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REVIEW

Protective effects of pharmacological therapies in animal models of multiple sclerosis: a review of studies 2014–2019

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Abstract

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system. The disability caused by inflammatory demyelination clinically dominates the early stages of relapsing-remitting MS and is reversible. Once there is considerable loss of axons, MS patients enter a secondary progressive stage. Disease-modifying drugs currently in use for MS suppress the immune system and reduce relapse rates but are not effective in the progressive stage. Various animal models of MS (mostly mouse and rat) have been established and proved useful in studying the disease process and response to therapy. The experimental autoimmune encephalomyelitis animal studies reviewed here showed that a chronic progressive disease can be induced by immunization with appropriate amounts of myelin oligodendrocyte glycoprotein together with mycobacterium tuberculosis and pertussis toxin in Freund's adjuvant. The clinical manifestations of autoimmune encephalomyelitis disease were prevented or reduced by treatment with certain pharmacological agents given prior to, at, or after peak disease, and the agents had protective effects as shown by inhibiting demyelination and damage to neurons, axons and oligodendrocytes. In the cuprizone-induced toxicity animal studies, the pharmacological agents tested were able to promote remyelination and increase the number of oligodendrocytes when administered therapeutically or prophylactically. A monoclonal IgM antibody protected axons in the spinal cord and preserved motor function in animals inoculated with Theiler's murine encephalomyelitis virus. In all these studies the pharmacological agents were administered singly. A combination therapy may be more effective, especially using agents that target neuroinflammation and neurodegeneration, as they may exert synergistic actions.

Key Words: *animal models; autoimmune encephalomyelitis disease; cuprizone-induced toxicity; multiple sclerosis; neurodegeneration; neuroinflammation; neuroprotection; pharmacological agents; progressive disease; Theiler's murine encephalomyelitis virus*

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Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). It is the main cause of non-traumatic neurological disability in young adults in the USA and Europe (Dutta and Trapp, 2011). Several phenotypes of MS have been recognized which include relapsing-remitting (RRMS) and progressive phenotypes (secondary and primary, SPMS and PPMS). Acute relapses are pathologically associated with inflammatory demyelination of the white matter, whereas disease progression is pathologically associated with irreversible damage to neurons. Inflammation may decrease in the late relapsing stage to levels found in age-matched controls; however, age- and disease-related neurodegeneration occurs which affects the damaged brain and spinal cord, causing further disability progression. In this stage of the disease, anti-inflammatory treatments are unlikely to be of benefit but neuroprotective and repair-inducing treatments may be successful (Lasssman, 2017). Relapses and new lesions in patients with RRMS have been prevented by disease-modifying treatments that primarily target adaptive immunity, but they have limited efficacy on disability progression (Zhang et al., 2013). In a recent review Kantarci et al. (2014) wrote "*No established treatment significantly impacts the neurodegenerative component of progressive disease course in MS. Any effect seen is seemingly related to prevention of ongoing relapses. Different and more effective strategies are needed to prevent lesions long before the progressive phase starts. The future prevention of MS-related disability will probably depend on how effectively we can design treatment protocols to prevent progressive disease course in MS.*" A drug that promotes endogenous remyelination and/or decreases axonal degeneration would reduce the rate and degree of disease progression (Moore et al., 2014a). All currently licensed immunomodulatory therapies for MS do not support remyelination directly (Williams, 2015; Strangel et al., 2017).

The use of animal models of MS allows for investigating and furthering an understanding of the pathological changes during the disease course and the testing of new therapies (Hochstrasser et al., 2018; Burrows et al., 2019). These models include animals (commonly rodents) in which experimental autoimmune encephalomyelitis (EAE) has been developed. They have been used to study white matter inflammatory demyelination which inhibits axonal conduction and suppression of inflammation causes recovery. They also allow a study of disease course viz. relapsing or progressive disease. Another model is the cuprizone (CPZ) model in which oligodendrocyte apoptosis leads to innate immune ac-

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tivation and finally demyelination. A third model is the Theiler's murine encephalomyelitis virus (TMEV) model and can be used to study progressive MS. The virus infects glial cells and macrophages during the chronic disease phase, which leads to inflammatory demyelination with oligodendrocyte apoptosis and axonal degeneration.

Neurodegeneration occurs to a variable extent in these models. In the EAE and TMEV models, tissue damage is mainly due to activation of the acquired immune system, whereas in the CPZ model activation of the innate immune system results in tissue damage. Interestingly, combining the immunomodulatory peptide, tuftsin, with benztropine, a remyelination-stimulating drug, alleviated symptoms in an EAE model and decreased pathological hallmarks in both EAE and CPZ models of MS. Tuftsin changed the inflammatory CNS environment normally present in the EAE model into anti-inflammatory one, and benztropin improved remyelination in the CPZ model (Thompson et al., 2018). Other toxin-induced animal models of MS include use of ethidium bromide and lysolecithin to cause myelin damage (van der Star et al., 2012).

We have performed a search of articles on the PubMed database for the period January 2014–May 2019 to evaluate the progress made in the testing of neuroprotective agents in animal models of MS and whether they are able to reduce the loss of neurons and axons in the chronic phase of the disease and impede disability progression which had been suggested as challenges in the review by Kantarci et al. (2014).

Animal Models of Multiple Sclerosis Disease

The most characterized animal models of MS are the EAE model, the toxin-induced model (CPZ model), and TMEV infection model (Procaccini et al., 2015).

Experimental autoimmune encephalomyelitis animal model

This is the most used animal model of MS. EAE is characterized by inflammatory infiltrates of T lymphocytes, B lymphocytes, macrophages and focal demyelinating plaques in the CNS. The cytokines and other factors released by the infiltrating cells cause oligodendrocyte degeneration. EAE can be induced in various species, including rodents and primates, either by active immunization using a myelin antigen in adjuvant (active EAE) or by the adoptive transfer of encephalitogenic T cells (passive EAE).

The commonly used EAE mouse models exhibit motor dysfunction as ascending paralysis, beginning with a flaccid or limp tail (Stromnes and Goverman, 2006a; Baxter, 2007). The paralysis progresses from the hind limbs to the fore limbs and is sometimes followed by urinary incontinence and fecal impaction (classical EAE models). In classical EAE, inflammatory infiltration begins at the lumbar region of the spinal cord (Juźwik et al., 2018). The standard EAE mouse model is induced using myelin proteolipid protein (PLP) peptide (amino acids 139–151), which causes relapse-remitting EAE in SJL mice. In addition, the myelin oligodendrocyte glycoprotein (MOG) peptide (amino acids 35–55) causes monophasic EAE in C57BL/6 mice with an incomplete recovery (Gold et al., 2006). Several studies have demonstrated real primary progressive or secondary progressive EAE, in which mice die as a result of disease progression (Tsunoda et al., 2000). Variations from classical EAE phenotype include ataxia or head rolling rather than limb paralysis, and are referred to as atypical EAE (Sobel, 2000). The observed clinical signs in atypical EAE models are associated with increased inflammation in the brain compared to classical EAE models (Takeuchi et al., 2013).

In the active EAE model, mice are immunized by subcutaneous injection (s.c.) of the myelin antigen with Freund's adjuvant and *Mycobacterium tuberculosis*. The incidence of EAE induction is increased by intravenous or intraperitoneal (i.p.) injection of pertussis toxin. The signs of motor dysfunction depend on the type of EAE model, and paralysis usually begins 9 to 14 days after sensitization (Stromnes and Goverman, 2006a). Passive EAE is induced in naïve mice by the adoptive transfer of T cells isolated from active EAE mice that have been sensitized with myelin antigens. Visible signs of EAE usually appear 10 to 15 days after induction (Stromnes and Goverman, 2006b). The severity of EAE is usually evaluated using an EAE scoring system, with mice being examined daily after the day of sensitization to accurately detect the time of disease onset and to monitor the progression of EAE. In most studies, a 0 to 5 point scale is used: 0, refers to no clinical signs; 1, paralyzed tail; 2, loss in coordinated movement, hind limb paralysis; 3, both hind limbs paralysis; 4, fore limbs paralysis; 5, moribund or death (Stromnes and Goverman, 2006a). The mice are weighed daily and scored for EAE signs after sensitization. A decrease in body weight that usually precedes paralysis is common in EAE, and lowered body weight remains during the recovery phase (Encinas et al., 2001). Body weight begins to increase during the chronic phase of the disease; hence, body weight loss is an important sign during the acute stage of EAE.

Cuprizone mouse model

Treatment with CPZ, a copper chelator, is the most frequently used of the toxin-induced models of MS. It is used to study mechanisms of oligodendrocyte turnover, astrogliosis and microgliosis (Blakemore and Franklin, 2008). As opposed to other toxin-induced models, in which the toxin is introduced into the brain by stereotactic microinjections and causes focal demyelination, oral administration of CPZ produces a global insult. Different rodent strains respond to CPZ treatment in distinctive ways and is best shown in the C57BL/6 strain. Unlike C57BL/6 mice, BALB/cJ mice develop a delayed and incomplete demyelination in specific brain regions following CPZ treatment (Skripuletz et al., 2008). Rats are resistant to CPZ-induced demyelination and are not a suitable model to study remyelination processes (Love, 1988). Female mice are more resistant to demyelination induced by CPZ (Taylor et al., 2009), so typically male C57BL/6 mice are fed CPZ, which leads mainly to selective oligodendrocyte death and reproducible demyelination in the brain (Hibbits et al., 2009; Steelman et al., 2012). Brain

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lesions in CPZ-fed mice can be shown by a MRI technique, but no dependable noninvasive method exists to distinguish the rate of remyelination in such mice from that in untreated animals. This necessitates the killing of animals in order to collect the corpus callosum and determine the effects of the toxicant, making it difficult to perform longitudinal studies. Consequently a large number of mice are required to sufficiently statistically power the studies.

