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REVIEW

G protein-coupled receptors as therapeutic targets for multiple sclerosis

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G protein-coupled receptors (GPCRs) mediate most of our physiological responses to hormones, neurotransmitters and environmental stimulants. They are considered as the most successful therapeutic targets for a broad spectrum of diseases. Multiple sclerosis (MS) is an inflammatory disease that is characterized by immune-mediated demyelination and degeneration of the central nervous system (CNS). It is the leading cause of non-traumatic disability in young adults. Great progress has been made over the past few decades in understanding the pathogenesis of MS. Numerous data from animal and clinical studies indicate that many GPCRs are critically involved in various aspects of MS pathogenesis, including antigen presentation, cytokine production, T-cell differentiation, T-cell proliferation, T-cell invasion, etc. In this review, we summarize the recent findings regarding the expression or functional changes of GPCRs in MS patients or animal models, and the influences of GPCRs on disease severity upon genetic or pharmacological manipulations. Hopefully some of these findings will lead to the development of novel therapies for MS in the near future.

***Keywords*:** G protein-coupled receptors (GPCRs); multiple sclerosis (MS); experimental autoimmune encephalomyelitis (EAE); agonist; antagonist; therapeutic targets

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Introduction

Multiple sclerosis (MS), also known as disseminated sclerosis or encephalomyelitis disseminata, is an inflammatory disease that is characterized by immune-mediated demyelination and neurodegeneration of the central nervous system (CNS). It leads to substantial disability through deficits of sensation and of motor, autonomic, and neurocognitive functions. The disease onset usually occurs in young adults between 20 to 40 years of age [1, 2] with a prevalence that ranges between 2 and 150 per 100 000 [3]. The disease is usually not life-threatening, but its socioeconomic importance is second only to trauma in young adults [4, 5].

$CD4^+$ T-cell-mediated autoimmunity has long been accepted as one of the most important aspects of MS pathogenesis, especially for the early initiation of the disease [6, 7]. T-helper type 1 (Th1) cells, characterized by the production of interferon-γ (IFN-γ), have been considered the type of effector T-helper cells that mediate the pathogenesis of MS. Subsequent studies have revealed that the IL-17-expressing T-helper cells (Th17) are also involved and are at least as critical as Th1 cells in this disease. Mice with fewer Th17 cells are less susceptible to experimental autoimmune encephalomyelitis (EAE) [8, 9], a mouse model of MS; and IL-17-expressing T cells have been found in lesions of brain tissues from patients with MS [10].

There are several types of MS, including Benign MS, Relapsing Remitting MS (RRMS), Secondary Progressive MS (SPMS), Primary Progressive MS (PPMS), and Malignant MS (also known as Marberg Variant MS). RRMS is the most frequent (85%-90%) form and affects women about twice as often as men. Patients tend to experience an attack or series of attacks (exacerba-

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tions) followed by complete or partial remission. Most RRMS patients later develop SPMS. At that stage, there are no real periods of remission, but only breaks in attack duration with no real recovery from symptoms. About 10%-15% of patients presenting with insidious disease onset and steady progression are termed PPMS [7]. It is characterized by gradual clinical decline with no real or distinct periods of remission. Magnetic resonance imaging (MRI) [11] or histopathological evaluation [12, 13] also revealed heterogeneity in morphological alterations of the brain in different patients. It is still not clear which factors may contribute to the different disease courses and the heterogeneity in clinical presentations. Complex genetic effects and environmental components that translate into different immune abnormalities and/or increased vulnerability of CNS tissue to inflammatory insults or reduced ability to repair damage are certainly involved. Relatives of people who have the disease have an increased risk; if a patient with MS has an identical twin, that twin's risk climbs to more than 25% [4, 14]. But when a team of US researchers compared the complete genomes of twin females with each other, they failed to find any genetic differences that might cause MS [15]. This observation indicates that the environmental stress might also play an important role in eventually triggering the pathogenesis of MS.

G protein-coupled receptors (GPCRs), also known as 7-transmembrane receptors, are the largest family of cell surface receptors involved in transmitting extracellular environmental signals into the cells. These receptors are activated by a wide variety of stimulations, including light, odorant molecules, peptide and non-peptide neurotransmitters, hormones, growth factors, lipids, etc. [16]. The GPCR family comprises approximately 2% of the human genome and remains a central focus in basic pharmacology studies and drug discovery efforts [17]. After agonist binding, the activated receptors catalyze the exchange of GDP for GTP on the α-subunit of heterotrimeric G proteins (composed of α-, β-, and γ-subunits), which in turn engages conformational changes that lead to the dissociation of the Gα from the dimeric Gβγ subunits [18]. Both the Gα- and Gβγ- subunits can convey the extracellular signals by activating or suppressing downstream effector molecules, such as adenylyl and guanylyl cyclases, phosphodiesterases, phospholipases, phosphoinositide 3-kinases (PI3K), ion channels and other signaling components [16].

GPCRs have emerged as the most important targets for human therapeutics due to their large numbers and critical roles in the physiology of vital systems, such as cardiovascular, nervous, immune, metabolic, and endocrine systems. The prominent roles of GPCRs in cancer are also well recognized [19, 20]. These receptors are the target of > 50% of the current therapeutic agents on the market, including more than a quarter of the 100 top-selling drugs with benefits in the range of several billion US dollars each year. Here, we review recent progress on the roles of GPCRs in the pathogenesis of MS and hope that some of these receptors might become new therapeutic targets for this disease in the near future.

GPCRs involved in MS or EAE

Adenosine receptors

Dysfunction of adenosinergic system has been implicated in the development of MS in humans and EAE in animals. Blood adenosine level decreases greatly in MS patients [21]. Among the four known subtypes of adenosine receptors – referred to as A1, A2A, A2B, and A3, the role of A1 in MS pathology has been intensively studied in both clinical samples and animal models. A1 was selectively diminished on cells of monocyte/macrophage lineage in both brain and blood samples from MS patients. This reduction potentially led to increased macrophage activation and CNS inflammation [21, 22]. In animal model, the A1 knockout mice developed a severe progressive-relapsing form of EAE with extensive inflammation and demyelination in CNS compared with the corresponding controls [23]. Conversely, treatment with the A1 receptor agonist ADAC reduced spinal cord injuries in EAE mice [23]. Caffeine, a non-selective antagonist of adenosine receptors, has also been shown to alleviate EAE in mice and rat [23-25]. It has been postulated that chronic treatment with caffeine may benefit EAE animals by upregulating A1 receptor and TGF-β, and suppressing IFN-γ [25]. These results suggest that adenosine might act through the A1 receptor to suppress inflammation and that dysfunction of A1 contributes to the pathogenesis of MS.

