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ORIGINAL ARTICLE

Analysis of quantitative trait loci in mice suggests a role of Enoph1 in stress reactivity

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Abstract

Significant progress in elucidating the genetic etiology of anxiety and depression has been made during the last decade through a combination of human and animal studies. In this study, we aimed to discover genetic loci linked with anxiety as well as depression in order to reveal new candidate genes. Therefore, we initially tested the behavioral sensitivity of 543 F2 animals derived from an intercross of C57BL/6J and C3H/HeJ mice in paradigms for anxiety and depression. Next, all animals were genotyped with 269 microsatellite markers with a mean distance of 5.56 cM. Finally, a Quantitative Trait Loci (QTL) analysis was carried out, followed by selection of candidate genes. The QTL analysis revealed several new QTL on chromosome 5 with a common core interval of 19 Mb. We further narrowed this interval by comparative genomics to a region of 15 Mb. A database search and gene prioritization revealed *Enoph1* as the most significant candidate gene on the prioritization list for anxiety and also for depression fulfilling our selection criteria. The Enoph1 gene, which is involved in polyamine biosynthesis, is differently expressed in parental strains, which have different brain spermidine levels and show distinct anxiety and depression-related phenotype. Our result suggests a significant role in polyamines in anxiety and depression-related behaviors.

Keywords: polyamines, mouse, stress, anxiety, depression, Enoph1.

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Stress is an important environmental factor contributing to the development of many common disorders, including psychiatric disorders (Holsboer and Ising 2010). Indeed, for depression it seems to be one of the major factors involved (Holsboer and Ising 2008). However, only a minority of individuals subjected to severe acute or chronic stress develop depressive symptoms, while the majority seems to be resilient. It is therefore likely that stress can exert its detrimental effects only in the context of other environmental factors or in genetically vulnerable individuals. Thus, it is important to identify genes involved in the modulation of stress sensitivity.

We have previously used mice from a C57BL/6J × C3H/HeJ intercross to study the possible interrelationship between a genetic predisposition to low- or high-stress sensitivity and operant nicotine self-administration behaviors (Bilkei-Gorzo *et al.* 2008). For this purpose, we first examined 622 F2 animals from a C57BL/6J x C3H/HeJ intercross in several behavioral paradigms for stress-responses and anxiety-related behaviors (zero maze, light–dark test, acoustic startle response, forced swim test). Every animal received a score for each test that reflected its behavior in relation to all other animals and cumulative scores across all tests were calculated. The highest and lowest scoring animals, representing the animals with the highest and lowest stress sensitivity, respectively, were then used to evaluate operant nicotine self-administration behavior. Although there was no difference in

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Abbreviations used: GOs, gene ontologies; QTL, quantitative trait loci; SAM, S-adenosylmethionine; SNP, single nucleotide polymorphism.

the acquisition and extinction of the operant behaviors, only mice with high stress sensitivity showed a stress-induced reinstatement of nicotine seeking behavior after extinction, indicating a genetic link between stress predisposition and relapse vulnerability.

We have now used this cohort of animals to search for Quantitative Trait Loci (QTL) associated with the increased stress vulnerability. QTL analysis is an effective method to dissect genetic factors involved in complex phenotypes in farm animals or in rodent models of human disorders (Moore and Nagle 2000; Peters *et al.* 2007). Previous studies demonstrated the power of QTL analyses for the identification of genetic loci in anxiety traits (Turri *et al.* 2001; Henderson *et al.* 2004; Singer *et al.* 2005; Laarakker *et al.* 2008) and depression-like behavior (Tomida *et al.* 2009). We first utilized a dense microsatellite marker set for genotyping and applied a comparative genomics approach subsequent to QTL analysis. Candidate genes were prioritized on the basis of gene ontologies (GOs) of a known disease gene. The best candidate gene (*Enoph1*) was evaluated by expression and single nucleotide polymorphism (SNP) analysis, as well as biochemical analysis.