CPZ causes demyelination by directly killing oligodendrocytes in the mouse brain. Treatment with CPZ is unlikely to mimic the complex pathology of relapsing-remitting MS and secondary progressive MS in humans (MacArthur and Papanikolaou, 2014).

As oligodendrocyte precursors mature, robust myelin repair can be observed in CPZ-fed mice after removal of CPZ from the chow. The pattern of demyelination and spontaneous remyelination occurs over a highly expected time course and within anatomically distinct areas, and can be evaluated using microscopic analysis. However, there are no overt signs such as paralysis or other observable nervous system impairments resulting from the CPZ-induced demyelination.

Theiler's murine encephalomyelitis virus model

TMEV is a single-stranded RNA virus that is a member of the *Picomaviridae* family. In susceptible strains of mice, it induces early acute disease resembling polioencephalomyelitis followed by late chronic demyelinating disease. During early acute disease the virus replicates in gray matter of the CNS but is eliminated to very low titers 2 weeks post-infection. Late chronic demyelinating disease occurs 2 weeks later and is characterized by extensive demyelinating lesions and mononuclear cell infiltrates, progressive spinal cord atrophy, and axonal loss (Oleszak et al., 2004).

Intracranial inoculation of susceptible strains of mice with the Theiler's DA strain induces biphasic disease, with early acute disease occurring within 3 to 12 days post-immunization (p.i.), followed by late chronic demyelinating disease at 30 to 40 days, and eventually causes the death of the animals (Oleszak et al., 1995; Dal Canto et al., 1996). Resistant strains of mice, such as C57BL/6, develop only slowly early acute disease, clear the virus completely in about 3 weeks p.i., and do not develop late chronic demyelinating disease (Lorch et al., 1981). Intracerebral infection of the C57BL/6J mouse strain causes acute seizures and epilepsy (DePaula-Silva et al., 2017).

Pharmacological Therapies for Multiple Sclerosis-Type Disease

The pharmacological therapies were with cerebrolysin, CTK 01512-2 (a calcium channel blocker), melatonin, naltrexone, diarylpropionitrile, ST266 (amnion cell secretome), trichostatin A, C-phycocyanin, siponimod, white grape juice extract, lanthionine ketamine ester, caffeine, adrenomedullin, glatiramer acetate (all in EAE mouse model); Pien Tze Huang (PZH), dihydrotestosterone, memantine hydrochloride, pregabalin (all in EAE rat model); bilobalide, linagliptin, BLZ945 (colony-stimulating factor 1 receptor kinase inhibitor), indomethacin (all in CPZ mouse model); recombinant human IgM12 antibody (in TMEV mouse model). There were fourteen studies using EAE mouse model and four studies using EAE rat model found in the PubMed search. All except three had used immunization with $MOG_{35–55}$ peptide emulsified in Freund's adjuvant, supplemented in most studies with mycobacterium tuberculosis and receiving pertussis toxin. In addition, four studies using CPZ mouse model and one study using TMEV mouse model were found in the PubMed search. In the mouse studies, the ages of the animals at which treatment was started ranged from 7–17 weeks for those studies in which ages were reported, and where gender was specified 13 studies had used females and 3 studies had used males. In the rat studies, the ages of the animals at which treatment was started ranged from 7–13 weeks, and where gender was specified 2 had used females and 2 had used males.

EAE animal studies

Cerebrolysin

Treatment i.p. with cerebrolysin (low molecular weight neuropeptide(s) prepared from extract of porcine brain tissue, Ghaffarpasand et al., 2018) for 28 days of female C57BL/6 mice, 10–11 weeks of age in which EAE had been induced by $MOG_{35–55}$ immunization, decreased the average clinical scores compared to mice treated with saline. Cerebrolysin alleviated demyelination in optic nerves and cervical spinal cord. In addition, cerebrolysin alleviated neurofilament loss in cervical spinal cord and microglia density (ionized calcium binding adaptor molecule 1 (Iba-1) staining) in the medulla. In this study a relapsing-remitting pattern of the disease was observed. The beginning of hind limb deficits in EAE mice was observed 8–12 days p.i. with increasing clinical scores up to 14 days after the beginning of the initial impairments, after which the scores began to decrease (Toader et al., 2018).

CTK 01512-2

Treatment with CTK 01512-2 (a calcium channel blocker) (50 and 100 pmol/site) intrathecal (i.t.) on days 4, 10, 15, 20, and 24 p.i. of female C57BL/6 mice, 6–8 weeks of age in which EAE had been induced by $MOG_{35–55}$ immunization, delayed the onset of symptoms and reduced the clinical manifestations of EAE, prevented the body weight loss, and significantly decreased neurological impairment compared to EAE-vehicle mice. CTK 01512-2 (50 pmol/site, i.t., days 4, 10, 15, 20, 24) significantly reduced the levels of tumor necrosis factor (TNF), interleukin (IL)-1β, interferon (IFN)-γ, IL-17 and IL-23; the values were close to those observed in the naïve group for spleen, brain and spinal cord except for IL-1β in the spleen and IL-23 in the brain and spleen. Treatment with CTK 01512-2 (50 pmol/site, i.t., days 4, 10, 15, 20, 24) significantly increased IL-10 levels in the spleen, brain and spinal cord at 25 days p.i. compared to EAE-vehicle group. Also, CTK 01512-2 treatment (50 pmol/site) significantly decreased inflammation and demyelination in the lumbar spinal cord and inflammation in the brain at 25

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days p.i.. CTK 01512-2 (50 pmol/site) significantly reduced the level of astrocyte activation (glial fibrillary acidic portein (GFAP) marker) in the lumbar spinal cord and microglia activation (Iba-1) in the brain at 25 days p.i. compared to EAE-vehicle group. CTK 01512-2 treatment (50 pmol/site, i.t., days 4, 10, 15, 20) significantly decreased the neuroinflammatory process in all evaluated brain regions, except for the hippocampus, on day 23 p.i. Administration of CTK 01512-2 by intravenous route at 0.2 mg/kg every 3 days, starting on day 7 post-$MOG_{35–55}$ injection, reduced all behavioral and non-behavioral parameters examined previously, and with a similar efficacy to i.t. route (Silva et al., 2018).

Melatonin

Female C57BL/6 mice, 8–10 weeks of age in which EAE had been induced by $MOG_{35–55}$ immunization, were treated i.p. with melatonin on day 0 p.i. to the peak period of the disease. Melatonin significantly decreased the neurological deficit score compared to EAE-vehicle mice and significantly delayed the first neurological signs of disease and shortened the clinical symptomatic stage. Treatment with melatonin decreased the number of inflammatory lymphocytes infiltrating into the spinal cord compared to EAE-vehicle mice. Melatonin significantly decreased the levels of thiobarbituric acid reactive substance (TBARS) and reactive oxygen species (ROS) and significantly increased the levels of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) compared with EAE-vehicle mice. Treatment with melatonin significantly increased the expression of nuclear factor erythroid 2-related factor 2, and its related antioxidant enzymes heme oxygenase-1 and NAD(P)H: quinione oxidoreductase 1 in the spinal cord at the peak of disease (Long et al., 2018).

Diarylpropionitrile

Treatment s.c. with diarylpropionitrile (an estrogen receptor β ligand) was initiated at 1 week prior to EAE induction and on alternate days to female ovariectomized C57BL/6 mice, 8–12 weeks of age in which disease had been induced by $MOG_{35–55}$ immunization. EAE mice treated with diarylpropionitrile exhibited alleviation of EAE during the chronic phase. Diarylpropionitrile protected axons in EAE mice as indicated by an increase in neurofilament 200 $(NF200)^+$ axons and a decrease in non-phosphorylated NF200 $(SMI32)^+$ and β-amyloid precursor protein $(βAPP)^+$ axons in spinal cord white matter. Myelin in EAE mice was protected by diarylpropionitrile and the g-ratio was decreased, consistent with increased myelin. Diarylpropionitrile treatment decreased the proinflammatory phenotype of myeloid cells in the CNS during EAE, and increased the percentages of $Olig2^+GSTπ^+$ mature and $Olig2^+CC1^+$ immature/mature oligodendrocytes (Kim et al., 2018).

Naltrexone

Naltrexone (an opioid receptor antagonist) was administered i.p. for 14 days to female C57BL/6 mice, 7–8 weeks of age in which EAE had been induced by $MOG_{35–55}$ immunization. Within 1 week p.i. and prior to observed clinical disease, EAE-PBS mice had significantly lower serum encephalin levels compared to controls. After 7 days of naltrexone treatment, EAE mice receiving naltrexone in the morning had significantly higher serum opioid growth factor ($[Met^5]$-enkephalin) when blood was collected in the morning and substantially less enkephalin in the serum when blood was collected in the afternoon. At week 3 (peak disease), treatment with naltrexone lowered the clinical score and increased serum enkephalin level compared to EAE-PBS mice. EAE mice injected with naltrexone for 7 days had a significant decrease in total leukocytes compared to mice receiving PBS. Naltrexone treated mice had significantly longer hot plate response at 7 days compared to EAE-PBS mice, but no difference at 2 or 3 weeks following a 7-day washout period. No difference was found in touch response over 3 weeks of testing between EAE-naltrexone and EAE-PBS mice (Ludwig et al., 2017).