On the other hand, a recent study unexpectedly discovered that mice with a genetic deficiency in CD73, an extracellular nucleotidase critical for the generation of extracellular adenosine, are highly resistant to MOG-induced brain and spinal cord injury [24]. Such reduction in EAE severity was not due to the lack of responsiveness of T cells, since $CD4^+$ T cells from $CD73^{-/-}$ mice secreted more proinflammatory cytokines than wild-type mice and were able to induce EAE when transferred into naive $CD73^{+/+}$ recipients. This correlates well with other reports that adenosine is an anti-inflammatory mediator. It seems that adenosine concentration in the CNS, possibly surrounding the choroid plexus epithelium, is critical for pathogenic T-cell infiltration, as the $CD73^{-/-}$ mice had fewer infiltrating lymphocytes in their CNS

compared with wild-type mice even though their T cells were highly activated. In the same study, the authors also found that pharmacological blockade of the A2A receptor with SCH58261 attenuates EAE pathology [24]. These results are quite controversial because A2A receptor is recognized as a major mediator of anti-inflammatory responses. Activation of A2A has been reported to suppress key components of the inflammatory process, including leukocyte recruitment, phagocytosis, pro-inflammatory cytokine production, and immune cell proliferation [26, 27]. These findings have already led to the clinical testing of A2A agonists in the treatment of inflammatory diseases such as chronic obstructive pulmonary disease (COPD) and diabetic foot ulcer [27-29]. The beneficial effect of A2A antagonist in EAE animals suggests that A2A receptors in the CNS might play an opposite role compared to the A2A receptors expressed on immune cells, though the functions of CNS A2A receptors are yet to be defined in autoimmune diseases. Using A2A knockout mice would certainly help to clarify the seemingly different roles of the periphery and CNS A2A receptors. It is quite surprising that no EAE studies have been conducted on the A2A knockout mice considering that these animals have been available for quite some time [30, 31].

Unique among the four adenosine receptors, A2B is a low-affinity receptor for adenosine. Adenosine activates A1, A2A, and A3 receptors with EC50 values between 10 nM and 1 μM, whereas A2B receptor activation generally requires adenosine levels that exceed 10 μM [32]. The physiological adenosine concentrations are lower than 1 μM, so activation of the A2B receptor is believed to require pathological conditions such as ischemia, trauma, inflammation or other types of stress [33]. Though its functions in MS or EAE are not clear, A2B has been reported to play pro-inflammatory roles in both rodent and human asthma and COPD. Activation of A2B has been demonstrated to increase the production of IL-6 and IL-19 from mast cells, bronchial smooth-muscle cells, bronchial epithelial cells, and lung fibroblasts [34]. In fact, CVT-6883, an A2B-selective antagonist, is under clinical investigation for the treatment of COPD [27]. A2B is also believed to be involved in inflammatory bowel diseases. A2B receptors are upregulated in gut tissue during both human and murine colitis [35], and A2B blockade [36] or knockout [37] suppresses intestinal inflammation and attenuates the course of disease in murine colitis.

Though it is still unclear whether the A3 receptor is involved in MS, this receptor has been implicated to mediate the inhibition of TNF-α production by adenosine [38, 39]. Anti TNF-α drugs are remarkably effective in several autoimmune diseases, including rheumatoid arthritis, Crohn's disease, psoriasis and ankylosing spondylitis [40]. Blocking TNF-α with antibodies or soluble TNF receptors also decreased EAE severity in animals [41]. Unfortunately, such treatments have been found to be harmful rather than beneficial in human MS trials [42, 43]. A3 has also been found to be overexpressed in inflammatory tissues [44] and the PBMCs of arthritic animals [45]. These findings warrant further investigations of this receptor in the pathogenesis of EAE or MS.

While the precise roles of adenosine and adenosine receptor subtypes in the development of MS and EAE remains to be clarified, the above findings clearly highlight the critical involvement of adenosine and adenosine receptors in inflammation and autoimmunity.

Adrenergic receptors

Accumulating evidence over the past few decades has documented that the brain communicates with the periphery immune system via two major pathways. The first pathway involves activation of the hypothalamic-pituitary-adrenal axis and the eventual secretion of corticosteroids from the adrenal cortex (reviewed in [46]). The second pathway involves activation of the sympathetic nervous system (SNS) and the release of the various neural transmitters (reviewed in [47]). As one of the major neural transmitters from the SNS, the catecholamine norepinephrine elicits its biological functions by activation of α1-, α2-, and β-adrenergic receptors [47].

Results from a series of studies show that the expression of adrenergic receptors, especially β-adrenergic receptors, changes significantly in both MS patients and EAE animals, when compared with the related controls. An early study with the Lewis rat acute EAE model indicated that in response to immune challenge, the splenic noradrenaline content fell significantly, accompanied by an increase in lymphocyte β-receptor density. These changes were considered as early indicators of immune reactivity [48]. Similarly, in the MRL-lpr/lpr mouse, a genetic model of the human autoimmune disease systemic lupus erythematosus, noradrenergic innervation and noradrenaline content were reduced in the spleen prior to the onset of observed splenomegaly and remained reduced at all ages examined [49]. In MS patients, increased β-adrenergic receptor density on PBMCs, including lymphocytes, has been well documented [50-55]. This increase in β-adrenoreceptor density has been shown to be correlated with the expression of high affinity IL-2 receptors (IL-2R) on PBMCs and disease activity of RRMS. *In vitro* studies showed that β-agonist stimulation of PBMCs reduces the IL-2R expression and suppresses cell proliferation following mitogenic stimulation [51]. This observation may indicate a recovery role for the enhanced β-adrenoceptor expression in MS. β2-adrenergic

receptor is expressed on Th1 but not on Th2 cells [56]. Given that cellular Th1 immune activity is considered to be one of the major contributors to disease activity in MS, increased expression of β2-adrenoreceptor may reflect activation of Th1 cells and a predominant cellular immune activity. Adrenergic receptors have also been reported to modulate cytokine production in dendritic cells (DCs) and affect their Th cell-priming ability [57]. In particular, activation of β2-adrenergic receptors in DCs hampered IL-12, but stimulated IL-10 production resulting in reduced migration and Th1 priming [58, 59]. In contrast, more recent studies indicated that activation of β2-adrenergic receptors in DCs might lead to a dominant Th2/Th17-promoting phenotype in response to immunogenic protein or pathogen stimulation [60].

