Supporting Information

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SI Materials and Methods

Human Brain and Spinal Cord Tissue. All human tissue studies were reviewed and approved by the appropriate ethical committee. Informed consent for donation of tissues was given by all patients or their next of kin. Tissue from multiple sclerosis (MS) patients and controls were acquired from the Netherlands Brain Bank as fresh frozen tissue blocks. Ten-micron sections were analyzed. Detection of hybridized probes used branched DNA/alkaline phosphatase/fast red-driven visualization. Precipitated and red punctuated dots indicate presence of target RNA molecule. Tissue sections were finally counterstained with Harris hematoxylin. For detection of lipid-filled phagocytes, cryosections were postfixed in formalin, stained with oil red O and finally counterstained with Mayer hematoxylin.

Experimental Autoimmune Encephalomyelitis Induction in Dark Agouti Rats, Scoring, and Treatment. All animal experimental procedures were performed in accordance with relevant guidelines and regulations provided by the Swedish Board of Agriculture. Each rat received an injection of 0.2 mL inoculum, containing 85–100 μg of recombinant rat myelin oligodendrocyte glycoprotein $(MOG)_{1\text{-}125}$. The score and body weight were monitored daily and treatment was blinded to the operator. AZD8797 was formulated in 30–35% (wt/wt) hydroxy-propyl-beta-cyklodextrin and administered s.c. through osmotic minipumps (Alzet) in 2 × 7 d (39 μmol/kg per day) or 2 × 14 d (78 μmol/kg per day). The plasma concentration of AZD8797 was analyzed twice from each animal (at pump exchange), and the limit of quatification was 64 nmol/L.

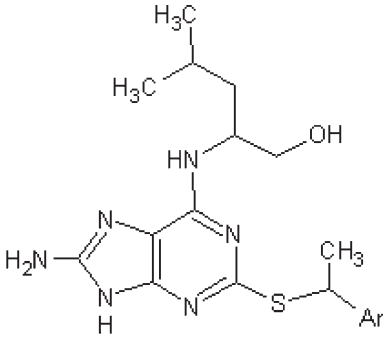
Histopathological Evaluation of EAE. Rats were perfused with phosphate-buffered 4% paraformaldehyde (pH 7.3), followed by immersion fixation in 4% paraformaldehyde overnight, and then transferred to PBS with 0.02% NaN_3. IHC was performed following microwave treatment using the primary antibodies PGP 9.5 (Affinity Research; 1:1,000), ED1 (CD68; Serotec; 1:500), or Iba-1 (Wako Biochemicals; 1:500). The avidin–biotin complex system was used as the detection system, with 3,3′-diaminobenzidine as the chromogen. The slides were then digitized using a Hamamatsu Nanozoomer slide scanner (20× objective, bright field illumination with standard settings, and a mercury lamp illumination source). The digitized images of the stained tissues were visualized at medium to high resolution, using the Hamamatsu NDP View software, and scored semiquantitatively for histopathological lesions, using a four-grade scale. Spinal cord expression of Iba-1 and CD68 was semiquantified using a three- or four-grade scale, respectively.

Evaluation of CX3CR1 mRNA, CD68 Protein Expression, and [^{3}H] PK11195 Binding on Consecutive Rat Spinal Cord Sections. IHC detection of CD68 (ED-1, Serotec) was performed on acetone fixed tissue sections.

For in situ hybridization, ^{35}S-labeled antisense and sense rat CX3CR1 ^{35}S-labeled cRNA probes (GenBank RN04808) were hybridized to the tissues over night at 60 °C, followed by high-stringency (0.1× SSC; 70 °C) washes and RNase digestion. Detection of bound, radiolabeled cRNA probes was achieved by exposure to NTB-2 emulsion (Kodak) for 3 wk.

