Identification of quantitative trait loci controlling cortical motor evoked potentials in experimental autoimmune encephalomyelitis: Correlation with incidence, onset and severity of disease.

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Abstract

Experimental autoimmune encephalomyelitis (EAE) is a polygenic chronic inflammatory demyelinating disease of the nervous system, commonly used as an animal model of multiple sclerosis. Previous studies have identified multiple quantitative trait loci (QTLs) controlling different aspects of disease pathogenesis. However, direct genetic control of cortical motor evoked potentials (cMEPs) as a straightforward measure of extent of demyelination or synaptic block has not been investigated before. Here we examined the genetic control of different traits of EAE in a F2 intercross population generated from the EAE susceptible SJL/J (SJL) and the EAE resistant C57BL/10.S (B10.S) mouse strains involving 400 animals. The genotypes of 150 microsatellite markers were determined in each animal and correlated to phenotypic data of onset and severity of disease, cell infiltration and cMEPs. Nine QTLs were identified. Three sex-linked QTLs mapped to chromosomes 2, 10 and 18 linked to disease severity in females, whereas QTLs on chromosomes 1, 8 and 15 linked to the latency of the cMEPs. QTLs affecting T-lymphocyte, B-lymphocyte and microglia infiltration mapped on chromosome 8 and 15.

The cMEP-associated QTLs correlated with incidence, onset or severity of disease, e.g. QTL on chromosome 8 cM 32-48 (EAE31) (LOD 6.9, p < 0.001) associated to cMEP latencies in non-immunized mice and correlated with disease onset; and EAE 32 on chromosome 15 linked to cMEP latencies 15 days post immunization and correlated with disease severity. Additionally, applying tissue micro array technology we identified QTLs associated to microglia and lymphocytes infiltration on chromosomes 8 and 15, which are different from the QTLs controlling cMEP latencies. There were no alterations in the morphological appearance of the myelin sheaths. Our findings suggest a possible role of myelin composition and/or synaptic transmission in susceptibility to EAE.
Introduction

Multiple sclerosis (MS) is a complex polygenic disease with prevalence dependent on age, gender, hormonal and environmental factors. Susceptibility to MS is influenced by genetic factors, as indicated by numerous studies showing higher rates of disease concordance in monozygotic than in dizygotic twins, and higher incidence in offspring of MS patients (1, 2). The primary genetic contribution to MS susceptibility is thought to be linked to the HLA locus (1). Identification of the non-MHC genetic loci regulating MS is a complex task due to genetic heterogeneity, incomplete penetrance and influence of environmental factors (3, 4). Hence, genetic analysis of well-defined experimental models such as Experimental Autoimmune Encephalomyelitis (EAE) and the search of syntenic regions between different species in these susceptible loci (5) have the potential to accelerate the genetic analysis of MS (6). In animals and humans most studies indicate that both non-MHC genes and MHC genes are associated with the susceptibility to EAE (7). The first QTL identified, EAE1, is located on chromosome 17 and includes the MHC. Nevertheless, inbred strains sharing the same MHC haplotype vary in their susceptibility indicating the existence of non-MHC susceptibility genes. The conservative estimate for the number of non-MHC susceptibility genes involved is suggested to be more than thirty (8-10). In the past few years extensive search localized 28 QTLs that regulate EAE in mice, mostly in crosses involving the SJL/J and C57BL/10.S strains, both sharing the same H2 haplotype (11).

We assumed that there could be additional QTLs that contribute to the susceptibility to EAE by controlling disease pathways that have not yet been identified. So we set out to identify such QTLs in a new wide genome screen of the F2 generation between C57BL/10.S and SJL/J strains. We focused on functional parameters of the central nervous system (CNS) of EAE mice as a primary trait. Impaired function reflected in electrophysiological changes was shown to be associated with disease severity and demyelination in earlier studies (12). The deterioration of the myelin sheath is one of the main pathological characteristics during the disease and temporal dispersion of neuronal conduction due to demyelination can result in altered cortical evoked responses. CMEP provide
quantitative data on the physiological status of myelinated cortical motor neuron projections and
synaptic transmission and are thus particularly appropriate for a functional study of the descending
tracts. With the measurement of latencies of cMEP, additional features such as cell infiltration in
the spinal cord, demyelination and axonal damage by implementing tissue microarrays of spinal
cord cylinders, disease onset and severity were performed. In this study we report the identification
of nine QTLs controlling disease severity, cellular infiltration and latencies of cMEPs.
Results

EAE incidence and severity in (SJL/J-C57Bl/10S) F2 progeny

We generated a total number of 42 (SJL/J×C57BL/10.S) F1 and 400 (SJL/J×C57BL/10.S) F2 mice and immunized all of them. 82.8 % (33 out of 42 animals) of the F1 progeny (SJL/J×C57BL/10.S) developed EAE with a mean disease severity score of $2.75 \pm 1.26$ and a mean duration until onset of $13.04 \pm 2.18$ days. More females were affected (93.3 %; 14 out of 15) than males (70.3 %; 19 out of 27). The (SJL/J×C57BL/10.S) F2 animals had an incidence of 58.6 % (248 out of 400), with a mean disease severity score of $2.69 \pm 1.27$ and a mean delay of onset of $12.98 \pm 3.22$ days post immunization. Again, more females were affected (74.6 %, 154 out of 207) than males (48.4 %, 94 out of 193). This is consistent with published studies (10).