Another interesting phenomenon is the lacking of the astrocytic β2-adrenergic receptor in MS patients. β2-adrenoceptor has been identified on all GFAP-positive astrocytes in white matter and the optic nerve of healthy human and normal animals [61, 62], and astrocytes are the main cellular target of norepinephrine terminals in the brain [63]. However, in MS patients, this receptor could neither be visualized on astrocytes in normal-appearing white matter nor in reactive astrocytes in chronic active and inactive plaques, although it was normally present on neurons [61, 64]. Astrocytes are considered the primary APCs of the CNS in EAE models. Mice astrocytes can express MHC class II and B-7 co-stimulatory molecules, which are necessary for the efficient activation of naive T cells [65], and have potential for processing and presenting CNS auto-antigens to pro-inflammatory T cells [66]. In normal conditions, the expression of MHC class II molecules are tightly suppressed by norepinephrine via β2-adrenergic receptor activation [67, 68]. Norepinephrine also inhibits the astrocytic expression of proinflammatory cytokines through the IκBα/NFκB pathway [69, 70]. The loss of astrocytic β2-adrenergic receptor might explain the presence of MHC class II on astrocytes and the increased pro-inflammatory cytokine levels in MS lesions. What causes the loss of astrocytic β2-adrenergic receptor in MS patients remains unclear, though a 'hit and run' viral infection model has been proposed [71].

Compounds regulating the adrenergic receptors have been used to treat EAE and MS. Nonselective β-adrenergic agonist isoproterenol and the β2-specific agonist terbutaline significantly suppressed both the first acute attack and the number of relapses in EAE Lewis rats [72]. Other β2-adrenergic agonists, such as salbutamol and albuterol, have been proposed to be used as add-on therapy in patients with MS [71, 73-75]. In a recent trial with albuterol as an add-on treatment to glatiramer acetate therapy, improvement in the MS functional composite and a delay in the time to the first relapse were observed in the glatiramer acetate plus albuterol group [76]. Other modulators of adrenergic receptors have also been reported to benefit EAE animals or MS patients. Prazosin, an α1-adrenergic receptor antagonist, suppressed the clinical and histological expression of EAE in the Lewis rat [77-79]. Tizanidine, an α2-adrenergic receptor agonist, is a very useful medication in patients suffering from spasticity caused by MS [80, 81].

Cannabinoid receptors

The medicinal use of *Cannabis sativa* (marijuana) can be traced back for centuries. But the existence of an 'endocannabinoid system' has only gained appreciation in the past few decades. This system consists of endocannabinoids (arachidonoylethanolamine (AEA), 2-arachidonoyl glycerol, 2-arachidonyl glyceryl ether (noladin ether), *N*-arachidonoyl-dopamine, virodhamine, etc.), their synthesizing/degradation enzymes and their receptors. There are two major types of cannabinoid receptors, termed CB1 and CB2. But other receptors, such as vanilloid receptor, GPR55 and GPR119 have also been reported to be activated by cannabinoids [82-84]. The CB1 receptor is mainly expressed in the CNS, but also in the lungs, liver and kidneys. The CB2 receptor is mainly expressed in the immune system and in hematopoietic cells. Recently, CB2 has also been described in microglia and neuronal progenitor cells, but with few exceptions, it is not expressed by neurons within the CNS [82, 85].

Both CB1 and CB2 are Gαi-coupled GPCRs. Activation of these receptors leads to inhibition of adenylate cyclase activity, reduced cAMP level, decreased activity of PKA, and eventual reduction in cytokine production and synaptic transmission [86]. Activation of these receptors showed protective effects in various EAE models. Using the Theiler's murine encephalomyelitis virus (TMEV) model, the synthetic cannabinoids WIN 55212-2, ACEA, and JWH-015 significantly improved the neurological deficits in a long-lasting way [87, 88]. In a rat EAE model, decreased endocannabinoid level was reported in the brain and activation of cannabinoid receptors reduced the neurological impairment [89]. The non-selective cannabinoid receptor agonist WIN-2 was found to ameliorate the clinical signs and diminish the cell infiltration into the spinal cord in a passive EAE rat model [90]

The CB1 receptor was the initial focus of attention for studies using cannabinoids to treat EAE, because the activation of CB1 was believed to inhibit synaptic transmissions, which might contribute to spasticity, tremor and paralysis in EAE [91-93]. These studies provide objective evidence to support the claims of MS patients that cannabinoids may have a benefit in symptom man-

agement [94]. The beneficial effects were further supported by recent clinical trials with medical cannabis extracts [95]. More direct evidence for the protective roles of CB1 receptor came from a study in a conditional knockout EAE model in which the neuronal CB1 was selectively deleted. In such animals, the cannabinoid-mediated EAE suppression was abolished [96].

In the same study, Maresz *et al*. [96] also reported that CB2 receptor expressed by the pathogenic T cells was critical for controlling inflammation associated with EAE. CB2-deficient T cells in the CNS of EAE animals exhibited reduced levels of apoptosis, a higher rate of proliferation, and increased production of inflammatory cytokines, resulting in more severe clinical symptoms. Palazuelos *et al*. [97] also found that CB2 knockout mice showed exacerbated clinical score of EAE; and the underlying mechanism might involve the extensive recruitment of immature bone marrow-derived $CD34^+$ myeloid progenitor cells towards the spinal cords in CB2 knockout EAE mice. The immunosuppressive effect of CB2 activation was also supported by studies with selective CB1/CB2 agonists/antagonists. Ni *et al*. [98] demonstrated that the therapeutic effect of WIN55212-2, a non-selective CB1/CB2 agonist, could be blocked by CB2 antagonist SR144528, but not by CB1 antagonist SR141716A. JWH-015, a cannabinoid with a relatively high selectivity for CB2, was reported to suppress microglial activation [99]. O-1966, a selective CB2 agonist [100], was found to significantly improve the motor function in the chronic EAE model, the remitting-relapsing model and the adoptive transfer model [101]. Administration of HU-308, with a selectivity of ~500× for CB2 vs CB1 [102], improved EAE symptoms and reduced spinal cord lesions and microglial activation [97]. Unlike CB1, CB2 activation is not associated with psychoactive effects. Therefore, targeting CB2 with selective agonists might be a more attractive way to treat MS.

