 5 and 7 after the SCI). Initially, the collected tissue was mechanically macerated so that the cellular proteins were fully lysed and homogenized in extraction buffer (Tris-HCl, pH 7.2) containing the protease inhibitor cocktail (Protease Inhibitor Cocktail Tablets - Roche Diagnostics, Indianapolis, USA). Immediately after extraction, the samples were centrifuged at 20,000 *g* for 40 min at 4 °C and the total protein concentration of the supernatant was measured by the BCA method using a spectrophotometer (Thermo Scientific, Washington, DC, USA). To perform the electrophoresis and transfer steps, 30 µg of protein per sample were solubilized in a buffered solution [20% glycerol, 1 M Tris (pH 6.8), 4% sodium dodecyl sulphate (SDS), 0.1 M dithiothreitol and 0.02% bromophenol blue; pH 6.8] submitted to SDS-polyacrylamide gel and subsequent transfer of the protein to the nitrocellulose membrane. To promote blocking of non-specific bonds, the membranes remained for 90 min in blocking solution [10% BSA dissolved in 1.5 M saline + 0.1% Tween 20 Tris-HCl buffer (TBS-T)].

Overnight, the membranes remained incubated with the primary antibodies anti-β-actin (rabbit, dilution 1:3000, Abcam, Germany), anti-IBA-1 (goat, dilution 1:500, Santa Cruz, USA), anti-GFAP (mouse, dilution 1:1000, Abcam, Germany), anti-BCL-2 (mouse, dilution 1:500, Santa Cruz, USA), anti-NeuN (rabbit,

dilution 1:1000, Millipore, USA), anti-CNPase (mouse, dilution 1:750, Millipore, USA) at 4 ℃ in a solution containing TBS-T and 5% BSA. After washing they remained incubated with secondary antibodies peroxidase-conjugated (Abcam, Germany) anti-rabbit (dilution 1:5000), or anti-goat (dilution 1:2000), or anti-mouse (dilution 1:1000) diluted in a solution containing 5% BSA in TBS-T for 1 h.

For detection of the bands, the membrane was incubated with chemiluminescence reagents (ECL; Bio-Rad, Hercules, CA, USA) and suffered 90 s of exposure in ChemiDoc XRS + Imaging System (Bio-Rad, Hercules, CA, USA). β-actin was applied in the same blot technique for densitometric measurements to normalize the intensities of specific bands using the ImageLab program of Bio-Rad (Bio-Rad, Hercules, CA, USA).

Real-time PCR

Spinal cord samples from animals of all groups and collected at different time points (days 1, 3, 5 and 7 after the SCI) were submitted to total RNA extraction using QIAzol Lysis Reagent (Qiagen) and eluted in 30 μL of RNAse free water. For mRNA analyzes, High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific) was used to prepare cDNA in a final concentration of 50 ng/μL, following the manufacturer's protocol. For qRT-PCR, the following reaction was prepared: 5.9 μL of nuclease-free water, 7.5 μL of Power SYBR® Green PCR Master Mix, 10 μM of forward and reverse primers and 5ng of diluted cDNA. Quantitative gene expression was normalized to the expression levels of housekeeping gene *GAPDH*. The target genes from the experimental group were compared with those from the control group using the 2-ΔΔCT method. Primer sequences for each of the mRNA targets were the following: Arginase-1 forward: 5' ATATCTGCCAAGGACATCGTG 3', reverse: 5' AGGTCTCTTCCATCACTTTGC 3'; iNOS forward: 5' GGAGCAGGTTGAGGATTACTTC 3', reverse: 5' TCAGAGTCTTGTGCCTTTGG 3'; TGF-β forward: 5' TGGCGTTACCTTGGTAACC 3', reverse: 5' GGTGTTGAGCCCTTTCCAG 3'; COX-2 forward: 5' TCAAGGGAGTCTGGAACATTG 3', reverse 5' GCTTCCCAACTTTTGTAACCG 3'; GAPDH forward: 5' CCATCAACGACCCCTTCATT 3', reverse: 5' GACCAGCTTCCCATTCTCAG 3'.

Statistical analysis

All statistical analyses and construction of the graphs were performed by GraphPad Prism 5.0 software (San Diego, CA, USA).To perform the BBB test analysis, the data were submitted to a two-way analysis of variance (ANOVA) for repeated measures followed by Bonferroni post-test. For the analysis of the Grid Walk test, Western blot and qRT-PCR, the data were submitted to one-way ANOVA followed by Bonferroni post-test. For data that did not present normal distribution, the Kruskal Wallis test was performed followed by Dunn's post-test. All dates from this experimental protocol were expressed as mean ± standard error of the mean. The statistics were considered significant only when $P < 0.05$.

RESULTS

Apipuncture promotes functional recovery in SCI rats

After SCI, rats show 0-1 scores in the BBB test, meaning no spontaneous movements in the hind limbs. Compared to CTL-SCI and BV (NP)-SCI groups, BV (ST36 + GV3)-SCI groups showed significant higher scores in BBB test, at 7, 14, 21 and 35 days after SCI (Two way Anova for repeated measures, $P < 0.001$). In the same way, the BV (ST36 + GV3)-SCI group had a significantly lower footfalls in the grid-walking test in comparison with the CTL-SCI and BV (NP)-SCI groups (One way ANOVA followed by Bonferroni test; $P < 0.001$) [Figure 1].

Apipuncture changes polarization phenotypes of M1 (iNOS) and M2 (Arg-1 and TGF-β)

BV (ST36 + GV3)-SCI group showed significant lower expression of iNOS mRNA (M1 marker) than CTL-SCI and BV (NP)-SCI groups in the 3rd and 5th day after SCI (One way ANOVA followed by Bonferroni

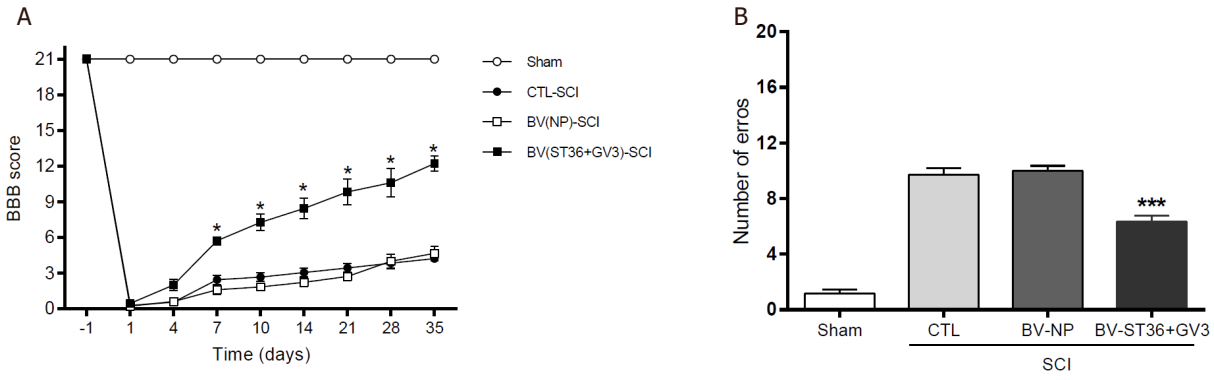

Figure 1. Apipuncture improves locomotor performance in SCI rats. The results of apipuncture at GV3 and ST36 acupoints [BV (ST36 + GV3)-SCI, *n* = 7] were compared to apipuncture at non-acupoints [BV (NP)-SCI, *n* = 8], control group without manipulation (CTL-SCI, *n* = 7) and Sham (*n* = 7) in rats submitted to compression SCI model. BV (ST36 + GV3)-SCI had higher BBB scores (A) at 7, 10, 14, 21, 28 and 25 days after SCI and lower footfalls in Grid-walking test (B) than CTL-SCI and BV (NP)-SCI groups at 35 days after SCI. Values are presented as mean ± SEM. **P* < 0.05 and ****P* < 0.001 compared to CTL-SCI and BV (NP)-SCI groups. BV: bee venom; NP: non-acupoints; BBB: Basso, Beattie, and Bresnahan; SCI: spinal cord injury

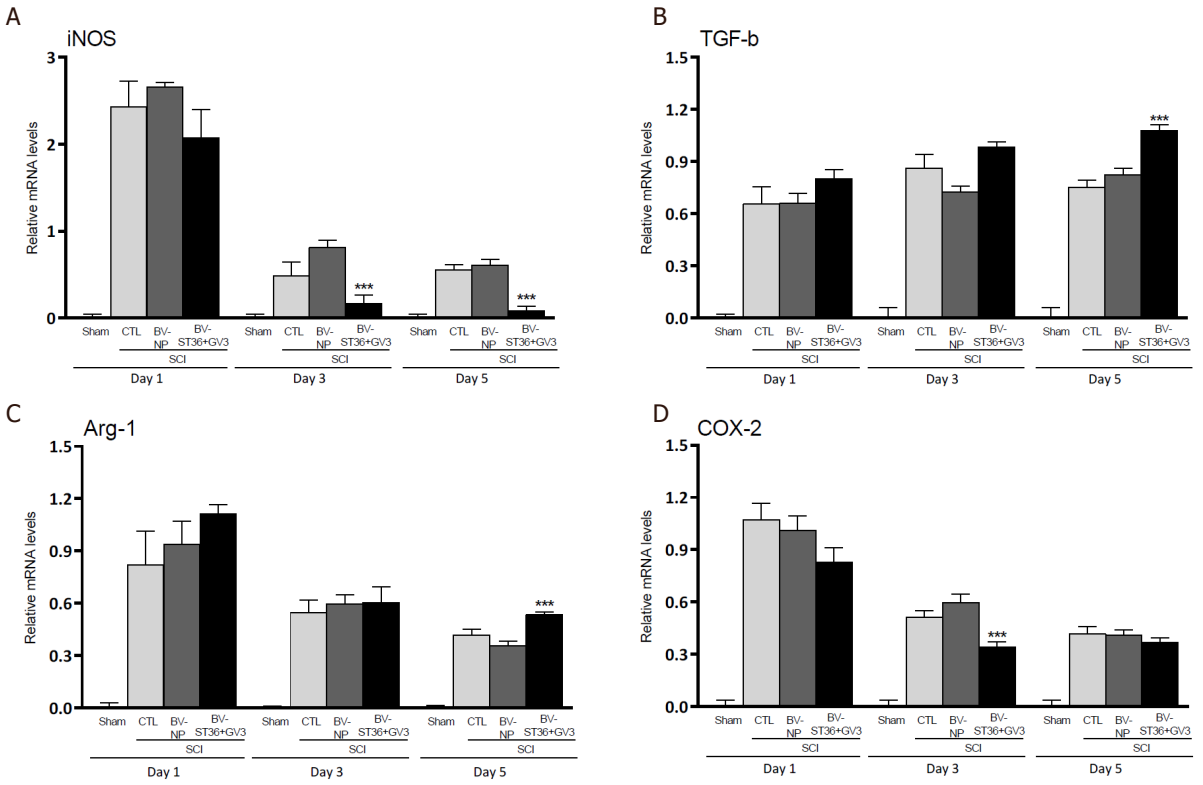

Figure 2. Influence of apipuncture in the mRNA expression of M1 (iNOS) and M2 (TGF-β and Arg-1) phenotype markers and COX-2 in the spinal cord 1, 3 and 5 days after SCI. The graph represents the iNOS (A), TGF-β (B), Arg-1 (C) and COX-2 (D) mRNA expression at the site of spinal cord injury in rats submitted to SCI and apipuncture at ST36 and GV3 points [BV (ST36)-SCI; *n* = 5], SCI and apipuncture at non-acupoints [BV (NP)-SCI, *n* = 5], only SCI (CTL-SCI, *n* = 5) and Sham-SCI (*n* = 5). Values are presented as mean ± SEM, ****P* < 0.001 compared to CTL-SCI and BV (NP)-SCI. BV: bee venom; NP: non-acupoints; SCI: spinal cord injury

test; $P < 0.001$ and $P < 0.05$, respectively), and higher expression of Arg-1 and TGF-β mRNA (M2 markers) than BV (NP)-SCI and CTL-SCI controls ($P < 0.05$) in the 5th day after SCI [Figure 2].

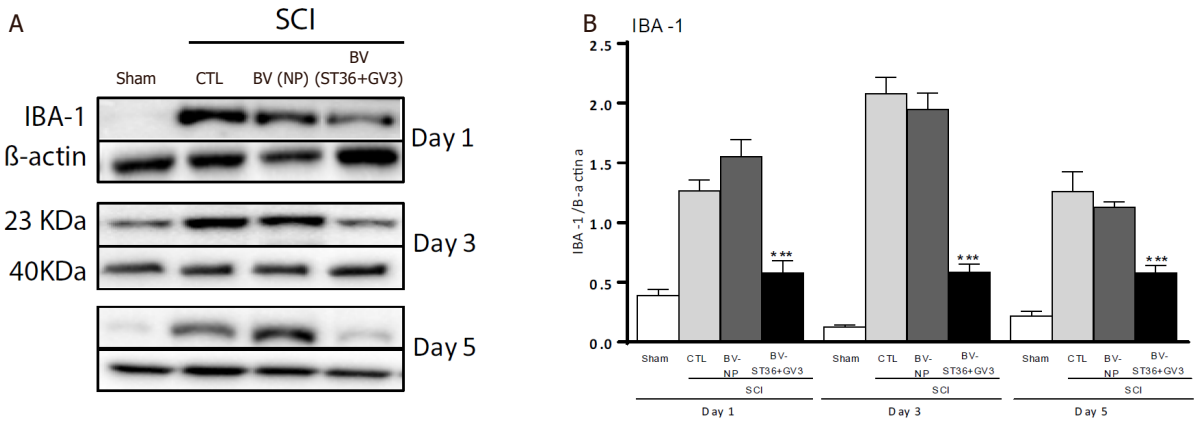

Figure 3. Influence of apipuncture on IBA-1 levels in the spinal cord 1, 3 and 5 days after SCI. In A: representative of the bands of proteins of IBA-1 by the groups Sham, CTL-SCI, BV (NP)-SCI and BV (ST36 + GV3)-SCI measured by densitometry, normalized by β-actin; In B: the comparison of the IBA-1 protein content at the spinal cord lesion site of rats submitted to SCI and apipuncture at ST36 and GV3 points [BV (ST36)-SCI; *n* = 6], SCI and apipuncture at non-acupoints [BV (NP)-SCI, *n* = 6], only SCI (CTL-SCI, *n* = 6) and Sham-SCI (*n* = 6). Values are presented as mean ± SEM. ***$P < 0.001$ compared to CTL-SCI and BV (NP)-SCI. BV: bee venom; NP: non-acupoints; SCI: spinal cord injury

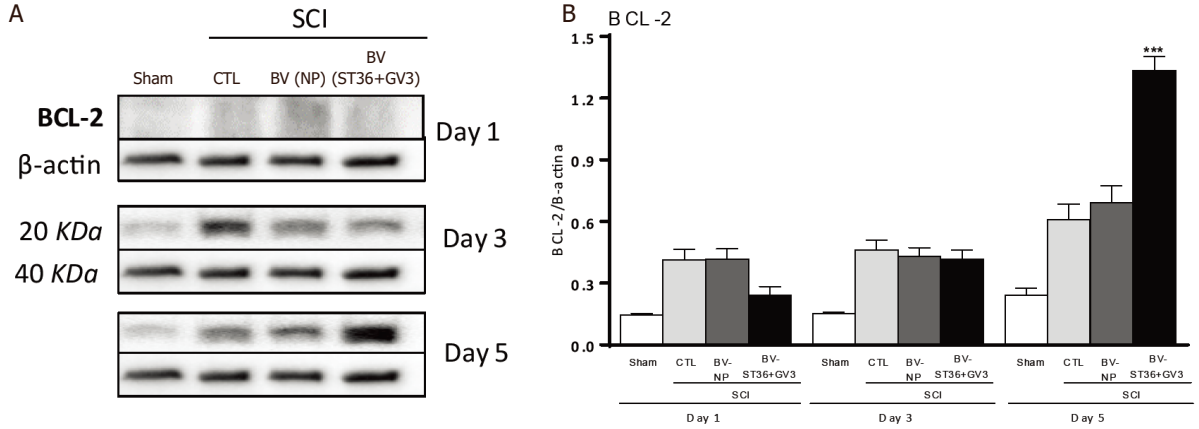

Figure 4. Influence of apipuncture on BCL levels in the spinal cord 1, 3 and 5 days after SCI. In A: representative of the bands of proteins of BCL-2 by the groups Sham, CTL-SCI, BV (NP)-SCI and BV (ST36 + GV3)-SCI measured by densitometry, normalized by β-actin; In B: the comparison of the BCL-2 protein content at the spinal cord lesion site of rats submitted to SCI and apipuncture at ST36 and GV3 points [BV (ST36)-SCI; *n* = 6], SCI and apipuncture at non-acupoints [BV (NP)-SCI, *n* = 6], only SCI (CTL-SCI, *n* = 6) and Sham-SCI (*n* = 6). Values are presented as mean ± SEM. ***$P < 0.001$ compared to CTL-SCI and BV (NP)-SCI. BV: bee venom; NP: non-acupoints; SCI: spinal cord injury

Apipuncture reduces activation of microglia/macrophages

The group BV (ST36 + GV3)-SCI showed significant lower levels of IBA-1 (microglia/macrophage active marker) protein compared to CTL-SCI and BV (NP)-SCI groups at all times evaluated (1st, 3rd and 5th days) after SCI (One way ANOVA followed by Bonferroni test; $P < 0.001$) [Figure 3].

Apipuncture increases anti-apoptotic factor BCL-2 and neuroprotection of neurons and oligodendrocytes

The group BV (ST36 + GV3)-SCI showed significant higher levels of BCL-2 (anti-apoptotic factor marker), compared to CTL-SCI and BV (NP)-SCI groups on the 5th day after SCI (One way ANOVA followed by Bonferroni test; $P < 0.001$) [Figure 4]. Furthermore the group BV (ST36 + GV3)-SCI showed significant higher levels of NeuN (neuron marker) and CNPase protein (enzyme marker expressed by viable oligodendrocytes), compared to CTL-SCI and BV (NP)-SCI groups on the 7th day after SCI (One way ANOVA followed by Bonferroni test; $P < 0.001$) [Figure 5].

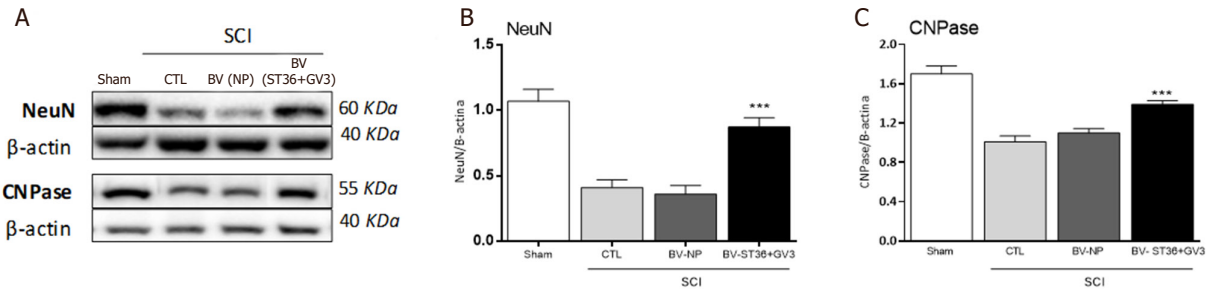

Figure 5. Influence of apipuncture on NeuN and CNPase levels in the spinal cord 7 days after SCI. In A: representative of the bands of proteins of NeuN and CNPase by the groups Sham, CTL-SCI, BV (NP)-SCI and BV (ST36 + GV3)-SCI measured by densitometry, normalized by β-actin; In B and C: the comparison of the NeuN and anti-CNPase protein content at the spinal cord lesion site of rats submitted to to SCI and apipuncture at ST36 and GV3 points [BV (ST36)-SCI; $n = 6$], SCI and apipuncture at non-acupoints [BV (NP)-SCI, $n = 6$], only SCI (CTL-SCI, $n = 6$) and Sham-SCI ($n = 6$). Values are presented as mean ± SEM. ***$P < 0.001$ compared to CTL-SCI and BV (NP)-SCI. BV: bee venom; NP: non-acupoints; SCI: spinal cord injury

DISCUSSION

Our results demonstrated that apipuncture treatment promoted improvement in locomotor function in the SCI compression model in rats. This improvement seems to be associated with neuroinflammation modulation through the reduction of the microglia/macrophage protein marker IBA-1, changes in the expression of M1 (iNOS) and M2 gene markers (TGF-β and ARG-1), and decrease of COX-2 expression. Furthermore, apipuncture increased protein levels of the anti-apoptotic marker BCL-2, as well as NeuN and CNPase markers indicating the reduction of the death of neurons and oligodendrocytes at the site of SCI.

The previous study conducted by our group, using the same experimental protocol showed that BV acupuncture at ST36 and GV3 acupoints was able to improve locomotor performance, reduced the area of tissue damage in the spinal cord and modulate the balance between cytokines by increasing the anti-inflammatory cytokine IL-10 and reducing the proinflammatory cytokine IL-6[17]. From these data, in the present study, we investigate whether the acupuncture mechanism could be associated with M1 and M2 status since apipuncture has shown to influence IBA-1 expression and the balance between proinflammatory and anti-inflammatory cytokine. Our data showed that apipuncture treatment reduced the expression of the iNOS gene a marker associated with M1 status whilst decreased TGF-β and ARG-1, associated with M2 status. Zhao *et al.*[7] demonstrated similar results in which electroacupuncture (EA) at Jizhong (GV6) and Zhiyang (GV9) acupoints was able to reduce IL-1β, IL-6 and TNF-α proinflammatory cytokines whilst increasing the anti-inflammatory cytokine IL-10. Additionally, EA reduced the proportion of $CD68^+$ and $CD86^+$ markers expressed by M1 status and increased $CD68^+$ and $CD206^+$ M2 markers in immunohistochemical analysis. From these results, the authors suggested that the mitigation of the inflammatory process occurs due to modulation on M1 and M2 status.

