Analysis of the QTL for sleep homeostasis in mice: Homer1a is a likely candidate

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Mackiewicz M, Paigen B, Naidoo N, Pack AI. Analysis of the QTL for sleep homeostasis in mice: Homer1a is a likely candidate. Physiol Genomics 33: 91–99, 2008. First published January 2, 2008; doi:10.1152/physiolgenomics.00189.2007.—Electroencephalographic oscillations in the frequency range of 0.5–4 Hz, characteristic of slow-wave sleep (SWS), are often referred to as the delta oscillation or delta power. Delta power reflects sleep intensity and correlates with the homeostatic response to sleep loss. A published survey of inbred strains of mice demonstrated that the time course of accumulation of delta power varied among inbred strains, and the segregation of the rebound of delta power in BxD recombinant inbred strains identified a genomic region on chromosome 13 referred to as the delta power in SWS (or Dps1). The quantitative trait locus (QTL) contains genes that modify the accumulation of delta power after sleep deprivation. Here, we narrow the QTL using interval-specific haplotype analysis and present a comprehensive annotation of the remaining genes in the Dps1 region with sequence comparisons to identify polymorphisms within the coding and regulatory regions. We established the expression pattern of selected genes located in the Dps1 interval in sleep and wakefulness in B6 and D2 parental strains. Taken together, these steps reduced the number of potential candidate genes that may underlie the accumulation of delta power after sleep deprivation and explain the Dps1 QTL. The strongest candidate gene is Homer1a, which is supported by expression differences between sleep and wakefulness and the SNP polymorphism in the upstream regulatory regions.

Homer1a; sleep; quantitative trait locus; haplotype; gene expression

IN A SEMINAL STUDY THAT INVOLVED in-depth phenotyping of sleep in inbred mice, Franken et al. (16) identified a quantitative trait locus (QTL) for sleep homeostasis. The phenotypic characterization involved the analysis of the time constant for the increase in delta power following 6 h of sleep deprivation. Delta power reflects sleep intensity and correlates with the homeostatic response to sleep loss (i.e., Process S) (47). The QTL for this trait was determined in DBA/2JxC57BL/6J, i.e., BxD recombinant inbred (RI) lines (16). The logarithm of odds (LOD) score for this QTL was 3.57 (genome-wide P < 0.01). This QTL was named delta power in slow-wave sleep (SWS) or Dps1 (MGI ID number 2135996). It was estimated that genes in this region account for 49% of the variance in this trait between B6 and D2 strains (16).

The previous approaches to narrowing QTL regions involved selective breeding strategies with characterization of the phenotype in the offspring (12, 15). This has not been performed for this QTL, given the time-consuming and expensive process of obtaining sleep phenotypic data. Recently, new strategies to narrow QTL regions have emerged based on in silico analysis of single nucleotide polymorphisms (SNPs) differences between inbred mouse strains (12, 37).

Dense SNP maps, available for various species and strains of laboratory animals, allow the narrowing of experimentally derived QTLs through haplotype analysis (8). This method assumes that the majority of genetic variation among inbred strains is ancestral (19), and thus, the regions of identity by descent between two strains used to identify a QTL are unlikely to contain polymorphisms underlying this QTL (54).

Analysis of polymorphisms in the QTL region can be combined with gene expression profiling data (31), since differences between strains may be related to different expression levels between strains as well as alterations in the protein. If such genes play a role in this QTL, one would expect SNPs in the regulatory regions of these genes. Moreover, we anticipated that such genes might have an altered expression in the brain in response to sleep deprivation in different inbred strains (e.g., B6 and D2).

MATERIALS AND METHODS

Bioinformatic Analyses

To verify the boundaries of the Dps1 QTL, fragments of DNA between PCR primers for D13Mit231, D13Mit126, and D13Mit107 markers defining the right, middle, and left boundary of the QTL (16) were mapped to mouse chromosome 13 with the BLAST (2). The number and location of genes in the Dps1 region were compiled using the Ensembl Genome Browser (3) (release 42, December 2006). Haplotype analysis was performed using the Mouse Phenome Database (4, 5). The search for protein domains was performed using the PROSITE database (24). Analysis of transcription factor binding sites in DNA sequences was performed using the Transcription Element Search System (41). The flow chart of data analysis and the number of candidate genes that emerged from each step is provided in Fig. 1. Table 1 contains URL addresses of the relevant databases.