It is also interesting to notice that the key components of the endocannabinoid system are all altered in MS patients. AEA and palmitoylethanolamide (PEA) were found to be higher in RRMS samples compared to controls. AEA, PEA, and oleoylethanolamide were also increased in the plasma of SPMS patients; PPMS patients had higher AEA plasma levels compared to controls. mRNA level of fatty acid amide hydrolase, the enzyme responsible for the degradation of endocannabinoids, was decreased in SPMS but not in RRMS or PPMS blood. CB1 and CB2 mRNAs were increased in the PPMS patients [103, 104]. The fact that all these alterations will lead to the activation of the endocannabinoid system suggests that the body might employ these as a mechanism to compensate for the over-activation of immune responses.

Chemokine receptors

Chemokines are cytokines initially characterized to be associated with leukocyte chemotaxis and inflammatory responses. Chemokines are classified on the basis of their structural properties, regarding the number and position of the conserved cysteine residues at the amino-terminal, into two major (CXC and CC) and two minor (C and CX3C) subfamilies [105, 106]. Chemokines were the first members of cytokine family to be shown to interact with GPCRs. Chemokine receptors comprise 10 CCR family members, 7 CXCR family members and other receptors including XCR1, CCRL1 and 2, and CX3CR1. Three decoy receptors, D6, DARC, and CCX-CKR (Chemocentryx-chemokine receptor), which bind chemokines with high affinity but do not elicit signal transduction, have also been reported [107]. Many chemokines bind multiple receptors and most receptors bind multiple chemokines, suggesting the possibility of functional redundancy, which is also likely to be modulated by both the spatial and temporal control of expression. Chemokine receptors signal through heterotrimeric G-proteins, which in turn regulate diverse signal transduction pathways, including intracellular calcium, mitogen-activated protein kinases, PLCβ, PI3K, Ras, and Rho GTPases pathways, etc. [108]. These signal mechanisms are believed to be responsible for cell movement beyond immune cell trafficking, as they also regulate other processes, such as hematopoiesis [109], angiogenesis [110], and organogenesis, including CNS formation [111].

The infiltration of leukocytes into the CNS is an essential step in the neuro-pathogenesis of MS. Leukocyte extravasation from the bloodstream is a multi-step process that depends on fluid dynamics within the vasculature and molecular interactions between circulating leukocytes and the vascular endothelium. An important step in this cascade is the binding of chemokines displayed on the vascular endothelial cell surface to chemokine receptors on circulating leukocytes, initiating intracellular signaling that leads to integrin activation, leukocyte arrest, and extravasation [112]. Indeed, during the pathogenesis of MS or EAE, the expression of many chemokines and/or their receptors has been found to be altered significantly in the demyelinating plaques or the periphery immune tissues (summarized in Table 1).

Modulating immune cell migration into the CNS may represent an ideal way of combating neuro-inflammation, and accurately determining which processes or physiological roles may be regulated by a given chemokine or its receptors is crucial. The best means of investigating the actual functions of chemokines and their receptors

Table 1 The expression changes of chemokines and their receptors during MS or EAE pathogenesis

Chemokine or receptor	MS patients vs healthy controls			EAE vs naive animals		
	Location	Expression change	Ref. no.	Location	Expression change	Ref. no.
CCR1	In early, actively demyelinating plaques	Elevated	[205-207]	Spinal cord	Elevated	[208]
CCR2	In chronic active MS lesions	Elevated	[209-211]	Spinal cord	Elevated	[208, 212, 213]
CCR3	In chronic active MS lesions	Elevated	[209]			
	$CD4^+$ and $CD8^+$ cells in CSF at relapse; CNS	Reduced	[214]			
CCR4	$CD4^+$ and $CD8^+$ cells in CSF at relapse	Reduced	[214]			
	Invading leukocytes	Elevated	[215]			
CCR5	MS lesions	Elevated	[207, 209, 216]	Spinal cord	Elevated	[208, 212]
	$CD4^+$, $CD8^+$, $CD19^+$, and $CD14^+$ cells in peripheral blood/ macrophages microglia	Elevated	[206, 217, 218]			
CCR7	MS lessions	Elevated	[219]	CNS	Elevated	[220]
CCR8	Phagocytic macrophages and activated microglia	Elevated	[221]	CNS	Elevated	[220]
CX3CR1	Peripheral mononuclear cells	Reduced	[222]	Spinal cord	Elevated	[205, 212, 223]
CXCR1	CNS	Elevated	[224, 225]			
CXCR2	MS lesions	Elevated	[224, 226]	Spinal cord	Elevated	[227]
CXCR3	CSF T cells	Elevated	[228]			
	$CD14^+$ cells in peripheral blood	Elevated	[211, 217]			
	CNS	Elevated	[224]			
	Active MS lesions	Elevated	[216, 229]			
CXCR4	$CD14^+$ cells in peripheral blood	Elevated	[217]	Spinal cord	Elevated	[227]
CCL1				Spinal cord	Elevated	[230]
CCL2	CSF/serum	Elevated	[231]	Spinal cord	Elevated	[230, 232]
	CSF	Reduced	[233, 234]			
CCL3	CSF	Elevated	[216, 231]	Spinal cord	Elevated	[213, 230, 232, 235, 236]
CCL4				Spinal cord	Elevated	[230, 232]
CCL5	CSF	Elevated	[216, 217, 231, 237]	Spinal cord	Elevated	[230, 232, 235]
CCL7				Spinal cord	Elevated	[232]
CCL17	CSF	Elevated	[238]			
CCL19	Brain tissue/CSF	Elevated	[239]	Venules surrounded by inflammatory cells	Elevated	[240, 241]
CCL21	Brain tissue/CSF	Unchanged	[239]	CNS	Unchanged	[240, 241]
CCL22	CNS	Elevated	[215]			
CX3CL1	CSF/serum	Elevated	[242]			
CXCL1	MS lesions	Elevated	[224-226]	Spinal cord	Elevated	[232, 235]
CXCL9	CSF	Elevated	[216]	CNS	Elevated	[243]
CXCL10	CSF	Elevated	[216, 217, 229, 231, 234]	Spinal cord/ cerebellum	Elevated	[232, 243]
CXCL11	Serum	Elevated	[244]			
CXCL12	Astrocytes in lesion areas/BBB	Elevated	[245-247]			
CXCL13	CSF	Elevated	[247]			