In the acute phase of the inflammatory response following SCI, M1 polarization phenotype markers are prevalent and pro-inflammatory factors such as IL1β, IL-6, TNF-α and iNOS contributing to positive feedback that triggers an exaggerated inflammatory response causing greater tissue damage around the SCI area[22,25]. However, the response generated by M1 status can be modulated by the M2 polarization phenotype that secretes anti-inflammatory factors such as IL-10, IL13, TGF-β and Arginase-1 (Arg-1)[18,27,34].

Previous studies have reported that the use of drugs, such as steroidal anti-inflammatory, that block the acute inflammatory response leads to more neurological damage in the SCI model than when this blockade does not occur[18,20,35]. One of the hypotheses for this worsening is intrinsically associated with the complexity of the phenotypic polarization that microglia and macrophages can acquire[18,21,25]. In SCI,

as well as in other neuroinflammatory diseases, there is deregulation between these states of polarization, causing greater death of neurons and oligodendrocytes due to greater initial activation of more cytotoxic status (M1) followed by a later activation of neuroprotective states (M2)[18,21,25,26]. Thus, it is believed that therapies that promote total blockade of the inflammatory response could inhibit both M1 and M2 status thus hampering tissue repair and aggravating damage after SCI[20,25]. Our data have shown that apipuncture was able to influence the reduction of iNOS, linked to M1 status and the increase of TGF-b and Arg-1, linked to M2 status, thus suggesting that just as EA, apipuncture treatment might modulate inflammation via M1/M2 status.

After the injury, an inflammatory response is initiated, in which microglia/macrophages quickly become active by chemical signals released by neural cell death[36,37], they are considered as one of the main initiators of the chronic response, triggering greater secondary damage[18,25,34]. When active, they express the ionized calcium-binding adapter molecule-1 (IBA-1) which has been widely used as a polarized microglia/macrophage marker[38].

Our data revealed significantly increased IBA-1 levels in the groups submitted to the surgical procedure compared to the sham group in the first 24 h, which remained increased until the 5th day. However, treatment with apipuncture at acupoints ST36 and GV3 significantly reduced the IBA-1 marker compared to control groups. This modulation in the IBA-1 levels is important in the reduction of neuroinflammation since after SCI there is an exacerbated neuroinflammatory response with microglia/macrophages activation[20,36,39,40], causing greater tissue damage, with the death of neurons and oligodendrocytes in the first hours after the trauma[18,25,41]. Similar results were shown by Kang *et al.*[8] using the SCI model followed by apipuncture treatment at ST36 which was able to reduce the IBA-1 marker detected by Western blotting. Moreover, the Kang and colleagues' study, the expression of the IBA-1 marker was very low in the sham group[8]. Manual acupuncture at ST36 acupoints can also contribute to the modulation of the inflammatory response due to a reduction of the IBA-1 marker in the ALS model using $hSOD1^{G93A}$ animals[3]. Our results suggest that apipuncture reduces microglia/macrophage polarization, but it is noteworthy that it is likely to be more involved with M1 status as it additionally reduced iNOS mRNA expression. More studies are needed to enhance these results.

In the present study, the apipuncture treatment was able to reduce COX-2 mRNA on the 3rd day after SCI. Although, in some tissues like the brain and the spinal cord COX-2 is constitutively expressed[42] after SCI occurs induction of COX-2[4], which increases mainly prostaglandin PGE2 that binds to prostaglandin E receptor subtypes in endothelial cells. It also stimulates the activation of NF-κB and consequently the nuclear transcription of chemokines such as MIP-1, attracting hematogenous macrophages to the lesion site. It is believed that this mechanism may contribute to a chronic inflammatory response in some situations[42,43]. Corroborating with our results, it has been reported that manual acupuncture reduces COX-2 expression 24 h after SCI and that EA also reduces COX-2 expression in the neuropathic pain model[4,44].

In the present study, apipuncture at ST36 + GV3 also increased BCL-2 levels on the 5th after SCI. Additionally, on the 7th day after SCI, apipuncture significantly minimized the reduction of NeuN protein content (a neuron marker) and of CNPase, an enzyme expressed by viable oligodendrocytes, indicating a lower death of these cell types. Previous studies also indicated that acupuncture and electroacupuncture were able to increase BCL-2 protein levels and reduce BAX and caspase-3 levels, maintaining a higher number of viable neurons after SCI[4,45]. After SCI, there is also an imbalance between pro and anti-apoptotic proteins. BAX is a pro-apoptotic protein that stimulates mitochondrial damage through the formation of pores in the mitochondrial membrane, releasing cytochrome C (Cyt-C) which stimulates the cleavage of caspase-3, an enzyme that leads to cell death[46,47]. BCL-2 is an important anti-apoptotic protein that acts by modulating BAX and reducing the stimulation of intrinsic apoptotic factors[48,49]. Corroborating

our data, Khalil *et al.*[50] demonstrated that apipuncture is capable of increasing BCL-2 expression in the PD model.

Despite the limitation of study in not having immunohistochemical analyses, the increase of anti-apoptotic factor BCL-2 caused by apipuncture can be related to neuroprotection and survival of neurons and oligodendrocytes resulting in better sensory and locomotor performance. Choi *et al.*[4] demonstrated through the immunohistochemical technique that manual acupuncture in GV26 and GB34 acupoints reduced caspase-3 expression, increased the expression of neuron markers and oligodendrocytes at the lesion site, suggesting that greater survival rate of these cells is related to better performance in the behavioral tests (BBB and Grid Walk test).

The clinical practice of different acupuncture modalities, such as BV acupuncture and EA, has become increasingly used as a complementary therapy for symptom relief in patients with PD, ALS, and SCI[16,51,52]. It is noteworthy that in apipuncture, the BV causes a local "irritation" at the acupoints producing a more intense and lasting effect than the simple needling[8,9]. In this experimental protocol, the BV was used in a very low dose, with this purpose[12,13]. The ST36 and GV3 acupoints were chosen from the combination of other acupoints that were more efficient in promoting the improvement of locomotor behavior in the SCI model (unpublished data).

The exact mechanism involved in the anti-inflammatory effects of acupoint stimulation has not yet been completely elucidated. Currently, it has been postulated the stimulation of acupuncture points acts through the autonomic nervous system, transmitting signals via the vagus nerve and promoting anti-inflammatory responses[53]. In experimental models of peripheral inflammation, such as sepsis and rheumatoid arthritis, acupuncture increases vagus nerve activity through a mechanism known as "cholinergic anti-inflammatory reflex"[54]. The stimulation of acupoints activates sensory inputs that are propagated by Aδ and C fibers, resulting in the activation of different brain nuclei involved with peripheral vagal tone regulation and increase of ACh release[54]. In macrophages, ACh down-regulates the NF-Kb pathway by stimulation alpha-7 nicotinic receptor, partially attenuating the inflammatory response. In the present study, the role of vagal activity was not investigated, although it could contribute to the lower macrophage infiltration from the peripheral circulation[6,54]. The physiological mechanisms of acupuncture in the SCI models are still uncertain with many questions that need to be clarified. One of the intriguing points about it is that SCI lesion *per se* does not seem to block the ascending propagation of the neuronal signal produced by acupoint stimulation (once in some studies, including ours, the acupoints used is located below the SCI lesion). Therefore, further studies will be needed to clarify these questions.

In conclusion, despite some limitations, our results indicate that apipuncture may modulate the neuroinflammatory response via alteration of M1/M2 polarization status, in addition to increasing the apoptotic factor and promoting neuroprotection, which may in part contribute to a reduction in locomotor sequelae in the compression SCI model. Thus, we believe that apipuncture may be a potential therapeutic target as a complementary therapy for the treatment of spinal cord injury.

DECLARATIONS

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Authors' contributions

Made substantial contributions to conception and design of the study: Souza RN, Medeiros MA
Performed data analysis and interpretation: Souza RN, Monteiro LRN, Medeiros MA.

Performed data acquisition, as well as provided administrative, technical, and material support: Souza RN, Lopes JMA, Monteiro LRN, Barbosa RAQ, Hollmann G, Allodi S, Reis LC, Medeiros MA

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

All procedures were approved by the Ethics Committee on Research of the Federal Rural University of Rio de Janeiro (23083.005880/2013).

Consent for publication

Not applicable.

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Review

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Evaluation, treatment, and surveillance of neurogenic detrusor overactivity in spinal cord injury patients

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Abstract

Neurogenic detrusor overactivity is a common urodynamic finding in patients with supra-sacral spinal cord injury. Early evaluation, stepped management, and close follow-up reduce the risk of upper urinary tract deterioration, renal failure and incontinence. In this article, we aim to outline the modern pathway of the management of this complex disease. Evaluation of patients with history, physical examination, renal function assessment, cystoscopy, and urodynamic study are essential. Management of neurogenic detrusor overactivity with adequate bladder drainage, medical therapy, intra-detrusor botulinum injections, and surgery can be offered in a stepwise manner. Follow-up after specific interventions should be done in a timely fashion to detect treatment response and to avoid complications of poorly managed neurogenic detrusor overactivity.

Keywords: Spinal cord injury, neurogenic lower urinary tract dysfunction, neurogenic bladder, neurogenic derusor overactivity, evalutation, treatment, surveillance

INTRODUCTION

Spinal cord injury (SCI) is one of the most common causes of neurogenic lower urinary tract dysfunction, which affects more than 291,000 individuals in the United States and an annual incidence rate of 17,730 cases[1]. It is estimated that 70%-84% of SCI patients have neurogenic lower urinary tract dysfunction[2].

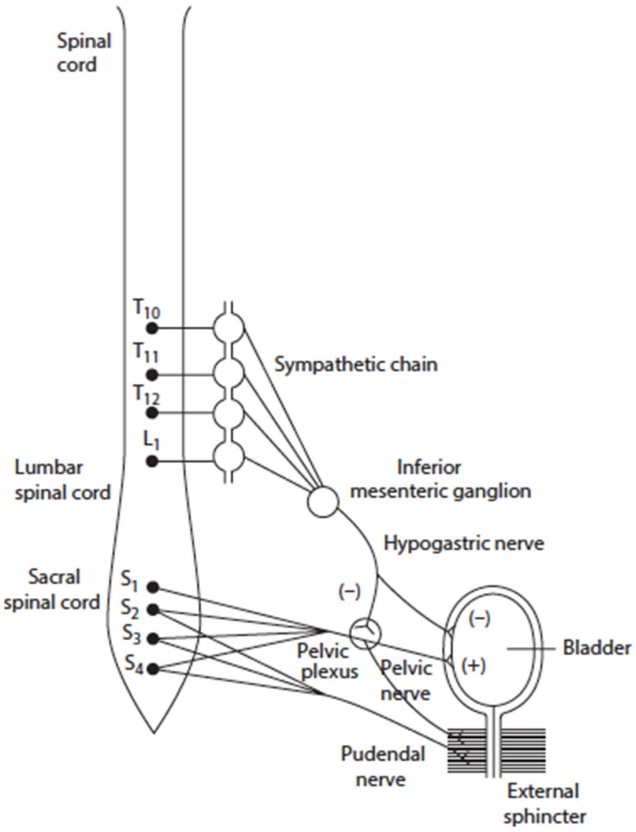

Figure 1. Lower urinary tract innervation (adopted from Aldousari *et al.*[4])

The effect of SCI on the lower urinary tract is variable and depends on the level and extent of the injury. Neurogenic detrusor overactivity (NDO) is a common urodynamic finding, reported to be present in up to 95% of supra-sacral SCI[3]. The ultimate goal of the management of neurogenic lower urinary tract dysfunction is to avoid morbidity and mortality secondary to renal failure and/or infections and to improve quality of life by controlling incontinence. The goal of this review article is to present an up-to-date pathway of the evaluation, treatment, and surveillance of NDO in SCI patients.

CLASSIFICATION OF NEUROGENIC LOWER URINARY TRACT DYSFUNCTION IN PATIENTS WITH SCI

The lower urinary tract is innervated by the hypogastric nerve, the pelvic nerve, and the pudendal nerve. The hypogastric nerve carries sympathetic innervation from spinal level T10-L1, while pelvic and pudendal nerves carry parasympathetic (pelvic), and motor and sensory innervation (pudendal) from the sacral spinal cord (S2-S4) [Figure 1][4]. Lower urinary tract dysfunction often follows certain patterns, based on the level of the injury, which can be classified into supra-pontine, infra-pontine supra-sacral, sacral, or infra-sacral[5]. The supra-sacral injury often results in NDO and infra-pontine supra-sacral lesions often result in detrusor-sphincter dyssynergia [Figure 2][5]. SCI at the vertebral level of T11 may cause sacral nerve damage which may cause underactive detrusor. Injury at the vertebral level of T7 may result in NDO. Injury in vertebral levels between T8 and T10 can generate either hypotonic or overactive detrusor[6]. It should be noted that those patterns do not happen in all patients, and further urological evaluation is mandatory to all patients to determine the exact neurogenic lower urinary tract dysfunction regardless of the level of SCI.

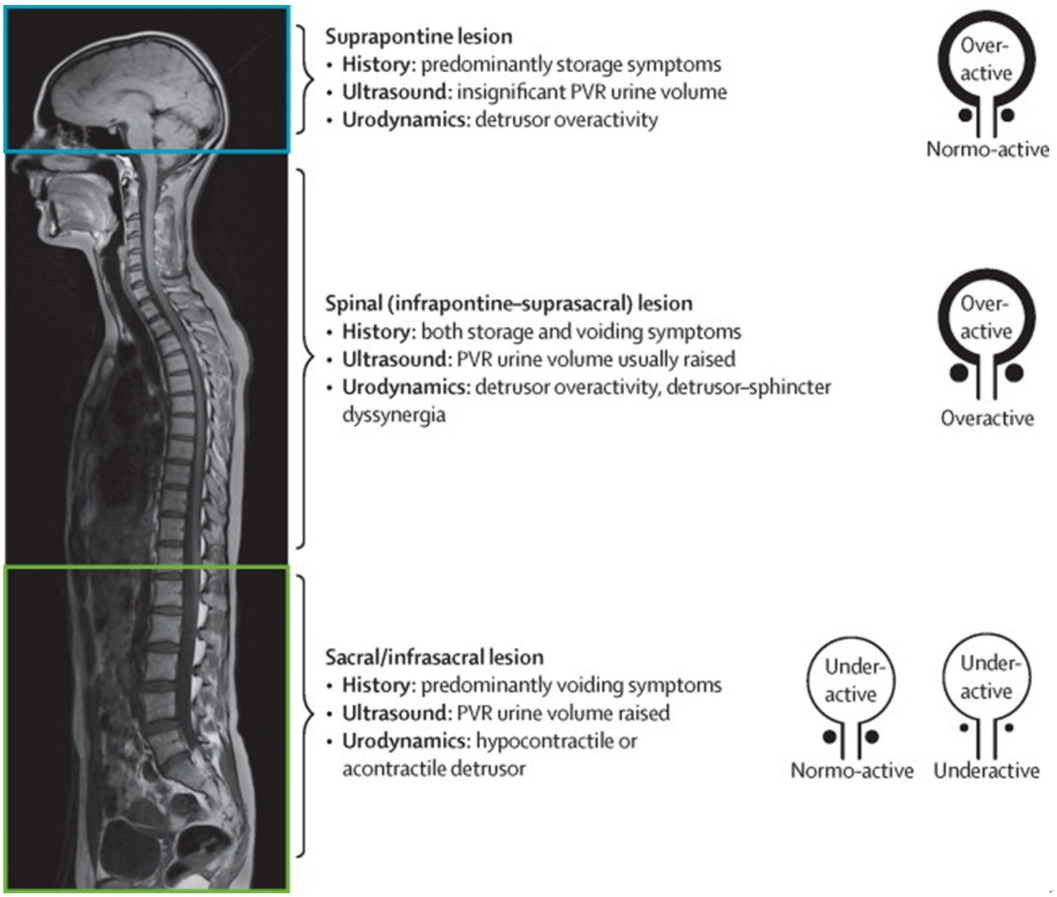

Figure 2. Expected lower urinary tract dysfunction based on the level of neurologic insult (adopted from Panicker *et al.*[5])

THE SEQUELAE OF UNTREATED LOWER URINARY TRACT DYSFUNCTION

The primary functions of the lower urinary tract are: (1) storage of urine under low vesical pressure without leakage; and (2) periodic complete expulsion of urine in socially acceptable time and location. Intact central and peripheral nervous systems are required to achieve these functions[7]. SCI and other neurologic disorders frequently affect the functions of the lower urinary tract. Untreated or poorly managed neurogenic lower urinary tract dysfunction may result in: (1) upper urinary tract deterioration resulting in end-stage renal disease; (2) urinary incontinence and urethral damage; (3) recurrent urinary tract infections and urolithiasis; and (4) autonomic dysreflexia[8]. In the last half-century, advancement in the management of neurogenic lower urinary tract dysfunction has led to decreased mortality caused by chronic kidney disease, from 50% in the 1950s and 1960s to less than 0.5% in the 1980s[9,10]. This emphasized the importance of prompt management of the neurogenic lower urinary tract dysfunction to prevent its sequelae. The 2019 report of the national SCI statistical center has reported a 2.9% mortality rate secondary to genitourinary diseases in SCI patients[11].

INITIAL EVALUATION AND MANAGEMENT AFTER ACUTE SCI

Early phase after SCI (the spinal shock)

After acute traumatic SCI, a spinal shock phase occurs. The bladder is hypotonic, and urinary retention and overflow incontinence happen in the absence of management. This phase resolves as early as 2 weeks, with an average of 8 weeks, and can be prolonged up to a year[6]. The extent and duration of this phase are variable and depend on the extension and the completeness of the spinal injury[12]. During this phase,

conducting a urodynamic study had been considered of limited clinical value[6]. Recent evidence has shown the presence of adverse urodynamic findings in those patients in the early phase (within 40 days of the injury)[12]. The best management at this phase is to ensure complete bladder drainage by intermittent catheterization (IC) or an indwelling urethral catheter[6-12]. Once the patient is stabilized medically, a trial of spontaneous voiding along with post-void residual measurement or self-intermittent catheterization, if the patient cannot void spontaneously, should be tried, while admitted in a rehabilitation facility[12].

Initial urological evaluation

This will take place at the first consultation and includes: detailed history, physical examination, bladder diary (often catheterization record), post-void residual, urinalysis and culture, renal function assessment, and upper urinary tract imaging[6,8,12]. Urodynamic assessment and cystoscopy may be indicated at the first evaluation, depending on the stage of evolution of the neurogenic bladder dysfunction.

History

Complete medical and surgical history is essential for further evaluation and potential management options and consideration of personalized treatment and follow up. Information about initial trauma and level and completeness of SCI (ASIA score), associated trauma to the urinary tract, and previous lower urinary tract diseases and treatments before spinal cord injury should be documented. The patient's mental and physical capacity should also be noted. History should also include the history of urinary tract infection frequency, symptoms, and treatments. Social history and social support, history of alcoholism or drug abuse are also important for long-term management. History of bowel management, past medical history of acute angle glaucoma, uncontrolled hypertension, and myasthenia gravis should be elicited as it might be a potential contraindication for medications that can be used for the treatment of NDO[6,12].

Physical examination

General physical examination is warranted, including a focused examination of the abdomen and genitalia. The ability to perform self-intermittent catheterization should be noted, along with previous abdominal scars or any contraindications that might complicate the insertion of suprapubic catheter insertion. If the clinician is in doubt regarding the patient's capability of performing IC, a consultation in ergotherapy could be very helpful. A focused neurologic exam is required, including anal tone, perineal sensation, and bulbocavernosus reflex[12].

Bladder diary

A bladder diary is highly recommended but not well studied[13]. It can add further information about how frequent the patient is urinating or catheterizing, the amount of urine drained, and the post-void residual. It can add further value in the monitoring of the treatment effect. Components of bladder diary recommended by ICS include voiding time, voided volume, incontinence episodes/use of pads, amount of fluids ingested, urgency, and incontinence degree, along with the method of emptying the bladder[12,14].

Urinalysis and culture

Urinalysis and microscopy are recommended in the initial visit and in follow up[8,12] to investigate the possibility of UTI in the presence of symptoms with subsequent urine cluture, and it can also detect microscopic hematuria, pyuria, or proteinuria, which might warrant further investigations. The presence of asymptomatic bacteriuria, which is frequent in patients performing IC, is not an indication of antibiotics in the majority of cases.

Post-void residual

Post void residual measurement is recommended in patients who void spontaneously, or uses valsalva voiding, reflexive voiding/crede voiding and/or condom catheter. The presence of elevated residual might

Table 1. Poor prognostic features on urodynamics study[6,8,12]

Urodynamic parameter	Abnormal value
Compliance	Low compliance (< 20 mL/cmH_2O)
Detrusor leak point pressure	Elevated (> 40 cmH_2O)
NDO	Any degree
Detrusor-sphincter dyssynergia	Any type
Vesico-ureteric reflex	Any grade
Bladder capacity	Reduced (<200 mL)
Sustained prolonged NDO	> 75 cmH_2O

NDO: neurogenic detrusor overactivity

trigger further evaluation and different management in those patients, as it increases the risk of UTIs and upper tract deterioration[8].

Urodynamic study

Urodynamic evaluation is the cornerstone in the evaluation of lower urinary tract dysfunction in SCI patients, but its technique and timing are essential[6,12,14]. Several urological authorities and guidelines recommend performing the first study 3 to 6 months after the injury[8,12,14], as recent evidence has shown that adverse urodynamic parameters can appear as early as 40 days after SCI[15]. However, in our practice, the first UDS is performed at the first sign of change in the urinary tract. It could be the onset of a UTI, the beginning of leakage between IC, the appearance or worsening of autonomic dysreflexia, *etc*. If the first study is done during the spinal shock phase, a repeat study is warranted after the resolution of spinal shock. Video Urodynamics, if available, is considered as the gold standard in the evaluation of patients with NLUTD after SCI, as it can detect vesicoureteric reflux and unmask hidden low bladder capacity and low compliance in patients with VUR[6,12,13]. Presence of poor prognostic features [Table 1] in the urodynamic study does require appropriate treatment and follow up urodynamics should be done to monitor the treatment effect and the need for further treatment[6,12]. Repeat urodynamic study should be selectively used based on the patient's course over time and with any change in clinical course[13]. There is no consensus in which intervals that urodynamics should be repeated in the high-risk population[6,8,12,13].