Quantitative trait Loci associated with disease onset, severity and cell infiltration

We genotyped all F2 mice for linkage analysis as described in materials and methods and we phenotyped for the latencies of cMEP, cell infiltration in spinal cord as well as disease onset and severity.

In the initial screen we identified three QTLs controlling disease severity in females located on chromosomes 2, 10 and 18 (Table 1, Figure 1). The first locus (EAE 33) on chromosome 2 had significant evidence of linkage to marker D2Mit32 (LOD score of 3.7). The second locus (EAE 34) on chromosome 10 was significantly linked to marker D10Mit261 (LOD score of 3.8). On chromosome 18 suggestive linkage to D18Mit144 was found (LOD score of 3.2). A QTL controlling disease onset in females on chromosome 10 with a peak linkage to a D10Mit271 was also identified (LOD score of 3.7). This locus overlaps with the severity controlling locus, EAE 34. Additionally, we identified a suggestive QTL (EAE 38) associated to disease severity in males (linked to area under the curve (AUC) as a trait) on chromosome 15 (linked to D15Mit171, LOD score of 3.3).

Using tissue microarrays and immunohistochemical stains for amyloid precursor protein (APP), myelin basic protein (MBP), neurofilament (NF200) as well as conventional Luxol-Nissl stain of
spinal cords cylinders revealed no significant difference in the myelination pattern and axonal integrity, thus pointing towards a minor involvement of obvious demyelination at this stage of disease. However, the stains for microglia (IBA1), B and T lymphocytes (B220 and CD3) revealed differences between the individual animals (Figure 4). Two new QTLs related to the amount of infiltrating T-lymphocytes and microglia (Table 1, Figure 3) were identified on chromosome 8 (EAE36, linked to D8Mit259) and on chromosome 15 (EAE37, linked to D15Mit35) respectively. A suggestive QTL associated to B-lymphocyte infiltration also map on chromosome 15 (EAE38, linked to D15Mit171) in a region associated to severity in males.

To validate the loci, a two-point analysis (F statistics, ANOVA) was performed (see supplementary Table 2).

Quantitative trait Loci associated with latencies of cortical motor evoked potential

In the light of the mismatch between severity and demyelination in the beginning of the disease we sought to determine a direct functional measure as provided by cortical motor evoked potentials (cMEP).

The cMEP consist on a biphasic wave following the stimulus at a mean latency of 2.66 ms for forelimb and 3.95 ms for hindlimb (Table 2). Interestingly we observed differences in cMEP latencies between non-immunized parental strains (Table 2, \( p < 0.05 \)) indicating that susceptible mice already have greater latencies in both, hindlimb and forelimb. Following disease induction in the F2 generation, there was a considerable prolongation of latencies (Table 2, Figure 2). A representative comparison between cMEP measured in a sick mouse at different time points during the disease is shown in figure 2. As obvious, disease progression leads to a progressive delay in the cMEP onset accompanied by a scattering of the cMEP.

We choose the latency of cMEP as a trait since it appeared to be the most reproducible and reliable parameter for both, myelin disturbance and functional impairment. By doing this, we identified three EAE loci associated to this trait (Table 1, Figure 1). A suggestive evidence of linkage on chromosome 1 (EAE 30) to marker D1Mit303 associated to the latency of cMEP 15 days after immunization (latency at day 15 recorded in the forelimb, LOD score of 3.4). The linkage became
statistically significant only when severity as a covariance was used in the analysis (LOD 3.7, Figure 1e).

A linkage on chromosome 8 (EAE 31), showed a high significance (peak marker D8Mit178, LOD score of 6.99). Surprisingly the locus influenced the latency of cMEP in the forelimb before immunization. A third locus on chromosome 15 (marker D15Mit67, LOD score 4.5) identified as EAE 32 influenced the latency of cMEP 15 days after immunization. In this case we use the difference in the latencies between days 0 (before immunization) and 15 as a trait and we recorded the cMEP in the hindlimb. The same locus linked to disease severity in males.

There is no overlap between QTL controlling histopathological changes and QTL controlling cMEP latencies.

A decrease of the incidence in mice used for the electrophysiology (42 %) comparing to the rest of F2 mice (58 %) was observed ($\chi^2 = 15.01 > 3.84$) but disease severity and onset were not affected by the neurological procedure. The mean disease severity score in mice for electrophysiology was $2.72 \pm 1.50$ which did not differ from the whole group, $2.67 \pm 1.29$ ($p = 0.8$). The mean onset values in the electrophysiological group and the whole group were $13.31 \pm 3.19$ days and $12.98 \pm 3.22$ days, respectively ($p = 0.8$). To validate the loci, a two-point analysis (F statistics, ANOVA) was performed (see supplementary Table 2).

**Association and correlation between QTLs influencing cortical motor evoked potentials and incidence, disease onset, disease severity and cell infiltration**

To explore the relevance of cMEP controlling alleles we investigated their effect i.e their relationship to incidence onset and severity of disease, respectively as well as to cell infiltration.