ST266

Female C57BL/6 mice, 8 weeks of age in which EAE had been induced by $MOG_{35–55}$ immunization, were treated intranasally with ST266 (secretome of amnion-derived multipotent progenitor cells) before onset of optic neuritis (day 1 to day 42) or after (days 15–30, 15–42, 22–42, and 30–42). ST266 treatment before the onset of optic neuritis significantly improved visual function of EAE mice. ST266 significantly decreased the loss of retinal ganglion cells and their axons in EAE mice at 6 weeks p.i. In addition, daily ST266 treatment significantly decreased inflammatory cell infiltration of optic nerve and significantly reduced the degree of demyelination induced by EAE optic neuritis at 6 weeks p.i. compared to EAE-vehicle mice. ST266 treatment initiated at day 15 significantly prevented retinal ganglion cell loss at day 42, and treatments started on day 22 or 30 showed a trend towards increased retinal ganglion cell survival. A small significant increase in retinal ganglion cell axon density was induced by ST266. Improved visual function and retinal ganglion cell survival in ST266 treated mice was associated with decreased optic nerve inflammation. Demyelination was also decreased in ST266 treated mice compared with vehicle treated mice. ST266 treatment started before or after onset of optic neuritis had little effect on ascending paralysis and related spinal cord inflammation and demyelination, consistent with the lower levels of ST266 that reach other parts of the CNS following intranasal delivery. ST266 treatment of EAE mice beginning on day 15 p.i. resulted in increased expression of NAD-dependent protein deacetylase sirtuin-1 (SIRT1) in the retina by day 22 compared to EAE-vehicle mice. This was associated with increased retinal and optic nerve levels of mitochondrial enzymes consistent with increased mitochondrial biogenesis in EAE-ST266 mice. In addition, ST266 treatment decreased ROS that accumulated in the optic nerve during optic neuritis (Khan et al., 2017).

Trichostatin A

Treatment s.c. 3 times/week for 8 weeks with trichostatin A (an inhibitor of histone deacetylase) of female NOD/ShiLtj

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mice, 9 weeks of age in which EAE had been induced by $MOG_{35–55}$ immunization, substantially decreased the clinical severity of the disease and protection was found after 9 weeks of trichostatin A cessation. The sustained disease course without pronounced remissions is similar to primary progressive MS. Trichostatin A treatment during the first 2 weeks of immunization was sufficient to lessen the progression of EAE. Also established EAE in NOD mice could be reversed by trichostatin A treatment started after the onset of severe disease (clinical score > 2.0) on day 15 and continued up to 45 days. The clinical score was decreased as soon as 5 days after the first dose of trichostatin A given therapeutically and protection lasted for at least 5 weeks following trichostatin A termination. Prophylactic treatment with trichostatin A significantly decreased the inflammation of the spinal cord both during the early (28 days) and chronic stage (54 days) and prevented demyelination and loss of axons during the chronic stage of the disease on day 54. Trichostatin A treatment reduced the infiltration of Th1 cells in NOD mice. During the late phase of the disease (97 days), in addition to Th1 cells, Th17 cells as well as those producing granulocyte-macrophage colony stimulating factor (GM-CSF) appeared in the spinal cord. Trichostatin A treatment decreased the frequency of the T cells infiltrating the CNS regardless of their lymphokine profiles, and mitigated neuronal damage (Jayaraman et al., 2017).

C-Phycocyanin

Female C57BL/6 mice, 6–8 weeks of age in which EAE had been induced by $MOG_{35–55}$ immunization, were treated i.p. with C-phycocyanin at disease onset for 15 days. C-Phycocyanin treatment appeared to decrease disease severity in EAE mice with maximum clinical scores lowered in mice treated with C-phycocyanin 4 mg/kg per day and 8 mg/kg per day. Mice treated with C-phycocyanin 4 mg/kg per day and 8 mg/kg per day had a significant reduction in the number of inflammatory foci present in the spinal cord at day 29 p.i. A significant reduction of the extent of white matter demyelination in the spinal cord was also identified in Luxol fast blue (LFB)-periodic acid Schiff (PAS) staining for C-phycocyanin doses 4 and 8 mg/kg per day. In Mac-3 staining for activated macrophages/microglia, there was a significant reduction of these cells in the lesions of C-phycocyanin 8 mg/kg per day treated EAE mice compared to vehicle treated EAE mice. At C-phycocyanin 4 mg/kg per day, there was a tendency for a reduction compared to the EAE vehicle group. However, C-phycocyanin 4 mg/kg per day treatment effectively counteracted axonal degeneration in EAE mice while there was only a tendency for a reduction with C-phycocyanin 8 mg/kg per day treatment. C-Phycocyanin treatment 8 mg/kg per day reduced the levels of oxidative stress parameters malondialdehyde, peroxidation potential, and CAT/SOD ratio, and significantly increased reduced glutathione (GSH) levels in blood lysates taken at day 15 p.i. In addition, C-phycocyanin treatment 8 mg/kg per day significantly decreased the relative expression of IL-17 mRNA and IL-6 mRNA in the brain compared to EAE vehicle animals at day 18 p.i. A set of genes associated with the remyelination process was upregulated by C-phycocyanin: Mal, Mog and Mobp, which are structural components of the myelin sheath; a transcription factor Olig1 present exclusively in oligodendrocytes; Nkx6-2 and Nkx2-2 genes related to the axon-glia interactions; and Bmp, involved in the processes of gliogenesis. Additionally, C-phycocyanin significantly decreased the expression of genes involved in the process of demyelination such as CD44 and PPAR (Pentón-Rol et al., 2016).

Siponimod

C57BL/6 mice were immunized with $MOG_{35–55}$ to induce EAE and 1 week prior to immunization received continuous intracerebroventricular (i.c.v.) infusion of siponimod for 4 weeks. Treatment with siponimod 4.5 μg/day significantly decreased the number of T lymphocytes in the blood of EAE-induced mice compared to EAE-vehicle mice at 18 days p.i., during the symptomatic phase of the disease. Siponimod 4.5 μg/day completely inhibited EAE development in treated mice compared to EAE-vehicle mice, suggesting that the effects of siponimod on peripheral lymphocytes mediated its disease-modifying activity when administered at this daily concentration. A siponimod dose of 0.45 μg/day had comparable effects on peripheral lymphocytes and significantly alleviated disease severity on days 21–24 p.i. compared to EAE-vehicle mice. In coronal striatal slices, the levels of GFAP (marker of astrocyte activation) and Iba-1 (marker of microglia/macrophage) were significantly decreased in EAE mice treated with siponimod 0.45 μg/day, as well as a decrease in the number of infiltrating lymphocytes. Quantitative analysis of Iba-1 mRNA and CD3 mRNA by qPCR confirmed significantly decreased microglia activation and lymphocyte infiltration in the striatum of EAE mice receiving siponimod compared to EAE-vehicle mice. Siponimod reduced GABAergic interneuron loss in the striatum of symptomatic EAE mice (Gentile et al., 2016).

White grape juice extract

Male C57BL/6 mice, 6–9 weeks of age in which EAE was induced by $MOG_{35–55}$ immunization, were treated p.o. with white grape juice extract (WGJe) given 1 week before EAE induction and continued until day 21. Mice treated daily with WGJE 40 mg/kg showed only slight signs of the disease (clinical score 0.9), while those receiving WGJe 20 mg/kg per day had decreased severity of disease (clinical score 2.4, compared to clinical score 4.8 for EAE control mice). An increase in body weight occurred in EAE mice receiving WGJe 40 mg/kg per day. A significant infiltration of T lymphocytes, monocytes and neutrophils was observed in the white matter of the spinal cord of EAE mice and demyelinating plaque formation. Treatment with WGJe 40 mg/kg per day significantly protected against EAE changes and a slight preventive effect occurred in WGJe 20 mg/kg per day treated mice in which there were some cells and leukocytes infiltrated areas of demyelination. High levels of TNF-α staining were observed in spinal cord tissues of EAE mice, but no positive staining for TNF-α was found in spinal cord tissues of mice treated with

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WGJe 20 or 40 mg/kg per day. A positive staining for nitrotyrosine (marker of oxidative stress) in spinal cord sections of EAE mice was found; treatment with WGJe significantly lowered the level of nitrotyrosine, with a dose of 40 mg/kg per day more effective than 20 mg/kg per day. WGJe at 20 mg/kg per day significantly decreased inducible nitric oxide synthase (iNOS) expression caused by EAE and no positive staining was observed for iNOS in WGJe 40 mg/kg per day treated mice. Regulatory T cells (Treg) are characterized by the expression of transcription factor Fork head box P3 (Foxp3). Spinal cord sections from EAE mice showed positive staining for Foxp3 but was not observed in WGJe 20 mg/kg per day and 40 mg/kg per day treated mice. A prounced presence of apoptotic cells was identified in spinal cord sections of EAE mice using TUNEL staining. The number of apoptotic cells was reduced in WGJe 20 mg/kg per day treated mice, and none were observed in WGJe 40 mg/kg per day treated mice. While no staining for Bcl-2 could be detected in spinal cord sections of EAE mice, positive staining was observed for WGJe 20 mg/kg per day and 40 mg/kg per day treated mice. Spinal cord sections of EAE mice had high levels of expression of caspase 3 and this was prevented by treatment with WGJe 20 mg/kg per day and 40 mg/kg per day (Giacoppo et al., 2015).