Imaging

Renal and bladder imaging is recommended in the initial evaluation of neurogenic bladder patients, preferably an ultrasound, to avoid the risk of exposure to radiation, at three months after the injury[6,8,12,13]. The ultrasound can detect complications of neurogenic lower urinary tract dysfunction, such as hydronephrosis, kidney or bladder stones, abnormal bladder morphology such as tumors, thickened bladder wall, or diverticulae, and renal atrophy or scarring[8,12]. The follow-up surveillance depends on the presence of adverse findings on urodynamics, which mandates more frequent imaging (every 6-12 months). The imaging might be delayed to 2-3 years in the absence of poor prognostic features on urodynamics[6].

Renal function assessment

Serum creatinine and estimated glomerular filtration rate are commonly used to assess renal function. It is less accurate than other methods such as creatinine clearance and nuclear GFR. Monitoring the changes of serum creatinine within the normal range is essential. It should be kept in mind that those patients might have less muscle mass, and GFR reduction might not reflect largely on serum creatinine level. Initial creatinine level and periodic follow-up are recommended, especially with unfavorable features on urodynamics, hydronephrosis, and febrile urinary tract infections[6,8,12,13].

Cystoscopy

Cystoscopy is an important, office-based evaluation of the lower urinary tract. It can detect bladder outlet obstruction due to urethral stricture or prostatic hypertrophy, and bladder abnormalities such as bladder

Table 2. Overview of management approach to neurogenic lower urinary tract dysfunction and NDO

Management Lines	Options
Assisted bladder drainage	Clean intermittent self catheterization, indwelling suprapubic catheters, indwelling urethral catheters
Systemic medications to reduce intravesical pressure	Anticholenergics, beta-3 agonists
Intra-detrusor botulinum toxin A injection	Onabotulinum toxin A, abobotulinium toxin A
Sacral nerve stimulation	N/A
Surgical treatment	Bladder augmentation, urinary diversion

NDO: neurogenic detrusor overactivity

tumor, trabeculation, bladder tumors, and bladder stones. Although the value of cystoscopy at initial evaluation has been questioned[8,12,13,16], We recommend doing cystoscopy at initial evaluation, and as diagnostic tool for patients who present with difficult catheterization to diagnose urethral stricture and false passage, or when presenting with recurrent urinary tract infection, increased incontinence, bladder spasticity and/or dysreflexia for possibility of finding a bladder stone. It should be noted that cystoscopy is a mandatory investigation for hematuria workup[7]. Screening cystoscopy for patients on a chronic indwelling catheter is recommended for early diagnosis of bladder cancer, although the value of such an approach has not been proven[17].

Treatment of NDO

The goals of NDO treatment are to reduce its risks on the urinary tract by reducing detrusor storage pressures, increasing bladder capacity, improving incontinence, and improving patient's quality of life. Table 2 summarizes management strategies of neurogenic lower urinary tract dysfunction and NDO. Treatment effect monitoring in a timely fashion (2-3 months) is essential to avoid long term complications of the poorly managed bladder[12].

Adequate bladder drainage using catheterization

It is estimated that around 75% of SCI injury patients cannot void spontaneously[18], which mandate assisted bladder drainage. Types of bladder drainage include: (1) clean intermittent self-catheterization (CISC); (2) indwelling suprapubic catheterization; and (3) indwelling urethral catheterization.

The patient should be aware of risks and benefits of the several methods of bladder drainage and advised to avoid indwelling urethral catheters if possible, to reduce risks of urinary tract infections, bladder stone formation, and urethral erosion[13,19]. It is recommended to keep patients, who cannot empty their bladder spontaneously, on CISC[8,12,13]. The frequency of CISC depends on many factors, such as fluid intake, bladder volume, and urodynamic parameters, and is recommended to do it 4 to 6 times/day[20]. CISC teaching is preferably done early during the rehabilitation phase, to evaluate the patient ability to perform it, and to evaluate for the possibility of spontaneous voiding[12].

CISC, although considered as the gold standard of assisted bladder drainage mechanism, have several limitations [Table 3], that treating physician should carefully assess and adjust those limitations if possible or shift the patient to alternative drainage options[18,21].

If CISC cannot be performed [Table 3], alternative management options include indwelling suprapubic catheterization or indwelling urethral catheterization. Suprapubic catheters are generally preferred over urethral catheters, with some studies supporting that preference. Suprapubic catheters have advantages over urethral catheters, which include the elimination of risks of urethral erosions and iatrogenic hypospadias[22], fewer risks of epididymitis and enable patients to perform sexual activities[23]. Some evidence showed a decreased risk of urinary tract infection with suprapubic catheters[24-26]. Indwelling catheters (urethral and

Table 3. Limitations of CISC[18,19]

Limitations of CISC	Examples
Limited upper extremity motor function	Quadriplegia
Anatomic limitation	Inability to reach urethra due to obesity or in female patients
Limited functional bladder capacity (below 300 mL)	Poor bladder compliance or untreated NDO
Obstructed urethra	Severe urethral damage, urethral stricture, non-relaxing sphincter

CISC: continuous intermittent self-catheterization; NDO: neurogenic detrusor overactivity

suprapubic) should be changed every 4-6 weeks, and the use of silicon catheters is recommended[22]. It should be kept in mind that indwelling catheters (urethral and suprapubic) associated with increased risk of bladder cancer (up to 10%) in the long term[27,28], therefore, screening cystoscopy has been recommended to start 10 years after indwelling catheter insertion. In cases of increased urethral leakage, recurrent urinary tract infection, or increased sediments and frequent catheter blockages, cystoscopy is also recommended to rule out the presence of bladder stones[22,28].

Therefore, we discourage the utilization of indwelling urethral catheterization and prefer the use of suprapubic catheters as a second-line option if clean IC (first line option) is not a feasible option for SCI patient.

Systemic medications to treat neurogenic detrusor over-activity

Systemic medications have been used to reduce detrusor storage pressure, increase bladder capacity, and improve urinary incontinence[8,12,13,22]. The most commonly used medications are anticholinergics (antimuscarinics), and beta-3 agonists. Systemic medications, along with CISC, constitute the first-line management of NDO in SCI patients[13,29]. Follow up after starting the systemic medication is warranted, which includes follow-up of symptoms improvement and urodynamic study in 3 months[12].

Antimuscarinics (anticholinergics), antimuscarinic receptor antagonists are the most commonly used medications in the treatment of NDO[8,12]. Several medications in this class are available and in multiple forms [Table 4]. It is expected to decrease the maximum detrusor pressure by 30%-40%, and to increase the bladder capacity by 30%-40%[30]. It increases the maximum cystometric capacity by 49.79 mL and lowers detrusor pressure at the strongest contraction by 38.3 cm/H_2O[31]. The possible adverse reactions should be monitored, along with improvement in symptoms and urodynamic parameters. Common adverse reactions include dry mouth, dry eyes, constipation, blurred vision, cognitive impairment (confusion), prolonged Q-T interval, and headache[29]. Anticholinergics should be avoided in patients with glaucoma, myasthenia gravis, gastrointestinal obstruction, and severe ulcerative colitis[32]. Another form of anticholinergics administration includes intravesical administration of 10 mL of 0.1% of oxybutynin three times daily, which shown to be equally effective to oral oxybutynin and with fewer systemic side effects[33]. The choice of specific anticholinergic medication is based on availability, tolerability, and side effect profile [Table 4].

Beta-3 agonists, mirabegron, the commercially available beta-3 agonist to treat detrusor overactivity, has been introduced recently. It acts on beta-3 receptors on the detrusor muscle to induce relaxation of the detrusor muscle[29]. Its use in NDO has been shown to increase the volume at the first detrusor contraction and to improve bladder compliance, with a non-significant increase in bladder capacity when compared to placebo[34]. The usual dose of Mirabegron is 25-50 mg/day, which can be increased to 100 mg/day[33]. The possible adverse reactions include hypertension, urinary tract infection, and headache[29]. The use of mirabegron is currently limited in neurogenic bladder patients, as limited evidence exists to support its use in this population[8,31]. It might be considered as an alternative in patients with contraindication to using anticholinergics, or as an add-on treatment to anticholinergics[35,36].

Table 4. Common antimuscarinic medications used in the treatment of NDO[12,29,60]

Name of drug	Form	Dosage	Advantages	Special precautions/disadvantages
Oxybutynin	Oral, immediate release	10-30 mg divided in 2-3 times/day	Antimuscarinic action with direct muscle relaxing effect and some local anesthetic effect No renal or hepatic dose adjustment	Highest side effect profile
	Oral, extended-release	Up to 30 mg/day	Avoids multiple daily dosing	
	Transdermal	3.9 mg/day patch, 2 patches/week	Lower anticholinergic side effect (avoid the first-pass metabolism)	Dermal side effects
Tolterodine	Oral, immediate release	2-8 mg divided twice per day	Non-selective anticholinergic Lower selectivity to the parotid gland	Needs dose reduction in renal impartment and hepatic dysfunction
	Oral, extended-release	2-8 mg/day	Avoids multiple daily dosing	Reduce dose in renal impairment and hepatic dysfunction
Propiverine	Oral	30-45 mg/day	Non-selective anticholinergic & musculotropic Less dry mouth	Reduce dose in severe renal impairment (30 mg/day) Avoid in severe hepatic dysfunction
Trospium	Oral, immediate release	20 mg twice per day	Does not cross BBB Minimal central side effect Minimal hepatic metabolism	Avoid use in moderate to severe hepatic dysfunction Maximum dose of renal impairment is 30 mg/day
	Oral, extended-release	60 mg once per day	Lower dry mouth than immediate-release form	
Solifenacin	Oral	5-10 mg once per day	Modest selectivity to M3 over M1 & M2 receptors Lower dry mouth than oxybutynin	Use low dose (5 mg) in renal impairment (CrCl < 30) and moderate hepatic dysfunction Avoid use in severe hepatic dysfunction
Darifenacin	Oral	7.5-15 mg once per day	Relative selectivity to M3 receptor No dose adjustment in renal impairment	No studies in NDO Use is not recommended in severe hepatic dysfunction
Fesoterodine	Oral	4-8 mg once per day	Selective M2, M3 receptor antagonist Equal affinity to M2 and M3 receptors Poor penetration to BBB	Not studied in NDO Use is not recommended in severe hepatic dysfunction Dose is reduced to 4 mg/day in renal impairment

BBB: blood-brain barrier; NDO: neurogenic detrusor overactivity; CrCl: creatinine clearance

Intravesical injection of botulinum toxin

Onabotulinumtoxin A (Botox) is a neurotoxin that causes detrusor muscle relaxation by preventing the release of acetylcholine on pre-synaptic parasympathetic nerve ending, resulting in a reduction of NDO, incontinence episodes, and increase of bladder capacity[37]. It has been approved as a treatment of neurogenic detrusor overactivity in SCI patients since 2011[38]. The usual dose is 200 units, injected into detrusor muscle in 20 sites delivered via cystoscope[37]. It increases the bladder capacity by 134.75 mL, volume at first involuntary detrusor contraction with a median difference of 163.42 mL, reduced maximal detrusor pressure at a median of 30.48 cm/H_2O, and reduced urinary incontinence episodes by 12.45/day, in compression to placebo[39]. It also improved the bladder compliance and reduced incidence of detrusor overactivity when compared to baseline (OR = 64.27; 95%CI: 12.17-339.28; $P < 0.00001$)[39].

The possible common adverse effects include urinary tract infections, hematuria, generalized weakness, and urinary retention (which is not a concern in patients using CISC)[37,38]. The effect usually lasts approximately nine months, and repeat injection is indicated after the disappearance of its effect[40].

Abobotulinum toxin A (750 IU) is another botulinum toxin that has also been used in the management of NDO with similar outcomes to onabotulinum toxin A[41]. Shifting to abobotulinium toxin A (Dysport) after failed onabotulinum toxin A therapy has been found successful in about 52%-57% of cases[41].

The clinician should evaluate the response of Botulinum Toxin A 2-3 months with symptomatic and

urodynamic evaluation 2-3 months after the first injection and then if there is a change of clinical course and recurrence of symptoms despite recent injection[12,40].

Sacral nerve stimulation

Several retrospective and observational studies have evaluated the role of dorsal rhizotomy (sacral deafferentation S2-S4/5), combined with anterior sacral root stimulation in the treatment of NDO[42-45]. This treatment has been shown to effectively reduce elevated detrusor pressure, improve compliance, increase bladder capacity, improve urinary incontinence, and achieve voluntary bladder and bowel emptying in patients with complete SCI[41-44]. This technique has a variable success rate in specialized centers but is limited by long-term complications and a high rate of surgical revision. It might be offered by experienced centers and in highly selected patients as a third-line option after failure of previous medical and minimally invasive options[8,13].

Surgical treatment of NDO

Surgical treatment options are considered when all medical and minimally invasive treatment options have failed to eliminate poor urodynamic parameters [Table 1]. Surgical options include bladder augmentation and urinary diversion.

Bladder augmentation, using a bowel segment, should be considered in patients who failed all medical and minimally invasive management for reduced bladder capacity and NDO[8,13]. It can eliminate urinary incontinence in 75%-100%, improve bladder compliance in 69%-100%, and improve quality of life in 90% of patients[8]. The addition of a continent catheterizable channel might be considered if the patient cannot catheterize through the urethra[8,46]. Contraindications to performing bladder augmentation include bladder malignancy, Chronic kidney disease (creatinine clearance less than 40 mL/min), bowel disease, previous significant bowel resection, inability to do CISC (such as quadriplegia), or unwillingness to perform CISC[8,46]. Long term complications include bladder stone formation, metabolic complications, intraperitoneal bladder perforation, urosepsis, vesicoureteric reflux, recurrence of NDO and adenocarcinoma or urothelial carcinoma in up to 6%[8,46-48]. Therefore, lifelong surveillance with cystoscopy is recommended. In case of recurrence of incontinence, video urodynamic is recommended, and in the presence of NDO, treatment with Botox injection into the augmented bladder can be tried before surgical revision or urinary diversion[49].

Urinary diversion, is considered as last resort option in the management of NDO if the patient is unfit or not a candidate for bladder augmentation. Urinary diversion options include continent and incontinent diversion[46].

Continent urinary diversion with continent catheterizable channels is performed when the patient can catheterize but cannot use native bladder due to a severely contracted bladder with severe vesicoureteral reflux or devastated bladder outlet, or bladder malignancy[8,47]. It carries a higher risk of long-term metabolic complications. It is contraindicated in patients with chronic kidney disease (creatinine clearance less than 40 mL/min) and in a patient who are not fit for CISC[8,48].

Incontinent urinary diversion is a last resort option[8,13,49], in which urine is diverted to the skin by using bowel segment (usually ileum) in patients who cannot perform CISC and in patients who are unfit or failed other surgical options[8,13,46]. An ileal conduit is a familiar procedure to urologists, the most commonly performed incontinent urinary diversion procedure, and the preferred incontinent urinary diversion procedure[8,46,50]. It results in renal function preservation in 88% to more than 90% of patients[8,50]. Possible complications include ureteral anastomotic stenosis, stomal or incisional hernia, stomal stenosis, bowel obstruction, urinary tract infections and pyelonephritis, urine leak, urolithiasis, and metabolic complications (acidosis)[46,51]. Overall major complications can reach up to 11%[8].

Other incontinent urinary diversion includes ileovesicostomy, which avoids ureteric reimplantation and cystectomy and related complications but has the disadvantage of leaving a bladder segment, which can increase the risk of malignancy or urethral incontinence[8,46,47,52]. It can be considered in select patients. Robust long-term results and quality of life data are lacking[8]. Reported complications include impaired bladder emptying, urethral incontinence, stomal stenosis, parastomal hernia, and urolithiasis[8,46,53-56].

Therefore, monitoring patients postoperatively is essential to detect complications, and surveillance is of paramount importance.

Monitoring, follow up, and surveillance

We recommend regular and tight surveillance after initial management, with clinical history, physical examination, and urodynamic evaluation after initiation of any intervention, to monitor response and to determine successful treatment and need for augmentation of medication dose or considering alternative management option. The time between the visit should be within 2-3 months after the initiation of medications or botulinum toxin A injection[8,12,40]. After controlling poor urodynamic features, annual follow up of symptoms, renal function, and upper tract ultrasound are recommended[6,8,13]. Repeat urodynamic and cystoscopy is recommended after a change in the clinical course, such as new incontinence or recurrent urinary tract infections[6].

Urinary tract related factors impacting the quality of life in SCI patients

Quality of life (QOL) in SCI was found to be associated with a poorly managed urinary tract. Worse QOL scores were found to be associated with urinary incontinence and recurrent UTIs[57-59]. Well managed bladder with surgery and indwelling catheters had better QOL scores than patients who use clean IC[58]. Worst QOL scores were associated with SCI patients who do not use catheters and leak on diapers[58]. This emphasizes the importance of proper management of urinary incontinence in SCI patients to improve their quality of life.

CONCLUSION

NDO secondary to SCI has a significant impact on patient quality of life, morbidity , and mortality. Early detailed evaluation and timely intervention are of paramount importance to avoid its long-term sequelae. Patients should be aware of the possible risks and benefits of each management option, and physicians should keep patients under close monitoring to act early upon any changes in patients' course of the disease. Research in NDO should continue to address several poorly studies areas in pathophysiology, treatments, and surveillance, as most of the available recommendations in this filed are largely based on retrospective studies.

DECLARATIONS

Authors' contributions

Wrote the first draft of the manuscript: Alsulihem A
Reviewed and edited the final manuscript: Corcos J

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Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

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Consent for publication

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Brain motor control assessment post intensive whole-body exercise *vs.* upper body exercise after spinal cord injury

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Abstract

Aim: The aim of this study was to assess the pattern of voluntary movements in patients with spinal cord injury (SCI) post intensive whole-body training *vs.* upper body training with brain motor control assessment (BMCA).

Methods: Twelve neurologically intact participants and 18 patients with SCI participated in this study as part of a multi-centre randomised controlled trial. All participants received 12 weeks training (three times per week), which comprised trunk, upper and lower limb exercises and locomotor training and functional electrical stimulation-assisted cycling in whole-body training group and an upper body strength and fitness program for upper body training group.

Results: Generalised linear model analysis showed significant effect of the main effect of the Task ($P < 0.001$) on the similarity index of voluntary movement patterns but not on the other factors or the interactions between them ($P > 0.05$). Some participants showed significant improvement in muscle strength post 12 weeks training; however, this improvement was not reflected in the pattern of muscle activation which was captured by BMCA.

Conclusion: BMCA is a valuable objective assessment tool that could add resolution to the clinical evaluation of patients with SCI post different therapeutic techniques.

Keywords: Brain motor control assessment, spinal cord injury, discomplete

INTRODUCTION

Spinal cord injury (SCI) is one of the most devastating disabilities which can affect a person's life significantly. Normal movement patterns are significantly impaired as a result of a spinal lesion, due to decreased/loss of supraspinal influences over the spinal cord and impaired appreciation of peripheral sensory inputs. One of the main aims of rehabilitation for patients with SCI is to assist them to become as independent as possible in daily activities[1] and facilitate normal movement patterns as much as possible. For patients with complete SCI, however, therapists usually do not focus on promoting neurological improvement in the paralysed extremities. For this group of patients, rehabilitation strategies are mainly focused on teaching compensatory strategies including using a variety of assistive devices during therapeutic sessions[1].

Many patients considered to have clinically complete SCI are neurophysiologically incomplete (discomplete)[2-6]. It has been argued that these connections are not detectable with clinical assessment; however, they are able to modulate the excitability of spinal sensorimotor connections below the level of injury[6-8]. In addition, there has been a case where the function of muscles below the level of injury could be improved by using functional electrical stimulation (FES) or locomotor training (LT) while body weight is partially supported[9-11].

Brain motor control assessment (BMCA) is a surface electromyography-based assessment that can add resolution to clinical assessment in patients with SCI[12]. In this assessment, motor outputs from the nervous system are recorded through a variety of reflexes and voluntary motor tasks of the lower limbs[13] performed under strictly controlled conditions. Sub-clinical evidence of translesional motor connections has been observed in patients considered to have a clinically complete lesion of the spinal cord using this type of assessment[2]. These subclinical responses can take various forms, for example repeatable responses to reinforcement manoeuvres or strong vibration[14] or the ability to volitionally suppress responses evoked by plantar surface stimulation[15,16].

This paper presents the results of the BMCA assessments conducted in patients at one site of a multi-centre, assessor-blinded, randomised controlled trial (Spinal Cord Injury and Physical Activity Full-On)[17], which investigated the effectiveness of an intensive activity-based therapy program for patients with clinically complete and incomplete SCI. For full details of the protocol, please refer to Galea *et al.*[17]. The trial was registered on ClinicalTrials.gov (NCT01236976).

METHODS

Twelve neurologically intact participants (six female and six male) and 18 patients who were at least 6 months post-SCI participated in this study. The demographic information of patients with SCI including neurological level (sensory and motor) and American Spinal Injury Association Impairment Scale (AIS) classification are presented in Table 1[18]. These measurements were generated using the International Standard for Neurological Classification of Spinal Cord Injury (ISNCSCI)[18,19].

All participants gave their written informed consent before the BMCA assessments were carried out (in addition to the consent for the clinical trial). All procedures used conformed with the Declaration of Helsinki, and the protocol was approved by the Human Research Ethics Committees at The University of Melbourne and Austin Health.