EAE 30 influenced the latency of cMEP at day 15 as recorded in the forelimb and was associated to the incidence of the disease since sick mice at day 15 showed longer conduction times than healthy mice ($p = 0.02$) (see Table 3 - 4, supplementary Table 1 and supplementary Figure 1). Additionally mice with severe disease had longer latencies than healthy mice ($p = 0.009$). When we divided the mice in groups depending on their alleles at this locus it was evident that the SJL allele is the major
contributor to this association. In this case mice with both mild and severe disease show differences with respect to healthy mice ($p = 0.036$, $p < 0.001$ respectively). Furthermore mice with the SJL allele at this locus with B-lymphocyte infiltration in their spinal cords show slower conduction times than mice without infiltration ($p < 0.001$) (see supplementary Figure 2). EAE 31 linked to the forelimb cMEP latencies before immunization. This is reflected in the significant differences between the B10S and the SJL (D8Mit178) mice ($p < 0.001$). Mice with the SJL allele had longer latencies than mice with the B10S allele. Early onset associates with longer latencies ($p = 0.018$). However, there was no association between the latencies before immunization and incidence or severity of the disease.

EAE 32 linked to the latency of cMEP in the hindlimb assessed by using the difference between the latencies at days 0 and 15 as a phenotype. The association of this trait with incidence of disease was very strong ($p < 0.001$) and was independent on the allele carried by the mice (see Table 3 and 4). We also observed differences in the latencies of cMEP between healthy mice with respect to mice suffering from mild disease and mice having severe disease (see Table 4). There is an association with severity showing that mice with severe disease had longer conduction times than mice presenting mild symptoms ($p = 0.006$). Regression analysis (Table 3) showed a good correlation between disease severity in mice with the B10S allele in this locus and latency of cMEP ($R = 0.87$ for B10S allele homozygous mice and $R = 0.77$ for mice with at least one copy of the B10S allele). There is also an association with B-lymphocyte infiltration in spinal cords since the mice with infiltration showed slower conduction times (measured as a difference between day 0 and day 15) than mice without infiltration. Again mice with at least one copy of the B10S allele at D15Mit67 locus and B-lymphocytes infiltration have longer conduction times than mice without B lymphocyte infiltration (see supplementary Figure 2).
Discussion

The most important finding of this study is the identification of 3 new QTLs controlling the latency of cMEPs, a phenotype that functionally reflects the disease activity and provides quantitative data additional to the semi quantitative clinical severity data.

EAE 30 on chromosome 1, cM 11-32, controlled the latency of cMEP as recorded in the forelimb. The linkage was disease dependent as significance was detected when using disease as a covariate in the analysis. This is concordant with the observation that the latency of forelimb cMEP was enhanced in diseased mice compared to healthy mice, with the SJL/J allele as the contributing allele. EAE 30 partially overlaps with an earlier described locus (Tmevd 6), which controls severity in Theiler’s murine encephalomyelitis, a virus-induced demyelinating disease (12-14). It also overlaps with the Idd5a locus identified in the NOD mice as predisposing to Diabetes (15) where there is strong evidence that CTLA4 and/or ICOS (16) are the likely susceptibility gene for this locus.

EAE 31 locus on chromosome 8 cM 32-48 controlled the latency of cMEPs before immunization. As shown for the EAE 30 the contributing allele originated from the susceptible strain, SJL/J, and had a dominant effect. A longer latency of cMEPs (measured in the forelimb) before immunization also correlates significantly with an earlier onset of the disease ($p < 0.001$). This suggests that at least one gene affecting axon structure, myelin composition or synapse transmission may predispose susceptible mice to an early onset of the disease. EAE 31 partially overlaps with the EAE 14 locus previously linked to incidence (9) of the disease and to demyelination (17). EAE 31 maps to a region that also contains several genes of putative relevance. One of those genes, the calcium channel alpha1 subunit gene (CACNA1a), encodes the alpha subunit of a P-type calcium channel (18-20). CACNA1a is associated with neurological symptoms in mice, like ataxia. Furthermore, it is polymorphic and earlier studies show that it is differentially expressed during the course of EAE (21). Another interesting, but less likely contributing gene, Caspase 3, is involved in apoptotic death of different neuronal cells (22-24) where apoptosis is one of the pathways that leads to demyelination (25). Genes like the Janus kinase 3 (JAK3) and Carboxypeptidase E (CPE) are
also putative contributors, although these genes cannot be linked to the phenotype studied. However both genes mapped to this interval are differentially expressed during the disease (26) and are polymorphic genes (27-30). CPE is a secretory granule enzyme involved in dibasic cleavage of pro-proteins and prohormones (31) whereas JAK3 is a tyrosine kinase involved in signal transduction processes (32).

The association between the susceptibility allele on chromosome 8 with a slower conduction and with an earlier onset of disease could have an important meaning because such a predictive allele could be used to screen populations at high risk of developing multiple sclerosis by the non invasive evoked potential measurements. This finding is supported by the fact that parental strain differences in cMEP latencies are already observed before immunization and are likely to be related to different myelin composition. Animals with attenuated biochemical properties of the myelin sheath may therefore be more prone to develop early EAE. The differences in cMEP measurements could unravel patients that show distinct alteration of the myelin sheath and it might therefore be a tool for either treatment monitoring or even risk assessment.