Lanthionine ketamine ester

Female C57BL/6 mice, 8 weeks mean age in which EAE was induced by $MOG_{35–55}$ immunization, were provided with lanthionine ketamine ester (LKE) 0.01% (w/w) in chow giving an average daily dose of 8 mg/kg beginning on day 36 p.i. and continued to day 57 p.i. Mice treated with LKE showed significant clinical recovery over this period with the average clinical score decreased from 2.3 to 1.3. Total neurodegeneration in the optic nerve and spinal cord of EAE mice was significantly reduced by LKE treatment. Treatment with LKE significantly decreased the percentage of axons with abnormal mitochondria and loss of contact in the optic nerve, and significantly decreased axons with condensation in the spinal cord. From EM images of the optic nerve there was a significant increase in myelin thickness and axon caliber in LKE-treated mice versus vehicle-treated mice. In the spinal cord, LKE treatment significantly increased myelin thickness compared to vehicle-treated EAE mice. No significant change in g-ratio due to LKE treatment occurred in either the optic nerve or spinal cord. While LKE treatment had modest, non-significant effects on CRMP2 and pCRMP2, the ratio of pCRMP2:CRMP2 which was greatly increased in the EAE mice was significantly lowered by LKE treatment (Dupree et al., 2015).

Caffeine

Treatment with caffeine 30 mg/kg per day in drinking water of female C57BL/6 mice, 8–10 weeks of age in which EAE had been induced by $MOG_{35–55}$ immunization, was performed over different time periods. The caffeine (0–10)-EAE group that received caffeine during the induction phase (0–10 days p.i.) showed a trend of worse neurological deficits and higher EAE scores and a relatively early peak of the neurological deficit compared to the water-EAE group, but was not statistically significant. By contrast, the caffeine (10–20)-EAE group received caffeine treatment at the effector phase (10–20 days p.i.) and had significantly decreased neurological deficits compared to the water-EAE group, with reduced maximal deficit score and relatively delayed disease onset. The most significant protection was found in the caffeine (−10–20)-EAE group that received caffeine for the entire course. Caffeine treatment given immediately prior to MOG immunization (i.e. −10–0 days p.i.) was not effective in modifying the disease development. Mice in the water-EAE group showed extensive entry of inflammatory cells into the spinal cord, and mice in the caffeine (0–10)-EAE group also showed inflammatory cell infiltration. Caffeine treatment for the entire EAE course (i.e. −10–20 days p.i.) protected against EAE pathology, with reduced infiltration of inflammatory cells into the spinal cord in association with decreased neurological deficits. Caffeine treatment at the effector phase (i.e. 10–20 days p.i.) significantly protected against EAE pathology, while caffeine treatment at the induction phase (i.e. 0–10 days p.i.) was not effective. While 10 days of caffeine treatment in caffeine (−10–0)-EAE, caffeine (0–10)-EAE, and caffeine (10–20)-EAE groups did not produce significant neuroprotective effects against demyelination in the cerebral cortex, the intensity of LFB staining was significantly higher for 30 days caffeine treatment in caffeine (−10–20)-EAE group. Comparing the expression of proinflammatory cytokines in the cerebral cortex to the water-CFA group, IL-17 mRNA in the brain was increased in the water-EAE group, caffeine (−10–0)-EAE group, and caffeine (0–10)-EAE group, but caffeine treatment in the caffeine (10–20)-EAE group and caffeine (−10–20)-EAE group significantly partially reversed this increase. Also the expression levels of IFN-γ mRNA in the brain were markedly increased in the water-EAE group and caffeine (0–10)-EAE group compared to the water-CFA group. The caffeine (−10–20)-EAE group had a significant lowering of the increase of IFN-γ, with caffeine (−10–0)-EAE group and caffeine (10–20)-EAE group showing similar reduction but without reaching statistical significance. The expression level of the anti-inflammatory cytokine transforming growth factor-β (TGF-β) was decreased in the water-EAE group compared to water-CFA group. Caffeine treatment in the caffeine (−10–20)-EAE group and caffeine (10–20)-EAE group significantly increased the TGF-β mRNA level in the brain compared to the water-EAE group (Wang et al., 2014).

Adrenomedullin

Female C57BL/6 mice, 8 weeks of age in which EAE was induced by $MOG_{35–55}$ immunization, were treated i.p. for 5 consecutive days in mice with a clinical score of 0.5–1 (onset) or 1–1.5 or > 2 (acute phase). Disease incidence and severity were greatly decreased by treatment with adrenomedullin after the onset or during the effector phase of the disease. Most of the adrenomedullin-treated EAE mice showed mild symptoms and a significant number recovered completely

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and were entirely asymptomatic 30–40 days after disease course. A short treatment for 5 days with adrenomedullin was sufficient to produce a long lasting protective effect. Adrenomedullin treatment markedly decreased the number of plaques with inflammatory infiltrates and areas of demyelination in both the lumbar and cervical regions of the spinal cord compared to EAE-vehicle group. Immunofluorescence analysis of CNS infiltrates in EAE animals at peak of disease revealed that the inflammatory cells ($CD45^+$) close to the perivascular area were mostly $CD4^+$ cells and $Iba\text{-}1^+$ macrophages. Treatment with adrenomedullin decreased the number of $CD45^+$, $CD4^+$, and $Iba\text{-}1^+$ cells infiltrating the CNS and correlated with a significant decrease in the levels of inflammatory cytokines and chemokines including IL-6, IFN-γ, CCL5, CCL2, CCXCL2 in the spinal cords at peak of disease. Also, adrenomedullin treatment significantly decreased the levels of GM-CSF and osteopontin in the spinal cords at peak of disease, two inflammatory markers that play a major role in the induction and progression of EAE and MS. Adrenomedullin treatment significantly decreased the number of effector T cells secreting IFN-γ but not IL-17 in draining lymph nodes and spleen, and significantly increased the percentage of IL-4- and IL-10-expressing CD4 cells. Adrenomedullin administration increased the number and percentage of IL-10-secreting $CD4^+CD25^+Foxp3^+$ Treg cells in draining lymph nodes and spleens of EAE mice. Despite lower numbers of CD4 T cells, adrenomedullin significantly increased the percentage of $Foxp3^+$ cells in the CNS. Treg cells confer significant protection against EAE by deactivating autoreactive T cells and their homing to the CNS (Kohm et al., 2002; Bynoe et al., 2003). Adrenomedullin decreased the production of inflammatory cytokines TNF-α, IL-12, and IL-6 from cultures of lipopolysaccharide-activated astrocytes, microglia and neuron-glia isolated from newborn mice, and of NO by lipopolysaccharide/IFN-γ-activated microglia. Adrenomedullin significantly decreased cell death induced by oxidative stress in precursor and mature oligodendrocytes. The gene expression of neuroprotective factors such as brain-derived neurotrophic factor (BDNF) and activity-dependent neuroprotective protein (ADNP) in the spinal cords was significantly increased by treatment of EAE mice with adrenomedullin at disease onset (Pedreño et al., 2014).

Glatiramer acetate

C57BL/6 mice, 8 weeks of age in which EAE was induced by $MOG_{35–55}$ immunization, were treated s.c. with glatiramer acetate 2 mg/day for 8 consecutive days beginning after the appearance of clinical disease (days ~12–15 p.i.) starting on day 16 (clinical score ~2, hind limb weakness) or day 21 (clinical score ~3, partial hind limb paralysis). Vehicle-treated EAE mice assumed a chronic, persistent EAE disability phenotype beginning at day ~20, as indicated by increased EAE clinical scores. Glatiramer acetate treatment started on day 16 caused a suppression of disease phenotype, with treated mice showing a pronounced decrease in EAE clinical scores. A significant but less pronounced and persistent decrease in EAE scores was observed when glatiramer acetate treatment was started on day 21. The persistent clinical benefit long after cessation of glatiramer acetate treatment indicated a durable response. Rotarod motor performance declined sharply as EAE clinical disability progressed, but glatiramer acetate treatment rescued performance within 10 days of treatment initiation (which was started on day 15 for females and day 18 for males). Increased 4′,6-diamidino-2-phenylindole$^+$ cell infiltration (which includes inflammatory infiltrates) was observed in dorsal column of thoracic spinal cord sections of EAE-vehicle mice, and which was decreased by glatiramer acetate treatment initiated on day 21. $CD3^+$ T cells were a major infiltrating cell population in vehicle-treated EAE spinal cords. Glatiramer acetate treatment significantly decreased T cell numbers in the spinal cord dorsal column of EAE mice. Activation of resident $CD45^+$ CNS microglia occurred in EAE lesions, and a decrease of $CD45^+$ CNS microglia was found in glatiramer acetate-treated EAE spinal cord dorsal column. GFAP astrogliosis was observed in both EAE groups, with a nonsignificant trend towards a decrease in the glatiramer acetate-treated group. The spinal cord dorsal column, a major myelinated ascending fiber tract, revealed a significant decrease in myelin basic protein (MBP) staining in vehicle-treated EAE mice compared with normal controls. The spinal cord dorsal column of glatiramer acetate-treated EAE mice had significantly greater MBP staining intensity compared to vehicle-treated EAE mice. By immunostaining mature myelin-producing oligodendrocytes for CC1, therapeutic glatiramer acetate induced an increase in $CC1^+$ mature oligodendrocyte cell numbers. Analysis using Olig2 of all oligodendrocyte lineage cells including oligodendrocyte progenitors showed a decrease in $Olig2^+$ cells in vehicle-treated EAE spinal cord dorsal column and a reversal of this effect in glatiramer acetate-treated EAE spinal cord dorsal column. Glatiramer acetate treatment significantly decreased the pronounced axonal loss (as measured by $NF200^+$ staining) observed in vehicle-treated EAE spinal cord (Moore et al., 2014b).

