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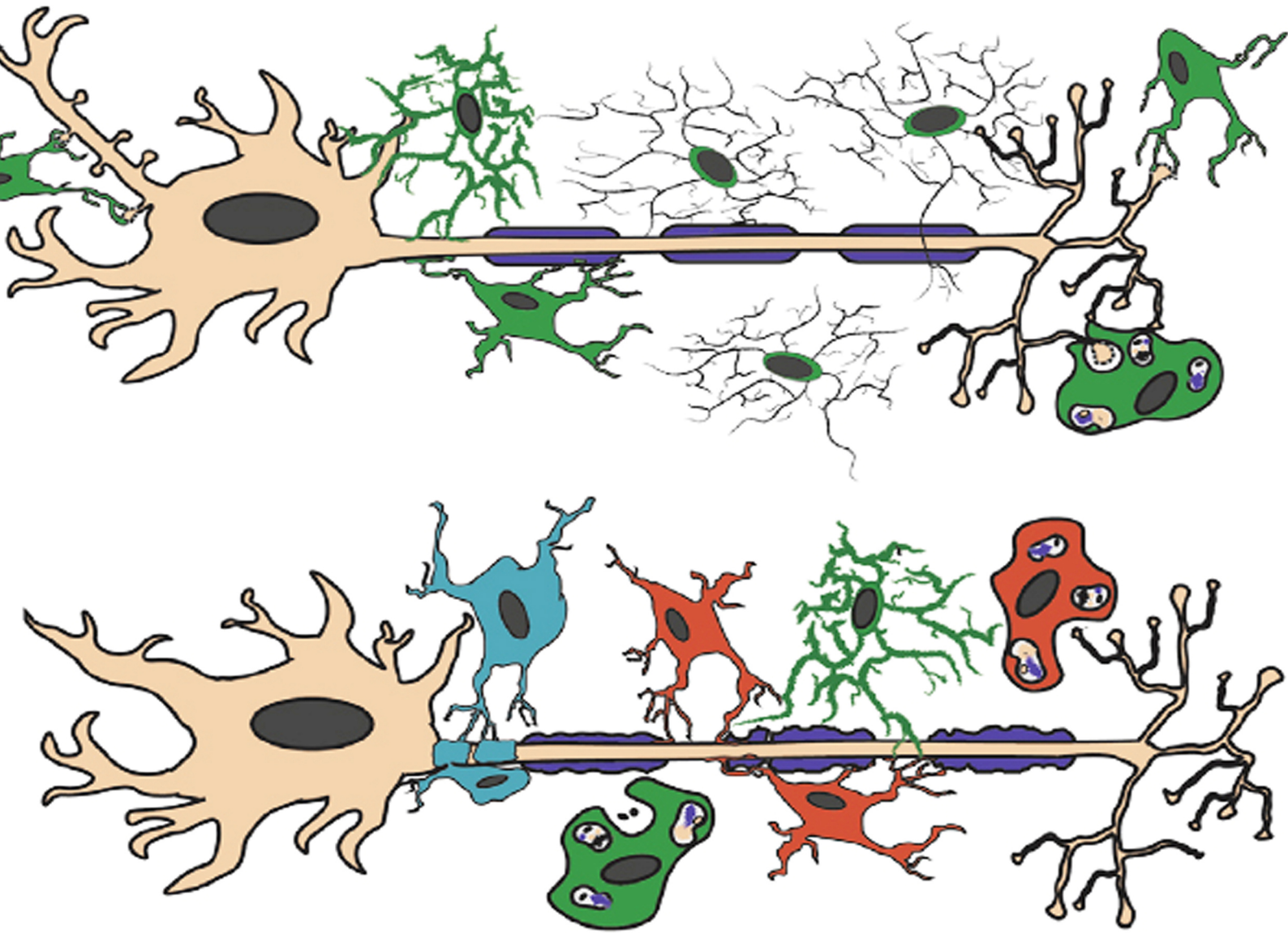

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Neuroimmunology and Neuroinflammation

Review

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Mesenchymal stromal cells as a choice for spinal cord injury treatment

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Abstract

Spinal cord injury (SCI) is a serious clinical problem that affects approximately 17,500 new patients per year in the United States. The main causes of SCI are vehicle collisions, falls, violence (mainly gunshot wounds), and sports/recreational activities. The final severity of the damage results from primary and secondary mechanisms that begin at the time of injury and last for months after trauma. To reduce the extent of damage, several treatments have been proposed. This review summarizes results from several studies that have pointed to cell therapy as the main form of neuroregenerative treatment. Mesenchymal stromal cells (MSCs) are important candidates for tissue regeneration due to the release of bioactive factors, as well as antiapoptotic effects, scar inhibitors, and angiogenic effects. Studies have shown that MSCs act in various ways on injured tissue, such as immunomodulation of the inflamed environment, release of bioactive factors, restoration of axon myelin, prevention of neuronal apoptosis, and neuroregeneration. Current research using MSCs aims to prevent secondary injury, promote regeneration, and replace destroyed spinal cord tissue. This review presents information about the damage from primary and secondary events after SCI, treatments usually used, and pre-clinical and clinical results aiming at the cell therapy using MSCs as a tissue regeneration strategy.

Keywords: Tissue regeneration, immunomodulation, neuroregeneration

SPINAL CORD INJURY

Spinal cord injury (SCI) is a very serious health problem, and available treatments are not capable of spinal cord regeneration[1]. SCI can lead to permanent neurological deficits, including motor and sensory disabilities, with high rates of physical disability and mortality. It can lead to serious damage to the physical and mental health of patients, which can cause serious socioeconomic issues[2,3]. According to the National

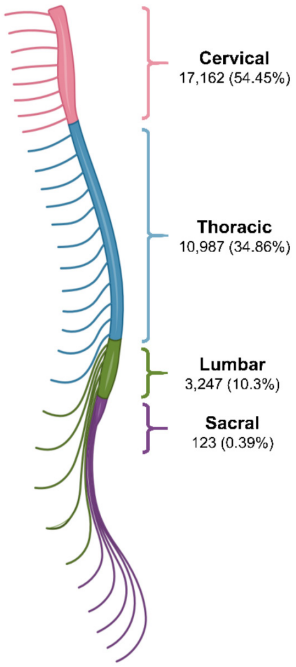

Figure 1. Number of cases of spinal cord injury according to trauma level. According to the National Spinal Cord Injury Statistical Center in the United States. The first region of the spine, the cervical region, is the most affected, accounting for more than half (54.45%) of the total number of cases, followed by the thoracic (34.86%) and lumbar (10.3%) regions. The sacral region is the least injured, accounting for 0.39% of cases

Spinal Cord Injury Statistical Center, in the United States, there are approximately 17,500 new cases per year, of which 81% are male. The average age of new cases has changed since the 1970s, from 29 to 43 years old. The main causes are vehicle collisions, falls, violence (mainly gunshot wounds), and sports or recreational activities[4]. In 2018, a survey conducted by the same institution about the frequency of SCI cases according to the level of the spinal cord showed that, of the total of 31,519 cases, 17,162 (54.45%) are lesions in the cervical region, 10,987 (34.86%) in the thoracic region, 3247 (10.3%) in the lumbar region, and 123 (0.39%) in the sacral region[4] [Figure 1].

According to the National Spinal Cord Injury Statistical Center, in the United States, the first region of the spine, the cervical region, is the most affected, accounting for more than half (54.45%) of the total number of cases, followed by the thoracic (34.86%) and lumbar (10.3%) regions, while the sacral region is the least injured, accounting for 0.39% of cases.

SCI results in disruption of the connection between the central nervous system and the rest of the body. Trauma, disease, and even spinal cord degeneration can compromise the sensory, motor, autonomic, and reflex functions of affected individuals, and only 0.4% of cases show complete recovery from their deteriorated functions[5]. The pathology of SCI results from two stages: (1) primary injury, which triggers damage to the spinal cord; and (2) secondary injury, characterized by events arising after the initial injury. Primary injury is usually the determining factor of the severity of the damage and the effects vary according to the affected site, which may be the cervical, thoracic, thoracolumbar, or sacral lumbar region[6].

After trauma, secondary events such as ischemia, anoxia, and inflammation further compromise the injured tissue. There is the migration of inflammatory cells to the lesion site, which release inflammatory cytokines; formation of reactive oxygen species (ROS), which lead to DNA damage and protein oxidation; and mitochondrial malfunction due to ionic imbalance[6,7].

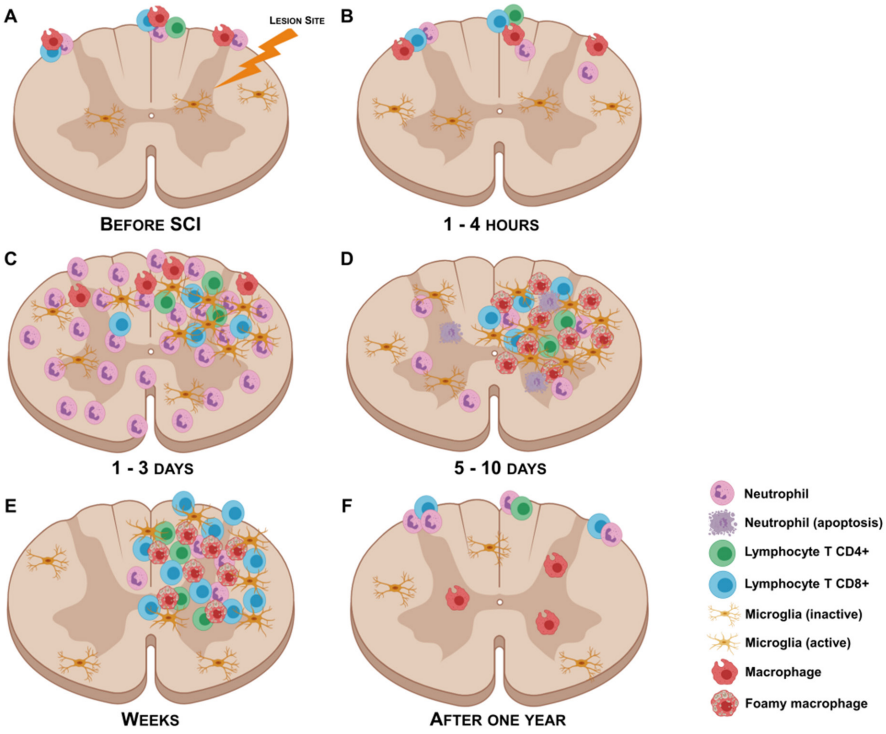

Figure 2. Immune cell migration in response to spinal cord injury (humans) (A-C): neutrophils migrate from vessels and perivascular region immediately after trauma, while lymphocytes, macrophages, and microglia migrate later. (D-F): the production of proinflammatory factors in response to the injured tissue results in tissue deterioration and damage spreading (secondary injury), which may compromise and determine the patient's grade of spinal cord injury recovery

Prior to the occurrence of SCI [Figure 2A], inflammatory cells, except for microglia, are found in the blood vessels and perivascular regions of the spinal cord. The microglia are distributed by gray and white matter. Mechanical damage to the injury (or trauma) results in immediate neuronal and glial death at the injury site. After the injury, an inflammatory process mediated by neutrophils, macrophages, lymphocytes, and microglia present in the vascular and medullary region develops. This secondary process leads to late deterioration of the spinal cord, resulting in worsening of the lesion condition. Immediately after injury, there is immediate neutrophil extravasation [Figure 2B] to the medulla, followed by late migration [Figure 2C] of lymphocytes and macrophages to the lesion site[8,9]. The microglia are activated [Figure 2C] and shorten and thicken their branches and migrate to the site of injury. Inactivated microglia remain in uninjured regions. During this period, there is production and release of proinflammatory factors (mainly activated microglia and macrophages), such as TNF-α and IL-6β, as well as proteases and lysosomal enzymes. The inflammatory environment promotes the spread of damage, inducing cell death and preventing any spontaneous spinal cord regeneration[10,11]. Within 5-10 days [Figure 2D], neutrophils enter apoptosis, while macrophages and microglia proliferate in the lesion region. After a few weeks [Figure 2E], the number of CD8+, CD4+, and T lymphocytes increases in the vessels of the injured region and the macrophage/microglial population remains in large numbers. The few remaining neutrophils accumulate in the necrotic region. One year after the injury [Figure 2F], neutrophils and lymphocytes are found in the intravascular region. The microglia remain in the region of white matter in their inactivated form, while macrophages are found in the gray matter[8-11] [Figure 2].

Secondary events mainly lead to neuron necrosis and apoptosis, which occur in the first hours after trauma[6,7]. At the same time, the body tries to prevent the injury from becoming more serious. In this

sense, repair cells act and try to reverse the damage caused, expressing factors responsible for the formation of new vessels, eliminating cell debris, and remodeling damaged neurons[5].

Treatment of the injury is limited by the low regenerative potential of the central nervous system, but spinal cord plasticity may support the recovery of some lost mechanisms after the injury. Spinal cord plasticity is related to factors such as synaptic reorganization, axonal sprouting, and neurogenesis[12]. There is little evidence of spontaneous axon regeneration after SCI but there is evidence for axonal sprouting as synaptic compensation. Regeneration is the growth of new axons, while sprouting involves the growth of collateral branches of the fibers. Due to the formation of a glial scar, which is a physical and chemical barrier to axonal regeneration, axonal sprouting is an alternative found because it can occur around a glial scar. To support SCI repair, studies have shown that functional exercise, neurotrophic factors, and cell therapy can effectively improve spinal cord neural plasticity response[12,13].

TREATMENTS

After SCI, mammals are unable to regenerate nervous tissue, which can lead to lifelong disability[14]. Some treatments may be used after SCI to try to reduce side effects and protect injured nerve tissue. Decompression surgery is one of the treatments used to relieve pressure, reducing hypoxia and ischemia caused by edema and hemorrhage[15,16]. Studies have shown that patients who underwent decompression surgery before 24 h after SCI showed an improvement compared to patients who underwent surgery more than 24 h after SCI[16-18]. Fehlings *et al.*[17] showed that more than half of the patients who underwent surgery (before or after 24 h) had at least one grade of improvement on the American Spinal Injury Association Impairment Scale (AIS) without statistical difference between the groups. However, a higher percentage of patients had two or three grade improvement on the AIS scale in the group who underwent surgery before 24 h after 6 months of follow-up. Sewell *et al.*[18] observed that patients with spinal cord injury (cervical level) who underwent surgery before and after 24 h showed no neurological improvement on the AIS scale with significant difference after 6 months of follow-up. However, there is a tendency for improvement in patients with early surgery, particularly in patients experiencing > 2-grade AIS improvement.

Another commonly used treatment after SCI is the intravenous application of methylprednisolone sodium succinate (MPSS). The central MPSS effect on SCI is the inhibition of posttraumatic lipid peroxidation occurring in neurons and blood vessels, directly compromising the function and integrity of neuronal and axonal membranes, causing microvascular damage and secondary ischemia that indirectly contribute to secondary neuronal injury. In addition to inhibiting lipid peroxidation, MPSS inhibits post-traumatic spinal cord ischemia, supports aerobic energy metabolism, and attenuates the neurofilaments loss[19,20]. However, the use of MPSS is not a consensus among professionals, because, even with improvement when applied up to eight hours after injury, this drug can cause gastrointestinal bleeding and infection[16,21]. Due to these associated complications, MPSS should be used with caution.

Neuroprotective agents are also a treatment option for spinal cord injury. These agents aim to prevent neuronal cell death by reducing side events that result in cell dysfunction and death[16,22]. Many of these neuroprotective agents have been studied, but without positive results for thoracic spinal cord injury patients[23,24]. Riluzole, a sodium channel blocker, and hypothermia, which decreases central nervous system metabolism, have been shown to be effective neuroprotective agents for the treatment of spinal cord injury[16,25-27]. Mu *et al.*[28] associated riluzole and MPSS in rats with spinal cord injury. The combined treatment preserved the tissue at the epicenter of the lesion but did not have a clear effect on the myelination index. The results of this study clearly demonstrate the potential beneficial effects of a combined approach in treating spinal cord injury.

Electroacupuncture/electrostimulation is another treatment that has long been used in spinal cord injury therapy and has been shown to inhibit inflammation, promote the secretion of neurotrophic factors, and reduce secondary injuries[29,30]. Chen *et al.*[31] performed electroacupuncture on rats with spinal cord injury and found that this treatment is effective to prevent oligodendrocyte apoptosis and to improve functional recovery after spinal cord injury. Krueger *et al.*[32] performed the association of electrostimulation with mesenchymal stromal cells derived from adipose tissue in dogs with SCI and observed improvement, but without statistical difference between the associated treatments (electrostimulation and MSCs) and isolated.

There are many studies developing different techniques to assist the recovery of spinal cord injury patients. These studies aim to combat the primary or secondary events of the injury, aiming at patient improvement, but without regenerating the nervous tissue. Cell-based therapy is the only promising treatment aimed at regeneration. Many cell types from different sources and infusion pathways have been studied or are being evaluated in ongoing studies.

STROMAL CELLS THERAPY

Cell therapy brought the promise of regenerating tissue after SCI, although the mechanism by which this type of cell therapy achieves neurological recovery have not yet been fully explained. Adult stem cells, such as MSCs, are stromal cells with potential self-renovation, multiple lineage differentiation, and immunomodulatory potential[33]. MSCs are major candidates for tissue regeneration due to release of bioactive factors, as well as anti-apoptotic, scar inhibitor, and angiogenic effects[34]. These cells also have the potential for differentiation into various adult cell types, including neurons[35,36]. The main source of MSCs is bone marrow, but other sources such as adipose tissue and umbilical cord, which are easily collected tissues, are also being used in preclinical and clinical studies. Following MSC transplantation, several repair processes occur, including: (1) the release of neurotrophic factors that may prevent nerve degeneration and apoptosis, as well as support neurogenesis, axonal growth, remyelination, and cellular metabolism; (2) reduction of neuroinflammation because MSCs can secrete a variety of soluble molecules, such as anti-inflammatory cytokines; (3) induction of angiogenesis, an important process by which new vasculature sprouts from pre-existing blood vessels; and (4) activation of endogenous spinal cord mechanisms capable of restoring some previously lost neurological functions[37-39] [Figure 3].

Although the precise mechanism by which MSCs transplantation promotes functional recovery after SCI is still unclear, it is widely accepted that most benefits of MSCs transplanted rely on the secretion of different factors and biomolecules[40]. MSCs release cytokines that may be neuroprotective and neuroregenerative. Some cytokines, e.g., neurotrophic factor, monocyte chemoattractant protein-1, and granulocyte-macrophage colony stimulating factor, play a role in neuroprotection; induce monocyte recruitment during inflammation, enhancing myelin debris clearance in central nervous system injuries; and inhibit apoptosis of neuronal cells and gliosis after SCI[41]. Other neurotrophic factors expressed by bone marrow derived mesenchymal stromal cell (BM-MSC) such as brain derived neurotrophic factor, glial-derived neurotrophic factor, and nerve growth factor can assist nervous tissue neuroregeneration including the formation of new synapses and myelination and promote axonal regeneration and functional recovery after SCI[42,43].

MSCs also reduce inflammation, which is a secondary event after trauma. These cells change the inflammatory profile to the anti-inflammatory one, which could have a beneficial effect on functional recovery after SCI[42]. Transplantation of MSCs also reduces the expression of glial scar marker (GFAP), a characteristic compatible with a resolutive inflammatory reaction[42], and increases the expression of Treg-gene[44].

Among the molecules secreted by MSCs, pro-angiogenic factors such as vascular endothelial growth factor (VEGF) are essential for repair of damaged tissue. VEGF/PDGF (platelet-derived growth factor) stimulated

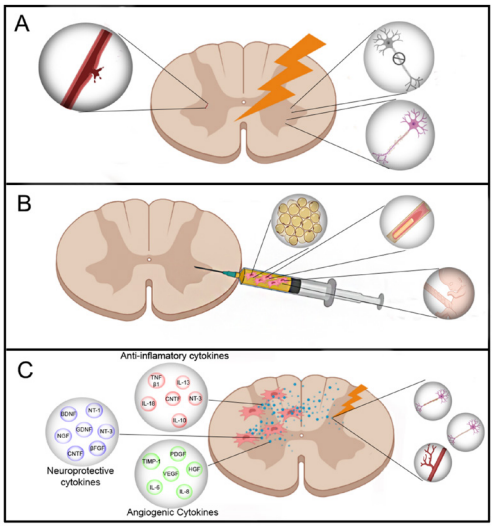

Figure 3. A: following injury, trauma and ruptured blood vessels result in ischemia, anoxia, and inflammation. This environment leads to neuronal death and degeneration; B: the infusion of MSCs can be done in different locations. There is still disagreement regarding the number of cells and infusions, but MSCs from different sources can be used for treatment (umbilical cord, adipose tissue, and bone marrow); C: after infusion, MSCs change the injured environment by releasing anti-inflammatory (TNF-β1, IL-13, IL-18, CNTF, NT-3, and IL-10), neuroprotective (BDNF, GDNF, NGF, NT-1, NT-3, CNTF, and bFGF), and angiogenic cytokines (TIMP-1, VEGF, HGF, PDGF, IL-6, and IL-8). Cell survival, remyelination, and vascular repair can also be observed. MSCs: mesenchymal stromal cells; TNF-β1: transforming growth factor β1; IL-13: interleukin 13; IL-18: interleukin 18; CNTF: ciliary neurotrophic factor; IL-10: interleukin 10; BDNF: brain-derived neurotrophic factor; GDNF: glial cell-derived neurotrophic factor; NGF: nerve growth factor; NT-1: neurotrophin 1; NT-3: neurotrophin 3; bFGF: basic fibroblast growth factor; TIMP-1: tissue inhibitor of metalloproteinase-1; VEGF: vascular endothelial growth factor; HGF: hepatocyte growth factor; PDGF: platelet-derived growth factor; IL-6: interleukin 6; IL-8: interleukin 8

angiogenesis results in a higher blood vessel density at the injured site, lesion size reduction, and white matter sparing with functional outcome after SCI[45].

Although most studies showed evidence that MSCs most likely act through their secretions (paracrine effect)[46-48]and not via their own integration/differentiation within the host tissue, some authors have reported the potential for MSCs transdifferentiation in cells of the nervous system and have shown that, after infusion into the spinal cord, these cells possibly promote regeneration of neurons because they have neuronal markers[49-52]. *In vitro* studies have shown that BM-MSC possess an intrinsic capacity to differentiate into neural-like and glial-like cells and express nestin, βIII-tubulin, neurofilaments, neuron-specific enolase, and glial fibrillary acidic protein (GFAP)[53-55].

A better understanding of the mechanisms underlying the regenerative effects of stromal/progenitor cells in the nervous system is essential for development of future cell-based therapies to treat SCI in humans.

Despite a lot of effort in recent years to develop new therapies using stromal cells to treat central nervous system trauma, there is no consensus on the cell type, source, number of cells, infusion pathways, and number of infusions suitable for achieving this goal[56].

Adult stromal cells have been used in preclinical research and clinical studies. These studies demonstrate how research uses different strategies for treating spinal cord injury using different sources of MSCs, multiple cell infusion pathways, and various models of SCI. Various types of SCI can be treated with cell therapy using MSCs, including even in patients with complete SCI[57-59]. MSCs can be transplanted intrathecally, intramedullary, intravenously, or intraarterially with different MSC sources (bone marrow, adipose tissue, umbilical cord blood, skin, and dental tissues)[5] [Tables 1 and 2].

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Table 1. Preclinical study of spinal cord injury using stromal cells

Study	SCI animal model	MSCs source		SCI site	MSCs infusion site	Number of transplanted MSCs	Infusion time	Results
Chen *et al.*[50] 2015	Wistar rat	BM-MSCs	Wistar Rats	T12	Tail vein and local transplantation	Tail vein: 1×10^6 (2 infusion) Local: 4×10^5 (1 infusion)	Acute (MSCs infusion after injury)	BMSC transplantation into the area of spinal cord injury can promote repair and regeneration of the SCI
Karaoz *et al.*[1] 2012	Wistar albino rats	BM-MSCs	Wistar Rats	T10-T11	Into the injured spinal cord	3×10^5	Acute (MSCs infusion after injury)	Cell transplantation into the contused spinal cord enhances the extent of myelination in the spared white matter and improved locomotor recovery
Quertainmont *et al.*[42] 2012	Wistar rats	BM-MSCs	Wistar Rats	T10	Caudal vein	1×10^6	7 days after injury	There has been an improvement in behavioral testing in mice transplanted with MSCs
Menezes *et al.*[36] 2014	Sprague-Dawley rats	ADSCs	Human fragments of subcutaneous adipose tissue and lipoaspirates	T8-T9	Cells were injected once, 1 cm rostrally to the lesion epicenter	Data not available	Acute (MSCs infusion after injury)	ADSCs are efficient in promoting regeneration after SCI and suggest laminin as a mediator of the beneficial effects of these cells
Chung *et al.*[60] 2016	Sprague-Dawley rats	UCB-MSCs	Human	T9	Injury site (in three spinal cord segments)	2×10^5	3 days after SCI	The therapeutic potency of transplanted UCB-MSCs occurs by increasing the levels of BDNF, NGF and NT-3 in the SCI
Melo *et al.*[53] 2017	Wistar rats	SD-MSCs	Human dermis of scalp tissue samples	T11	Intrathecal injection	10^4	One hour after SCI	Transplanted MSCs reduced the severity of tissue loss and improved functional recovery through the attenuation of immune responses and promotion of neuronal protection in the acute phase of SCI
Yang *et al.*[61] 2017	Sprague-Dawley rats	Dental stem cells	Human dental follicle, apical papilla, dental pulp	T10	Injury site (rostral and the caudal stumps) and a cell pellet was grafted into the transected gap lesion	2.5×10^5	Acute (MSCs infusion after injury)	Dental stem cells presented remarkable tissue regenerative capability after spinal cord injury through immunomodulation, differentiation, and protection capacities

BM-MSCs: bone marrow derived mesenchymal stromal cells; ADSCs: adipose-derived mesenchymal stromal cells; UCB-MSCs: umbilical cord blood-derived mesenchymal stromal cells; SD-MSCs: skin-derived mesenchymal stromal cells; SCI: spinal cord injury; BDNF: brain-derived neurotrophic factor; NGF: nerve growth factor; NT-3: neurotrofin-3; BMSC: bone marrow mesenchymal stem cell

As the results of preclinical trials have shown that MSCs are effective in the treatment of SCI, clinical studies have been conducted showing the safety and efficacy of MSC therapy. There are currently seven trials enrolled in the clinical trials platform that are recruiting patients for MSC therapy[62].

The results from both preclinical and clinical trials show that MSC transplantation seems to help mainly in sensory recovery. Studies demonstrated a significant enhancement in bladder compliance, bladder sensation, and bowel function, which may be an early indication of future improvements in urinary

Table 2. Clinical trials of spinal cord injury using stromal cells

Study	MSCs source	Injury type	SCI site	MSCs infusion site	SCI time	Results
Cristante *et al.*[63] 2009	BMSC	Complete	Cervical or thoracic	Peripheral bloodstream	Chronic	There was a positive response in 66.7% of patients for SSEP, regardless of whether the patient had paraplegia and quadriplegia
Frolov and Bryukhovetskiy[57] 2012	PHSC	Complete e incomplete	Cervical (C4-C8)	Intrathecal	Chronic	SEP and MEP improved in patients treated with MSCs
Yoon *et al.*[64] 2007	BMSC	Complete	Cervical or thoracic	Injury site	Acute	Neuropathic pain was observed in 20% of patients and 7.7% of control group; 20% of the treated group showed improvement from AIS A to B or C
Pal *et al.*[65] 2009	BMSC	Complete	Cervical or thoracic (C4-T10)	Lumbar puncture	Acute and Chronic	Two patients showed significant improvement for SSEP, MEP and NCV, being able to walk and sit with the aid of supports; three patients had improvements in bladder function
Sharma *et al.*[66] 2012	BMSC	-	-	Intrathecal	-	Improved muscle strength, balance, urine control, and sensation and reduced spasticity in 100% of patients
Mendonça *et al.*[67] 2014	BMSC	Complete	Thoracic or lumbar	Injury site	Chronic	Improvement in lower limb motor function was observed in eight patients; seven patients had sensation in the anal region, of whom six changed to AIS B and one to AIS C
Vaquero *et al.*[68] 2017	BMSC	Incomplete	Cervical, thoracic, or lumbar	Lumbar puncture	Chronic	There was significant motor improvement in 60% of cases; improvement in sexual function in 25% of men; 88.8% improvement in bladder function
Karamouzian *et al.*[69] 2012	BMSC	Complete	Thoracic or lumbar (T1-L1)	Lumbar puncture	Acute	ASIA A to C improved in 45.5% of treated patients, increasing motor and sensory score (patients were able to walk with support)
Kumar *et al.*[70] 2009	BMSC	Complete e incomplete	Cervical, thoracic, lumbar, or sacral	Lumbar puncture	Chronic	There was an improvement in 32.66% of the cases; ASIA A score progressed to B-D in 30.5% of patients
Shin *et al.*[71] 2015	hNSPC	Complete e incomplete	Cervical (C3-C8)	Injury site	Acute and Chronic	In the treated group, 26.32% of patients improved the AIS A score to B or C, compared to 6.67% in the control group; increase in recovery of motor levels was observed
Hur *et al.*[59] 2016	ADMSC	Complete e incomplete	Cervical, thoracic, or lumbar	Intrathecal	-	Motor ASIA score improved by 35.71%, voluntary anal contraction by 14.29%, and sensory ASIA score by 71.43% of patients
Oh *et al.*[72] 2016	BMSC	Incomplete	Cervical	Injury site	Chronic	There was motor improvement in the upper extremities of 12.5% of the cases
Vaquero *et al.*[58] 2016	BMSC	Complete	Thoracic	Injury site	Chronic	There was evolution from complete to incomplete lesion in 30% of patients; SSEP appeared in 58.3%, while MEP in 25%; voluntary contraction of muscles below the lesion was achieved in 58.3%; urinary tract functions improved in 83%

hNSPC: human neural stromal/progenitor cells; PHSC: peripheral hematopoietic stromal cells; ADMSC: adipose-derived mesenchymal stromal cells; SSEP/SEP: somatosensory evoked potentials; MEP: motor evoked potentials; NCV: nerve conduction velocity; ASIA: American Spinal Injury Association; AIS: ASIA Impairment Scale; MSCs: mesenchymal stromal cells; SCI: spinal cord injury; BMSC: bone marrow mesenchymal stem cell

function[68,69]. The motor function is shown in few patients and with no significant improvement. Studies suggest that motor improvement is associated with multiple MSC applications, which may be an important factor in therapeutic effectiveness[56,58].

CHALLENGES AND PERSPECTIVES

SCI has been extensively studied and its mechanism is already known. Many preclinical and clinical studies have already been performed using drugs associated with SCI, neurotrophic factors, and stem cells. In cell therapy, several cell types and sources have already been tested. Embryonic stem cells involve ethical issues and chromosomal instability that make them difficult to use in clinical trials. MSCs have emerged as an alternative, but with a more limited differentiation capacity. Studies have already demonstrated the effectiveness of these MSCs in SCI, but the next challenges are to identify the type of cell that has the most appropriate potential to support SCI regeneration and develop an infusion methodology that can overcome the hostile microenvironment and facilitate MSCs delivery in damaged neural tissue. Understanding how the reorganization of injured neural tissues associated with MSCs is also crucial for restoring neural function but remains largely unknown and needs further clarification. While addressing these challenges, it is still necessary to maintain the safety of patients involved in the studies, as the mechanisms of action of stem cells are not yet fully described.

CONCLUSION

SCI is a serious disease which generates disability with unknown cure. Different treatments have already been developed but none of them has tissue regeneration as a result. Mesenchymal stromal cells seem to be a promising alternative because, in addition to tissue regeneration, they can act to improve the inflamed environment through immunomodulation, release of bioactive factors, and restoration of axon myelin. Preclinical and clinical research studies will enable the definition of the best source of MSCs, cell number, route of infusion, and number of infusions that may lead to clinical improvement for SCI patients.

Animal model and human clinical studies have shown the regenerative and neuroprotective potential of MSCs from different sources. In addition, it is interesting to note the absence of adverse effects after MSCs infusion. MSCs emerge as a new alternative therapy because they are not limited by the time of injury, showing promising results in patients with acute and chronic lesions, or by the type of injury, resulting in improvements in patients with complete and incomplete SCI.

DECLARATIONS

Authors' Contributions

Designed of the work, summarized the references and wrote the manuscript: Fracaro L
Summarized the references, wrote the manuscript, prepared the figures: Zoehler B
Discussed paper writing and revised the manuscript: Rebelatto CLK

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

Not applicable.

Consent for publication

Not applicable.

