See related Commentary on page 2369**BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION**

Nerve Conduction Velocity Is Regulated by the Inositol Polyphosphate-4-Phosphatase II Gene

Susanne Lemcke,^{*} Susen Müller,^{*†} Steffen Möller,^{*} Arne Schillert,[‡] Andreas Ziegler,[‡] Sabine Cepok-Kauffeld,[§] Manuel Comabella,[¶] Xavier Montalban,[¶] Thomas Rüdlicke,^{||} Kutty Selva Nandakumar,^{**} Bernhard Hemmer,[§] Rikard Holmdahl,^{**} Jens Pahnke,^{†,††‡‡} and Saleh M. Ibrahim^{*}

From the Department of Dermatology, Venereology and Allergology,^{*} University of Lübeck, Lübeck, Germany; the Neurodegeneration Research Lab,[†] Department of Neurology, University of Magdeburg, Magdeburg, Germany; the Institute of Medical Biometry and Statistics,[‡] University of Lübeck, University Hospital Schleswig-Holstein, Campus Lübeck, Lübeck, Germany; the Department of Neurology,[§] University Hospital, Technical University of Munich, Munich, Germany; the Department of Neurology-Neuroimmunology,[¶] Multiple Sclerosis Center of Catalonia, University Hospital Vall d'Hebron, Barcelona, Spain; the Institute of Laboratory Animal Science,^{||} University of Veterinary Medicine, Vienna, Austria; the Medical Inflammation Research Division,^{**} Karolinska Institute, Stockholm, Sweden; the German Center for Neurodegenerative Diseases Magdeburg,^{††} Magdeburg, Germany; and the Department of Behavioral Neurology,^{‡‡} Leibniz Institute for Neurobiology, Magdeburg, Germany

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Address correspondence to
Susanne Lemcke, Ph.D.,
Department of Dermatology,
Venereology and Allergology,
University of Lübeck, Ratze-
burger Allee 160, 23538
Lübeck, Germany. E-mail:
susanne.lemcke@uksh.de.

Impairment of nerve conduction is common in neurodegenerative and neuroinflammatory diseases such as multiple sclerosis (MS), and measurement of evoked potentials (visual, motor, or sensory) has been widely used for diagnosis and recently also as a prognostic marker for MS. We used a classical genetic approach to identify novel genes controlling nerve conduction. First, we used quantitative trait mapping in F₂ progeny of B10/SJL mice to identify EAE31, a locus controlling latency of motor evoked potentials (MEPs) and clinical onset of experimental autoimmune encephalomyelitis. Then, by combining congenic mapping, *in silico* haplotype analyses, and comparative genomics we identified inositol polyphosphate-4-phosphatase, type II (*Inpp4b*) as the quantitative trait gene for EAE31. Sequence variants of *Inpp4b* (C/A, exon 13; A/C, exon 14) were identified as differing among multiple mouse strains and correlated with individual cortical MEP latency differences. To evaluate the functional relevance of the amino acid exchanges at positions S474R and H548P, we generated transgenic mice carrying the longer-latency allele (*Inpp4b*^{474R/548P}) in the C57BL/6J background. *Inpp4b*^{474R/548P} mice exhibited significantly longer cortical MEP latencies (4.5 ± 0.22 ms versus 3.7 ± 0.13 ms; $P = 1.04 \times 10^{-9}$), indicating that INPP4B regulates nerve conduction velocity. An association of an *INPP4B* polymorphism (rs13102150) with MS was observed in German and Spanish MS cohorts (3676 controls and 911 cases) ($P = 8.8 \times 10^{-3}$). (*Am J Pathol* 2014, 184: 2420–2429; <http://dx.doi.org/10.1016/j.ajpath.2014.05.021>)

Reduced nerve conduction velocity resulting from, for example, axonopathy or demyelination strongly affects quality of life. For neurodegenerative and neuroinflammatory diseases such as multiple sclerosis (MS), impairment of nerve conduction (measured by visual, motor, and somatosensory

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evoked potentials) is a common feature. The value of evoked potentials in establishing the diagnosis in MS has been addressed in several studies.^{1–3} Although measurement of visual evoked potentials is one of the most sensitive diagnostic tool in early MS,^{4,5} combined pathological somatosensory evoked potential and motor evoked potential (MEP) findings at first presentation best predict clinical disability after 5 years.⁶ This was confirmed by a recent study showing that multimodal evoked potentials correlate well with clinical disability in early MS and allow prediction of disease evolution.⁷ Interestingly, no defined pathway controlling MEPs has been identified to date.