Pien Tze Huang

Male Lewis rats, 9–11 weeks of age in which EAE was induced by immunization with guinea pig MBP, were treated intragastrically with PZH daily for 3 weeks from day 10 p.i. (EAE disease onset). PZH 0.162 g/kg and 0.486 g/kg decreased the clinical score especially in the remission phase. Treatment with PZH markedly reduced the extent of inflammatory lesions in the brain, brainstem, and spinal cord. PZH 0.162 g/kg and 0.486 g/kg significantly decreased the levels of the proinflammatory cytokines and chemokines IL-17A, IL-23, CCL3, and CCL5 in the spinal cord and serum compared to EAE-vehicle rats. Furthermore, the levels of p-P65 and p-STAT3 in the spinal cord were significantly decreased by PZH 0.162 g/kg and 0.486 g/kg compared to EAE-vehicle rats. Treatment with PZH 0.162 g/kg and 0.486 g/kg significantly increased the Olig2 and MBP levels in the brain compared to EAE-vehicle rats (Qiu et al., 2018).

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Dihydrotestosterone

Treatment of male Dark Agouti rats, 7–9 weeks of age in which EAE was induced by inoculation with syngenic spinal cord homogenate, with dihydrotestosterone 1 mg s.c. on alternate days from the day after EAE induction changed the disease course after the peak (on day 14–15 p.i.), decreasing the clinical score from day 16 until the end of the study (day 45 p.i.). In addition, the weight of the rats recovered faster in EAE-dihydrotestosterone rats compared to EAE-vehicle rats. At 45 days p.i., dihydrotestosterone levels in spinal cord were significantly higher in EAE-dihydrotestosterone rats compared to EAE-vehicle rats, but there was no difference in the levels of its direct metabolites, 3α-diol or 3β-diol. These results suggested that the effect on clinical score of dihydrotestosterone treatment was not mediated by conversion into 3α-diol or 3β-diol. At 45 days p.i. there are few $CD3^+$ (T cells) and $ED1^+$ cells (activated macrophages) present in the spinal cord of EAE-Dark Agouti rats (Giatti et al., 2013). Therefore, at this stage neuroinflammation seems to be mainly sustained by resident cells. Spinal cord sections revelaled that GFAP staining (marker of astrocytes) was significantly increased in EAE-vehicle rats compared to control rats. Dihydrotestosterone treatment significantly decreased GFAP staining compared to EAE-vehicle rats, with the levels being similar to control animals. MHC-II immunoreactive cells (microglia/macrophages) were significantly increased in the white matter of spinal cord of EAE-vehicle rats compared to control animals, and these cells showed a reactive morphology with some of them exhibiting amoeboid forms characteristic of highly activated states. Dihydrotestosterone treatment significantly reduced MHC-II staining and the reactive phenotype of MHC-II immunoreactive cells. Transcription analysis of TSPO gene in the spinal cord indicated a significant increase in EAE-vehcle rats compared to control animals and a significant decrease following dihydrotestosterone administration.

Dihydrotestosterone treatment significantly decreased the transcription of the proinflammatory cytokine IL-1β in the spinal cord, and also significantly decreased the transcription of both Toll-like receptor 4 and the p50 subunit of nuclear factor kappa B (NF-κB) which are two factors involved in IL-1β expression. Treatment with dihydrotestosterone significantly decreased TBARS in the spinal cord compared to EAE-vehicle rats, indicating an amelioration of oxidative stress. Dihydrotestosterone treatment reversed the lowering of mtDNA levels observed in the spinal cord of EAE-vehicle rats (Giatti et al., 2015).

Memantine hydrochloride

Female Brown Norway rats, 8–10 weeks of age in which EAE was induced by $MOG_{35–55}$ immunization, were treated i.p. with memantine hydrochloride on alternate days starting on the day of immunization. EAE disease onset was significantly delayed in the rats treated with memantine 60 mg/kg compared to rats receiving vehicle. EAE rats receiving vehicle developed clinical signs on day 15.6 ± 0.7, memantine 20 mg/kg treated rats developed signs on day 17.0 ± 0.8, and the first neurologic signs appeared in memantine 60 mg/kg treated rats on day 23.5 ± 5.5. Clinical scores were significantly lower in both memantine groups. Disease incidence for immunized rats treated with vehicle was 67%, while for memantine 20 mg/kg and 60 mg/kg treated rats it was 45% and 25%, respectively. Levels of TNF-α and TGF-β1 in sera on day 8 of EAE were significantly increased in the EAE-vehicle group compared to normal controls, but no significant differences in TNF-α or TGF-β1 were found for the memantine treatment groups. The extent of demyelination of the optic nerve on day 8 of EAE was significantly decreased in both EAE-memantine groups compared to EAE-vehicle group. Also, the degree of inflammatory infiltration was significantly reduced in both EAE-memantine groups compared to EAE-vehicle group, with significantly lower numbers of $CD3^+$ cells and $ED1^+$ cells per square millimeter in whole optic nerve cross section in both EAE-memantine groups compared to EAE-vehicle group. Axonal loss was significantly lower in both EAE-memantine groups compared to EAE-vehicle group. In the axons remaining at this time point, there was a tendency toward decreased βAPP deposition in the memantine 60 mg/kg group, but not in the 20 mg/kg group, compared to EAE-vehicle group. An anti-pan-neurofilament antibody was used to further investigate differences in surviving axons between treatment groups. Axonal densities were increased in the memantine-treated groups, especially the 60 mg/kg treatment group (Sühs et al., 2014).

Pregabalin

Pregabalin 30 mg/kg per day was administered p.o. to female Lewis rats, 7–8 weeks of age in which EAE was induced by immunization with MBP, starting immediately and continued until the time the animals were euthanized. Treatment with pregabalin delayed the onset of clinical signs compared to the vehicle treated group. Immunostaining of synaptophysin around the motoneuron cell bodies at lamina IX of the ventral horn of spinal cord showed that pregabalin treatment preserved presynaptic terminals in apposition to motoneurons. Iba-1 immunolabeling was used to assess the microglial reactivity as well as the infiltration of macrophages derived from monocytes to the spinal cord microenvironment. Microglia at lamina IX of Rexed (motor nucleus) showed a decrease in reactivity in response to pregabalin treatment, restricted to the exacerbation phase of EAE. A significant increase in the number of activated macrophages during peak disease in EAE-vehicle rats was found compared to normal rats, with the motoneurons surrounded by such cells. Treatment with pregabalin significantly decreased the microglial response. In the remission phase, a general reduction of Iba-1 immunoreactivity was detected in all groups. However, both EAE-vehicle and EAE-pregabalin groups showed significantly higher expression of Iba-1 compared to normal controls. GFAP immunolabeling was used to evaluate astroglial reactivity during the course of the disease. In the exacerbation phase during peak disease, there was a significant decrease of GFAP labeling of EAE-pregabalin group and normal controls compared to EAE-vehicle group,

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with no significant difference between normal controls and EAE-pregabalin group. In the remission phase, the GFAP labeling of both the EAE-vehicle and EAE-pregabalin groups was significantly higher than the normal controls, with no significant difference between EAE-vehicle and EAE-pregabalin groups. Treatment with pregabalin did not significantly alter the levels of IL-6, IL-10, IL-27 and TGF-β in lymph nodes from the inguinal region, indicating a non-immunomodulatory role of pregabalin (Silva et al., 2014).