are probably those using gene-manipulated (transgenic or knockout) mice and specific pharmacological blockers of the receptors (summarized in Table 2). Even though the attempt to translate knowledge from the animal models to the human situation is criticized, these models have rapidly led to an understanding of chemokine signaling pathways and have provided the basis for their use as therapeutic targets.

The recent failures of CCR1 antagonists BX471 (Berlex/Schering), MLN 3701 and MLN 3897 (Millennium) and CCR2 antagonist MK-0812 (Merck), which showed promising results in animal models [113], in treating MS in phase II clinical trials highlighted the difficulty of this animal-to-human translation [114]. Functional redundancy of chemokines and their receptors might contribute to this problem. Moreover, the redundancy could vary between species, making it difficult to predict what the outcome of an antagonist tested in animal models will be in humans. Finally, the heterogeneity of MS [114, 115] might further complicate the problem. Despite these difficulties, pharmaceutical companies still consider chemokine receptors as promising therapeutic targets. Almost every major company has a list of potential blockers in clinical development for different indications, including MS (summarized in Table 3).

Leukotriene receptors

Leukotrienes are potent pro-inflammatory mediators

Table 2 Pharmacological and genetic modulations of the chemokine system mediate disease severity in EAE animal models

Chemokine or receptor	Modulation or intervention		EAE severity	Ref. no.
	Pharmacological	Genetic		
CCR1		Knockout	Alleviated	[248]
	Antagonists (2-2-diphenyl-5-(4-chlorophenyl) piperidin-1-yl)valeronitrile; BX 471)		Alleviated	[249, 250]
CCR2		Knockout	Alleviated	[251-254]
	Antibody		Alleviated	[211]
CCR5		Knockout	Comparable	[255]
	Antagonist (TAK-779)		Alleviated	[256]
CCR6		Knockout	Enhanced	[257, 258]
		Knockout	Alleviated	[259, 260]
CCR7		Knockout	Alleviated	[261]
CMKLR1		Knockout	Alleviated	[262]
CX3CR1		Knockout	Enhanced	[263]
CXCR2	Anti-CXCR2 antiserum	Knockout	Alleviated	[264]
	Antagonists		Alleviated	[265]
CXCR3		Knockout	Enhanced	[266, 267]
	Antagonist (CXCL11(4-79))		Alleviated	[268]
CXCR4	Antagonist (CXCL12(P2G2))		Alleviated	[268]
	Antagonist (AMD3100)		Enhanced	[269]
CCL2		Knockout	Alleviated	[270]
	Antibody		Alleviated	[236, 271]
		Transgenic expression	Alleviated	[272]
CCL3		Knockout	Comparable	[255]
	Antibody		Alleviated	[236, 271]
CCL5	Antibody		Comparable	[236]
CCL19/CCL21	Exogenous CCL19/CCL21 treatment		Alleviated	[261]
CXCL10	CXCL10 antibody		Alleviated	[273]
	CXCL10 antibody		Enhanced	[274]
		Knockout	Enhanced	[275]
CXCL13		Knockout	Alleviated	[276]

derived from arachidonic acid via the sequential actions of cytosolic phospholipase A2α (cPLA2α), 5-lipoxygenase (5-LO), and LTA4 hydrolase (LTA4H) for LTB4, or LTC4 synthase (LTC4S) for cysteinyl leukotrienes (CysLTs, including LTC4, LTD4, and LTE4) [116]. There have been contradictory reports regarding the change of leukotriene levels in MS patients and animal models. Some studies found that the LTB4 and LTC4 levels are significantly increased in the cerebrospinal fluid (CSF) of MS patients compared with the controls [117, 118]. But no significant difference exists in LTC4 production between MS and control peripheral blood monocytes and macrophages [118, 119]. These results were also verified in the animal model of EAE [120]. Other studies, however, found normal CSF concentrations of leukotrienes [121]. Such discrepancies have been attributed to the difficulties in measuring leukotrienes accurately in body fluids.

However, the studies of the key enzymes of the leukotriene biosynthesis pathways revealed important roles of leukotrienes in the pathogenesis of MS. The biosynthesis of leukotrienes in inflammatory cells begins with the cleavage of arachidonic acid from nuclear membrane glycerophospholipids by cPLA2α. Marusic *et al*. [122, 123] reported that cPLA2α-deficient mice are resistant to EAE and blocking cPLA2α with specific inhibitors prevents EAE development and greatly reduces antigen-induced production of Th1-type cytokines and IL-17. Another key enzyme, 5-LO, which catalyses the conversion of arachidonic acid to 5-hydroperoxyeicosatetraenoic acid and subsequently the unstable precursor LTA4, was found to be upregulated in both MS lesions and EAE brains by microarray analysis [124]. 5-LO-specific inhibitors-treated guinea pigs showed significantly lower histological inflammation and better clinical outcome compared with controls upon EAE induction [120, 125, 126].