REFERENCES

1. Karaoz E, Kabatas S, Duruksu G, Okcu A, Subasi C, et al. Reduction of lesion in injured rat spinal cord and partial functional recovery of motility after bone marrow derived mesenchymal stem cell transplantation. Turk Neurosurg 2012;22:207-17.
2. Yi XM, Chen Y, Tu GJ. Neuregulin-1 impacting bone marrow mesenchymal stem cell migration is conducive to functional recovery following spinal cord injury. Mol Med Rep 2019;20:41-8.
3. Jain NB, Ayers GD, Peterson EN, Harris MB, Morse L, et al. Traumatic spinal cord injury in the United States, 1993-2012. Jama 2015;313:2236-43.
4. Center NSCIS. Facts and Figures at a Glance. University of Alabama at Birmingham 2018.
5. Shende P, Subedi M. Pathophysiology, mechanisms and applications of mesenchymal stem cells for the treatment of spinal cord injury. Biomed Pharmacother 2017;91:693-706.
6. Ahuja CS, Nori S, Tetreault L, Wilson J, Kwon B, et al. Traumatic spinal cord injury-repair and regeneration. Neurosurgery 2017;80:S9-22.
7. Kjell J, Olson L. Rat models of spinal cord injury: from pathology to potential therapies. Dis Model Mech 2016;9:1125-37.
8. Fleming JC, Norenberg MD, Ramsay DA, Dekaban GA, Marcillo AE, et al. The cellular inflammatory response in human spinal cords after injury. Brain 2006;129:3249-69.
9. Beattie MS. Inflammation and apoptosis: linked therapeutic targets in spinal cord injury. Trends Mol Med 2004;10:580-3.
10. Okada S. The pathophysiological role of acute inflammation after spinal cord injury. Inflamm Regen 2016;36:20.
11. Rust R, Kaiser J. Insights into the dual role of inflammation after spinal cord injury. J Neurosci 2017;37:4658-60.
12. Liu J, Yang X, Jiang L, Wang C, Yang M. Neural plasticity after spinal cord injury. Neural Regene Res 2012;7:386-91.
13. Darian-Smith C. Synaptic plasticity, neurogenesis, and functional recovery after spinal cord injury. Neuroscientist 2009;15:149-65.
14. Qu J, Zhang H. Roles of mesenchymal stem cells in spinal cord injury. Stem Cells Int 2017;2017:5251313.
15. Ahuja CS, Martin AR, Fehlings M. Recent advances in managing a spinal cord injury secondary to trauma. F1000Res 2016;5.
16. Rouanet C, Reges D, Rocha E, Gagliardi V, Silva GS. Traumatic spinal cord injury: current concepts and treatment update. Arq Neuropsiquiatr 2017;75:387-93.
17. Fehlings MG, Vaccaro A, Wilson JR, Singh A, W Cadotte D, et al. Early versus delayed decompression for traumatic cervical spinal cord injury: results of the Surgical Timing in Acute Spinal Cord Injury Study (STASCIS). PLoS One 2012;7:e32037.
18. Sewell MD, Vachhani K, Alrawi A, Williams R. Results of early and late surgical decompression and stabilization for acute traumatic cervical spinal cord injury in patients with concomitant chest injuries. World Neurosurg 2018;118:e161-5.
19. Wilson JR, Forgione N, Fehlings MG. Emerging therapies for acute traumatic spinal cord injury. CMAJ 2013;185:485-92.
20. Hall ED, Springer JE. Neuroprotection and acute spinal cord injury: a reappraisal. NeuroRx 2004;1:80-100.
21. Bracken MB, Shepard MJ, Holford TR, Leo-Summers L, Aldrich EF, et al. Administration of methylprednisolone for 24 or 48 hours or tirilazad mesylate for 48 hours in the treatment of acute spinal cord injury. Results of the Third National Acute Spinal Cord Injury Randomized Controlled Trial. National Acute Spinal Cord Injury Study. JAMA 1997;277:1597-604.
22. Schapira AHV. Chapter 18 - Neuroprotection in Parkinson's Disease. In: Schapira AHV, Samuels MA, editors. Blue Books of Neurology: Butterworth-Heinemann; 2010. pp. 301-20.
23. Casha S, Zygun D, McGowan MD, Bains I, Yong VW, et al. Results of a phase II placebo-controlled randomized trial of minocycline in acute spinal cord injury. Brain 2012;135:1224-36.
24. Ulndreaj A, Badner A, Fehlings MG. Promising neuroprotective strategies for traumatic spinal cord injury with a focus on the differential effects among anatomical levels of injury. F1000Res 2017;6:1907.
25. Wang J, Pearse DD. Therapeutic hypothermia in spinal cord injury: the status of its use and open questions. Int J Mol Sci 2015;16:16848-79.
26. Levi AD, Green BA, Wang MY, Dietrich WD, Brindle T, et al. Clinical application of modest hypothermia after spinal cord injury. J Neurotrauma 2009;26:407-15.
27. Grossman RG, Fehlings MG, Frankowski RF, Burau KD, Chow DS, et al. A prospective, multicenter, phase I matched-comparison group trial of safety, pharmacokinetics, and preliminary efficacy of riluzole in patients with traumatic spinal cord injury. J Neurotrauma 2014;31:239-55.
28. Mu X, Azbill RD, Springer JE. Riluzole and methylprednisolone combined treatment improves functional recovery in traumatic spinal cord injury. J Neurotrauma 2000;17:773-80.
29. Chen XM, Xu J, Song JG, Zheng BJ, Wang XR. Electroacupuncture inhibits excessive interferon-gamma evoked up-regulation of P2X4 receptor in spinal microglia in a CCI rat model for neuropathic pain. Br J Anaesth 2015;114:150-7.
30. Zhang YT, Jin H, Wang JH, Wen LY, Yang Y, et al. Tail nerve electrical stimulation and electro-acupuncture can protect spinal motor neurons and alleviate muscle atrophy after spinal cord transection in rats. Neural plasticity 2017;2017:7351238.
31. Chen W, Wu Y. Electro-acupuncture (EA) mediated downregulation of microRNA-181a alleviates spinal cord neuronal apoptosisby inhibition of p38 MAPK pathway. Int J Clin Exp Med 2017;10:7806-15.
32. Krueger E, Magri LMS, Botelho AS, Bach FS, Rebellato CLK, et al. Effects of low-intensity electrical stimulation and adipose derived stem cells transplantation on the time-domain analysis-based electromyographic signals in dogs with SCI. Neurosci Lett 2019;696:38-45.
33. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J Cell Physiol 2007;213:341-7.
34. Caplan AI. Why are MSCs therapeutic? New data: new insight. J Pathol 2009;217:318-24.
35. Scuteri A, Miloso M, Foudah D, Orciani M, Cavaletti G, et al. Mesenchymal stem cells neuronal differentiation ability: a real

perspective for nervous system repair? Curr Stem Cell Res Ther 2011;6:82-92.
36. Menezes K, Nascimento MA, Goncalves JP, Cruz AS, Lopes DV, et al. Human mesenchymal cells from adipose tissue deposit laminin and promote regeneration of injured spinal cord in rats. PLoS One 2014;9:e96020.
37. Cofano F, Boido M, Monticelli M, Zenga F, Ducati A, et al. Mesenchymal stem cells for spinal cord injury: current options, limitations, and future of cell therapy. Int J Mol Sci 2019;20.
38. Paradisi M, Alviano F, Pirondi S, Lanzoni G, Fernandez M, et al. Human mesenchymal stem cells produce bioactive neurotrophic factors: source, individual variability and differentiation issues. Int J Immunopathol Pharmacol 2014;27:391-402.
39. Garbossa D, Boido M, Fontanella M, Fronda C, Ducati A, et al. Recent therapeutic strategies for spinal cord injury treatment: possible role of stem cells. Neurosurg Rev 2012;35:293-311.
40. Martín-Martín Y, Fernández-García L, Sanchez-Rebato MH, Marí-Buyé N, Rojo FJ, et al. Evaluation of neurosecretome from mesenchymal stem cells encapsulated in silk fibroin hydrogels. Sci Rep 2019;9:8801.
41. Bouhy D, Malgrange B, Multon S, Poirrier AL, Scholtes F, et al. Delayed GM-CSF treatment stimulates axonal regeneration and functional recovery in paraplegic rats via an increased BDNF expression by endogenous macrophages. FASEB J 2006;20:1239-41.
42. Quertainmont R, Cantinieaux D, Botman O, Sid S, Schoenen J, et al. Mesenchymal stem cell graft improves recovery after spinal cord injury in adult rats through neurotrophic and pro-angiogenic actions. PLoS One 2012;7:e39500.
43. Milczarek O, Jarocha D, Starowicz-Filip A, Kwiatkowski S, Badyra B, et al. Multiple autologous bone marrow-derived CD271(+) mesenchymal stem cell transplantation overcomes drug-resistant epilepsy in children. Stem Cells Transl Med 2018;7:20-33.
44. Fu Q, Liu Y, Liu X, Zhang Q, Chen L, et al. Engrafted peripheral blood-derived mesenchymal stem cells promote locomotive recovery in adult rats after spinal cord injury. Am J Transl Res 2017;9:3950-66.
45. Sundberg LM, Herrera JJ, Narayana PA. Effect of vascular endothelial growth factor treatment in experimental traumatic spinal cord injury: in vivo longitudinal assessment. J Neurotrauma 2011;28:565-78.
46. de Almeida FM, Marques SA, Ramalho Bdos S, Massoto TB, Martinez AM. Chronic spinal cord lesions respond positively to tranplants of mesenchymal stem cells. Restor Neurol Neurosci 2015;33:43-55.
47. Chudickova M, Vackova I, Machova Urdzikova L, Jancova P, Kekulova K, et al. The effect of wharton jelly-derived mesenchymal stromal cells and their conditioned media in the treatment of a rat spinal cord injury. Int J Mol Sci 2019;20.
48. Hakim R, Covacu R, Zachariadis V, Frostell A, Sankavaram SR, et al. Mesenchymal stem cells transplanted into spinal cord injury adopt immune cell-like characteristics. Stem Cell Res Ther 2019;10:115.
49. Lee KH, Suh-Kim H, Choi JS, Jeun SS, Kim EJ, et al. Human mesenchymal stem cell transplantation promotes functional recovery following acute spinal cord injury in rats. Acta Neurobiol Exp (Wars) 2007;67:13-22.
50. Chen YB, Jia QZ, Li DJ, Sun JH, Xi S, et al. Spinal cord injury in rats treated using bone marrow mesenchymal stem-cell transplantation. Int J Clin Exp Med 2015;8:9348-54.
51. Cui B, Li E, Yang B, Wang B. Human umbilical cord blood-derived mesenchymal stem cell transplantation for the treatment of spinal cord injury. Exp Ther Med 2014;7:1233-6.
52. Gao S, Guo X, Zhao S, Jin Y, Zhou F, et al. Differentiation of human adipose-derived stem cells into neuron/motoneuron-like cells for cell replacement therapy of spinal cord injury. Cell Death Dis 2019;10:597.
53. Melo FR, Bressan RB, Forner S, Martini AC, Rode M, et al. Transplantation of human skin-derived mesenchymal stromal cells improves locomotor recovery after spinal cord injury in rats. Cell Mol Neurobiol 2017;37:941-7.
54. Ma K, Fox L, Shi G, Shen J, Liu Q, et al. Generation of neural stem cell-like cells from bone marrow-derived human mesenchymal stem cells. Neurol Res 2011;33:1083-93.
55. Blecker D, Elashry MI, Heimann M, Wenisch S, Arnhold S. New insights into the neural differentiation potential of canine adipose tissue-derived mesenchymal stem cells. Anat Histol Embryol 2017;46:304-15.
56. Thuret S, Moon LDF, Gage FH. Therapeutic interventions after spinal cord injury. Nat Rev Neurosci 2006;7:628.
57. Frolov AA, Bryukhovetskiy AS. Effects of hematopoietic autologous stem cell transplantation to the chronically injured human spinal cord evaluated by motor and somatosensory evoked potentials methods. Cell Transplant 2012;21:49-55.
58. Vaquero J, Zurita M, Rico MA, Bonilla C, Aguayo C, et al. An approach to personalized cell therapy in chronic complete paraplegia: The Puerta de Hierro phase I/II clinical trial. Cytotherapy 2016;18:1025-36.
59. Hur JW, Cho TH, Park DH, Lee JB, Park JY, et al. Intrathecal transplantation of autologous adipose-derived mesenchymal stem cells for treating spinal cord injury: a human trial. J Spinal Cord Med 2016;39:655-64.
60. Chung HJ, Chung WH, Lee JH, Chung DJ, Yang WJ, et al. Expression of neurotrophic factors in injured spinal cord after transplantation of human-umbilical cord blood stem cells in rats. J Vet Sci 2016;17:97-102.
61. Yang C, Li X, Sun L, Guo W, Tian W. Potential of human dental stem cells in repairing the complete transection of rat spinal cord. J Neural Eng 2017;14:026005.
62. ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US), National Institutes of Health; 2000 - [cited 2019 Aug 25]. Available from https://clinicaltrials.gov/ct2/home [Last accessed on 20 Jan 2020]
63. Cristante AF, Barros-Filho TE, Tatsui N, Mendrone A, Caldas JG, et al. Stem cells in the treatment of chronic spinal cord injury: evaluation of somatosensitive evoked potentials in 39 patients. Spinal Cord 2009;47:733-8.
64. Yoon SH, Shim YS, Park YH, Chung JK, Nam JH, et al. Complete spinal cord injury treatment using autologous bone marrow cell transplantation and bone marrow stimulation with granulocyte macrophage-colony stimulating factor: phase I/II clinical trial. Stem Cells 2007;25:2066-73.
65. Pal R, Venkataramana NK, Bansal A, Balaraju S, Jan M, et al. Ex vivo-expanded autologous bone marrow-derived mesenchymal stromal cells in human spinal cord injury/paraplegia: a pilot clinical study. Cytotherapy 2009;11:897-911.
66. Sharma A, Gokulchandran N, Chopra G, Kulkarni P, Lohia M, et al. Administration of autologous bone marrow-derived mononuclear cells in children with incurable neurological disorders and injury is safe and improves their quality of life. Cell Transplant 2012;21

Suppl 1:S79-90.
67. Mendonça MVP, Larocca TF, de Freitas Souza BS, Villarreal CF, Silva LFM, et al. Safety and neurological assessments after autologous transplantation of bone marrow mesenchymal stem cells in subjects with chronic spinal cord injury. Stem Cell Res Ther 2014;5:126.
68. Vaquero J, Zurita M, Rico MA, Bonilla C, Aguayo C, et al. Repeated subarachnoid administrations of autologous mesenchymal stromal cells supported in autologous plasma improve quality of life in patients suffering incomplete spinal cord injury. Cytotherapy 2017;19:349-59.
69. Karamouzian S, Nematollahi-Mahani SN, Nakhaee N, Eskandary H. Clinical safety and primary efficacy of bone marrow mesenchymal cell transplantation in subacute spinal cord injured patients. Clin Neurol Neurosurg 2012;114:935-9.
70. Kumar AA, Kumar SR, Narayanan R, Arul K, Baskaran M. Autologous bone marrow derived mononuclear cell therapy for spinal cord injury: a phase I/II clinical safety and primary efficacy data. Exp Clin Transplant 2009;7:241-8.
71. Shin JC, Kim KN, Yoo J, Kim IS, Yun S, et al. Clinical trial of human fetal brain-derived neural stem/progenitor cell transplantation in patients with traumatic cervical spinal cord injury. Neural Plast 2015;2015:630932.
72. Oh SK, Choi KH, Yoo JY, Kim DY, Kim SJ, et al. A phase III clinical trial showing limited efficacy of autologous mesenchymal stem cell therapy for spinal cord injury. Neurosurgery 2016;78:436-47.

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Review

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Physiological sex differences in microglia and their relevance in neurological disorders

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Abstract

Microglia are the resident immune cells in the brain and maintain homeostasis and functionality of this tissue. These cells are key producers of immune mediators, such as cytokines and chemokines, are critical for normal brain development, and affect neurogenesis, axonal migration, synapse formation and function, and programmed cell death, among others. Sex differences exist in many of these processes throughout brain development up to adulthood and the aged brain. In the last few years, sex differences in microglia responses, brain colonization, and number and morphology within the developing brain have drawn the attention of researchers as a potential explanation to the sex differences in the brain and due to their potential relevance in the incidence, prevalence, and outcome of many neurological disorders. In this review, we summarize the sex differences of microglial cell functions and their potential relevance in physiological as well as pathological conditions in the brain.

Keywords: Microglia, sex differences, functional responses, neurological disorders

INTRODUCTION

There is a differential sex-susceptibility, penetrance, and outcome in neurological disorders. An important inflammatory component goes along with these disorders, and microglia, the immune resident cells of the Central Nervous System (CNS), play a pivotal role in the triggering and resolution of neuroinflammatory processes[1]. As the main regulators of immune responses in the CNS, they have come into focus recently due to their potential contribution to the sex differences found in neurological disorders. In this review,

we aim to summarize the sex differences in functional responses of microglia described thus far and their relevance in pathology.

MICROGLIA

Microglia represent 10%-15% of the cells in the brain[2]. In recent years, microglia functions have extensively widened, ranging from mere local immune defense of the CNS to being key players in brain development and physiology. They have been shown to regulate dynamic surveillance of the environment through their active processes, maintaining homeostasis and modulating neuroinflammation[3-6]. They also mediate phagocytosis and clearance of debris and apoptotic cells in disease and neurogenic niches[5,7-10], shape brain development through synapse pruning, and allow brain wiring of neuronal circuits[11-13].

Unlike most cell types in the CNS, microglia proceed from myeloid precursors that migrate from the yolk sack to the CNS in early embryological stages [Embryonic Day 8.5 (E8.5)][2,14], before the closure of the blood-brain barrier, which occurs around E13-E14.5 in mice[14,15]. Interestingly, microglial brain colonization during embryonic development is highly conserved across vertebrate species[16-19].

Microglial lineage differs from that of macrophages, as it is driven by the cytokine macrophage colony stimulating factor (M-CSF), as well as the transcription factors Pu.1, Irf8, and Sall1[14,20,21]. Microglia are self-renewed from local proliferation of CNS resident cells, and the turnover is relatively low in both humans and rodents. This suggests that these cells are likely to be primed, or even have a memory, due to the different events they are exposed to through their lifespan[22-24]. This microglia priming will be determinant in their responses, both in physiological conditions and in disease[25].

Microglia represent most of the fetal glial population, especially in early developmental stages[26]. Microglia roles at this time are likely to be sex-specific as sex differences may arise as early as hematopoiesis in the embryonic yolk sac or when CNS colonization occurs during early embryonic development[26-28]. Moreover, male and female microglia follow temporarily different trajectories during development[29,30]. Microglia influence sexual differentiation; indeed, masculinization of the brain is dependent on the activation stage of these cells[28,31-34].

Fetal gonads develop early in development, and in males are fully active (except for spermatogenesis) by mid to late gestation, showing a surge in fetal testis androgen production beginning the last few days of gestation and enduring until shortly after birth in rodents. In primates, androgen production occurs from the end of the first trimester and well into the second with another peak at birth[35,36]. Once in the brain, testosterone (T) can be either aromatized to estradiol (E2) or 5-α reduced to dihyrdotestosterone (DHT). Both T and DHT induce some masculine endpoints but it is E2 that is the dominant masculinizing hormone in the rodent brain, through a prostaglandin E2 (PGE2)-mediated process[37,38]. Morphologically, in certain sex differentiated brain regions, such as the preoptic area, males have more microglia with an "activated" morphology characterized by an increase in cell body size and a decrease in process length and branching[33].

Recent studies have demonstrated that microglia density and phenotype vary between male and female rodents in several brain areas[33,39,40]. Mid adolescent changes lead to a higher blood flow in women compared to men, which is maintained throughout life until the 60s, when this difference is milder. These differences in blood flow may play a role in differential microglia density in certain brain areas[41-43]. Despite this, not much attention has been paid to the relevance of sex differences in blood flow or vasculature in differential microglia infiltration during fetal development. Subtle changes in the timing and density of microglia arrival to certain brain regions, as a result of differential blood flow, would lead to differential interaction of these cells with progenitors at different stages of microglia or neural progenitor

Table 1. Sex differences in the incidence of neurological disorders in humans

Male brain	Female brain
Autism Spectrum Disorders (4:1)	Alzheimer's Disease (3:1)
Parkinson's Disease (3:1)	Depression (2:1)
Attention Deficit Hyperactivity Disorder	Anxiety (2:1)
Attention Deficit Hyperactivity Disorder (3:1)	Multiple Sclerosis (2-3:1)
Schizophrenia (1.4:1)	
Amyotrophic Lateral Sclerosis (1.6:1)	Adult-onset neurological disorders
Early-onset neurological disorders	

Most frequent neurological disorders in humans. Global prevalence of each disorder is shown in parenthesis as the ratio of men *vs.* women (left side) or women *vs.* men (right side)

differentiation, resulting in sex specific microglia subpopulations in different brain areas. This is relevant because microglia phenotypes vary across regions of the CNS, in disease as well as in physiological conditions at different stages in life, especially in early development and aging, which are two critical life stages for the appearance of neurological disorders, in both humans and rodents[44-47].

Sex differences in neurological disorders

There is an increasing concern for the real relevance of experimental results obtained in current research. Experimental procedures are often done using only one sex, and results are often extrapolated to both sexes without solid grounds. Several funding agencies, such as the European Commission, the Canadian Institutes of Health Research, and the US National Institutes of Health, have tried to influence researchers to integrate sex/gender not only in clinical research, but also in basic and preclinical research, especially since they identified a sex bias in most clinical trials, usually done in male subjects, in which females are under-represented, leading to mistreatment of women[48,49]. In the specific case of neurological disorders, there is a well described sex bias in the prevalence, severity, progression, and outcome of these diseases [Table 1][29]. Therefore, there is a need of development, implementation, and prioritization of treatments and preventive interventions specific for sex, age, and population to reduce the burden from these disorders[50].

Many early-onset neurodevelopmental disorders show a strong sex-bias toward males[51] while adult-onset neurological disorders are female biased[52]. As microglia play an important role in both sexual differentiation of the brain and progression of most neurological disorders[27,33,53,54], it is critical to understand how the dynamics and potential dysfunction of microglia at certain developmental points affect the onset and progression of these disorders.

Women have a higher prevalence of Alzheimer's disease (AD, 1.6-3:1 ratio compared to men)[55,56], autoimmune diseases such as multiple sclerosis (MS, 2-3:1 ratio)[57], or mood related disorders such as depression or anxiety disorders (2:1)[58,59]. On the other hand, men are more prone to suffer from Parkinson's disease (PD, 3.5:1 compared to women)[60,61], motor neuron disorders such as amyotrophic lateral sclerosis (ALS, 1.6:1)[62,63], autism spectrum disorders (ASD, 4:1)[64-66], attention deficit hyperactivity disorder (3:1)[67-70], or schizophrenia (1.4:1)[71,72].

Beyond the prevalence of these disorders, women show greater cognitive decline than men with AD[73] and a slower rate of decline when suffering from PD[60,61]. In this line, women show increased severity of depression or anxiety disorder symptoms, and men show earlier onset of schizophrenia and more severe symptoms along with worse response to antipsychotic drugs than women[58,59,71,74]. On the other hand, men suffering from MS have a faster progression of the disease than women[57,75], and women suffering from ALS have worse survival rates than men[62,63].

Microglia and sexual differentiation of the brain

Sexual differentiation of the brain is orchestrated by sex chromosomes, gonadal hormones, and early postnatal environment. X chromosome contains the largest number of immune-related genes in the human genome, including Toll-like receptor pathways (*BTK, IRAK1*, and *IKKγ*) and microRNAs involved in immune regulation[76-78].

X chromosome inactivation to match gene expression levels between males and females is not random, as previously thought. Indeed, it is the paternal X chromosome that is consistently inactivated in neonatal brains[79]. Fifteen percent of the genes in the X chromosome, particularly immune-related genes such as toll-like receptor 7 (*Tlr7*), escape inactivation in females[80,81]. TLR7 is implicated in miRNA-mediated increased TNFα release, and different expression of *Tlr7* in females may contribute to intrinsic differences in immune response[82]. Therefore, male and female microglia are differentially influenced by these factors since early developmental stages[83].

Sex hormones are likely key players in microglia sexual differentiation, independently of their genetic background. Microglia physiologically express steroid hormone receptors, and are therefore sensitive to the effects of both estrogens and testosterone[84]. Indeed, hormones are necessary to establish initial sex differences in microglia. Studies by Villa *et al.*[30] showed that masculinization of female brain at E2 in mice resulted in transcriptionally male microglia in adulthood in those females. Indeed, once differentiated, microglia retain their sex-specific transcriptional profiles even after transplantation into the brain of the opposite sex in adulthood[30].

Interestingly, young adult female microglia maintain their sex differences in the absence of hormones, as their transcriptome is not drastically affected after ovariectomy[30]. However, hormone depletion in aged female mice (over 13 months old) induces profound transcriptome changes in these cells, with increased inflammatory phenotypes[85,86]. Further studies are required to determine if changes in circulating hormones during aging are responsible for these differences. It would be especially relevant to determine the relevance of this in the incidence of neurodegenerative disorders in women, as these often appear in the postmenopausal period.

In addition, early pre- and postnatal environment is key in the sexual differentiation of the brain. Development at this point involves rapid myelination of neuronal fibers and synaptogenesis, arborization, and pruning. This time of extensive growth is also a critical period where environmental factors, such as nutritional factors (folate and palmitic acid), early postnatal stress, or smoking, can influence optimal CNS development[87,88]. Indeed, some functional sex differences in early postnatal microglia are lost upon exposure to palmitic acid[89].

Basal sex differences in microglia functional responses

There are well described sex differences in microglia in the male and female brain. These differences range from cell density and morphology to different transcription profiles and functions. Transcriptomic data have shown that microglia transcriptome during brain development is characterized by temporal maturation steps that follow different trajectories in males and females: male microglia are developmentally delayed compared with female microglia, starting from E18[90,91]. Besides, the maturation process has features that resemble the pro-inflammatory activation programs typical of adult cells. This is of special relevance, as it suggests a higher sensitivity to inflammatory events in male microglia, which could lead to a faster aging of these cells and affect the risk of disorders[91] [Figure 1].

Microglia density varies significantly across different subregions in the brain in a spatiotemporal fashion. In early developmental stages, microglia density in specific brain areas such as the hippocampus is higher in

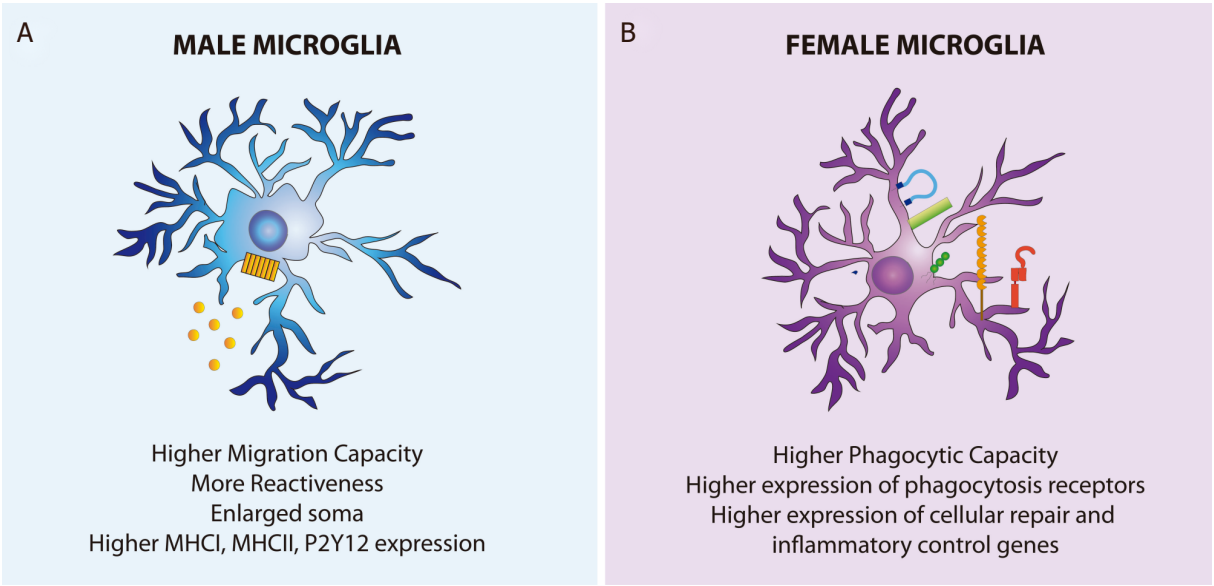

Figure 1. Physiological sex differences in male and female microglia. A: male microglia have an enlarged soma and more reactiveness in physiological conditions than female microglia. These cells have more pro-inflammatory responses, higher migration capacity, and enhanced MHCI, MHCII, and P2Y12 constitutive expression; B: female microglia, on the other hand, have a higher phagocytic capacity and higher gene expression of cell repair and inflammatory control genes

female brains, whereas it is higher in the amygdala in male brains. Other areas, such as the cortex, striatum, and cerebellum, show similar densities in both sexes at these stages. At later developmental stages (early adulthood), there is a higher microglia density in the cortex, hippocampus, and amygdala of male brains, while there are no differences in the striatum and cerebellum[39,92].

Similar to cell density, significant regional heterogeneity has also been found in microglial structural complexity. During development, the soma size of female microglia is larger in the cortex, hippocampus, and amygdala, while there were no changes in microglia size in other areas[39,92]. However, in adult stages, male microglia show enlarged somas throughout the brain[39].

Microglial phagocytic activity has been shown to be enhanced in early postnatal female microglia compared with males in both mice and rats[89,93]. On the other hand, male microglia show a higher P2Y12 receptor expression and higher motility capacity at this time[89,94]. Interestingly, male microglia also show higher MHCI and MHCII expression, as well as enhanced P2X receptor-mediated signaling, which are indicative of more reactiveness than female microglia already under physiological conditions[39] [Figure 1].

Functional sex differences in microglia may have important functional consequences for disease progression. It is likely that each sex uses different mechanisms to achieve similar baseline functional states adapted to their sex-specific environments, and therefore microglial cells would have equivalent cellular functionality regardless the sex[95].

Recent work has shown that microglia contribute to sex differences in social behavior[83] and further research will determine to what extent microglia partake in the brain sexual dimorphism. How such intrinsic differences contribute to disease susceptibility also remains to be elucidated[30,39].

Sex differences in microglia responses in disease

Microglia dysfunction is implicated in every single brain disease. Unveiling microglia functional sex differences in non-physiological states may explain differences in disease susceptibility that result from sex-

specific inefficient responses. Sexual differentiation of the brain during early development likely underlies the strong sex biases prevalent in many neurological conditions, as they acquire their sex specific identity early in development, which persists during the injury response[30]. Therefore, studying sex differences in this context could shed some light on sex-specific disease mechanisms.

Beyond the neuroprotective effect of estrogens *per se*[96,97], RNA-seq analysis revealed that female microglia express more genes involved in cellular repair and inflammatory control than male microglia, which likely contributes to a more favorable outcome in several injuries[30]. Besides, recent studies have shown that male microglia seem to be more reactive already under physiological conditions as well as have a shorter lifespan[39]. For example, female microglia show a higher mRNA expression of Shank 3, Fxyd1, Aqp1, or Timp3 and a decreased mRNA expression of Akt1s1, Trem1, S100a9, or Cxcl2, as well as decreased NF-κB activity levels, compared to male microglia[30].

Sex differences in microglia immunomodulatory response to lipopolysaccharide (LPS), a potent pro-inflammatory agent, have been studied both *in vitro* and *in vivo*, and in both conditions male microglia display a higher immune response of male microglia after LPS stimulation[90,98], which is accompanied by greater IL-1β mRNA and MHCI/II expression in male microglia, and decreased CD14 mRNA expression in female microglia[39,98].

Mouse models of forebrain or focal ischemia have shown that young adult female mice and rats sustain lesser injury than males[99-101]. Moreover, female microglia display a neuroprotective phenotype in ischemic stroke; indeed, when transplanted in male brains, they protect them from this disease[30].

Something important to keep in mind is that different subsets of microglia respond to various insults such as aging or immune challenges differently. Microglia can be classified into gene expression clusters through the lifespan of the individual. For example, on Postnatal Days 4 and 5, female microglia are enriched for the genes Cd74, chemokine (C-C motif) ligand 24 (Ccl24), and Arg1[102]. Interestingly, as the brain ages, there is a progressive expansion of clusters that typically have few very cells in adolescent and adult samples, which are enriched in inflammatory genes and are more responsive to interferon[102]. Combination of deep single-cell transcriptome analysis, fate mapping, clonal analysis, *in vivo* imaging, and transgenic mouse lines have allowed the identification of microglia subsets in different CNS compartments during neuroinflammation[102-104].

Single-cell sequencing of microglia in an Alzheimer's disease mouse model revealed a unique AD-related microglial phenotype, generated by a two-step process involving triggering receptor expressed on myeloid cells 2 (*Trem2*). Activation is initiated in a TREM2-independent manner involving downregulation of microglia checkpoints, followed by activation of a TREM2-dependent manner[104]. The relevance of sex in these unique microglial subsets such as disease-associated microglia remains to be elucidated.

Interestingly, some genes have been linked with sex-specific phenotypes in the case of AD. One such gene is *Apoe*, which codes apolipoprotein E (ApoE), a modulator of the CNS immune system that can have differential outcomes on microglial function depending on the variant[105-107]. The ε4 variant of the gene, which is expressed more strongly in females, has been linked with a higher risk of developing late-onset AD in humans[56,108,109]. Microglia are a major source of plaque-associated ApoE, which is modulated by TREM2 in AD mouse models[110].

A sex-specific differential expression of Apoe in disease associated microglia has been found in a mouse model of ALS[104,111,112]. Microglia isolated from female aged mice also have upregulation of ApoE transcripts compared to males[113]. Overall, these findings suggest that *ApoE* is a gene that could partially explain the sex differences found in AD and maybe other neurodegenerative disorders.

CONCLUSION

Hormonal and genetic environments determine microglia fate to be sex-specific. There are several sex differences in microglia physiology, distribution throughout the brain, functional responses, transcriptional profiles, and sex chromosome composition. Some of these are maintained throughout the lifespan of the individual; however, most of them are dynamic and vary over time. As microglia play a key role in every neurological disease, it is likely that the differences they present contribute to sex differences in the course and incidence of these disorders. Therefore, sex differences in microglia are a new and promising research field to explain the differences in neurological disorders in humans and potentially lead to sex-specific strategies to treat these patients.

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Availability of data and materials

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REFERENCES

1. Wolf SA, Boddeke HWGM, Kettenmann H. Microglia in physiology and disease. Annu Rev Physiol 2017;79:619-43.
2. Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. Physiology of microglia. Physiol Rev 2011;91:461-553.
3. Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, et al. ATP mediates rapid microglial response to local brain injury in vivo. Nat Neurosci 2005;8:752-8.
4. Nimmerjahn A, Kirchhoff F, Helmchen F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 2005;308:1314-8.
5. Zhao Y, Wu X, Li X, Jiang LL, Gui X, et al. TREM2 Is a Receptor for beta-amyloid that mediates microglial function. Neuron 2018;97:1023-31.e7.
6. Colonna M, Butovsky O. Microglia function in the central nervous system during health and neurodegeneration. Annu Rev Immunol 2017;35:441-68.
7. Asai H, Ikezu S, Tsunoda S, Medalla M, Luebke J, et al. Depletion of microglia and inhibition of exosome synthesis halt tau propagation. Nat Neurosci 2015;18:1584-93.
8. Bolós M, Perea JR, Terreros-Roncal J, Pallas-Bazarra N, Jurado-Arjona J, et al. Absence of microglial CX3CR1 impairs the synaptic integration of adult-born hippocampal granule neurons. Brain Behav Immun 2018;68:76-89.
9. Fourgeaud L, Través PG, Tufail Y, Leal-Bailey H, Lew ED, et al. TAM receptors regulate multiple features of microglial physiology. Nature 2016;532:240-4.
10. Sierra A, Encinas JM, Deudero JJ, Chancey JH, Enikolopov G, et al. Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. Cell Stem Cell 2010;7:483-95.
11. Kettenmann H, Kirchhoff F, Verkhratsky A. Microglia: new roles for the synaptic stripper. Neuron 2013;77:10-8.

12. Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, et al. Synaptic pruning by microglia is necessary for normal brain development. Science 2011;333:1456-8.
13. Paolicelli RC, Gross CT. Microglia in development: linking brain wiring to brain environment. Neuron Glia Biol 2011;7:77-83.
14. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, et al. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science 2010;330:841-5.
15. Hoeffel G, Ginhoux F. Ontogeny of tissue-resident macrophages. Front Immunol 2015;6:486.
16. Hanisch UK, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat Neurosci 2007;10:1387-94.
17. Herbomel P, Thisse B, Thisse C. Zebrafish early macrophages colonize cephalic mesenchyme and developing brain, retina, and epidermis through a M-CSF receptor-dependent invasive process. Dev Biol 2001;238:274-88.
18. Schlegelmilch T, Henke K, Peri F. Microglia in the developing brain: from immunity to behaviour. Curr Opin Neurobiol 2011;21:5-10.
19. Swinnen N, Smolders S, Avila A, Notelaers K, Paesen R, et al. Complex invasion pattern of the cerebral cortex bymicroglial cells during development of the mouse embryo. Glia 2013;61:150-63.
20. Buttgereit A, Lelios I, Yu X, Vrohlings M, Krakoski NR, et al. Sall1 is a transcriptional regulator defining microglia identity and function. Nat Immunol 2016;17:1397-406.
21. Kierdorf K, Erny D, Goldmann T, Sander V, Schulz C, et al. Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. Nat Neurosci 2013;16:273-80.
22. Askew K, Li K, Olmos-Alonso A, Garcia-Moreno F, Liang Y, et al. Coupled proliferation and apoptosis maintain the rapid turnover of microglia in the adult brain. Cell Rep 2017;18:391-405.
23. Réu P, Khosravi A, Bernard S, Mold JE, Salehpour M, et al. The lifespan and turnover of microglia in the human brain. Cell Rep 2017;20:779-84.
24. Tay TL, Mai D, Dautzenberg J, Fernández-Klett F, Lin G, et al. A new fate mapping system reveals context-dependent random or clonal expansion of microglia. Nat Neurosci 2017;20:793-803.
25. Perry VH, Teeling J. Microglia and macrophages of the central nervous system: the contribution of microglia priming and systemic inflammation to chronic neurodegeneration. Semin Immunopathol 2013;35:601-12.
26. Thion MS, Ginhoux F, Garel S. Microglia and early brain development: an intimate journey. Science 2018;362:185-9.
27. VanRyzin JW, Pickett LA, McCarthy MM. Microglia: driving critical periods and sexual differentiation of the brain. Dev Neurobiol 2018;78:580-92.
28. Lenz KM, McCarthy MM. A starring role for microglia in brain sex differences. Neuroscientist 2015;21:306-21.
29. Hanamsagar R, Bilbo SD. Sex differences in neurodevelopmental and neurodegenerative disorders: focus on microglial function and neuroinflammation during development. J Steroid Biochem Mol Biol 2016;160:127-33.
30. Villa A, Gelosa P, Castiglioni L, Cimino M, Rizzi N, et al. Sex-specific features of microglia from adult mice. Cell Rep 2018;23:3501-11.
31. Welberg L. Microglia maketh the male. Nat Rev Neurosci 2013;14:226.
32. Garden GA, Campbell BM. Glial biomarkers in human central nervous system disease. Glia 2016;64:1755-71.
33. Lenz KM, Nugent BM, Haliyur R, McCarthy MM. Microglia are essential to masculinization of brain and behavior. J Neurosci 2013;33:2761-72.
34. Schafer DP, Lehrman EK, Stevens B. The "quad-partite" synapse: microglia-synapse interactions in the developing and mature CNS. Glia 2013;61:24-36.
35. Forest MG, Cathiard AM, Bertrand JA. Total and unbound testosterone levels in the newborn and in normal and hypogonadal children: use of a sensitive radioimmunoassay for testosterone. J Clin Endocrinol Metab 1973;36:1132-42.
36. Reyes FI, Winter JS, Faiman C. Studies on human sexual development. I. Fetal gonadal and adrenal sex steroids. J Clin Endocrinol Metab 1973;37:74-8.
37. Wright CL, McCarthy MM. Prostaglandin E2-induced masculinization of brain and behavior requires protein kinase A, AMPA/kainate, and metabotropic glutamate receptor signaling. J Neurosci 2009;29:13274-82.
38. McCarthy MM. Estradiol and the developing brain. Physiol Rev 2008;88:91-124.
39. Guneykaya D, Ivanov A, Hernandez DP, Haage V, Wojtas B, et al. Transcriptional and translational differences of microglia from male and female brains. Cell Rep 2018;24:2773-83.e6.
40. Bilbo SD. Sex differences in microglial appetites during development: Inferences and implications. Brain Behav Immun 2017;64:9-10.
41. Aanerud J, Borghammer P, Rodell A, Jónsdottir KY, Gjedde A. Sex differences of human cortical blood flow and energy metabolism. J Cereb Blood Flow Metab 2017;37:2433-40.
42. Esposito G, Van Horn JD, Weinberger DR, Berman KF. Gender differences in cerebral blood flow as a function of cognitive state with PET. J Nucl Med 1996;37:559-64.
43. Satterthwaite TD, Shinohara RT, Wolf DH, Hopson RD, Elliott MA, et al. Impact of puberty on the evolution of cerebral perfusion during adolescence. Proc Natl Acad Sci U S A 2014;111:8643-8.
44. Böttcher C, Schlickeiser S, Sneeboer MAM, Kunkel D, Knop A, et al. Human microglia regional heterogeneity and phenotypes determined by multiplexed single-cell mass cytometry. Nat Neurosci 2019;22:78-90.
45. Grabert K, Michoel T, Karavolos MH, Clohisey S, Baillie JK, et al. Microglial brain region-dependent diversity and selective regional sensitivities to aging. Nat Neurosci 2016;19:504-16.
46. Li Q, Cheng Z, Zhou L, Darmanis S, Neff NF, et al. Developmental heterogeneity of microglia and brain myeloid cells revealed by deep single-cell rna sequencing. Neuron 2019;101:207-23.e10.
47. Masuda T, Sankowski R, Staszewski O, Böttcher C, Amann L, et al. Spatial and temporal heterogeneity of mouse and human microglia at single-cell resolution. Nature 2019;566:388-92.
48. Lee SK. Sex as an important biological variable in biomedical research. BMB Rep 2018;51:167-73.