EAE 32 on chromosome 15 controlled the latency of cMEPs measured in the hindlimb (LOD score 4.5). Interestingly the C57BL/10.S allele was responsible for the delay of cMEPs after 15 days and had a dominant effect. The delay of the cMEPs correlated with the incidence disease severity and B lymphocyte infiltration. A candidate gene in this locus is the calcium channel gamma subunit gene (CACNG2), which encodes stargazing, a transmembrane protein, which may act as both neuronal voltage-dependent calcium channel gamma subunit and AMPA receptor regulatory proteins (TARPs) and is highly expressed in cerebellum, cerebral cortex, hippocampus and thalamus. CACNG2 is differentially expressed during EAE (21) and it has been associated with neurological disorders (33). Another interesting gene in this region is peripherin, an intermediate filament protein, which is upregulated in inflammatory processes and leads to a degeneration of motor axons in amyotrophic lateral sclerosis (ALS) (34).

Our morphological analysis of the spinal cords of the F2 population did not reveal obvious differences in the myelination pattern, however, already minor changes in the biochemical
composition of the isolating myelin sheath is known to influence the electrophysiological properties of spinal cord tracts. These biochemical changes may be explained by the inflammation produced by the cell infiltration since our study reveals an association between the cMEP latencies fifteen days post immunization with the B-lymphocyte infiltration. (supplementary Figure 2)

Additionally to the cMEP QTLs, we identified new QTLs controlling traits like disease severity, disease onset and cell infiltration.

Two new QTLs on chromosomes 2 (D2Mit32) and 10 (D10Mit271) were associated with severity in females (Table 1). EAE 34 is a new QTL that although it partially overlaps with EAE 17, which has previously been shown to be associated with disease severity and spinal cord demyelination in females (9). EAE 33 on chromosome 2 represents a new locus that is mapped to the distal p-fragment of the chromosome. This appears to be a common QTL for other autoimmune diseases e.g. collagen induced arthritis (CIA2) (35). There is evidence suggesting that the complement C5 is the probable susceptibility gene in this area as many strains showing linkage to arthritis are C5-deficient due to a two base pair mutation. However, there are no known polymorphisms or mutations in the C5 gene in C57Bl/10 or SJL/J. This observation and additional evidence from arthritis linkage analysis studies suggest that other genes in this locus might be involved. On chromosome 18 we identified a suggestive QTL (EAE 35). This locus overlaps with the EAE 25, a QTL previously described contributing to spinal cord lesions in EAE (17).

Onset of the disease was linked to D10Mit233, suggesting that the same QTL may influence severity and onset of the disease in our F2 study. Several genes of importance are mapped in this region like interferon gamma, a determinant factor of disease (36).

Furthermore, we describe two QTLs controlling for the infiltration of microglia and B-lymphocytes in a distal position on chromosome 15. The presence of both cell types is a sign of spinal cord inflammation and both cell types probably target myelin sheath epitopes. Recently it was show that microglia contact myelin membranes before the onset of demyelination suggesting that this contact could lead to a later demyelination and inflammation. (37). Indeed, microglia is the major effectors cell in the CNS and cytokines like IFN-gamma and TNF activate microglia into migratory and
phagocytic cells. However there is also evidence for a role of extracellular matrix proteins as a regulator or modulator proteins of microglia activity probably by increasing the local accessibility of fibronectin due to different pathological conditions in the central nervous system, like MS, (38) and this in turn induces microglia activation and expression of integrins like alpha-4-beta1 and alpha-5-beta1 integrin (37). Integrin alpha-5 (fibronectin receptor-alpha-5) maps at this region on chromosome 15. The fact that this region also associates to the severity in males supports the suggested role of this area in the development of the disease.

In conclusion the cMEP-associated QTLs correlated with incidence, onset or severity of disease and did not overlap with QTL controlling cell infiltration, suggesting a possible role of genetic control of basic myelin composition or patterns and synaptic structure mediated by polymorphic genes on chromosomes 1, 8 and 15 in susceptibility to EAE. In addition our data provide evidence for the view that not the entire functional impairment is reflected by observable morphological changes on light or electron microscopy level but rather strengthens the reliability of our new strategy to choose cMEPs as a trait for QTL analysis in diseases involving demyelination.

Further strategies must be done to confirm the role of these genes in EAE. Search for polymorphism in the candidate genes by sequencing, congenic or subcongenic strains generation for fine mapping, gene expression profiling in these QTL, comparative genomics or functional analyses by knock out or transgenesis are some of theses strategies that we are developing to point out to relevant genes in the disease.
Material and Methods