A strong tool for investigating the pathophysiology of a complex disease is identification of the underlying genetic control. Linkage analyses in animal models may yield quantitative trait loci (QTLs), and the experimental autoimmune encephalomyelitis (EAE) as a rodent model of MS has been extensively studied. Genetic analyses led to the identification of more than 39 QTLs,^{8–15} which suggests the complex genetic basis for this disease. We elected to contribute to this effort by mapping genes that are not only linked to EAE but also control the latency of cortical MEPs (cMEPs) in F₂ progeny of a cross between the MHC-compatible susceptible SJL/J and resistant C57BL/10SnSg mouse strains.¹⁴ This led to the identification of three QTLs controlling the latency of cMEPs.

The central challenge for interpreting QTLs is that the genomic regions identified are generally rather large, and extensive work is required to positionally clone the quantitative trait gene (QTG) involved. So far, despite the introduction of novel technologies such as large-scale sequencing, gene expression profiling, and advanced bioinformatics,^{16,17} only a few QTGs have been identified.^{18–22}

Our aim in this study was to identify the QTG for EAE31 associated with disease onset and forelimb cMEP latency.¹⁴ This locus partially overlaps with EAE17,⁹ a QTL involved in disease severity and spinal cord injury. The allele conferring increased forelimb cMEP latency with a dominant mode of inheritance is derived from the EAE-susceptible SJL/J mice strain. Using congenic mice, high-resolution mapping, and *in silico* strategies combined with haplotype analyses and comparative genomics, we identified *Inpp4b*, a Mg²⁺-independent phosphatase involved in the phosphoinositide 3-kinase signaling pathway,²³ as the gene controlling the latency of cMEP. We also demonstrated an association of *INPP4B* with increased risk of developing MS.

Materials and Methods

Mouse Strains

The mice used for electrophysiological measurements and for sequencing of *Inpp4b* were maintained under standard laboratory conditions. The local state's Animal Care Committee approved all experimental procedures in agreement with the European Communities Council Directive of November 24,

1986 (86/609/EEC). Mice of strains C3H/HeJ, C57BL/6J, C57BL/10S, DBA/1J, YBR/EiJ, NZB/J, FVB/NJ, and SJL/J were obtained from Charles River Laboratories International (Wilmington, MA), Harlan Winkelmann (Borchen, Germany), and the Jackson Laboratory (Bar Harbor, ME).

Generation of Congenic Strains

Eight congenic mouse strains were generated to fine-map the QTL EAE31 on chromosome 8. Three microsatellite markers were chosen to genotype the 20-cM region: D8Mit258 at 23 cM, D8Mit178 at 33 cM and D8Mit267 at 43 cM. After backcrossing for five generations of the B10/SJL F₂ (C57BL/10SnSg × SJL/J) population into the SJL/J background, mice positive (homozygous) for the microsatellite markers were selected for analysis. To keep the variability in the QTL region while reducing it to the SJL/J background, mice with less heterozygous content in the non-QTL region were chosen and backcrossed into the SJL/J strain. Heterozygous content was determined by genetic markers placed outside the QTL region, chosen by combining systematic and random selection.

Recording of cMEPs

To apply the cMEPs, mice of different strains were anesthetized by intraperitoneal injection of 2.5% 1.7 mg/g of an avertin formulation (2,2,2-tribromoethanol–amylene hydrate) and were placed in a conventional stereotactic head frame for insertion under sterile conditions of 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), as described previously.¹⁴ The cMEPs were elicited by constant current anodal square-wave pulses of 150 μ s 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) in anesthetized mice. Intensity was adjusted to 1.5 times the level inducing threshold contraction. We recorded the electromyographic response with needle electrodes (Medtronic Functional Diagnostics, Skovlunde, Denmark) positioned in the small muscles of the forelimb and the hindlimb proximal to the elbow against a reference located at the dorsum pedis of the paw. A ground electrode was placed subcutaneously between stimulating and recording electrodes. For a recording session, 5 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 bandpass between 30 Hz and 1.3 kHz and amplified with a gain of 1 V/mV by an EXT-10C amplifier (npi electronic, Tamm, Germany). Further digitalizing (at 10 kHz) and analysis were performed using LabView-based software (National Instruments).