For receptor autoradiography [^{3}H]PK11195 at 83.5 Ci/mmol (Perkin-Elmer) and unlabeled PK11195 (Tocris) were used. Tissue sections were incubated with 1 nM [^{3}H]PK11195 with or without competing cold ligand for 60 min at room temperature, washed in 50 mM Tris (pH 7.4) at 1 °C, and dipped in cold deionized water. Tissue sections and tritium standards were exposed to imaging plates (Fuji BAS-TR2040) for 5 d and the processed with a FLA-7000 Image reader (Fujifilm). Each spinal cord section was outlined, and the intensity was measured over the entire section.

Photomicrographs of emulsion-coated slides in bright field were captured with a Sony DXC-S500 digital camera mounted on a Leica microscope. Digital images were imported into Adobe Photoshop CS 8.0, where they were adjusted to balance and optimize brightness, contrast, and sharpness. CD68 staining is presented as area fraction positive cells quantified with Image J software (National Institutes of Health), whereas [^{3}H]PK11195 binding and CX3CR1 in situ hybridizations was quantified with Multi Gauge V3.0 software (Fujifilm).



AZD8797

Fig. S1. Structural formula of the CX3CR1 inhibitor AZD8797.

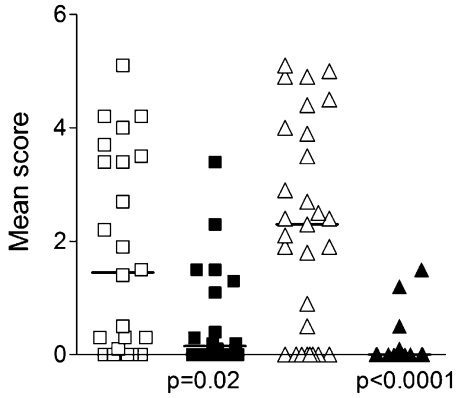


Fig. S2. Distribution of the mean clinical score in the respective treatment group in the same study as in Table S2 (□, vehicle; ■, 39 μmol/kg; △, vehicle; ▲, 78 μmol/kg).

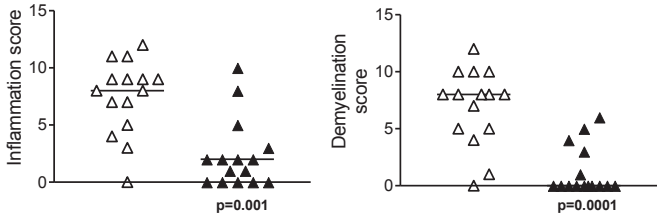


Fig. S3. Distribution of the inflammation score (*Left*) and demyelination score (*Right*) in spinal cords from rats in the same study as in Table S2 at termination (day 30) (△, vehicle; ▲, 78 μmol/kg).

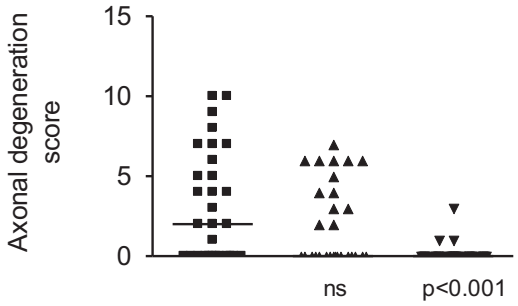
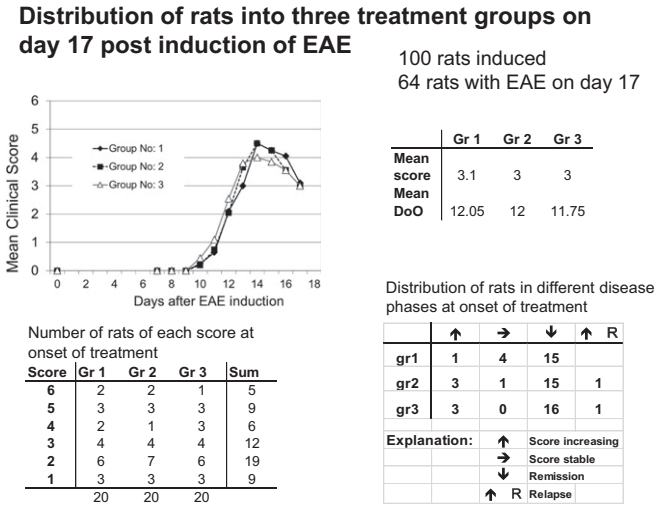