Neurologically intact participants were assessed using BMCA to generate prototype response vectors for two bilateral voluntary tasks (hip/knee flexion-extension) and four unilateral voluntary tasks (hip/knee flexion/extension and ankle dorsiflexion/plantar flexion). These values were used to calculate the similarity

Table 1. Demographics of patients with spinal cord injury

Participant/Ax	Neurological level				AIS
	Sensory right	Sensory left	Motor right	Motor left	
P1	T6	T6	T6	T6	D
P2	C8	C7	T1	T1	B
P3	C1	C3	C6	C3	D
P4	T6	T7	T1	T1	A
P5	C7	T12	T1	T12	D
P6	C2	C2	C2	C2	D
P7	C5	C7	C5	S1	D
P8	T2	T3	T2	T3	A
P9	C5	C5	C6	C6	A
P10	T8	T8	T8	T8	A
P11	C3	C3	C6	C6	A
P12	T5	T6	T5	T6	A
P13	T4	T5	T4	T5	A
P14	C8	C8	T1	T1	B
P15	T1	T2	T1	T2	C
P16	C8	C8	T1	T1	A
P17	T4	T4	T4	T4	A
P18	T3	T3	T3	T1	A

T: thoracic; C: cervical; S: sacral; Ax: assessment; AIS: association impairment scale; P: participant

index (SI) value for each task[20,21]. The relative distribution of surface EMG (sEMG) activity across the chosen muscles for each lower limb task (presented according to SI) in patients with SCI were compared to SI values for each task in neurologically intact participants.

Inclusion criteria for patients with SCI[22] were: ≥ 18 years old and able to give informed consent; sustained a traumatic SCI ≥ 6 months prior to consent and had completed their primary rehabilitation; and had a complete or incomplete SCI (C6-T12)[17]. Exclusion criteria for patients with SCI[22] were: brachial plexus, cauda equina or peripheral nerve injury; Stage 3 or 4 pressure ulcer[23]; had recent major trauma or surgery (up to six months prior to this trial); were post-menopausal at the time of injury (females); had a BMI < 25; had endocrinopathy or metabolic disorders of the bone; had a medical history of exposure to medication(s) known to affect mineral or bone metabolism; had chronic systemic diseases; had significant impairment or disability; had severe spasticity; had uncontrolled neuropathic pain; were likely to experience clinically significant autonomic dysreflexia and/or orthostatic hypotension in response to electrical stimulation or prolonged upright postures; or had any contraindications to FES such as a cardiac pacemaker, lower limb fracture or pregnancy.

Patients with SCI were randomly allocated to whole body exercise or upper body exercise groups [Table 2]. Twenty-four potential participants were screened for this trial at this centre. One failed to meet the inclusion criteria. Three were withdrawn after the first assessment based on personal reasons and two were not available for the BMCA assessments. Therefore, data from 18 patients with SCI were included in data analysis. Participants in the whole-body exercise group (n = 12; 7 AIS A complete; 5 AIS B-D incomplete) participated in training sessions three times per week for 12 weeks, which comprised trunk, upper and lower limb exercises, LT and FES-assisted cycling. LT sessions[22] were provided using a Therastride system (Innoventor, Inc., St Louis, MO, USA). Participants were supported in a harness. One therapist stood behind the participant to assist them to maintain optimal posture and facilitate rotation of the pelvis, while two therapists/assistants moved the lower limbs during the training session. The treadmill speed was adjusted individually based on the stepping pattern and body weight load. It was progressively increased as appropriate to a normal walking speed range (0.89-1.34 m/s). In addition, as participants were improving, the amount of body weight support was gradually reduced[24]. FES-assisted cycling was provided using a

Table 2. Group allocation of each participant and time of each assessment post injury

Participant	Group WBT or UBT	Date of injury	Level of injury	First Ax WPI	Second Ax WPI	Third Ax WPI	Fourth Ax WPI
P1	WBT	06/2009	D Incomplete	91	107	120	147
P2	WBT	03/2009	B Incomplete	152	167	NA	NA
P3	WBT	04/2002	D Incomplete	527	543	553	NA
P4	WBT	07/2010	A Complete	83	113	124	NA
P5	UBT	10/2009	D Incomplete	93	108	122	148
P6	WBT	12/1960	D Incomplete	2641	2657	2673	2697
P7	UBT	08/2010	D Incomplete	91	107	118	NA
P8	WBT	06/2006	A Complete	243	262	273	300
P9	WBT	05/2001	A Complete	592	605	NA	NA
P10	UBT	11/2002	A Complete	511	525	NA	NA
P11	WBT	04/2006	A Complete	320	337	346	NA
P12	UBT	12/2008	A Complete	115	133	146	172
P13	UBT	08/2010	A Complete	109	122	NA	NA
P14	UBT	06/2011	B Incomplete	6	22	41	61
P15	WBT	03/1999	C Incomplete	647	663	678	703
P16	WBT	10/2001	A Complete	554	570	580	NA
P17	WBT	08/2003	A Complete	418	433	NA	NA
P18	WBT	09/1992/	A Complete	961	979	991	1019

WBT: whole body training; UBT: upper body training; WPI: weeks post injury; NA: not assessed; Ax: assessment; P: participant

RT300 cycle (Restorative Therapies, Baltimore, MD, USA). Surface electrodes were attached on quadriceps, gluteal and hamstrings muscles. The parameters of the FES were: pedal cadence, 5-50 rev/min; stimulus intensity, maximum 140 mA; pulse width, 0.3 ms; frequency, 35 Hz; and duration, up to 30 min[24].

Participants in the upper body exercise group (n = 6, 3 AIS A complete and 3 AIS B-D incomplete) received an upper body strength and fitness program three times per week for 12 weeks. This upper body training program included a circuit-based exercise program incorporating resistance and cardiorespiratory training. None of the participants had participated in an intensive exercise program during the three-week period before starting this trial.

The participants with SCI were assessed up to four times over a period of one year. The assessment sessions are reported based on the number of weeks post-SCI [Table 2].

The following assessments were performed on participants in both groups before training (baseline), after 12 weeks of training and 6 months and 12 months post-recruitment.

Lower limb BMCA

The lower limb BMCA protocol was performed with participants lying supine. The protocol included: voluntary tasks, tendon-tap responses and vibration responses. The sEMG of 14 muscles (seven muscles from each lower limb and trunk) were recorded continuously throughout the protocol[21] with self-adhesive pre-gelled disposable surface electrodes (Noraxon Dual electrodes, Scottsdale AZ, USA). The muscles were lumbar paraspinal muscles, rectus abdominis, quadriceps, adductors, tibialis anterior, hamstring and gastrocnemius. EMG signals were amplified (1000 ×) by ZeroWire electrodes (Cometa, Milan, Italy) and then filtered (20-500 Hz) and digitised online (1 kHz sampling rate) using a PowerLab recording system (ADInstruments Ltd).

Two bilateral voluntary tasks (hip/knee flexion-extension) and four unilateral voluntary tasks (hip/knee flexion/extension and ankle dorsiflexion/plantar flexion) were assessed on both sides. All voluntary tasks were cued by two 5-s tones with a brief pause (less than 1 s) between them. Participants were asked to start the first task at the tone and not to start the second task until they heard the second tone. A customised

tendon hammer was used to record ten tendon responses quadriceps and triceps surae bilaterally with similar strike (consistent energy and independent of orientation and relative position)[21]. Tonic vibratory responses (TVR) of quadriceps and triceps surae muscles on both sides were also assessed by applying 30-s vibration over the tendon. The vibrator was custom-constructed from a pneumatic hand-grinder fitted with an offset weight and protective barrel (frequency of 115 Hz and a motion amplitude of 0.8 mm peak to peak). All assessments were completed by an assessor blinded to group allocation.

ISNCSCI

The motor scores were derived from part of the ISNCSCI assessment[19]. It involved testing the strength of ten key muscles on each side of the body in the supine position (elbow flexors, wrist extensors, elbow extensors, finger flexors, finger abductors, hip flexors, knee extensors, ankle dorsiflexors, long toe extensors and ankle plantar flexors) on a scale from 0 = no contraction to 5 = normal resistance through full range of motion. Scores were summed to give a total possible score of 50 for the upper extremities and 50 for the lower extremities.

Data reduction

A prototype response vector for each phase of each voluntary task in the protocol was generated from 12 neurologically intact participants (24 limbs)[20,21]. The muscles selected for hip and knee tasks in prototype calculations were quadriceps, hip adductors, hamstrings, lumbar paraspinal muscles and rectus abdominis from both sides. Those selected for ankle tasks in prototype calculations were quadriceps, hamstrings, tibialis anterior and gastrocnemius from both sides.

These values were used to calculate the SI, which compares the relative distribution of sEMG activity across the above chosen muscles for each voluntary tasks[21] and to evaluate the progression of participants with SCI during the trial. If SCI participants were able to recruit the prime movers for a specific task and decrease unnecessary muscle activity in the other muscles, their SI scores approximated neurologically intact participants' values, indicating better control of their movements. A value of 1.0 for the SI means that the test participant had an identical distribution of sEMG activity across muscles to the neurologically intact group for that task.

Generalised linear model (GLM) analysis was used to assess the main effects of Group: whole body training *vs.* upper body training; Side: right *vs.* left; Tasks: four unilateral tasks on both sides; and Assessment timepoint (Ax): first Ax (baseline), second Ax (after 12 weeks training), third Ax (6 months post-randomisation) and fourth Ax (12 months post-randomisation), on SI. GLM analysis was also conducted to assess the main effects of Group (whole body training *vs.* upper body training) and Ax [first Ax (baseline), second Ax (after 12 weeks training), third Ax (6 months post-randomisation) and fourth Ax (12 months post-randomisation)] on ISNCSCI motor score for right upper and lower limbs, left upper and lower limbs and total ISNCSCI motor score for upper and lower limbs. A significance level of $P < 0.05$ was adopted for all comparisons. This analysis was conducted using SPSS 22 software.

RESULTS

The main effect of Group, Side or Assessment time point on SI was not significant. GLM analysis only showed a significant main effect of Task ($P < 0.001$) on SI. There were no significant interactions between the factors ($P > 0.05$). The individual SI changes over time for two tasks in both groups are shown in Figure 1.

GLM analysis showed a significant main effect of Group ($P < 0.05$) on ISNCSCI motor scores of right, left and total ISNCSCI motor score for the upper limb [Figure 2]. However, the main effect of Assessment time point, the interaction between Group and Assessment time point and the analyses of ISNCSCI motor scores from lower limb were not significant ($P > 0.05$). The strength of the key lower limb muscles in 12

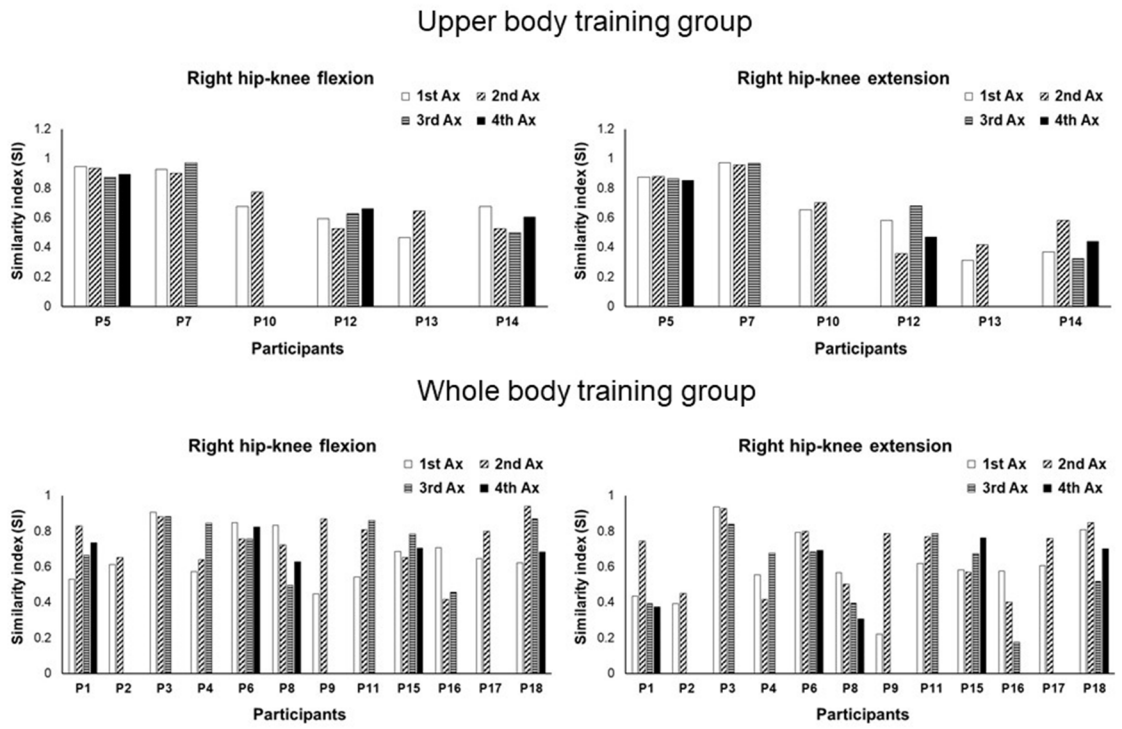

Figure 1. Individual SI changes over the 12 month-period and four assessment sessions for two tasks in both groups. Please note that some participants did not attend all the assessment sessions. Different participants showed different SI values for different tasks over time. P: participant; Ax: assessment; SI: similarity index

participants was documented as 0 at all assessment time points. Participants 1, 3, 5, 6, 7 and 15 showed some changes in lower limb muscle strength throughout the trial. Among these participants, Participant 5, who was in the upper body training group, showed a decrease in ISNCSCI motor scores; however, the other participants who showed no changes (Participant 7) or some improvements (Participants 3 and 6) in ISNCSCI motor scores were in the whole body training group.

Figure 3 shows the pattern of muscle activation during right and left hip-knee flexion and extension in a neurologically intact participant [Figure 3A] and two SCI participants (Participants 6 and 3) [Figure 3B and C] at four different assessment sessions throughout the trial in addition to the lower limb muscle strength changes during the trial for these two SCI participants [Figure 3C]. Figure 3C shows the total of manual muscle testing scores for five key muscles (hip flexors, knee extensors, ankle dorsiflexors, long toe extensors and ankle plantar flexors) on right and left sides plus the total score for both sides for Participants 6 and 3 at four different assessment time points.

Figure 3A shows the pattern of muscle activation during right and left hip-knee flexion and extension in a neurologically intact participant. Figure 3B shows the pattern of muscle activation during the same tasks in Participant 6 (one of the participants with SCI) at four different assessment sessions throughout the trial. Figure 3C shows similar data as Figure 3B in Participant 3 (another participant with SCI). Figure 3C shows the total of manual muscle testing scores for five muscles (hip flexors, knee extensors, ankle dorsiflexors, long toe extensors and ankle plantar flexors) on right and left side plus the total score for both sides for Participants 3 and 6 at four different assessment sessions. As can be seen in this figure, both Participants 3 and 6 showed some improvements in lower limb muscle strength throughout the trial at each assessment session; however, the increased strength of these muscles did not have any effect on the pattern of muscle activation during the assessed tasks (right and left hip-knee flexion and extension). For instance, the

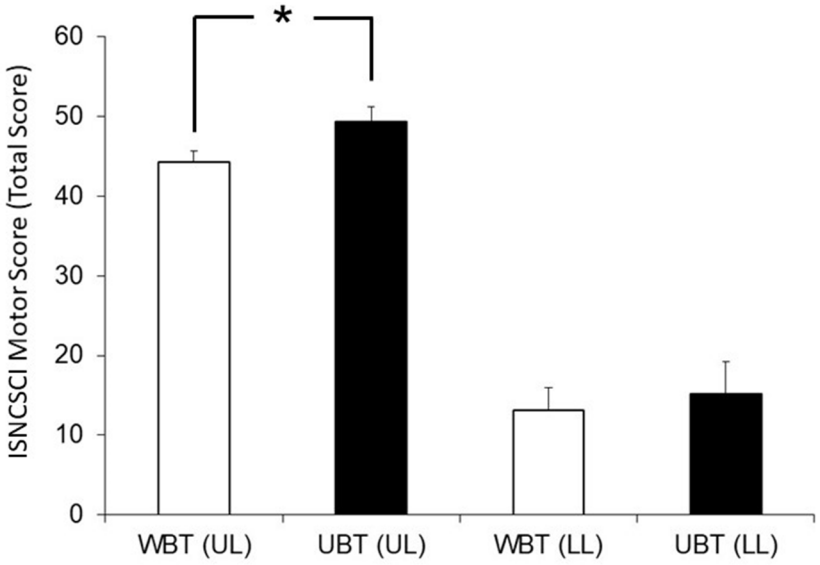

Figure 2. Total ISNCSCI motor score for upper and lower limbs in experimental and control group. WBT: whole-body training; UBT: upper body training; LL: lower limb; UL: upper limb; ISNCSCI: international standard for neurological classification of spinal cord injury

patterns of muscle activation during left hip-knee flexion in Participant 6 throughout the trial are very similar to each other and very different from the normal pattern of muscle activation [Figure 3A]. It can be seen that left quadriceps is more active than left hamstring and right hamstring does not show enough activity. The other example is during right hip-knee flexion: Participant 3 showed significant co-contraction of right hip adductors and left quadriceps even though they needed to be quiet during this task [Figure 3A].

In this study, 10 participants were assessed as having clinically complete SCI. Of these, nine participants showed tendon tap responses in 1-4 of the assessed muscles (Participants 4, 8, 10, 11, 12, 13, 16, 17 and 18) [Table 3]. Participants 4 and 11 did not show any TVR in any of the four assessed muscles. However, Participants 8, 10, 12, 13, 16, 17 and 18 showed TVR in 1-4 targeted muscles [Table 4].

Tendon tap responses are markers that can be used to indicate the existence of supraspinal influences over the motor circuitry of the examined muscle. Multi-level tendon-tap responses can be seen in some patients in both groups over time.

Vibration responses are markers that can be used to indicate the existence of supraspinal influences over the motor circuitry of the examined muscle. These responses were seen in some patients who were categorised as clinically complete SCI.

DISCUSSION

Eighteen participants with different levels of SCI (C6-T12) from one site who were participating in a multi-centre randomised controlled trial were assessed up to four times with the BMCA protocol. Twelve of these participants received whole body training while the other six participants received an upper body strength and fitness program three times per week for 12 weeks. Five of the six participants in upper body training group had the maximum total ISNCSCI motor scores of 50 throughout the study as their injury levels were at the thoracic level or at C8 level (incomplete). The training provided to the whole-body group had no effect on lower limb ISNCSCI motor scores. Twelve of the 18 participants in this group were classified as AIS A-complete (10 participants) or AIS B (2 participants) with the strength of the assessed lower limb muscles recorded as 0 throughout the trial.

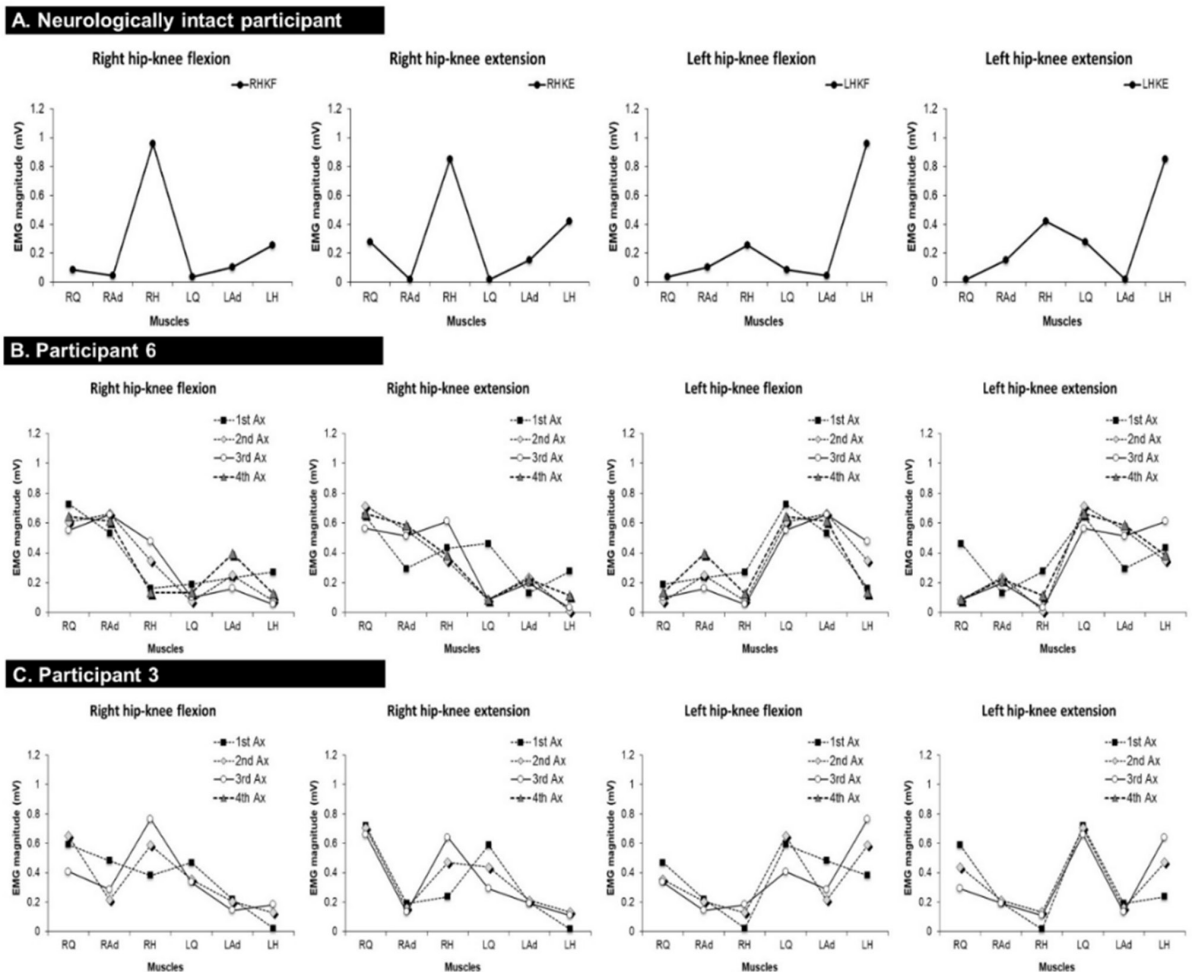

Figure 3. Pattern of muscle activation during right and left hip-knee flexion and extension in a neurologically intact participant (A) and 2 SCI participants (B and C). The lower limb muscle strength changes during the trial for these two SCI participants (C). RQ: right quadriceps; RAd: right hip adductors; RH: right hamstring; LQ: left quadriceps; LAd: left hip adductors; LH: left hamstring; Ax: assessment; SCI: spinal cord injury

BMCA can provide objective information regarding the pattern of muscular activation during lower limb tasks in patients with SCI during rehabilitation and how the treatment strategies can shift this pattern towards the normal pattern of movements in lower limbs. This assessment can be even more valuable when other functional clinical assessments, e.g., gait assessment (10-m walk test, timed up and go, 6-min walk test, *etc.*), cannot be completed due to the level of injuries, e.g., for patients with complete lesion at cervical or thoracic levels. Available data post BMCA are very limited, which makes it very difficult to compare these data with those of previous studies.