49. GAO. Women's health-FDA needs to ensure more study of gender differences in prescription drug testing. 1992.
50. GBD 2016 Neurology Collaborators. Global, regional, and national burden of neurological disorders, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet Neurol 2019;18:459-80.
51. Thibaut F. The role of sex and gender in neuropsychiatric disorders. Dialogues Clin Neurosci 2016;18:351-2.
52. Zagni E, Simoni L, Colombo D. Sex and gender differences in central nervous system-related disorders. Neurosci J 2016;2016:2827090.
53. Kopec AM, Smith CJ, Ayre NR, Sweat SC, Bilbo SD. Microglial dopamine receptor elimination defines sex-specific nucleus accumbens development and social behavior in adolescent rats. Nat Commun 2018;9:3769.
54. McCarthy MM, Pickett LA, VanRyzin JW, Kight KE. Surprising origins of sex differences in the brain. Horm Behav 2015;76:3-10.
55. Plassman BL, Langa KM, McCammon RJ, Fisher GG, Potter GG, et al. Incidence of dementia and cognitive impairment, not dementia in the United States. Ann Neurol 2011;70:418-26.
56. Seshadri S, Wolf PA, Beiser A, Au R, McNulty K, et al. Lifetime risk of dementia and Alzheimer's disease. The impact of mortality on risk estimates in the Framingham Study. Neurology 1997;49:1498-504.
57. Voskuhl RR, Gold SM. Sex-related factors in multiple sclerosis susceptibility and progression. Nat Rev Neurol 2012;8:255-63.
58. Nolen-Hoeksema S, Girgus JS. The emergence of gender differences in depression during adolescence. Psychol Bull 1994;115:424-43.
59. Altemus M, Sarvaiya N, Neill Epperson C. Sex differences in anxiety and depression clinical perspectives. Front Neuroendocrinol 2014;35:320-30.
60. Baldereschi M, Di Carlo A, Rocca WA, Vanni P, Maggi S, et al. Parkinson's disease and parkinsonism in a longitudinal study: two-fold higher incidence in men. ILSA Working Group. Italian Longitudinal Study on Aging. Neurology 2000;55:1358-63.
61. Elbaz A, Bower JH, Maraganore DM, McDonnell SK, Peterson BJ, et al. Risk tables for parkinsonism and Parkinson's disease. J Clin Epidemiol 2002;55:25-31.
62. del Aguila MA, Longstreth WT Jr, McGuire V, Koepsell TD, van Belle G. Prognosis in amyotrophic lateral sclerosis: a population-based study. Neurology 2003;60:813-9.
63. McCombe PA, Henderson RD. Effects of gender in amyotrophic lateral sclerosis. Gend Med 2010;7:557-70.
64. Fombonne E. Epidemiological surveys of autism and other pervasive developmental disorders: an update. J Autism Dev Disord 2003;33:365-82.
65. Hattier MA, Matson JL, Tureck K, Horovitz M. The effects of gender and age on repetitive and/or restricted behaviors and interests in adults with autism spectrum disorders and intellectual disability. Res Dev Disabil 2011;32:2346-51.
66. Hiller RM, Young RL, Weber N. Sex differences in pre-diagnosis concerns for children later diagnosed with autism spectrum disorder. Autism 2016;20:75-84.
67. Bálint S, Czobor P, Komlósi S, Mészáros A, Simon V, et al. Attention deficit hyperactivity disorder (ADHD): gender- and age-related differences in neurocognition. Psychol Med 2009;39:1337-45.
68. Catalá-López F, Peiró S, Ridao M, Sanfélix-Gimeno G, Gènova-Maleras R, et al. Prevalence of attention deficit hyperactivity disorder among children and adolescents in Spain: a systematic review and meta-analysis of epidemiological studies. BMC Psychiatry 2012;12:168.
69. Cole WR, Mostofsky SH, Larson JC, Denckla MB, Mahone EM. Age-related changes in motor subtle signs among girls and boys with ADHD. Neurology 2008;71:1514-20.
70. Willcutt EG. The prevalence of DSM-IV attention-deficit/hyperactivity disorder: a meta-analytic review. Neurotherapeutics 2012;9:490-9.
71. Aleman A, Kahn RS, Selten JP. Sex differences in the risk of schizophrenia: evidence from meta-analysis. Arch Gen Psychiatry 2003;60:565-71.
72. Goldstein JM, Cherkerzian S, Tsuang MT, Petryshen TL. Sex differences in the genetic risk for schizophrenia: history of the evidence for sex-specific and sex-dependent effects. Am J Med Genet B Neuropsychiatr Genet 2013;162B:698-710.
73. Irvine K, Laws KR, Gale TM, Kondel TK. Greater cognitive deterioration in women than men with Alzheimer's disease: a meta analysis. J Clin Exp Neuropsychol 2012;34:989-98.
74. McGrath J, Saha S, Chant D, Welham J. Schizophrenia: a concise overview of incidence, prevalence, and mortality. Epidemiol Rev 2008;30:67-76.
75. Confavreux C, Vukusic S, Adeleine P. Early clinical predictors and progression of irreversible disability in multiple sclerosis: an amnesic process. Brain 2003;126:770-82.
76. Klein SL, Flanagan KL. Sex differences in immune responses. Nat Rev Immunol 2016;16:626-38.
77. Pinheiro I, Dejager L, Libert C. X-chromosome-located microRNAs in immunity: might they explain male/female differences? The X chromosome-genomic context may affect X-located miRNAs and downstream signaling, thereby contributing to the enhanced immune response of females. Bioessays 2011;33:791-802.
78. Fish EN. The X-files in immunity: sex-based differences predispose immune responses. Nat Rev Immunol 2008;8:737-44.
79. Wang X, Soloway PD, Clark AG. Paternally biased X inactivation in mouse neonatal brain. Genome Biol 2010;11:R79.
80. Carrel L, Willard HF. X-inactivation profile reveals extensive variability in X-linked gene expression in females. Nature 2005;434:400-4.
81. Souyris M, Cenac C, Azar P, Daviaud D, Canivet A, et al. TLR7 escapes X chromosome inactivation in immune cells. Sci Immunol 2018;3.
82. Lehmann SM, Krüger C, Park B, Derkow K, Rosenberger K, et al. An unconventional role for miRNA: let-7 activates Toll-like receptor 7 and causes neurodegeneration. Nat Neurosci 2012;15:827-35.
83. VanRyzin JW, Marquardt AE, Pickett LA, McCarthy MM. Microglia and sexual differentiation of the developing brain: a focus on extrinsic factors. Glia 2019.
84. Sierra A, Gottfried-Blackmore A, Milner TA, McEwen BS, Bulloch K. Steroid hormone receptor expression and function in microglia. Glia 2008;56:659-74.

85. Benedusi V, Meda C, Della Torre S, Monteleone G, Vegeto E, et al. A lack of ovarian function increases neuroinflammation in aged mice. Endocrinology 2012;153:2777-88.
86. Sárvári M, Hrabovszky E, Kalló I, Solymosi N, Likó I, et al. Menopause leads to elevated expression of macrophage-associated genes in the aging frontal cortex: rat and human studies identify strikingly similar changes. J Neuroinflammation 2012;9:264.
87. Buss C, Entringer S, Swanson JM, Wadhwa PD. The role of stress in brain development: the gestational environment's long-term effects on the brain. Cerebrum 2012;2012:4.
88. Levesque ML, Fahim C, Ismaylova E, Verner MP, Casey KF. The impact of the in utero and early postnatal environments on grey and white matter volume: a study with adolescent monozygotic twins. Dev Neurosci 2015;37:489-96.
89. Yanguas-Casás N, Crespo-Castrillo A, de Ceballos ML, Chowen JA, Azcoitia I, et al. Sex differences in the phagocytic and migratory activity of microglia and their impairment by palmitic acid. Glia 2018;66:522-37.
90. Hanamsagar R, Bilbo SD. Environment matters: microglia function and dysfunction in a changing world. Curr Opin Neurobiol 2017;47:146-55.
91. Hanamsagar R, Alter MD, Block CS, Sullivan H, Bolton JL, et al. Generation of a microglial developmental index in mice and in humans reveals a sex difference in maturation and immune reactivity. Glia 2017; 65:1504-20.
92. Bordt EA, Ceasrine AM, Bilbo SD. Microglia and sexual differentiation of the developing brain: a focus on ontogeny and intrinsic factors. Glia 2019.
93. Nelson LH, Warden S, Lenz KM. Sex differences in microglial phagocytosis in the neonatal hippocampus. Brain Behav Immun 2017;64:11-22.
94. Wu LJ, Vadakkan KI, Zhuo M. ATP-induced chemotaxis of microglial processes requires P2Y receptor-activated initiation of outward potassium currents. Glia 2007;55:810-21.
95. McCarthy MM, Arnold AP. Reframing sexual differentiation of the brain. Nat Neurosci 2011;14:677-83.
96. Green PS, Simpkins JW. Neuroprotective effects of estrogens: potential mechanisms of action. Int J Dev Neurosci 2000;18:347-58.
97. Raghava N, Das BC, Ray SK. Neuroprotective effects of estrogen in CNS injuries: insights from animal models. Neurosci Neuroecon 2017;6:15-29.
98. Loram LC, Sholar PW, Taylor FR, Wiesler JL, Babb JA, et al. Sex and estradiol influence glial pro-inflammatory responses to lipopolysaccharide in rats. Psychoneuroendocrinology 2012;37:1688-99.
99. Smith AL, Alexander M, Rosenkrantz TS, Sadek ML, Fitch RH. Sex differences in behavioral outcome following neonatal hypoxia ischemia: insights from a clinical meta-analysis and a rodent model of induced hypoxic ischemic brain injury. Exp Neurol 2014;254:54-67.
100. Demarest TG, Schuh RA, Waddell J, McKenna MC, Fiskum G. Sex-dependent mitochondrial respiratory impairment and oxidative stress in a rat model of neonatal hypoxic-ischemic encephalopathy. J Neurochem 2016;137:714-29.
101. Mrdjen D, Pavlovic A, Hartmann FJ, Schreiner B, Utz SG, et al. High-dimensional single-cell mapping of central nervous system immune cells reveals distinct myeloid subsets in health, aging, and disease. Immunity 2018:48:599.
102. Hammond TR, Dufort C, Dissing-Olesen L, Giera S, Young A, et al. Single-cell RNA sequencing of microglia throughout the mouse lifespan and in the injured brain reveals complex cell-state changes. Immunity 2019;50:253-71.
103. Jordão MJC, Sankowski R, Brendecke SM, Sagar, Locatelli G, et al. Single-cell profiling identifies myeloid cell subsets with distinct fates during neuroinflammation. Science 2019;363:6425.
104. Keren-Shaul H, Spinrad A, Weiner A, Matcovitch-Natan O, Dvir-Szternfeld R, et al. A unique microglia type associated with restricting development of Alzheimer's disease. Cell 2017;169:1276-90.
105. Laskowitz DT, Thekdi AD, Thekdi SD, Han SK, Myers JK, et al. Downregulation of microglial activation by apolipoprotein E and apoE-mimetic peptides. Exp Neurol 2001;167:74-85.
106. Lynch JR, Tang W, Wang H, Vitek MP, Bennett ER, et al. APOE genotype and an ApoE-mimetic peptide modify the systemic and central nervous system inflammatory response. J Biol Chem 2003;278:48529-33.
107. Colton CA, Brown CM, Vitek MP. Sex steroids, APOE genotype and the innate immune system. Neurobiol Aging 2005;26:363-72.
108. Altmann A, Tian L, Henderson VW, Greicius MD; Alzheimer's Disease Neuroimaging Initiative Investigators. Sex modifies the APOE-related risk of developing Alzheimer disease. Ann Neurol 2014;75:563-73.
109. Cambronero FE, Liu D, Neal JE, Moore EE, Gifford KA, et al. APOE genotype modifies the association between central arterial stiffening and cognition in older adults. Neurobiol Aging 2018;67:120-7.
110. Parhizkar S, Arzberger T, Brendel M, Kleinberger G, Deussing M, et al. Loss of TREM2 function increases amyloid seeding but reduces plaque-associated ApoE. Nat Neurosci 2019;22:191-204.
111. Krasemann S, Madore C, Cialic R, Baufeld C, Calcagno N, et al. The TREM2-APOE pathway drives the transcriptional phenotype of dysfunctional microglia in neurodegenerative diseases. Immunity 2017;47:566-81.
112. Butovsky O, Jedrychowski MP, Cialic R, Krasemann S, Murugaiyan G, et al. Targeting miR-155 restores abnormal microglia and attenuates disease in SOD1 mice. Ann Neurol 2015;77:75-99.
113. Kang SS, Ebbert MTW, Baker KE, Cook C, Wang X, et al. Microglial translational profiling reveals a convergent APOE pathway from aging, amyloid, and tau. J Exp Med 2018;215:2235-45.

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Review

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Microglial process convergence on axonal segments in health and disease

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Abstract

Microglia dynamically interact with neurons influencing the development, structure, and function of neuronal networks. Recent studies suggest microglia may also influence neuronal activity by physically interacting with axonal domains responsible for action potential initiation and propagation. However, the nature of these microglial process interactions is not well understood. Microglial-axonal contacts are present early in development and persist through adulthood, implicating microglial interactions in the regulation of axonal integrity in both the developing and mature central nervous system. Moreover, changes in microglial-axonal contact have been described in disease states such as multiple sclerosis (MS) and traumatic brain injury (TBI). Depending on the disease state, there are increased associations with specific axonal segments. In MS, there is enhanced contact with the axon initial segment and node of Ranvier, while, in TBI, microglia alter interactions with axons at the site of injury, as well as at the axon initial segment. In this article, we review the interactions of microglial processes with axonal segments, analyzing their associations with various axonal domains and how these interactions may differ between MS and TBI. Furthermore, we discuss potential functional consequences and molecular mechanisms of these interactions and how these may differ among various types of microglial-axonal interactions.

Keywords: Microglia, multiple sclerosis, traumatic brain injury, microglia-axonal interactions

INTRODUCTION

Microglia are the innate immune cells of the central nervous system (CNS) and the primary mediators of the neuroinflammatory response. They are derived from a pool of primitive macrophages from the yolk sac that appear during early embryonic development[1-3]. Microglia are ontogenetically distinct from

the peripheral blood-derived monocytes/macrophages that reside outside the CNS and mediate the peripheral inflammatory response[4,5]. Peripheral blood-derived immune cells are not typically found in the healthy CNS. However, peripheral monocytes/macrophages can infiltrate the CNS and exacerbate the neuroinflammatory response under pathological conditions. The distinct developmental origin of microglia from peripheral monocyte-derived macrophages and the exclusion of peripheral immune cells from the CNS underscores the immunological privilege of the CNS and the unique functions microglia might exert in the healthy brain and in pathological processes[6].

Microglia are cells with highly dynamic process networks that rapidly remodel to survey the microenvironment and maintain tissue homeostasis[7-9]. The surveying processes of microglia respond to CNS perturbations through rapid protrusion onto the site of insult/interest[7,10] and microglia undergo "activation", a complex series of alterations including changes in enzyme, receptor, and immune factor expression and altered cellular morphology[3,11,12]. Microglia exhibit a variety of morphologies ranging from small cell bodies with long highly-branched processes to enlarged cell bodies with short, thick processes[3,13]. The spectrum of microglial morphologies is indicative of their activation state and is commonly used to characterize activated *vs.* non-activated microglia in histological samples. Surveying (non-activated) microglia exhibit long, highly-branched or "ramified" processes that sample the surrounding environment. However, upon activation, microglia retract their processes and increase their cell body size, exhibiting morphologies defined by short, thick processes and large somas[3,14]. Highly activated, phagocytic microglia tend to lose distinctive processes all together and exhibit an ameboid shape[3,14].

Many studies have investigated microglial-neuronal interactions via secreted factors. Activated microglia exhibit extensive changes in the expression of their inflammatory profile[15]. While some of these secreted factors may provide neurotrophic functions, pro-inflammatory factors exhibit deleterious effects[16,17]. Various neurotrophic secreted factors released from microglia induce neurite outgrowth and have been shown to be involved in regulating the cytoarchitecture of the developing brain[18-20]. Pro-inflammatory microglia, however, up-regulate cytokines and enzymes that produce reactive oxygen species, which have been implicated in axonal injury and disruption[16,21-32].

Microglia also interact with neurons through physical contact under homeostatic conditions[7,9,11,33-36]. Microglia have recently been shown to contact dendrites and neuronal cell bodies in the normal adult brain[37,38]. Both contact types require purinergic signaling through the P2Y12 receptor and appear to be protective in nature[37-40]. In the developing somatosensory cortex, it was recently found that microglial process contacts onto dendrites precipitates filipodia formation, linking microglia process contacts with synaptic formation[38]. Microglia are also key mediators of synaptic pruning, which alters the neuronal excitatory/inhibitory balance[41]. Microglia contact pre- and postsynaptic neuronal elements in an activity-dependent manner, and synapses that are contacted by microglia more frequently and for longer durations of time are subsequently removed [Figure 1A][9,42,43]. Specifically, studies have demonstrated that early during development (Postnatal Day 5 in mice) phagocytic microglia engulf synapses of neurons with reduced activity/input in a complement-dependent manor[42,43]. Alternatively, later during development (Postnatal Day 15 in mice) microglia only appear to remove parts of synapses in a process called "trogocytosis"[44]. Another study using zebrafish larva demonstrated that microglial-synaptic contacts increased with increased neuronal spontaneous activity. Further, the zebrafish neurons that were contacted by microglia exhibited a decrease in activity, while noncontacted neurons maintained an increased firing rate[36].

Microglia may also influence neuronal excitability through contact with the axon initial segment (AIS), the axonal domain responsible for action potential initiation and modulation [Figure 1A][45]. Microglia appear to establish contact with the AIS early in development and maintain this contact through adulthood,

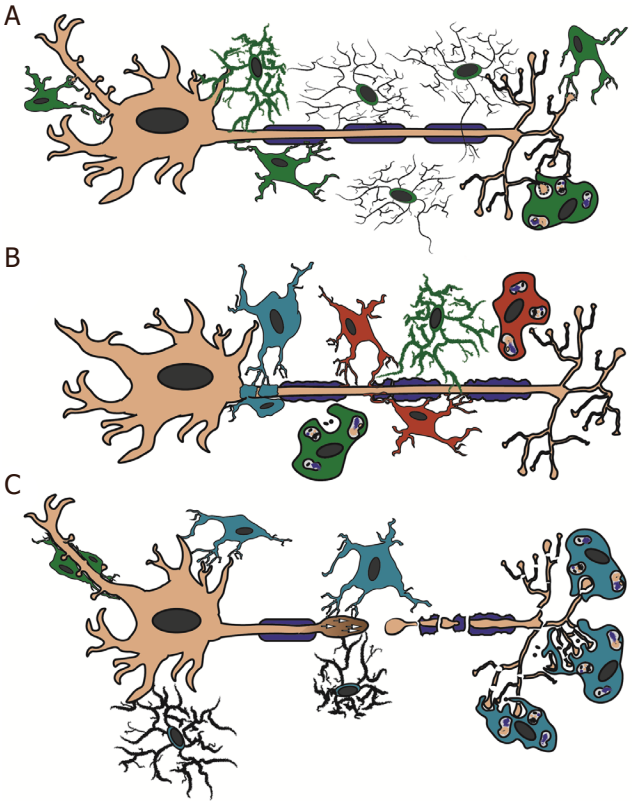

Figure 1. Schematic representation of microglial process contacts in health and disease. Illustration demonstrating various microglial and monocytic contacts onto axonal segments. A: in the healthy brain, resident microglia (green) contact the neuronal cell body and axon initial segment. These microglia potentially express TNF-α and CSF1 and are involved in reduction of hyperexcitability in neurons. The dynamic surveying processes of non-activated ramified microglia also contact various areas of the axon in the healthy CNS. During development, contacts by resident microglia are involved in pre- and postsynaptic pruning; B: in MS, both resident microglia (green) and infiltrating peripheral monocytes (red) contact the nodes of Ranvier. Note that the processes of monocytes are found between the layers of myelin and the axon sheath, while the resident microglial processes are primarily in contact with adjacent monocytes and/or involved in debris clearance. Neuroinflammatory cells that have yet to be identified as either resident microglia or infiltrating monocytes (teal) that express TNF-α, INOS, Nox2, and higher levels of activated calpain, wrap the axon initial segment. This wrapping is involved in a notable reduction in the length of the axon initial segment; C: following TBI, macrophages (monocytes and/or microglia) phagocytosis the Wallerian debris from the degenerating distal axonal segments of an injured axons. Potential hyperexcitability of neurons following TBI induces microglial process convergence onto the neuronal soma via elevated ATP levels and/or glutamate levels. Rod microglia (green) are also common along the apical dendrite following injury; however, their function is currently unknown. Microglial process convergence onto the proximal injured axonal segment is associated with P2Y12 and potentially confers neuroprotective effects on the damaged axon leading to axonal sprouting. CNS: central nervous system; MS: multiple sclerosis; TBI: traumatic brain injury; TNF: tumor necrosis factor; CSF1: colony stimulating factor 1; INOS: inducible nitric oxide synthase; Nox2: NADPH oxidase 2

strongly suggesting that microglia play a role in regulating AIS structure and function[45]. Additionally, when repeated stimulations were used to induce neuronal hyperexcitability, microglia extended their processes and wrapped around axons[35]. This induced a rapid repolarization in the neuron back to resting levels which was lost when microglia were pharmacologically blocked[35].

Neuroinflammatory microglial changes are associated with various pathologies, including, but not limited to, spinal cord injury, neurodegenerative diseases, and early-life stress[46-49]. Alterations in the form and frequency of physical microglial-axonal contacts, however, have been described in the most detail in multiple sclerosis (MS) and traumatic brain injury (TBI), therefore this review focuses on these two disease states[45,50-52]. Many of the changes in microglial-neuronal contacts appear to be dependent on the disease state, in which there are alterations in microglial associations with specific axonal segments. Below, we review the interactions of microglial processes with axonal segments, focusing on their associations with various axonal domains and the unique alterations of these physical interactions in MS and TBI.

NEUROINFLAMMATION IN MS

MS is an autoimmune-mediated disease of the CNS that is characterized by inflammation and demyelination. While the cause of MS is not fully understood, it is accepted that neuroinflammation, resulting from the accumulation and activation of macrophages (derived from microglia or infiltrating monocytes) in the human CNS, is a crucial step in MS pathogenesis, which culminates in injury to myelin and axons and disrupts the flow of information[53-56]. The autoimmune nature of MS and the role of autoreactive peripheral T cells is highly complex and has been reviewed previously[57]. Therefore, we do not discuss the autoreactive peripheral immune cells in this review. Furthermore, destruction of myelin and axons, as well as oligodendrocyte cell-death, are directly related to the numbers of activated inflammatory cells[53,58-60]. The symptoms of MS range widely based on the CNS region affected and include a variety of motor or sensory dysfunctions such as muscle weakness, spasticity, tremor, unexplained pain or numbness, vision problems, and cognitive deficits[61]. While demyelination is a hallmark of MS, axonal injury is also a prominent pathological feature and is a major contributor of chronic disability in patients[59,60,62-64]. The types of axonal injuries in MS and its models include the formation of axonal swellings, reduced levels of Na^+/K^+ ATPase, synaptic damage, axon transection, and disruption of axonal domains, such as the node of Ranvier (NOR) and the AIS[65-69]. These axonal injuries may occur as either a consequence of demyelination[65,70] or as a primary event, independent of myelin loss[27,71], although the mechanisms driving primary axonal pathology are not fully understood. It is appreciated that soluble factors produced by resident microglia and infiltrating monocytes and their interactions with peripheral immune cells play a pivotal role in driving axonal injury[59,60,72-75]; however, recent studies have implicated a mechanistic role for microglia/monocytes through physical interactions with axonal domains[6,27,28].

Studies investigating axonal contact by microglia and/or infiltrating monocytes have utilized two common models of MS: a toxin-induced demyelinating model, cuprizone[27], and an immune-mediated model, experimental autoimmune/allergic encephalomyelitis (EAE)[6,27]. In the cuprizone model, a copper-chelating toxin, cuprizone, is administered through chow resulting in oligodendrocyte cell death and, consequently, loss of myelin[75]. Demyelination is detectable 1-2 weeks after cuprizone treatment with peak demyelination occurring by 5-6 weeks of exposure[76-78]. The cuprizone model yields substantial demyelination and, upon removal of toxin-containing chow, spontaneous remyelination occurs. While this model does not recapitulate immune-mediated aspects of MS, it does allow for the investigation of fundamental mechanistic questions of the demyelination/remyelination process and roles of myelin in the stability of axonal domains[75]. The EAE model is an immune-mediated model that is induced through subcutaneous injection of myelin proteins accompanied by pertussis toxin and an adjuvant to ignite an inflammatory response[75,79,80]. The resulting neuroinflammation recapitulates key pathological features of MS such as inflammation, demyelination, and neuronal insults[75,80,81]. These two models allow for the rigorous assessment of MS-associated alterations in microglial-axonal interaction due to demyelination both in the presence of and independent from the autoreactive inflammatory response.

MICROGLIAL CONTACT WITH THE NOR IN MODELS OF MS

Axonal function requires maintenance of the NOR[82], and a major regulator of nodal axonal domain stability is myelin integrity[77,83-89]. For example, cuprizone-induced demyelination resulted in loss of nodal and paranodal clustered proteins[77]. Other studies have also demonstrated loss of nodal protein clustering as a downstream consequence of demyelination in mouse models of MS and postmortem MS tissue[67,69,90,91]. In addition to NOR disruption, analyses of human MS tissues have revealed that prominent microglia/macrophage accumulation correlates with active demyelination[56,59,60,67]. Indeed, myelin is required for NOR stability; however, NOR protein clustering can also be disrupted independent of demyelination. Howell *et al.*[67] used immunohistochemical techniques to study NOR integrity in normal-appearing white matter of MS cases and in EAE and found NOR disruption correlated with local microglial inflammation but was independent of demyelinating lesions and did not correlate with the density of infiltrating lymphocytes. This was

consistent with other studies demonstrating that numbers of microglia/macrophages correlate to EAE severity[27,72-74]. However, the cellular mechanisms by which microglia/infiltrating macrophages promote disease progression and whether these cells play differential roles in initiating demyelination or promoting repair remain unknown[61,92,93]. Yamasaki *et al.*[6] began to elucidate the roles these cell types play in the disease course of EAE and their differential roles in myelin disruption. Serial block-face scanning electron microscopy of mice, in which the resident microglia fluoresced green and the infiltrating monocyte-derived macrophages fluoresced red, was utilized to distinguish the two inflammatory cell populations and to investigate their role in demyelination[6]. It was demonstrated that both microglia and infiltrating peripheral monocyte-derived macrophages contact the axo-glial unit at the NOR in the spinal cord of EAE-induced mice at disease onset [Figure 1B][6]. They found that most (73%) of the NOR investigated (both intact and disrupted) were physically contacted by some sort of macrophage[6]. Interestingly, microglial association with the axo-glial unit was limited, while monocyte-derived infiltrating macrophage contact at the NOR was more extensive[6]. Monocyte-derived macrophage processes were found extended between the myelin and axolemma, potentially uprooting paranodal contacts and initiating demyelination [Figure 1B][6]. In contrast, microglial processes contacted the axo-glial unit at the NOR, but the microglial processes did not extend beneath the axolemma and, instead, appeared to primarily interact with adjacent macrophages and appeared to be involved in debris clearance [Figure 1B][6]. Gene expression profiles supported that infiltrating monocyte-derived macrophages were highly phagocytic and pro-inflammatory, whereas microglia demonstrated a suppressed cellular metabolism and activation phenotype[6]. These findings suggest that, at disease onset, infiltrating macrophages initiate active demyelination while microglia perform myelin debris clearance, a function that supports tissue regeneration and affects the maturation of oligodendrocyte progenitor cells[3].

The differential mechanisms underlying microglial contact at the NOR is still to be fully determined. It was shown that C-C chemokine receptor type 2 (CCR2), a chemokine receptor essential for monocyte recruitment to CNS tissues during immune-mediated inflammation[94,95], was important for recognition of disrupted NOR by infiltrating monocyte-derived macrophages[6]. Mice lacking CCR2 demonstrated reduced NOR contact by monocyte-derived macrophages and significantly less demyelination at EAE onset. Interestingly, CCR2-deficient mice displayed similar nodal pathology during the pre-onset stage of EAE (post-EAE induction but prior to onset of motor clinical symptoms), suggesting that inflammatory nodal disruption could be reversible if monocyte-derived macrophages were prevented from initiating demyelination at those sites[6].

MICROGLIAL CONTACT WITH THE AIS

Microglia contact the AIS during normal development and throughout life, indicating that these cells likely play a role in the regulation of AIS structure and/or function in both the developing and mature CNS [Figure 1A][45]. A recent study utilizing both EAE and cuprizone models of MS to assess MS-related axonal injury and their underlying mechanisms found that inflammatory microglia and/or Macrophages physically contact the AIS[27]. It was found that the AIS is a primary target in disease pathogenesis of EAE[27]. In this study, mice were induced with either myelin-oligodendrocyte glycoprotein + EAE or cuprizone and AIS integrity of cortical neurons was assessed using immunohistochemical techniques. The integrity of the AIS was assessed by immunolabeling for ankyrinG (AnkG), a protein critical for AIS establishment and maintenance[96-98]. Upon EAE induction, it was found that the number and length of AISs were significantly reduced and that the number of disrupted AISs was associated with disease severity and progression[27]. This loss of AIS integrity, however, was not associated with demyelination, neuronal death, or axonal damage, rather appeared to be mediated by inflammatory factors[27-29]. Specifically, AIS disruption was preceded by microglial morphological changes suggestive of enhanced reactivity and increased contact by Iba-1 positive inflammatory cells but occurred independently of demyelination[27]. The nature of microglial interaction with the AIS changed substantially following EAE, transitioning from microglial process

alignment along the AIS and periodic process ends contacting the AIS [Figure 1A] to microglial processes completely wrapping around the AIS [Figure 1B][27,99]. Treatment with anti-inflammatory Didox, a free-radical scavenger and NF-κB modulator[100-102], resulted in enhanced AIS structural integrity and reduction in microglial-AIS contact, indicating that EAE-induced inflammation is the driver for AIS disruption and enhanced microglial-AIS contact.

Microglial-AIS contact increased prior to and concomitant with changes in AIS structure, although it does not appear that contact alone drives AIS disruption. In the cuprizone model, demyelination and inflammation are present in the cortex; however, AISs were spared, suggesting the AIS, unlike the NOR, is not maintained by myelin presence[27,103]. Interestingly, in the cortex of cuprizone-fed mice, reactive microglia also enhanced contact with AISs but AIS structure was preserved[27]. Thus, the consequence of microglial-AIS contact appears to be stimulus dependent. In other models, microglia are recruited to and make contact with the initial portion of the axon and soma of hyperexcitable cells[35,36]. Microglial-axonal contact is activity dependent and results in a protective phenotype, preventing the neuron from excitotoxic death[35,36]. While live-imaging and physiological experiments have not been performed in MS models, analysis of AIS plasticity in EAE revealed structural changes of the AIS, such as decreased length[27], which can occur in response to hyperexcitable environments[104-106]. Thus, the nature of microglial-AIS contacts may be context dependent and could either drive disruption or confer protection. Since the AIS is the axonal domain where action potentials are generated, this consistent microglial-AIS contact in both health and disease strongly implicates microglia as a regulator and/or modulator of neuronal function and further studies are needed to investigate the role of enhanced microglial interactions with the AIS in MS and its models.

The mechanisms mediating microglial contact with either the NOR or AIS remain undefined; however, as the molecular architecture is highly conserved between these two axonal segments, it is likely that the molecular mechanisms involved in associations with either region are similar. The fractalkine receptor CX3CR1 mediates microglial synaptic pruning and microglial contact with neuronal somatic-dendritic domains, and was, therefore, a prime candidate for mediating microglial-AIS contact[107-109]. However, absence of CX3CR1 fractalkine receptors on microglia did not alter contact with the AIS in the healthy mouse brain, suggesting that microglial-AIS interactions are not mediated through the fractalkine receptor[45]. Loss of brevican and versican, specialized extracellular matrix molecules surrounding the AIS, also did not alter microglial contacts onto the AIS[45]. In an effort to determine if AIS proteins are necessary for microglial contact, the AIS master scaffolding protein AnkG was knocked down, which disrupted AIS protein clustering and significantly reduced the number of microglial-AIS contacts, suggesting that molecules normally restricted to the AIS are important for microglial-AIS contact[45]. Thus, some progress has been made in eliminating candidates that mediate microglial-AIS contact and in determining that an intact AIS is important for microglial contact, but these experiments[45] focused on microglial contact specifically with the AIS.