Morphology

Tissue preparation, staining, and digitizing were performed as described previously.^{24–29} For tissue preparation, mice were sacrificed by cervical dislocation and transcardially perfused first with phosphate-buffered saline and then with buffered 4% paraformaldehyde. The brain was removed and stored in buffered 4% paraformaldehyde for paraffin embedding. Spinal cords were removed together with the embedding bone structures and postfixed in buffered 4% paraformaldehyde. After final fixation, spinal cord tissue was gently removed from the embedding bones. Brain and spinal cord samples were embedded in paraffin after adequate pretreatment. Spinal cords were manually arranged in a single tissue array. Sections (4 μ m thick) cut from the brains and spinal cord array were stained with conventional hematoxylin and eosin and Luxol fast blue eosin stain. Finally, whole tissue slides were digitized at 230-nm resolution using a Mirax Midi slide scanner (Carl Zeiss MicroImaging, Jena, Germany).

DNA Isolation and Genotyping

The genomic DNAs used for genotyping the mice were isolated from a tail biopsy according to standard isolation protocols.³⁰ The mice were genotyped by PCR with the microsatellite markers D8Mit259, D8Mit178, and D8Mit267 according to the protocol published by Mazón Peláez et al.¹⁴

RT-PCR

RNA from cerebrum, cerebellum, spinal cord, and skeletal muscle was extracted and cDNA was prepared by using 1 μ g RNA, 200 U SuperScript II Reverse Transcriptase (Life Technologies, Karlsruhe, Germany; Carlsbad, CA) and 0.5 μ mol/L hexamer primers (Sigma-ARK, Darmstadt, Germany) in a final volume of 25 μ L. The reaction was incubated at 42°C for 2 hours. For the detection of splice variants of the *Inpp4b* gene, the following primers were used: forward *Inpp4b*- α , - β 5'-GATTAAAGAAGGCCAGTTGC-3', *Inpp4b*- α reverse 5'-ACTTGGGGAAAGCCCATCAGC-3', *Inpp4b*- β reverse 5'-AGAAGTGTGCGAGAAGTTGGC-3', *Inpp4b*- α short forward (exon 2) 5'-AACCGGAGCTCAT-TATTGGTACCATTG-3', and *Inpp4b*- α short reverse (exon 6) 5'-GGCACAGCCCAGGAAGCTTC-3'. The PCR reactions were performed in a final volume of 22.5 μ L containing 2.5 U AmpliTaq Gold (Life Technologies), primers (0.4 pmol each), 50 mmol/L KCl, 10 mmol/L Tris, 1.5 mmol/L MgCl₂, and 0.8 mmol/L dNTP. Thermal cycling was performed with initial denaturation for 5 minutes at 94°C, followed by 35 cycles of denaturation for 30 seconds at 94°C, primer annealing for 30 seconds at 60°C for α and β and 64.5°C for α short, and synthesis for 1 minute at 72°C. A final primer extension was performed for 5 minutes at 72°C. Each amplicon was controlled for PCR yield and specificity by UV transillumination of ethidium bromide-stained agarose gels.

Generation of cDNA for Cloning

The full-length cDNA clone IRAVp968F08118D obtained by the German Resource Center for Genome Research [Deutsches Ressourcenzentrum für Genomforschung (RZPD); now Source BioScience imaGenes, Berlin, Germany] was used to amplify the coding DNA sequence region of the murine *Inpp4b* gene (NM_001024617). PCR was performed using the forward *Inpp4b*Nsi primer 5'-TATTATATGC-ATACCACCATGGAAATCAAAGAGGAAGGAACATC-AG-3' and the reverse *Inpp4b*Nsi primer 5'-TATTATAT-**GC**ATTTATTGGTATTTGGCTAAGAGTAATGC-3' containing an NsiI restriction site (in bold) for later cloning in the pJP3-Nsilon-DEL vector. In brief, 100 ng cDNA was amplified in a final volume of 50 μ L containing 2.5 U Stratagene Pfu-Turbo DNA polymerase (Agilent Technologies, Darmstadt, Germany; Santa Clara, CA), 250 ng of each forward and reverse primers (Eurofins MWG Operon, Ebersberg, Germany; Huntsville, AL), 50 mmol/L Tris-Cl, 0.1 mmol/L EDTA, 1 mmol/L dithiothreitol, and 2 mmol/L each dNTP. Amplification conditions were as follows: 95°C for 2 minutes, followed by 35 cycles of 95°C for 30 seconds, 66°C for 30 seconds, and 72°C for 3 minutes, with a final extension at 72°C for 10 minutes. The reactions were performed using an ABI Prism 2000 PCR cyclor (Life Technologies).