Fig. S4. Distribution of spinal cord axonal degeneration in another EAE treatment study with AZD8797. Rats treated from day 6–35 and terminated at day 35. AZD8797 at a dose of 61 μmol/kg ▼ caused a significant reduction ($P < 0.001$) in axonal degeneration compared with vehicle control (■). Treatment at 20 μmol/kg ▲ did not have a significant effect.



	Gr 1	Gr 2	Gr 3
Mean score	3.1	3	3
Mean DoO	12.05	12	11.75

Score	Gr 1	Gr 2	Gr 3	Sum
6	2	2	1	5
5	3	3	3	9
4	2	1	3	6
3	4	4	4	12
2	6	7	6	19
1	3	3	3	9
	20	20	20	

	↑	→	↓	↑ R
gr1	1	4	15	
gr2	3	1	15	1
gr3	3	0	16	1

Fig. S5. Graphic illustration of the distribution of DA rats with EAE (at day 17 after induction) into three groups (with equal mean score and distribution of disease patterns). The groups then received either of three treatments (vehicle or high or low dose of AZD8797). Treatment was blinded for the experimentalists until termination of the study. (*Upper Left*) Mean clinical score of the three groups of rats from d6 to d17. (*Upper Right*) Mean score and mean day of onset (DoO) of the three goups of rats at d17. (*Lower Left*) Number of rats at each score (between 1–6) allocated to the different groups. (*Lower Right*) Number of rats in different disease phases (increasing score, stable score, remission, and relapse).