Mice, immunization and score of the disease. The mice used in this study were obtained from the Jackson Laboratory and were kept under standard laboratory conditions. The local state's Animal Care Committee previously approved all experimental procedures in agreement with the European Communities Council Directive of November 24, 1986 (86/609/EEC). We inoculate the disease according to established protocols (7). In brief, SJL/J, C57BL/10.S, (SJL/J×C57BL/10.S) F1 and 400 (SJL/J×C57BL/10.S) F2 mice were immunized at 8-12 weeks of age at the base of the tail with 100 µg of PLP139-151 (American Peptide Company, Sunnyvale, California, USA) dissolved in water and mixed with an equal volume (50 µl) of CFA (IFA with 4 mg/ml Mycobacterium tuberculosis; DIFCO Laboratories Detroit, USA). 200 ng of Bordetella pertussis toxin (SIGMA, Saint Louis, Missouri, USA) were injected to each mouse the day of immunization and 48 hours later. Mice were followed for 4 weeks post immunization. The clinical scoring (severity) of EAE commenced 8 days after immunization and animals were monitored daily according to the current protocol: 0 normal, 1 flaccid tail, 2 waddle, 3 moderate paraparesis, 4 severe paraparesis, 5 tetraparesis. We analyzed the severity as the maximal score observed in each individual mouse or the area under the curve (AUC), a continuous trait that measure the accumulative severity from the disease onset until the day of sacrifice. The day of onset was also analyzed as a quantitative trait.

CMEP recordings. We used a total of 15 mice of each parental strain (SJL/J and C57BL/10.S) and 125 (SJL/J×C57BL/10.S) F2 mice for electrophysiological analysis. The F2 mice were randomly chosen and divided into five groups. Ten days proceeding immunization we anaesthetized the 125 F2 mice for surgery by intraperitoneal injection of Avertin (1.7 mg/g), an anesthetic composed of 2,2,2-Tribromoethanol (SIGMA, Saint Louis, Missouri, USA) and 2-methyl-2-butanol (SIGMA, Saint Louis, Missouri, USA). Subsequently, we placed the mice in a conventional stereotactic head frame and we inserted two stainless steel screws (AgnTho’s, Lidingö, Sweden) 1.5 mm right of the midline and 1.5 mm behind the bregma suture with the tip just above the pia mater (anode) and on the nasal bone (cathode) under sterile conditions (39). At days 0, 10 and 15 post immunization we
anaesthetized the mice and we elicited cMEP by constant current anodal square wave pulses of 150 µs duration and a frequency of 0.2 Hz applied to the brain surface of the motor cortex by a stimulator (A320, World Precision Instruments, Berlin, Germany) triggered by LabView-based software (National Instruments, Austin, TX, USA). Intensity was adjusted to 1.5 times the level inducing threshold contraction (40). We recorded the electromyographic response by needle electrodes (Medtronic Functional Diagnostics, Skovlunde, Denmark) positioned in the small muscles of the forelimb and the hindlimb proximal to the elbow (12) against a reference located at the dorsum pedis of the paw. We also placed a ground electrode subcutaneously between stimulating and recording electrodes. Three recording sessions were performed over a period of 15 days. Five to 10 traces for each time point and stimulation were recorded and the shortest onset latency of the first deflection latency was taken as a measure of the conduction time of cMEP. We filtered the data with a band pass between 30 Hz and 1.3 kHz and amplified with a gain of 1V/mV by an EXT-10C amplifier (npi electronics, Tamm, Germany). Further digitalizing (at 10 kHz) and analysis were done using LabView-based software (National Instruments) on a standard PC computer.

**DNA isolation and genotyping.** The genomic DNAs used for genotyping the mice were isolated from a 1 cm tail clip by using standard isolation protocols (41). We genotyped by PCR amplification all F2 generation mice we used 150 informative microsatellite markers covering the genome to the extent of 98 % of the genome at approximately 10 cM inter-marker distance. The mean inter-marker distance ranged from 5.5 to 21.5 cM for the different chromosomes. The accuracy of our loci order and interval maps was verified by comparing the genetic map calculated from our data with the Mouse Genome Informatics map (www.jax.org). The protocol for the genotyping was the following: Genomic DNA (20 ng) was amplified in a final volume of 10 µl containing Hot Start Taq polymerase (0.25 U) (QIAGEN, Hilden, Germany), primers (0.1 µM each) (Metabion GmbH, Planegg-Martinsried, Germany), 50 mM KCl, 10 mM Tris, 2.5 mM MgCl₂, 0.25 mM dNTP, and 0.02 µM M13-IRD700 or M13-IRD800 (LI-COR, Lincoln, Nebraska, USA).
Amplification conditions were as follows: 95 °C for 10 min, followed by 2 cycles of 94 °C for 30 s, 59 °C for 1 min, 72 °C for 1 min, 2 cycles of 94 °C for 30 s, 57 °C for 1 min, 72 °C for 1 min, then another 35 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 7 min. The reactions were performed using GeneAmp PCR System 9700 cycler (Applied Biosystems, Inc., Foster City, California, USA). The PCR products were resolved on denaturing polyacrylamide gels and were detected by using a LI-COR Model 4200L automated DNA sequencer (LI-COR, Inc., Lincoln, Nebraska, USA). The genotypes were scored independently by at least two people using the Saga software supplied by LI-COR.