Materials and methods

Animals

A common strategy in mouse QTL studies is based on the breeding of a suitable genetically divers population of mice, followed by phenotype and genotype analyses of the individuals. Typically, two or more inbred strains of mice are crossed for at least two generations. The progenitor mouse strains should have sufficient variation for the traits of interest and they should be genetically diverse enough to enable genetic mapping. In addition, a high degree of DNA sequence variation facilitates the choice of polymorphic markers (Liu 1997). The C57BL/6J and C3H/HeJ mouse strains fulfilled these requirements, so that these mice were used to create a mapping population. In addition to that, C57BL/6J and C3H/HeJ mice meet the condition of a low degree of genetic relationship, as they represent different branches of the family tree of laboratory mice (Witmer *et al.* 2003). The phenotypic variance for anxiety- and depression-related behavior of the parental strains was verified in several studies (Crawley *et al.* 1997; Griebel *et al.* 2000; Lucki *et al.* 2001; Crowley *et al.* 2005; Milner and Crabbe 2008). Thus, the QTL study was carried out with DNA samples of a F2 generation of mice from a C57BL/6J x C3H/HeJ intercross using 543 animals. Expression studies, SNP, histological, western blot as well as HPLC analyses were done with samples obtained from parental mouse strains. Animals at the age of 2–3 months were kept in groups of three to five mice under a reversed light–dark cycle (lights on: 7.00 pm; lights off: 9:00 am). They received water and food *ad libitum*. Animal procedures followed the guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) as well as the German Animal Protection Law and were approved by local animal care and use committee.

Behavioral analysis

Phenotypic data of the behavioral experiments were assessed within the scope of another study, which has already been published (Bilkei-Gorzo *et al.* 2008). Briefly, trait anxiety was assessed by the zero maze and the light–dark test. In both tests, time spend (s) and distance travelled (cm) in open, lighted areas were measured. The longer an animal stayed or travelled in the open area, the less anxious it is. State anxiety levels were detected by the startle response test and the animal's startle response (g) before as well as after an acoustic stimulus was recorded. Generally, an animal with low anxiety levels exhibits less movement after the stimulus. Depression-like behavior was determined with the forced swim test by measuring the time (s) the animal was immobile showing no flight behavior. Animals with less depressive behavior usually present longer flight behavior. Each animal was analyzed once in all behavioral tests and was left undisturbed for 7 days between two experiments. The behavioral examination was achieved in the following order: (i) zero maze, (ii) light–dark test, (iii) startle response test, (iv) forced swim test.

Genotyping

DNA samples of the F2 generation and the parental mouse strains were extracted from tail biopsies with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). We genotyped the F2 progeny using 269 microsatellite markers with a mean distance of 5.56 cM as previously described (Drews *et al.* 2010). PCRs were carried out in 96-well plates using HotStarTaq polymerase (Qiagen) with 3.5 mM $MgCl_2$ according to the manual. PCR products were loaded on 384-well plates, separated with the ABI 3130xl Genetic Analyzer (Applied Biosystems, Darmstadt, Germany) and analyzed with the ABI GeneMapper® software version 3.7 (Applied Biosystems). Microsatellite markers of different allele size ranges and fluorescent dye labeling were loaded into the same capillary of the sequencer to allow multiplexing of PCR products. A Janus pipetting robot (PerkinElmer, Rodgau, Germany) was used for liquid handling.

Data analysis

We performed QTL mapping with the software R/qtl version 1.14-2 (www.rqtl.org), using the Haley–Knott regression method or the non-parametrical method, if behavioral data were not normally distributed and could not be normalized (Broman *et al.* 2003). We applied a single-QTL genome scan utilizing the scanone function of R/qtl that is based on marker regression by comparing the genotypes of each marker individually with the phenotype data and applying the analysis of variance for the linkage calculation. This function also includes the interval mapping for the handling of missing genotype data and we utilized the Haley–Knott regression method (Haley and Knott 1992) for this purpose because of the faster calculation and robustness. For not normally distributed data, we applied the non-parametrical method, which was specifically design for this type of input data (Broman *et al.* 2003). We determined QTL combined in both sexes and also in sex separated subsets. For the calculation of significance thresholds, we performed 10 000 permutations to receive genome-wide adjusted *p*-values. In the permutation, the replicates of the genotypic data are analyzed for logarithm (base 10) of odds (LOD) score rising above a pre-defined threshold under the hypothesis that there is no linkage