The biological effects of LTB4 are mediated via two GPCRs, BLT1 and BLT2 [127]. CysLTs also activate two GPCRs, namely CysLT1 and CysLT2 [128]. Inhibition of LTB4 receptors by antagonists or gene knockout alleviates disease pathology in EAE. An early study found that LTB4 receptor antagonist treatment significantly reduced, but did not completely inhibit the cachectic response in a guinea pig EAE model [129]. Similar results were also observed in a murine model of EAE upon antagonist treatment [130]. A recent study with BLT1-knockout mice found that BLT1 deficiency led to delayed onset and less severe symptoms of EAE, and $BLT1^{-/-}$ lymphocytes showed impaired proliferation ability and decreased cytokine production [127]. Our recent study with two anti-asthmatic drugs (montelukast and zafirlukast) targeting CysLT1 indicated that blocking CysLT1 could alleviate CNS inflammatory cell infiltration and pathogenesis of EAE by reducing the permeability of the blood brain barrier (BBB) and the chemotaxis of pathogenic T cells [131].

Opioid receptors

Opioid compounds such as morphine modulate nociceptive pathways in the nervous system and produce powerful analgesia, and are used to treat various types of pain. It has also been noticed for a long time that opioids can alter the immune responses. Acute or chronic administration of opioids is known to have inhibitory effects on humoral and cellular immune responses, including antibody production, natural killer-lymphocyte activity, cytokine expression, and phagocytic activity [132-134]. Increased morbidity and mortality due to artificial infection and faster cancer progression have been well docu-

Table 3 Chemokine receptor modulators in clinical investigation for MS

Chemokine receptor	Modulators	Activity	Phase	Company	Status	Ref. no.
CCR1	AVE9897	Antagonist	Phase I completed	SanofiAventis	Discontinued	[277]
CCR1	MLN3897	Antagonist	Phase I completed	Millenium Pharmaceuticals	Discontinued	[277]
CCR1	BX471	Antagonist	Phase II completed	Schering AG	No significant activity	[114]
CCR2	CCX915	Antagonist	Phase II planned	ChemoCentryx		[277]
CCR2	INCB8696	Antagonist	Phase I ongoing	Incyte		[277]
CCR2	MK-0812	Antagonist	Phase II	Merck	Completed	[188]
CCR2	MLN1202	Antibody	Phase II	Millenium Pharmaceuticals	Completed	[278]

mented in animal studies with morphine treatment [134, 135]. However, from other studies it emerges that not all opioids induce the same immuno-suppressive effects [136, 137].

The endogenous opioid system is comprised of native opioid peptides and four opioid receptors: delta, mu, kappa receptors, and opioid receptor-like 1 [138]. Preclinical investigations utilizing animal models, as well as clinical observations with MS patients, suggested alteration of endogenous opioid systems in the disease. In the TMEV model of MS, mRNA levels of the mu, delta, and kappa opioid receptors were significantly decreased in the spinal cord at days 90, 150, and 180 post infection [139]. The loss of opioid receptors might partially explain the common central neuropathic pain in MS patients [140]. Pregnant woman usually have higher levels of endogenous opioids [141]. They experience remission of MS and have fewer relapses during their pregnancy. However, these women exhibit a marked increase in relapse rate 3 months after delivery, when endogenous opioid levels are decreased [142, 143]. These findings suggest that one or more of the elevated opioids are acting with relevant receptors to attenuate the pathogenesis of MS. However, Gironi *et al*. [144] have reported a reduction of β-endorphin levels in PBMCs from patients with clinically inactive MS, but demonstrated an increase of β-endorphin in PBMCs from patients experiencing a relapse. The same group also found that β-endorphin level varies in different forms of MS. The lowest PBMC β-endorphin level was observed in primary and secondary progressive forms of MS, while the highest level was found in patients with benign and relapsing remitting forms of MS [145]. Treatment with IFN-β seems to induce an increase of this opioid in MS patients [144]. The increase of β-endorphin concentration during a clinical relapse may represent a possible control mechanism aimed at downregulating the inflammatory process.

Opioids have been used to treat EAE or MS. Met-enkephalin, an endogenous opioid, inhibited the onset and progression of EAE [146]. A preliminary clinical trial showed that intrathecally given met-enkephalin exerted a beneficial effect on 13 patients with chronic severe progressive MS [147]. Rats treated with MR 2034, a kappa opioid receptor agonist, showed a pronounced suppression of EAE clinical signs, CNS histological lesions, and anti-myelin basic protein antibody production [148]. However, other reports have shown that naltrexone (NTX), a non-selective opioid receptor antagonist, has protective effects on both EAE animals and MS patients. Zagon *et al.* [149] demonstrated that low-dose NTX (LDN, 0.1 mg/kg) markedly reduced the severity and disease index of the treated mice, and over 33% of the MOG-treated animals receiving LDN treatment did not exhibit behavioral signs of disease. On the other hand, high-dose NTX (HDN, 10 mg/kg) displayed no beneficial effect. A 6-month phase II multi-center pilot trial with LDN has been carried out in 40 patients with PPMS. A significant reduction of spasticity was observed at the end of the trial [150]. The therapeutic effect of LDN might be explained by the elevated endogenous opioids and opioid receptors due to the temporary blockade of the opioid receptors [151-153].

These results suggest that endogenous opioids and their receptors may play important roles in the development of EAE and MS, though the exact ligand, receptor or their mechanisms remain to be elucidated. Nevertheless, LDN, which has been successful in the treatment of Crohn's disease [154], would represent a safe, non-toxic, and generically available agent for attenuating MS and possibly other autoimmune diseases.

Sphingosine-1-phosphate receptors

Sphingolipids were first identified in the ethanolic brain extracts in the 1870s and were named after the Greek mythological creature, Sphinx, because of their enigmatic nature [155]. Sphingosine-1-phosphate (S1P) represents a minor constituent of total sphingolipids. It is found abundantly in vertebrate blood and lymph. With the discovery of five S1P receptors [156, 157], designated S1P1-5, S1P is recognized as an important extracellular lipid mediator.

S1P receptors are ubiquitously but differentially expressed on all cells, including a wide range of cells that are involved in the development of MS. Genetic deletion of S1P1 in mice demonstrated that this receptor plays key roles in angiogenesis and vascular maturation [158], immune cell trafficking [159], endothelial barrier function [160], and vascular tone [110]. Like S1P1, S1P2 and S1P3 are also widely expressed. S1P4 has a more restricted expression pattern and is detectable predominantly within immune compartments and leukocytes [161], and may play a role in regulating T-cell cytokine production [162]. S1P5 is expressed primarily in the white matter of the CNS, but its precise role remains to be clarified.