**Morphology.** Spinal cord tissue was arranged in two tissue micro arrays (TMA). TMAs were constructed as recently described (42). Shortly, multiple spinal cord sections of each animal were poured into a paraffin block. Spinal cord pieces in a TMA were arranged in a TMA with 200 punch holes of 1.3 mm each was established. Spinal cord tissue cylinders of the mice were transferred from the initial block to the TMA using a puncher. 4 µm thick sections were then transferred to epoxyd resin covered slides and pre incubated in Xylol for 2 hours prior to the staining procedure. Slides were conventional stained with a Luxol-Nissl stain to highlight myelin irregularities. A Ventana machine with the appropriate pretreatment (0.1 M Citrate or 1 mM EDTA) was used for immunohistochemical determination of damaged and regenerated axons, irregular myelin, demyelination and cell infiltration. Antibodies against amyloid precursor protein (APP) (Boehringer, Mannheim, Germany), myelin basic protein (MBP) (DAKO, Carpinteria, California, USA), neurofilament (NF200) (Sigma, Saint Louis, Missouri, USA), IBA1 (Wako Chemicals GmbH, Neuss, Germany), CD3 (LabVision, Freemont, California, USA) and B220 (CD45R) (BDBioscences, Heidelberg, Germany) were used; Slides were developed using the Ventana DAB MAP kit or the DAKO iView kit.

The staining was evaluated double blind by establishing a 1 to 5 point evaluation scale for the different staining intensities (Luxol-Nissl, APP, MBP, and NF200). Staining intensity in control
spinal cords was used as a reference. The number of CD3 and B220 positive cells was counted on a representative spinal cord diameter of 1.3mm.

**Linkage analysis.** All linkage analyses have been performed using the imputation model in the R/qtl software package (43, 44). The order of the loci was obtained from the mouse genome informatics database of the Jackson Laboratory ([http://www.informatics.jax.org](http://www.informatics.jax.org)). EAE severity and onset of the disease, latencies of cortical motor evoked potentials (cMEP) and spinal cord cell infiltration were taken as phenotypes. Continuous values were checked for normal distribution using QTL Cartographer Software and logarithmic values were used when necessary. For the significant and suggestive linkage threshold values, we have followed the guidelines for the permutation test of data (number of permutations = 1000) and significance level 95 % (p = 0.05) was used to determine linkage Intervals containing significant evidence of linkage were reanalyzed by using Analyse-it software ([http://www.analyse-it.com](http://www.analyse-it.com)) testing the association between marker and phenotype using analysis of variation (ANOVA).

**Statistics.** Results are expressed as mean value ± Standard Deviation (SD) or Standard Error of the Mean (SEM) where appropriate. A p-value < 0.05 was considered significant. Linear regression and ANOVA were used to test the correlation or association between the latencies of cortical motor evoked potentials and the onset, severity incidence of the disease and cell infiltration.
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References


Figure Legends

**Fig. 1 QTL identified in this study**

QTL graphics showing the linkage between the different traits and the corresponding microsatellite marker. LOD score values represent the values for which each association became statistically significant. A. EAE33 QTL associated to disease severity in females. B. EAE34 QTL linked to disease onset in females. C. EAE32 QTL associated to latency of cMEP in hindlimb (HLCMEP) 15 days after immunization (trait study as a difference (Δ) between the latency at day 15 and the latency at day 0 for each mouse) D. EAE31 QTL associated to latency of cMEP measured in the forelimb (FLCMEP) before immunization (day 0) E. EAE30 QTL associated to latency of cMEP measured in the forelimb 15 days post immunization. Full lines represent the association graphic while segment lines represent the association graphic using the severity as a covariant. F. EAE37 QTL linked to microglia infiltration in spinal cord. G. EAE36 QTL linked to T-cell infiltration in spinal cord.

**Fig. 2 CMEP latencies - changes during the course of disease.**

Representative traces of cMEP recorded in A. the hindlimb and B. the forelimb before immunization (grey), 10 days (red) and 15 days post immunization (green) in a sick mouse. CMEP are overlayed for clarity. Arrows indicate the time point of stimulation. Note the progressive increment in latencies and the decomposition with time post immunization. C and D. Effect of sex and disease on the forelimb (C) and on the hindlimb cMEP latencies (D). Black bars represent the mean of the values of the F2 male population at different time points (days 0, 10 and 15) while white bars represent the mean values of the F2 female mice. Significant differences in the latencies of forelimb and hindlimb cMEP were observed in both groups, males and females, during the course of disease.

**Fig. 3 Comparison between published EAE loci and those QTL identified in this study**

Red lines correspond to the 28 QTL described in the literature while green lines represent the QTL described in our study.
Fig. 4 Morphological analysis of spinal cord cylinders arranged in tissue microarrays (TMA)

Subset a. shows A. Hematoxilin-Eosin stain (H&E) of a representative spinal cord cylinder of 1.3mm diameter B. B lymphocytes infiltration (B220, 20x)) C. T lymphocytes infiltration (CD3, 20x) D, E, F. Low microglia infiltration (IBA-1) G, H, I. High microglia infiltration (IBA-1) (magnifications D, E 1x; E, F 5x; F, I 40x)

Subset b. represents myelination properties in A, B. stain of the myelin basic protein (MBP) stain and number of axons in C, D. neurofilament (NF200) axonal stain (magnifications A, B 1x; B, D 10x; left white matter, right grey matter) Myelin and axonal stains revealed no significant individual differences between the mice.
Table 1. Summary of the EAE linked quantitative trait loci identified in this study.