NEUROINFLAMMATION IN TBI

TBI affects millions of people and is associated with devastating financial and societal costs linked to the long-term morbidities that develop and persist for years after the initial insult[110-113]. Recent studies have demonstrated the impact of inflammatory cascades in regulating many of these TBI-mediated outcomes[114-118]. While astrocytes and infiltrating peripheral monocytes/macrophages do play a role in TBI-induced neuroinflammation, microglia are thought to be the critical mediators of these TBI-induced neuroinflammatory processes and, therefore, have been the primary focus of TBI-related neuroinflammatory investigations. However, as it is difficult to specifically identify resident microglia from peripheral infiltrating monocytes following TBI, many studies call both populations "microglia" for simplicity. In the following sections, we do the same unless the population is specifically known to be infiltrating monocytic in origin.

Studies have also demonstrated neuroinflammation in various brain regions within the human population following TBI[119-122]. Molecular imaging studies have demonstrated microglial activation in populations of TBI patients as visualized via positron emission tomography using ligands for the mitochondrial translocator protein, TSPO, following brain injury[114,119-121]. While the TSPO ligands used in these studies have been shown to significantly increase binding to activated microglia post-TBI, they also bind to other neuroinflammatory cells following trauma[114,119-121]. Complementary histopathological studies investigating the extent and localization of various neuroinflammatory makers, including microglial CD68 and/or complement receptors, as well as morphological indications of microglial activation also demonstrated significant inflammation following brain injury in humans[123-125]. Many of these studies also indicate that neuroinflammation persists and evolves years after the initial head injury and that inflammation may become more severe with time post-injury[117,121,125,126].

The majority of preclinical TBI models can be divided into focal and diffuse injury models, with some of the most used models being the controlled cortical impact (focal), central fluid percussion injury (diffuse), lateral fluid percussion injury (mixed focal and diffuse), and head rotational (diffuse) models; however, the specific models used to induce TBI are highly varied. For a review of the different types of TBI preclinical models, please see[127,128]. While the occurrence of microglial activation following TBI is rather well accepted, the role of activated microglia in the post-injury brain is far more enigmatic. A wide range of studies using various rodent models of brain injury have demonstrated that activated microglia can have a host of functions. For simplicity's sake, these functions were lumped into two historical categories: M1, or pro-inflammatory microglia, that were involved with cytokine release that lead primarily to neuronal damage and M2, or anti-inflammatory microglia, that were associated with release of neurotrophic factors and cytokines downregulating the inflammatory responses[129-132]. These binary definitions, however, appear too simplistic for the complex interactions between the pro- and anti-inflammatory signals coming from activated microglia following TBI[133]. While the nomenclature for microglia falling along the inflammatory spectrum is still up for debate, studies do indicate that location, time following TBI, and systemic factors, including stress and infection, can push activated microglia toward a more pro-inflammatory state[131,132,134,135]. Information regarding these microglial populations is covered in greater detail in the following reviews[129,133,134].

Many well-designed studies using rodents have indicated that reduction of activated microglial and/or targeting various neuroinflammatory signaling pathways ameliorates downstream pathology and behavioral morbidity[136-149]. One of the most common compounds used to assess the role of microglial activation following TBI is the second generation tetracycline drug, minocycline[129]. Minocycline is traditionally used clinically as an antibiotic; however, it has various other uses/effects including as a powerful anti-inflammatory compound[140]. Various studies demonstrate significant reductions in damaged or dying neurons, reduced lesion volumes, enhanced behavioral scores, and drastic reduction in pro-inflammatory cytokine expression following administration of minocycline, indicating that interactions between activated microglia and neurons could precipitate neurodegeneration[141-143]. In fact, minocycline is currently being assessed for safety in clinical trials for the treatment of TBI-associated morbidities thought to be regulated by inflammation[144]. However, other studies indicate that prolonged microglial inhibition via minocycline administration precipitates enhanced neurodegeneration and inflammation or no effect at all, demonstrating the complexity of neuroinflammatory responses following TBI[145-147]. Based on the fact that minocycline has a multitude of effects, it is also possible that the variability in these studies' findings highlight the potential that non-inflammatory minocycline-induced reductions in TBI-mediated pathology in turn reduce inflammation and microglial activation[140,147,148]. In support of this possibility are studies showing little or no effect of genetic microglial elimination or direct microglial inhibition using compounds targeting the CSF1 receptor in altering TBI-induced pathology[135,149,150]. Additionally, administration of pro-inflammatory stimuli into the ventricle, surpassing induction of peripheral inflammatory responses,

does not result in enhanced post-injury neurodegeneration, indicating that the peripheral inflammatory response, more than direct microglial activation, precipitates proinflammation-mediated secondary insults[134,151]. Overall, these studies underscore the intricacies of TBI-induced microglial activation and our limited understanding of microglial-neuronal interactions following brain injury.

PHAGOCYTOSIS FOLLOWING TBI

One of the most well-studied physical interactions between microglia and neuronal segments following TBI is phagocytic engulfment. As in the non-injured brain, activated microglia serve a prominent and vital role in the clearance of cellular debris following brain injury. Upon the initial TBI insult, a multitude of cellular pathologies progress. One of the most well studied pathologies, and the hallmark of diffuse brain injury following TBI, is diffuse axonal injury/traumatic axonal injury[125,152-156]. Axonal injury first manifests as disruption of molecular transport anterogradely down the axon and progresses over hours, days, and months following injury to a disconnection at the point of initial transport disruption, resulting in a proximal axonal segment that remains connected to the neuronal cell body and a distal axonal segment that undergoes Wallerian degeneration[157-159]. Phagocytosis by activated microglia is required to engulf and clear away the axonal and myelin debris from the Wallerian degeneration of the distal axonal segment and involves the toll-like receptors, TREM-2, complement receptors 3 and 4, as well as MAC-2, for the engulfment of myelin, and the purinergic receptor P2RY6 [Figure 1C][160,161]. Ultrastructural assessments of the injured brain have demonstrated significant phagocytosis of Wallerian debris by activated microglia following TBI[52,116,162]. Microglia with ameboid morphologies, indicative of phagocytic activity, were found primarily in proximity to the distal axonal segment sustaining dieback, but not the proximal axonal segments, following TBI-induced optic nerve damage[163]. Further, expression of mRNA indicative of phagocytic activity is significantly increased following trauma[150]. It should be noted, however, that both microglia and astrocytes containing phagocytic material have been observed, demonstrating that, while microglia may be the primary phagocytic cells in the brain, astrocytes also phagocytosis debris following injury[162]. Additionally, not all activated microglia were observed to be phagocytic following TBI, indicating that phagocytosis is not the only microglial-axonal interaction upregulated following TBI[116].

ROD MICROGLIA AND TBI

The readily identifiable, yet mysterious, "rod microglia" have been noted following TBI in a variety of pre-clinical models and in the human population. This subset of microglia appear following injury and are defined exclusively by their rod-like morphology and chain-like associations that form long microglial trains of several rod microglia lined up end-to-end [Figure 1C][164]. These rod-shaped microglia have been described following a variety of neurological diseases, including neurosyphilis, and appear to be both non-phagocytic and reversible[165]. Both rod microglia and microglial trains appear primarily in brain regions in which the fiber tracks are linear, such as the neocortex, brainstem, and hippocampus[124,166-168]. This subset of rod microglia, however, appear to be absent in areas that are not linearly arranged, such as the thalamus[166]. The formation of microglial trains appears to be associated with p38; however the function of these microglial trains remains unknown[169]. Recently, it was found that microglial trains formed by rod microglia align with the apical dendrite, but not the axon as was previously thought, of pyramidal neurons in the rodent cortex [Figure 1C] and spatially associate with astrocytes, indicating that this subset of microglial-neuronal interaction is neuronal-segment specific and could be involved in an additional interplay between neuroinflammatory cell types[150]. However, the study of rod microglia following TBI is still in its infancy and requires further investigation into the timing and function of this microglial-axonal interaction subtype.

TBI-INDUCED PROCESS CONVERGING AND DIVERGING MICROGLIA

Over the last several years, another subtype of microglial-neuronal interaction has been observed following brain injury. This interaction subtype manifests as physical contacts between activated microglia and

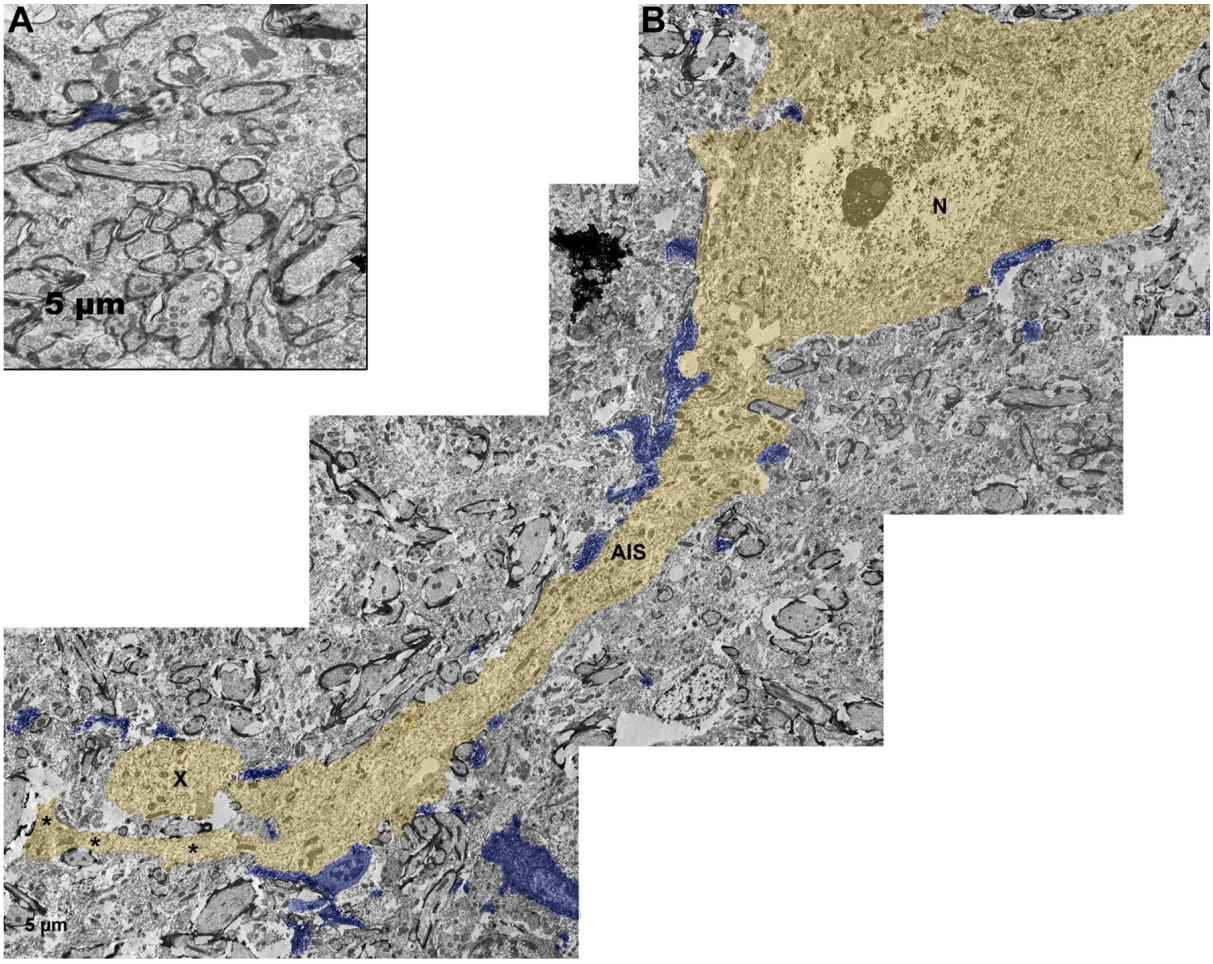

Figure 2. Ultrastructure of microglial process contacts onto axonal segments following TBI. Electron micrographs of Iba-1 immuno-labeled microglia contacting: A: intact non-injured axons in sham-injured micro pig thalamus; B: injured axonal segment in the thalamus of micro pigs acutely (one day) following diffuse TBI. Iba-1-labeled microglial processes are pseudo-colored blue and the injured axon is pseudo-colored yellow for clarity. While few microglial processes were observed in direct contact with axons normally, microglial processes were observed in direct contact various segments of the neuron, including the soma (N = nucleus), AIS, and the proximal axonal swelling (X) of the injured neuron. Note that the proximal axonal segment of the injured neuron demonstrates ultrastructural characteristics of axonal sprouting (*). Scale bar: 5 µm. AIS: axon initial segment; TBI: traumatic brain injury

the proximal axonal segment of injured axons following TBI[51]. Using a micro pig model of central fluid percussion-generated diffuse TBI, paired with multiplexed immunohistochemical quantitative image analysis, recent studies found processes from activated microglia converge onto adjacent injured thalamic axons acutely (hours to one day post-injury) following injury, a phenomenon termed "microglial process convergence" (MPC; Figure 1C)[50,52]. These process converging (PC) microglia were neither ameboid nor rod shaped, rather they displayed shortened processes, with fewer process branches, morphological changes indicative of activation without progression to phagocytosis[13,51,52]. Ultrastructural assessments confirmed that PC microglia were non-phagocytic in nature[50,52]. Additionally, a single injured axon could have processes converging from multiple PC microglia[50,52]. As the majority of diffuse axonal injury following TBI appears to occur in or adjacent to the AIS, it is likely that the proximal axonal swellings in which this subtype of PC microglia are converging are nearer to the AIS than to more distal points along the axon [Figure 2][170,171]. Another group using a micro pig model of head-rotation-induced diffuse TBI found indications of potential PC microglia associated with injured neurons following brain injury[172]. Specifically, also using multiplexed immunohistochemical quantitative image analysis, they observed that microglia were in closer proximity to injured neuronal soma in multiple brain regions following TBI compared to

neurons in sham injured micro pigs [Figure 1C][172]. These PC microglia also appeared activated without falling into the morphological categories of phagocytic or rod microglia[172]. Another recent study found that MPC onto cell bodies of injured neurons is associated with protection. Specifically inhibition of this MPC increased ischemia-induced lesion volume, behavioral morbidity, and calcium influx[37]. Additionally, ischemic injury results in microglial process contacts with injured synapses that are nearly 10 times longer in duration than the 4-5-min-long contacts observed in non-injured animals using a thinned-skull live imaging approach[9]. Therefore, it is likely that MPC could involve an increase in both the number of microglial processes as well as the duration of these contacts onto injured axons.

The mechanisms involved in regulating MPC onto neuronal and axonal segments has primarily been studied in mouse models of epilepsy. The number of microglial process contacts appear to be directly related to the level of neuronal activity, in that MPC was significantly reduced upon reduction in neuronal activity via either temperature reduction or tetrodotoxin administration in thinned-skull live-imaging studies[9]. Induction of neuronal hyperexcitability to the point of excitotoxicity also promoted MPC[173]. Hyperexcitability-induced MPC resulted in reduced neuronal activity and overall increased neuronal survival in the face of otherwise excitotoxic events that were not seen following microglia elimination or inhibition of MPC[173,174]. Neuronal excitation precipitates higher extracellular and lower intracellular Ca^{2+} concentrations and increased extracellular ATP concentrations around the active neuron, which appear to be primary molecular mediators of hyperexcitability-induced MPC[173-176]. ATP-mediated MPC was found to promote polarization of microglial process outgrowth toward the location with high ATP levels [Figure 1C][177]. Elimination of the purinergic receptor P2Y12 or the fractalkine receptor CX3CR1 drastically reduced MPC onto hyper-excitable neurons, indicating that microglial P2Y12 and CX3CR1 are required for ATP-mediated MPC[176,178]. Excitatory neurons also release glutamate upon excitation. Concentration of extracellular glutamate has also been found to mediate hyperexcitability-induced MPC potentially via activation of N-methyl-D-aspartate (NMDA) receptors[174,178]. Glutamate/NMDA-mediated MPC was also found to require microglial P2Y12, but not CX3CR1[177]. Glutamate-mediated MPC also promoted nonpolarized outgrowth of microglial processes, indicating that different molecular mechanisms of hyperexcitability-induced MPC may result in different forms of MPC [Figure 1C][177]. Further, these mechanisms appear distinct from those involved in microglial phagocytosis, as knocking out or inhibiting P2Y12 or NMDA inhibited MPC without affecting phagocytosis[178]. While epilepsy and TBI are different CNS diseases with distinct neuropathologies, the molecules and mechanisms discussed above are prime candidates for regulation of TBI-induced MPC. In fact, the Jacobs group found that both axotomized and intact neurons demonstrate hyperexcitability one day following TBI in mice that appears to resolve in the axotomized population, but not the intact neurons, by two days post-injury[179,180]. While TBI-induced MPC onto the proximal axonal segments of axotomized neurons has yet to be thoroughly investigated, these findings indicate the potential that similar mechanisms might be at play in TBI and epilepsy-induced MPC.

It appears that TBI-induced MPC may be species dependent, as it was found that rats sustaining the same central fluid percussion injury paradigm as their pig counterparts did not demonstrate MPC[50]. Rather, at the same time points following injury, there was a significant decrease in microglial contacts onto injured proximal axonal segments in the rats, indicating microglial processes that diverged from injured axons or microglia process divergence (MPD)[50]. This MPD observed in rats is in alignment with previous observations in injured rats and mice that activated microglia do not physically associate with proximal segments of injured axons following brain injury[116,163]. TBI-associated MPD was also observed by a group assessing the occurrence of microglia associations with the AIS, regardless of axonal injury, following TBI in mice[45]. They demonstrated that microglial contacts onto the AIS of axons significantly decreased following TBI in mice, indicating MPD similar to that observed by the other groups following TBI in rodents[45,50,116,163]. In contrast to those studies, however, these AIS-associating, or "AXIS", microglia were not specific to injured axonal segments and appeared ramified (morphologically not activated), indicating that

these AXIS microglia could represent a distinct subtype of MPD microglia[45,50]. The interaction between the AXIS microglia and the AIS appears to be ankrin-G and GABA mediated, while the fractalkine receptor, CX3CR1, does not appear necessary for the AXIS microglial interactions[45].

There are reports indicating that microglia physically interact with injured axons following TBI in the human brain. In 2014, a study demonstrated co-labeling of microglia with injured proximal axonal swellings in brains of veterans who had histories of blast injury exposure[181]. Another study showed potential PC microglia contacting injured axonal swellings when employing double-labeling techniques in human TBI tissue[182]. These studies indicate that microglial processes may contact axonal swellings in the human brain following TBI; however, further investigation is needed to comprehensively assess potential alterations in microglial-neuronal physical interactions in the human population and address how those changes compare to those observed pre-clinically.

Additionally, a study investigating the expression of neuronal outgrowth marker, GAP43, in injured axonal segments as it related to the density of microglia in brain tissue from people diagnosed with MS or TBI demonstrated a positive correlation between neuronal regeneration and microglial density following TBI in clinical samples [Figure 1C][183]. Other studies have also observed GAP43 expression in proximal axonal swellings following injury in both human tissue and following induction of TBI in pre-clinical models[184-186]. Ultrastructural assessments further demonstrated morphological alterations indicative of active axonal sprouting of proximal axonal swellings following TBI, demonstrating that axonal process outgrowth following TBI is possible and potentially likely [Figure 2][184,185]. Microglia have been shown to express neurotrophic factors, such as nerve growth factor, following TBI, supporting a potential role for MPC in post-injury axonal outgrowth[187]. Microglia may also release exosomes that induce neurite outgrowth[19]. The role of MPC and/or MPD in potential post-injury axonal sprouting, however, remains speculative.

CONCLUSION

It is well accepted that microglia mediate neuroinflammatory processes in health and disease via pro- and anti-inflammatory cytokines and chemokines. However, microglia also appear to mediate neuronal function through physical contacts onto various neuronal segments, including dendrites, synapses, cells bodies, and axons. While the study of microglial-axonal contacts is still in its infancy, there are indications that these contacts play diverse and important roles during normal development and in the healthy CNS as well as following TBI or in disease states, such as MS. Analysis of microglia in experimental and human tissues demonstrate that microglia exhibit a spectrum of morphologies including ramified, rod-like, hypertrophied, and ameboid that all exert unique contact subtypes onto axonal segments indicative of the diverse roles microglial-axonal interactions play. Microglia and infiltrating monocytes contact various axonal segments in unique and specific ways that appear tied to the axonal region contacted, the morphology of the microglia, and the disease state. Further, it appears that the presence of microglia contacts at axonal domains may confer protection. Some of the immediate questions for this burgeoning field focus on the potential ameliorative effects of microglial contacts onto axonal and other neuronal segments as well as the timing of these interactions following various pathologies. Future examinations of axonal interactions using functional assessments and live imaging techniques could refine the distinction between axonal contacts of resident microglia and those formed by peripheral monocyte-derived infiltrating macrophages and help elucidate the nature of these interactions. Furthermore, identifying the molecules mediating contact between microglia and the axon will point toward new strategies to treat disease and promote repair in diverse inflammatory pathologies. The studies reviewed herein underscore the importance of microglial-axonal contacts in the regulation of neural signaling and the need for further investigation into these variable interactions in both the healthy and injured CNS.

DECLARATIONS

Authors' contributions

Conceptualized and wrote this review: Benusa SD, Lafrenaye AD

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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.

REFERENCES

1. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, et al. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science 2010;330;841-5.
2. Schulz C, Gomez Perdiguero E, Chorro L, Szabo-Rogers H, Cagnard N, et al. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. Science 2012;336:86-90.
3. Wolf SA, Boddeke HW, Kettenmann H. Microglia in Physiology and Disease. Annu Rev Physiol 2017;79:619-43.
4. Bennett ML, Bennett FC. The influence of environment and origin on brain resident macrophages and implications for therapy. Nat Neurosci 2020;23:157-66.
5. Prinz M, Priller J. The role of peripheral immune cells in the CNS in steady state and disease. Nat Neurosci 2017;20:136-44.
6. Yamasaki R, Lu H, Butovsky O, Ohno N, Rietsch AM, et al. Differential roles of microglia and monocytes in the inflamed central nervous system. J Exp Med 2014;211:1533-49.
7. Nimmerjahn A, Kirchhoff F, Helmchen F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 2005;308:1314-8.
8. Sierra A, Tremblay MÈ, Wake H. Never-resting microglia: physiological roles in the healthy brain and pathological implications. Front Cell Neurosci 2014;8:240.
9. Wake H, Moorhouse AJ, Jinno S, Kohsaka S, Nabekura J. Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. J Neurosci 2009;29:3974-80.
10. Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, et al. ATP mediates rapid microglial response to local brain injury in vivo. Nat Neurosci 2005;8:752-8.
11. von Bernhardi R, Heredia F, Salgado N, Muñoz P. Microglia function in the normal brain. Adv Exp Med Biol 2016;949:67-92.
12. Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. Physiology of microglia. Physiol Rev 2011;91:461-553.
13. Byrnes KR, Loane DJ, Stoica BA, Zhang J, Faden AI. Delayed mGluR5 activation limits neuroinflammation and neurodegeneration after traumatic brain injury. J Neuroinflammation 2012;9:43.
14. Tay TL, Mai D, Dautzenberg J, Fernández-Klett F, Lin G, et al. A new fate mapping system reveals context-dependent random or clonal expansion of microglia. Nat Neurosci 2017;20:793-803.
15. Crotti A, Ransohoff RM. Microglial physiology and pathophysiology: insights from genome-wide transcriptional profiling. Immunity 2016;44:505-15.
16. Chen SH, Oyarzabal EA, Hong JS. Critical role of the Mac1/NOX2 pathway in mediating reactive microgliosis-generated chronic neuroinflammation and progressive neurodegeneration. Curr Opin Pharmacol 2016;26:54-60.
17. Ransohoff RM, Khoury JE. Microglia in health and disease. Cold Spring Harb 2016;8:a020560.
18. Chen Z, Trapp BD. Microglia and neuroprotection. J Neurochem 2016;136 Suppl 1:10-7.
19. Raffo-Romero A, Arab T, Al-Amri IS, Le Marrec-Croq F, Van Camp C, et al. Medicinal leech CNS as a model for exosome studies in

the crosstalk between microglia and neurons. Int J Mol Sci 2018;19:4124.
20. Pósfai B, Cserép C, Orsolits B, Dénes Á. New insights into microglia–neuron interactions: a neuron's perspective. Neuroscience 2019;405:103-17.
21. Kumar A, Barrett JP, Alvarez-Croda DM, Stoica BA, Faden AI, et al. NOX2 drives M1-like microglial/macrophage activation and neurodegeneration following experimental traumatic brain injury. Brain Behav Immun 2016;58:291-309.
22. Hool LC. The L-type Ca(2+) channel as a potential mediator of pathology during alterations in cellular redox state. Heart Lung Circ 2009;18:3-10.
23. Hool LC, Arthur PG. Decreasing cellular hydrogen peroxide with catalase mimics the effects of hypoxia on the sensitivity of the L-type Ca2+ channel to β-adrenergic receptor stimulation in cardiac myocytes. Circ Res 2002;91:601-9.
24. Hudasek K, Brown ST, Fearon IM. H2O2 regulates recombinant Ca2+ channel α1C subunits but does not mediate their sensitivity to acute hypoxia. Biochem Biophys Res Commun 2004;318:135-41.
25. Mossakowski AA, Pohlan J, Bremer D, Lindquist R, Millward JM, et al. Tracking CNS and systemic sources of oxidative stress during the course of chronic neuroinflammation. Acta Neuropathol 2015;130:799-814.
26. Benned-Jensen T, Christensen RK, Denti F, Perrier JF, Rasmussen HB, et al. Live imaging of Kv7.2/7.3 cell surface dynamics at the axon initial segment: high steady-state stability and calpain-dependent excitotoxic downregulation revealed. J Neurosci 2016;36:2261-6.
27. Clark KC, Josephson A, Benusa SD, Hartley RK, Baer M, et al. Compromised axon initial segment integrity in EAE is preceded by microglial reactivity and contact. Glia 2016;64:1190-1209.
28. Benusa SD, George NM, Sword BA, DeVries GH, Dupree JL. Acute neuroinflammation induces AIS structural plasticity in a NOX2-dependent manner. J Neuroinflammation 2017;14:116.
29. Clark K, Sword BA, Dupree JL. Oxidative stress induces disruption of the axon initial segment. ASN Neuro 2017;9:1759091417745426.
30. Del Puerto A, Fronzaroli-Molinieres L, Perez-Alvarez MJ, Giraud P, Carlier E, et al. ATP-P2X7 receptor modulates axon initial segment composition and function in physiological conditions and brain injury. Cereb Cortex 2015;25:2282-94.
31. Evans MD, Sammons RP, Lebron S, Dumitrescu AS, Watkins TB, et al. Calcineurin signaling mediates activity-dependent relocation of the axon initial segment. J Neurosci 2013;33:6950-63.
32. Schafer DP, Jha S, Liu F, Akella T, McCullough LD, et al. Disruption of the axon initial segment cytoskeleton is a new mechanism for neuronal injury. J Neurosci 2009;29:13242-54.
33. Bilimoria PM, Stevens B. Microglia function during brain development: new insights from animal models. Brain Res 2015;1617:7-17.
34. Bordt EA, Ceasrine AM, Bilbo SD. Microglia and sexual differentiation of the developing brain: a focus on ontogeny and intrinsic factors. Glia 2019; Epub ahead of print. doi: 10.1002/glia.23753.
35. Kato G, Inada H, Wake H, Akiyoshi R, Miyamoto A, et al. Microglial contact prevents excess depolarization and rescues neurons from excitotoxicity. eNeuro 2016;3.
36. Li Y, Du XF, Liu CS, Wen ZL, Du JL. Reciprocal regulation between resting microglial dynamics and neuronal activity in vivo. Dev Cell 2012;23:1189-202.
37. Cserép C, Pósfai B, Lénárt N, Fekete R, László ZI, et al. Microglia monitor and protect neuronal function through specialized somatic purinergic junctions. Science 2020;367:528-37.
38. Miyamoto A, Wake H, Ishikawa AW, Eto K, Shibata K, et al. Microglia contact induces synapse formation in developing somatosensory cortex. Nat Commun 2016;7:12540.
39. Eyo UB, Murugan M, Wu LJ. Microglia-neuron communication in epilepsy. Glia 2017;65:5-18.
40. Eyo UB, Wu LJ. Bidirectional microglia-neuron communication in the healthy brain. Neural Plast 2013;2013:456857.
41. Kettenmann H, Kirchhoff F, Verkhratsky A. Microglia: new roles for the synaptic stripper. Neuron 2013;77:10-8.
42. Schafer DP, Lehrman EK, Kautzman AG, Koyama R, Mardinly AR, et al. Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. Neuron 2012;74:691-705.
43. Tremblay MÈ, Lowery RL, Majewska AK. Microglial interactions with synapses are modulated by visual experience. PLoS Biol 2010;8:e1000527.
44. Weinhard L, di Bartolomei G, Bolasco G, Machado P, Schieber NL, et al. Microglia remodel synapses by presynaptic trogocytosis and spine head filopodia induction. Nat Commun 2018;9:1228.
45. Baalman K, Marin MA, Ho TS, Godoy M, Cherian L, et al. Axon initial segment-associated microglia. J Neurosci 2015;35:2283-92.
46. Catale C, Gironda S, Iacono LL, Carola V. Microglial function in the effects of early-life stress on brain and behavioral development. J Clin Med 2020;9:E468.
47. Koellhoffer EC, McCullough LD, Ritzel RM. Old maids: aging and its impact on microglia function. Int J Mol Sci 2017;18:769.
48. Akhmetzyanova E, Kletenkov K, Mukhamedshina Y, Rizvanov A. Different approaches to modulation of microglia phenotypes after spinal cord injury. Front Syst Neurosci 2019;13:37.
49. Gaudet AD, Fonken LK. Glial cells shape pathology and repair after spinal cord injury. Neurotherapeutics 2018;15:554-77.
50. Gorse KM, Lafrenaye AD. The importance of inter-species variation in traumatic brain injury-induced alterations of microglial-axonal interactions. Front Neurol 2018;9:778.
51. Lafrenaye AD. Physical interactions between activated microglia and injured axons: do all contacts lead to phagocytosis? Neural Regen Res 2016;11:538-40.
52. Lafrenaye AD, Todani M, Walker SA, Povlishock JT. Microglia processes associate with diffusely injured axons following mild traumatic brain injury in the micro pig. J Neuroinflammation 2015;12:186.
53. Bitsch A, Schuchardt J, Bunkowski S, Kuhlmann T, Brück W. Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation. Brain 2000;123:1174-83.
54. Hemmer B, Kerschensteiner M, Korn T. Role of the innate and adaptive immune responses in the course of multiple sclerosis. Lancet Neurol 2015;14:406-19.

55. Sørensen TL, Ransohoff RM. Etiology and pathogenesis of multiple sclerosis. Semin Neurol 1998;18:287-94.
56. Trebst C, Sørensen TL, Kivisäkk P, Cathcart MK, Hesselgesser J, et al. CCR1+/CCR5+ mononuclear phagocytes accumulate in the central nervous system of patients with multiple sclerosis. Am J Pathol 2001;159:1701-10.
57. Thompson KK, Tsirka SE. The diverse roles of microglia in the neurodegenerative aspects of central nervous system (CNS) autoimmunity. Int J Mol Sci 2017;18:504.
58. Bitsch A, Wegener C, da Costa C, Bunkowski S, Reimers CD, et al. Lesion development in Marburg's type of acute multiple sclerosis: from inflammation to demyelination. Mult Scler 1999;5:138-46.
59. Ferguson B, Matyszak MK, Esiri MM, Perry VH. Axonal damage in acute multiple sclerosis lesions. Brain 1997;120:393-9.
60. Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mörk S, et al. Axonal transection in the lesions of multiple sclerosis. N Engl J Med 1998;338:278-85.
61. Reich DS, Lucchinetti CF, Calabresi PA. Multiple sclerosis. N Engl J Med 2018;378:169-80.
62. Bjartmar C, Kinkel RP, Kidd G, Rudick RA, Trapp BD. Axonal loss in normal-appearing white matter in a patient with acute MS. Neurology 2001;57:1248-52.
63. Kornek B, Lassmann H. Axonal pathology in multiple sclerosis. A historical note. Brain Pathol 1999;9:651-6.
64. Lassmann H, van Horssen J. The molecular basis of neurodegeneration in multiple sclerosis. FEBS Lett 2011;585:3715-23.
65. Black JA, Newcombe J, Trapp BD, Waxman SG. Sodium channel expression within chronic multiple sclerosis plaques. J Neuropathol Exp Neurol 2007;66:828-37.
66. Dutta R, Trapp BD. Mechanisms of neuronal dysfunction and degeneration in multiple sclerosis. Prog Neurobiol 2011;93:1-12.
67. Howell OW, Rundle JL, Garg A, Komada M, Brophy PJ, et al. Activated microglia mediate axoglial disruption that contributes to axonal injury in multiple sclerosis. J Neuropathol Exp Neurol 2010;69:1017-33.
68. Peterson JW, Bö L, Mörk S, Chang A, Trapp BD. Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions. Ann Neurol 2001;50:389-400.
69. Pomicter AD, Shroff SM, Fuss B, Sato-Bigbee C, Brophy PJ, et al. Novel forms of neurofascin 155 in the central nervous system: alterations in paranodal disruption models and multiple sclerosis. Brain 2010;133:389-405.
70. Waxman SG. Axonal dysfunction in chronic multiple sclerosis: meltdown in the membrane. Ann Neurol 2008;63:411-3.
71. Calabrese M, Reynolds R, Magliozzi R, Castellaro M, Morra A, et al. Regional distribution and evolution of gray matter damage in different populations of multiple sclerosis patients. PloS One 2015;10:e0135428.
72. Ajami B, Bennett JL, Krieger C, McNagny KM, Rossi FM. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. Nat Neurosci 2011;14:1142-9.
73. Huitinga I, van Rooijen N, de Groot CJ, Uitdehaag BM, Dijkstra CD. Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages. J Exp Med 1990;172:1025-33.
74. Huitinga I, Damoiseaux JG, Döpp EA, Dijkstra CD. Treatment with anti-CR3 antibodies ED7 and ED8 suppresses experimental allergic encephalomyelitis in Lewis rats. Eur J Immunol 1993;23:709-15.
75. Ransohoff RM. Animal models of multiple sclerosis: the good, the bad and the bottom line. Nat Neurosci 2012;15:1074-7.
76. Denic A, Johnson AJ, Bieber AJ, Warrington AE, Rodriguez M, et al. The relevance of animal models in multiple sclerosis research. Pathophysiology 2011;18:21-9.
77. Dupree JL, Mason JL, Marcus JR, Stull M, Levinson R, et al. Oligodendrocytes assist in the maintenance of sodium channel clusters independent of the myelin sheath. Neuron Glia Biol 2004;1:179-92.
78. Torre-Fuentes L, Moreno-Jiménez L, Pytel V, Matías-Guiu JA, Gómez-Pinedo U, et al. Experimental models of demyelination and remyelination. Neurologia 2017:S0213-4853:30236.
79. Beeton C, Garcia A, Chandy KG. Induction and clinical scoring of chronic-relapsing experimental autoimmune encephalomyelitis. J Vis Exp 2007:224.
80. Kipp M, Nyamoya S, Hochstrasser T, Amor S. Multiple sclerosis animal models: a clinical and histopathological perspective. Brain Pathol 2017;27:123-37.
81. Williams KC, Ulvestad E, Hickey WF. Immunology of multiple sclerosis. Clin Neurosci 1994;2:229-45.
82. Buttermore ED, Thaxton CL, Bhat MA. Organization and maintenance of molecular domains in myelinated axons. J Neurosci Res 2013;91:603-22.
83. Bhat MA, Rios JC, Lu Y, Garcia-Fresco GP, Ching W, et al. Axon-glia interactions and the domain organization of myelinated axons requires neurexin IV/Caspr/Paranodin. Neuron 2001;30:369-83.
84. Dupree JL, Girault JA, Popko B. Axo-glial interactions regulate the localization of axonal paranodal proteins. J Cell Biol 1999;147:1145-52.
85. Ishibashi T, Ikenaka K, Shimizu T, Kagawa T, Baba H. Initiation of sodium channel clustering at the node of Ranvier in the mouse optic nerve. Neurochem Res 2003;28:117-25.
86. Pillai AM, Thaxton C, Pribisko AL, Cheng JG, Dupree JL, et al. Spatiotemporal ablation of myelinating glia-specific neurofascin (Nfasc NF155) in mice reveals gradual loss of paranodal axoglial junctions and concomitant disorganization of axonal domains. J Neurosci Res 2009;87:1773-93.
87. Rasband MN, Peles E, Trimmer JS, Levinson SR, Lux SE, et al. Dependence of nodal sodium channel clustering on paranodal axoglial contact in the developing CNS. J Neurosci 1999;19:7516-28.
88. Rosenbluth J, Dupree JL, Popko B. Nodal sodium channel domain integrity depends on the conformation of the paranodal junction, not on the presence of transverse bands. Glia 2003;41:318-25.
89. Suzuki A, Hoshi T, Ishibashi T, Hayashi A, Yamaguchi Y, et al. Paranodal axoglial junction is required for the maintenance of the Nav1.6-type sodium channel in the node of Ranvier in the optic nerves but not in peripheral nerve fibers in the sulfatide-deficient mice. Glia 2004;46:274-83.