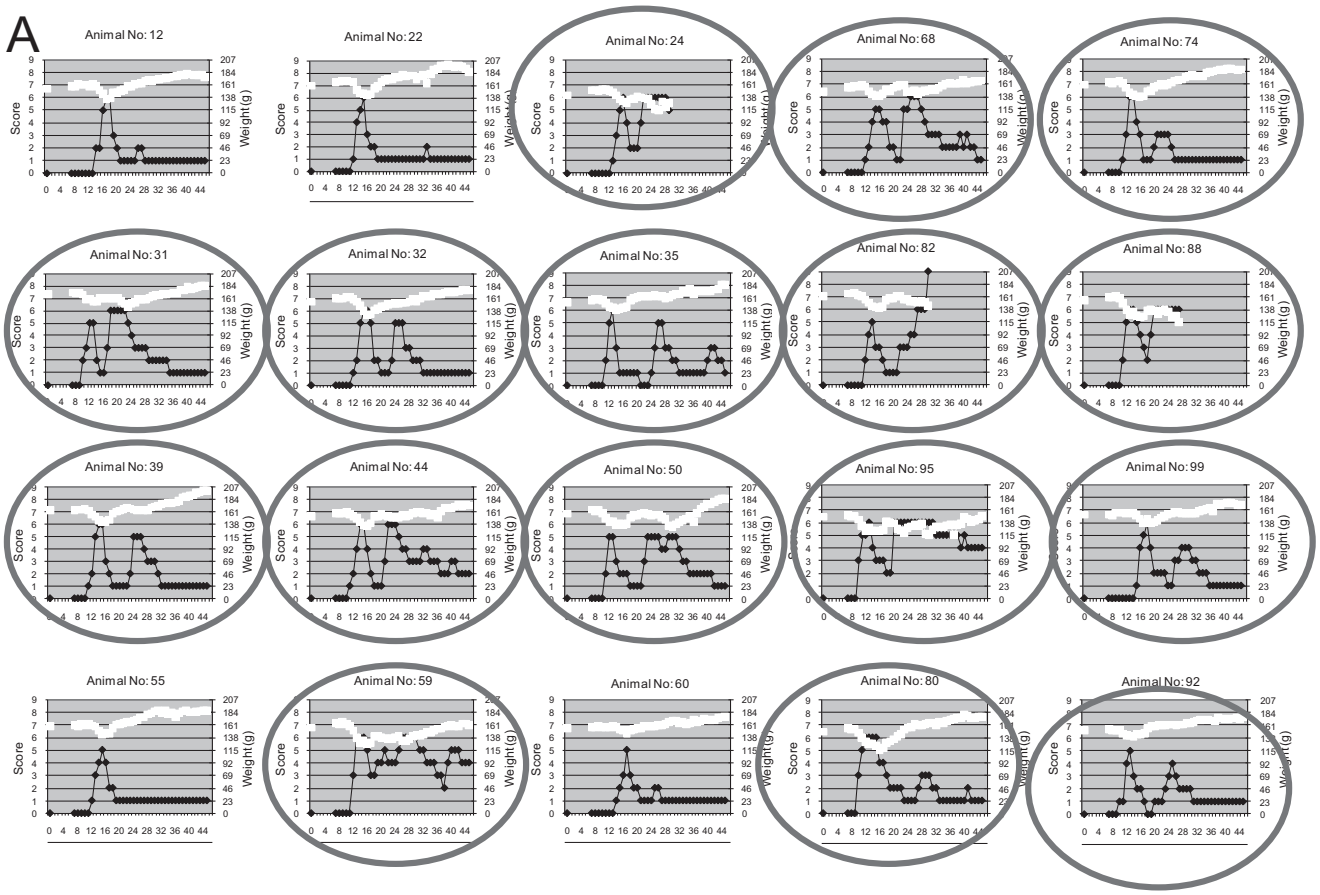


Fig. S6. (Continued)