Cuprizone animal studies

Bilobalide

Male C57/BL6 mice, 11–13 weeks of age, were fed 0.2% (w/w) CPZ in chow diet for 6 weeks to induce acute demyelination, and after CPZ feeding for 4 weeks were treated i.p. with bilobalide 40 mg/kg per day for 2 consecutive weeks during CPZ feeding. The CPZ diet significantly increased touchdown time and decreased the percentage of time in the open arm compared to normal mice in both the pole test and elevated plus maze test. Bilobalide administration significantly improved behavioral performance of CPZ mice. The demyelination induced by CPZ was assessed by LFB, Black Gold II and MBP staining of corpus callosum of the brain after CPZ feeding for 4 weeks, and the intensity of staining was significantly decreased in CPZ-fed mice compared to normal mice. Bilobalide treatment of CPZ mice significantly increased the intensity of staining for myelin compared to CPZ-saline mice. The MBP expression and $O4^+$ mature oligodendrocytes were decreased in the brain of CPZ-fed mice compared with normal mice. Bilobalide treatment significantly increased MBP expression and $O4^+$ oligodendrocytes compared to CPZ-saline mice. CPZ fed-mice exhibited splenic atrophy and decreased splenic weight compared to normal mice. Bilobalide treatment increased the volume of the spleen and significantly increased the weight of the spleen compared to CPZ-saline mice. As oligodendrocytes are the main target cells in the CPZ model, $MOG_{35–55}$ specific antibody was measured in serum, supernatant of cultured splenocytes and brain extract from CPZ mice. $MOG_{35–55}$ specific antibody was significantly increased in serum, supernatant of cultured splenocytes and brain extract from CPZ-saline mice compared to normal mice. Bilobalide treatment of CPZ mice significantly decreased MOG antibody in serum, supernatant of cultured splenocytes and brain extract homogenate. There were no significant differences in the percentage of $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}17^+$ T cell subsets in the absence or presence of MOG stimulation. No difference was found in the levels of IFN-γ, IL-10 and IL-17 among the three groups. These findings suggested that CPZ feeding or bilobalide treatment did not alter T cell subsets. IHC showed that a few of $CD4^+$ T cells, $CD68^+$ macrophages and $B220^+$ B cells invaded the surrounding brain parenchyma of CPZ-saline mice, and this was significantly decreased by bilobalide treatment. Compared to normal mice, Iba-1^+ microglia were significantly increased in CPZ-saline mice, and which was significantly reduced by bilobalide treatment. iNOS is an important marker of inflammatory M1 microglia, while NF-κB is the major inflammatory signaling pathway in microglia. The number of Iba-1^+iNOS^+ and Iba-$1^+NF\text{-}\kappa B^+$ microglia in CPZ-saline mice was significantly higher than that of normal mice. Bilobalide treatment significantly decreased the number of Iba-1^+iNOS^+ and Iba-$1^+NF\text{-}\kappa B^+$ microglia, indicating that the anti-inflammatory effect of bilobalide was by inhibiting the activation of microglia or M1 polarization in CPZ mice. Inflammatory and cytotoxic mediators are released by activated microglia or M1 polarized microglia, leading to cell death and neurological dysfunction. Compared to normal mice, the levels of cytokines IL-1β, IL-6 and TNF-α were significantly increased in the brain of CPZ-saline mice, and bilobalide treatment significantly decreased the production of IL-1β and IL-6 compared to CPZ-saline mice. These findings indicated that CPZ-induced demyelination is associated with neuroinflammation caused by the activation of M1 microglia, and which was decreased by bilobalide treatment. The migration and enrichment of Iba-1^+ microglia in different regions of myelin sheath was examined. Compared with normal mice, the migration and enrichment of Iba-1^+ microglia toward the corpus callosum, striatum and medial septal nucleus was increased in CPZ-saline mice and had a negative correlation with $O4^+$ oligodendrocytes, suggesting that the migration and enrichment of Iba-1^+ microglia may be closely related to the damage of $O4^+$ oligodendrocytes. Bilobalide treatment of CPZ mice decreased the migration and accumulation of Iba-1^+ microglia toward the myelin sheath (Sui et al., 2019).

Linagliptin

Male C57BL/6 mice, 6 weeks of age, were fed 0.7% (w/w) CPZ in chow for 7 days followed by 0.2% CPZ in chow. CPZ mice were treated p.o. with linagliptin 10mg/kg per day starting from the second week. Linagliptin treatment of CPZ mice significantly increased body weight and brain weight compared to CPZ-saline mice. CPZ mice showed behavioral and motor abnormalities as revealed by the open field, rotarod, and grip strength tests. Linaglipin treatment ameliorated these behavioral and motor abnormalities. CPZ-saline mice had significantly decreased MBP, PLP and Olig2 gene expression levels as well as significantly decreased LFB staining in corpus callosum sections compared to normal controls. Linagliptin treatment significantly increased MBP, PLP and Olig2 gene expression levels and LFB staining intensity in CPZ mice. In addition, CPZ-saline mice had a significant increase in TBARS level and a significant decrease in GSH content of the brain tissue compared to normal controls. Linagliptin treatment significantly decreased TBARS level and significantly increased GSH content of the brain tissue compared to CPZ-saline mice. CPZ feeding induced an inflammatory state as shown by a significant 4-fold increase in TNF-α level in the brain compared to normal controls. Linagliptin treatment significantly decreased brain TNF-α level compared to CPZ-saline mice. CPZ-saline mice had significant large increases in p-JAK2, p-STAT3 and NF-κB p65 protein expression levels compared to normal controls. Linagliptin treatment significantly decreased the p-JAK2, p-STAT3 and NF-κB p65 protein expression levels compared to CPZ-saline

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mice. Also CPZ mice had significantly lower p-AMPK protein and SIRT1 gene expression levels compared to normal control mice. Linagliptin treatment significantly increased p-AMPK protein and SIRT1 gene expression levels compared to CPZ-saline mice (Elbaz et al., 2018).

BLZ945

Female C57BL/6 mice, 9–10 weeks of age, were fed 0.2% (w/w) CPZ in chow for 5 weeks to induce a pronounced demyelination together with a large involvement of microglia and astrocytes as observed by histology. CPZ mice were treated p.o. once per day with BLZ945 (colony-stimulating factor 1 receptor kinase inhibitor) at different doses. MBP and oligodendrocytes (glutathione S-transferase π, (GST-π) a marker for mature oligodendrocytes) were significantly decreased in cortex/striatum after 5-week CPZ-ingestion compared to normal controls, and 169 mg/kg per day BLZ945 treatment for 2 weeks after 5-week CPZ-ingestion significantly increased MBP and GST-π in the cortex/striatum. In corpus callosum/external capsule there was a significant decrease in MOG, GST-π, as well as myelin (LFB staining) in CPZ-vehicle group compared to controls, but no difference following 2 weeks of BLZ945 treatment. Thus, BLZ945 treatment enhanced remyelination and increased the number of mature oligodendrocytes in cortex and striatum but not in corpus callosum/external capsule of CPZ mice. Microglia and astrocytes were highly activated and significantly increased after 5-week CPZ-induced demyelination in cortex, striatum and corpus callosum/external capsule. A 2-week therapeutic treatment with BLZ945 significantly decreased the number of Iba-1$^+$ microglia in cortex, striatum and corpus callosum/external capsule compared to CPZ-vehicle mice. BLZ945 treatment for 2 weeks after CPZ feeding significantly increased the number of GFAP$^+$ astrocytes in cortex, striatum and tended to increase the number in corpus callosum/external capsule compared to CPZ-vehicle mice. Microglia size, microglia form factor, and microglia activation were mostly increased after CPZ in all brain areas examined. These microglia parameters in cortex and striatum were further increased by therapeutic BLZ945 treatment, revealing a higher morphological activation status of the remaining microglia. These parameters were not changed at all or even decreased in corpus callosum and external capsule. To assess the effect of BLZ945 given prophylactically, mice were treated with 169 mg/kg per day BLZ945 p.o. for 1 week before as well as during the 5-week CPZ feeding period. CPZ-vehicle mice showed the expected demyelination (LFB staining) and increase of microglia (Iba-1) in the corpus callosum and external capsule compared to normal controls. BLZ945 treatment significantly increased myelin and decreased Iba-1$^+$ microglia in the corpus callosum and external capsule compared to CPZ-vehicle mice. In addition, a significantly increased number of oligodendrocytes was detected in the corpus callosum in CPZ-BLZ945 mice compared to CPZ-vehicle mice, but a non-significant increase in the external capsule. No significant difference of SMI312$^+$ axon fibers was observed in corpus callosum of CPZ-BLZ945 mice compared to CPZ-vehicle mice, whereas a significant decrease was observed in the external capsule. In the external capsule, Iba-1$^+$ microglia were significantly slightly decreased in number, but a significant increase in myelin debris accompanied by a significant decrease in axonal fibers (SMI312) was detected in CPZ-BLZ945 mice compared to CPZ-vehicle mice. In the cortex, similar observations of increased myelin debris, axonal pathology and decreased NeuN$^+$ cells in CPZ-BLZ945 mice were made (Beckmann et al., 2018).

Indomethacin

C57BL/6 mice were fed 0.2% (w/w) CPZ in chow for 10 weeks to induce demyelination in the corpus callosum, then fed normal chow and treated i.p. with 2.5 mg/kg per day indomethacin for up to 2 weeks. Mice that received indomethacin treatment after cessation of CPZ diet showed significantly increased levels of Cnp, Mag, Mbp and Plp1 genes in corpus callosum during the remyelination phase compared to mice injected with vehicle alone. This was associated with higher numbers of NogroA$^+$ mature oligodendrocytes, increased remyelination (LFB/PAS), higher numbers of Caspr$^+$ paranodes and increased numbers of myelinated axons as indicated by electron microscopy. The g-ratio was significantly decreased in indomethacin-treated mice compared to vehicle-treated mice after 1 week of treatment suggesting enhanced remyelination. There were no differences in the total number of oligodendroglial lineage cells (oligodendroglial progenitor cells and mature oligodendrocytes) expressing Olig2$^+$ or in the number of proliferating Olig2$^+$ cells between the two treatment groups. Axonal damage was examined by staining for amyloid precursor protein (APP) that accumulates in transected axons as well as in axons with a disturbed axonal transport. After 10 weeks of demyelination, as well as during remyelination, low numbers of APP$^+$ axons were detected. Indomethacin treatment did not affect the numbers of damaged APP$^+$ axonal spheroids or the numbers of astrocytes or microglia. The influence of indomethacin on several M1 or M2 markers expressed by microglia was investigated and no M2 shift *in vivo* was found (Preisner et al., 2015).