Mutagenesis of the *Inpp4b* Construct

The pJP3-Nsilon-DEL plasmid containing the complete coding DNA sequence of *Inpp4b* (NM_001024617) were used to insert single-nucleotide changes at gene positions 1421 (C/A) and 1642 (A/C). The mutagenesis was performed with a Stratagene QuikChange XL system (Agilent Technologies), using the following primers according to the manufacturer's protocol (SNP indicated in bold): *Inpp4b*-mut1 forward 5'-CCTTAAGAAGCCACCCTCTCCTAATGTCAGAACAG-AGGAGAAAAGTACTCAGC-3', *Inpp4b*-mut1 reverse 5'-GCTGAGTACTTTTCTCCTCTGTTCTGACATTAGGAG-AGGGTGGCTTCTTAAGG-3', *Inpp4b*-mut2 forward 5'-GGCAGCAGTAGCAAGGATGGAGAGGCAGACCCTA-CCCTGGAAGATTCCATCACATCTCACCC-3', and *Inpp4b*-mut2 reverse 5'-GGGTGAGATGTGATGGAAT-CTCCAGGGTAGGGTCTGCCTCTCCATCCTTGCTA-CTGCTGCC-3'.

Generation of Transgenic Mice

After sequence verification of the pJP3-Nsilon-DEL plasmid containing the mutated fragment, the *Inpp4b* coding DNA sequence was cloned into a mouse transformation vector under the control of the *Pmp* promoter.³¹ Before use for generation of transgenic mice (as described previously³²), plasmid DNA was digested with NotI and SalI heated for 5 minutes at 65°C, electrophoresed through a 1% agarose gel, and then purified using a QIAquick gel extraction kit (Qiagen, Hilden, Germany; Valencia, CA). The *Inpp4b* sequence with the introduced

mutations was microinjected into the pronucleus of zygotes of C57BL/6N mice, resulting in five founder lines. One was chosen for further analysis, designated as B6N-Tg(Pnp-*Inpp4b*)210Biat and backcrossed with C57BL/6J mice.

Analysis of *Inpp4b* Expression in Transgenic Mice

To determine the expression of the mutagenized *Inpp4b* gene in the transgenic mice, a new restriction site based on the mutation of AGC to AGA was used to distinguish between the wild-type and mutagenized gene. RNA was extracted from different tissues to generate cDNA. PCR was performed to amplify a gene fragment of 250 bp that includes the mutation, using the *Inpp4b* Hpy188I forward primer 5'-CTCTACACTGCAAGGCCAGG-3' and *Inpp4b* Hpy188I reverse primer 5'-TGCCTCCAGCACCTTCTTCG-3'. After the PCR, 1 µg of the product was restricted by using 1 U of Hpy188I (New England Biolabs, Frankfurt am Main, Germany; Ipswich, MA). The reaction was incubated at 37°C for 1 hour.

Association Study

For association analysis, genotypes of two independent MS case-control studies were investigated: a Spanish cohort with 349 cases and 362 controls and a German cohort with 562 cases and 3314 controls. All MS patients were classified according to the criteria of Poser et al.³³

Genomic DNA was extracted from whole blood using standard methods. The genotyping of the single-nucleotide polymorphism (SNP) rs13102150 was performed with a 5' exonuclease assay (TaqMan Assays-on-Demand; Life Technologies) according to standard protocols. All individuals participated in this study on the basis of informed consent. Genotype counts for rs13102150 for healthy control subjects from the Gutenberg Health Study were retrieved *in silico*. The present study was approved by the local ethics committee. All individuals under study gave informed consent.

Statistical Analysis

The *U*-test was used to test significance in differences found between strains in the measurements of cMEP latencies. Clinical scoring data were adjusted by the Student's *t*-test. A value of $P < 0.05$ was considered significant. Association with MS was tested using a logistic regression with an additive model. Pooling of the two stages was performed using inverse variance weighting.

Results

Fine-Mapping of EAE31

The EAE31 Locus Controls Latency of cMEP in Congenic Strains

To confirm the findings from the original F₂ screen, congenic and subcongenic mice for EAE31 were generated.

The QTL region was backcrossed onto susceptible SJL/J mice for five generations. The congenic fragment on chromosome 8 covered the region from 23 to 43 cM (46 to 110 Mb), including three microsatellite markers (D8Mit258, D8Mit178, and D8Mit267.1). Forty congenic mice were phenotyped for cMEP latencies before immunization in forelimbs and hindlimbs. Mice carrying the C57BL/10S alleles in these three loci showed faster conduction than mice carrying the SJL/J alleles (data not shown), confirming the results obtained using F₂ population (see data of Mazón Peláez et al.¹⁴). For further fine-mapping, subcongenic strains were generated by backcrossing for additional five generations onto the SJL/J background. The mice that expressed this phenotype had the congenic fragment spanning from 33 to 43 cM (70 to 110 Mb).