<table>
<thead>
<tr>
<th>QTL</th>
<th>Chr</th>
<th>Flanking markers</th>
<th>Effect marker</th>
<th>Allele</th>
<th>Peak (cM)</th>
<th>C.I (cM)</th>
<th>LOD Score</th>
<th>LOD Score (s.level, p=0.05)</th>
<th>Trait</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAE30</td>
<td>1</td>
<td>D1Mit380 D1Mit236</td>
<td>D1Mit303*</td>
<td>SJL/J</td>
<td>22</td>
<td>11-32</td>
<td>3.4*</td>
<td>4.7*</td>
<td>3.5</td>
<td>Forelimb cMEP latency day 15</td>
</tr>
<tr>
<td>EAE31</td>
<td>8</td>
<td>D8Mit24 D8Mit88</td>
<td>D8Mit258 D8Mit259 D8Mit178 D8Mit15 D15Mit67 D15Mit171</td>
<td>SJL/J</td>
<td>42</td>
<td>32-48</td>
<td>6.9</td>
<td>3.7</td>
<td></td>
<td>Forelimb cMEP latency day 0</td>
</tr>
<tr>
<td>EAE32</td>
<td>15</td>
<td>D15Mit126 D15Mit35</td>
<td>C57BL/10.S</td>
<td>38</td>
<td>24-56</td>
<td>4.5</td>
<td>3.8</td>
<td></td>
<td>Hindlimb cMEP latency, difference day 15-day 0</td>
<td>New</td>
</tr>
<tr>
<td>EAE33</td>
<td>2</td>
<td>D2Mit11 D2Mit32</td>
<td>C57BL/10.S</td>
<td>20</td>
<td>6-30</td>
<td>3.7</td>
<td>3.6</td>
<td></td>
<td>Severity females</td>
<td>New</td>
</tr>
<tr>
<td>EAE34</td>
<td>10</td>
<td>D10Mit2 D10Mit233</td>
<td>C57BL/10.S</td>
<td>56</td>
<td>42-58</td>
<td>3.8</td>
<td>3.6</td>
<td></td>
<td>Severity females</td>
<td>New, partially overlaps with EAE17 Blankenhorn et al (1999)</td>
</tr>
<tr>
<td>EAE35</td>
<td>18</td>
<td>D18Mit186.1 D18Mit144.1*</td>
<td>C57BL/10.S</td>
<td>68</td>
<td>54-69</td>
<td>3.26*</td>
<td>3.6</td>
<td></td>
<td>Severity females</td>
<td>Overlaps with EAE25, Blankenhorn et al (1999)</td>
</tr>
<tr>
<td>EAE36</td>
<td>8</td>
<td>D8Mit124 D8Mit178</td>
<td>D8Mit258 D8Mit259</td>
<td>SJL/J</td>
<td>26</td>
<td>2-34</td>
<td>4.1</td>
<td>3.6</td>
<td></td>
<td>T-lymphocytes infiltration in spinal cord</td>
</tr>
<tr>
<td>EAE37</td>
<td>15</td>
<td>D15Mit171</td>
<td>D15Mit35</td>
<td>C57BL/10.S</td>
<td>69</td>
<td>58-69</td>
<td>3.6</td>
<td>3.5</td>
<td></td>
<td>Microglia infiltration in spinal cord</td>
</tr>
<tr>
<td>EAE38</td>
<td>15</td>
<td>D15Mit67.1 D15Mit35</td>
<td>C57BL/10.S</td>
<td>54</td>
<td>18-72</td>
<td>3.3*</td>
<td>3.6</td>
<td></td>
<td>AUC males B-lymphocytes infiltration in spinal cord</td>
<td>New</td>
</tr>
</tbody>
</table>

AUC: area under the curve, C.I: Confidence Intervals, LOD score s.level: significance level 95 % (threshold values calculated based on 1000 permutations), *suggestive locus, ¹ sex influence, ² severity influence
Table 2. Comparison of cMEP latencies between parental strains and F2 generation

<table>
<thead>
<tr>
<th>Strain</th>
<th>Days post inoculation</th>
<th>All mice forelimb (ms)</th>
<th>All mice hindlimb (ms)</th>
<th>Females forelimb (ms)</th>
<th>Male forelimb (ms)</th>
<th>Female hindlimb (ms)</th>
<th>Male hindlimb (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/10.S</td>
<td>0</td>
<td>2.57 ± 0.17</td>
<td>3.90 ± 0.14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SJL/J</td>
<td>0</td>
<td>2.71 ± 0.12</td>
<td>4.03 ± 0.14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2 generation (SJL/J×C57BL/10.S)</td>
<td>0</td>
<td>2.66 ± 0.27</td>
<td>3.96 ± 0.12</td>
<td>2.63 ± 0.28</td>
<td>2.72 ± 0.41</td>
<td>3.88 ± 0.54</td>
<td>4.01 ± 0.54</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.92 ± 0.32</td>
<td>4.13 ± 0.43</td>
<td>2.91 ± 0.33</td>
<td>2.99 ± 0.32</td>
<td>4.01 ± 0.43</td>
<td>4.27 ± 0.60</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>3.00 ± 0.34</td>
<td>4.26 ± 0.34</td>
<td>2.95 ± 0.34</td>
<td>3.09 ± 0.34</td>
<td>4.26 ± 0.34</td>
<td>4.55 ± 0.60</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD

1. Significant differences between the strains P<0.05
2. Significant differences between days 0 and 10 postimmunization P<0.05
3. Significant differences between days 0 and 15 postimmunization P<0.05
4. Significant differences between days 10 and 15 postimmunization P<0.05
5. Significant differences between latencies (day 10-day 0) and latencies (day 15-day 0) P<0.05
Table 3. Linear regression correlation between latencies of cMEP with severity and onset

<table>
<thead>
<tr>
<th>QTL</th>
<th>Trait</th>
<th>Locus</th>
<th>Genotype in locus (allele)</th>
<th>Severity *</th>
<th>Onset *</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAE30</td>
<td>Latency of forelimb cMEP 15 days post immunization</td>
<td>D1Mit303</td>
<td>All mice</td>
<td>C57BL/10.S Heterocygous SJL/J</td>
<td>R= 0.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R= 0.19</td>
<td>R= 0.01</td>
</tr>
<tr>
<td>EAE31</td>
<td>Latency of forelimb cMEP before immunization</td>
<td>D8Mit178</td>
<td>All mice</td>
<td>C57BL/10.S Heterocygous SJL/J</td>
<td>R= -0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R= -0.1</td>
<td>R= -0.198</td>
</tr>
<tr>
<td>EAE32</td>
<td>Latency of hindlimb cMEP day 15 (day 15 - day0)</td>
<td>D15Mit67</td>
<td>All mice</td>
<td>C57BL/10.S Heterocygous SJL/J</td>
<td>R= 0.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R= 0.87</td>
<td>R= 0.76</td>
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</tbody>
</table>

* Correlation
<table>
<thead>
<tr>
<th>QTL</th>
<th>Linkage marker</th>
<th>Effect allele</th>
<th>Sex Effect</th>
<th>Genotypes in locus (allele)</th>
<th>Disease association</th>
<th>Severity association</th>
<th>Onset association</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAE30</td>
<td>D1Mit303*</td>
<td>SJL/J</td>
<td>No</td>
<td>All mice</td>
<td>p=0.02</td>
<td>No</td>
<td>No</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C57BL/10.S</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Heterozygous</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SJL/J</td>
<td></td>
<td></td>
<td>p&lt;0.001</td>
<td>p=0.036 (healthy-mild)</td>
<td>No</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>p&lt;0.001 (healthy-sev)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p=0.003 (healthy-sev)</td>
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<td></td>
<td>p=0.003 (mild-sev)</td>
<td>No</td>
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<td></td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>EAE31</td>
<td>D8Mit178</td>
<td>SJL/J</td>
<td>No</td>
<td>All mice</td>
<td>No</td>
<td>No</td>
<td>p&lt;0.001 (early-late)</td>
</tr>
<tr>
<td></td>
<td>(p=0.001)</td>
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<td></td>
<td>C57BL/10.S</td>
<td>No</td>
<td>No</td>
<td>No (early-late)</td>
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<td>(p=0.001)</td>
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<td>Heterozygous</td>
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<td>No</td>
<td>No</td>
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<tr>
<td></td>
<td>(p=0.05)</td>
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<td></td>
<td>SJL/J</td>
<td>No</td>
<td>No</td>
<td>No (early-late)</td>
</tr>
<tr>
<td></td>
<td>Hetero-SJL/J</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p=0.03 (early-late)</td>
<td>No</td>
</tr>
<tr>
<td>EAE32</td>
<td>D15Mit67</td>
<td>C57BL/10.S</td>
<td>Yes</td>
<td>All mice</td>
<td>p&lt;0.001</td>
<td>p=0.008 (healthy-mild)</td>
<td>No</td>
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<tr>
<td></td>
<td>(p=0.008)</td>
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<td>C57BL/10.S</td>
<td>p=0.001</td>
<td>p=0.001 (healthy-mild)</td>
<td>No</td>
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<tr>
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<td>(p=0.046)</td>
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<td>Heterozygous</td>
<td>p=0.06</td>
<td>p=0.006 (mild-sev)</td>
<td>No</td>
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<td>Hetero-SJL/J</td>
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<tr>
<td></td>
<td>(p=0.03)</td>
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<td>SJL/J</td>
<td>p=0.003</td>
<td>p=0.001 (healthy-mild)</td>
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<tr>
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<td>p=0.03 (healthy-sev)</td>
<td>No</td>
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<td>No</td>
</tr>
</tbody>
</table>

*Statistical analysis by ANOVA
Fig. 1 QTLs identified in this study.
Fig. 2 CMEP latencies - changes during the course of disease
Fig. 3 Comparison between published EAE QTL and those QTL identified in this study.
Fig. 4 Morphological analyses of spinal cord cylinders arranged in tissue microarrays

Subset a.

Subset b.
**Abbreviations.**

cMEP: cortical motor evoked potential

QTL: quantitative trait loci

MS: multiple sclerosis

EAE: experimental autoimmune encephalomyelitis

AUC: area under the curve

FLCMEP: forelimb cortical motor evoked potential

HLCMEP: hindlimb cortical motor evoked potential