Experimental Procedures

The expression of genes located in the Dps1 QTL during sleep and sleep deprivation in the cerebral cortex and hypothalamus of B6 mice was published previously (31). Expression of candidate genes in the cerebral cortex and hypothalamus of B6 and D2 mice during sleep and in sleep deprivation was established by RT-PCR using the TaqMan Expression Assay (Applied Biosystems). The following sets of primers and probes for the relevant genes (listed in parentheses) were used: Mm00545877_m1 (Arc), Mm00802167_m1 (Arsb), Mm00516275_m1 (Cem3), Mm00488952_m1 (Hpln1), Mm00600423_m1 (Mef2c), Mm00516275_m1 (Homer1, exons 3 and 4); Mm01290043_m1 (Homer1, exons 9 and 10); Mm00661927_g1 (Zc3hc9), Mm01352847_m1 (Homer1a)}
All experiments were performed on male mice, 10 wk of age ± 1 wk. Animals were housed in a light/dark cycle of 12 h, in temperature- and humidity-controlled room (22°C and 45–55%, respectively) with water and food available ad libitum. Mice were killed following 6 h of total sleep deprivation (n = 7). Deprivation was initiated at lights-on (7:00 AM), and performed through gentle handling (28), following an acclimatization period for handling procedures. Sleeping animals, which were left undisturbed, were killed at the same diurnal time points as sleep deprived mice (n = 7). An additional control group of mice (n = 7) were killed at time zero, i.e., at the time of lights-on at 7:00 AM. All mice were behaviorally monitored using the AccuScan infrared activity monitoring system. The amount of sleep was computed as described previously (36).

Mice were killed by cervical dislocation. Brain dissection was performed according to atlas of Franklin and Paxinos (17). The sample of cerebral cortex was composed of M1 and M2 areas, but also included the midline-located Cg1 and Cg2 regions, as well as parts of S1FL areas. Total RNA was isolated with TRIzol (Invitrogen) and further cleaned using RNeasy purification kit (Qiagen).

The relative transcript level was established by the “ΔΔk” method as described previously (30). The Apc gene was used as an internal standard; this gene was identified as unchanged in its expression during sleep or sleep deprivation in the cerebral cortex and hypothalamus of B6 mice (31).

The Western blot experiments were performed according to our previously described protocol (34), using samples of the cerebral cortex from animals described above. Homer1a-specific antibodies were obtained from Santa Cruz Biotechnology [antibody Homer-1a (M-13): sc8922]. These were the goat polyclonal antibodies raised against the peptide mapping at the COOH terminus of the Homer1a protein of mouse origin. Antibodies against the NH2 terminus of the Homer1 gene, recognizing Homer1a, 1b, and 1c, as well as 2a and 2b (antibody number GTX11157), were obtained from GeneTex.

The verification of sequence polymorphism of selected genes (i.e., Cspg2 - SNP identification number rs29526320 and rs29929323, Homer1 – SNP rs29874758, Tmem161b – SNP rs29251726, and 2900024O10Rik – SNP rs2928182) in B6 and D2 parental strains was performed via the dideoxynucleotide sequencing as described previously (53). In addition, sequencing of the promoter region of the Homer1 gene in the vicinity of the SNP rs29874758 was performed in all 25 RI strains used by Franken et al. (16) for QTL mapping.

RESULTS

In Silico Analysis of the Dps1 Region

Mapping and annotation of genes located in the Dps1 region. To verify the flanking edges of the Dps1 interval, the key QTL mapping markers were placed on the mouse genomic map. Marker D13Mit231 mapped to 77.51 Mbp of chromosome 13 and defined the left flanking border of the Dps1

Table 1. Bioinformatics tools and the URL addresses of databases used in data analyses