In Silico Fine-Mapping

After congenic mapping, the QTL region of EAE31 consisted of a 40-Mb region containing a substantial number of candidate genes. To further reduce the number of genes, we used a combination of haplotype analyses and comparative genomics (intergenomics) *in silico* strategies.

Comparative genomic analysis of the human MS locus (chromosome 4, 141.17 to 145.56 Mb)³⁴ and the rat susceptibility locus EAE8 (chromosome 19, 38.37 to 45.28 Mb)⁸ and mouse EAE31 identified a syntenic region among the three species (Supplemental Figure S1), so that the QTL region on chromosome 8 was significantly reduced, to a region of approximately 3 Mb (79.96 to 83.43 Mb) containing only 16 genes. To further reduce the fragment, we used *in silico* haplotype mapping by identifying inbred mouse strains carrying various haplotypes in this region. These strains were phenotyped for cMEP latencies before immunization (Figure 1) and were divided into two groups

Figure 1 cMEP latencies in different mouse strains. YBR/EiJ, SJL/J, DBA/1J, C3H/HeJ, and NZB/J mice exhibit larger cMEP latencies than C57BL/10S, FVB/NJ, and C57BL/6J mice in both forelimb (A) and hindlimb (B) Data are expressed as means $\pm$ SEM. $n = 15$ mice per strain. * $P < 0.05$.

Figure 2 Fine-mapping with haplotype analysis. Two groups of mice strains were defined by their cMEP latencies, and a haplotype analysis was performed. The FVB/NJ, C57BL/10S, and C57BL/6J strains (light gray) exhibit shorter latencies, and the C3H/HeJ, SJL/J, and DBA/1J (dark gray) strains exhibit longer latencies. The two haplotype groups of strains differ for the SNPs shown.

according to their nerve conduction velocity: one with faster conduction times (C57BL/10S, FVB/NJ, and C57BL/6J) and the other with slower conduction times (YBR/EiJ, SJL/J, DBA/1J, C3H/HeJ, and NZB/J). Six strains were screened for a large number of markers on the chromosome 8 locus, including the QTL region. Additionally, 15 SNPs for which both groups differ were identified in a region within EAE31, positioned from 80.87 to 84.14 Mb. In this smaller QTL region, only nine genes are localized: *Gab1*, *Usp38*, *Inpp4b*, *Il15*, *Rnf150*, *Tbc1d9*, *Clgn*, *Gpsn2*, and *Cc2d1a* (Figure 2).

The syntenic fragment analyzed by the comparative genomic approach overlapped only partially with the region identified with the haplotype analysis; with the combination of both strategies, the QTL EAE31 region was reduced to a 2.6-Mb fragment containing only six genes of interest (Figure 3). To confirm these findings, we genotyped and phenotyped heterogeneous stock (HS) mice^{35–37} descended from eight inbred progenitor strains (A/J, AKR/J, BALB/CJ, LP/J, CBA/2J, C3H/HeJ, C57BL/6J, and DBA/2J). Mice carrying a haplotype block shared with the SJL/J mice had longer cMEP latency. This block had only two genes, *Inpp4b* and *Il15* (data not shown). Because *Il15* is not differentially expressed in EAE and is not polymorphic (in contrast to *Inpp4b*), the latter is the likely QTG for EAE31.

The *Inpp4b* Gene Is Polymorphic

To identify potential structural or regulatory polymorphism responsible for the phenotype, the *Inpp4b* gene-coding region was sequenced in the relevant mice strains (NZB/NJ, C3H/HeJ, SJL/J, C57BL/6J, C57BL/10S, and FVB/NJ). *Inpp4b* has a 9.5-kb coding region.³⁸ Two SNP variants were identified in the coding region of exon 13 at position 1421 (C/

A) and in exon 14 at position 1642 (A/C), resulting in amino acid exchanges from serine to arginine (S474R) and histidine to proline (H548P), respectively (Figure 4). Interestingly the strains carrying the longer-latency SJL/J allele (C3H/HeJ, NZB/NJ, DBA/1J, YBR/EiJ) had the amino acid RP, whereas the mouse strains carrying the shorter-latency C57BL/10S allele (C57BL/6J, FVB/NJ) had the amino acid SH. These data suggest that *Inpp4b* structural polymorphism is associated with the speed of neuronal conduction.

Confirmation in Transgenic Mice

To confirm the hypothesis that the RP/SH polymorphism is responsible for cMEP latency changes, we generated transgenic mice carrying the longer-latency allele *Inpp4b*RP

Figure 3 Fine-mapping of EAE31, a QTL controlling latency of cMEP and onset of EAE, using congenic mapping and haplotype sharing analysis.