between genome and trait. This LOD scores were counted and divided by the total number of replicates in order to reveal the genome-wide significance of the applied threshold. Confidence intervals were calculated with the Bayesian algorithm integrated in R/qtl. Analysis of syntenic regions was carried out using the Ensembl database (Hubbard *et al.* 2009).

The software FunSimMat was used for prioritization of candidate genes based on GO data (Schlicker and Albrecht 2010). This prioritization is based on comparison of GOs of candidate genes with GOs of a reference gene, which was associated with a specific phenotype or disease in earlier studies. Subsequently, the software prioritizes the candidate genes. For the identification of possible candidate genes we chose the biological process score representing the similarity of biological process component of the GOs. We analyzed two different values of this score (Lin & simRel); Lin's measure of semantic similarity is based on the information content of GO terms and simRel is a functional similarity measure for comparing two GO terms with each other. The third score we chose was the rfunSim score, which is the square-root of a calculation from the biological process score and the MF score (molecular function component of the GOs) of a pair of proteins or protein families. These scores were identified in simulation studies as the most interesting and robust ones for the prioritization process (A. Schlicker, personal communication). An average similarity is indicated by a score above 0.5 and strong similarity by a score higher than 0.8. A score under 0.5 indicates that there is no similarity. Therefore, those genes for which at least one of these scores (Lin, simRel & rfunSim) was equal or above 0.5 were considered possible candidate genes. Five percent of the genes in the QTL region were above this threshold, thus they showed the highest similarity with the reference gene. For the forced swim test we determined the 'susceptibility to unipolar depression' phenotype (no. 607478) as reference phenotype; the 'anxiety' phenotype (no. 607834) for anxiety tests. Both references were obtained from the 'online Mendelian inheritance in man' (OMIM) database (http://www.ncbi.nlm.nih.gov/omim). These phenotypes matched best with the anxiety- and depression-related behavior analyzed in our paradigms.

RNA preparation, RT-PCR, & TaqMan assays

Amygdala, cingulate cortex, hippocampus, and hypothalamus were rapidly dissected from mouse brains of the parental strains, snap frozen in isopentane and stored at −80°C. Liver was also dissected from the parental strains and frozen. Total RNA was prepared by the Trizol method (Invitrogen, Darmstadt, Germany). Superscript II reverse transcriptase (Invitrogen) was applied for generation of cDNA according to the manual. TaqMan assays were performed using an ABI 7900 sequence detector (Applied Biosystems, Rodgau, Germany) on cDNA samples as recommended in the manual. TaqMan primer and probe sets were purchased from Applied Biosystems: Mm01207771_m1 for *Enoph1*, Mm00504406_m1 for *Hsd17b11*, Mm01203271_m1 for *Hsd17b13,* and Mm00446973_m1 for TATA binding protein. For *Hsd17b11* and *Hsd17b13*, we analyzed expression levels in the liver, because this organ plays a major role in steroid metabolism. *Enoph1* expression was analyzed in amygdala, cingulate cortex, hippocampus, and in hypothalamus, which represent brain regions involved in the regulation of emotional behavior (Price and Drevets 2010; Shin and Liberzon 2010). Results were presented as relative expression levels after normalization to TATA binding protein and calculated with the $2^{-\Delta C(t)}$-method. For each brain region, 10 mice of each parental strain were used.