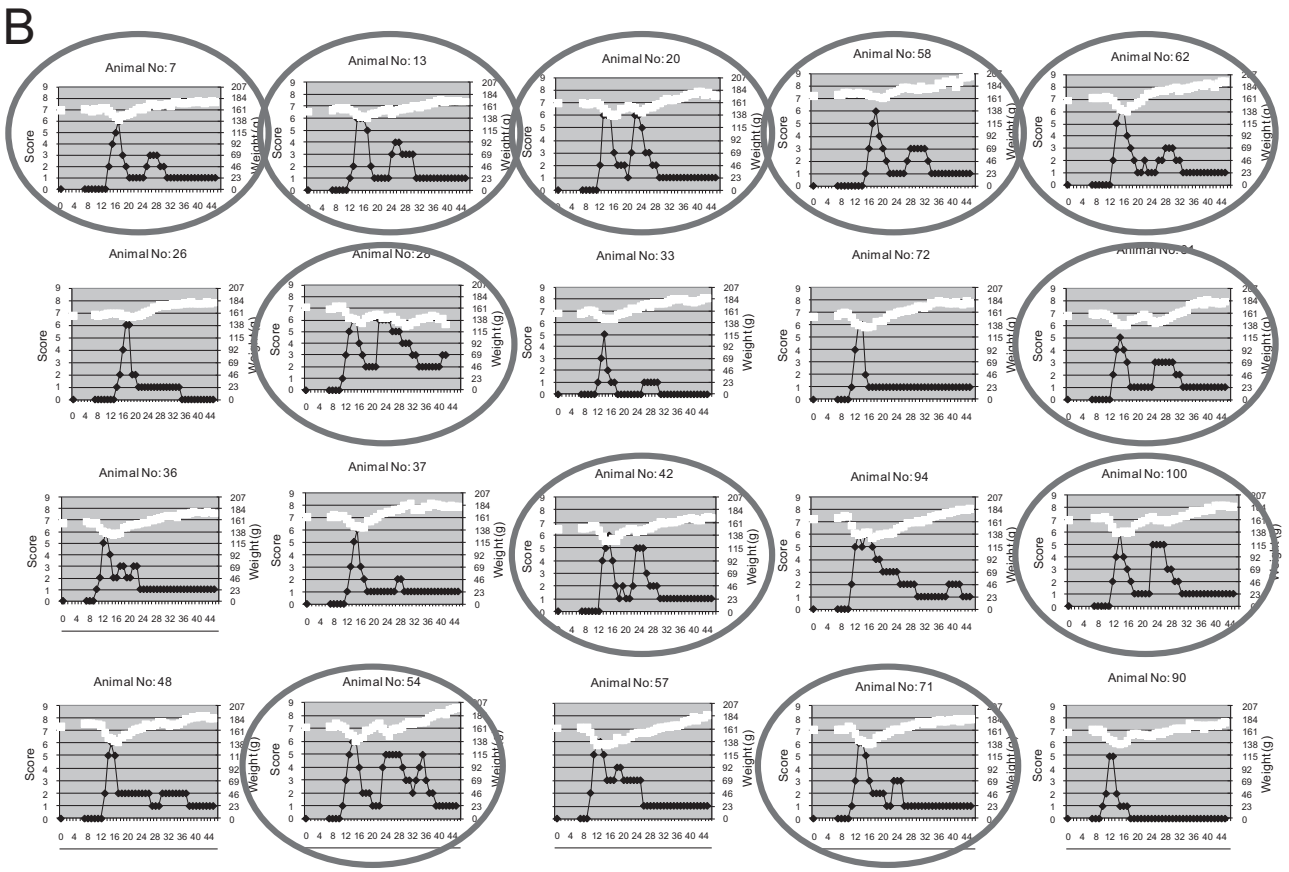


Fig. S6. (Continued)