<table>
<thead>
<tr>
<th>Task Performed</th>
<th>Database Used</th>
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<tbody>
<tr>
<td>Gene mapping</td>
<td><a href="http://www.ensembl.org/Mus_musculus/index.html">http://www.ensembl.org/Mus_musculus/index.html</a></td>
</tr>
<tr>
<td>Haplotypal analysis</td>
<td><a href="http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rtn=snps/door">http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rtn=snps/door</a></td>
</tr>
<tr>
<td>Search for protein domains</td>
<td><a href="http://expasy.org/prosite/">http://expasy.org/prosite/</a></td>
</tr>
<tr>
<td>Database of transcription factors</td>
<td><a href="http://www.gene-regulation.de/">http://www.gene-regulation.de/</a></td>
</tr>
<tr>
<td>Gene expression in the CNS</td>
<td><a href="http://www.alleninstitute.org">http://www.alleninstitute.org</a></td>
</tr>
<tr>
<td>Gene expression in inbred strains</td>
<td><a href="http://www.genenetnetwork.org">http://www.genenetnetwork.org</a></td>
</tr>
</tbody>
</table>

SNP, single nucleotide polymorphism; CNS, central nervous system.
region. Marker D13Mit126 mapped to 85.80 Mbp and defined a peak of the QTL. Marker D13Mit107 mapped to 96.69 Mbp of chromosome 13 and defined the right flanking border of the QTL. Since the 95% confidence interval (i.e., 1.5 LOD score drop) was not provided by Franken et al. (16), the list of genes compiled in this paper are from the confidence interval defined by the 2.0 LOD score. Thus, the Dps1 region was between 38.0 and 53.0 cm, i.e., between 72,702,852 and 108,597,573 base pairs. The Dps1 region spans ~34 Mb and contains 236 known or predicted genes.

**Haplotypic structure of the Dps1 region in the RI strains.** To narrow the QTL, the BxD RI strains used by Franken et al. (16) were arranged in the order of their responses to sleep deprivation, from the lowest to highest values of accumulation of delta power, and the haplotype pattern in the QTL region was assessed. All strains exhibiting the B6 phenotype (i.e., BXD-22/TyJ, BXD-25/TyJ, BXD-19/TyJ, BXD-6/TyJ, BXD-8/TyJ, BXD5/TyJ) had comparable haplotype patterns from 81 to 95 Mb which differed from those strains with the D2 phenotype (Fig. 2), except for BXD-29/TyJ, for which Franken and colleagues (16) maintained that the phenotype did not fit the genotype. There was no clear correlation between the haplotype structure and phenotype in regions other than the region between 81.2 and 94.9 Mb. Thus, the haplotype analysis of the RI strains narrowed the QTL from ~34 to 13 Mb, and this reduced the number of genes from 236 to 44 protein-encoding genes. Supplemental Table S1 contains a list of genes located within the narrowed Dps1 interval (Fig. 2).

**Sequence analysis of the Dps1 coding regions.** We limited the sequence analyses of coding regions to B6 and D2 strains (i.e., parental strains from which the BxD RI strains were derived and used for QTL mapping), concentrating on genes located in the region spanning 81–95 Mb.

We assessed polymorphic sites that led to nonsynonymous substitutions (Cn) in amino acids of relevant proteins and, in parallel, the assessment of synonymous changes (Cs). There were five genes within the narrowed Dps1 region with Cn differences within their coding sequences: the transmembrane protein Tmem161b (Tmem161b), predicted gene EG667132 (EG667132), chondroitin sulfate proteoglycan 2 (Cspg2), mutS homolog 3 (Msh3), and arylsulfatase B (Arsb) (Fig. 3). The SNPs rs29251726 and rs29251727 in the Tmem161b and Cspg2 genes, respectively, lead to pronounced changes in the properties of the relevant amino acids. For example, the G→A change in the Tmem161b gene leads to a substitution of threonine with alanine, a hydrophilic amino acid to an amino acid with strong hydrophobic properties (Fig. 3).

Cross-species sequence comparisons between distantly related genomes have been instrumental in defining evolutionarily conserved elements with biological roles (18). To assess the likely functional consequences of amino acid substitutions, we searched amino acid sequences with such substitutions for the highly conserved protein domains and functional sites in regions containing the Cn. The alignment of protein sequences with Cn changes revealed that a number of SNPs occurred in regions that are highly conserved among different species, although none were present within a known functional site for a posttranslational modification (data not shown).