NZB/BINJ	ILKKAPSPNVRTTEKSTQHDTPLRRQDSIPHHSDYDEEEWDRVWVWVWGKSLNCIAKVD
C3H/HeJ	ILKKPPSPNVRTTEKSTQHDTPLRRQDSIPHHSDYDEEEWDRVWVWVWGKSLNCIAKVD
DBA/1J	ILKKPPSPNVRTTEKSTQHDTPLRRQDSIPHHSDYDEEEWDRVWVWVWGKSLNCIAKVD
SJL/J	ILKKPPSPNVRTTEKSTQHDTPLRRQDSIPHHSDYDEEEWDRVWVWVWGKSLNCIAKVD
YBR/EIJ	ILKKPPSPNVRTTEKSTQHDTPLRRQDSIPHHSDYDEEEWDRVWVWVWGKSLNCIAKVD
C57BL/6J	ILKKPPSPNVSTEEKSTQHDTPLRRQDSIPHHSDYDEEEWDRVWVWVWGKSLNCIAKVD
C57BL/10.S	ILKKPPSPNVSTEEKSTQHDTPLRRQDSIPHHSDYDEEEWDRVWVWVWGKSLNCIAKVD
FVB/NJ	ILKKPPSPNVSTEEKSTQHDTPLRRQDSIPHHSDYDEEEWDRVWVWVWGKSLNCIAKVD

NZB/BINJ	KLIERSHNEEGAGGSSSKDGEADPTLEDSITSHSPREDWYEQHLPLTLKECMGEVNR
C3H/HeJ	KLIERSHNEEGAGGSSSKDGEADPTLEDSITSHSPREDWYEQHLPLTLKECMGEVNR
DBA/1J	KLIERSHNEEGAGGSSSKDGEADPTLEDSITSHSPREDWYEQHLPLTLKECMGEVNR
SJL/J	KLIERSHNEEGAGGSSSKDGEADPTLEDSITSHSPREDWYEQHLPLTLKECMGEVNR
YBR/EIJ	KLIERSHNEEGAGGSSSKDGEADPTLEDSITSHSPREDWYEQHLPLTLKECMGEVNR
C57BL/6J	KLIERSHNEEGAGGSSSKDGEADHTLEDSITSHSPREDWYEQHLPLTLKECMGEVNR
C57BL/10.S	KLIERSHNEEGAGGSSSKDGEADHTLEDSITSHSPREDWYEQHLPLTLKECMGEVNR
FVB/NJ	KLIERSHNEEGAGGSSSKDGEADHTLEDSITSHSPREDWYEQHLPLTLKECMGEVNR

Rs 33528344 (S→R)
Rs 38061815 (H→P)

Figure 4 The INPP4B protein sequence. Analysis of eight mice strains reveals two amino acid changes in the INPP4B protein sequence. The eight strains can be divided in two groups according to the allele presented at each polymorphic position (highlighted in bold type).

coding region under the control of the *Prnp* promoter. We used the restricted *Inpp4b* β isoform, which has a restricted expression pattern with the highest level in brain, in contrast to the wide expression pattern of the α isoform.³⁹ By using the prion protein promoter and the *Inpp4b* beta isoform, we expected a higher expression level in the murine central nervous system of the transgenic mice. The expression of the mutated *Inpp4b* gene was analyzed in 11 different tissues by extracting the RNA and using RT-PCR (Figure 5). To distinguish between the wild-type *Inpp4b* and the mutated *Inpp4b* transgene carrying both SNPs from the SJL/J genotype, PCR products were restricted by Hpy188I. This resulted in two nearly similar size fragments (124 and 126 bp) of the C57BL/6J *Inpp4b* gene and three fragments (55, 71, and 124 bp) of the transgenic cDNA. The mutated *Inpp4b* transgene was widely expressed in different tissues, such as brain, spinal cord, skeletal muscle, lung, liver, testis, and kidney (Figure 5).

To analyze whether the genotype of the SJL/J allele is responsible for the longer-latency phenotype, transgenic C57BL/6J mice carrying both SNPs of the SJL/J allele were analyzed. cMEPs were measured in hindlimb of 13 transgenic mice harboring the mutant SJL/J *Inpp4b* gene and 12 C57BL/6J wild-type control mice at day 0 (before immunization). The latency of measured cMEPs of transgenic mice was 4.5 ± 0.22 ms, which is significantly higher ($P = 1.04 \times 10^{-9}$) than the 3.7 ± 0.13 ms latency of the littermate C57BL/6J control mice (Figure 6). Thus, INPP4B influences neuronal conduction, and polymorphisms in *Inpp4b* result in altered cortical motor evoked response.