90. Coman I, Aigrot MS, Seilhean D, Reynolds R, Girault JA, et al. Nodal, paranodal and juxtaparanodal axonal proteins during demyelination and remyelination in multiple sclerosis. Brain 2006;129:3186-95.
91. Zoupi L, Markoullis K, Kleopa KA, Karagogeos D. Alterations of juxtaparanodal domains in two rodent models of CNS demyelination. Glia 2013;61:1236-49.
92. Prinz M, Priller J, Sisodia SS, Ransohoff RM. Heterogeneity of CNS myeloid cells and their roles in neurodegeneration. Nat Neurosci 2011;14:1227-35.
93. Steinman L, Martin R, Bernard C, Conlon P, Oksenberg JR. Multiple sclerosis: deeper understanding of its pathogenesis reveals new targets for therapy. Annu Rev Neurosci 2002;25:491-505.
94. Fife BT, Huffnagle GB, Kuziel WA, Karpus WJ. CC chemokine receptor 2 is critical for induction of experimental autoimmune encephalomyelitis. J Exp Med 2000;192:899-905.
95. Izikson L, Klein RS, Charo IF, Weiner HL, Luster AD. Resistance to experimental autoimmune encephalomyelitis in mice lacking the CC chemokine receptor (CCR)2. J Exp Med 2000;192:1075-80.
96. Buffington SA, Rasband MN. The axon initial segment in nervous system disease and injury. Eur J Neurosci 2011;34:1609-19.
97. Hedstrom KL, Ogawa Y, Rasband MN. AnkyrinG is required for maintenance of the axon initial segment and neuronal polarity. J Cell Biol 2008;183:635-40.
98. Jenkins SM, Bennett V. Developing nodes of Ranvier are defined by ankyrin-G clustering and are independent of paranodal axoglial adhesion. Proc Natl Acad Sci U S A 2002;99:2303-8.
99. Hartley RK. Differential reactivity of microglia in two mouse models of multiple sclerosis. Theses Diss 2016.
100. Inayat MS, El-Amouri IS, Bani-Ahmad M, Elford HL, Gallicchio VS, et al. Inhibition of allogeneic inflammatory responses by the Ribonucleotide Reductase Inhibitors, Didox and Trimidox. J Inflamm (Lond) 2010;7:43.
101. Matsebatlela TM, Anderson AL, Gallicchio VS, Elford H, Rice CD. 3,4-Dihydroxy-benzohydroxamic acid (Didox) suppresses pro-inflammatory profiles and oxidative stress in TLR4-activated RAW264.7 murine macrophages. Chem Biol Interact 2015;233:95-105.
102. Turchan J, Pocernich CB, Gairola C, Chauhan A, Schifitto G, et al. Oxidative stress in HIV demented patients and protection ex vivo with novel antioxidants. Neurology 2003;60:307-14.
103. Hamada MS, Kole MH. Myelin loss and axonal ion channel adaptations associated with gray matter neuronal hyperexcitability. J Neurosci 2015;35:7272-86.
104. Evans MD, Dumitrescu AS, Kruijssen DLH, Taylor SE, Grubb MS. Rapid modulation of axon initial segment length influences repetitive spike firing. Cell Rep 2015;13:1233-45.
105. Grubb MS, Burrone J. Activity-dependent relocation of the axon initial segment fine-tunes neuronal excitability. Nature 2010;465:1070-4.
106. Kuba H, Adachi R, Ohmori H. Activity-dependent and activity-independent development of the axon initial segment. J Neurosci 2014;34:3443-53.
107. Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, et al. Synaptic pruning by microglia is necessary for normal brain development. Science 2011;333:1456-8.
108. Rogers JT, Morganti JM, Bachstetter AD, Hudson CE, Peters MM, et al. CX3CR1 deficiency leads to impairment of hippocampal cognitive function and synaptic plasticity. Version 2. J Neurosci 2011;31:16241-50.
109. Zhan Y, Paolicelli RC, Sforazzini F, Weinhard L, Bolasco G, et al. Deficient neuron-microglia signaling results in impaired functional brain connectivity and social behavior. Nat Neurosci 2014;17:400-6.
110. Coronado VG, McGuire LC, Sarmiento K, Bell J, Lionbarger MR, et al. Trends in traumatic brain injury in the U.S. and the public health response: 1995-2009. J Safety Res 2012;43:299-307.
111. Gardner RC, Yaffe K. Epidemiology of mild traumatic brain injury and neurodegenerative disease. Mol Cell Neurosci 2015;66:75-80.
112. Langlois JA, Rutland-Brown W, Wald MM. The epidemiology and impact of traumatic brain injury: a brief overview. J Head Trauma Rehabil 2006;21:375-8.
113. Taylor CA, Bell JM, Breiding MJ, Xu L. Traumatic brain injury-related emergency department visits, hospitalizations, and deaths - United States, 2007 and 2013. MMWR Surveill Summ 2017;66:1-16.
114. Coughlin JM, Wang Y, Minn I, Bienko N, Ambinder EB, et al. Imaging of glial cell activation and white matter integrity in brains of active and recently retired national football league players. JAMA Neurol 2017;74:67-74.
115. Das M, Mohapatra S, Mohapatra SS. New perspectives on central and peripheral immune responses to acute traumatic brain injury. J Neuroinflammation 2012;9:236.
116. Kelley BJ, Lifshitz J, Povlishock JT. Neuroinflammatory responses after experimental diffuse traumatic brain injury. J Neuropathol Exp Neurol 2007;66:989-1001.
117. Morganti-Kossmann MC, Satgunaseelan L, Bye N, Kossmann T. Modulation of immune response by head injury. Injury 2007;38:1392-400.
118. Nizamutdinov D, Shapiro LA. Overview of traumatic brain injury: an immunological context. Brain Sci 2017;7.
119. Velázquez A, Ortega M, Rojas S, González-Oliván FJ, Rodríguez-Baeza A. Widespread microglial activation in patients deceased from traumatic brain injury. Brain Inj 2015;29:1126-33.
120. Coughlin JM, Wang Y, Munro CA, Ma S, Yue C, et al. Neuroinflammation and brain atrophy in former NFL players: an in vivo multimodal imaging pilot study. Neurobiol Dis 2015;74:58-65.
121. Ramlackhansingh AF, Brooks DJ, Greenwood RJ, Bose SK, Turkheimer FE, et al. Inflammation after trauma: microglial activation and traumatic brain injury. Ann Neurol 2011;70:374-83.
122. Zhou Y, Lui YW, Zuo XN, Milham MP, Reaume J, et al. Characterization of thalamo-cortical association using amplitude and connectivity of functional MRI in mild traumatic brain injury. J Magn Reson Imaging 2014;39:1558-68.
123. Boche D, Perry VH, Nicoll JA. Review: activation patterns of microglia and their identification in the human brain. Neuropathol Appl

Neurobiol. 2013;39:3-18.
124. Engel S, Schluesener H, Mittelbronn M, Seid K, Adjodah D, et al. Dynamics of microglial activation after human traumatic brain injury are revealed by delayed expression of macrophage-related proteins MRP8 and MRP14. Acta Neuropathol 2000;100:313-22.
125. Johnson VE, Stewart JE, Begbie FD, Trojanowski JQ, Smith DH, et al. Inflammation and white matter degeneration persist for years after a single traumatic brain injury. Brain 2013;136:28-42.
126. Engel S, Isenmann S, Ständer M, Rieger J, Bähr M, et al. Inhibition of experimental rat glioma growth by decorin gene transfer is associated with decreased microglial infiltration. J Neuroimmunol 1999;99:13-8.
127. Marklund N. Rodent models of traumatic brain injury: methods and challenges. Methods Mol Biol 2016;1462:29-46.
128. Xiong Y, Mahmood A, Chopp M. Animal models of traumatic brain injury. Nat Rev Neurosci 2013;14:128-42.
129. Donat CK, Scott G, Gentleman SM, Sastre M. Microglial activation in traumatic brain injury. Front Aging Neurosci 2017;9:208.
130. Gensel JC, Zhang B. Macrophage activation and its role in repair and pathology after spinal cord injury. Brain Res 2015;1619:1-11.
131. Kigerl KA, Gensel JC, Ankeny DP, Alexander JK, Donnelly DJ, et al. Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. J Neurosci 2009;29:13435-44.
132. Loane DJ, Kumar A, Stoica BA, Cabatbat R, Faden AI. Progressive neurodegeneration after experimental brain trauma: association with chronic microglial activation. J Neuropathol Exp Neurol 2014;73:14-29.
133. Jassam YN, Izzy S, Whalen M, McGavern DB, El Khoury J. Neuroimmunology of traumatic brain injury: time for a paradigm shift. Neuron 2017;95:1246-65.
134. Tapp ZM, Godbout JP, Kokiko-Cochran ON. A tilted axis: maladaptive inflammation and HPA axis dysfunction contribute to consequences of TBI. Front Neurol 2019;10:345.
135. Zanier ER, Marchesi F, Ortolano F, Perego C, Arabian M, et al. Fractalkine receptor deficiency is associated with early protection but late worsening of outcome following brain trauma in mice. J Neurotrauma 2016;33:1060-72.
136. Weber MD, McKim DB, Niraula A, Witcher KG, Yin W, et al. The influence of microglial elimination and repopulation on stress sensitization induced by repeated social defeat. Biol Psychiatry 2019;85:667-78.
137. d'Avila JC, Lam TI, Bingham D, Shi J, Won SJ, et al. Microglial activation induced by brain trauma is suppressed by post-injury treatment with a PARP inhibitor. J Neuroinflammation 2012;9:31.
138. Siopi E, Llufriu-Dabén G, Fanucchi F, Plotkine M, Marchand-Leroux C, et al. Evaluation of late cognitive impairment and anxiety states following traumatic brain injury in mice: the effect of minocycline. Neurosci Lett 2012;511:110-5.
139. Eyo UB, Gu N, De S, Dong H, Richardson JR, et al. Modulation of microglial process convergence toward neuronal dendrites by extracellular calcium. J Neurosci 2015;35:2417-22.
140. Garrido-Mesa N, Zarzuelo A, Gálvez J. Minocycline: far beyond an antibiotic. Br J Pharmacol. 2013;169:337-52.
141. Homsi S, Federico F, Croci N, Palmier B, Plotkine M, et al. Minocycline effects on cerebral edema: relations with inflammatory and oxidative stress markers following traumatic brain injury in mice. Brain Res 2009;1291:122-32.
142. Sanchez Mejia RO, Ona VO, Li M, Friedlander RM. Minocycline reduces traumatic brain injury-mediated caspase-1 activation, tissue damage, and neurological dysfunction. Neurosurgery 2001;48:1393-9.
143. Stirling DP, Khodarahmi K, Liu J, McPhail LT, McBride CB, et al. Minocycline treatment reduces delayed oligodendrocyte death, attenuates axonal dieback, and improves functional outcome after spinal cord injury. J Neurosci 2004;24:2182-90.
144. Meythaler J, Fath J, Fuerst D, Zokary H, Freese K, et al. Safety and feasibility of minocycline in treatment of acute traumatic brain injury. Brain Inj 2019;33:679-89.
145. Bye N, Habgood MD, Callaway JK, Malakooti N, Potter A, et al. Transient neuroprotection by minocycline following traumatic brain injury is associated with attenuated microglial activation but no changes in cell apoptosis or neutrophil infiltration. Exp Neurol 2007;204:220-33.
146. Hanlon LA, Raghupathi R, Huh JW. Differential effects of minocycline on microglial activation and neurodegeneration following closed head injury in the neonate rat. Exp Neurol 2017;290:1-14.
147. Lopez-Rodriguez AB, Siopi E, Finn DP, Marchand-Leroux C, Garcia-Segura LM, et al. CB1 and CB2 cannabinoid receptor antagonists prevent minocycline-induced neuroprotection following traumatic brain injury in mice. Cereb Cortex 2015;25:35-45.
148. Simon DW, Aneja RK, Alexander H, Bell MJ, Bayır H, et al. Minocycline attenuates high mobility group box 1 translocation, microglial activation, and thalamic neurodegeneration after traumatic brain injury in post-natal day 17 Rats. J Neurotrauma 2018;35:130-8.
149. Bachstetter AD, Zhou Z, Rowe RK, Xing B, Goulding DS, et al. MW151 inhibited IL-1β levels after traumatic brain injury with no effect on microglia physiological responses. PLoS One 2016;11:e0149451.
150. Witcher KG, Bray CE, Dziabis JE, McKim DB, Benner BN, et al. Traumatic brain injury-induced neuronal damage in the somatosensory cortex causes formation of rod-shaped microglia that promote astrogliosis and persistent neuroinflammation. Glia 2018;66:2719-36.
151. Henry RJ, Ritzel RM, Barrett JP, Doran SJ, Jiao Y, et al. Microglial depletion with CSF1R inhibitor during chronic phase of experimental traumatic brain injury reduces neurodegeneration and neurological deficits. J Neurosci 2020:2402-19.
152. Büki A, Povlishock JT. All roads lead to disconnection?--Traumatic axonal injury revisited. Acta Neurochir (Wien) 2006;148:181-93.
153. Gentleman SM, Nash MJ, Sweeting CJ, Graham DI, Roberts GW. Beta-amyloid precursor protein (beta APP) as a marker for axonal injury after head injury. Neurosci Lett 1993;160:139-44.
154. Johnson VE, Stewart W, Weber MT, Cullen DK, Siman R, et al. SNTF immunostaining reveals previously undetected axonal pathology in traumatic brain injury. Acta Neuropathol 2016;131:115-35.
155. Povlishock JT, Erb DE, Astruc J. Axonal response to traumatic brain injury: reactive axonal change, deafferentation, and neuroplasticity. J Neurotrauma 1992;9:S189-200.
156. Sherriff FE, Bridges LR, Gentleman SM, Sivaloganathan S, Wilson S. Markers of axonal injury in post mortem human brain. Acta

Neuropathol 1994;88:433-9.
157. Koliatsos VE, Alexandris AS. Wallerian degeneration as a therapeutic target in traumatic brain injury. Curr Opin Neurol 2019;32:786-95.
158. Stoll G, Müller HW. Nerve injury, axonal degeneration and neural regeneration: basic insights. Brain Pathol 1999;9:313-25.
159. Yin TC, Voorhees JR, Genova RM, Davis KC, Madison AM, et al. Acute axonal degeneration drives development of cognitive, motor, and visual deficits after blast-mediated traumatic brain injury in mice. eNeuro 2016;3.
160. Fu R, Shen Q, Xu P, Luo JJ, Tang Y. Phagocytosis of microglia in the central nervous system diseases. Mol. Neurobiol 2014;49:1422-34.
161. Neumann H, Kotter MR, Franklin RJM. Debris clearance by microglia: an essential link between degeneration and regeneration. Brain J Neurol 2009;132:288-95.
162. Bechmann I, Nitsch R. Astrocytes and microglial cells incorporate degenerating fibers following entorhinal lesion: a light, confocal, and electron microscopical study using a phagocytosis-dependent labeling technique. Glia 1997;20:145-54.
163. Wang J, Fox MA, Povlishock JT. Diffuse traumatic axonal injury in the optic nerve does not elicit retinal ganglion cell loss. J Neuropathol Exp Neurol 2013;72:768-81.
164. Holloway OG, Canty AJ, King AE, Ziebell JM. Rod microglia and their role in neurological diseases. Semin Cell Dev Biol 2019;94:96-103.
165. Graeber MB. Changing face of microglia. Science 2010;330:783-8.
166. Cao T, Thomas TC, Ziebell JM, Pauly JR, Lifshitz J. Morphological and genetic activation of microglia after diffuse traumatic brain injury in the rat. Neuroscience 2012;225:65-75.
167. Thomas TC, Ogle SB, Rumney BM, May HG, Adelson PD, et al. Does time heal all wounds? Experimental diffuse traumatic brain injury results in persisting histopathology in the thalamus. Behav Brain Res 2018;340:137-46.
168. Ziebell JM, Taylor SE, Cao T, Harrison JL, Lifshitz J. Rod microglia: elongation, alignment, and coupling to form trains across the somatosensory cortex after experimental diffuse brain injury. J Neuroinflammation 2012;9:247.
169. Bachstetter AD, Rowe RK, Kaneko M, Goulding D, Lifshitz J, et al. The p38α MAPK regulates microglial responsiveness to diffuse traumatic brain injury. J Neurosci 2013;33:6143-53.
170. Greer JE, Hånell A, McGinn MJ, Povlishock JT. Mild traumatic brain injury in the mouse induces axotomy primarily within the axon initial segment. Acta Neuropathol 2013;126:59-74.
171. Lifshitz J, Kelley BJ, Povlishock JT. Perisomatic thalamic axotomy after diffuse traumatic brain injury is associated with atrophy rather than cell death. J Neuropathol Exp Neurol 2007;66:218-29.
172. Wofford KL, Harris JP, Browne KD, Brown DP, Grovola MR, et al. Rapid neuroinflammatory response localized to injured neurons after diffuse traumatic brain injury in swine. Exp Neurol 2017;290:85-94.
173. Szalay G, Martinecz B, Lénárt N, Környei Z, Orsolits B, et al. Microglia protect against brain injury and their selective elimination dysregulates neuronal network activity after stroke. Nat Commun 2016;7:11499.
174. Eyo UB, Peng J, Murugan M, Mo M, Lalani A, et al. Regulation of physical microglia-neuron interactions by fractalkine signaling after status epilepticus. eNeuro 2017;3.
175. Eyo UB, Gu N, De S, Dong H, Richardson JR, et al. Modulation of microglial process convergence toward neuronal dendrites by extracellular calcium. J Neurosci 2015;35:2417-22.
176. Swiatkowski P, Murugan M, Eyo UB, Wang Y, Rangaraju S, et al. Activation of microglial P2Y12 receptor is required for outward potassium currents in response to neuronal injury. Neuroscience 2016;318:22-33.
177. Dissing-Olesen L, LeDue JM, Rungta RL, Hefendehl JK, Choi HB, et al. Activation of neuronal NMDA receptors triggers transient ATP-mediated microglial process outgrowth. J Neurosci 2014;34:10511-27.
178. Eyo UB, Peng J, Swiatkowski P, Mukherjee A, Bispo A, et al. Neuronal hyperactivity recruits microglial processes via neuronal NMDA receptors and microglial P2Y12 receptors after status epilepticus. J Neurosci 2014;34:10528-40.
179. Greer JE, Povlishock JT, Jacobs KM. Electrophysiological abnormalities in both axotomized and nonaxotomized pyramidal neurons following mild traumatic brain injury. J Neurosci 2012;32:6682-7.
180. Hånell A, Greer JE, Jacobs KM. Increased network excitability due to altered synaptic inputs to neocortical layer v intact and axotomized pyramidal neurons after mild traumatic brain injury. J Neurotrauma 2015;32:1590-8.
181. Ryu J, Horkayne-Szakaly I, Xu L, Pletnikova O, Leri F, et al. The problem of axonal injury in the brains of veterans with histories of blast exposure. Acta Neuropathol Commun 2014;2:153.
182. Oehmichen M, Theuerkauf I, Meissner C. Is traumatic axonal injury (AI) associated with an early microglial activation? Application of a double-labeling technique for simultaneous detection of microglia and AI. Acta Neuropathol 1999;97:491-4.
183. Schirmer L, Merkler D, König FB, Brück W, Stadelmann C. Neuroaxonal regeneration is more pronounced in early multiple sclerosis than in traumatic brain injury lesions. Brain Pathol Zurich Switz 2013;23:2-12.
184. Christman CW, Grady MS, Walker SA, Holloway KL, Povlishock JT. Ultrastructural studies of diffuse axonal injury in humans. J Neurotrauma 1994;11:173-86.
185. Christman CW, Salvant JB, Walker SA, Povlishock JT. Characterization of a prolonged regenerative attempt by diffusely injured axons following traumatic brain injury in adult cat: a light and electron microscopic immunocytochemical study. Acta Neuropathol 1997;94329-37.
186. Harris NG, Mironova YA, Hovda DA, Sutton RL. Pericontusion axon sprouting is spatially and temporally consistent with a growth-permissive environment after traumatic brain injury. J Neuropathol Exp Neurol 2010;69:139-54.
187. Venkatesan C, Chrzaszcz M, Cho N, Wainwright MS. Chronic upregulation of activated microglia immunoreactive for galectin-3/Mac-2 and nerve growth factor following diffuse axonal injury. J Neuroinflammation 2010;7:32.

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Improved quality of life and body satisfaction in response to activity-based therapy in adults with spinal cord injury

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Abstract

Aim: The decline in ambulation characteristic of spinal cord injury (SCI) dramatically modifies quality of life and body composition. To examine changes in quality of life, body satisfaction, and body composition in response to 6 months of activity-based therapy in individuals with spinal cord injury (SCI).

Methods: Men and women with complete or incomplete SCI (12 with tetraplegia and 13 with paraplegia; mean age and duration of injury of 35.8 ± 12.9 years and 3.8 ± 5.5 years, respectively) completed 6 months of activity-based therapy consisting of load bearing, locomotor training, whole-body resistance training, functional electrical stimulation, and assisted/unassisted walking for 8.5 ± 4.3 h/week. At baseline and at 3 and 6 months of training, body satisfaction, perceived quality of life, depression, and bodily pain were assessed using various questionnaires, and whole-body and regional fat mass and fat-free mass were determined with dual-energy X-ray absorptiometry. One-way analysis of variance with repeated measures was used to examine changes in outcome measures during the study.

Results: Measures of body satisfaction (+23%) and quality of life (+8%) were improved ($P < 0.05$) in response to training, yet no change in depression or pain was demonstrated ($P > 0.05$). Percent body fat increased ($P = 0.02$), yet no change ($P > 0.05$) was seen in whole-body or regional fat free mass.

Conclusion: Data suggest that chronic high-volume activity-based therapy enhances various indices of quality of life in men and women with SCI, but may be an ineffective approach to reduce fat deposition and increase muscle mass after SCI.

Keywords: Body satisfaction, depression, rehabilitation, fat free mass, paralysis

INTRODUCTION

The paralysis associated with spinal cord injury (SCI) compromises locomotion and, in turn, diminishes physical function and leads to various secondary complications including obesity and insulin resistance[1]. Above and beyond the physical effects of SCI is the onset of various psychological issues that may affect perceived quality of life (PQOL), which encompasses aspects of happiness, health, quality of family relationships, and financial and physical independence[2]. In fact, in men and women with SCI, well-being is negatively associated with self-reported overweight status and onset of secondary complications[3], which emphasizes the need to also consider participants' psychological state during rehabilitation. Data on persons with SCI demonstrate that aerobic and resistance exercise training improves PQOL compared to non-exercising controls[4], although PQOL was reduced after a decline in physical activity three months later[5]. Incidence of pain is also common in SCI, as, in a survey of 200 individuals, 25% had more severe pain and 44% reported that it interfered with daily activities[6]. Moreover, pain decreases quality of life and serves as a barrier to regular exercise participation in SCI[6]. In addition, more pain is consequent with greater incidence of depression[7], which is widely studied in persons with SCI[8], and incidence of substantial pain may negatively influence participation in rehabilitation. Moreover, depression is associated with body image disturbances and body dissatisfaction, which are common in persons with disability such as SCI[9]. With exception of one study showing positive effects on quality of life[4], little is known about efficacy of exercise training to modify quality of life and body satisfaction in SCI.

As stated above, quality of life is associated with body composition that is severely altered after SCI. For example, Moore *et al.*[10] reported that persons with chronic incomplete or complete SCI have 14%-32% lower muscle cross sectional area in the calf than controls. Moreover, lower extremity fat deposition is up to fourfold higher in SCI versus able-bodied persons[11], and there is a loss of lean body mass in the trunk and arms[12] that reduces physical function and capacity for day-to-day activities including wheelchair ambulation, all leading to altered quality of life. In addition, visceral adipose tissue is typically higher[13], which increases the risk of cardiovascular disease and diabetes[14,15]. Chronic aerobic and resistance exercise training can modify body composition in SCI, but the majority of data showing positive effects are from studies employing lower extremity functional electrical stimulation (FES) using leg cycling[16], resistance training[17], or subtetanic contractions[18,19] that are often inaccessible for people with SCI. Recent studies show no change in body composition in response to completion of chronic upper-body resistance training[13] or arm ergometry[20], two widely-used exercise modes in this population. Consequently, a recent review[21] concluded that there is insufficient evidence to support exercise training to modify body composition in SCI.

One rehabilitation modality frequently used in this population is activity-based therapy (ABT), which targets activation of the neuromuscular system below the injury level, with a goal of retraining the nervous system to recover a specific motor task such as locomotion[22]. Institution of ABT using modalities including load bearing, locomotor training, resistance training, and/or FES typically targets the paralyzed or partially paralyzed muscles and may also aid in prevention or treatment of various secondary health complications seen after SCI. In a randomized controlled trial, Jones *et al.*[23] showed that ABT significantly improved walking-related outcomes in persons with incomplete SCI. Nevertheless, exercise training such as ABT requiring voluntary contractions may have a lesser ability to modify body composition[24,25], although this is not universal[26]. Therefore, it is unclear if body composition can be modified with voluntary exercise in SCI such as ABT, and if any change in body composition is consequent with a change in quality of life. If ABT does not elicit changes in body composition, alternative exercise modalities may need to be implemented to improve this outcome in this population.

The current study examined effects of chronic ABT on perceived quality of life, pain, body satisfaction, and body composition in persons with SCI, which are related to subjective well being. Data from a recent review[27] of four studies containing 139 participants indicated no effect of ABT on quality of life, yet most included studies were of low quality. Data from Dolbow *et al.*[28], Sharif *et al.*[29], and Sadowsky *et al.*[30] show greater quality of life after FES training, yet it is unknown if similar results can occur with multi-modal ABT. It was hypothesized that ABT would improve measures of quality of life and body satisfaction, but not pain, fat free mass (FFM), or fat mass (FM) in men and women with SCI. Resultant data can be used towards identifying effective rehabilitation strategies targeting these outcomes, which are associated with physical and psychological function and overall well-being of persons with SCI.

METHODS

Design: this study was a within-subjects longitudinal project

Participants initiated 6 months of exercise training, which was continuously supervised by personnel trained in SCI rehabilitation at a local ABT facility. During a single session at baseline and at three and six months, a survey was completed to assess variables including body satisfaction, pain, depression, and quality of life, and participants underwent dual-energy X-ray absorptiometry (DXA) scans to determine whole body and regional body composition. This session was overseen by the primary investigator. In addition, a four-day food log was completed. Assessments were performed a minimum of 18 h after their last training session. Time of day was maintained across all trials within participants. Compliance to training was monitored each day by staff at the facility.

Participants

Twenty-nine men and women with SCI were initially recruited and subsequently initiated this study; they were recruited by word-of-mouth. They comprised a convenience sample of patients completing ABT at a local rehabilitation facility. Eighteen individuals were identified as Caucasian, six as Hispanic, three as Middle Eastern, and two as African-American. Initially, participants' health history was examined with a brief survey to ensure they met these inclusion criteria: complete or incomplete SCI, injury level lower than C2, non-ventilator dependent, and physician's permission to engage in an intense exercise program. Prospective participants were excluded if they completed regular ABT (locomotor training, assisted/unassisted walking, *etc.*) in the preceding 12 months or were unwilling to abstain from additional exercise other than wheelchair ambulation outside the study; lacked the physical function or had excess pain to complete training; were taking medications altering body composition or mental status (such as testosterone, antidepressants, and/or diabetic and/or cardiovascular drugs) other than calcium or vitamin D supplements; had medical conditions besides paralysis that alter body composition, such as diabetes or hyperthyroidism; were peri- or post-menopausal; or suffered an acute infection. Participants provided informed consent to participate in the study, which was approved by the University Institutional Review Board and was conducted in accordance with the Declaration of Helsinki.

Assessment of quality of life

Using an interview format, the primary investigator read each item to the participant and recorded his/her response, as not all participants could use pen and paper. Initially, the 10-item body satisfaction survey (BSS)[31] was completed, with scores ranging from -3 (very dissatisfied) to +3 (very satisfied). This survey has a range of scores from -30 to +30 and included questions pertaining to physical function (seven items) and appearance (BSS-A) (three items), which were scored separately. Next, participants were asked "how much bodily pain have you had in the last four weeks" and "how much did pain interfere with your normal work/day-to-day activities", which were scored from 1 (none) to 6 (very severe). These were taken from the Short Form-36 survey (SF-36) scale[32] and have been previously utilized in SCI[2]. The 11-item PQOL scale[2] containing four additional items related to quality of life in SCI[33] was then completed, in which participants were asked their degree of satisfaction on a scale of 0-100.

Subsequently, we asked participants questions regarding the severity of onset of secondary complications commonly seen in SCI including spasms, joint stiffness, constipation, urinary tract infection, pain, *etc.*, using the following scale previously developed in this population[34]: (1) "I don't have this problem"; (2) "Problem is not at all bothersome"; (3) "Problem is slightly bothersome"; (4) "Problem is moderately bothersome"; and (5) "Problem is greatly bothersome".

Lastly, the Center for Epidemiological Studies Depression scale[35] was completed. Participants were asked to rank on a 0 (rarely or none of the time) to 3 (most or all of the time) scale how they felt about each of 20 items during the last week. The overall score was the sum of all 20 items for a total possible score equal to 60. This survey is reliable and valid in persons with SCI[36].

Assessment of body composition

Participants arrived at the laboratory after an overnight fast (> 10 h) wearing exercise attire without metal. Initially, the participant was placed on the dual-energy X-ray absorptiometer (DXA software version 13.5, Lunar Prodigy Advance, GE Healthcare, Madison, WI, USA) for a few minutes to minimize muscle spasm. They were instructed to remain motionless and not talk during the scan, which was used to estimate whole-body and regional (arm, trunk, and legs) FM and FFM. Body weight (in kg) was calculated from the summation of FM, FFM, and bone mineral content. Body composition changes during the study were expressed in absolute units (kg). Analyses were performed by the same technician who followed standard quality control procedures developed by the manufacturer. Intraclass correlation coefficients and coefficients of variation for whole-body and regional determinations of FM and FFM obtained in five individuals with SCI measured three months apart were equal to 0.98 and 0.99 and 0.7% and 0.8%, respectively. In addition, waist circumference was obtained in duplicate in the supine position according to standardized procedures[37].

Assessment of dietary intake

Participants completed a four-day food log (including two weekend days) at baseline and at three and six months. They were encouraged to actively report all food and drink ingested (including supplements) each day with specific instructions to describe method of preparation, portion sizes, and brands where applicable. This information was reviewed during each visit and used to determine total caloric intake as well as fat, carbohydrate (CHO), and protein intake (in g) using a commercially-available website (http://ndb.nal.usda.gov/ndb/foods/list). They were asked to maintain their dietary practices during the study.

Intervention

Participants performed 2-3 h sessions of supervised ABT targeting the lower extremities (80% for those with tetraplegia and 100% for paraplegia) a minimum of two days/week to a maximum of five days/week. Activity-based therapy was shown to enhance motor gains in persons with chronic SCI[38]. This regimen elicits energy expenditure between 5 and 8 mL/kg/min[39], which is similar to circuit training and FES leg cycling[40,41] yet lower than arm ergometry, wheelchair ambulation[40], or exoskeleton-assisted walking[42]. ABT as performed in the current study consisted of these modalities as previously described[38,43]: 1.5-2.0 h/week of active assistive exercise, 1.5 h/week of upper/lower body and core resistance training, 1 h/week of load bearing, 30 min/week of arm/cycle ergometry, 1.0-2.0 h/week of gait training including assisted and unassisted walking as well as body weight-supported mechanized elliptical training, 10-30 min/week of vibration training, and 30 min/week of FES of the quadriceps, gluteals, and hamstrings. Training was individualized for each client based on their baseline function, and progression was instituted daily based on participant tolerance to training and level of adaptation. During the study, time performing active assistive exercises and passive gait training generally decreased while time performing resistance training and active gait training increased. Training volume differed across participants as rehabilitation costs were paid out-of-pocket.

Table 1. Physical characteristics of participants completing six months of activity-based therapy (*n* = 25)

Parameter	Mean (SD)	Range
Age (year)	35.8 ± 12.9	18-59
Height (cm)	178.7 ± 7.1	158-188
Mass (kg)	77.0 ± 13.1	53.7-101.0
DOI (year)	3.8 ± 5.5	0.2-10.0
Complete/incomplete	9/16	NA
Tetraplegia/paraplegia	12/13	NA
Injury level	NA	C5-L1
Gender (male/female)	22/3	

SD: standard deviation; cm: centimeters; kg, kilograms; DOI: duration of injury; NA: non-applicable

Data analysis

Data are reported as mean ± standard deviation (SD) and were analyzed using SPSS Version 20.0 (Chicago, IL). Initially, normality of all variables was examined. Two-way (one within-subjects factor representing training (zero, three, and six months) and one between-subjects factor including injury completeness, severity, and duration of injury as well as training volume) analysis of variance with repeated measures was used to examine changes in all variables in response to training. Overall, data were combined across participants as there were few baseline differences or group × time interactions in most outcome measures when variables including duration of injury, injury completeness, or injury severity were considered. The Greenhouse-Geisser correction was used to account for the sphericity assumption. If a significant *F* ratio was obtained, Tukey's *post hoc* test was used to identify differences between means. Partial eta-squared (η^2_p) was used as an estimate of effect size. Multiple regression was used to examine predictors of the change in body satisfaction and quality of life. Statistical significance was set at $P < 0.05$.

Data availability statement

De-identified data from this study are available upon request.

RESULTS

One woman and three men dropped out after one month ($n = 2$) and five months ($n = 2$) due to injury unrelated to training and moving out of the area. Our results are from 22 men and 3 women who completed six months of ABT and were assessed at baseline and at three and six months of training. Fourteen participants were within one year post-SCI. Participants' injury level included C5-C6 ($n = 7$), C4 ($n = 3$), T3-T4 ($n = 5$), T6-T10 ($n = 4$), T11 ($n = 1$), T12 ($n = 4$), and L1 ($n = 1$). Participant characteristics are demonstrated in Table 1. Adherence to training was equal to 100%. Training volume across participants ranged 4-17 h/week, with a mean value equal to 8.5 ± 4.3 h/week. However, when training volume was used as a between subjects factor in all analyses, there was no effect ($P > 0.05$) of this factor on our outcomes. Where applicable, we separated participants by low (< 8 h/week, $n = 14$) and high volume of ABT (> 8 h/week, $n = 11$).

Change in body satisfaction and pain in response to ABT

The results show a main effect of training on BSS ($P = 0.03$, $\eta^2_p = 0.13$) in that it increased during the study. *Post hoc* analyses showed that the six-month score was higher than at baseline by 0.60 (Table 2, d = 1.0). When injury completeness was used as a between-subjects factor, there was a main effect of training ($P = 0.04$, $\eta^2_p = 0.13$) and training × completeness interaction ($P = 0.02$, $\eta^2_p = 0.15$). *Post hoc* analyses showed that BSS increased from baseline to six months of training in persons with incomplete SCI (-0.87 ± 1.30 *vs.* +0.38 ± 1.40, d = 1.5) but not in complete SCI (0.68 ± 1.63 *vs.* 0.78 ± 1.32, d = 0.1). For BSS-A, there was a main effect of training ($P = 0.01$, $\eta^2_p = 0.17$) and *post hoc* analyses showed that three- (+0.8, d = 0.8) and six-month values (+0.9, d = 0.8) were greater than baseline. BSS-A was higher in persons with paraplegia versus tetraplegia ($P = 0.02$, $\eta^2_p = 0.19$), although no training × group interactions were found across injury duration, severity, or completeness. Pain was unchanged ($P = 0.67$) in response to training.