As shown in Figure 3, both Participants 3 and 6 (both in experimental group, incomplete D) showed some improvements in lower limb muscle strength throughout the trial at each assessment sessions; however, the increased strength of these muscles did not have any effect on the pattern of muscle activation during the assessed tasks (right and left hip-knee flexion and extension). For instance, the patterns of muscle activation during left hip-knee flexion in Participant 6 throughout the trial are very similar to each other and very different from the normal pattern of muscle activation [Figure 3A]. It can be seen that the left quadriceps are more active than the left hamstrings, and the right hamstrings do not show sufficient activity. Furthermore, during right hip-knee flexion, Participant 3 showed significant co-contraction of the right hip adductors and left quadriceps even though these muscles should have been quiet during this task

Table 3. Tendon tap responses from right and left quadriceps and triceps surae during four assessment sessions in 18 participants with SCI

Participant/Complete *vs.* Incomplete	First Ax	Second Ax	Third Ax	Fourth Ax
1/Incomplete	RQ: MLR RTS: MLR LQ: MLR LTS: MLR	RQ: MLR RTS: MLR LQ: MLR LTS: MLR	RQ: MLR RTS: MLR LQ: MLR LTS: MLR	RQ: MLR RTS: MLR LQ: MLR LTS: MLR
2/Incomplete	RQ: RP RTS: RP LQ: RP LTS: RP	RQ: MLR RTS: MLR LQ: MLR LTS: MLR	NA	NA
3/Incomplete	RQ: MLR RTS: RP LQ: MLR LTS: RP	RQ: MLR RTS: RP LQ: MLR LTS: RP	RQ: MLR RTS: RP LQ: MLR LTS: RP	NA
4/Complete	RQ: RP RTS: NR LQ: NR LTS: RP	RQ: RP RTS: NR LQ: NR LTS: RP	RQ: NR RTS: NR LQ: NR LTS: RP	NA
5/Incomplete	RQ: NR RTS: NR LQ: NR LTS: NR	RQ: MLR RTS: NR LQ: MLR LTS: NR	RQ: NR RTS: NR LQ: NR LTS: NR	RQ: MLR RTS: NR LQ: MLR LTS: NR
6/Incomplete	RQ: MLR RTS: NR LQ: MLR LTS: NR	RQ: MLR RTS: NR LQ: MLR LTS: RP	RQ: MLR RTS: NR LQ: RP LTS: NR	RQ: MLR RTS: NR LQ: MLR LTS: NR
7/Incomplete	RQ: MLR RTS: RP LQ: NR LTS: RP	RQ: MLR RTS: RP LQ: MLR LTS: RP	RQ: MLR RTS: RP LQ: MLR LTS: RP	NA
8/Complete	RQ: MLR RTS: RP LQ: NR LTS: RP	RQ: MLR RTS: RP LQ: MLR LTS: RP	RQ: NR RTS: RP LQ: NR LTS: NR	RQ: NR RTS: RP LQ: NR LTS: NR
9/Complete	RQ: MLR RTS: MLR LQ: MLR LTS: MLR	RQ: MLR RTS: MLR LQ: MLR LTS: MLR	NA	NA
10/Complete	RQ: RP RTS: MLR LQ: MLR LTS: MLR	RQ: NR RTS: MLR LQ: MLR LTS: MLR	NA	NA
11/Complete	RQ: NR RTS: NR LQ: NR LTS: NR	RQ: NR RTS: RP LQ: NR LTS: NR	RQ: NR RTS: NR LQ: NR LTS: NR	NA
12/Complete	RQ: NR RTS: NR LQ: RP LTS: RP	RQ: NR RTS: NR LQ: RP LTS: RP	RQ: NR RTS: NR LQ: MLR LTS: RP	RQ: NR RTS: NR LQ: MLR LTS: RP
13/Complete	RQ: NR RTS: NR LQ: RP LTS: NR	RQ: NR RTS: NR LQ: RP LTS: NR	NA	NA
14/Incomplete	RQ: NR RTS: NR LQ: MLR LTS: MLR	RQ: NR RTS: NR LQ: RP LTS: MLR	RQ: NR RTS: NR LQ: RP LTS: MLR	RQ: NR RTS: NR LQ: RP LTS: MLR
15/Incomplete	RQ: RP RTS: RP LQ: RP LTS: RP	RQ: RP RTS: NR LQ: RP LTS: NR	RQ: RP RTS: NR LQ: RP LTS: RP	RQ: MLR RTS: NR LQ: MLR LTS: MLR
16/Complete	RQ: NR RTS: RP LQ: NR LTS: NR	RQ: NR RTS: MLR LQ: RP LTS: NR	RQ: NR RTS: MLR LQ: MLR LTS: NR	NA

17/Complete	RQ: NR RTS: NR LQ: NR LTS: NR	RQ: RP RTS: NR LQ: NR LTS: NR	NA	NA
18/Complete	RQ: NR RTS: RP LQ: NR LTS: NR	RQ: NR RTS: RP LQ: RP LTS: NR	RQ: NR RTS: NR LQ: NR LTS: RP	RQ: NR RTS: NR LQ: NR LTS: NR

MLR: multi-level response; RP: response present; NR: no response; NA: not assessed; Ax: assessment; SCI: spinal cord injury; RQ: right quadriceps; RTS: right triceps surae; LQ: left quadriceps; LTS: left triceps surae

Table 4. Vibration responses from right and left quadriceps and triceps surae during four assessment sessions in 18 participants with SCI

Participant/Complete *vs.* Incomplete	First Ax	Second Ax	Third Ax	Fourth Ax
1/Incomplete	RQ: RP RTS: RP LQ: RP LTS: NR	RQ: RP RTS: RP LQ: RP LTS: RP	RQ: RP RTS: RP LQ: RP LTS: RP	RQ: RP RTS: NR LQ: RP LTS: NR
2/Incomplete	RQ: NR RTS: NR LQ: NR LTS: NR	RQ: MLR RTS: NR LQ: NR LTS: NR	NA	NA
3/Incomplete	RQ: MLR RTS: NR LQ: NR LTS: NR	RQ: MLR RTS: NR LQ: MLR LTS: NR	RQ: MLR RTS: NR LQ: MLR LTS: NR	NA
4/Complete	RQ: NR RTS: NR LQ: NR LTS: NR	RQ: NR RTS: NR LQ: NR LTS: NR	RQ: NR RTS: NR LQ: NR LTS: NR	NA
5/Incomplete	RQ: RP RTS: NR LQ: RP LTS: NR	RQ: NR RTS: NR LQ: RP LTS: NR	RQ: NR RTS: NR LQ: NR LTS: NR	RQ: NR RTS: NR LQ: NR LTS: NR
6/Incomplete	RQ: NR RTS: NR LQ: RP LTS: NR	RQ: RP RTS: NR LQ: NR LTS: NR	RQ: NR RTS: NR LQ: NR LTS: NR	RQ: NR RTS: NR LQ: RP LTS: NR
7/Incomplete	RQ: NR RTS: NR LQ: NR LTS: NR	RQ: NR RTS: NR LQ: NR LTS: NR	RQ: NR RTS: NR LQ: NR LTS: NR	NA
8/Complete	RQ: MLR RTS: MLR LQ: MLR LTS: NR	RQ: MLR RTS: MLR LQ: MLR LTS: MLR	RQ: MLR RTS: MLR LQ: MLR LTS: MLR	RQ: NR RTS: NR LQ: NR LTS: NR
9/Complete	RQ: MLR RTS: NR LQ: MLR LTS: NR	RQ: NR RTS: NR LQ: NR LTS: NR	NA	NA
10/Complete	RQ: NR RTS: NR LQ: MLR LTS: MLR	RQ: NR RTS: NR LQ: NR LTS: MLR	NA	NA
11/Complete	RQ: NR RTS: NR LQ: NR LTS: NR	RQ: NR RTS: NR LQ: NR LTS: NR	RQ: NR RTS: NR LQ: NR LTS: NR	NA
12/Complete	RQ: NR RTS: RP LQ: NR LTS: NR	RQ: RP RTS: RP LQ: NR LTS: NR	RQ: NR RTS: RP LQ: NR LTS: NR	RQ: NR RTS: NR LQ: NR LTS: NR
13/Complete	RQ: NR RTS: NR LQ: NR LTS: NR	RQ: NR RTS: RP LQ: NR LTS: NR	NA	NA

14/Incomplete	RQ: NR RTS: NR LQ: NR LTS: NR	RQ: RP RTS: RP LQ: NR LTS: RP	RQ: NR RTS: NR LQ: NR LTS: NR	RQ: NR RTS: NR LQ: NR LTS: NR
15/Incomplete	RQ: MLR RTS: MLR LQ: MLR LTS: NR	RQ: NR RTS: NR LQ: RP LTS: NR	RQ: NR RTS: NR LQ: RP LTS: NR	RQ: NR RTS: NR LQ: MLR LTS: NR
16/Complete	RQ: NR RTS: NR LQ: NR LTS: NR	RQ: NR RTS: RP LQ: NR LTS: NR	RQ: NR RTS: RP LQ: NR LTS: NR	NA
17/Complete	RQ: RP RTS: RP LQ: MLR LTS: MLR	RQ: RP RTS: NR LQ: MLR LTS: NR	NA	NA
18/Complete	RQ: MLR RTS: NR LQ: MLR LTS: NR	RQ: NR RTS: RP LQ: RP LTS: NR	NA	RQ: NR RTS: NR LQ: NR LTS: NR

MLR: multi-level response; NR: no response; RP: response present; NA: not assessed; SCI: spinal cord injury; RQ: right quadriceps; RTS: right triceps surae; LQ: left quadriceps; LTS: left triceps surae

[Figure 3A]. These results show that the ISNCSCI strength assessment only provides information about one element for evaluating treatment efficacy properly in this population. The ISNCSCI "improvement" noted in the current study may be non-specific for indicating clinically useful improvement. Thus, neurophysiological assessments similar to BMCA can increase the resolution of assessment, enabling clinicians to more reliably understand changes in motor control in their patients.

Significant functional recovery after incomplete SCI depends on the plasticity that is occurring through propriospinal network, intraspinal circuits and supraspinal influences through descending systems. Many factors can influence the effectiveness of different rehabilitation strategies in this group of patients, e.g., the level of injury, onset of training and the intensity of the training (how much, how often and how long). To be able to understand which strategy would maximise the activity-dependent plasticity in these patients with significant functional recovery, it would be desirable to undertake routine neurophysiological assessment to collect valuable information from this population during their rehabilitation period. The results reported here illustrate the variability of responses between patients and highlight the importance of collection of larger datasets for interpretation of changes over time and in response to different rehabilitation strategies.

In this study, all 10 participants who were categorised as having complete SCI showed some sub-clinical supraspinal influences over the muscles below the level of injury. This is in line with previous studies[25]. It has been shown that a TVR response in people with clinically complete SCI can be considered as a sub-clinical supraspinal response so they should be classified as having discomplete SCI[2,26,27]. In the present study, Participants 8, 10, 12, 13, 16, 17 and 18 showed TVR in 1-4 targeted muscles, which indicates that they should be categorised as discomplete. Gillies *et al.*[28] showed that the TVR could be observed in a cat with SCI only if the lateral vestibulospinal and pontine reticulospinal tracts were intact. It has been argued that, during vibration, the sensory information is transmitted to the brainstem, the reticular formation and associated tracts, as well as other parts of the brain that all are involved in controlling this response[27]. In the present study, Participants 8, 10, 12, 13, 16, 17 and 18 showed TVR in 1-4 targeted muscles. These subclinical responses should not be ignored as they might open a new window for exploring new rehabilitation techniques to improve the supraspinal influences over the muscles under the level of injury that these patients could benefit[3,29].

Another marker for the existence of supraspinal influences is the tendon tap response[25,30]. In this study, Participants 4, 8, 10, 11, 12, 13, 16, 17 and 18 showed tendon tap responses in at least one of the assessed muscles without extending to other spinal segments. This response has been reported in previous studies as well[14,31]. There is significant supraspinal influence on inhibitory interneurons at different spinal segments and propriospinal neurons that can extend to other segmental levels, as well as a direct influence on alpha and gamma motoneurons. It has been shown that a reduction of supraspinal influences over propriospinal interneuron networks increases their excitability, which in turn increases the possibility of motor unit activation in other spinal levels including on the contralateral side[32].

In the present study, participants in the whole-body training group completed 12 weeks of training including trunk, upper and lower limb exercises and LT, FES-assisted cycling. These participants received FES, which increased the sensory inputs to the propriospinal network and intraspinal circuits through dromic and anti-dromic currents in the stimulated nerves and sensory feedback from the contracted muscles and joint receptors post-muscle contractions[33]. Plasticity of these networks plays a significant role in functional recovery in patients with incomplete SCI by forming new connections and re-establishing corticospinal connections to the affected muscles[34]. The increased sensory inputs to the spinal cord could increase the excitability of the propriospinal network and promote multi-level muscle co-activations or reflex responses, which can adversely decrease the SIs for different tasks. However, it is unlikely that this was the case in the present study, as these responses were seen in participants in both groups. As we assessed a small number of patients, this speculation needs to be confirmed in larger studies.

Limitations of the study

BMCA requires specialised equipment and expertise in collecting and analysing the data, which may not be readily available at all sites. The sample size in this study was low as the BMCA assessments were limited to participants at only one site of a multi-centre trial. The number of tasks was limited to just four unilateral tasks. In future studies, other lower limb movements should also be assessed, e.g., hip abduction/adduction. In addition, all the tasks were completed in the supine position in order to standardise the testing position. This could affect the control of anti-gravity movements, e.g., hip and knee flexion, and increase the inter-subject variability significantly. Other factors were the variability in time post-injury within this group of participants (1.5-50 years), and their unique patterns of injury, which may also affect the interpretation of the data.

In conclusion, knowledge about how to improve function in people with SCI is growing, with new therapeutic approaches, modification of previous approaches and new technologies to facilitate compensatory function. In line with this, the need for objective evaluation of the effectiveness of these therapeutic approaches will also grow. Neurophysiological assessment will assist clinicians to monitor their patients' progress during rehabilitation programs with more resolution and potentially lead to individualised adjustment to optimise rehabilitation outcomes. BMCA is a valuable objective assessment tool that can refine the clinical evaluation of patients with SCI and assist in maximising their functional capabilities. Reporting the BMCA findings after different therapeutic techniques and rehabilitation programs, even in a small number of patients, will help to increase our knowledge of the effects of those interventions on movement patterns and residual supraspinal effects.

DECLARATIONS

Authors' contributions

Designed the study, obtained funding, collected and interpreted the data, and revised the manuscript: Galea M

Collected, analysed and interpreted data, prepared the manuscript and all tables and figures, and revised the manuscript: Zoghi M

Availability of data and materials

Data can be made available on application to the authors.

Financial support and sponsorship

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The study was approved by the Human Research Ethics Commitees of Austin Health and the University of Melbourne.

Consent for publication

Not applicable.

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Meeting Abstracts

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1. Yeast studies on the benefit of simvastatin in reducing levels of amyloid betaian

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A large-scale epidemiology study on statins previously showed that simvastatin was unique among statins in reducing the incidence of dementia. Since amyloid beta (Aβ42) is the protein that is most associated with Alzheimer's disease, this study has focused on how simvastatin influences the turnover of native Aβ42 and Aβ42 fused with green fluorescent protein (GFP), in the simplest eukaryotic model organism, saccharomyces cerevisiae. Previous studies have established that yeast constitutively producing Aβ42 fused to GFP offer a convenient means of analyzing yeast cellular responses to Aβ42. Young cells clear the GFP fusion protein and do not have green fluorescence while the older population of cells retains the fusion protein and exhibits green fluorescence, offering a fast and convenient means of studying factors that affect Aβ42 turnover. In this study the proportion of cells having GFP fused to Aβ after exposure to simvastatin, atorvastatin and lovastatin was analyzed by flow cytometry. Simvastatin effectively reduced levels of the cellular Aβ42 protein in a dose-dependent manner. Simvastatin promoted the greatest reduction as compared to the other two statins. A comparison with fluconazole, which targets that same pathway of ergosterol synthesis, suggests that effects on ergosterol synthesis do not account for the reduced amounts of Aβ42 fused to GFP. The levels of native Aβ42 following treated with simvastatin were also examined using a more laborious approach, quantitative MALDI TOF mass spectrometry. Simvastatin efficiently reduced levels of native Aβ42 from the population. This work indicates a novel action of simvastatin in reducing levels of Aβ42 providing new insights into how simvastatin exerts its neuroprotective role. This reduction is likely to be due to protein clearance.

2. The segmented brain - why the hypothalamus is not part of the diencephalon and other surprises

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The traditional approach to classifying parts of the brain has been challenged by new findings on developmental gene expression. The columnar model espoused by Herrick in the early 20th century has been replaced by the prosomeric model of Puelles and Rubenstein (Puelles *et al.* TINS 2013;36:570). The prosomeric model shows that the brain is made up of a series of distinct segments - the hypothalamus (two segments), diencephalon (three segments), and midbrain (two segments), and the hindbrain (twelve segments).

The hypothalamus forms the rostral end of the neural tube and is divided into terminal and peduncular segments. The "new" diencephalon is divided into three rostro-caudal segments - the prethalamus, the thalamus, and the pretectal area. The midbrain, which is now included in the forebrain on gene expression grounds, can be divided into a large rostral segment (mesomere 1) and a much smaller pre-isthmic segment (mesomere 2). The hindbrain is also segmented, consisting of the isthmus and 11 rhombomeres (r1 to r11). In all areas of the brain, each segment contains all the dorsoventral elements of the neural tube: roof plate, alar plate, basal plate, and floor plate.

The most striking new features of the prosomeric model are seen in the hypothalamus. Firstly, it is now evident that the hypothalamus is not a part of the diencephalon (as was previously assumed), but a separate region which sits rostral to the diencephalon developmentally. The terminal segment of the hypothalamus forms the rostral end of the neural tube. The apparent ventral position of the hypothalamus (its name means "under the thalamus") in the adult brain is simply due to the sharp bend in the neural axis created by the cephalic flexure. This 180° bend even gives the illusion that the hypothalamus is continuous with the midbrain. Secondly, it is now evident that the subpallial and pallial components of the telencephalon are derived from the alar plate of the peduncular hypothalamus. The alar plate of the terminal hypothalamus gives rise into the preoptic area and the eye vesicle.

In the newly defined diencephalon, gene expression evidence now shows that the posterior commissure and related pretectal nuclei belong to the caudal diencephalon and not to the midbrain, as is popularly supposed. Within the hindbrain, the cerebellum arises from the alar plate of the isthmus and r1, and the pontine nuclei migrate from the alar plate of r6 to a ventral position in r3 and r4.

The traditional picture of brain anatomy has been based on a superficial interpretation of topography as seen in the adult human brain, without regard to the underlying ontological realities. The new discoveries concerning the underlying segmental nature of the brain give us a new understanding of the real interrelationships of brain structures. It will probably take years before the old formulations are abandoned; in the meantime it is important that medical students are presented with this new evidence, instead of being fed outdated ideas based on simplistic interpretations of brain topography.

3. The circadian system plays a major role in the aetiology, progression and treatment of parkinson's disease: ending an era of "forcing nature" with dopamine replacement

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There is an ever increasing body of research demonstrating that the circadian system plays an important role in the motor and non-motor symptoms of Parkinson's disease (PD). Historical evidence supporting this hypothesis can be found in the early work of Parkinson, Charcot and others, whereby the symptoms of this disease can vary in accordance with the phase of the circadian cycle. More recent work, examining the role of anatomical substrates of the circadian system, has shown that the retina, hypothalamus and pineal are important locations whereby underlying functional changes may well contribute to the aetiology of PD. Equally compelling are the recent preclinical and clinical findings demonstrating that more effective, less invasive therapeutic intervention may well be achieved if chronotherapeutics target these sub-anatomical parts of the circadian system. In our exploration these substrates we have identified the retina and the pineal as two such important locations where chronotherapeutics are most effectively delivered to produce the optimal therapeutic benefit while minimising adverse side effects. In particular, we will demonstrate how treatments, such as minute intravitreal injections of anti-PD drugs, produce robust therapeutic effects, that are normally attributed to deep brain structures. In contrast, the chronotherapeutic intervention observed in in the disease itself using strategic light therapy, provide additional evidence that circadian function plays a major role in the aetiology, progression and treatment of PD. It is time for a reappraisal of the underlying anatomical substrates reflexively attributed exclusively to the Nigro-striatal dopamine system. It is time to sojourn that endless search for the magic bullet of dopamine replacement and pursue the highly significant, but less invasive, contribution of chronotherapeutics in correcting this disorder.