Inpp4b RP Expression Does Not Alter Neuronal Structure

Reasons for cortical MEPs abnormalities such as absence or delay of the MEP may differ. The causes of MEP modifications are not clear, but they could be dependent on structural or metabolic alterations of the myelin, modifications of myelinated pathways, axonal loss, or changes in the synaptic

transmission. To clarify whether latency of cMEPs was due to demyelinating processes, histopathological analysis of brain and spinal cord tissues were performed, but no histological alterations were detected (Supplemental Figure S2).

Association Analysis of MS Samples versus Healthy Controls

The human *INPP4B* gene is located on chromosome 4, in a region associated with MS.³⁴ Therefore, we investigated the association of *INPP4B* polymorphisms with susceptibility to MS in a large case–control cohort. Using the Tagger algorithm in Haploview version 4.1 (<http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview>), 39 Tag-SNPs covering all haplotypes inside the *INPP4b* genomic region were selected. All selected SNPs had a minor allele frequency (MAF) value of >0.05 . In 349 Spanish MS patients and 362 healthy controls, tests for association were performed using logistic regression assuming additive genotype coding. The strongest association was obtained for SNP rs13102150 ($P = 8.8 \times 10^{-3}$). To support these findings, a haplotype analysis using a sliding window approach was performed, with a window size of three SNPs. The haplotype with the strongest signal ($P = 8.92 \times 10^{-4}$) included the SNP rs13102150, thus supporting the findings from the single-marker analysis. A replication analysis was performed with 562 German MS cases⁴⁰ and 3314 German population-based controls from the Gutenberg Health Study.⁴¹ The results of both stages were pooled using inverse variance weighting; the

Figure 5 Verification of *Inpp4b* mRNA expression in the transgenic mouse line. **A:** Scheme of the additional restriction site of Hpy188I resulting from the mutation of *Inpp4b* in the transgenic mice. **B:** mRNA expression analysis in different tissues of the transgenic mice by semi-quantitative RT-PCR followed by Hpy188I restriction. Neg., negative; Pos., positive; Sk., skeletal; tg, transgenic; wt, wild type.

Figure 6 cMEP latencies in hindlimb. cMEP measurements were performed in 13 transgenic C57BL/6J mice carrying the SJL/J allele of *Inpp4b* including both SNPs and 12 C57BL/6J wild-type mice at day 0 before immunization. Data are expressed as means $\pm$ SEM. * $P = 1.04 \times 10^{-9}$, Student's *t*-test. WT, wild type.

combined P value of rs13102150 was $P = 0.022$ (pooled odds ratio = 0.88; CI, 0.78–0.98) (Figure 7).

Discussion

With the present study, we have identified *Inpp4b* as the QTG of EAE31, a QTL associated with the latency of cMEP before immunization in mice. We have also demonstrated that application of *in silico* strategies (eg, comparative genomics and haplotype analysis) is a fast and economical method for fine-mapping a QTL region. The first dissection of the QTL region was performed by using congenic and subcongenic mouse strains, which reduced the EAE31 region to 10 cM (16.9 Mb). To avoid time-consuming backcrosses and/or the generation of advanced intercross lines for dissecting the chromosome region by further recombination events, we focused on *in silico* strategies such as comparative genomics and haplotype analysis. Comparative genomic analysis of MS and EAE loci in human, rat, and mouse provided evidence for conservation of gene order over evolutionary time in a corresponding chromosomal region. With consideration of the corresponding human³⁴ and rat chromosomal consensus region,⁸ the QTL EAE31 was refined to a 3-Mb genomic region containing only 16 genes.

In parallel, we performed a haplotype sharing analysis as a second fine-mapping approach.^{42,43} This approach is based on identifying shared alleles by many distantly related individuals who are separated by many meioses.⁴⁴ This increased number of meiotic events between individuals is important, to increase the resolution and to narrow the expected segment length. The success of haplotype analysis is dependent on the large phenotypic effect of the QTL and on the ability of selected phenotypes to have low intrastrain variation but sufficient variance within the strain set selected. By combining different *in silico* methods, the genomic QTL region was reduced by 96% (from 64 Mb to 2.6 Mb), and we identified *Inpp4b* as the putative QTG for this QTL. It is likely that the strong QTL effect with the high LOD score of

6.9¹⁴ (where LOD score is the base 10 logarithm of odds) made it possible to identify a single candidate gene inside this region with a combination of multiple *in silico* methods.