Table 2. Changes in indices of quality of life (mean ± SD) in response to six months of activity-based therapy in persons with SCI

Parameter	Zero months	Three months	Six months
BSS	-0.36 ± 1.62	0.22 ± 1.48	0.44 ± 1.38*
BSS-A	-0.82 ± 1.59	-0.01 ± 1.80*	0.04 ± 1.54*
PQOL	60.4 ± 18.2	59.7 ± 19.8	64.5 ± 20.6a
CESD	21.3 ± 6.9	20.8 ± 5.7	19.7 ± 5.3
Pain	4.5 ± 1.3	4.4 ± 1.4	4.5 ± 1.3
Don't have secondary complications (%)	48.1 ± 16.8	41.9 ± 21.7	43.0 ± 18.6
Bothersome secondary complications (%)	7.2 ± 9.9	4.4 ± 7.4	3.6 ± 5.6

BSS: body satisfaction survey; BSS-A: body satisfaction survey - appearance; PQOL: perceived quality of life; CESD: Center for Epidemiological Studies depression scale; SCI: spinal cord injury; SD: standard deviation. *$P < 0.05$ *vs.* zero-month value; [a]$P < 0.05$ *vs.* three-month value

Change in perceived quality of life and depression in response to ABT

Perceived quality of life differed with training ($P = 0.04$, $\eta^2_p = 0.11$) and *post hoc* analyses showed that the six-month value was higher than at three months [Table 2] by approximately five units (d = 0.8). Change in PQOL from baseline to six months was higher in persons with acute (+7.6 ± 12.9) or incomplete injury (+7.5 ± 11.2) compared to chronic (+0.7 ± 7.4) or complete injury (+0.9 ± 11.6), although it failed to reach significance ($P = 0.10$). The results show no change in depression ($P = 0.30$) from baseline to six months and there were no effects of injury level, completeness, or volume of physical activity on this response.

Regression data

Various two-predictor models were developed to identify the best predictors of change in PQOL and body satisfaction in response to training. A model ($r = 0.63$, $F = 7.05$, $P = 0.004$) consisting of age ($r = -0.41$, $P = 0.02$) and change in pain ($r = -0.48$, $P = 0.007$) explained 39% of the variance in change in PQOL. Although percent body fat was correlated with change in BSS ($r = 0.33$, $P = 0.049$), no significant models were found. For change in BSS-A, a significant model ($r = 0.504$, $F = 4.08$, $P = 0.03$) consisted of body fat ($r = 0.36$, $P = 0.03$) and baseline pain ($r = 0.27$, $P = 0.08$).

Change in body composition in response to ABT

Body composition results are revealed in Table 3. Body mass ($P = 0.30$) did not change but %BF (body fat) increased ($P = 0.02$, $\eta^2_p = 0.17$) from baseline to six months by 1%. Whole-body FFM did not change across time ($P = 0.11$), but there was a training × group interaction in that it declined by approximately 2 kg in individuals with complete SCI ($n = 9$, 50.8 ± 7.9 kg to 48.7 ± 7.0 kg), but did not change in participants with incomplete injury ($n = 16$, 47.7 ± 7.4 kg to 47.5 ± 7.5 kg). Leg FFM ($P = 0.88$), leg %BF ($P = 0.08$), and waist circumference ($P = 0.80$) were unchanged during the study. There was a tendency for trunk FFM to decline during the study ($P = 0.06$). Trunk %BF increased ($P = 0.03$), and *post hoc* analyses showed that three- and six-month values were higher by 1.2%-1.3% than at baseline. There were no differences in arm FFM ($P = 0.20$) or %BF ($P = 0.13$) during the study. Arm FFM was higher ($P = 0.003$) in persons with paraplegia versus tetraplegia. From baseline to six months of training, whole-body %BF declined by more than the coefficient of variation of the measure in 24% of participants, and 32% of participants showed increases in whole-body FFM.

Change in dietary intake

Data revealed that total energy intake declined from baseline (1769.1 ± 349.3 kcal, 1650.3 ± 410.4 kcal, and 1660.9 ± 366.9 kcal, $P = 0.03$), whereas fat (64.9 ± 15.0 g, 59.7 ± 15.8 g, and 60.6 ± 16.2 g, $P = 0.20$), CHO (211.6 ± 53.0 g, 197.2 ± 53.6 g, and 201.0 ± 54.5 g, $P = 0.17$), and protein intake (78.3 ± 21.1 g, 80.6 ± 26.0 g, and 74.2 ± 28.5 g, $P = 0.27$) were unaltered.

Table 3. Changes in body weight and body composition (mean ± SD) in response to six months of activity-based therapy in persons with SCI

Parameter	Zero months	Three months	Six months
Mass (kg)	76.3 ± 13.2	77.1 ± 13.1	77.0 ± 13.4
Whole-body FFM (kg)	48.8 ± 7.9	48.6 ± 7.4	47.9 ± 7.2
%BF	32.7 ± 12.2	33.6 ± 11.7*	33.8 ± 11.2*
WC (cm)	91.0 ± 13.0	90.7 ± 14.6	90.1 ± 13.4
Leg FFM (kg)	14.3 ± 3.0	14.3 ± 3.1	14.3 ± 2.9
Leg %BF	36.4 ± 11.9	37.3 ± 11.3	37.5 ± 10.8
Trunk FFM (kg)	24.2 ± 3.6	23.9 ± 3.3	23.6 ± 3.4
Trunk %BF	33.4 ± 13.5	34.6 ± 13.4*	34.7 ± 12.7*
Arm FFM (kg)	6.1 ± 1.9	6.3 ± 1.8	6.3 ± 1.9
Arm %BF	27.1 ± 13.7	27.6 ± 14.3	28.6 ± 13.2

kg: kilograms; FFM: fat-free mass; BF: body fat; WC: waist circumference; SD: standard deviation; SCI: spinal cord injury. *$P < 0.05$ *vs.* baseline

DISCUSSION

Despite no significant improvements in FFM or FM, bodily pain, or depression, individuals with SCI undergoing six months of ABT revealed small but significant increases in PQOL and body satisfaction. Although our data cannot explain what led to this improved quality of life, previous reports indicate that exercise improves sense of control and mastery that people have regarding their physical function[44]. Due to the potential link between quality of life and exercise participation in SCI[3], structuring exercise programs targeting these outcomes may help promote exercise adherence in this population.

Supporting our findings, improved PQOL has been reported in response to exercise training in SCI. In men and women at least one year post-SCI[4], nine months of resistance training and arm cycling improved PQOL, which was coincident with reduced pain, depression, and greater muscle strength and arm cycling performance that, in turn, might elicit an improved mental health profile. Similarly, improved PQOL occurred in patients undergoing 12 weeks of FES ambulation training despite no change in depression[29]. Nevertheless, in persons with complete SCI, 18 months of incorporation of ABT into daily activities had no effect on functional independence or quality of life (measured with the Short Form-36)[45]. Jones *et al.*[23] reported no change in quality of life despite increased walking speed in men and women with incomplete SCI undergoing six months of ABT. Potential explanations for no change in PQOL can be due to a ceiling effect or inclusion of participants with varying injury duration, as persons with chronic SCI may come to terms with their injury and may believe that it no longer alters their quality of life. Alternatively, we had many individuals within one year of injury who likely struggle with the challenges of acute SCI and report a low quality of life. Our ABT regime was also held in a facility providing social interactions to clients that also may enhance PQOL.

In 695 men and women with SCI[3], a greater incidence of pain and depression and lower life satisfaction was found in overweight versus normal weight individuals, likely due to greater difficulty in completing activities of daily living. Previous studies also suggest that body satisfaction may be higher[46] or lower[47] in persons with greater physical activity. Our body satisfaction values are lower than those reported in men approximately 15 years post-SCI[48], likely due to their more recent injury status and higher levels of body fat. Similar to our findings, 10 weeks of exercise training in a heterogeneous group of men and women with SCI improved functional and appearance-related body satisfaction[49]. Overall, various modes of exercise including ABT have the potential to improve body satisfaction in persons with SCI. Because of our small sample and lack of a control group, our results showing enhanced body satisfaction are preliminary and require further study to confirm.

Our results show no change in bodily pain, which may be due to the fact that many participants reported no or minimal pain at baseline. This finding opposes previous results; for example, in individuals with chronic SCI, 12 weeks of FES-ambulation training reduced bodily pain[29], similar to findings seen in response to nine months of resistance/aerobic training[4]. In persons with paraplegia, four months of circuit training reduced shoulder pain, which was consequent with increased total body strength[50]. In addition, a single bout of locomotor training may reduce pain perception in persons with incomplete SCI[51]. However, findings from one study[5] demonstrated no change in pain after nine months of aerobic and resistance training, which was seen as a positive response considering that non-exercising controls showed greater pain. Baseline pain was also associated with change in BSS-A, and our participants' change in pain was a significant predictor of change in PQOL, which supports previous findings[3]. As pain is related to exercise adherence[3,52], mobility[53], and onset of depressive symptoms[54], rehabilitation and fitness professionals should consider this outcome when treating persons with SCI who have elevated pain.

In the present study, we used the Center for Epidemiological Studies Depression scale to assess potential changes in depression in response to ABT. It is evident[55] that scores above 16 may identify individuals at risk for clinical depression. Although our participants' scores declined by two units from baseline to six months, this change was not significant. Our heterogeneous sample may have been too small to detect changes in depression considering that greater than 40 participants may be needed for adequate statistical power[55]. Moreover, examination of change in depression with between-subjects factors equal to injury severity, duration, completeness, and volume of training did not reveal any differences between groups. In another study[4], fewer depressive symptoms were noted after nine months of exercise training in individuals with SCI compared to non-exercising controls, although their baseline score did not indicate clinical depression and, in addition, the value did not increase from pre- to post-training.

Our data do not support the efficacy of ABT to improve body composition measured via DXA, as whole body and regional %BF increased and there was no change in FFM [Table 3]. However, the observed increase in %BF is minimal and may not be clinically meaningful in regards to enhancing risk of comorbidities associated with SCI. In contrast, decreased FM and increased FFM occurred[17,19] when long-term FES is performed by persons with acute as well as chronic paraplegia and tetraplegia. There are a few explanations for the lack of change in body composition in response to ABT. First, our ABT regime required more core and upper-body resistance training than exposure to FES, which may minimize potential for muscle hypertrophy. Second, energy expenditure of ABT is lower than other exercise modes[40], which may be insufficient to induce negative energy balance and thus weight or fat loss. In the present study, 56% of participants were less than or equal to 1 year post-injury, during which there is a considerable loss in FFM and rise in FM[56], and it could be that our minimal changes are a result of continued changes in body composition that were not slowed by our intervention. Third, despite wide use of DXA to assess body composition[16,57] and data showing DXA-derived increases in FFM and/or decreases in regional body fat in SCI in response to electrical stimulation training[19,58], its ability to detect small changes in FM or FFM after exercise training is less than magnetic resonance imaging. It is plausible that DXA should only be used in studies when relatively robust changes in energy balance and/or body composition are expected, such as those using high-volume FES-based exercise or manipulation of both exercise and dietary intake to improve health status in this population. Fourth, individual variability in these responses occurred, as FFM declined in persons with complete injury and was unchanged in men and women with incomplete injury. Overall, by itself ABT does not seem to induce significant changes in body composition, especially in persons with acute injury.

Our study had a few limitations. We used a convenience sample composed of individuals who were already completing ABT at the facility. A non-exercising control group was not recruited, thus we are uncertain if the changes seen in this study are truly due to exercise training. Participants differed in injury duration,

severity, and completeness that may reduce our ability to gauge the efficacy of ABT, especially in persons with acute SCI who are experiencing changes in body fat and FFM. However, in a previous study[4], regular physical activity improved various physical and psychological outcomes in persons with SCI irrespective of their level or completeness of injury. The Short Form-36 is widely used in populations including SCI to monitor changes in health-related quality of life[29]. However, other than its two pain-related items, we did not use it as it was found to be too burdensome on our sample. Although all participants received comprehensive ABT, the make-up and volume of said training varied based on participants' existing function, their progress through training, and, lastly, their ability to pay for training, which was not covered by insurance. This requirement to pay for training may have led to more favorable outcomes related to QOL. The latter factor led to different doses of training performed by each participant. However, there was no relationship between weekly volume of training and the change in %BF, FFM, or any of the psychological variables. In addition, the increases in PQOL and body satisfaction were evident irrespective of whether participants completed a low or high volume of ABT. These findings suggest that disparate volumes of training had little impact on our results. In addition, FES comprised a small portion of habitual training, which may have led to the non-significant changes in body composition. However, our study is strengthened by a sample size that is greater than those used in most studies examining PQOL. Our sample included persons of varied injury level and injury duration, which allows generalization of our findings to the entire population with SCI rather than one homogeneous group. In addition, we tracked food intake through dietary logs, and data showed minimal changes in energy intake during the study, which gives us greater confidence that observed changes in body composition were not due to variations in dietary patterns.

In conclusion, Six months of ABT slightly improved various indices of quality of life but did not induce changes in body fat or FFM. Pain was also associated with the changes in quality of life and body satisfaction observed in response to training. The changes seen in this study are small. Due to our small and heterogenous sample recruited by convenience, lack of a control group, and non-standardized training regimen, additional work is needed to confirm these data. Future studies should explore the potential for alternative modalities of exercise to enhance quality of life due to its relationship with exercise adherence in SCI.

DECLARATIONS

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Authors' contributions

Conceived the study: Astorino TA, Harness ET
Analyzed the data and wrote the final draft of the manuscript: Astorino TA, which Harness ET reviewed
ETH supervised all training sessions; whereas: Harness ET
Supervised all assessments: Astorino TA

Availability of data and materials

De-identified data could be made available to readers upon request.

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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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REFERENCES

1. Krassioukov A, Claydon VE. The clinical problems in cardiovascular control following spinal cord injury: an overview. Prog Brain Res 2006;152:223-9.
2. Patrick DL, Danis M, Southerland LI, Hong G. Quality of life following intensive care. J Gen Int Med 1988;3:218-23.
3. Hetz SP, Latimer AE, Arbour-Nicitopoulos KP, Martin Ginis KA. Secondary complications and subjective well-being in individuals with chronic spinal cord injury: associations with self-reported adiposity. Spinal Cord 2011;49:266-72.
4. Hicks AL, Martin KA, Ditor DS, Latimer AE, Craven C, et al. Long-term exercise training in persons with spinal cord injury: effects on strength, arm ergometry performance, and psychological well-being. Spinal Cord 2003;41:34-43.
5. Ditor DS, Latimer AE, Martin Ginis KA, Arbour KP, McCartney N, et al. Maintenance of exercise participation in individuals with spinal cord injury: effects on quality of life, stress, and pain. Spinal Cord 2003;41:446-50.
6. Nepomuceno C, Fine PR, Richards JS, Gowens H, Stover SL, et al. Pain in patients with spinal cord injury. Arch Phys Med Rehabil 1979;60:605-9.
7. Ataoğlu E, Tiftik T, Kara M, Tunç H, Ersöz M, et al. Effects of chronic pain on quality of life and depression in patients with spinal cord injury. Spinal Cord 2013;51:23-6.
8. Elliott TR, Frank RG. Depression following spinal cord injury. Arch Phys Med Rehabil 1996;77:816-23.
9. American Psychological Association. Diagnostic and statistical manual of mental disorders. 4th edn. Washington, DC: American Psychological Association; 1994.
10. Moore CD, Craven BC, Thabane L, Laing AC, Frank-Wilson AW, et al. Lower-extremity muscle atrophy and fat infiltration after chronic spinal cord injury. J Musculoskelet Neuronal Interact 2015;15:32-41.
11. Elder CP, Apple DF, Bickel CS, Meyer RA, Dudley GA. Intramuscular fat and glucose tolerance after spinal cord injury--a cross-sectional study. Spinal Cord 2004;42:711-6.
12. Fisher JA, McNelis MA, Gorgey AS, Dolbow DR, Loetz LL. Does upper extremity training influence body composition after spinal cord injury? Aging Dis 2015;6:271-81.
13. Gorgey AS, Mather KJ, Gater DR. Central adiposity associations to carbohydrate and lipid metabolism in individuals with complete motor spinal cord injury. Metabolism 2011;60:843-51.
14. Bauman WA, Spungen AM. Coronary heart disease in individuals with spinal cord injury: assessment of risk factors. Spinal Cord 2008;46:466-76.
15. Cragg JJ, Noonan VK, Dvorak M, Krassioukov A, Mancini GB, et al. Spinal cord injury and type 2 diabetes: results from a population health survey. Neurology 2013;81:164-8.
16. Griffin L, Decker MJ, Hwang JY, Wang B, Kitchen K, et al. Functional electrical stimulation cycling improves body composition, metabolic, and neural factors in persons with spinal cord injury. J Electromyogr Kinesiol 2009;19:614-22.
17. Gorgey AS, Shepherd C. Skeletal muscle hypertrophy and decreased intramuscular fat after unilateral resistance training in spinal cord injury: case report. J Spinal Cord Med 2011;33:90-5.
18. McCormack K, Carty A, Coughlan G, Crowe L, Caulfield B. The effects of a neuromuscular electrical stimulation training intervention on physiological measures in a spinal cord injured population. Physiother Ireland 2010;31:30-5.
19. Carty A, McCormack K, Coughlan GF, Crowe L, Caulfield B. Alterations in body composition and spasticity following subtetanic neuromuscular electrical stimulation training in spinal cord injury. J Rehabil Res Dev 2013;50:193-202.
20. Bresnahan JJ, Farkas GJ, Clasey JL, Yates JW, Gater DR. Arm crank ergometry improves cardiovascular disease risk factors and community mobility independent of body composition in high motor complete spinal cord injury. J Spinal Cord Med 2019;42:272-80.
21. Hicks AL, Martin Ginis KA, Pelletier CA, Ditor DS, Foulon B, et al. The effects of exercise training on physical capacity, strength, body composition and functional performance among adults with spinal cord injury: a systematic review. Spinal Cord 2011;49:1103-27.
22. Sadowsky CL, McDonald JW. Activity-based restorative therapies: concepts and applications in spinal cord injury-related neurorehabilitation. Dev Disabil Res Rev 2009;15:112-6.
23. Jones ML, Evans N, Tefertiller C, Backus D, Sweatman M, et al. Activity-based therapy for recovery of walking in individuals with chronic spinal cord injury: results from a randomized clinical trial. Arch Phys Med Rehabil 2014;95:2239-46.e2.
24. Ung RV, Lapointe NP, Rouleau P, Guertin PA. Non-assisted treadmill training does not improve motor recovery and body composition in spinal cord-transected mice. Spinal Cord 2010;48:750-5.
25. Giangregorio L, Craven C, Richards K, Kapadia N, Hitzig SL, et al. A randomized trial of functional electrical stimulation for walking in incomplete spinal cord injury: effects on body composition. J Spinal Cord Med 2012;35:351-60.
26. Rosety-Rodriguez M, Rosety I, Fornieles G, Rosety JM, Elosegui S, et al. A short-term arm-crank exercise program improved testosterone deficiency in adults with chronic spinal cord injury. Int Braz J Urol 2014;40:367-72.

27. Quel de Oliveira C, Refshauge K, Middleton J, de Jong L, Davis GM. Effects of activity-based therapy interventions on mobility, independence, and quality of life for people with spinal cord injuries: a systematic review and meta-analysis. J Neurotrauma 2017;34:1726-43.
28. Dolbow DR, Gorgey AS, Ketchum JM, Gater DR. Home-based functional electrical stimulation cycling enhances quality of life in individuals with spinal cord injury. Top Spinal Cord Inj Rehabil 2013;19:324-9.
29. Sharif H, Gammage K, Chun S, Ditor D. Effects of FES-ambulation training on locomotor function and health-related quality of life in individuals with spinal cord injury. Top Spinal Cord Inj Rehabil 2014;20:58-69.
30. Sadowsky CL, Hammond ER, Strohl AB, Commean PK, Eby SA, et al. Lower extremity functional electrical stimulation cycling promotes physical and functional recovery in chronic spinal cord injury. J Spinal Cord Med 2013;36:623-31.
31. Reboussin BA, Rejeski WJ, Martin KA, Callahan K, Dunn AL, et al. Correlates of satisfaction with body function and body appearance in middle- and older-age adults: the activity counseling trial (ACT). Psychol Health 2000;15:239-54.
32. Ware JE, Sherbourne CD. The MOS 36-item short form health survey (SF-36). Conceptual framework and item selection. Med Care 1992;30:473-83.
33. Gerhart KK, Johnson RL, Whiteneck GG. Health and psychosocial issues of individuals with incomplete and resolving spinal cord injuries. Paraplegia 1992;30:282-7.
34. Radloff JS. The CES-D scale: a self-report depression scale for research in the general population. Appl Psychol Measure 1977;1:385-401.
35. Miller WC, Anton HA, Townson AF. Measurement properties of the CESD scale among individuals with spinal cord injury. Spinal Cord 2008;46:287-92.
36. Dudley-Javoroski S, Shields RK. Assessment of physical function and secondary complications after complete spinal cord injury. Disabil Rehabil 2006;28:103-10.
37. Heyward VH. Advanced fitness assessment and exercise prescription. 5th Ed. Human Kinetics, Champaign, IL. 2006.
38. Harness ET, Yozbatiran N, Cramer SC. Effects of intense exercise in chronic spinal cord injury. Spinal Cord 2008;46:733-7.
39. Harness ET, Astorino TA. Acute energy cost of multi-modal activity-based therapy in persons with spinal cord injury. J Spinal Cord Med 2011;34:495-500.
40. Collins EG, Gater D, Kiratli J, Butler J, Hanson K, et al. Energy cost of physical activities in persons with spinal cord injury. Med Sci Sports Exerc 2010:42:691-700.
41. Hasnan N, Ektas N, Tanhoffer AI, anhoffer R, Fornusek C, et al. Exercise responses during functional electrical stimulation cycling in individuals with spinal cord injury. Med Sci Sports Exerc 2013;45:1131-8.
42. Evans N, Hartigan C, Kandilakis C, Pharo E, Cleeson I. Acute cardiorespiratory and metabolic responses during exoskeleton-assisted walking overground among persons with chronic spinal cord injury. Top Spinal Cord Inj Rehabil 2015;21:122-32.
43. Astorino TA, Harness ET, Witzke KA. Effect of chronic activity-based therapy on bone mineral density and bone turnover in persons with spinal cord injury. Eur J Appl Physiol 2013;113:3027-37.
44. Rejeski WJ, Ettinger WH, Martin K, Morgan T. Treating disability in knee osteoarthritis with exercise therapy: a central role for self-efficacy and pain. Arthritis Care Res 1998;11:94-101.
45. Padula N, Costa M, Batista A, Gaspar R, Motta C, et al. Long-term effects of an intensive interventional training program based on activities for individuals with spinal cord injury: a pilot study. Physiother Theory Pract 2015;31:568-74.
46. Hausenblas HA, Fallon EA. Exercise and body image: a meta-analysis. Psychol Health 2006;21:33-47.
47. Hienberg LJ, Thompson JK, Matzon JL. Body image dissatisfaction as a motivator for healthy lifestyle change: is some distress beneficial? In: Striegel-Moore RH, Smolak L, editors. Eating disorders: innovative directions in research and practice. Washington, DC: American Psychological Association; 2001. pp. 215-32.
48. Bassett RL, Martin Ginis KA. More than looking good: impact on quality of life moderates the relationship between functional body image and physical activity in men with SCI. Spinal Cord 2009;47:252-6.
49. Semerjian TZ, Montague SM, Dominguez DF, Davidian AM, de Leon RD. Enhancement of quality of life and body satisfaction through the use of adapted exercise devices for individuals with spinal cord injuries. Top Spinal Cord Inj Rehabil 2006;11:95-108.
50. Nash MS, van de Ven I, van Elk N, Johnson BM. Effects of circuit resistance training on fitness attributes and upper-extremity pain in middle-aged men with paraplegia. Arch Phys Med Rehabil 2007;88:70-5.
51. Martin Ginis K, Latimer AE. The effects of single bouts of body-weight supported treadmill training on the feeling states of people with spinal cord injury. Spinal Cord 2007;45:112-5.
52. Alschuler KN, Jensen MP, Sullivan-Singh SJ, Borson S, Smith AE, et al. The association of age, pain, and fatigue with physical functioning and depressive symptoms in persons with spinal cord injury. J Spinal Cord Med 2013;36:483-91.
53. Marcondes BF, Sreepathi S, Markowski J, Nguyen D, Stock SR, et al. Pain severity and mobility one year after spinal cord injury: a multicenter, cross-sectional study. Eur J Phys Rehabil Med 2016;52:626-36.
54. Latimer AE, Martin Ginis K, Hicks AL, McCartney N. An examination of the mechanisms of exercise-induced change in psychological well-being among people with spinal cord injury. J Rehabil Res Dev 2004;41:643-52.
55. Fuhrer MJ, Rintala DH, Hart KA, Clearman R, Young ME. Depressive symptomatology in persons with spinal cord injury who reside in the community. Arch Phys Med Rehabil 1993;74:255-60.
56. Wilmet E, Ismail AA, Heilporn A, Welraeds D, Bergmann P. Longitudinal study of the bone mineral content and of soft tissue composition after spinal cord section. Paraplegia 1995;33:674-7.
57. Sköld C, Lönn L, Harms-Ringdahl K, Hultling C, Levi R, et al. Effects of functional electrical stimulation training for six months on body composition and spasticity in motor complete tetraplegic spinal cord-injured individuals. J Rehabil Med 2002;34:25-32.
58. Gorgey AS, Caudill C, Khalil RE. Effects of once weekly of NMES training on knee extensors fatigue and body composition in a person with spinal cord injury. J Spinal Cord Med 2016;39:99-102.

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Original Article

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Substantial subpial cortical demyelination in progressive multiple sclerosis: have we underestimated the extent of cortical pathology?

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Abstract

Aim: Multiple sclerosis (MS) is an inflammatory demyelinating and neurodegenerative disease. Much of the complex symptomatology relates to pathology outside the classic white matter plaque, whereby lesions of the cortical grey matter, which are difficult to resolve by conventional clinical imaging, are in part predictive of outcome. We investigated the extent of grey matter pathology in whole coronal macrosections to reassess the contribution of cortical pathology to total demyelinating lesion area in progressive MS.

Methods: Twenty-two cases of progressive MS were prepared as whole bi-hemispheric macrosections for histology, immunostaining and quantitative analysis of lesion number and relative area, leptomeningeal inflammation and microglial/macrophage activation.

Results: Cortical grey matter demyelination was seen in all cases, which was more extensive than in white and deep grey matter (hippocampus, thalamus and basal ganglia) and accounted for 0.8%-60.2% of the entire measurable cortical ribbon. The pattern of cortical grey matter demyelination was predominantly subpial (mean 90.9%, range 60%-100%, of total cortical grey matter lesion area) and cases with the largest areas of subpial cortical lesions had more and larger deep grey matter lesions, greater numbers of activated microglia/macrophages, both in lesions as well as in normal cortical grey matter, together with elevated leptomeningeal inflammation and lymphoid-like

structures. White matter lesion area was unchanged when compared with the progressive MS cases with little subpial cortical demyelination.

Conclusion: Analysis of whole coronal macrosections reveals cortical demyelination is more extensive than reported by conventional histological methods. Cases of progressive MS with substantial subpial cortical demyelination that is independent of underlying white matter lesion area support the implications that these lesions may in-part arise through different pathogenetic mechanisms. Biomarkers and/or imaging correlates of this subpial pathology are required if we are to fully comprehend the clinical disease process.

Keywords: Leptomeninges, B cell follicle, microglia activation, normal appearing grey matter.

INTRODUCTION

Neocortical grey matter demyelination, microglial activation and neurodegeneration, which may in-part be driven by inflammation of the overlying leptomeninges, are important pathological processes influencing the clinical severity and outcome of multiple sclerosis (MS)[1]. No biomarker or widely available imaging technology can fully report the extent of neocortical pathology, which is essential for optimal disease management.

Neocortical and cerebellar cortical pathology is evident from the earliest stages of MS[2-5]. It is a pathological hallmark of progressive MS[1,2] and closely associates with clinical severity at all stages[6]. For example, the extent of cortical pathology is better at predicting disease outcome than white matter pathology[7]. Radiological imaging of grey matter and cortical atrophy are predictive of the conversion to clinically definite MS[8-10] or the risk of transitioning to the progressive phase and disability[7,11]. Nevertheless, even high fidelity, non-routine, imaging technologies fail to identify lesions of the most superficial cortical layers, whilst neuropathological assessment of standard size tissue blocks often underestimates the extent of cortical lesions, which can occupy the cortical ribbon over multiple contiguous gyri and sulci[2,12].

Lesions of the superficial layers of the neocortex (subpial cortical grey matter lesions) are more numerous and sometimes associate topographically with leptomeningeal foci of immune cell aggregates that resemble lymphoid-like structures (alternatively termed ectopic B cell follicle structures) seen in other autoimmune, chronic inflammatory and infectious diseases[13,14]. Progressive MS cases with leptomeningeal lymphoid-like structures exhibit a gradient of cortical tissue damage extending away from the pial surface, suggestive of factor(s) in the CSF that promote underlying cortical inflammation and injury[15-17], possibly through the activation of microglia. The degree of leptomeningeal inflammation correlates with the extent of cortical demyelination, neurodegeneration and microglial activation in acute and progressive stages[4,13,18,19], whilst white matter lesion area is not changed. These findings suggest that subpial cortical lesions may arise through partly independent mechanisms compared with lesions located further from the CSF-filled spaces of the pia or ventricular lumen[17].

The study of whole bi-hemispheric coronal macrosections, although technically challenging, can reveal hitherto undisclosed aspects of MS pathology and better report the extent of global pathology. We analysed the distribution and histological characteristics of cortical grey matter, white matter and deep grey matter lesions, together with inflammation of the brain and overlying leptomeninges, using whole brain coronal sections from 22 cases of progressive MS. We demonstrate the sometimes surprising extent of subpial cortical grey matter demyelinating pathology that can be seen and highlight the association between subpial and periventricular grey matter lesions, the dissociation between subpial lesion load and white matter lesion area, and confirm the close relationship between leptomeningeal inflammation and subpial

Table 1. Cases and sections used in this study

Case	Sections analysed	Sex	Age at death (years)	Disease duration (years)	MS type	Cause of death
MS202	A2, P1	F	58	23	SPMS	Pulmonary embolism
MS204	A3, P1, P3	M	58	19	SPMS	Leukaemia and MS
MS212	A3, P1, P3	F	47	29	SPMS	Multiple sclerosis/bronchopneumonia
MS214	P1	F	51	31	SPMS	Multiple sclerosis
MS217	A3, P1, P3	F	57	15	SPMS	Suicide
MS223	A2	F	45	2	SPMS	Multiple sclerosis/bronchopneumonia
MS224	A2, P1, P3	F	59	33	SPMS	Multiple sclerosis/bronchopneumonia
MS226	A2, P3	M	64	27	SPMS	Multiple sclerosis/pneumonia
MS253	A3, P1, P3	F	37	16	SPMS	Multiple sclerosis/pulmonary embolism
MS257	A2, P1, P3	F	49	22	SPMS	Aspiration pneumonia
MS258	A2, P1, P3	M	46	20	SPMS	Multiple sclerosis
MS278	A3, P1, P3	M	30	21	SPMS	Pneumonia
MS293	P1, P3	F	53	18	SPMS	Multiple sclerosis
MS295	P1, P3	F	71	15	SPMS	Bronchopneumonia
MS323	A4, P1	F	62	31	SPMS	Multiple sclerosis/sepsis
MS336	A2, P1, P3	F	57	27	SPMS	Multiple sclerosis/resp failure
MS344	P1	F	57	15	SPMS	Multiple sclerosis/septicaemia
MS360	A2, P1, P3	M	55	40	SPMS	Multiple sclerosis
MS361	P1	F	60	34	SPMS	Multiple sclerosis
MS366	P1	F	61	19	SPMS	Multiple sclerosis/bronchopneumonia
MS387	P3	F	43	11	SPMS	Multiple sclerosis
MS395	A3, P3	M	63	26	SPMS	Multiple sclerosis/chest infection

List of cases, number of coronal planes analysed, disease course and principal cause of death. Sex (Female/Male), age of death and disease duration reported in years and disease type (secondary progressive MS). MS: multiple sclerosis; SPMS: secondary progressive MS

cortical lesion pathology. This data support efforts to develop brain imaging and biomarker technologies for the identification of subpial grey matter lesions to improve disease prediction and monitoring.

METHODS

Post-mortem cohort

Formalin fixed, whole brains [n = 22; median age 57 years (range 30-71 years), median disease duration 21.5 years (2-40 years), female = 17] were available from clinically and neuropathologically validated cases of secondary progressive MS (see Table 1 for details). All cases were provided by the UK Multiple Sclerosis Society Tissue Bank, Imperial College London, with appropriate research ethics approval (08/MRE09/31+5). Case selection was based on availability of whole brains with well-preserved leptomeninges and accompanying detailed clinical and neuropathology summaries, collected between February 2004 and December 2008. Some of these cases have previously been reported[18], but all analysis and data presented here are unique to this manuscript.

Individual progressive MS brains were dissected into 1-cm-thick coronal sections, cut in an anterior direction from the mammillary bodies as coronal bi-hemispheric sections (A1, A2 and A3), or posteriorly from the mammillary bodies towards the occipital lobe (sections P1, P2 and P3) such that each coronal section contained several different cyto-architectonic areas [Figure 1]. For example, coronal section A3 includes frontal cortex and poles of the temporal gyrus; coronal section P1 includes motor and somatosensory cortex, thalamus and anterior hippocampus; and coronal section P3 includes parietal and occipital lobe and occipital horn of the lateral ventricle. Areas of interest for comparison were subdivided into: (1) cortical (neocortex); (2) white matter; and (3) hippocampus and deep grey matter [comprising caudate, pallidum (interna and externa), putamen, thalamus, hypothalamus and hippocampus and dentate gyrus].

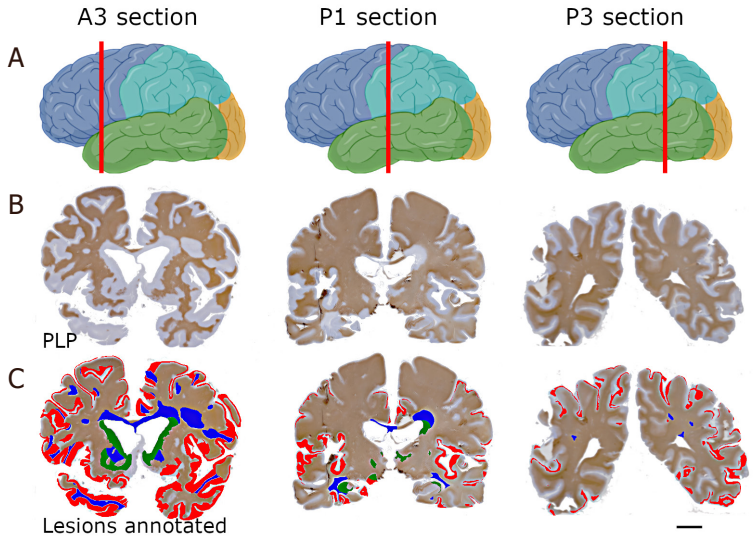

Figure 1. Study setup and sampling protocol. Whole coronal tissue slabs sectioned along coronal planes A3, P1 and P3 were prepared at three levels from immersion fixed brains (A) (created with Biorender.com). Histology and immunohistochemistry (anti-PLP immunostaining) revealed tissue anatomy and areas of frank demyelination (B) (MS278 section A3, MS202 section P1 and MS360 section P3). Stained and mounted tissue sections were digitised and colour masks depicting demyelinating lesions involving the neocortical grey matter (red colour mask), deep grey matter and hippocampus (green colour mask) and white matter (blue colour mask) were annotated for lesion quantification (C). (B, C) Scale bar = 2 cm. PLP: proteolipid protein

Tissue preparation, staining and characterising demyelinated lesions

Whole coronal tissue sections were processed under vacuum and paraffin wax embedded prior to sectioning at 10 µm onto 3-Aminopropyltriethoxysilane-silane (Sigma, UK) coated glass slides using a Reicheart-Jung tetrander microtome. Following dewaxing and rehydration, coronal sections from each case were stained with luxol fast blue (LFB) and cresyl violet to identify anatomical landmarks (grey and white matter) and to facilitate the assessment of total cortical, hippocampal and deep grey matter and white matter areas. Subsequent sections were immunostained with anti-proteolipid protein (PLP) and anti-CD68 primary antisera [Table 2] and revealed by sequential anti-species specific biotinylated secondary and avidin-biotin complex (Vector Labs. Ltd.) horse-radish peroxidase conjugated tertiary detection reagent with ImmPACT DAB as the reporter chromogen (Vector). All slides were counterstained, dehydrated, cleared in xylene and DePeX mounted under glass coverslip (Ted Pella Inc. USA).