Within the narrowed Dps1 region, a number of nucleotide changes did not affect the amino acid sequences in the relevant proteins. Since each Cs modifies an amino acid codon, we evaluated the codon abundance to rule out the presence of “rare” codons that may impact on the efficiency of mRNA translation (42). No Cs substitutions were found with significant impact on codon abundance defined as a change from the abundance in the range 20–50% to <10%. Similarly, the search for SNPs in all genes in the Dps1 interval in the splice regions defined as the first or last two bases of an intron revealed no such SNPs. We included all five genes with Cn changes on the list of candidates to explain the Dps1 QTL. However, taking into consideration that some substitutions involved amino acids with a significant difference in their physical properties, we gave the Tmem161b and Cspg2 genes higher priority on this list of candidate genes.

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1 The online version of this article contains supplemental material.
Expression of genes located in the Dps1 interval and the haplotype structure of the Dps1 regulatory regions. The identification of genes that are both located in the Dps1 region and differentially expressed between sleep and wakefulness was established with microarrays in the brain of B6 mice (31). Genes located in the narrowed Dps1, with differences in their transcript level between wakefulness and sleep, are shown in Table 2. The significance of the difference in gene expression between behavioral states is expressed as a false discovery rate (FDR) (46). There are seven genes located in the narrowed Dps1 region that also exhibit transcript-level differences in their transcript levels between sleep and sleep deprivation in the cerebral cortex and/or hypothalamus with FDR <1% (Table 2).

Genes differentially expressed between sleep and sleep deprivation were subjected to sequence analysis of their regulatory areas. The significance of difference between B6 and D2 mice in the first intron of all genes that may indicate the presence of regulatory sequences. The SNP chosen to explain the gene may lead to the substitution of tyrosine with various AA whose nature is currently unknown.

Table 2. Genes in the narrowed Dps1 region of chromosome 13 that exhibit changes in transcript level between sleep and/or sleep deprivation in the cerebral cortex and/or hypothalamus

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Affymetrix ID Number</th>
<th>% FDR in the Cerebral Cortex</th>
<th>% FDR in the Hypothalamus</th>
</tr>
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<tbody>
<tr>
<td>2900024010Rik</td>
<td>1431207</td>
<td>0.51</td>
<td>9.5</td>
</tr>
<tr>
<td>Cetn3</td>
<td>1417239</td>
<td>0.002</td>
<td>6.1</td>
</tr>
<tr>
<td>Mef2c</td>
<td>1446844</td>
<td>0.2</td>
<td>9.9</td>
</tr>
<tr>
<td>Hapl1</td>
<td>1426294</td>
<td>45</td>
<td>0.5</td>
</tr>
<tr>
<td>Zcche9</td>
<td>1424557</td>
<td>0.6</td>
<td>5.8</td>
</tr>
<tr>
<td>Homer1</td>
<td>1425671</td>
<td>0.0006</td>
<td>10</td>
</tr>
<tr>
<td>Arsb</td>
<td>1458281</td>
<td>21</td>
<td>0.7</td>
</tr>
</tbody>
</table>

In boldface are genes with polymorphic differences between B6 and D2 in the 5'-upstream regulatory (promoter) regions. The significance of difference in gene expression between sleep and sleep deprivation is expressed in terms of the false discovery rate (FDR).

To further assess the significance of polymorphic changes in the promoter regions, we analyzed how putative transcription factor(s) bind to various alleles defined by SNPs. Figure 4 demonstrates selected binding sites for putative transcription factors along the 50-nucleotide sequence of the 2900024010Rik and Homer1 containing SNP. In both genes the nucleotide substitution significantly alters the binding site for putative transcription factors (Fig. 4).

Since some regulatory sequences may be located within the first intron of a gene, we assessed the distribution of SNPs among B6 and D2 mice in the first intron of all genes that exhibit differential expression between sleep and sleep deprivation. There were two genes (i.e., Homer1 and Arsb) with differences among B6 and D2 strains in the SNP content of the first intron. To assess the significance of such changes, the nucleotide sequence of 50 nucleotides surrounding the SNP was aligned with the relevant human sequences in the search for regions of cross-species conservation; such a homology may indicate the presence of regulatory sequences. The SNP present in the first intron of Homer1 and Arsb genes did not occur in a region that displayed cross-species conservation.

Finally, we assessed whether the candidate genes that were chosen to explain the Dps1 QTL based on the combination of the gene expression pattern and SNP of regulatory sequences contained cis-acting expression QTLs (eQTLs) (13). We collected all eQTLs in the Integrative Neuroscience Initiative on Alcoholism M430 robust multiarray average whole brain data set (January 06, freeze) of BxD RI strains and identified those that were located in the narrowed Dps1 region. We established that none of the genes located in the narrowed Dps1 region had
an eQTL in its vicinity when LOD scores of 4.3 and 10 Mb exclusion buffer were used as search parameters.