MS is generally believed to be caused by the invasion of autoreactive T cells into the CNS, which leads to demyelination and axonal damage. The S1P1 receptor has been shown to regulate the recirculation of lymphocytes [163-165] and their egress from secondary lymphatic organs [159]. Therefore, targeting S1P1 to reduce circulating T cells might be an effective treatment for MS and other autoimmune diseases.

Recently, fingolimod (FTY720), a non-selective S1P

receptor modulator, has been approved by the US FDA as the first oral, first-line treatment for RRMS. It outperformed the established first-line therapy IFN-β1a in a 1-year, double-blind, and double-dummy phase III study (known as TRANSFORMS) [166]. In animal studies, prophylactic administration of fingolimod completely prevented development of EAE features, whereas therapeutic administration significantly reduced the clinical severity of EAE [167, 168]. As a structural analog of sphingosine [169], fingolimod is phosphorylated *in vivo* by sphingosine kinase 2 [170] to produce fingolimod-phosphate, which binds to four of the five S1P receptors (S1P1 and S1P3-5) with high affinity. Fingolimod-phosphate initially activates lymphocyte S1P1 but subsequently induces S1P1 internalization and down-regulation, which prevents lymphocyte egress from lymphoid tissues, therefore reducing pathogenic lymphocyte infiltration into the CNS [163, 171]. Fingolimod is also reported to ameliorate EAE by suppressing both cellular and humoral immune responses [172].

Fingolimod is able to cross the BBB [173], and may therefore have direct CNS effects, which is unique compared to other immunologically targeted MS therapies. S1P receptors are also expressed in many CNS cells and have been shown to influence cell proliferation, morphology, and migration [174, 175]. S1P and S1P1 have been implicated to mediate the migration of neural stem cells towards sites of injury in the spinal cord [175]. Recent studies in EAE also suggested a key role of the neuronal S1P1 in disease progression [176]. Re-myelination has been documented in human MS lesions and animal models [177, 178]. S1P1 and S1P5 are both expressed on oligodendrocytes and may be involved in the re-myelination process. Fingolimod can increase the number of both progenitor and mature oligodendrocytes *in vitro*. It can protect oligodendrocytes from cell death induced by cytokines or the withdrawal of growth factors, and modulate process outgrowth [179, 180]. High levels of S1P1 and S1P3 are also found in astrocytes [181], a glial cell type that might act like immune cells to enhance the immune responses and inhibit myelin repair. S1P induces activation and proliferation of astrocytes *in vitro*, while injection of S1P into the striata of mouse brains induced astrogliosis [182]. In a recent study with conditional knockout mice, EAE was attenuated and fingolimod efficacy was lost in mutants lacking S1P1 on GFAP-expressing astrocytes but not on neurons [183]. Receptor rescue and pharmacological experiments supported the loss of S1P1 on astrocytes via functional antagonism by fingolimod-phosphate as a primary mechanism of fingolimod. S1P1 is also expressed and plays important physiological roles in neurons. Genetic deletion of S1P1 resulted in defective neuronal development [184]. Fingolimod has been shown to display neuroprotective effect in both *in vitro* and EAE animal models [185].

Fingolimod thus represents a new generation of medicines for MS treatment with the advantages of oral administration, and beneficially affecting not only the immune system to reduce inflammatory damage but also the CNS to promote neuroprotection and repair. Fingolimod is a non-selective sphingosine receptor modulator. Targeting other receptors, especially the S1P3 receptor, has been reported to induce certain cardiovascular side effects [186]. Pharmaceutical companies are currently developing more specific S1P1 agonists to avoid such potential problems. For example, ACT-128800, an orally available S1P1 receptor agonist ~650-fold more selective for human S1P1 over S1P3 than the natural ligand, is currently under phase II clinical investigation to treat RRMS [187-189].

Other GPCRs

Many other GPCRs have also been reported to be involved in the pathogenesis of EAE. For example, blocking dopamine D2-like-receptors (including D2, D3, and D4) with antagonist L750667 promoted DC-mediated Th17 differentiation [190]. This is consistent with a previous report that D2 receptor agonist bromocriptine displayed therapeutic effect on acute and relapsing EAE models [191]. In contrast, SCH23390, a D1-like-receptor antagonist, inhibited DC-mediated Th17 differentiation and prevented EAE in mice [190].

Histamine also plays a key regulatory role in EAE and exerts its effect through four GPCRs designated H1, H2, H3, and H4 receptors. Histidine decarboxylase is the necessary enzyme to make histamine. The histidine decarboxylase-deficient mice are genetically unable to make histamine. EAE was found to be significantly more severe in these animals [192]. H3R knockout mice also developed a more severe EAE and neuro-inflammation compared with the wild-type animals [193], indicating that the immunosuppressive effect of histamine might be mediated via H3R. However, H1R-deficient mice have been found to be more resistant to EAE induction than wild-type controls [194]. Treatment with pyrilamine or hydroxyzine, the H1R antagonists, led to reduced clinical signs of EAE and brain mast cell activation [195-197]. Results from a pilot open-label clinical trial also indicated that hydroxyzine can partially inhibit brain mast cell activation and reduce MS symptoms [198].

Increasing evidence suggests that the platelet-activating factor (PAF) and its receptor (PAFR) are involved in EAE and MS pathogenesis. PAF was upregulated in peripheral-blood leukocytes during EAE induction, and

the receptor antagonist treatment or gene knockout led to alleviation of clinical signs [195, 196, 199, 200]. Polymorphism of PAFR gene has also been identified to be correlated with the susceptibility to MS in human [201].

Apart from leukotrienes, prostaglandins form another family of important signaling molecules derived from arachidonic acid. Prostaglandin E2 (PGE2) was found to be increased in the CSF of MS patients [118, 202]. Among the four PGE2 receptors, EP1-EP4, only the EP4-knockout significantly suppressed EAE induction. This was mimicked in wild-type mice and to a greater extent, in EP2-knockout mice by administration of the EP4 antagonist ONO-AE3-208 during the immunization or preclinical phase [203, 204]. But ONO-AE3-208 administration at EAE onset had little effect on disease severity. In contrast, administration of the EP4 agonist ONO-AE1-329 at EAE onset delayed and suppressed disease progression as well as inhibited the associated increase in permeability of the BBB [204]. Thus, PGE2 exerts dual functions in EAE, facilitating Th1 and Th17 cell generation redundantly through EP4 and EP2 during immunization and attenuating invasion of these cells into the brain by protecting the BBB integrity through EP4 [203, 204].