4. Multicentric cryptococcomas mimicking neoplasia

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Fungal mass lesions in the central nervous system are, as a group, extremely rare. In this group, cryptococcomas are the most commonly seen and are often included in the differential diagnosis of the multicentric space occupying lesions in immunocompromised hosts. While crypococcomas are known to occur in both healthy and immunocompromised individuals, they are more commonly seen in the latter where *Cryptococcus neoformans* is the typical agent. This contrasts the species seen in immunocompetent hosts where *Cryptococcus gatti* occurs more commonly.

These lesions are commonly 3-10 mm in diameter and occur in the basal ganglia due to the organism spreading via the Virchow-Robbins spaces surrounding the small perforator vessels as part of contiguous spread from a basal meningitis. Although most frequently associated with HIV infection, patients with chronic renal disease, vascular conditions, hepatitis B or C, alcoholism, diabetes mellitus, and oncological diseases may also succumb to this infection and present with cryptococcomas.

In rare cases, a chronic granulomatous process may lead to formation of a mass lesion (cryptococcoma) that has a tumoral appearance. Metabolites released by *cryptococcus* can inhibit the migration and function of leukocytes and promote survival and localized replication of the pathogen, thus facilitating chronic granulomatous inflammation and giant cryptococcoma formation.

5. Analysis of repetitive element expression in the blood and skin of patients with parkinson's disease identifies differential expression of satellite elements

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Repetitive elements (RE) constitute the majority of the human genome and have a range of functions both structural and regulatory on genomic function and gene expression. RE overexpression has been observed in several neurodegenerative diseases, consistent with the observation of aberrant expression of RE posing a mutagenic threat. Despite reports that associate RE expression with Parkinson's disease (PD) no study has comprehensively analysed the role of these elements in the disease. This study presents the first genome-wide analysis of RE expression in PD to date. Analysis of RNA-sequencing data of 12 PD patients and 12 healthy controls identified tissue-specific expression differences and more significantly, differential expression of four satellite elements; two simple satellite III (repName = CAttC_nand_GAATG_n) a high-copy satellite II (HSATII) and a centromeric satellite (ALR_Alpha) in the blood of PD patients. In support of the growing body of recent evidence associating REs with neurodegenerative disease, this study highlights the potential importance of characterization of RE expression in such diseases.

6. Alzheimer's disease: is the inflammasome the missing link?

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Alzheimer's disease (AD) is a devastating neurodegenerative disease characterised by widespread neuronal cell death and progressive dementia. Genetic and molecular studies have confirmed the central role of amyloid-β (Aβ) production in the pathogenesis of AD. However, therapies eliminating Aβ from AD have unfortunately failed to stem progressive cognitive decline. The association of several immune responsive genes with increased AD risks have in recent years revealed that inflammatory mechanisms are also a powerful pathogenic forces in the process of neurodegeneration.

Inflammasome, a multiprotein complex, is implicated in the execution of inflammatory responses and pyroptotic death leading to neurodegeneration. Inflammasomes serve as platforms for the recruitment and activation of caspase-1, the de facto executioner of a diverse downstream inflammatory processes

including the maturation of two major pro-inflammatory cytokines, interleukin-1β (IL-1β) and interleukin-18. Increased IL-1β, a member of the IL-1 cytokine family, has been implicated in the response to Aβ deposition and up-regulated in specimens from patients with AD. Since IL-1β secretion is critically dependent on the activation of inflammasomes, inflammasomes have been inferred as the missing link for Aβ-induced IL-1β secretions.

Within the central nervous system (CNS), several types of inflammasome have been identified, of which the best characterised are the absent in melanoma 2, NOD-like receptor (NLR)-family pyrin domain-containing 1 (NLRP1), NLRP3 and NLR-family caspase recruitment domain (CARD)-containing 4 inflammasomes. Different subsets of inflammasomes contain different cytosolic pattern-recognition receptors and their assembly is initiated by different stimuli. Once activated, inflammasome induces an inflammatory cell death mode termed as pyroptosis. Pyroptosis is a process of programmed cell death closely associated with inflammasome activation. However, in contrast to apoptosis, in pyroptotic cell, the integrity of the cell membrane is affected and micro-pores are formed resulting in intracellular and extracellular ion imbalance cell swelling and rupture. Meanwhile, the pro-inflammatory cytokines are released to the extracellular space causing focal inflammation and cell death. Multiple potential targets upstream of pyroptosis signaling may pave the way for newly therapeutic drugs that may rescue inflammation in neurological diseases. This has incited us to study the response of human neurons to Aβ and to determine whether specific neuronal molecular events initiated link neuronal degeneration to an inflammatory response.

In our studies, Aβ was found to induce inflammasome activation and inflammasome-mediated pyroptosis. Using gene-trap mutagenesis approach, candidate genes, which could play an important role in regulating inflammasome-mediated pyroptosis have been identified. We also demonstrated that neural stem cells (NSCs) regulated the NLRP3 inflammasome, and inhibited the production of IL-1β and caspase-1 in activated microglia, as well as subsequently attenuating neurotoxicity caused by microglial neuroinflammation, adding to the inherent benefits of NSCs in AD treatment. By understanding precisely how inflammasomes work in the CNS under both physiological and pathological conditions, as well as determining how these inflammasomes can be pharmacologically targeted, we may be one major step closer towards developing a proper cure for AD.

7. Colocalization of iron and aluminum in nuclei of nerve cells in brains of patients with sporadic Alzheimer's disease

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The etiology of Alzheimer's disease (sporadic Alzheimer's disease, AD) remains to be clarified. However, growing lines of evidence indicate that metal-induced oxidative stress plays a key role in the pathogenesis of AD[1]. Recently, the presence of 8-hydroxydeoxyguanosine, a biomarker of oxidative DNA damage, was demonstrated in nuclear DNA (nDNA) in the AD brain. It has also been reported that accumulation of DNA damage is one of the earliest detectable events during the progression from healthy aging to dementia.

Iron (Fe) is a pro-oxidant metal capable of generating hydroxyl radicals that can oxidize DNA through Fenton reaction. Aluminum (Al) has been reported to facilitate Fe-mediated Fenton reaction, as shown in the chemical formulae below. These cyclic reactions continuously generate hydroxyl radicals and can cause severe oxidative damage to nDNA.

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^-$ (Fenton reaction)
$Al^{3+} + O_2^-\bullet \leftrightarrow AlO_2^{2+}\bullet$ [2]
$Fe^{3+} + AlO_2^{2+}\bullet \rightarrow Fe^{2+} + Al^{3+} + O_2$ [2]

Since hydroxyl radicals are highly reactive, the half-lives of hydroxyl radicals have been reported to be as short as 10^{-9} s (1 ns). Therefore, to oxidize nDNA, Fe must be localized at close proximity to nDNA. To facilitate Fe-mediated oxidative reactions, Al must be colocalized at close proximity with Fe. However, the colocalization of Fe and Al in neuronal nuclei remains to be clearly demonstrated in the AD brain.

In this study, we examined the colocalization of Fe and Al in the nuclei of nerve cells in the AD brain using scanning electron microscopy (SEM) coupled with energy-dispersive X-ray spectroscopy (EDS). SEM-EDS analysis allows the concurrent imaging of subcellular structures with high spatial resolution and detection of small quantities of elements contained in the same subcellular structures.

Our results demonstrate that Al and Fe were colocalized in the nuclei of nerve cells in the AD brain. Within the nuclei, the highest levels of both Al and Fe were measured in the nucleolus. The SEM-EDS analysis also revealed the colocalization of Al and Fe in the heterochromatin and euchromatin in neuronal nuclei in AD brains. Notably, the levels of Al and Fe in neuronal nuclei in AD brains were markedly higher than those in age-matched control brains.

Additionally, it has been reported that metals, including Fe and Al, which bind to DNA or DNA-binding proteins, inhibit the repair of oxidatively damaged DNA. We hypothesize that the colocalization of Al and Fe in the nucleus of nerve cells might induce oxidative damage to nDNA and concurrently inhibit the repair of oxidatively damaged nDNA. An imbalance caused by the increase in DNA damage and the decrease in DNA repair activities might lead to the accumulation of unrepaired damaged DNA, eventually causing neurodegeneration and the development of AD.

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8. The role of high-flow bypass in the treatment of delayed complications of head and neck radiation

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Radiation therapy is the mainstay of treatment for head and neck cancers. Due to the prolonged survival of patients who have received radiation therapy, the incidence of delayed radiation-induced complications, including carotid stenosis and transient ischemic attacks, is expected to increase. Some of the survivors present with rare but devastating carotid blowout syndrome as a result of the ruptured carotid artery pseudoaneurysms. The best treatment of these delayed complications remains to be elucidated. We here

report our experience in treating seven patients with delayed radiation-induced complications using high-flow extracranial-intracranial bypass.

9. Spinal cord stimulation improves the microvascular perfusion insufficiency caused by critical limb ischemia

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Aim: The study aimed to identify the benefit and efficacy of spinal cord stimulation (SCS) in patients with perfusion problem caused by critical limb ischemia (CLI) compared with those who did not receive CLI.

Methods: Seventy-eight patients were diagnosed as having perfusion problem, with perfusion difference < 0.95, by using lower-limb 201TI scintigraphy. Thirty-seven patients were treated with SCS and 41 treated without. All patients took the same medications. The outcomes of walking distance, walking time, and sleeping quality were interrogated and recorded. The pain intensities were evaluated via visual analog scale score.

Results: The outcomes in SCS treatment group were dramatically ameliorated. The visual analog scale (VAS) score evidenced improvement immediately following one-week of SCS implantation. On the other hand, the outcomes in non-SCS group were exacerbated. Indeed, the increased intensities of microcirculation were observed in the lower extremities after SCS implantation compared with pre-implantation by using lower-limb 201TI scintigraphy. Most importantly, 10 of 41 patients were on wheelchairs in Non-SCS group, and no one on wheelchairs in SCS group after one-year follow-up.

Conclusion: Early diagnosis of perfusion problem in patients with CLI and treating with SCS immediately are crucial for the patients' improved outcomes and limb salvage.

10. Plasma biomarkers and neurodegenerative diseases

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Neurodegenerative diseases are now considered as proteinopathies of various combinations. Amyloid-β and tauopathies are the major pathognomonic pathological changes of Alzheimer's disease, α-synucleinopathies are for Parkinson's disease, and TDP-43 proteinopathies and tauopathies are for frontotemporal dementia. Other tauopathies include Pick's disease, corticobasal disease, and progressive supranuclear palsy. Synucleinopathies include multiple system atrophy and Lewy body disease. TDP-43 proteinopathies include amyotrophic lateral sclerosis. However, co-pathology of neurodegenerative diseases exist: Lewy bodies

commonly occur in Alzheimer's disease and Alzheimer's disease pathology is frequently found in Lewy body diseases, but the extent of such co-pathologies across neurodegenerative diseases remains undefined. The prevalences of proteinopathies of most neurodegenerative diseases were such that tau was nearly universal, amyloid-β was common, α-synuclein was less common, and TDP-43 was the least common. Recent development in cerebro-spinal fluid (CSF) biomarkers of neurodegenerative diseases demonstrated that tau proteins (both total tau and phosphorylated-tau) increased and amyloid-β (Aβ42) decreased in patients with Alzheimer's disease; α-synuclein increased in patients with Parkinson's disease; and so on. Nevertheless, the collection of CSF is invasive and is not without risk. Thus, blood-based biomarkers warrant further development. In our previous study, we developed a panel of plasma biomarkers by using immunomagnetic reduction assay technology including Aβ42, Aβ40, total tau, phosphorylated-tau, α-synuclein, phosphorylated α-synuclein, and TDP-43. Individual plasma biomarkers Aβ42 and total tau and combined biomarkers such as Aβ42/Aβ40 and Aβ42/tau performed well in differentiating older controls from patients with dementia due to Alzheimer's disease. α-synuclein and phosphorylated α-synuclein helped separate older controls from patients with Parkinson's disease, as well as assisted the differential diagnosis between Parkinson's disease and other atypical Parkinsonism. In this study, we demonstrated IMR assay results for Aβ42, total tau, phosphorylated-tau, α- synuclein, phosphorylated α-synuclein, and TDP-43 in five groups of patients including control (n = 39), mild cognitive impairment due to Alzheimer's disease (n = 40), dementia due to Alzheimer's disease (n = 34), Parkinson's disease (n = 28), and frontotemporal dementia (n = 30). We demonstrated the capacity of IMR blood-based (plasma) biomarkers in assisting diagnosis of individual neurodegenerative disease and showed the proteinopathy co-pathology in the blood.

11. Critical role of brain-specific gangliosides in the pathogenesis of traumatic brain injury and Alzheimer's disease

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Major brain glycosphingolipids, also called brain gangliosides, are localized within neuronal lipid rafts (NLR) of neuronal axons and synapses and their role in neurodegenerative diseases remains unknown. Here, we compared the outcome of traumatic brain injury (TBI) and Alzheimer's disease (AD) pathology in wild-type and glycosphingolipid-deficient animals. The *st3gal5* gene encodes for ST3 β-galactoside alpha-2,3-sialyltransferase 5, which is responsible for the biosynthesis of complex a, b, and c series gangliosides in the brain. We found that uninjured st3gal5-deficient mice exhibit normal cognitive and social behaviors, but also exhibit some very mild motor deficits. After TBI, st3gal5-deficient animals exhibit marked deficits in cognitive and motor functions, which was associated with increased hemorrhage and neuronal damage owing to the failure of NLR-induced platelet activation and serotonin secretion. The decrease in NLR-induced platelet-derived platelet activating factor release also resulted in reduced microglial activation and central nervous system macrophage infiltration in the st3gal5-deficient animals after TBI. Further investigation demonstrated that the interaction of platelets with NLR stimulated neurite growth, increased the number of dendritic spines, and increased neuronal activity during TBI. To understand the role of gangliosides in Alzheimer's disease pathology, we crossed st3gal5-deficient mice with 5XFAD transgenic mice that overexpress three mutant human amyloid proteins AP695 and two presenilin PS1 genes. We found that st3gal5-deficient 5XFAD mice had a significantly reduced burden of amyloid depositions, low level of neuroinflammation, and did not exhibit neuronal loss or synaptic dysfunction as compared to wild-

type 5XFAD mice. st3gal5-deficient 5XFAD mice also performed significantly better in a cognitive test than the wild-type 5XFAD control group. Finally, the treatment of wild-type 5XFAD mice with the sialic acid-specific *Limax flavus* lectin resulted in substantial improvement of AD pathology. Thus, our study establishes an important role for major brain glycolipids in the regulation of neuroinflammation, neuronal plasticity, synaptic functions, and cognitive ability after a neuronal injury during TBI- and AD-related neurodegeneration.

12. Analysis of the association of MIR124-1 and its target gene *RSG4* polymorphisms with major depressive disorder and antidepressant response

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Increasing evidence has indicated that dysfunction of miR-124 and target gene regulator of G protein signaling 4 (RGS4) may be involved in the etiology and treatment of major depressive disorder (MDD). However, the molecular mechanisms are not fully understood. This study aimed to investigate whether common genetic variations in these two genes are associated with MDD and therapeutic response to antidepressants in the Chinese population. Three polymorphisms including rs531564 [a functional single-nucleotide polymorphism (SNP) in MIR124-1], rs10759 (a microRNA-binding site SNP in RGS4), and rs951436 (a promoter SNP in RGS4) were genotyped in 225 Chinese MDD patients and 436 controls. Among the MDD patients, 147 accepted antidepressant treatment for eight weeks with therapeutic evaluation at baseline, Week 2, Week 4, Week 6, and Week 8 using the 17-item Hamilton Rating Scale for Depression. Multifactor dimensionality reduction (MDR) was used to identify gene-gene interactions. No significant association with MDD was discovered in single-SNP analyses. However, in the optimal model containing rs531564, rs10759, and rs951436 SNPs by MDR analysis, the *P*-value was 0.0024, the accuracy of the sample test was 0.49, and the cross-validation consistency was 10/10. Values of ORs and 95% confidence interval (CI) indicated that the combined action could increase the risk of MDD (OR = 1.67, 95%CI: 1.20-2.33). In pharmacogenetic study, a significant association was found in genotypic frequencies of rs951436 between the responder and non-responder groups (x^2 = 6.191, P = 0.045, correction P = 0.135) as well as between the remitter and non-remitter groups (x^2 = 7.216, P = 0.026, correction P = 0.078). For further analysis, the rs951436 heterozygote carriers had threefold probabilities of achieving clinical complete remission (OR = 3.00, 95%CI: 1.33-6.76, P = 0.007, correction P = 0.021) and 3.21-fold probabilities of achieving clinical response (OR = 3.21, 95%CI: 1.13-9.14, P = 0.022, correction P = 0.066) as compared with rs951436 homozygotes (AA + CC) after eight-week treatment. Moreover, the homozygous (AA + CC) of rs951436 showed a worse response to antidepressant treatment and had lower percent reduction of HAM-D scores over eight weeks than heterozygous AC, and significant associations were found at Week 6 (AA + CC *vs.* AC: 53.58 ± 27.04 *vs.* 61.34 ± 21.54, t = -2.08, P = 0.040) and Week 8 (AA + CC *vs.* AC: 60.17 ± 29.56 *vs.* 70.19 ± 20.41, t = -2.404, P = 0.018) after the adjusting for age and gender. In conclusion, an interaction effect of MIR124-1 and RGS4 polymorphisms may play a more important role than individual factors for MDD development. Moreover, RGS4 gene polymorphisms may be associated with antidepressant response among the Han population such as Weighted Correlation Network Analysis to explore pathogenic genes related to MDD, schizophrenia, and other psychiatric disorders. All directions of her research program are supported by the China National Major Project.

13. Ketogenic diets in parkinson's and alzheimer's

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Aging is accompanied by a mild decline in bioenergetic capacity on many structural levels (molecule, organelle, and cell) in cells throughout the body. In neurons afflicted by Parkinson's (PD) and Alzheimer's (AD), this decline is pathologically accelerated. Given that they alter multi-targeted nutrient sensors, metabolic therapies such as calorie restriction, intermittent fasting, and high-fat, low-carbohydrate ketogenic diets may be able to restore the bioenergetic decline at all these structural levels; ketogenic diets are probably the most sustainable of these options in PD and AD. In 2017, we developed a protocol to support 47 people with PD randomized to either a low-fat or ketogenic diet for 8 weeks. Primary outcomes were between-group changes in motor and nonmotor scores from baseline to week 8. By the end of the study, the ketogenic group showed clinically and significantly greater nonmotor score baseline improvements (41% compared to 11%), particularly in urinary problems, pain, fatigue, daytime sleepiness, and cognitive impairment. In mid-2019, Waikato Hospital will coordinate a similar randomized controlled study in patients with mild AD. Primary outcomes will be between-group changes in cognition, function, and quality of life scores from baseline to week 12.

14. Pharmacological annotation of polygenic risk in individuals with psychiatric disorders

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With high rates of heritability, the genetic analysis of psychiatric disorders is seen as an important strategy to identify the molecular determinants of its pathogenesis and therefore more specific targets for therapeutic intervention. In many respects, large genome wide association studies have delivered on this expectation, by revealing hundreds of genomic loci. Several significant challenges, however, remain to be overcome before we can more effectively capitalize on these discoveries, as most of the known genetic risk is complex and involves hundreds of genes. Where risk loci can be mapped to individual genes, their small effect size and/or low frequency may, by themselves, not present a compelling case for therapeutic development. To address this challenge, we are investigating an approach that exploits systems biology to aggregate genetic burden of complex traits into clinically actionable pathways. We have been exploring this concept with both SNP array and whole genome sequencing data for individual participants in the Australian Schizophrenia Research Bank cohort and identify several existing compounds that could potentially be directed with more biological specificity to patients with higher levels of risk in associated pathways. While some of these drugs have been used in schizophrenia, or are under investigation for use in the disorder, many are approved for use in other conditions and have not been considered in the context of psychiatric treatment. This approach has the potential to provide mechanism for precision treatment of schizophrenia and other psychiatric disorders, particularly in difficult treatment resistant cases.

15. Plasma-biomarker panel for discriminating Alzheimer's disease, Parkinson diseases, and Frontotemporal dementia

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Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson diseases (PD), and Frontotemporal dementia (FTD) sometimes show similar change in one kind of biomarker as compared to healthy controls. Use of individual biomarker may cause low specificity to identify the disease type. The demand for a biomarker panel is growing to achieve a high-degree discrimination among different types of neurodegenerative diseases. In this work, the assay technology "immunomagnetic reduction" was applied to assay Amyloid Beta Peptide (Ab) 1-40, total Tau protein (T-Tau), phosphorylated Tau protein (p-Tau181), a-synuclein, phosphorylated a-synuclein (pS181), TDP-43, and Neurofilament Light (NfL) in human plasma for HC (n = 91), amnesic mild cognitive impairment (n = 41), AD (n = 35), PD with normal cognition (n = 47), PD dementia (PDD) (n = 62), and FTD (n = 25). For each kind of biomarkers, hematocrit (HC) shows a significantly lower level as compared to disease groups. It was found that FTD shows the highest levels of T-Tau (41.5 ± 20.5 pg/mL), p-Tau181 (6.67 ± 1.34 pg/mL), and TDP-43 (0.356 ± 0.202 pg/mL). AD shows the highest levels of Ab1-42 (21.2 ± 7.2 pg/mL). PDD shows the highest levels of a-synuclein (4.76 ± 1.10 pg/mL) and pS181 (12.4 ± 18.6 fg/mL). According to these data, a plasma-biomarker panel constructed with Ab1-42, T-Tau, and a-synuclein is promising for differentiating AD, PD, and FTD.