**Experimental Verification of Key In Silico Results**

The verification of selected SNPs by the nucleotide sequencing. The verification of sequence polymorphism was performed on selected genes; the Cspg2 and Tmem161b genes were chosen for the verification since they carried the Cn acid substitutions, and the Homer1 and 290024010Rik genes were chosen for the nucleotide sequencing since they displayed an SNP in their promoter regions. We verified the following alleles by the nucleotide sequencing: A/T in Cspg2 gene - SNP rs29929323; A/G in Tmem161b gene - SNP rs29251726; C/G in Homer1 gene - SNP rs29874758; and C/T in 290024010Rik gene - SNP rs29928182 (data not shown). We did not verify the A/G polymorphism in Cspg2 defined by the SNP rs29526320; therefore the SNP rs29526320 is not further considered as a potential candidate to explain the QTL.

**Homer1 gene is a likely candidate to explain the Dps1 QTL.** We performed nucleotide sequencing of the promoter region in all RI strains used for QTL mapping and combined the information on nucleotide sequences with the behavioral data published previously by Franken et al. (16) on the accumulation of sleep need after sleep deprivation. These comparisons revealed that all RI strains carrying the C allele in the promoter region of the Homer1 gene defined by the SNP rs29874758 accumulate sleep need with a time course similar to DBA/2J mice. In contrast, all RI strains containing the G allele exhibit C57BL/6J-like phenotype (see Fig. 5). The one exception to this is BXD29/TyJ, which has a G allele but a DBA/2J phenotype.

**State-dependent expression of candidate genes in the central nervous system of B6 and D2 mice.** The expression pattern of candidate genes that emerged from expression studies and sequence analysis of regulatory regions was assessed with RT-PCR in B6 and D2 mice in spontaneous sleep and after sleep deprivation. Although only two genes exhibited an SNP in regulatory sequences, all seven genes identified through gene expression analysis (Table 2) were subjected to RT-PCR. The abundance of transcripts in all genes was assessed at the time of lights-on (i.e., 7:00 AM) and following 6 h of total sleep deprivation or 6 h of spontaneous sleep.

Among seven genes subjected to studies of their expression, only the Homer1 and 290024010Rik genes showed differences in mRNA at 7:00 AM (lights-on) between B6 and D2 mice. The expression level of the 290024010Rik gene was different between sleep and sleep deprivation in both inbred strains. However, the magnitude of this difference was not significantly different between B6 and D2 mice. Therefore, we concentrated our efforts on assessing changes in mRNA and protein levels of Homer1.

**Homer1 is a complex gene with three splice variants (1a, 1b, 1c).** Homer1a is the short form of Homer1 that is upregulated with neuronal activity (7). Full-length Homer1 gene-1c has more exons. Using the primer-specific to Homer1a variant, we find that this gene is upregulated with sleep deprivation (see Fig. 6A). (Identical results were found with a primer to exons 3 and 4 of the Homer1 gene; data not shown.) There are differences in the nature of the change in Homer1a expression with sleep deprivation in C57BL/6J and DBA/2J mice (see Fig. 6A). In both mouse strains there is a decline in expression of this gene in mice left to sleep undisturbed for 6 h
from lights-on (7:00 AM) compared with the 7:00 AM point. Compared with these sleeping-undisturbed controls, there is increased expression of \textit{Homer1a} gene with sleep deprivation in both inbred strains. However, the magnitude of this difference is greater in C57BL/6J compared with DBA/2J. It is only in C57BL/6J that sleep deprivation results in higher levels of \textit{Homer1a} gene expression compared with the 7:00 AM lights-on value.

Somewhat different results are found for a primer to exons 9 and 10 that recognizes the long form of \textit{Homer1} gene (see Fig. 6B). There are again reductions in expression of the long form of \textit{Homer1} gene when animals are left to sleep undisturbed for 6 h. The magnitude of the differences between sleep-deprived and sleeping mice are, however, much smaller than for \textit{Homer1a} (Fig. 6A), and in neither strain is there an increase in expression with sleep deprivation that is significant compared with the 7:00 AM level.