Other GPCRs that have been reported to be involved in EAE or MS pathogenesis, such as serotonin receptors, GPR30, and a list of peptide receptors, are summarized in Table 4.

Table 4 Other GPCRs that may play a role in EAE pathogenesis

Receptor or ligand	Modulation			EAE severity	Ref. no.
	Pharmacological		Genetic		
	Agonist	Antagonist			
AT1R		Losartan		Alleviated	[279, 280]
Bdkrb1	R838			Alleviated	[281]
Bdkrb2			Knockout	Alleviated	[282]
D1-like-R		SCH23390		Alleviated	[190]
EDNRA		BQ-123		Alleviated	[283]
EP1			Knockout	Comparable	[204]
EP2			Knockout	Comparable	[204]
EP3			Knockout	Comparable	[204]
EP4			Knockout	Alleviated	[204]
		ONO-AE3-208		Alleviated	[203, 204]
G2A receptor			Knockout	Comparable	[284]
GALR2			Loss-of-function mutant	Enhanced	[285]
GPR30		Luzindole		Alleviated	[286]
H1R		pyrilamine, hydroxyzine		Alleviated	[195-198]
			Knockout	Alleviated	[194]
H3R			Knockout	Enhanced	[193]
HTR1A		WAY100635		Alleviated	[287]
NK1R		CP-96,345		Alleviated	[288]
NPY1R	[F^7,P^{34}]NPY; [D-His^{26}]NPY			Alleviated	[289]
		BIBO3304		Enhanced	[289]
NPY5R	[Ala^{31},Aib^{32}]NPY			Comparable	[289]
Galanin			Transgenic expression	Alleviated	[285]
			Loss-of-function mutant	Enhanced	[285]
Neurokinin-1			Knockout	Alleviated	[290]
PACAP			Knockout	Enhanced	[291]

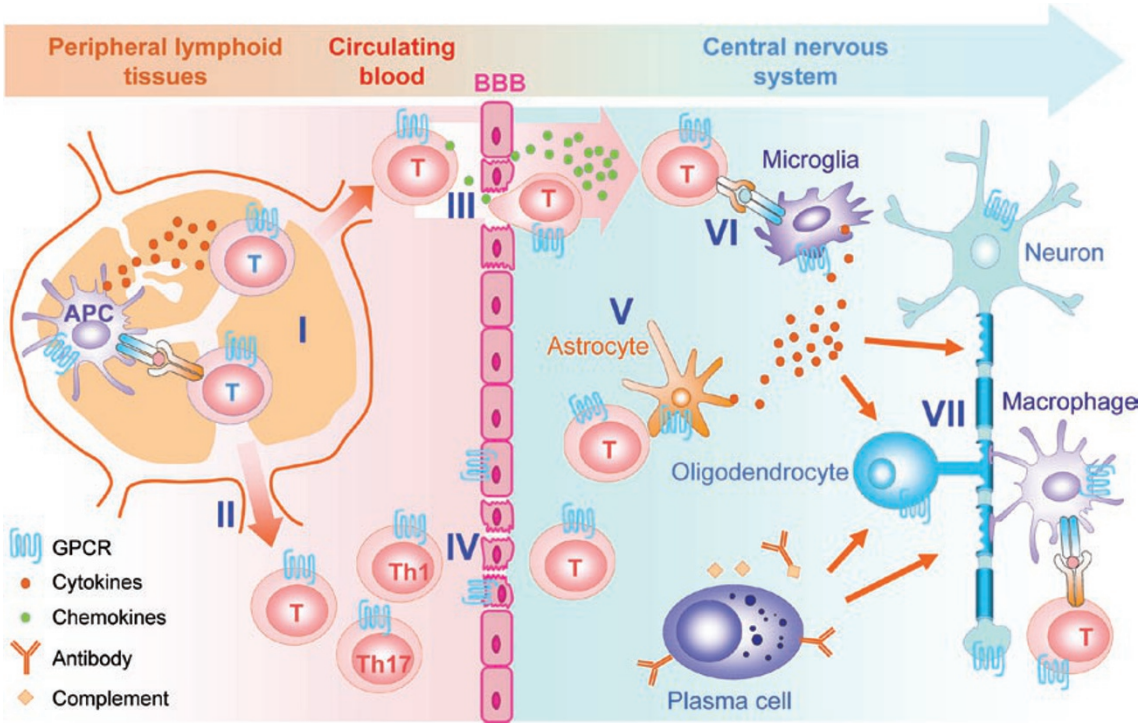

Figure 1 Pathogenesis of multiple sclerosis and critical roles of GPCRs. Studies with genetic manipulations and/or pharmacological interventions suggest that GPCRs mediate important processes in the development of EAE or MS: (I) T-cell activation; (II) T-cell egress from lymphoid tissues; (III) migration and infiltration of inflammatory cells from the periphery to the CNS; (IV) BBB integrity maintenance; (V) astrocyte activation; (VI) microglia activation; (VII) demyelination and neurotoxicity.

Conclusion

As summarized herein, a large body of evidence from both *in vivo* and *in vitro* studies suggests that GPCRs mediate important physiological or pathological functions in the development of MS (Figure 1). Targeted blockade or activation of GPCR-mediated signaling may provide novel approaches to treating MS. Given the current lack of effective pharmacological targets for the treatment of MS, the continued identification and study of GPCRs in MS pathogenesis may eventually lead to major breakthroughs and new pharmacological strategies. One of the best examples of targeting GPCRs to treat MS is the case of fingolimod (FTY720), an S1P1 receptor modulator. Application of this drug significantly reduced the relapse rates, the risk of disability progression, and MRI measures of disease activity in MS patients, as compared with IFN-β1a or placebo. More interestingly, as a wide variety of drugs or compounds targeting GPCRs have already been developed for the treatment of other human diseases, repositioning of these agents might greatly facilitate the development of novel therapies for MS or other autoimmune diseases. We anticipate an exciting future for the discovery of new drugs for MS by targeting GPCRs.

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