Theiler's murine encephalomyelitis virus animal study

rHIgM12

SJL/J mice, 8 weeks of age, received intracerebral injection of 2.0×10^5 plaque-forming units of Daniel's strain TMEV. At 45 days post infection, the time point when demyelination in the spinal cord is well established and before substantial axon loss, SJL mice were treated with a single i.p. dose of 0.25, 2.5, 10, or 20 mg rHIgM12/kg. Improved horizontal nocturnal motor function in rHIgM12-treated mice became statistically significant on days 6, 9, 3, and 14 post-treatment for 0.25, 2.5, 10, and 25 mg/kg doses, respectively, compared to control IgM. Improved horizontal nocturnal motor function of rHIgM12-treated mice persisted until the end of the study at 8 weeks. Improved vertical nocturnal motor function in rHIgM12-treated mice became statistically significant at days 12, 15, and 23 post-treatment for 2.5, 10, and 25 mg/kg

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doses, respectively, compared to control IgM. In a retrograde labeling study, SJL mice at 90 days post infection, the time point when axonal degeneration is present and progressing, were treated with a single i.p. dose of 10 mg rHIgM12/kg (n = 9). At 9 weeks post treatment, retrograde labeling was performed using the tracer, Fluoro-Gold. There were fewer fluorescently labeled neuron cell bodies in the brainstem of TMEV mice compared to uninfected mice. The average number of labeled neurons in TMEV-rHIgM12 mice was significantly higher than in the TMEV-saline group. At 10 weeks post-treatment, significantly more axons were found in the spinal cord in the rHIgM12-treated group compared to the saline-treated group (Wootla et al., 2016).

Disease-Modifying Effects of Pharmacological Agents

Experimental autoimmune encephalomyelitis animal studies

Different profiles of disease were identified among the various EAE studies reviewed. In the mouse studies, many had identified a chronic progressive type of EAE disease from the daily clinical scores that was similar to progressive MS (Moore et al., 2014b; Pedreño et al., 2014; Dupree et al., 2015; Jayaraman et al., 2017; Kim et al., 2018; Silva et al., 2018). However, some studies showed a relapsing-remitting pattern of disease (Pentón-Rol et al., 2016; Toader et al., 2018). It has been suggested that the $MOG_{35–55}$ peptide dose may influence the course of the disease in C57BL/6 mice with a large dose of peptide and adjuvant causing a non-remitting EAE form, while a small dose induces relapse-remitting disease (Berard et al., 2010). Some of the mouse studies had examined the effects of certain pharmacological agents on optic neuritis. In the rat studies, a chronic progressive disease was induced by immunization with rat $MOG_{1–125}$ peptide (Sühs et al., 2014), while immunization with MBP led to a peak of disease followed by a marked decline, reaching baseline clinical score (Silva et al., 2014; Qiu et al., 2018), and immunization with syngenic spinal cord homogenate caused a protracted relapsing course of the disease with all rats having at least one relapse during the follow-up period (Giatti et al., 2015).

In the EAE mouse studies all of the pharmacological agents tested, except for ST266 administered intranasally, caused a significant lowering of clinical scores when administered therapeutically, and some delayed the onset of disease (e.g. melatonin). Noteworthy, siponimod 4.5 μg/day administered i.c.v. completely inhibited EAE development, and a significant number of EAE mice receiving adrenomedullin 1 nmol/day i.p. for 5 consecutive days recovered completely and were asymptomatic 30–40 days after disease course. In addition, some agents were able to exert protection against chronic progressive EAE disease when administered prophylactically (e.g. trichostatin A).

In the EAE rat studies, PZH, dihydrotestosterone and memantine hydrochloride caused a significant lowering of clinical scores when administered therapeutically, and memantine hydrochloride and pregabalin delayed the onset of clinical signs. Interestingly, the incidence of disease was considerably lower in rats treated with memantine hydrochloride 60 mg/kg (started on the day of immunization with MOG) than in animals receiving vehicle (25% and 67%, respectively).

The disease-modifying effects of the agents in the EAE mouse and rat studies are summarized in **Table 1**, and included protection by preventing/reducing axonal, neuronal, myelin and oligodendrocyte damage, apoptosis, oxidative stress, excitotoxicity, infiltration of inflammatory cells, and proinflammatory cytokines/chemokines.

Cuprizone mouse studies

These studies examined whether the pharmacological therapies tested were able to promote remyelination when administered therapeutically to CPZ-fed mice, or prevent demyelination and oligodendrocyte loss when given prophylactically. The disease-modifying effects of therapeutic administration of these agents are summarized in **Table 1**. Prophylactic administration of BLZ945 resulted in increased myelin and decreased microglia in the corpus callosum and external capsule, increased number of oligodendrocytes in the corpus callosum, and decreased number of microglia in the external capsule.

Theiler's murine encephalomyelitis virus mouse study

This study examined pharmacological therapy in a model with a progressive form of demyelination. The effects of therapeutic administration of monoclonal antibody rHIgM12 were improved nocturnal motor activity, increased neuron cell bodies in the brainstem, and increased number of axons in the spinal cord (**Table 1**).

Future Perspectives

Two pathogenic processes are responsible for the neurological deficits in MS patients: acute inflammatory demyelination and axonal degeneration. The disability caused by inflammatory demyelination clinically dominates the early stages of relapsing-remitting MS and is reversible. Once a certain level of axonal degeneration is exceeded, MS patients enter an irreversible secondary progressive stage. In secondary progressive MS, axonal loss is caused by chronic demyelination and may be irreversibly progressive (Trapp et al., 1999). Disease-modifying drugs currently in use for MS suppress the immune system and reduce relapse rates but are ineffective in the progressive phase of the disease. For example, in clinical trials it was found that IFN-β significantly decreases the annualized relapse rates and the appearance of new brain lesions in relapsing-remitting MS, but has little or no effect on established progressive MS (Annibali et al., 2015). Moreover, about 40% of MS patients have no or only a low response to IFN-β treatment (Rudick et al., 2004). Therefore, it is necessary to develop novel therapies that target more specifically the inflammatory demyelinating and neurodegenerative components of the disease (Pentón-Rol et al., 2016).

Various animal models of MS have been established and

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Table 1 Disease-modifying effects of pharmacological agents in EAE, CPZ and TMEV animal models of MS

Effect of pharmacological agent	Pharmacological agent
EAE mouse studies	
Improved behavioral performance	CTK 01512-2, glatiramer acetate
Alleviated demyelination of spinal cord	Cerebrolysin, CTK 01512-2, C-phycocyanin, lanthionine ketamine ester, adrenomedullin
Alleviated demyelination of optic nerve	ST266, lanthionine ketamine ester
Protected against demyelination of cerebral cortex	caffeine
Decreased the number of microglia in medulla	Cerebrolysin
Decreased inflammation in the brain and spinal cord by altering the balance of proinflammatory/anti-inflammatory cytokines	CTK 01512-2, C-phycocyanin, caffeine, adrenomedullin
Decreased astrocyte activation in the spinal cord and microglia activation in the brain	CTK 01512-2
Decreased astrocyte activation and microglia/macrophage activation in the striatum	Siponimod
Decreased activated microglia/macrophages in the spinal cord	C-phycocyanin
Decreased the number of macrophages in CNS infiltrates	Adrenomedullin
Decreased infiltration of inflammatory cells into the spinal cord and optic nerve	Melatonin, ST266, trichostatin A, caffeine, adrenomedullin, glatiramer acetate
Decreased the numbers of infiltrating CD4 T cells into the brain, particularly of IFN-γ- and IL-17-producing Th1 and Th17 cells	Adrenomedullin
Increased the percentage of IL-10-secreting $CD4^+CD25^+FoxP3^+$ Treg cells in the CNS	Adrenomedullin
Decreased the levels of oxidative stress in the brain and spinal cord	Melatonin, white grape juice extract
Decreased the levels of ROS in optic nerve during optic neuritis	ST266
Decreased parameters of oxidative stress in blood lysates	C-phycocyanin
Decreased the number of injured axons in the spinal cord	Diarylpropionitrile
Decreased proinflammatory phenotype of myeloid cells in the CNS	Diarylpropionitrile
Increased the percentages of mature and immature oligodendrocytes in dorsal white matter of spinal cord	Diarylpropionitrile
Decreased T-lymphocytes in the blood	Siponimod
Decreased the number of infiltrating lymphocytes in the striatum	Siponimod
Rescued GABAergic interneuron loss in the striatum	Siponimod
Decreased infiltration of T lymphocytes, monocytes and neutrophils in the white matter of the spinal cord	White grape juice extract
Decreased TNF-α in the spinal cord	White grape juice extract
Decreased the number of apoptotic cells in the spinal cord	White grape juice extract
Decreased neurodegeneration in the optic nerve	Lanthionine ketamine ester
Decreased the percentage of axons with abnormal mitochondria and loss of contact in the optic nerve	Lanthionine ketamine ester
Decreased the number of axons with condensation in the spinal cord	Lanthionine ketamine ester
Decreased the ratio between MOG-specific IgG2a and IgG1 antibodies	Adrenomedullin
Increased the gene expression of neuroprotective factors BDNF and ADNP in the spinal cord	Adrenomedullin
Increased MBP staining intensity in spinal cord dorsal column	Glatiramer acetate
Reversed a decrease in oligodendrocyte lineage cells in the spinal cord dorsal column	Glatiramer acetate
Decreased axonal loss and the number of damaged axons in the spinal cord ventral column	Glatiramer acetate
Decreased inflammatory lesions in the corpus callosum	Glatiramer acetate
Decreased $CD45^+$ activated microglia and $CD3^+$ T cell infiltrates in the corpus callosum	Glatiramer acetate
EAE rat studies	
Decreased demyelination of the optic nerve	Memantine hydrochloride
Decreased inflammatory cell infiltration in the brain, brainstem, and spinal cord	Pien Tze Huang
Decreased inflammatory cell infiltration in the optic nerve	Memantine hydrochloride
Decreased axonal loss in the optic nerve	Memantine hydrochloride
Decreased proinflammatory cytokines and chemokines in the spinal cord	Pien Tze Huang, dihydrotestosterone
Increased the Olig2 and MBP levels in the brain	Pien Tze Huang
Decreased astrocytes in the spinal cord	Dihydrotestosterone
Decreased microglia/macrophages and their reactive phenotype in the white matter of the spinal cord	Dihydrotestosterone
Decreased reactivity of microglia at lamina IX of ventral horn of the spinal cord in exacerbation phase of EAE	Pregabalin
Decreased astrocyte reactivity at lamina IX of ventral horn of the spinal cord in the exacerbation phase during peak disease	Pregabalin
Decreased oxidative stress in the spinal cord	Dihydrotestosterone
Reversed the decrease in mtDNA levels in the spinal cord	Dihydrotestosterone