The level of Homer1 proteins was determined by Westerns in the cerebral cortex of C57BL/6J and DBA/2J mice after 6 h of sleep deprivation and 6 h of spontaneous sleep such that mice were killed at the same diurnal time point. There were two different antibodies used in this experiment: 1) antibody specific for NH$_2$ terminus of \textit{Homer1} and \textit{Homer2} genes and all their splice variants, including Homer1a; and 2) antibody specific for \textit{Homer1a} variant of \textit{Homer1} gene; this antibody was raised against 11 amino acids that distinguish Homer1a and Homer1c proteins. Both antibodies showed increased protein levels with sleep deprivation in B6 but not in D2 mice (Fig. 7). That this difference is due to a differential response of

Fig. 6. Changes in the expression of the \textit{Homer1} gene in the cerebral cortex of B6 and D2 mice after 6 h of total sleep deprivation and 6 h of spontaneous sleep. A: percentage change in the steady state level of the \textit{Homer1a} gene as determined using a probe and primers specific for \textit{Homer1a} splice variant. The transcript level at lights-on (i.e., 7:00 AM) is presented as 100%. Sleep deprivation significantly upregulates the expression of the gene in B6 but not D2 mice (**$P = 0.001$). These results are in full agreement with the data obtained using primers and a probe for exons 3 and 4 (data not shown). Using sets of primers specific for the \textit{Homer1a} variant (as well as from exons 3–4; data not shown) we determined that spontaneous sleep significantly down-regulates \textit{Homer1a} transcripts in B6 and D2 mice (**$P = 0.003$ and 0.0001, respectively). B: percentage change in the steady state level of the \textit{Homer1c} gene transcript as determined using a probe and primers for exons 9 and 10. The transcript level at lights-on (i.e., 7:00 AM) is presented as 100%. Sleep deprivation does not modulate the expression of the \textit{Homer1c} gene, compared with the 7:00 AM time-point. However, during sleep there is a decrease in the transcript of the \textit{Homer1c} gene in D2 and B6 mice (*$P < 0.05$ for both strains).

Somewhat different results are found for a primer to exons 9 and 10 that recognizes the long form of \textit{Homer1} gene (see Fig. 6B). There are again reductions in expression of the long form of \textit{Homer1} gene when animals are left to sleep undisturbed for 6 h. The magnitude of the differences between sleep-deprived and sleeping mice are, however, much smaller than for \textit{Homer1a} (Fig. 6A), and in neither strain is there an increase in expression with sleep deprivation that is significant compared with the 7:00 AM level.

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Fig. 7. The level of Homer1 proteins as determined by Western blot using an antibody against the NH$_2$-terminal part of the Homer1 protein (A) or an antibody specific for the Homer1a variant (B). These results indicate that sleep deprivation upregulates the NH$_2$-terminal part of Homer1 (i.e., Homer1c proteins or its variants) and Homer1a in B6 but not D2 mice ($P < 0.05$ for both antibodies in B6 mice) and fully support mRNA quantitation data.
Homer1a is supported by results using the antibody specific to Homer1a (Fig. 7B). Thus, the Western blot studies support the concept that the expression of the Homer1a splice variant and Homer1a protein increase more with sleep deprivation in the cerebral cortex of C57BL/6J mice compared with DBA/2J.

DISCUSSION

The QTL analysis is an important method for identifying a genomic region underlying a phenotype; however, finding a causal relationship between a gene in this region and a phenotype remains difficult (1). The narrowing of a QTL region can be performed through additional mouse crosses or using bioinformatics and statistical tools (12, 15). Dense SNP maps available for various species and strains of laboratory animals allow the narrowing of an experimentally derived QTL through a haplotype analysis (8, 44, 51).

One candidate gene described by Franken et al. (16) in the Dps1 region was the Ntrk2 gene encoding a receptor that binds to the brain-derived neurotrophic factor (BDNF). Ntrk2 might be an important gene to explain the Dps1 QTL since the expression of the Bdnf gene parallels expression of delta power and increases with wakefulness and decreases during recovery sleep (23). However, based on current data, the Ntrk2 gene is not located within the interval defined by the D13Mit26 and D13Mit288 markers defining the 95% confidence interval of Dps1 QTL. This prompted us to perform a comprehensive annotation of genes located within the Dps1 interval; we identified 236 known or predicted genes. The haplotype analysis of parental and BxD RI strains narrowed the interval from ~34 Mb to 13 Mb, and the number of genes was reduced to 44.