Cortical grey matter lesions (GMLs), revealed by anti-PLP immunohistochemistry, were grouped as Type I (leukocortical), Type II (intracortical and not contacting the pia or grey/white matter boundary), or Type III (extending from the pia, sometimes involving the entire breadth of the cortex and stopping at the grey/white matter boundary (Bo Type IV cortical GML[20]). Areas of sparse and patchy anti-PLP immunostaining, which reflect incomplete demyelination and/or remyelination, but are notably different to the most "normal" grey matter, were labelled as partially de/re-myelinated grey matter[4].

White matter lesions (WMLs) were separately characterised as active inflammatory (confluent in CD68+ microglia/macrophages), chronic active (activated microglia/macrophages restricted to the lesion edge containing PLP+ and/or LFB+ myelin breakdown products) and chronic inactive lesions (few activated microglia/macrophages at the lesion edge without evidence of recent myelin phagocytosis). Additional areas of putative remyelinating/shadow plaque (by PLP+ myelin internodes and a classic LFB+ shadow appearance) were identified but not quantified. Demyelinating lesions of the hippocampus and deep grey matter were identified based on a clear loss of LFB and/or PLP+ immunostain and were characterised as active, chronic active or chronic inactive inflammatory demyelinating lesions[21].

Table 2. List of primary antibodies used

Antibody	Dilution	Target antigen	Company and product details
Anti-CD68	1:400	CD68 (macrosialin)	Dako; clone kp1
Anti-CD20	1:125	CD20	Dako; clone l26
Anti-PLP	1:1500	Myelin proteolipid protein	BioRad; clone plpc1

Primary antibody, working dilution, principal target and product details. PLP: proteolipid protein

Slide digitisation, lesion masks and quantitative analysis

Individual coronal sections were reviewed using an Olympus SZ60 stereo microscope 0.1-10 × magnification) and a Zeiss AxioImager Z1 (40-400 × magnification) to identify normal and pathological regions of interest in each slide, which were marked on A4-sized print-outs of the same slide captured using a conventional document scanner (HP Scanjet 300) at 1200 dpi.

Quantifying the number and area of cortical, white matter and hippocampus and deep grey matter lesions

The scanned images were used to guide our tracing of white and grey matter areas (from the LFB stained section), and areas of cortical, white matter and hippocampus and deep grey matter lesions (PLP+ slide) as colour-mask overlays using GNU image manipulation software (GIMP 2.10; see Figure 1). The modified high-resolution TIFF images were analysed in ImageJ (https://imagej.net/Fuiji/Downloads) to record the total number and area (mm^2) of cortical grey matter, white matter and hippocampus and deep grey GML area per section, per case. We defined the maximum extent of a Type III lesion for lesion counting as an area of complete demyelination that extended over a maximum of two entire sulci and gyri, as some Type III lesions extended across three or more gyri or involved the entire superficial cortical grey matter in a single hemisphere. Therefore, our Type III lesion count represents individual, small, Type III lesions, as well as large subpial lesions, subdivided and quantified as two or more separate lesions. In addition to measuring the area of cortical GMLs, the area and relative extent of cortex identified as de/re-myelinated cortical grey matter was recorded per section, per case.

To produce illustrative "heat maps" that depict the burden of cortical GMLs in cases defined by lymphoid-like structure status (absence/presence), we first identified all cortical GMLs in section P1 per case and superimposed these lesions as "layer" images on a line-drawn representative whole coronal brain section (adapted from plate 34[22]) in GIMP. The final overlaid schema, comprising the "layer" masks of each case sampled at the P1 coronal level, revealed the absolute number of lesions by the relative depth of colour at that site.

Determining leptomeningeal inflammation

A measure of relative leptomeningeal immune cell infiltration per case was reported. Briefly, meningeal infiltrates were graded by assessing the extent of Nissl+ counter-stained cellular infiltrates of the intact cerebral leptomeninges, with the most notable infiltrate per case, rather than the average extent of infiltration, being reported. None to mild leptomeningeal inflammation was rated 0+ (0-5 cells per 100 × microscopic field of view; equivalent to 440-μm length of leptomeningeal tissue); diffuse and modest rated ++ (equivalent to an infiltrate of 5-50 loosely packed cells); or substantial infiltration rated +++ (based on > 50 cells in a tightly packed infiltrate[18]). *Bona fide* leptomeningeal lymphoid-like structures characterised by the presence of an anti-CD35+ reticular network, proliferating B cells (Ki67 antigen+) and immunoglobulin+ plasma cells[23] were previously reported in a subset of these cases[18] and the lymphoid-like structure status (presence or absence of detectable structures) is detailed in is detailed in the results section.

Quantifying activated microglia/macrophages

The density of CD68+ microglia/macrophages in cortical GML centres, normal appearing cortical grey matter or WML centre or normal appearing white matter was quantified from four 40 × images (Zeiss

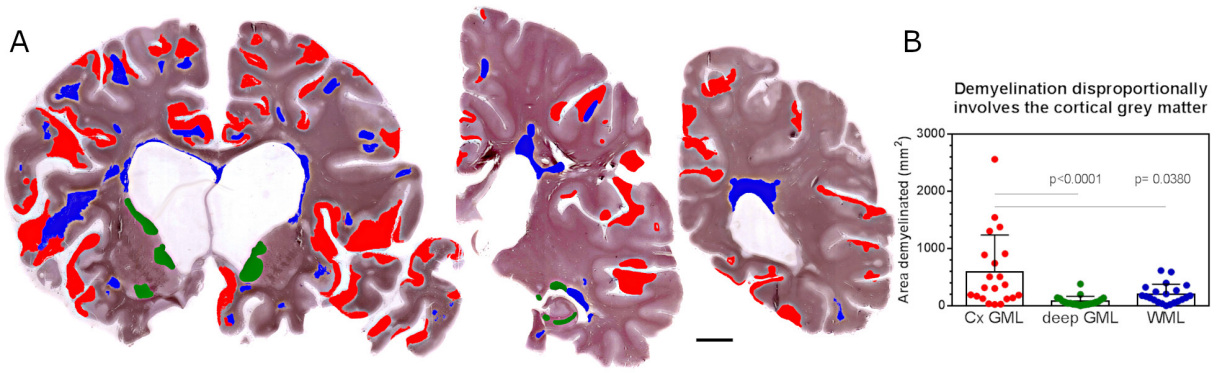

Figure 2. Demyelinating lesions of the cortical grey matter, deep grey matter and hippocampus and white matter. Anti-PLP immunostaining of sections through the rostral-caudal extent of MS212, at coronal levels A3, P1 and P3 (A). Note the widespread demyelination of the cortical grey matter (red colour mask). Quantification of the area of demyelinated tissues revealed the total area of demyelinated cortical grey matter to exceed that of the total measured area of deep grey matter and white matter (B). Data points represent the sum of all demyelinated lesions, per region of interest per case with mean and SD depicted. Groups compared by Kruskal-Wallis and Dunn's multiple comparison post-test. Cx: cortical; GML: grey matter lesion; WML: white matter lesion. Scale bar = 1 cm

AxioImager Z1 and Axiocam Hrc camera) per region of interest captured from cingulate, pre-central gyrus (including pre- and post-central superior gyrus), insula cortex in parietal lobe and temporal lobe (medial) of each available P1 coronal macrosection, per case. In the absence of a demyelinating lesion, only data from the normal appearing tissue were reported for that region, per case. All data were averaged for lesion or normal appearing status across the sampled regions and reported as density of CD68+ cells/mm^2, for comparison between groups.

Statistical analysis

Group means or medians were compared and plotted using GraphPad Prism 7. All data were assumed to be non-normally distributed given the small sample size[24]. The unpaired Kruskal-Wallis with Dunn's multiple comparison post-test was used for three-group comparisons [i.e., when comparing the average area (mm^2) of cortical and subcortical lesions per section, per case], whilst the Mann-Whitney *U* test was used when comparing two groups (i.e., per cent hippocampus and deep GML in cortical High *vs*. Low MS). Fischer's exact test compared the actual *vs*. anticipated proportion of males/females and other clinical variables, such as seizures (yes/no) or lymphoid-like structure status (absent/present), between groups. Correlative analysis used Spearman's method and Spearman *r* and *P* values were reported. A two-tailed *P* value of > 0.05 was considered significant.

RESULTS

We conducted a study of whole coronal sections to better understand the burden of demyelination and inflammation in a cohort of 22 cases of secondary progressive MS (SPMS).

Cortical grey matter is disproportionality demyelinated in progressive MS

Cortical grey matter (GM), hippocampus and deep GM and WMLs were seen in all 22 cases analysed, with lesions noticeable across the coronal planes sampled [Figures 1 and 2A]. The area of demyelination, determined from the analysis of LFB and anti-PLP immuno-stained sections, varied considerably amongst the MS cohort [Figure 2B]. Quantification of the total area occupied by cortical GMLs, hippocampus and deep GML and WMLs revealed the total area of cortical GML (mean 589 mm^2, range 25-2563 mm^2) to be significantly greater than the total hippocampus and deep GML area (mean 81 mm^2, range 0-382 mm^2) and subcortical WML area (mean 203 mm^2, range 2-617 mm^2; Figure 2B). The relative area of cortical GML (per cent GML of total cortical GM) was almost twice that of the relative area of WML of total section WM per

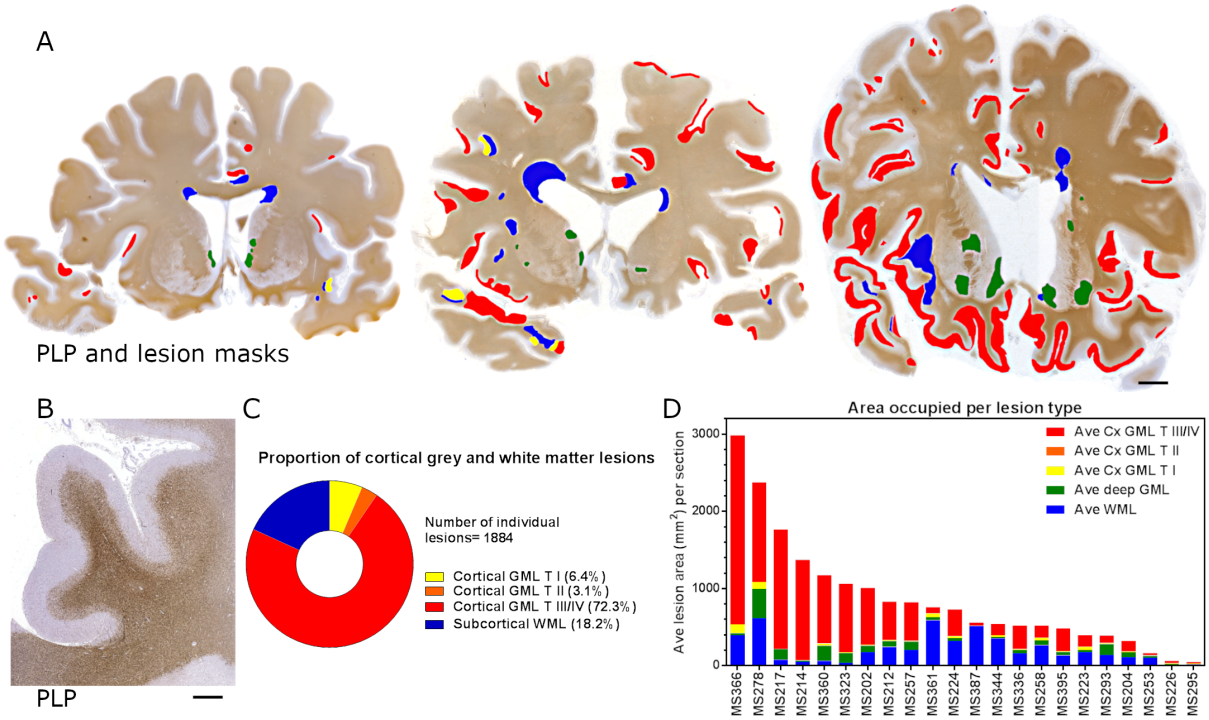

Figure 3. Subpial cortical grey matter lesions are the most frequent and occupy the greatest area. Anti-PLP immunostaining of section A3 of MS253, MS224 and MS257 revealed subpial (Type III/IV cortical lesions, red mask), intracortical (Type II cortical lesion, orange mask) and leukocortical (Type I cortical lesion, yellow mask) lesions. Lesions of the deep grey matter (green) and lesions of white matter (blue mask) are also shown (A). Subpial (Type III/IV) lesions were predominant and often spanned multiple gyri (B). Of 1884 separate lesions, 72.3% were subpial cortical grey matter lesions (C). By plotting the average area of each lesion type (calculated per section) and represented per case, we show the heterogeneity of lesion size across our cohort (D), and how subpial cortical lesions contribute a large component of the lesion burden, especially in those cases most affected (from MS366 to MS257, for example). Ave: average; Cx: cortical; GML: grey matter lesion; PLP: proteolipid protein immunohistochemistry; WML: white matter lesion. (A) Scale bar = 1 cm; (B) Scale bar = 1 mm

case (17.9% ± 2.6% compared with 8.9% ± 1.3% for cortical GML and subcortical WML, respectively, P = 0.023, non-parametric Mann-Whitney U test).

Type III cortical grey matter lesions are the most frequent and can affect the entire cortical ribbon in a whole coronal section

We noted cases with little and others with expansive areas of cortical GM demyelination in our progressive MS cohort [Figure 3A]. We counted the number of separate lesions of the neocortex and white matter and showed that the subpial cortical GMLs (Type III and IV lesions; Figure 3B) were more numerous than any other cortical grey matter lesion type or white matter lesion (Figure 3C, $P < 0.0001$). In total, 1884 lesions were identified, of which 1363 (72.3%) were subpial (Type III or IV) cortical GMLs.

Subpial cortical GMLs accounted for the largest area of all lesion types measured. We plotted the measured area of each lesion type, with the bar height representing the total average area (mm^2) of demyelination per section, per case, to show the substantial contribution subpial GMLs made to the total lesion area [Figure 3D]. Arranging cases according to the total measured area of demyelination [from left (highest), to right (lowest)] highlighted that subpial GMLs overwhelmingly accounted for the different total lesion areas between cases with relatively high or low total demyelinated lesion area. In cases with relatively lower lesion area (cases MS361-MS295), the area of subpial GML as a per cent of total measured lesion area was far less than in cases with the higher total lesion area (34.5%, range 8.0%-60.1% *vs.* 74.3%, range 54.3%-94.9%; $P < 0.0001$, non-parametric Mann-Whitney U test). We compared the relative extent of demyelination in

Table 3. Correlating cortical, white matter and hippocampus and deep grey matter lesions

	% Cx GML III/IV	% Hippo and deep GML	% WML	% Total Cx GML
% Cx GML I/II	r = -0.005 P = 0.982	r = 0.224 P = 0.328	r = 0.771 P < 0.0001	r = 0.051 P = 0.827
% Cx GML III/IV		r = 0.458 P = 0.037	r = 0.092 P = 0.684	r = 0.988 P < 0.0001
% Hippo and deep GML			r = 0.169 P = 0.464	r = 0.495 P = 0.023
% WML				r = 0.135 P = 0.549

Correlation analysis revealed the relative area of intracortical and leukocortical grey matter lesions (Type I/II cortical lesions) associated with the extent of white matter demyelination. Subpial cortical grey matter lesions (Type III/IV) associated with the extent of hippocampus and deep grey matter lesion area. Row and column headings refer to: % Cx GML I/II: relative area of intracortical/leukocortical grey matter lesions; Cx GML III/IV: relative area of subpial cortical grey matter lesions; % Hippo and deep GML: relative area of grey matter lesions affecting hippocampus and deep grey matter; % WML: relative area of demyelinated white matter; % Total Cx GML: relative area of demyelinated neocortex. Spearman analysis with r and P values shown. Significant associations are underlined. Cx: cortical; GML: grey matter lesion; WML: white matter lesion

cortical and subcortical tissues and showed that the relative area of total cortical GML was not statistically associated with the relative area of WML (r = 0.135, P = 0.549; Table 3). The area of subpial cortical GML was significantly associated with the extent of hippocampus and deep GML area (Spearman r = 0.458, P = 0.036), whilst leukocortical/intracortical GML area was significantly associated with WML area (Spearman r = 0.771, P < 0.0001; Table 3).

Pathological correlates of a high subpial cortical grey matter lesion load

To understand the clinical and pathological associations that might co-exist with subpial cortical GM demyelination, we subdivided the cohort based on relative extent of cortical GML area per section, per case [Table 4 and Figure 4]. Cases with 15% or greater relative cortical GML of total cortical GM (n = 9 cases in total; Figure 4A, which represent cases MS366-MS257 in graph Figure 3D) were designated cortical GML High MS (mean cortical GML area 35.6%; range 15.6%-60.2%), whilst the others (n = 13 cases, cortical GML less than 12% total cortical GM area) were designated as cortical GML Low MS (mean cortical GML area 5.5%; range 0.9%-12.0%).

The relative area of hippocampus and deep GML was greater in cortical GML High MS [Figure 4B], whilst the relative area of WML was not different [Figure 4C]. Cortical GML High MS cases had a greater density of CD68+ microglia/macrophages in both GML centre and normal appearing GM regions, in comparison to cortical GML Low MS [Figure 4D and G], whilst the density of CD68+ microglia/macrophages was only increased in normal appearing WM of cortical GML High cases [Figure 4E and H]. Inflammation of the forebrain leptomeninges is associated topographically with subpial cortical demyelination and cases with semi-organised lymphoid-like structures are characterised by a more extensive cortical GML load[13]. Our designated cortical GML High MS cohort presented with a substantially increased median rating of leptomeningeal immune cell infiltrates (P = 0.003, Figure 4F), with seven of nine cases containing at least one *bona fide* lymphoid-like structure [Table 4]. Examples of leptomeningeal infiltrates rated as mild [+; Figure 4I and L], modest [++, Figure 4J and M] and substantial [+++, Figure 4K and N] are provided in Figure 4. Immune cell aggregates were associated both with areas of partially de/re-myelinated subpial cortical GM as well as areas of subpial GM demyelination [Figure 4].

Areas of cortical GM defined as partially de/re-myelinated GM ranged from < 1% (0.9%, MS223) to 13.5% (MS257) of total cortical GM area per case but were not different between the cortical GML High and Low groups (area of partially de/re-myelinated GM = 8.01% *vs.* 6.13%, for cortical High *vs.* Low GML cases, respectively, P = 0.387). Rudimentary measures of gross brain pathology, such as brain weight at death and the measured area of grey and white matter per P1 section (as an indication of regional grey or white

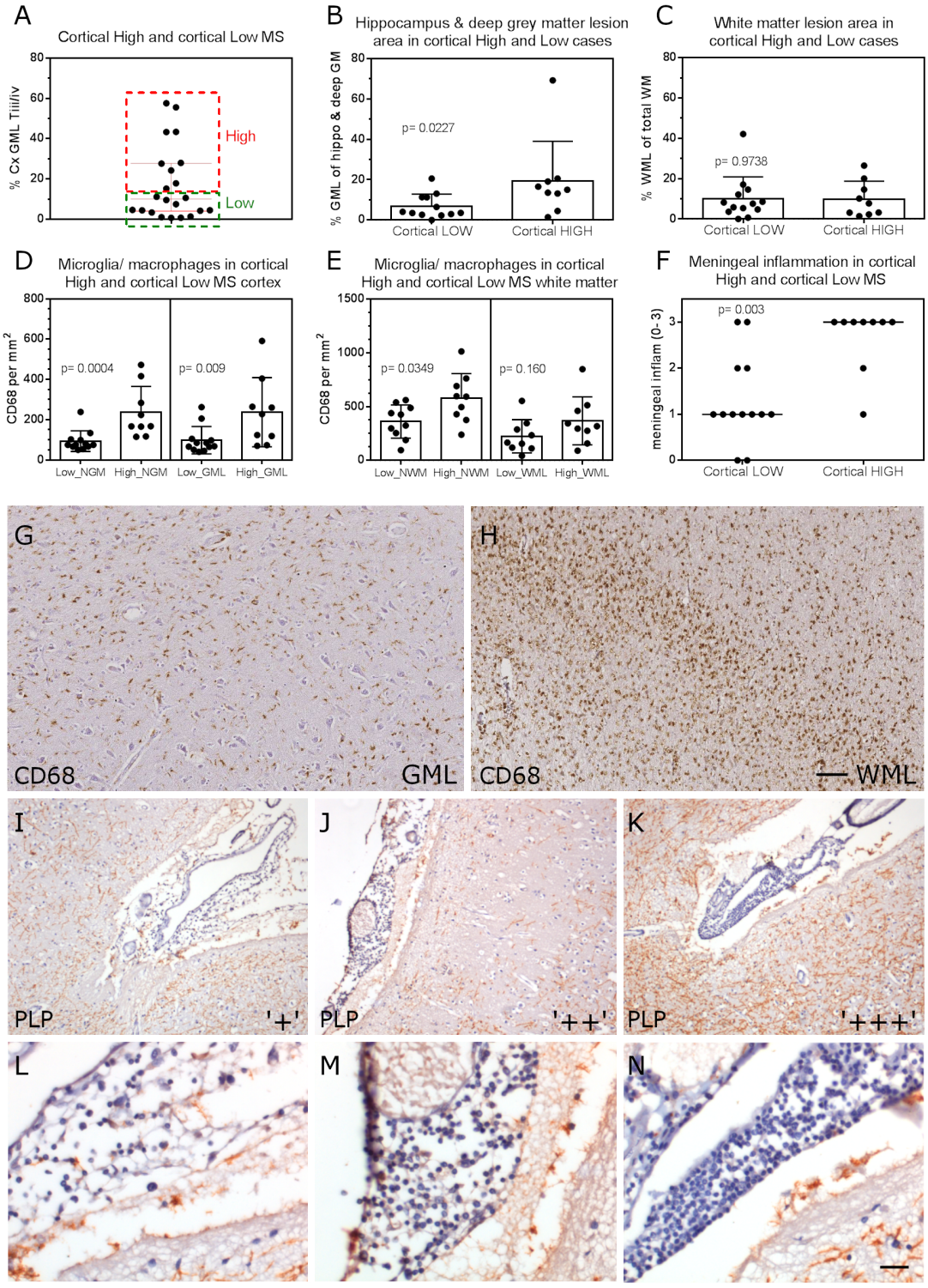

Figure 4. Defining a cohort of cortical High grey matter lesion MS. Nine progressive MS cases with the greatest relative area of subpial cortical demyelination were labelled as Cortical High GML MS and the remaining cases as Cortical Low GML MS (A). Cortical High GML MS had a greater area of demyelinated deep grey matter and hippocampus (B), but unchanged white matter lesion areas (C). The density of CD68+ microglia/macrophages was increased in both normal appearing GM (NGM) and GML of cortical High GML MS (D) but was only different in the underlying normal appearing WM (NWM) (E). Semi-quantitative evaluation of meningeal inflammation revealed cortical High GML MS to have increased inflammation of the leptomeninges (F). Examples of CD68+ microglia of a subpial GM lesion (G) and CD68+ microglia/macrophages in a white matter lesion (H). Examples of 1-3 rated inflammatory infiltrates ("+" to "+++") of the forebrain meninges [I-N with (L-N) being higher power images of (I-K), respectively]. Please note that infiltrates (I, L, K, N) are near partially de/re-myelinated cortex of cortical High GML Case MS257. Cx: cortical; GML: grey matter lesion; NGM: normal appearing grey matter; NWM: normal appearing white matter; PLP: proteolipid protein immunohistochemistry; WML: white matter lesion. (G-K) Scale bar = 100μm; (L-N) Scale bar = 25 μm

Table 4. Progressive MS cases defined by relative cortical grey matter lesion load

Case	Sex	2yr RR	Onset	Prog	W'chair	Died	Duration	Pm delay	Brain weight (g)	Seizures (Y/N)	LLS	Menin inflam (0-3)
Cortical GM High MS												
MS202	F	2	35	46	47	58	23	39	1200	N	Y	3
MS212	F	1	18	33	35	47	29	66	1314	N	Y	3
MS214	F	2	20	31	38	51	31	20	1140	n/d	N	2
MS217	F	1	42	49	50	57	15	29	1200	N	Y	3
MS257	F	6	27	31	39	49	22	28	1168	Y	Y	3
MS278	M	1	9	23	23	30	21	60	1402	Y	Y	3
MS323	F	2	31	38	44	62	31	13	1207	Y	N	1
MS360	M	1	15	40	51	55	40	18	1063	Y	Y	3
MS366	F	3	42	45	53	61	19	14	1090	N	Y	3
n = 9	7F:2M	2	27	38	44	55	23	31.9	1198.2	4Y:4N	7/9	3
Cortical GM Low MS												
MS204	M	3	39	44	48	58	19	35	1180	N	N	1
MS223	F	4	43	43	45	45	2	72	1004	Y	N	1
MS224	F	1	26	35	36	59	33	27	1100	N	N	1
MS226	F	5	37	51	58	64	27	27	1300	N	N	0
MS253	F	3	21	23	33	37	16	24	1259	N	N	2
MS258	M	2	26	35	38	46	20	44	1460	N	N	1
MS293	F	2	35	42	43	53	18	44	1250	Y	Y	3
MS295	F	5	56	60	62	71	15	42	1166	N	N	0
MS336	F	1	30	47	51	57	27	24	1226	N	Y	3
MS344	F	6	42	43	47	57	15	14	1062	N	N	1
MS361	F	2	26	38	42	60	34	10	956	Y	N	1
MS387	F	6	32	35	36	43	11	13	1115	N	N	2
MS395	M	1	37	45	52	63	26	4	958	N	N	1
n = 13	10F:3M	3	35	43	45	57	19	24.3	1161.3	3Y:10N	2/13	1

Associated clinical and pathological data of Cortical High and Cortical Low grey matter lesion MS. Sex (Female/Male), 2yr RR (number of relapses in first two years of clinical disease), Onset (retrospectively determined age at first MS symptoms, Prog (age at which disease became progressive), Died (age at death), Duration (time from first symptom onset to death), PM delay (post-mortem delay in hours), Brain weight (wet brain weight), Seizures (one or more seizures recorded in the clinical notes - Yes or No), LLS [positive (Yes) or negative (No)], Menin inflam (relative extent of meningeal inflammation). n/d, not determined, data not available. Text in bold represents median or mean (brain weight only) values for each group. LLS: lymphoid-like structure; GM: grey matter; MS: multiple sclerosis; W'chair: age at which they required a wheelchair

matter tissue atrophy; data not shown), did not reveal a difference between the cortical GML High and Low MS cases [Table 4].

Clinical correlations of a high subpial cortical grey matter lesion load

There was no difference in the proportion of males to females or of any reported clinical measure (such as the number of relapses in the first two years, the report of seizures or age of death) between cortical GML High and Low groups [Table 4]. There was no difference between groups with regards age of disease onset (P = 0.11), confirmed age at onset of progressive phase (P = 0.27) or disease duration (P = 0.15). Post-mortem delay did not differ between the groups (P = 0.75; Table 4).

Leptomeningeal inflammation, lymphoid-like structures and cortical demyelination

We identified four separate "+++" rated foci of substantial leptomeningeal infiltrates in a single P1 section of Case MS217, which displayed massive cortical, and exclusively subpial, GM demyelination (59.6% of cortical GM defined as GML; Figure 5A and B). Progressive MS cases with moderate-to-high leptomeningeal inflammation (rated ++/+++ or +++/+++ for immune cell infiltrates) had an increased relative cortical GML area compared with those cases with little-to-mild leptomeningeal infiltrates 7.8% (range 0.8%-27.7%) *vs.* 26.2% (range 0.8%-60.2%), P = 0.012, Mann-Whitney U test). The relative area of hippocampus and deep GML area (7.4%, range 0%-19.1% *vs.* 16.4% range 1.4%-69.2%, P = 0.159) and WML

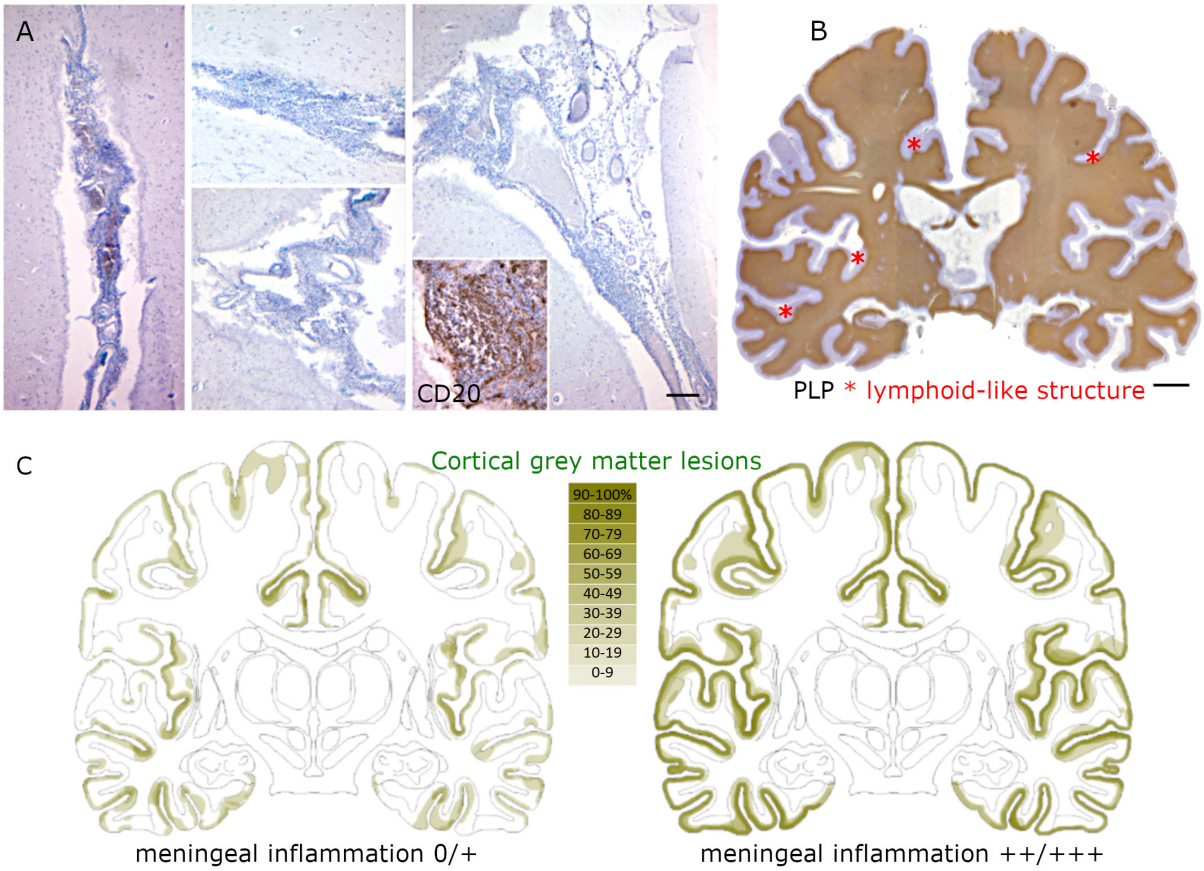

Figure 5. Meningeal inflammation and subpial cortical demyelination. Example infiltrates of the leptomeninges overlying the neocortex in a single coronal macrosection. Aggregates visualised by LFB/haematoxylin histology were rich in CD20+ B cells (A). The location of the infiltrates in (A) are marked as asterisks (*) on the serial anti-PLP stained section (B) (Case MS217). By grouping the cases based on their relative extent of leptomeningeal inflammation (0/+ *vs*. ++/+++), we constructed schema depicting all neocortical grey matter lesions noted per P1 section, per case (n = 18). Depth of colour (see heatmap representing percent lesion occurrence with 100% indicating tissue was demyelinated in every P1 section in that group) illustrates how frequently an area of neocortex was demyelinated in meningeal inflammation rated 0/+ and meningeal inflammation rated ++/+++ MS (C). PLP: proteolipid protein. (A) Scale bar = 200 μm; (B) Scale bar = 1 cm

lesion area (10.1%, range 0.6%-42.1% *vs*. 10.0%, range 2.3%-26.5%, P = 0.981) was unchanged between leptomeningeal rated moderate-high cases *vs*. those rated with little-to-mild infiltrates, respectively. The relationship between the extent of leptomeningeal inflammation and cortical GML area is presented schematically as a heat map of all cortical GMLs observed (in olive green colour) in section P1 of cases with mild (rated 0 to +, n = 9) *vs*. modest/substantial infiltrates (++ to +++, n = 9; Figure 5C). Please note that four cases did not have P1 sections available for analysis [Table 1].

DISCUSSION

Our quantitative histological analysis of whole coronal macrosections demonstrates cortical grey matter demyelination can be the preeminent pathology in some cases of progressive MS and can be used to identify a post-mortem cohort with a more severe pathological disease. Subpial cortical grey matter lesions associated with the extent of demyelinated deep grey matter and with infiltrates of the leptomeninges but importantly did not associate with white matter lesion load. Our study reinforces the concept of a CSF-derived (outside-in) driver of tissue pathology in SPMS.

Substantial subpial cortical grey matter demyelination defines a subset of progressive MS cases

Quantitative image analysis of whole coronal macrosections revealed the proportion of demyelinated cortical grey matter to be almost twice that of the proportion of demyelinated white matter. In those cases, with the greatest cortical pathology, labelled as cortical GML High MS, there was a near four-fold increase in relative area of cortical GML compared to WML. These data are in accordance with the work of Carassiti *et al.*[25] and are supportive of an MS post-mortem cohort characterised by a dominant neocortical demyelinating pathology[26]. Amongst our samples, we observed instances of hugely divergent grey over white matter pathology (for example, Case MS214 WML accounted for 3.2% of total WM, whilst cortical GML area was 43.4% of total cortex; Case MS217 WML area was 3.1% and cortical GML was 59.6%). Cortical GML pathology was overwhelmingly subpial in location, with an average of 90.9% of total cortical GML being characterised as subpial (Type III/IV) in distribution. Such extensive and disproportionate demyelination exceeds that previously reported by ourselves and others and highlights the benefit of working with whole coronal macrosection preparations[2,12,18] to better understand MS pathology.

We have previously noted associations between lymphoid-like structure status and clinical disease measures, such as age of onset, age at progression and age at death[13,18]. Our cortical GML High and Low MS cases had a similar age of onset to that seen in our earlier studies (median age at onset of 27 and 35 years, respectively) but this did not represent a significant difference in our relatively small study. No other clinical-pathological correlation was noted, which in part reflects the complex and highly variable pathology of this disease, whereby synaptic, neuritic and neuronal loss, alongside demyelination and gliosis, variable impact on disease outcome.