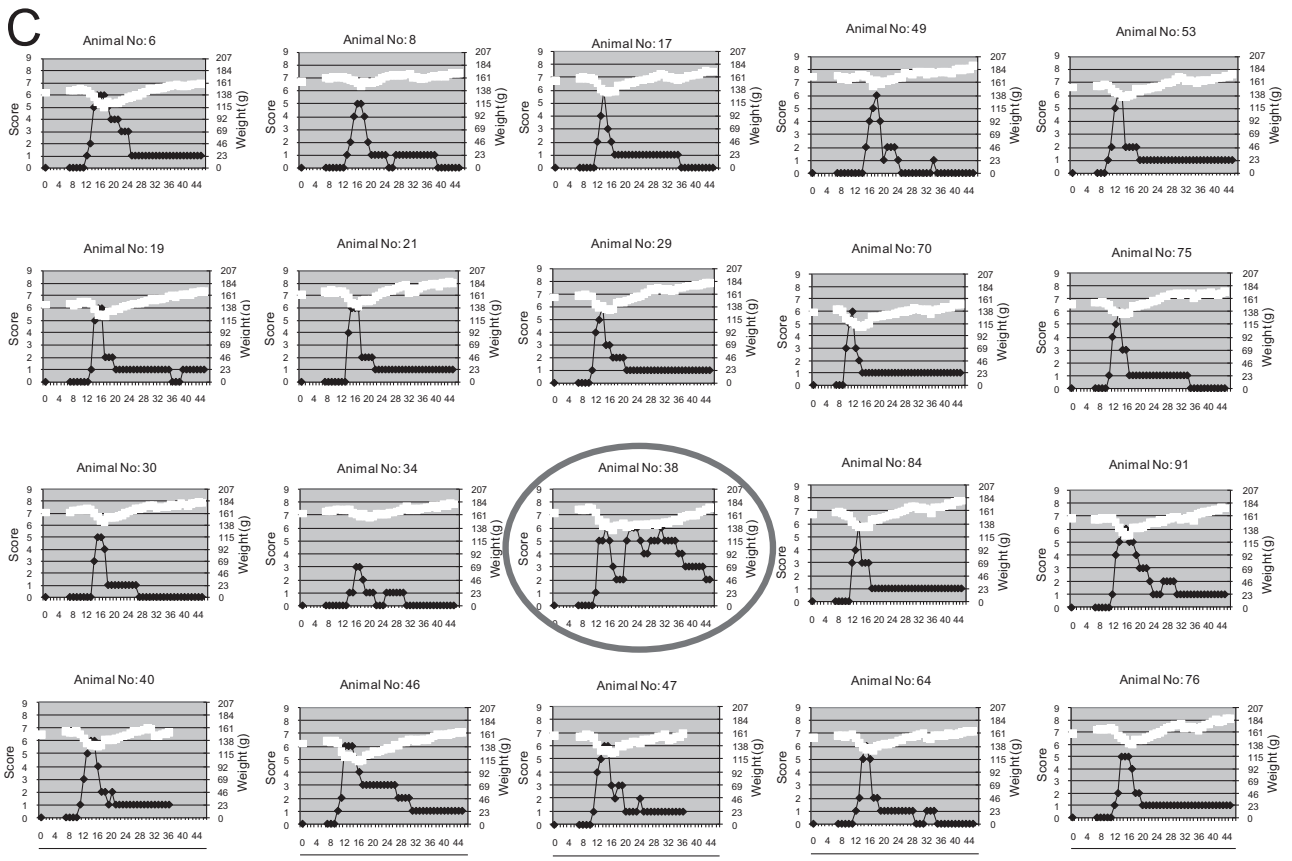


Fig. S6. Individual scoring (black) and weight (white) curves and incidence of relapses defined as animals with a second period of clinical score of 3 or above (circled), following a period of remission. This is the same study as outlined in Fig. S5. (*A*) Vehicle. (*B*) AZD8797 at 32 μmol/kg. (*C*) AZD8797 at 64 μmol/kg.

Table S1. Summary of study design, exposure, and mean average score during the relapse phase in the rat EAE studies

Study no.	Dose, μmol/kg per 24 h	Treatment period*	No. of induced rats	MOG, μg	Sampling time,* day	Mean C_{ss}, μmol/L	± SD C_{ss}	Mean of individual average score during the relapse phase
Exp 1	0	8–31	25	90	22 and 31	—	—	1.8
Exp 1	39	8–31	25	90	22 and 31	2.5	1.3	0.6
Exp 1	78	8–22	30	90	15 and 21	5.9	1.8	0.12
Exp 1	0	8–22	30	90	15 and 21	—	—	2.2
Exp 2	78	8–22	30	90	15 and 21	7.3	2.1	0
Exp 2	0	8–22	30	90	15 and 21	—	—	1.16
Exp 3	0	8–22	30	140	15 and 21	—	—	2.77
Exp 3	60	8–22	30	140	15 and 21	4.1	1.6	0.6
Exp 3	15	8–22	30	140	15 and 21	1.5	0.4	1.54
Exp 4	0	6–35	30	100	20 and 35	—	—	2.64
Exp 4	6.8	6–35	30	100	20 and 35	0.5	0.1	2.95
Exp 4	20	6–35	30	100	20 and 35	1.4	0.4	1.96
Exp 4	61	6–35	30	100	20 and 35	2.2	2.0	0.009

Control rats received only vehicle and are given a "0" in the second column (Dose) and they do not show any exposure as indicated by a dash in column number seven and eight. Exp, experiment.
*Defined as day number postimmunization.

Table S2. Effect of AZD8797 treatment on clinical score and other disease parameters in DA rats with EAE

Estimated dose, μmol/kg per 24 h	Pumps	Average score, day 17–31 (median)	*P*	Incidence of disease	Median day of onset	Mortality*
—	2 × 14 d (day 8–31)	1.4		18/24 (75%)	15.5	0/24
39	2 × 14 d (day 8–31)	0.2	0.020	18/24 (75%)	17.5	0/20
78	2 × 7 d (day 8–22)	0	<0.0001	20/30 (67%)	12	0/30
—	2 × 7 d (day 8–22)	2.3		22/30 (73%)	16.5	3/29

Control animals, receiving only vehicle, are indicated by dashes in the first column.
*No. of rats that were killed or died because of EAE-related causes. (Some rats were removed during the study for non-EAE or treatment-related causes.)