There were five genes [i.e., the transmembrane protein 161B (Tmem161b), predicted gene EG667132 (EG667132), chondroitin sulfate proteoglycan 2 (Cspg2), mutS homolog 3 of Escherichia coli (Msh3), and arylsulfatase B (Arsb)] in the narrowed Dps1 interval that contained within their exons polymorphic differences between B6 and D2 mouse strains that lead to Cn changes in amino acid composition of the relevant proteins (Fig. 3). The Tmem161b and EG667132 genes encode hypothetical proteins for which there is no information available as to their functions. There is no information available on the expression of EG667132 gene in the mouse central nervous system; however, the Tmem161b gene is expressed in selected areas of the mouse brain (see www.alleninstitute.org).

The remaining genes, i.e., Cspg2, Msh3, and Arsb, are well characterized. Although the Cn changes in these genes did not arise at known protein domains, they did occur in highly conserved protein regions. The Cspg2 gene encodes chondroitin sulfate proteoglycan 2 (versican), an extracellular matrix proteoglycan (26). The Msh3 gene encodes a mouse homolog of the bacterial (E. coli) mismatch repair protein MutS. Msh3 functions in the repair of DNA mismatches (10, 27). ARSB is a lysosomal enzyme involved in the degradation of sulfatides (29).

The assessment of the functional significance of SNP differences in regulatory regions presents a particular challenge. There is a plethora of proteins involved in the regulation of gene transcription, and regulatory sequences are diverse and can be localized in various places in the genome in relation to the core promoter or transcription start site (43, 52). We argue that the polymorphism of regulatory sequences among inbred strains should be analyzed in conjunction with the analysis of data on gene expression during sleep and wakefulness.

We identified seven genes located in the narrowed Dps1 region that exhibited differences in gene expression in the cerebral cortex and/or hypothalamus between sleep and sleep deprivation (Table 2). Among genes differentially expressed between behavioral states, two genes (i.e., 2900024010Rik and Homer1) contained an SNP within the upstream regulatory region (Fig. 4). We postulate that genes that exhibit significant differences among B6 and D2 strains in binding of a putative transcription factor are candidates to explain the Dps1 QTL. In the 2900024010Rik gene, the binding site impacted by the SNP involves the transcription factors GATA-1 and N-Oct-3.

The SNP for the Homer1 gene involves the transcription factors D1 and c-Ets-1. The SNP in the promoter of the Homer1 gene is in the immediate vicinity (one nucleotide apart) of an E-box, a promoter element that regulates circadian oscillations (21). The Ets1 E26 avian leukemia oncogene 1 (c-Ets-1) transcription activator exhibits a high log-likelihood binding score for the region containing a C/G polymorphism (Fig. 4). The G allele retains the Ets1 binding site, whereas the C allele loses the capability to bind it. There are multiple Ca2+-dependent phosphorylation sites within the transcription activator Ets1 that act additively to produce graded DNA binding affinity (38). Since the cellular physiology underlying delta power relies on the intracellular level of calcium (see review in Ref. 45), the variable, Ca2+-dependent phosphorylation of the Ets-1 may serve as a ‘rheostat’ for fine-tuning of transcription at the level of DNA binding relative to delta power. Upregulation of the Homer1 gene involves the MAPK/ERK cascade (40) and activation of the MAPK/ERK impacts the expression of various genes including those that utilize the Ets-1 transcription factor (9).

There were no cis-acting eQTLs in the vicinity of those genes that were differentially expressed in sleep and after sleep deprivation. Such eQTLs, however, only examine basal expression, whereas the key question is what genes change expression when animals are sleep deprived?