Demyelinating pathology at subpial and subependymal territories

The correlation between the extent of demyelination of the hippocampus and deep grey (including the caudate, putamen, pallidum and thalamus) with that of subpial cortical GML suggests, at least in part, a shared pathological mechanism of lesion formation and/or expansion between these regions lining superficial surfaces of the pia and ventricles. Subpial demyelinating lesions are a pathological hallmark of MS[27] and clinical imaging shows that the thalamus of children with MS, a structure which is severely affected in progressive MS[28], displays a specific imaging abnormality as a surface-in gradient pattern from the ventricular margin[29]. Adult MS patients also display a gradient of magnetisation transfer ratio signal change from the superficial surfaces of the brain: a gradient of signal change, declining with distance across the white matter[30,31] when measured from the ventricular surface, and across the neocortex from the pial surface, which are most altered in progressive MS[32,33]. A magnetic resonance imaging (MRI) signature of gadolinium leptomeningeal enhancement, which may partly reflect inflammation of meninges[34], relates to the number and volume of cortical and thalamic lesions[35]. These imaging studies support the concepts of meningeal inflammation and outside-in CSF factor(s) in lesion genesis. Numerous post-mortem studies have demonstrated a topographical association between the extent of immune cell infiltration of the leptomeninges with cortical GM demyelination[13,23,36-40], which is also true for subpial tissue of the cerebellar cortex and spinal cord[41-44]. This subpial demyelinating pathology of the neocortex is associated with microglia activation, a gradient of tissue injury and disruption of the pial glial limiting membrane[16,45]. Therefore, the pattern of neocortical lesion location and deep GM pathology is suggestive, at least in part, of an effect of soluble cytotoxic mediators from the overlying CSF-filled spaces that contributes to the underlying disease process.

Soluble mediators of subpial lesions

Recently, we have demonstrated the overexpression of RNA transcripts associated with pleiotropic chemokines and cytokines in the isolated meningeal tissue from cases characterised by extensive leptomeningeal inflammation and subpial demyelination[46,47]. The profile of elevated mediators, many

of which are associated with processes of lymphoid neogenesis, were mirrored by the finding of elevated protein levels of many of the same factors in the matched post-mortem CSF, which were also differentially expressed in an independent cohort of newly diagnosed MS patients with a clinical and radiological signature of substantial cortical pathology[47]. TNF and IFNγ are amongst those differentially expressed in patients with a cortical phenotype and these cytokines mediate a rapid pattern of cortical demyelination and microglia/macrophage activation when injected into the subarachnoid space of animals with subclinical autoimmune encephalitis[46]. These findings imply that lymphoid follicle-like structures, and infiltrates outside of these semi-organised structures, are a source of damaging factors that drive subpial pathology. Sampling CSF may reveal disease-relevant biomarkers of activity to aid therapeutic decision making[48].

Subpial cortical GMLs are associated with the loss of neurons of the superficial cortical layers, an elevated number of complement activated neurons and glia, together with substantial neuritic and synaptic loss[13,16,45,49-53]. The most striking pathological changes are found in the superficial cortical laminae, with a gradient of lessening neuronal damage with distance from the pia[16]. Neurons and glia of the MS cortex are exposed to elevated excitotoxins and reactive free radicals[54,55], are energy depleted and display mitochondrial pathology, which may contribute to further neurodegeneration[56-58]. There is an imbalance between TNF receptor 1/2 anti-apoptotic pathways *vs.* pro-death signals in the progressive MS cortex and oligodendrocytes are vulnerable to degenerate by a TNF receptor type 1/Receptor-interacting protein kinase type 1 necroptotic cascade[59,60]. Immunoglobulins derived from meningeal plasma cells are enriched in the MS neocortex, and products of central CD20+ B effector cells are directly toxic to cultured neurons and oligodendrocytes[61-63]. Alongside myriad immune mediators, MS CSF is enriched in bio-active lipids, whose levels associate with disease severity[64-66]. Lipid sterols, including key products of cholesterol metabolism and ceramide, can be directly neurotoxic. For example, C16:0 and C24:0 ceramides are enriched in MS patient CSF and can mediate neuroaxonal pathology and mitochondrial dysfunction[65], whilst simvastatin, a cholesterol-reducing therapy that enters the CNS, is associated with a slowing of brain atrophy in SPMS[67]. We currently have identified neither the combination of damaging factors that are causative of injury nor the relationship between these mediators with clinical progression on an individual level.

Lesions of the deeper cortical laminae (leukocortical or Type I cortical GMLs) are centred on cortical veins, typically contain greater numbers of activated microglia/macrophages than subpial lesions[53], and their relative area correlated with the extent of white matter lesion area in the same case. The statistical association between cortical and subcortical lesions, both characterised by an inflamed central vein, suggests they both share similar a mechanism of formation, which is in part different to lesions of the superficial grey matter structures. MRI is adept at resolving leukocortical GMLs, whilst even ultra-high field MRI detects only a fraction of all subpial lesions[68]. Our finding of an association between the relative area of leukocortical GML and WML area may explain the correlation between white matter and cortical grey matter lesions reported by MRI.

Methodological considerations and study limitations

The analysis of whole coronal macrosections allows the study of cortical lesions in continuity, improves the accuracy of their interpretation and the relationship with other pathological features, such as meningeal inflammation or lesions in different anatomical sites[19,69,70]. The handling of such large tissue samples is not trivial and this restricted the *n* number for our work, which may have meant our clinical-pathological comparisons were statistically underpowered [for instance, to observe a significant difference in age of onset between our GML High and Low MS cases, *post-hoc* power calculations ($\beta = 0.8$) predicted a cohort of at least 40 cases would be required ($\alpha = 0.05$)]. Whole brain immersion fixation often led to some deformation of tissues, such that it was not always possible to align anatomical structures of

interest, meaning that we could not, for example, report lesion measures for separate deep GM structures. Nevertheless, the use of whole coronal sections reduces observer variation between individual cases, increases the accuracy of observations, and ensures comparisons between different forebrain areas, which are invaluable for the study of this heterogeneous disease.

In conclusion, our quantitative histological analysis revealed global grey matter demyelination and meningeal inflammation to be substantial in a subset of progressive MS brains. Cases defined by a substantial cortical GML load displayed greater microglia/macrophage activation, larger areas of deep grey matter pathology but little change in white matter lesion area, which highlights the partially separate pathogenetic mechanisms of lesion evolution (or susceptibility to damage) in these compartments. The distribution of subcortical and deep GML in MS is consistent with the presence of soluble proinflammatory factors in the subarachnoid space and ventricular CSF and furthers the need to identify companion biomarkers of cortical pathology to aid patient monitoring and therapeutic choice.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study: Griffiths L, Reynolds R, Howell OW
Performed data analysis, interpretation and contributed to writing the manuscript and approved the final submitted document: Griffiths L, Reynolds R, Evans R, Bevan RJ, Rees MI, Gveric D, Neal JW, Howell OW

Availability of data and materials

Data generated during the current study is available on reasonable request.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The use of human post mortem tissue is covered by UK Research Ethics committee approval (study approval number 08/MRE09/31+5).

Consent for publication

Not applicable.

REFERENCES

1. Reynolds R, Roncaroli F, Nicholas R, Radotra B, Gveric D, et al. The neuropathological basis of clinical progression in multiple sclerosis. Acta Neuropathol 2011;122:155-70.

2. Kutzelnigg A, Lucchinetti CF, Stadelmann C, Brück W, Rauschka H, et al. Cortical demyelination and diffuse white matter injury in multiple sclerosis. Brain 2005;128:2705-12.
3. Lucchinetti CF, Popescu BFG, Bunyan RF, Moll NM, Roemer SF, et al. Inflammatory cortical demyelination in early multiple sclerosis. N Engl J Med 2011;365:2188-97.
4. Bevan RJ, Evans R, Griffiths L, Watkins LM, Rees MI, et al. Meningeal inflammation and cortical demyelination in acute multiple sclerosis. Ann Neurol 2018;84:829-42.
5. Kutzelnigg A, Faber-Rod JC, Bauer J, Lucchinetti CF, Sorensen PS, et al. Widespread demyelination in the cerebellar cortex in multiple sclerosis. Brain Pathol 2007;17:38-44.
6. Calabrese M, Magliozzi R, Ciccarelli O, Geurts JJ, Reynolds R, et al. Exploring the origins of grey matter damage in multiple sclerosis. Nat Rev Neurosci 2015;16:147-58.
7. Calabrese M, Romualdi C, Poretto V, Favaretto A, Morra A, et al. The changing clinical course of multiple sclerosis: a matter of gray matter. Ann Neurol 2013;74:76-83.
8. Dalton CM, Chard DT, Davies GR, Miszkiel KA, Altmann DR, et al. Early development of multiple sclerosis is associated with progressive grey matter atrophy in patients presenting with clinically isolated syndromes. Brain 2004;127:1101-7.
9. Calabrese M, Rinaldi F, Mattisi I, Bernardi V, Favaretto A, et al. The predictive value of gray matter atrophy in clinically isolated syndromes. Neurology 2011;77:257-63.
10. Fisniku LK, Chard DT, Jackson JS, Anderson VM, Altmann DR, et al. Gray matter atrophy is related to long-term disability in multiple sclerosis. Ann Neurol 2008;64:247-54.
11. Scalfari A, Romualdi C, Nicholas RS, Mattoscio M, Magliozzi R, et al. The cortical damage, early relapses, and onset of the progressive phase in multiple sclerosis. Neurology 2018;90:e2107-18.
12. Vercellino M, Plano F, Votta B, Mutani R, Giordana MT, et al. Grey matter pathology in multiple sclerosis. J Neuropathol Exp Neurol 2005;64:1101-7.
13. Magliozzi R, Howell O, Vora A, Serafini B, Nicholas R, et al. Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. Brain 2007;130:1089-104.
14. Pitzalis C, Jones GW, Bombardieri M, Jones SA. Ectopic lymphoid-like structures in infection, cancer and autoimmunity. Nat Rev Immunol 2014;14:447-62.
15. Bø L, Vedeler C, Nyland H, rapp BD, Mørk SJ. Intracortical multiple sclerosis lesions are not associated with increased lymphocyte infiltration. Mult Scler 2003;9:323-31.
16. Magliozzi R, Howell OW, Reeves C, Roncaroli F, Nicholas R, et al. A Gradient of neuronal loss and meningeal inflammation in multiple sclerosis. Ann Neurol 2010;68:477-93.
17. Lassmann H. Pathogenic mechanisms associated with different clinical courses of multiple sclerosis. Front Immunol 2019;10:1-14.
18. Howell OW, Reeves CA, Nicholas R, Carassiti D, Radotra B, et al. Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis. Brain 2011;134:2755-71.
19. Haider L, Zrzavy T, Hametner S, Höftberger R, Bagnato F, et al. The topograpy of demyelination and neurodegeneration in the multiple sclerosis brain. Brain 2016;139:807-15.
20. Bø L, Vedeler C a, Nyland HI, Trapp BD, Mørk SJ. Subpial demyelination in the cerebral cortex of multiple sclerosis patients. J Neuropathol Exp Neurol 2003;62:723-32.
21. Lassmann H, Raine CS, Antel J, Prineas JW. Immunopathology of multiple sclerosis: report on an international meeting held at the Institute of Neurology of the University of Vienna. J Neuroimmunol 1998;86:213-7.
22. Mai JK, George Paxinos TV. Atlas of the Human Brain. 3rd edition. Academic Press; 2007.
23. Serafini B, Rosicarelli B, Magliozzi R, Stigliano E, Aloisi F. Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis. Brain Pathol 2004;14:164-74.
24. Motulsky HJ. 'When to choose a nonparametric test'. GraphPad Stat. Guid. Available from: https://www.graphpad.com/guides/prism/8/statistics/index.htm [Last accessed on 2 Mar 2020]
25. Carassiti D, Altmann DR, Petrova N, Pakkenberg B, Scaravilli F, et al. Neuronal loss, demyelination and volume change in the multiple sclerosis neocortex. Neuropathol Appl Neurobiol 2018;44:377-90.
26. Trapp BD, Vignos M, Dudman J, Chang A, Fisher E, et al. Cortical neuronal densities and cerebral white matter demyelination in multiple sclerosis: a retrospective study. Lancet Neurol 2018;17:870-84.
27. Moll NM, Rietsch AM, Ransohoff AJ, Cossoy MB, Huang D, et al. Cortical demyelination in PML and MS: similarities and differences. Neurology 2008;70:336-43.
28. Cifelli A, Arridge M, Jezzard P, Esiri MM, Palace J, et al. Thalamic neurodegeneration in multiple sclerosis. Ann Neurol 2002;52:650-3.
29. Fadda G, Brown RA, Magliozzi R, Aubert-Broche B, O'Mahony J, et al. A surface-in gradient of thalamic damage evolves in pediatric multiple sclerosis. Ann Neurol 2019;85:340-51.
30. Liu Z, Pardini M, Yaldizli O, Sethi V, Muhlert N, et al. Magnetization transfer ratio measures in normal-appearing white matter show periventricular gradient abnormalities in multiple sclerosis. Brain 2015;138:1239-46.
31. Kearney H, Yiannakas MC, Samson RS, Wheeler-Kingshott CA, Ciccarelli O, et al. Investigation of magnetization transfer ratio-derived pial and subpial abnormalities in the multiple sclerosis spinal cord. Brain 2014;137:2456-68.
32. Samson RS, Cardoso MJ, Muhlert N, Sethi V, Wheeler-Kingshott CA, et al. Investigation of outer cortical magnetisation transfer ratio abnormalities in multiple sclerosis clinical subgroups. Mult Scler 2014;20:1322-30.
33. Mainero C, Louapre C, Govindarajan ST, Giannì C, Nielsen AS, et al. A gradient in cortical pathology in multiple sclerosis by in vivo

quantitative 7 T imaging. Brain 2015;138:932-45.
34. Absinta M, Vuolo L, Rao A, Nair G, Sati P, et al. Gadolinium-based MRI characterization of leptomeningeal inflammation in multiple sclerosis. Neurology 2015;85:18-28.
35. Zurawski J, Tauhid S, Chu R, Khalid F, Healy BC, et al. 7T MRI cerebral leptomeningeal enhancement is common in relapsing-remitting multiple sclerosis and is associated with cortical and thalamic lesions. Mult Scler 2020;26:177-87.
36. Pirko I, Lucchinetti CF, Sriram S, Bakshi R. Gray matter involvement in multiple sclerosis. Neurology 2007;68:634-42.
37. Lucchinetti CF, Popescu BFG, Bunyan RF, Moll NM, Roemer SF, et al. Inflammatory cortical demyelination in early multiple sclerosis. N Engl J Med 2011;365:2188-97.
38. Guseo A, Jellinger K. The significance of perivascular infiltrations in multiple sclerosis. J Neurol 1975;211:51-60.
39. Adams CW. Pathology of multiple sclerosis: progression of the lesion. Br Med Bull 1977;33:15-20.
40. Dawson JW. XVIII.-the histology of disseminated sclerosis. Trans R Soc Edinburgh 1916;50:517-740.
41. Howell OW, Schulz-Trieglaff EK, Carassiti D, Gentleman SM, Nicholas R, et al. Extensive grey matter pathology in the cerebellum in multiple sclerosis is linked to inflammation in the subarachnoid space. Neuropathol Appl Neurobiol 2015;41:798-813.
42. DeLuca GC, Alterman R, Martin JL, Mittal A, Blundell S, et al. Casting light on multiple sclerosis heterogeneity: the role of HLA-DRB1 on spinal cord pathology. Brain 2013;136:1025-34.
43. Androdias G, Reynolds R, Chanal M, Ritleng C, Confavreux C, et al. Meningeal T cells associate with diffuse axonal loss in multiple sclerosis spinal cords. Ann Neurol 2010;68:465-76.
44. Brown DA, Sawchenko PE. Time course and distribution of inflammatory and neurodegenerative events suggest structural bases for the pathogenesis of experimental autoimmune encephalomyelitis. J Comp Neurol 2007;502:236-60.
45. Choi SR, Howell OW, Carassiti D, Magliozzi R, Gveric D, et al. Meningeal inflammation plays a role in the pathology of primary progressive multiple sclerosis. Brain 2012;135:2925-37.
46. Gardner C, Magliozzi R, Durrenberger PF, Howell OW, Rundle J, et al. Cortical grey matter demyelination can be induced by elevated pro-inflammatory cytokines in the subarachnoid space of MOG-immunized rats. Brain 2013;136:3596-608.
47. Magliozzi R, Howell OW, Nicholas R, Cruciani C, Castellaro M, et al. Inflammatory intrathecal profiles and cortical damage in multiple sclerosis. Ann Neurol 2018;83:739-55.
48. Magliozzi R, Hametner S, Facchiano F, Marastoni D, Rossi S, et al. Iron homeostasis, complement, and coagulation cascade as CSF signature of cortical lesions in early multiple sclerosis. Ann Clin Transl Neurol 2019;6:2150-63.
49. Schirmer L, Velmeshev D, Holmqvist S, Kaufmann M, Werneburg S, et al. Neuronal vulnerability and multilineage diversity in multiple sclerosis. Nature 2019;573:75-82.
50. Watkins LM, Neal JW, Loveless S, Michailidou I, Ramaglia V, et al. Complement is activated in progressive multiple sclerosis cortical grey matter lesions. J Neuroinflammation 2016;13:161.
51. Klaver R, Popescu V, Voorn P, Galis-de Graaf Y, van der Valk P, et al. Neuronal and axonal loss in normal-appearing gray matter and subpial lesions in multiple sclerosis. J Neuropathol Exp Neurol 2015;74:453-8.
52. Jürgens T, Jafari M, Kreutzfeldt M, Bahn E, Brück W, et al. Reconstruction of single cortical projection neurons reveals primary spine loss in multiple sclerosis. Brain 2016;139:39-46.
53. Peterson JW, Bö L, Mörk S, Chang A, Trapp BD. Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions. Ann Neurol 2001;50:389-400.
54. Fischer MT, Wimmer I, Höftberger R, Gerlach S, Haider L, et al. Disease-specific molecular events in cortical multiple sclerosis lesions. Brain 2013;136:1799-815.
55. Haider L, Fischer MT, Frischer JM, Bauer J, Höftberger R, et al. Oxidative damage in multiple sclerosis lesions. Brain 2011;134:1914-24.
56. Witte ME, Nijland PG, Drexhage JAR, Gerritsen W, Geerts D, et al. Reduced expression of PGC-1α partly underlies mitochondrial changes and correlates with neuronal loss in multiple sclerosis cortex. Acta Neuropathol 2013;125:231-43.
57. Lassmann H, van Horssen J, Mahad D. Progressive multiple sclerosis: pathology and pathogenesis. Nat Rev Neurol 2012;8:647-56.
58. Campbell G., Ziabreva I, Reeve A, Krishnan KJ, Reynolds R, et al. Mitochondrial DNA deletions and neurodegeneration in multiple sclerosis. Ann Neurol 2011;69:481-92.
59. Ofengeim D, Ito Y, Trapp B, Zhang Y, Shan B, et al. Activation of necroptosis in multiple sclerosis. Cell Rep 2015;10:1836-49.
60. Magliozzi R, Howell OW, Durrenberger P, Aricò E, James R, et al. Meningeal inflammation changes the balance of TNF signalling in cortical grey matter in multiple sclerosis. J Neuroinflammation 2019;16:259.
61. Lisak RP, Benjamins JA, Nedelkoska L, Barger JL, Ragheb S, et al. Secretory products of multiple sclerosis B cells are cytotoxic to oligodendroglia in vitro. J Neuroimmunol 2012;246:85-95.
62. Lisak RP, Nedelkoska L, Benjamins JA, Schalk D, Bealmear B, et al. B cells from patients with multiple sclerosis induce cell death via apoptosis in neurons in vitro. J Neuroimmunol 2017;309:88-99.
63. Torkildsen Ø, Stansberg C, Angelskår SM, Kooi EJ, Geurts JJ, et al. Upregulation of immunoglobulin-related genes in cortical sections from multiple sclerosis patients. Brain Pathol 2010;20:720-9.
64. Crick PJ, Griffiths WJ, Zhang J, Beibel M, Abdel-Khalik J, et al. Reduced plasma levels of 25-hydroxycholesterol and increased cerebrospinal fluid levels of bile acid precursors in multiple sclerosis patients. Mol Neurobiol 2017;54:8009-20.
65. Vidaurre OG, Haines JD, Katz Sand I, Adula KP, Huynh JL, et al. Cerebrospinal fluid ceramides from patients with multiple sclerosis impair neuronal bioenergetics. Brain 2014;137:2271-86.
66. Castle D, Wynford-Thomas R, Loveless S, Bentley E, Howell OW, et al. Using biomarkers to predict clinical outcomes in multiple sclerosis. Pract Neurol 2019;19:342-9.

67. Chataway J, Schuerer N, Alsanousi A, Chan D, MacManus D, et al. Effect of high-dose simvastatin on brain atrophy and disability in secondary progressive multiple sclerosis (MS-STAT): a randomised, placebo-controlled, phase 2 trial. Lancet 2014;383:2213-21.
68. Kilsdonk ID, Jonkman LE, Klaver R, van Veluw SJ, Zwanenburg JJ, et al. Increased cortical grey matter lesion detection in multiple sclerosis with 7 T MRI: a post-mortem verification study. Brain 2016;139:1472-81.
69. Haider L, Simeonidou C, Steinberger G, Hametner S, Grigoriadis N, et al. Multiple sclerosis deep grey matter: the relation between demyelination, neurodegeneration, inflammation and iron. J Neurol Neurosurg Psychiatry 2014;85:1386-95.
70. Bramow S, Frischer JM, Lassmann H, Koch-Henriksen N, Lucchinetti CF, et al. Demyelination versus remyelination in progressive multiple sclerosis. Brain 2010;133:2983-98.

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Case Report

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Paraneoplastic cerebellar degeneration associated with somatic mutations in ultra-early diagnosis of small cell lung cancer: a case report

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Abstract

Paraneoplastic cerebellar degeneration (PCD) can occur in patients with underlying cancer, such as small cell lung cancer (SCLC). Anti-CV2/CRMP5 antibodies are well-established biomarkers of PCD associated with SCLC, but cannot be detected in most situations. Recently, next-generation sequencing has been a promising technology to discover cancer-driven mutations, which provide an alternative strategy to accomplish ultra-early diagnosis of those patients. Here, we report the case of a 75-year-old man diagnosed with SCLC, who primarily presented with anti-CV2/CRMP5 antibodies positive PCD. Eight high-frequency gene mutations (*TSC2*, *DNMT1*, *CIC*, *FGF6*, *NSD1*, *TSHR*, *CRLF2*, and *EPPK1*) were detected 7 months before diagnosis with no abnormalities of imaging or cerebrospinal fluid examination found initially. Therefore, this case suggests the possibility of detecting certain somatic mutations for the ultra-early diagnosis of patients presenting with PCD associated with SCLC.

Keywords: Paraneoplastic cerebellar degeneration, small cell lung cancer, next-generation sequencing, ultra-early diagnosis

INTRODUCTION

As an autoimmune-mediated complication of cancer, paraneoplastic cerebellar degeneration (PCD) is associated with tumors such as lung cancer, gynecologic and breast cancers, and Hodgkin lymphoma[1].

It is characterized by subacute onset gait instability that further develops into a pancerebellar syndrome with vertigo, diplopia, dysarthria, and truncal and appendicular ataxia[2]. Characterized by severe loss of cerebellar Purkinje cells, PCD is related to several antineuronal antibodies, such as anti-Yo, anti-Hu, and anti-CV2/CRMP5 antibodies[3]. Anti-CV2 antibodies, also known as anti-CRMP5 (collapsing response-mediator protein 5) antibodies, are well-established biomarkers of PCD associated with small cell lung cancer (SCLC), thymoma, and probably prostatic adenocarcinoma[4]. However, no antibodies are identified in approximately 40% of patients with a PCD due to unclear etiology of PCD or the limited detective technologies utilized[5,6].

The cancer is primarily driven by the accumulation of somatic mutations in the genome, along with the contributions of epigenetic and transcriptomic alterations over one's lifetime. Revolutionary high-throughput DNA sequencing has become a promising and indispensable technique to study cancer[7]. On rare occasions, patient presents with a PCD months to years before cancer is diagnosed[8,9]. Therefore, whether the detection of certain mutations is available for clinical use in the ultra-early diagnosis of patients with PCD is worthy of further investigation.

This case highlights the fact that the detection of certain mutations by next-generation sequencing might plays a key role in ultra-early diagnosis of malignancy in a patient only displaying a PCD.

CASE REPORT

A 75-year-old man presented with a four-month history suggestive of progressive cerebellar symptoms in the form of nausea, vomiting, unsteadiness upon walking, unclear speech, and occasional choking when drinking water. He was treated in a local hospital. Cervical and cranial magnetic resonance imaging examinations and an electroencephalogram showed no obvious abnormalities. The cerebrospinal fluid (CSF) biochemical test showed a protein level of 0.5 g/L (the rest is unknown) at this time.

He was admitted to our hospital on four occasions subsequently. Lumbar puncture was performed twice, and the results showed normal intracranial pressure. Cytology test results showed 6-11 white blood cells/mm^3, with a higher proportion of lymphocytes. Anti-CV2 antibodies were detected in both serum and CSF utilizing immunoblot techniques, but not immunofluorescence. The cancer-driven mutations were captured at the first visit by next-generation sequencing technology in CSF, and the results showed that eight genes (*TSC2*, *DNMT1*, *CIC*, *FGF6*, *NSD1*, *TSHR*, *CRLF2*, and *EPPK1*) had high-frequency mutations. Positron emission tomography-computed tomography scanning was performed in the third visit and no obvious lesions were seen in the parenchyma of the bilateral cerebellar hemisphere, with no signals of density or glucose metabolism changes in those areas.

Chest CT scanning was performed four times [Figure 1]. The first two scans only showed a right lower lobe inflammatory focus. The third scan showed old inflammation, along with enlargement of primary lymph nodes and the fourth scan performed on his fourth visit showed that the enlarged lymph nodes under the aortic arch were larger than before, and a shadow of soft tissue around the aortic arch of the left lower lobe probably reflected a malignant lesion. Histopathological evaluation of a biopsy specimen obtained from the posterior segment of the lung tissue revealed features of small cell cancer. Immunohistochemistry of the lung tissue was positive for CD117, CD56, CK8/18, Syn, and TTF-1 and negative for CgA, CK7, and P40, with a Ki-67 value-added index of 90%. Therefore, morphological combined with immunohistochemical results supported the diagnosis of SCLC, seven months after the cancer-driven mutations were detected. The patient was given small doses of dexamethasone and rituximab (100 mg), and the further treatment was administered at his local hospital's oncology department.

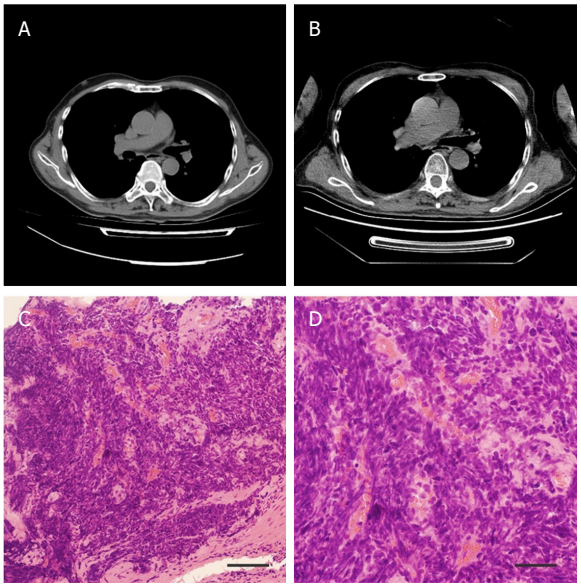

Figure 1. Chest CT scans and pathological result. A: only a right lower lobe inflammatory focus can be found. The chest CT scan was performed at the same time the eight cancer-driven mutations were detected (May 2017); B: the enlarged lymph nodes under the aortic arch can be seen, and a shadow of soft tissue around the aortic arch of the left lower lobe probably reflects a malignant lesion (January 2018); C, D: morphological results reflect the possibility of small cell lung cancer. Scale bar: 400 μm (C); and 200 μm (D)

DISCUSSION

Given that PCD is associated with uncommon neuroendocrine tumors, such as SCLC, breast cancer, and prostate cancer, the possibility of malignancy in patients with a subacute cerebellar syndrome should be considered and excluded by clinicians. Several antineuronal antibodies are related to PCD, and more than 90% of patients harboring those antibodies are confirmed to have a tumor, mostly SCLC[10]. In patients with positive anti-CV2/CRMP5 antibodies, cerebellar ataxia, uveitis, and peripheral neuropathy are the most commonly occurring symptoms[6]. CRMP5, a protein belonging to a family of developmentally regulated neural proteins, expresses in regions of the central nervous system undergoing postnatal plasticity or in the peripheral nervous system mediating Schwann cell differentiation and axon repair[10]. In both anti-Hu and anti-CV2/CRMP5 antibodies positive patients, SCLC was the most commonly found cancer, while malignant thymoma was found in patients only positive for anti-CV2 antibodies[11]. However, considering that the detection of those antibodies has very low sensitivity and specificity[6], clinicians should be more vigilant, especially in cases with initial negative clinical examining results but with high suspicion of malignancy.

Together with epigenetic and transcriptomic alterations, cancer is primarily driven by the accumulation of somatic mutations in the genome over one's lifetime. In recent years, the identification of somatic mutations in cancer genomes has been revolutionized by high-throughput DNA sequencing and all types of somatic mutations can be revealed by whole-genome sequencing. In light of these advances, precision medicine and precision oncology have become possible, and treatments tailored based on an individual's mutational profile could be made.

In this case, no abnormalities were found via imaging or CSF examination until morphological and immunohistochemical results supported the diagnosis of SCLC, seven months after eight somatic mutations were detected. To be specific, we collected 278 somatic cancer-driven mutations from a cancer database and used target region sequencing to find candidate mutations on this occasion. Mutation frequency, variation prediction, and other analytic methods were utilized to analyze the data, and eight high-frequency gene mutations were identified in our patient: *TSC2*, *DNMT1*, *CIC*, *FGF6*, *NSD1*, *TSHR*, *CRLF2*,

and *EPPK1* (the method utilized here showed that the sensitivity, specificity, and diagnostic coincidence rate in diagnosing cancer of the central nervous system were 83.64%, 76.32%, and 80.65%, respectively, but the related article has not been published yet). Many of them have gained great attention in cancer studies. For example, *TSC2* was reported to be related to sporadic pulmonary lymphangioleiomyomatosis[12]. The interaction between *NSD1* and *FLT3/ITD* mutations determines the poor outcome of acute myeloid leukemia patients[13]. Aberrant expression of CRLF2, associated with mutated *JAK2*, was found to underlie the occurrence of acute lymphoblastic leukemia in Down syndrome[14]. *CIC* mutation is among the most studied of these genes, and has been well established to be tumorigenic in glioblastoma[15]. Hence, we hypothesize that the certain mutations found in this case may be related to SCLC with PCD, possibly mediated by the production of anti-CV2 antibodies.

Moreover, in this case, modest efficacy was initially achieved employing a treatment regimen utilizing a combination of dexamethasone and rituximab; however, there was no more significant clinical improvement achieved after the second treatment. A similar scenario was reported in a prior report about a patient presenting with PCD associated with squamous cell carcinoma of the tongue. A likely mechanism to account for this could be the irreversible neuronal tissue damage or the failure to normalize the cellular dysfunction caused by anti-CV2 antibodies in the long term[16]. Therefore, it calls for an alternative method of treatment for these patients targeting high anti-CV2 antibodies or their causative mutations.

Above all, our findings reveal certain mutations that might be related to PCD associated with SCLC to accomplish ultra-early diagnosis. To fulfill this goal, researchers need to overcome several challenges including analysis and interpretation of the sequencing data to understand the underlying mechanisms. Although this field is still in its infancy, pathogenesis, diagnosis, treatment, and prevention of cancer based on somatic mutations deserve more attention.

DECLARATIONS

Acknowledgments

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Authors' contributions

Conception and design of the study: Zhao G, Du F
Performed data acquisition and provided technical and material support: Wu R, He Y
Performed data analysis and interpretation and drafted the work: Shi XD, Li Y
Read and approved the final manuscript: Shi XD, Li Y, He Y, Wu R, Du F, Zhao G

Availability of data and materials

The data and material could be available to readers upon request.

Financial support and sponsorship

The work was supported by National Key Research and Development Program of China (No. 2016YFC0904500) and Shanxi National Science Foundation (No. 2018kw-15).

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Our research proposal has been reviewed and approved by the Independent Ethics Committee (I.E.C.), First Affiliated Hospital of Fourth Military Medical University on 19 May 2016. Number of ethics approval is No. KY20163367-1. The written informed consent was obtained from the patient.

Consent for publication

Not applicable.

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REFERENCES

1. May ML, Dent S. Anti-Yo antibody-mediated paraneoplastic cerebellar degeneration associated with cognitive affective syndrome in a patient with breast cancer: a case report and literature review. Curr Oncol 2018;25:e585-91.
2. Mitoma H, Adhikari K, Aeschlimann D, Chattopadhyay P, Hadjivassiliou M, et al. Consensus paper: neuroimmune mechanisms of cerebellar ataxias. Cerebellum 2016;15:213-32.
3. Shams'ili S, Grefkens J, de Leeuw B, van den Bent M, Hooijkaas H, et al. Paraneoplastic cerebellar degeneration associated with antineuronal antibodies: analysis of 50 patients. Brain 2003;126:1409-18.
4. Aliprandi A, Terruzzi A, Rigamonti A, Bazzigaluppi E, Tremolizzo L, et al. Paraneoplastic cerebellar degeneration with anti-CV2/CRMP5 antibodies and prostate adenocarcinoma. Neurol Sci 2015;36:1501-3.
5. Mason WP, Graus F, Lang B, Honnorat J, Delattre JY, et al. Small-cell lung cancer, paraneoplastic cerebellar degeneration and the Lambert-Eaton myasthenic syndrome. Brain 1997;120:1279-300.
6. Sabater L, Saiz A, Dalmau J, Graus F. Pitfalls in the detection of CV2 (CRMP5) antibodies. J Neuroimmunol 2016;290:80-3.
7. Levy SE, Boone BE. Next-generation sequencing strategies. Cold Spring Harb Perspect Med 2019;9.
8. Bhardwaj S, Khasani S, Benasher D, Stein EG, Meghal T, et al. Paraneoplastic cerebellar degeneration in nasopharyngeal carcinoma: a unique association. Cerebellum 2019;18:1126-9.
9. Abdulaziz ATA, Yu XQ, Zhang L, Jiang XY, Zhou D, et al. Paraneoplastic cerebellar degeneration associated with cerebellar hypermetabolism: case report. Medicine (Baltimore) 2018;97:e10717.
10. Antoine JC. Peripheral neuropathies associated with antibodies directed to intracellular neural antigens. Rev Neurol (Paris) 2014;170:570-6.
11. Antoine JC, Camdessanche JP. Paraneoplastic neuropathies. Curr Opin Neurol 2017;30:513-20.
12. Badri KR, Gao L, Hyjek E, Schuger N, Schuger L, et al. Exonic mutations of TSC2/TSC1 are common but not seen in all sporadic pulmonary lymphangioleiomyomatosis. Am J Respir Crit Care Med 2013;187:663-5.
13. Ostronoff F, Othus M, Gerbing RB, Loken MR, Raimondi SC, et al. NUP98/NSD1 and FLT3/ITD coexpression is more prevalent in younger AML patients and leads to induction failure: a COG and SWOG report. Blood 2014;124:2400-7.
14. Hertzberg L, Vendramini E, Ganmore I, Cazzaniga G, Schmitz M, et al. Down syndrome acute lymphoblastic leukemia, a highly heterogeneous disease in which aberrant expression of CRLF2 is associated with mutated JAK2: a report from the International BFM Study Group. Blood 2010;115:1006-17.
15. Bunda S, Heir P, Metcalf J, Li ASC, Agnihotri S, et al. CIC protein instability contributes to tumorigenesis in glioblastoma. Nat Commun 2019;10:661.
16. Saloustros E, Zaganas I, Mavridis M, Vamvakas L, Plaitakis A, et al. Anti-CV2 associated cerebellar degeneration after complete response to chemoradiation of head and neck carcinoma. J Neurooncol 2010;97:291-4.

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