16. Circulating circular RNAs as biomarkers in the acute phase of ischemic stroke

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Currently, there are no valuable blood-based biomarkers that can be used for diagnosing acute ischemic stroke (AIS) and predicting stroke outcomes. circRNAs show promise as stroke biomarkers because of their participation in various pathophysiological processes associated with stroke and stability in peripheral blood. To explore circulating circRNAs associated with AIS, their utility as an early diagnostic marker and their significance in predicting stroke outcomes, a circRNA microarray was used to identify differentially expressed circulating circRNAs in a discovery cohort of three patients with AIS and three matched healthy control subjects (HCs). Validation was performed in an independent validation cohort (36 patients with AIS and 36 matched HCs) by quantitative real-time polymerase chain reaction (qRT-PCR). The replication cohort (200 patients with AIS and 100 HCs) was used for large sample verification, and the

copy numbers per microliter plasma were calculated by qRT-PCR. We identified, validated, and replicated three differentially expressed circRNAs, which were upregulated in patients with AIS compared with HCs (circFUNDC1: $P = 0.00014$; circPDS5B: $P = 4.13 \times 10^{-9}$; circCDC14A: $P = 1.86 \times 10^{-9}$). With an area under the curve (AUC) of 0.875 corresponding to a specificity of 91% and a sensitivity of 71.5%, the combination index of these three circRNAs had diagnostic power for stroke. The baseline circRNA levels showed poor significance, but the change rate in the level of circRNAs within the first seven days of treatment showed significance in predicting stroke outcomes (AUCs of circFUNDC1, circPDS5B, circCDC14A, and the overall circRNA set were 0.884, 0.953, 0.943, and 0.960, respectively). The elevation levels of circRNAs after stroke might be due to increasing levels in lymphocytes and granulocytes. In conclusion, a set of circulating circRNAs - circFUNDC1, circPDS5B, and circCDC14A - could not only serve as biomarkers for AIS diagnosis but also be applied in predicting stroke outcomes.

17. Stress-induced changes of NMDA and AMPA receptor expression in the rat brain are aggravated by neonatal bacterial endotoxin exposure

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Deregulated glutamatergic transmission is known to be implicated in neurological-psychiatric disorders, including stress-evoked schizophrenia and post-traumatic stress disorder (PTSD). Structural changes of NMDA and AMPA receptors can affect glutamatergic transmission. These receptors have a heterotetramer structure: NMDA-Rs consist of obligatory GluN1 subunit and variable GluN2 (a-d) or GluN3 (a and b) subunits; AMPA-Rs are composed of obligatory GluA2-dimer and a dimer of two other subunits, GluA1, GluA3, or GluA4. The expression of NMDA-R and AMPA-R subunit genes in regards to vital stress has only been explored in few studies without investigation of long-term changes, even though this seems important for understanding the mechanisms of PTSD. Moreover, these disturbances of glutamic receptor subunit expression are hypothesized to become even more vulnerable to stress effects after early-life immune challenges. According to the "two-hit" hypothesis, neonatal pro-inflammatory activation ("first hit") can affect brain maturation, thus making stressful events later in life ("second hit") have a more pronounced negative effect on brain function that can cause severe mental disorders, including schizophrenia.

The present study was aimed at the investigation of NMDA-R and AMPA-R subunit gene expression in the rat brain in a model of vital stress alone or combined with neonatal lipopolysaccharide exposure.

Two series of experiments were performed: male three-month-old Wistar rats were subjected to stress associated with contact with a predator (a black-tailed python) for 40 min either without neonatal manipulation (Study I) or after treatment with lipopolysaccharide (LPS), 25 or 50 μg/kg, i.p., at P15, P18, and P21 (Study II). qRT-PCR analysis of mRNA expression of NMDA (GluN1, GluN2a, and GluN2b) and

AMPA (GluA1 and GluA2) glutamate receptors was performed in the brain structures of rats at 6 or 24 h, 3, 9, and 25 days after stress for Study I, and seven days after stress for Study II.

In Study I, the most pronounced alterations of gene expression were revealed 25 days after stress: mRNA level of GluN2a NMDA-R subunit was upregulated in the amygdala of stressed animals compared to non-stressed control; GluN2b expression increased in the ventral hippocampus (VH) and medial prefrontal cortex and decreased in the dorsal hippocampus (DH) of rats exposed to vital stress in comparison with control. Expression of GluA1 and GluA2 decreased in DH and increased in VH after stress.

In Study II, mRNA expression of GluN2a, GluN2b, and GluA2 subunits was upregulated in mPFC of non-stressed animals injected with 25 μg/kg LPS. Levels of GluA1 subunit mRNA in DH of LPS-treated (50 μg/kg) rats increased, with no changes in VH of non-stressed LPS-treated animals. Stress-induced changes were more prominent in animals injected with 50 μg/kg LPS. In mPFC of stressed LPS-treated rats, when compared with vehicle-treated control groups, the levels of GluN1, GluN2a, GluN2b, GluA1, and GluA2 mRNA, as well as GluN2a/GluN2b ratio, were increased, while, in DH, GluN2a and GluA1 subunit mRNA levels were downregulated, and GluN2a/GluN2b ratio decreased.

Thus, early-life LPS treatment aggravates stress-induced disturbances of NMDA-R and AMPA-R subunit expression, which may contribute to severe mental illnesses.

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18. The pathogenic role of complement C5a receptor, C5aR1 in motor neuron disease

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The complement system is upregulated in MND, with recent studies indicating that the activation product C5a may accelerate disease progression via its receptor, C5aR1. This study examined the pathological role of C5aR1 in $SOD1^{G93A}$ mice, using $SOD1^{G93A}$ mice lacking C5aR1 and by means of pharmacological inhibition of C5aR1 using PMX205. C5aR1 deficient mice were backcrossed to $SOD1^{G93A}$ mice to generate $SOD1^{G93A}$ mice lacking C5aR1. The selective and orally active C5aR1 antagonist, PMX205, was also administered to $SOD1^{G93A}$ mice via their drinking water, both pre- and post-disease onset. The effect of C5aR1 genetic ablation and/or pharmacological inhibition using PMX205 on disease progression of $SOD1^{G93A}$ mice was determined using body weight, hind limb grip strength, survival time and molecular analysis of spinal cord, tibialis anterior and blood. $SOD1^{G93A}$ mice lacking C5aR1 and $SOD1^{G93A}$ mice treated with PMX205 prior to disease onset, both had significantly improved hind-limb grip strengths, slower disease progression and extended survival, compared with control or vehicle treated $SOD1^{G93A}$ mice. These improvements in the $SOD1^{G93A}$ mice lacking C5aR1 and PMX205-treated group were associated with reductions in pro-inflammatory monocytes/macrophages/microglia in the peripheral blood, tibialis anterior and spinal cord. There was also a reduction in pro-inflammatory cytokines in the lumbar spinal cord. Importantly, PMX205 treatment beginning several weeks following disease onset also had an attenuating effect on disease progression, significantly extending survival. These results confirm that C5aR1 plays a pathogenic role in

$SOD1^{G93A}$ mice, further validating the C5a-C5aR1 signalling axis as a potential therapeutic target to slow disease progression in MND.

19. Gap junction network within an olfactory sensory unit for colony identification in the Japanese carpenter ant: 3D structure and putative function

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The environment is filled with chemical information, and animals, including human beings, have developed adaptive chemosensory systems. It is thought that the chemical information is integrated in the brain before making a decision. Here, I talk about a complicated olfactory sensory system of a tiny insect, the carpenter ant, *Camponotus japonicus*, because we recently found an information integration network at this very peripheral system. For colony identification, worker ants utilize a colony-specific body odor consisting a characteristic blend of cuticular hydrocarbons (CHCs) as a social pheromone. C. *japonicus* workers appeal to their own colony identification with the colony-specific body odor comprising 18 species-specific CHCs. Thus, the accurate difference detection among such colony-specific body odors of workers is indispensable for their social life while inaccurate difference detection is sometimes fatal in competition among colonies. The body odor CHCs is sensed in a particular type of olfactory organ called Sensilla basiconica on the antennae. The number of *S. basiconica* responding to own colony's CHCs was significantly smaller than that responding to other colony's CHCs. This suggests that the very peripheral tiny sensory system possesses a whole basic machinery for colony identification via odor difference detection. To investigate the functional design of this type of sensilla, we observed its ultra-structures, using a serial block-face scanning electron microscope (SBF-SEM). Based on the serial images of 352 cross sections of SBF-SEM, we reconstructed a 3D model of the sensillum. This model reveals that each S. basiconica houses > 100 unbranched dendritic processes, which extend from the same number of olfactory receptor neurons (ORNs). The dendritic processes have characteristic beaded-structures and form a twisted bundle within the sensillum. At the beaded-structures, the cell membranes of the processes are closely adjacent in the interdigitated profiles, suggesting functional interactions via gap junctions (GJs). Immunohistochemistry with anti-innexin (invertebrate GJ protein) antisera revealed positive labeling in the antennae of C. *japonicus*. Innexin 3, one of the five antennal innexin subtypes, was detected as a dotted signal within the S. basiconica as a sensory organ for colony identification. The fluorescence intensity of innexin 3 shows a characteristic twin-peak-distribution similar to the distribution of adhesion regions at beaded-structures. These morphological results suggest that the beaded-structure provides a platform for functional connection among ORNs via close apposition of membranes and ORNs form an electrical network via GJs between dendritic processes. To reveal the function of the ORNs network via GJs, we examined a simplified mathematical simulation for the inter-dendritic neural network based on cable theory and proposed possible modification of its responsiveness to virtual stimulation. The mathematical simulation showed that the information network acts as a "stronger-input-spread or weaker-input-cut filter" in a GJ-distribution-dependent manner. This novel "filter" supports that ORNs in the S. basiconica generate few impulses when they respond to own colony's CHCs (weak stimulation) and generate many impulses when they respond to other colony's CHCs (strong stimulation). Therefore, the ORN network via GJs possibly contributes to the distinct identification of colony-specific blends of CHCs.

20. Tissue-engineered electrodes for brain-machine interfaces

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State-of-the-art neural interfaces rely on conventional metallic electrodes. Ideally, bionic devices should safely operate for a lifetime; However, the fibrotic tissue encapsulation leads to inefficient stimulation and formation of toxic by-products, ultimately compromising the electrical and biological performance of these bionic interfaces. This limitation further challenges the development of smaller and more densely packed electrodes aiming for a more specific neuronal stimulation. Conductive hydrogel (CH) coatings, based on poly (vinyl alcohol) polymers modified with conductive polymers, can provide enhanced electrical properties, superior to those of traditional platinum (Pt) electrodes. These coating materials can be further modified to include an overlaying layer of neural progenitors encapsulated within a 3D biosynthetic hydrogel. This study tested the hypothesis that a CH coated electrode decorated with a loaded cell coating provides a more physiological interface able to integrate electrodes with the neural tissue without significantly reducing the charge transfer properties. The aim of this study was to develop and assess a tissue-engineered, living electrode (LE) coating for brain-machine interfaces. LEs were fabricated by first coating intra-cortical Pt electrodes with CH followed by an overlaying degradable bio-synthetic hydrogel coat loaded with primary neural progenitor cells. The electrical performance of LEs was compared with conventional Pt electrodes *in vitro* and *in vivo*. *in vitro* studies confirmed that overlaying a neural cell-loaded coat on the CH did not significantly impacted the electrode impedance and charge storage capacity. These results suggest that the electrical performance of LEs was comparable to standalone CH coated electrodes and significantly superior to Pt electrodes. *In vivo* studies showed that implanted LEs and uncoated Pt electrodes in a rat brain model did not cause any adverse events over 8 weeks. LEs presented a significantly higher signal to noise ratio than Pt electrodes. On-going research is assessing the tissue response to implanted LEs. These results demonstrate the potential for LEs to support the development of more robust neural interfaces.

21. Golgi fragmentation induced by cyclin-dependent kinase 5 overactivation is associated with isoflurane-induced cognitive decline

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Isoflurane is a widely used anesthetic. Isoflurane exposure induces cognitive decline, especially in elderly patients, while the underlying mechanism remains to be elucidated. In the present study, we explored whether Golgi fragmentation is relevant to isoflurane-induced cognitive decline and the underlying molecular mechanism in aged mice. Sixteen-month-old C57BL/6J mice inhaled 1.4% isoflurane for 2 h daily for three consecutive days. To inhibit aberrant cyclin-dependent kinase 5 (Cdk5), 10 mg/kg roscovitine was given 30 min before isoflurane treatment. The Golgi structure, Cdk5 activity, and level of p25/p35 were assessed 2 h after isoflurane exposure. Spatial learning and memory ability of mice were evaluated

by Morris water maze one day after isoflurane treatment. Our results show that the number of fragmented Golgi and Cdk5 activity increased. Learning and memory ability were impaired in aged mice after isoflurane exposure, while Cdk5 inhibitor roscovitine rescued the Golgi structure and improved learning and memory performances. In addition, after isoflurane exposure, the levels of p25/p35 increased, while Cdk5 levels unchanged. Our study reveals that the cleavage of p35 into p25 may contribute to aberrant Cdk5 activation, and Cdk5 overactivation-induced Golgi fragmentation may mediate isoflurane-induced cognitive decline in aged mice. Inhibition of aberrant Cdk5 activation alleviates Golgi fragmentation and cognitive decline, which provides a potential therapeutic approach for isoflurane-induced cognitive decline.

22. A novel analytical method for detection of phosphorylated a-synuclein S129 in Parkinson's disease

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Parkinson's disease (PD) is characterized by the intraneuronal α-synuclein inclusions called Lewy bodies. Increase of phosphorylation of a-synuclein in Serine 129 (pS129) has been correlated with the aggregation, toxicity, protein interaction, and turnover of α-synuclein. Thus, pS129 can indicate the pathogenesis of PD. Since the concentration of pS129 in the plasma (femtogram level) is far lower than the normal detection range of ELISA, we developed an ultrasensitive immunomagnetic reduction assay to detect the trace amount of pS129 in limited volume of human plasma (60 μL). The pS129 assay covered a range of concentration (0.00048-144.78 pg/mL) with a limit of detection of 0.065 fg/mL. Furthermore, we analyzed the pS129 level from healthy control (n = 10) and patients with PD (n = 23) and found a significant increase of pS129 in plasma from PD ($P < 0.0001$). The cut-off value of pS129 for discriminating control from PD was 0.505 fg/mL with corresponding clinical sensitivity and specificity of 95.65% and 100%, respectively. In conclusion, we developed a novel plasma pS129 assay that is convenient, sensitive, sample saving, and useful for identifying PD patients.

23. Linking autism to an imbalanced catabolism of synaptic monoamine

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An interdisciplinary study of autism led to implicate a relatively poor catabolism of one of the monoamines released in the synapse, namely *serotonin*. This deficit would result from persistent epigenetic regulations of two enzymes (i.e., MAOA- and COMT+) across neural differentiation, for counteracting an accidental excess of MAOA in the early gestation. Epigenetic traits would outlast this temporary excess and be inherited by generations of neurons, and possibly by next human generations. In addition, the late occurrence of autistic symptoms may be consistent with the increase of the monoamine oxidase B (MAOB)

enzyme that degrades another monoamine (dopamine), but only significantly around two years after birth. The consequent long-term imbalance of synaptic monoamines is assumed here to impact the architecture of sleep and learning[1], inducing a range of developmental problems.

This theory is drawn on Guided Propagation Networks (GPNs), the computer simulations of which show the growth of aberrant structures when modulation parameters akin to monoamines do not satisfy inner learning constraints. Comparisons are made between a reference well-tuned network and others grown with shifted parameters, all using the same learning data. Unlike the reference network, impaired GPNs display features that have been observed in the autistic brain: (1) more local connections (here underlying either repetitive behavior or over-activity); (2) missing or impaired long-distance connections (which convey emotional conditioning towards decision-making modules); and (3) overgrowth: the overall connectivity can involve 1.5 more cells and links. Apart from these computer experiments[2], the 4:1 sex ratio observed in autism can be calculated in a family tree which combines genetic variants and epigenetic regulations. According to this calculation, which involves two types of genetic masking of the relevant epigenetic traits (i.e., X-silencing and low-COMT), in addition to 1.5% of the population having developed an overt form of autism, about 6% of men and 24% of women would be "healthy carriers" of the enzymatic (dys) regulation at issue.

On the medical side, an epileptic 11-year-old boy with severe autism received sodium valproate daily for its ability to both stimulate MAOA and treat epilepsy. In this case study, behavioral changes have been recorded for one year by parents and caregivers unaware of the autism target. This one-year monitoring showed improvement of sleep and then gaze, followed by a gradual decrease of stereotypy among other behavioral changes arising nine months after the treatment initiation. Hyperactivity, which hindered learning across this treatment, could afterwards be reduced by low-dose of the methylphenidate psychostimulant. The proposed dual therapy thus involves a MAOA inducer and a psychostimulant, together with re-education, all monitored by relevant biomarkers. If validated by future investigations, this approach is first intended to prevent early gestation from environmental factors that are likely to stimulate the production of MAOA, including small-sized fatty acids.

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24. Neuropsychology intervention in Williams syndrome: a clinic case

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Williams syndrome (WS) is a neurodevelopmental disorder caused by the removal of 7q11.23. Patients with WS present neuroanatomical alterations that are reflected in neuropsychological alterations mainly in visuospatial abilities, attention, and executive functions. The objective of the study was to apply a neuropsychological intervention program to improve attention and visuospatial skills. The program was applied for 10 months in sessions of 1.5 h to an 8-year-old girl with WS from Mexico City. The tasks were designed based on neuropsychological clinical models. The pre- and post-intervention results were compared with a clinical sample of five patients with WS, which allowed identifying if there were an

improvement and clinical recovery. The results were analyzed using the reliable change index. The results reveal that the patient improved her intellectual abilities, attention, and visuospatial abilities. They also improved the skills of abstraction and memory. In the literature, it is related that the neuropsychological intervention stimulates the activation of alternative or inactive neural networks that begin to have a greater implication in the affected cognitive processes. These findings demonstrate that neuropsychological intervention is an effective therapeutic strategy for patients with congenital brain damage.

25. Caspr2/CNTNAP2 (or cadm1) forms a complex with GPR37 and Mupp1 but not with autism-related mutated ones

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Autism spectrum disorder (ASD) is one of the developmental brain disorders. Mutations in the synaptic components including NLGN, Nrx, Cadm1, Caspr2/CNTNAP2 (Contactin-associated protein 2), and GPR37 (G-protein-coupled receptor 37) have been found in ASD patients. Caspr2 and Cadm1 have a PDZ binding domain at C-terminal region and form a complex with receptors via interaction with Multiple PDZ domain protein 1 (Mupp1). However, little is known about the impaired Caspr2 (Cadm1)-Mupp1-receptor complex related to the pathogenesis of ASD. Recently, we found mutations (R558Q and Del312F) of GPR37 gene in the ASD patients. Caspr2 (Cadm1) and GPR37 mainly interacted with PDZ3 and PDZ11 domains of Mupp1 via their C-terminal PDZ binding domains, respectively, while the ASD-associated mutated GPR37 (R558Q) more weakly interacted with Mupp1 and was much less transported to the cell surface by Mupp1. In the present study, we found two missense mutations in *Mupp1* gene of the ASD patients and investigated the density and morphology of PSD95-positive dendritic spines and protrusions in cultured hippocampal neurons overexpressing the mutated Mupp1. Compared to the Mupp1, mutated Mupp1 significantly reduced the density of PSD95-positive dendritic spines and decreased the width of dendritic spines. Thus, ASD-related Mupp1 missense mutations influence the dendritic spine morphogenesis, causing the pathogenesis of ASD. The impaired Caspr2 (Cadm1)-Mupp1-receptor complex including Gpr37 or serotine receptors may be related to the pathogenesis of ASD.

26. The prognostic factors affecting the occurrence of subsequent unprovoked seizure in patients who present with febrile seizure after 6 years of age

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Aim: Few reports have described the prognostic factors affecting the occurrence of subsequent unprovoked seizure in patients who present with febrile seizure (FS) after six years of age. We investigated the prognostic factors affecting the development of unprovoked seizures after FS among patients from Jeju Island.

Methods: We included patients who developed FS after six years of age and presented to our outpatient clinic between January 2011 and June 2017. Clinical data were obtained through chart reviews and phone call interviews. We used logistic regression analysis to analyze the risk factors associated with the occurrence of subsequent unprovoked seizure.

Results: Of the 895 patients, 83 developed FS after six years of age. Among these 83, three patients were prescribed antiepileptic drugs before the onset of the unprovoked seizure and four patients developed an unprovoked seizure before six years of age. Thus, overall, 76 patients were included in the study. Fifty-one patients developed first FS before six years of age. In the remaining patients, the first FS developed after six years of age. The mean observational period since the last outpatient follow-up visit was 3.2 years (median 3.04 years, range: 1.42-4.71 years). Among them, 21% developed an unprovoked seizure. Logistic regression analysis showed that electroencephalographic (EEG) abnormalities serve as an independent risk factor for a subsequent unprovoked seizure.

Conclusion: EEG is the proper diagnostic tool to predict the risk of a subsequent unprovoked seizure in patients with FS after six years of age.