That Homer1a mRNA is upregulated with sleep loss was originally identified by Nelson et al. (35) in the rat. This finding has been reproduced in rat (23) and in mice (32). Data presented here show differential response of Homer1a gene to sleep loss in the C57BL/6J and DBA/2J inbred strains, extending the recent observations of Maret et al. (32). They conclude, based on taking a ratio of expression levels between sleeping and sleep-deprived mice at the same diurnal time, that C57BL/6J shows a lower Homer1a response to sleep deprivation than DBA/2J. A ratio, however, may not be the best way to assess this. If one looks at the absolute magnitude of change, i.e., difference between expression in sleep and sleep-deprived mice, this is greater in C57BL/6J than DBA/2J. Although this is not discussed by Maret et al. (32), their actual data are similar to ours with different interpretation. In addition, we find that at the protein level there is upregulation of Homer1a in cerebral cortex with sleep loss in C57BL/6J, but not in DBA/2J mice. We further show that the C allele in the Homer1 regulatory region shows a strong correlation with large SWS response in recombinant strains. The one exception to this otherwise perfect correlation is BXD-29, which has the G allele but a low SWS recovery response. This discrepancy between the phenotypic response to sleep deprivation and the
genotype of this particular recombinant was noted in the original publication of Franken et al. (16) using MIT markers. Our more refined analysis of a relevant specific polymorphism does not provide an explanation of this discrepancy.

The mechanism by which altered expression of Homer1a gene might affect sleep homeostasis is currently unknown. Homer1a is a member of the Homer family of proteins that are scaffolding proteins localized in postsynaptic densities of excitatory synapse (for reviews, see Ref. 56). They interact with group I metabotropic glutamate receptors, IP3 receptors, and ryanodine receptors (49), as well as with Shank protein (48) forming multimeric complexes. Homer1a is a short form of Homer1. It lacks the COOH-terminal domain that is required for multimerization. It therefore antagonizes the activity of full length Homer proteins by competing for binding to metabotropic glutamate receptors (56).

The Homer1a gene is rapidly activated by neuronal activity (7), and its expression is increased by many stimuli including exploratory behavior in a novel environment that increases its mRNA in hypothalamus and neocortex (50). Both promotion of AMPA receptor function by Homer1a (a positive feedback mechanism) (22) and an inhibition of synaptic transmission (negative feedback) (39) have been proposed. Homer1a protein alters the change in Ca2+ in response to stimulation, although effects in different directions are found in different neurons (55). The basis for these differences is unknown.

Although the Homer1a variant is a short form of Homer1 gene, its level can be directly transcriptionally controlled. Its transcription is upregulated by BDNF (40). Increased expression of BDNF is also found in cortex during wakefulness (23), with the degree of increase in BDNF being correlated both with the amount of exploratory behavior during wakefulness, and the amount of slow-wave activity during subsequent sleep (23). The promoter region of the Homer1 gene also contains multiple CRE sites that will bind the cyclic-AMP response element binding protein (CREB) (6). Studies of cultured striatal neurons indicate that the increased expression of Homer1 produced by dopamine is mediated by CREB (57). Mice with low levels of CREB due to deletion of the alpha and delta isoforms of CREB have reduced wakefulness during the early part of their nighttime active period (20). Whether this reduction in wakefulness is mediated, at least in part, by reduced increases in Homer1a with wakefulness in CREB hypomorph mice, is currently unknown.

These observations imply that the effect of altered Homer1a on sleep homeostasis is likely to be mediated by alterations in the nature of wakefulness and the processes that occur during this state. Ultimately it will be important to determine whether a Homer1a knockout mouse has altered wakefulness and sleep and, in particular, an altered amount of delta power following a period of sleep deprivation. The knockout of the long form of Homer1 gene has no alteration in sleep-wake behavior (32) even though it has a number of other behavioral abnormalities (25). This, however, does not exclude Homer1a being important in sleep homeostasis since its function is different from that of the long form of Homer1.

In summary, using interval-specific haplotype analysis, we narrowed the Dpsl region from 34 Mb to 13 Mb to include 44 protein-encoding genes. Sequence analysis of the coding regions of genes within the Dpsl interval identified four potential candidates. Further studies need to address whether alterations in expression of these genes alter the sleep/wake phenotype of mice. For example, the unequivocal proof that the G/C polymorphism in the promoter region of the Homer1 affects the accumulation of sleep need after sleep deprivation would require a conversion of C to G in the promoter of Homer1 gene in DBA/2J mice. The proof of the functional significance of this SNP would be the demonstration of a change of DBA/2J response to sleep deprivation to that found in C57BL/6J. There is some evidence that Homer1 is likely to be causally involved in sleep/wake control based on studies in Drosophila. Drosophila has only one Homer gene (D-Homer) unlike mammalian species (11, 14) and flies lacking D-Homer have fragmented sleep (33).

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