Using transgenic mice, we then confirmed *Inpp4b* as a gene controlling the latency of cMEP. The transgenic mice carrying the SJL/J allele had slower latency than wild-type mice. Changes to the conductance of MEP could be caused by structural alterations of the myelin or by modifications of signaling pathways. Histological investigation of brain and spinal cord tissues in mice carrying the longer-latency allele did not indicate any differences in terms of demyelination. At the same time, cortical MEP measurements revealed the strong influence of the protein on the neuronal conduction velocity. This suggests a new pathway controlled by this phosphatase in neuronal conduction. *Inpp4b* is not well characterized; however, it is the homolog of *Inpp4a*, a gene responsible for neuronal survival. Targeted disruption of *Inpp4a* in mice leads to neurodegeneration in the striatum, a region of the brain with a central role in motor and cognitive behaviors.⁴⁵ Mutations in human *INPP4A* also lead to neuronal loss in the cerebellum⁴⁶ and to Lowe oculocerebrorenal syndrome, causing cataracts and intellectual disabilities.^{47,48}

INPP4B is involved in the phosphoinositide 3-kinase signaling pathway and can influence other inositol phospholipids in this cascade, resulting in different cellular responses such as cell differentiation, apoptosis, or cell migration.^{23,49} Iino⁵⁰ showed that inositol 1,4,5-trisphosphate-induced Ca^{2+} release plays a pivotal role in central synapses and that intracellular Ca^{2+} signals are important for regulation of synaptic functions. INPP4B catalyzes hydrolysis of the 4-position phosphate of different inositol phospholipids, which present only a minor fraction of lipids in the membrane but have a major function in membrane traffic and signaling pathways. Phosphorylation and dephosphorylation of these phospholipids represents a mechanism used to regulate

Figure 7 Forest plots of odds ratios of the association of *INPP4B* (rs13102150) with MS of two independent stages: stage 1, the Spanish cohort (349 cases and 362 controls); stage 2, the German cohort (562 cases and 3314 controls). Data are expressed as effect with confidence interval (squares with lines) and as the pooled summary measure with confidence interval (diamond, center and tips). The 1.0 value for no effect is marked by the dashed line.

recruitment of coat proteins, cytoskeletal scaffolds, signaling complexes, and membrane proteins. Traffic at the synapse is also controlled by this mechanism. The reversible phosphorylation of inositol phospholipids is one of the mechanisms governing the timing and vectorial progression of synaptic vesicle membranes during their exocytic–endocytic cycle.⁵¹ Using a *Drosophila* model system, Acharya et al⁵² showed that an inositol polyphosphatase (ie, inositol polyphosphatase 1-phosphatase) plays a role in synaptic transmission, its mutants show an increase in the probability of neurotransmitter release.

Neuronal conduction is also influenced by neuronal excitability orchestrated by ion channels of the plasma membrane, of which some (eg, KCNQ channels) need plasma-membrane-specific phosphatidylinositol-4,5-bisphosphate to function. The activity of KCNQs decreases when the abundance of these phosphoinositides falls,⁵³ demonstrating the regulatory function of phosphoinositides. Receptor-mediated suppression, for instance, increases the responses to excitatory synaptic inputs.⁵⁴ Different pathways and signal cascades are conceivable for the involvement of INPP4B in neuronal conduction, and further molecular and biochemical analyses would be necessary to dissect the molecular pathways of INPP4B and to fully understand its physiological function.

With the present study, we have shown that polymorphisms in *Inpp4b* may alter the cortical motor evoked response and influence the speed of neuronal conduction (probably via synaptic transmission). Changes in the amplitude or speed of evoked potentials such as MEP, visual evoked potential, and others are typical for MS patients and are used as diagnostic markers.^{6,55,56}

Whether polymorphisms in the human gene, *INPP4B*, can influence the speed of evoked potential remains to be investigated. Because this human gene is localized within a chromosome region linked to MS,³⁴ polymorphisms in *INPP4B* were analyzed for association with susceptibility to MS.

Jointly analyzing the German and Spanish cohorts, we determined that a polymorphism in the *INPP4B* gene is associated with MS. The effect was much stronger in the Spanish cohort, for which the SNP rs13102150 was selected as a tag SNP for a strongly associated haplotype. Despite its larger numbers, by itself the German cohort only confirms a trend for this particular SNP. The exact reason for the diverging effect across these populations remains unresolved. Contributing factors may be differences in the allele frequencies in both populations [MAF = 0.25 in central Europe (<http://www.1000genomes.org>, last accessed May 1, 2014), compared with MAF = 0.46 in Spain, (unpublished data)].

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2014.05.021>.

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