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Table 1 Continued

Effect of pharmacological agent	Pharmacological agent
CPZ mouse studies	
Improved behavioral performance	Bilobalide, linagliptin
Increased myelin in the corpus callosum	Bilobalide, linagliptin
Increased MBP and mature oligodendrocytes in the brain	Bilobalide
Decreased MOG35-55 specific antibody in serum and the brain	Bilobalide
Decreased infiltration of $CD4^+$ T cells, $CD68^+$ macrophages and $B220^+$ B cells into the brain	Bilobalide
Decreased the number of microglia in the brain	Bilobalide, BLZ945
Inhibited the activation of microglia or M1 polarization of microglia in the brain	Bilobalide
Decreased the levels of the proinflammatory cytokines IL-1β, IL-6 and TNF-α in the brain	Bilobalide, linagliptin
Decreased the migration and accumulation of microglia toward the myelin sheath in the corpus callosum, striatum and medial septal nucleus	Bilobalide
Decreased oxidative stress in the brain	Linagliptin
Increased MBP-positive remyelination and mature oligodendrocytes in cortex/striatum	BLZ945
Increased the numbers of mature oligodendrocytes, accelerated remyelination, and increased the numbers of myelinated axons	Indomethacin
TMEV study	
Improved nocturnal motor activity	rHIgM12
Increased neuron cell bodies in the brainstem, and increased number of axons in the spinal cord	rHIgM12

ADNP: Activity-dependent neuroprotective protein; BDNF: brain-derived neurotrophic factor; CNS: central nervous system; CPZ: cuprizone; EAE: experimental autoimmune encephalomyelitis; GABA: gamma-aminobutyric acid; IFN-γ: interferon-γ; IL: interleukin; MBP: myelin basic protein; MOG: myelin oligodendrocyte glycoprotein; MS: multiple sclerosis; TMEV: Theiler's murine encephalomyelitis virus; TNF-α: tumor necrosis factor-α.

proved useful in studying the disease process and response to different therapies. The EAE animal studies reviewed here have shown that a chronic progressive disease can be induced by immunization with appropriate amounts of MOG, mycobacterium tuberculosis, and pertussis toxin in Freund's adjuvant (Moore et al., 2014b; Pedreño et al., 2014; Dupree et al., 2015; Jayaraman et al., 2017; Kim et al., 2018; Silva et al., 2018). They have enabled specific pathological processes to be studied and modified at different stages of the disease by administration of pharmacological agents. For example, administration of glatiramer acetate on day 16 (at onset, mouse EAE) resulted in suppression of disease phenotype and a marked decrease in EAE clinical scores. Furthermore, initiation of glatiramer acetate treatment on day 21 brought about a significant and persistent decrease in clinical scores (Moore et al., 2014b). Adrenomedullin administered after the onset or during the effector phase of the disease greatly reduced disease incidence and severity. Most of the adrenomedullin-treated EAE mice showed mild symptoms and a significant number recovered completely and were entirely asymptomatic 30–40 days after disease course (Pedreño et al., 2014). In another study, EAE mice were provided with lanthionine ketamine ester in chow on day 36, at which time the progressive nature of the disease was well established, and showed significant clinical recovery up to day 57 with a marked decrease in clinical scores. Improvements in clinical signs were observed in mice that had moderate to severe clinical signs (scores of 3 or 4) and also in those with milder signs (scores of 1 or 2) (Dupree et al., 2015). Treatment with trichostatin A for 8 weeks starting at the peak of disease was shown to decrease the clinical severity during the period of protracted disease in EAE NOD mice (between days 82 and 115) and protection continued for 9 weeks after cessation of treatment (Jayaraman et al., 2017). Intrathecal administration of CTK 01512-2 on days 4, 10, 15, 20, and 24 following MOG immunization prevented the occurrence of symptoms associated with the progression of EAE (Silva et al., 2018). These studies have shown that the clinical manifestation of EAE disease can be prevented or reduced by treatment with certain pharmacological agents given prior to, at, or after peak disease, and for some of these agents have confirmed the findings of previous studies e.g. glatiramer acetate (Aharoni et al., 2008, 2011). The CPZ mouse studies reviewed have shown that a marked demyelination of the CNS was induced in this animal model and the pharmacological agents tested were able to promote remyelination and increase the number of oligodendrocytes, and improve behavioral performance when administered therapeutically (Preisner et al., 2015; Beckmann et al., 2018; Elbaz et al., 2018; Sui et al., 2019). Prophylactic treatment with BLZ945 also promoted remyelination and increased the number of oligodendrocytes in CPZ mice (Beckmann et al., 2018). In the TMEV mouse study, it was shown that rHIgM12 administration preserved motor function by protecting axons in this model of progressive MS (Wootla et al., 2016). Demyelination has been shown to contribute to axonal loss in MS (DeLuca et al., 2006). Neuroprotective strategies aim at preventing axonal and neuronal loss, myelin and oligodendrocyte damage and cell death in MS patients (Villoslada, 2016). If such findings can be translated to clinical practice, they have relevance for delaying the progression of disease in patients with relapsing-remitting MS, for reducing clinical severity in patients with progressive phenotypes of MS, and for treating patients with clinically isolated syndrome (CIS) to prevent progression to MS. A person with CIS, accompanied or not accompanied by MRI brain lesions that are similar to those seen in MS, has a 60–80% or 20% chance, respectively, of developing MS within several years (National Multiple Sclerosis Society). Of note is the recent approval by the US FDA to approve siponimod for treating adults with RRMS, SPMS, and CIS.

Where age and gender were reported, most of the stud-

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ies had used young adult female animals. While this might be related to a higher prevalence of MS in female patients (Wallin et al., 2019), there is a need for additional studies to be performed with male animals especially using the EAE model of MS. Furthermore studies are warranted using aged animals as chronic low-grade inflammation is an important contributor to various age-related pathologies and natural processes in aging tissues, including the nervous system (Franceschi and Campisi, 2014; Sanada et al., 2018). Also most animals used in research studies are bred and housed in clean holding facilities, and do not have the same bacteria, viruses and pathogens that humans have, making them less than ideal models to study human disease. It has been suggested that laboratory animals born to wild parents or kept with animals from pet shops may be a way of achieving a more humanlike immune response (Frederick, 2019).

While therapies with single pharmacological agents have been shown to have benefit in the various types of animal models of MS, it may be that a combination therapy may be more effective, especially using agents that target neuroinflammation and neurodegeneration. The utilization of other neuroprotective agents with an immunomodulator was suggested as a way of enhancing the effectiveness of current drugs in treating MS, and one possible suggested neuroprotective agent was pregabalin (Silva et al., 2014). Similarly, a reagent that protects axons without associated remyelination could be paired with an immunomodulatory or remyelination-promoting agent (Wootla et al., 2016). A combination treatment using an immunomodulatory agent tuftsin and a drug promoting remyelination benztropine improved MS-like pathologies in both EAE and CPZ models (Thompson et al., 2018). The importance of oxidative stress in MS is becoming increasingly recognized (Mossakowski et al., 2015), and a combination therapy using an immunomodulator and an antioxidant drug has been investigated. Combination treatment with glatiramer acetate and epigallocatechin-3-gallate significantly delayed disease onset, strongly reduced clinical severity, even after onset of symptoms, and reduced inflammatory infiltrates in an EAE relapsing-remitting mouse model (Herges et al., 2011) but not in an EAE progressive disease mouse model (Janssen et al., 2015). Future studies should determine the effectiveness of combining agents shown to be effective singly in alleviating processes such as neuroinflammation, axonal loss, and oxidative stress in animal models of chronic progressive disease as they may exert synergistic effects, and may provide more effective therapies for patients with MS.

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