27. Comparative effects of hydrogen sulfide-releasing compounds on [3H]D-aspartate release from bovine isolated retinae

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Previously known as an industrial toxicant, hydrogen sulfide (H_2S) has evolved into a signaling gasotransmitter that possess physiological roles in the central nervous, cardiovascular and the immune systems (Kimura Molecules 2014;19:16146). It is endogenously derived from *L*-cysteine and *D*-cysteine by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) enzymes, with some contribution from cysteine aminotransferase and *D*-aminotransferase in combination with 3-mercaptosulfurtransferase using *L*-cysteine or homocysteine as substrates (Abe & Kimura. J Neurosci 1996;16:1066; Nagai Y *et al*. FASEB J 2004;18:557). In ocular tissues, a deficiency of CBS due to a mutation in the gene encoding the enzyme is associated with several eye disorders such as glaucoma, cataracts and retinal detachment [Kraus JP, Kozich V. In Carmel & Jacobsen DW (eds) Homocysteine in health and disease. Cambridge University Press; 2001. p. 223]. Furthermore, H_2S has been shown to exert pharmacological effects on mammalian ocular tissues from both anterior and posterior segments *in vitro* and *in vivo* (Ohia *et al*. J Ocul Pharmacol Ther 2018;34:61). In the present study, we used the Superfusion Method to (1) compare the pharmacological actions GYY 4137, a slow-releasing H_2S donor to that of *L*-cysteine, a substrate for H_2S biosynthesis on K^+-evoked [3H]D-aspartate release, and (2) examine the role of KATP channels and nitric oxide (NO) in the responses elicited by these compounds on neurotransmitter release in isolated bovine retinae. GYY 4137 (10 nM - 10 μM), *L*-cysteine (100 nM - 10 μM) and its prodrug, *N*-acetyl cysteine (10 μM -1 mM) attenuated K^+-evoked [3H]D-aspartate release in a concentration-dependent manner from isolated bovine retinae without affecting basal tritium efflux. The rank order of activity observed at an equimolar concentration of 10 μM was: *L*-cysteine > GYY 4137 > *N*-acetyl cysteine. Interestingly, the dual inhibitor of the biosynthetic enzymes for H_2S, CBS and CSE, amino-oxyacetic acid (3 mM) reversed the inhibitory

responses caused by the three H2S donors on K^+-evoked [3H]D-aspartate release. Glibenclamide (300 μM), an inhibitor of KATP channels reversed the inhibitory action elicited by GYY 4137 and *L*-cysteine but not that of *N*-acetyl cysteine on K^+-induced [3H]D-aspartate release, suggesting a distinct and unique mechanism for the *L*-cysteine prodrug. The inhibitory effect of GYY 4137 and *L*-cysteine on neurotransmitter release was reversed by the non-specific inhibitor of NO synthase (NOS), L-NAME (300 μM). Furthermore, a specific inhibitor of inducible NOS, aminoguanidine (10 μM) mitigated the inhibitory action of *L*-cysteine on K^+-evoked [3H]D-aspartate release. We conclude that both donors and substrates for H2S production can inhibit amino acid neurotransmission in bovine isolated retinae, an effect that is dependent, at least in part, upon the intramural biosynthesis of this gas, and on the activity of KATP channels and NOS enzyme (Bankhele *et al*. Neurochem Res 2018;43:692).

28. Surgical outcome of pediatric spinal cord tumor

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Aim: The objective of this study was to examine the long-term surgical outcomes of pediatric spinal cord tumor in a prospective multicenter database.

Methods: Of 48,901 surgical cases in our database, 1046 (2.1%) involved patients under 20 years old. Among these, 47 cases (0.1%; male 28, female 19; mean age 11.1 years; and mean follow-up: five years) were spinal cord tumors with clinical records, plain radiographs, and MRI. The patient characteristics, symptoms at onset, tumor resection, surgical procedure, postoperative radiotherapy and chemotherapy, surgical outcome, and kyphotic change at final follow-up were examined. Statistical analysis was performed by unpaired t-test and Fisher exact test.

Results: Intradural extramedullary, intramedullary, and extradural tumors accounted for 50%, 33%, and 17% of the 47 cases, respectively. A thoracic spine tumor was most common (40%). The common pathological diagnoses were ependymoma (n = 8), neurinoma (n = 7), and neurofibroma (n = 6), including high-grade malignant spinal tumor. The most common symptom at onset was pain (50%), followed by motor palsy (34%), gait disturbance (18%), and bladder disturbance (15%). In 35% of the cases, pain was the only preoperative symptom. Total resection was achieved in 61% and subtotal resection in 22% of cases, and radiotherapy and chemotherapy were performed postoperatively in 18% and 14%, respectively. The recurrence rate was 24%, and these cases were treated with additional surgery and chemotherapy. Postoperative improvement of symptoms occurred in 38 cases (81%), but there were four deaths due to a malignant tumor. Progression of spinal kyphosis (> 5°) occurred in 18 cases (38%), with an average of 11°. Postoperative kyphosis was significantly related to postoperative radiation therapy ($P < 0.05$), but not to the number of laminectomy levels.

Conclusion: In pediatric spinal tumor, the main symptom at onset was pain without neurological deficit. Postoperative radiotherapy may be effective, but postoperative kyphotic changes are a concern in these patients.

29. The Di Bella method's biological multi-therapy has improved the objective response, expectation and quality of life of brain malignancies: Statistical evaluation at seven years

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The interaction between growth hormone (GH) and prolactin receptor (PRL) acts on physiological and neoplastic growth, which uses these factors on multiple physiological measures, with dose-dependent relationship. From a review of the literature, GH and growth hormone receptor (GHR) overexpression in tumors is constant. In more than a thousand cases published on www.pubmed.gov by Giuseppe and Luigi Di Bella of various neoplasms, an improvement in the objective response, quality of life, and survival was documented, compared to conventional oncological protocols, inhibiting GH and GF correlated through somatostatin and analogs in the context of biological multi-therapy "Metodo Di Bella" (MDB).

Rationale-Mechanism of action of therapy. The Di Bella Foundation has been treating and monitoring for nine years several cerebral neoplasms including oligodendrogliomas 2° and 3°, astrocytomas, glioblastomas 3°, anaplastic gliomas 2° and 3°, and anaplastic oligoastrocytomas.

- Somatostatin 3 mg - subcutaneous (every night - 12 h infusion)
- Slow Octreotide 20 mg intramuscular release (every three weeks)
- Conjugated Melatonin (Melatonin 12%, Adenosine 51%, and Glycine 37%) 100 mg (daily-oral)
- Retinoid solution - 8 mL oral (three times a day) (ATRA 0.5 g, Axeroftole Palm. 0.5 g, Beta Carotene 2 g, and Alpha Tocopherol Acet. 1000 g)
- Vit. D3, 1.25; OH-Tachysterol, 10-12 drops (three times a day - oral)
- Tetracosactide Acetate 0.25 mg intramuscular (three times a week)
- Cabergoline 0.5 mg (½ cps) - oral (twice a week)
- Bromocriptine 2.5 mg (½ cps) - oral (twice a day)
- Temozolamide (20 mg/morning and evening) daily metronomic administration (morning and evening) Hydroxyurea 500 mg at midday meal
- Ac. Slow release valproic 500 mg morning and evening
- Calcium Levofolinate capsules 22 mg per day
- Vit. C 2 gr (2 times a day)

Somatostatin + Octreotide with antiproliferative function and receptor saturation are interactive with dopamine 2 receptor (D2R) agonist prolactin inhibitors, whose PRLR receptor is co-expressed with GHR on cytosolic membranes. MDB multi-therapy with inhibition of GH-PRL proliferative axis and GH-dependent growth factors (IGF1-FGF-VEGF-EGF) with anti-proliferative effect have increased life expectancy, on average over six years, in the mentioned neoplasms.

The myelotoxicity of the metronomic use of Temozolamide-Hydroxyurea is contained by the myeloprotective properties of melatonin and retinoid solution, which, with vitamins D3 and C, exert a differentiating synergism, with antioxidant and immunomodulating activities; instead, with somatostatin and prolactin inhibitors, they exert a cytostatic effect. For the still reduced survival of malignant brain tumors (Glioblastoma, 15 months), we consider useful this biological multi-therapy of synergistic and factorially interactive molecules, singularly managed by non-toxic antitumor activity, which act centripetally on the myriad biological reactions of the tumoral life, bringing back to normal the vital reactions deviated due to cancer.

30. Thrombolysis with rhTNK-tPA at different therapeutic time windows in animal model of embolic stroke

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It is essential for us to investigate the effects and characteristics of thrombolytics on different therapeutic time windows (TTW) in an animal model of embolic stroke. In our research, we investigated the thrombolysis with recombinant human TNK-tPA (rhTNK-tPA) on thromboembolic stroke in an animal model at different TTW. Rats were subjected to embolic middle cerebral artery occlusion. RhTNK-tPA and positive control drugs rt-PA were administered 1, 2, 3, 4.5, and 6 h after inducing thromboembolic stroke. Neurological deficit scoring (NDS) was evaluated at 6 and 24 h after the treatment. The lesion volume in cerebral hemispheres was measured by MRI using MRI scanning machine after 6 h of thrombolysis, and the infarct volume was measured by TTC stain, together with hemorrhagic volume quantified by a spectrophotometric assay after 24 h of thrombolysis. The results show that rhTNK-tPA 1.6 mg/kg significantly improved the NDS after cerebral thromboembolism in rats at different TTW. RhTNK-tPA improved the NDS by 36.4% ($P < 0.05$), 41.7% ($P < 0.01$), 37.5% ($P < 0.01$), 36.0% ($P < 0.05$), and 26.1% ($P > 0.05$) at 1, 2, 3, 4.5, and 6 h TTW with 6 h treatment, respectively, and by 45.8% ($P < 0.01$), 50.0% ($P < 0.01$), 48.0% ($P < 0.05$), 37.5% ($P > 0.05$), and 28.0% ($P > 0.05$)at 1, 2, 3, 4.5, and 6 h TTW with 24 h treatment, respectively. Rt-PA improved the NDS by 40.9% ($P < 0.05$), 37.5% ($P < 0.05$), 33.3% ($P < 0.05$), 28.0% ($P > 0.05$), and 8.7% ($P > 0.05$) at 1, 2, 3, and 4.5 h TTW with 6 h treatment, respectively, and by 50.0% ($P < 0.01$), 50.0% ($P < 0.01$), 48.0% ($P < 0.05$), 33.3% ($P > 0.05$), and 12.0% ($P > 0.05$) at 1, 2, 3, 4.5, and 6 h TTW with 24 h treatment, respectively. RhTNK-tPA significantly reduced the extent of brain lesions examined by MRI at different TTW. At 1, 2, 3, 4.5, and 6 h TTW, lesion volume was reduced by 73.3% ($P < 0.001$), 64.2% ($P < 0.01$), 62.1% ($P < 0.01$), 46.1% ($P < 0.05$), and 39.6% ($P > 0.05$) at rhTNK-tPA dose of 1.6 mg/kg, respectively, and by 61.7% ($P < 0.01$), 59.4% ($P < 0.05$), 48.1% ($P < 0.05$), 49.1% ($P < 0.05$), and 17.8% ($P > 0.05$) at rt-PA dose of 9 mg/kg, respectively. RhTNK-tPA significantly reduced the extent of cerebral infarction examined by TTC at different TTW. At 1, 2, 3, 4.5, and 6 h TTW, infarction volume was reduced by 63.8% ($P < 0.01$), 71.0% ($P < 0.05$), 63.2% ($P < 0.05$), 58.0% ($P < 0.01$), and 35.7% ($P > 0.05$) at rhTNK-tPA dose of 1.6 mg/kg, respectively, and by 53.4% ($P < 0.01$), 60.9% ($P < 0.05$), 51.7% ($P < 0.05$), 54.6% ($P < 0.05$), and 20.4% ($P > 0.05$) at rt-PA dose of 9 mg/kg, respectively. The amount of hemorrhage in rhTNK-tPA rats increased slightly with the prolongation of the TTW ($P > 0.05$), and the amount of bleeding at 6 h TTW increased approximately 1.6 folds compared with the model group ($P = 0.0748$). The amount of hemorrhage in rt-PA rats increased with the prolongation of the TTW, and the amount of bleeding at 6 h TTW increased more than two folds compared with the model group ($P < 0.01$). Thus, rhTNK-tPA had an obvious therapeutic effect on ischemic stroke caused by thrombosis, and could be started within 4.5 h TTW.

31. Role of glutamate in the pathogenesis of acquired epilepsy in alzheimer's disease

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Alzheimer's disease (AD) can increase the risk of epileptogenesis up to 10-fold in the patient, compared to healthy age-matched controls. However, the relationship between acquired epilepsy and AD is yet to

be elucidated. Here we proposed that changes in the brain that occur early in the AD pathology may lead to this higher susceptibility to epileptogenesis. Disruption in brain's glutamate homeostasis has been reported in both disease and therefore it has the potential to link the two diseases together. This study aimed to explore the potential role of glutamate in the pathogenesis of acquired epilepsy in AD. It also aimed to identify potential early biomarkers or a diagnostic tool for acquired epilepsy in AD. Six month-old Tg2576 AD mice along with their wild-type (WT) littermate were utilised in this study. The cortex and the hippocampus were extracted from the animal, then western blotting and mass spectrometry were performed. Tg2576 had significantly lower amounts of GLT-1 and Glutamine synthetase in the cortex, compared to the WT. Additionally, mass spectrometry have shown that metabolites such as glutamate and glutamine have the potential to be the early biomarker for acquired epilepsy in AD. The results suggest that the astrocytic function could be impaired early in AD and this include the glutamate-glutamine cycle. This impairment might lead to a higher susceptibility of the brain to epileptogenesis via the excessive extracellular glutamate. The findings from the metabolomics analysis also suggest that there are changes in different brain's metabolites early in AD.

32. Late preterm infants' social competence, motor development, and cognition

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A preterm birth with a GA of 34 weeks 0 days to 36 weeks 6 days is called a late preterm birth; 70% of preterm births fall into this gestation period. Until a few years ago, late preterm birth was considered of no importance in the regular monitoring of babies' health, neurodevelopment, and social development. The aim of this study was to compare the social competence, motor development, and cognition of late preterm infants (LPIs) with full-term infants. Several studies in the recent past indicated that LPIs are at high risk of social development problems. We compared the development of motor skills, cognition, and social competency of LPIs with full-term infants at between 2 and 2.5 years old. The Chinese versions of the Gesell development diagnosis scale and the normal development of social skills from infants to junior high school children scale were used for the assessment. LPIs were not more socially competent than their full-term counterparts. Each skill, namely adaptability, gross motor, fine motor, language, and personal-social responses, was separately associated with the total level of social skills. It was found that gross motor skills had a positive correlation with the self-help and locomotive abilities, and fine motor skills had a positive association with locomotion abilities. LPIs had risk factors due to their delayed social skills in areas including motor disorders and physiological and perinatal factors. LPIs under three were at a higher risk of impairment in social competency. Therefore, it is recommended that they be monitored regularly to identify the development of social and cognitive disorders at an early stage.

33. The interaction of N-terminal motif of acetylcholinesterase and β-amyloid triggers β-amyloid aggregation and deposition

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Acetylcholinesterase (AChE) is one of the molecular chaperones inducing β-amyloid (Aβ) aggregation and deposition in the pathological process of Alzheimer's disease (AD). Peripheral anionic site (PAS) of AChE is

a binding site of Aβ with AChE. Our previous study found for the first time that the 7-20 amino acid region on the N-terminal of AChE (AChE7-20) can induce the formation of Aβ oligomer. However, the binding mode and pathophysiological effects of AChE7-20 and Aβ interaction remains unclear. In this study, protein-protein docking and molecular dynamics simulation were used to probe the interaction between AChE7-20 and Aβ. The residues of Arg13, Glu7, and Arg18 on AChE7-20 were the main contacts of AChE7-20 with Aβ. His13/14, Glu22/Asp23, and Asn27 on α-helix Aβ and Glu11, Phe19/20, Glu22/Asp23, and Met35 on β-sheet Aβ were the key residues of Aβ binding with AChE7-20, respectively. Compared with Aβ alone, AChE7-20-Aβ interaction triggered the transformation of Aβ from α helix to β sheet. The residues of Aβ participating in aggregation became fluctuant due to the presence of AChE7-20. The TEM morphology of Aβ aggregation confirmed that AChE7-20 induced more Aβ fibrils than control AChE7-20 in scramble sequence (Sc-AChE7-20). The binding affinity of AChE7-20 with Aβ oligomer was higher than Sc-AChE7-20 determined by SPR assay. Compared with Aβ alone, AChE7-20 co-incubation enhanced the apoptosis of primary hippocampal neurons induced by Aβ. Intervention of AChE-Aβ interaction was further studied with Bis-(9)-(-)-Meptazinol (BisMep), a novel dual-binding AChE inhibitor developed by our group. Results of molecular docking suggested that BisMep could not only interact with Tyr341 and Asp74 residues in the PAS of AChE through hydrogen bond and ionic bond, but also Arg13 and Arg16 residues in AChE7-20 through H-π and cation-π interaction, which are the key residues in AChE7-20-Aβ interaction. Subsequently, in Aβ-induced AD model mice, BisMep could significantly decrease the Aβ deposits, the activation of astrocytes and microglia, the levels of pro-inflammatory factors, and the AChE catalytic activity in the hippocampus, and eventually improve the performance of learning and memory of AD model mice. In conclusion, this study revealed the key residues and binding mode between AChE7-20 and Aβ and confirmed the triggering effect of AChE7-20 on Aβ aggregation and neurotoxicity, which provided structural biological information for the discovery of a lead compound intervening AChE-Aβ interaction.

34. Perceptual closure analysis: preliminary study with event-related potentials

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The perceptual closure is a complex visual skill that allows perceiving an incomplete pattern or object as complete or whole. A better knowledge of the electrophysiological bases of the perceptual closure would be useful to better understand the visuospatial alterations that occur in pathologies such as Williams syndrome, schizophrenia, and autism. Event-related potentials (ERP) studies in tasks with fragmented objects or faces have evidenced the presence of a component called Closure Negativity.

The objective of this study was to identify differences in ERP obtained from correctly or incorrectly fragmented figures in a perceptual closure task. Participants were 12 healthy male adults, university students between 20 and 31 years of age, who performed a visual closure task in which they had to decide if an incomplete figure corresponded (congruent condition) or not (incongruent condition) to a complete figure that was presented before. ERPs were recorded in 32 electrodes with a NeuroScan equipment. The ERP showed a negative peak around 170 ms (N1) without differences between the conditions, and a positive peak between 240 and 270 ms (P2) with greater amplitude for the incongruent condition than for the congruent one. Additionally, a negativity was observed around 400 ms that also had a greater amplitude for the incongruent *vs*. congruent condition. These preliminary findings give evidence of a differentiated processing for figures perceived as congruent *vs*. incongruent from a pattern, in latencies that could be related to late visual processing (P2) and probably to a perceptual semantic evaluation. This pattern should be confirmed with more studies, to be applied later in clinical populations such as those mentioned.

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Neuroimmunology and Neuroinflammation

Correction

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Correction: Neuroinflammatory modulators of oligodendrogenesis

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The first author declared he changed his name. His present name is Adam Armada-Moreira. Therefore, he needed to change his name in his publication in our journal.

According to the evidence provided by the author, Adam Armada-Moreira and Ana Armada-Moreira are the same person, and all of the other authors of this papers agreed with the change mentioned above. Therefore, we publish this correction to announce this change.

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Manuscript Type	Definition	Abstract	Keywords	Main Text Structure

Original Article	An Original Article describes detailed results from novel research. All findings are extensively discussed.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
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Authors' full names should be listed. The initials of middle names can be provided. Institutional addresses and email addresses for all authors should be listed. At least one author should be designated as corresponding author. In addition, corresponding authors are suggested to provide their Open Researcher and Contributor ID upon submission. Please note that any change to authorship is not allowed after manuscript acceptance.

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Methods should contain sufficient details to allow others to fully replicate the study. New methods and protocols should be described in detail while well-established methods can be briefly described or appropriately cited. Experimental participants selected, the drugs and chemicals used, the statistical methods taken, and the computer software used should be identified precisely. Statistical terms, abbreviations, and all symbols used should be defined clearly. Protocol documents for clinical trials, observational studies, and other non-laboratory investigations may be uploaded as supplementary materials.

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This section should discuss the implications of the findings in context of existing research and highlight limitations of the study. Future research directions may also be mentioned.

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It should state clearly the main conclusions and include the explanation of their relevance or importance to the field.

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to conception and design of the study and performed data analysis and interpretation: Salas H, Castaneda WV; performed data acquisition, as well as provided administrative, technical, and material support: Castillo N, Young V.
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Organization as author	Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. Hypertension 2002;40:679-86. [PMID: 12411462]

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Books	Sherlock S, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub; 1993. pp. 258-96.
Book chapters	Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. The genetic basis of human cancer. New York: McGraw-Hill; 2002. pp. 93-113.
Online resource	FDA News Release. FDA approval brings first gene therapy to the United States. Available from: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm. [Last accessed on 30 Oct 2017]
Conference proceedings	Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.
Conference paper	Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. pp. 182-91.
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Figures can be submitted in format of tiff, psd, AI or jpeg, with resolution of 300-600 dpi;
Figure caption is placed under the Figure;

Diagrams with describing words (including, flow chart, coordinate diagram, bar chart, line chart, and scatter diagram, etc.) should be editable in word, excel or powerpoint format. Non-English information should be avoided;
Labels, numbers, letters, arrows, and symbols in figure should be clear, of uniform size, and contrast with the background;
Symbols, arrows, numbers, or letters used to identify parts of the illustrations must be identified and explained in the legend;
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2.4.6 Tables

Tables should be cited in numeric order and placed after the paragraph where it is first cited;
The table caption should be placed above the table and labeled sequentially (e.g., Table 1, Table 2);
Tables should be provided in editable form like DOC or DOCX format (picture is not allowed);
Abbreviations and symbols used in table should be explained in footnote;
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2.4.7 Abbreviations

Abbreviations should be defined upon first appearance in the abstract, main text, and in figure or table captions and used consistently thereafter. Non-standard abbreviations are not allowed unless they appear at least three times in the text. Commonly-used abbreviations, such as DNA, RNA, ATP, *etc*., can be used directly without definition. Abbreviations in titles and keywords should be avoided, except for the ones which are widely used.

2.4.8 Italics

General italic words like *vs*., *et al., etc*., *in vivo*, *in vitro*; *t* test, *F* test, *U* test; related coefficient as *r*, sample number as *n*, and probability as *P*; names of genes; names of bacteria and biology species in Latin.

2.4.9 Units

SI Units should be used. Imperial, US customary and other units should be converted to SI units whenever possible. There is a space between the number and the unit (i.e., 23 mL). Hour, minute, second should be written as h, min, s.

2.4.10 Numbers

Numbers appearing at the beginning of sentences should be expressed in English. When there are two or more numbers in a paragraph, they should be expressed as Arabic numerals; when there is only one number in a paragraph, number < 10 should be expressed in English and number > 10 should be expressed as Arabic numerals. 12345678 should be written as 12,345,678.

2.4.11 Equations

Equations should be editable and not appear in a picture format. Authors are advised to use either the Microsoft Equation Editor or the MathType for display and inline equations.

2.5 Submission Link

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