SNP analysis

SNP analysis was performed as described previously (Ditzen *et al.* 2010). Briefly, mouse brain tissues of parental mice were immediately frozen in liquid nitrogen and ground into fine powder under liquid nitrogen using mortar and pestle. A maximum amount of 30 mg frozen tissue powder was then homogenized and lysed in 600 µL RLT buffer (Qiagen) with QIAshredder spin columns (Qiagen). RNA extraction was performed with RNeasy spin columns (Qiagen). The cDNA synthesis as well as PCR was carried out using the SuperScript™ One-Step RT-PCR with the help of the Platinum® Taq system (Invitrogen). Reverse transcription and PCR were performed in one reaction using *Enoph1* specific primers (sense primer: 5′-GTG TTG CCC TCC TTA ACC A-3′; antisense primer: 5′-GGT CTT CAT GGA ACG GAC A-3′). After cDNA synthesis and amplification the products were separated in an agarose gel and stained with ethidium bromide. The 996 bp PCR products were confirmed by DNA sequencing (Medigenomix, Ebersberg, Germany).

Biochemical analysis

Five brains of each parental mouse strain were quickly dissected and sagittally sliced into two equal halves. One half was used for S-adenosylmethionine (SAM) measurements, the other one for polyamine assays. For SAM measurements, the brain tissue was homogenized in 10x volume 5% trichloroacetic acid by sonication (40 W, 90 s) on ice. N^6-methyladenosine was added as an internal standard. Homogenates were centrifuged at 10 000 *g* for 30 min at 4°C. The supernatant was washed three times with 10× volume water-saturated diethyl ether and vacuum dried to remove diethyl ether residues. The remaining solution was directly applied to a high-performance liquid chromatography (HPLC) system (Knauer, Berlin, Germany). Results were corrected with the internal standard. For the polyamine assays, the brain tissue was treated with 3× volume 4% perchloric acid and sonicated (40 W, 60 s) on ice, followed by extraction of polyamines overnight at 4°C. After centrifugation at 10 000 *g* for 20 min, 100 µL of the supernatants were mixed with 300 µL of 2N NaOH and 3 µL of benzoyl chloride. After incubation for 20 min at 23°C, the reaction was stopped by the addition of 500 µL of a saturated NaCl solution. Polyamines were extracted in 500 µL chloroform. After centrifugation at 10 000 *g* for 10–20 min, the chloroform layer was collected, evaporated to dryness, and redissolved in 100 µL 55% methanol.

SAM and polyamine levels were detected on a Smartline Series HPLC system (Knauer). For this purpose, 20 mL aliquots were injected onto a 250 × 2 mm Eurospher 100-3 C18 column with precolumn (Knauer). SAM and polyamine separation was done as previously described (Ditzen *et al.* 2010).

Western blotting

Six male mice of each of the C57BL/6J and C3H/J mouse strains were exposed to 10 min of forced swim stress (23°C water temperature). Three hours after this acute stress the mice were decapitated, their brains removed and shock frozen in liquid nitrogen.

Brains of three unstressed male mice of each strain served as control. The whole brains were sonicated in ristocetin-induced platelet agglutination buffer containing protease inhibitors. Protein content was determined using a commercially available bicinchoninic acid assay kit from Pierce (Thermo Scientific, Rockford, IL, USA). The absorbance was measured at 570 nm on a Revelation Microtiter Plate Reader (Dynex Technologies, Chantilly, VA, USA). Protein concentration was calculated from the optical density values according to the standard curve of bovine serum albumine. Twenty-five microgram of total protein was then used for western blot analyses. The samples were run on polyacrylamide gels, blotted onto nitrocellulose membranes and subsequently probed with primary antibody to Enoph1 (1 : 200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ß-actin (1 : 10000; Sigma). Blots were then incubated with peroxidase-conjugated secondary antibodies, and positive signals were detected using chemiluminescent substrates of peroxidase (ECL, Pierce). Quantification was performed using the ImageLab software (Bio-Rad Laboratories, Hercules, CA, USA). Signal intensities were normalized to ß-actin. Groups were compared using two-way ANOVA (stress and genotype as main effects).

Results

Behavioral phenotyping and QTL analysis

We used a 543 F2 generation animals obtained from a C57BL/6J × C3H/HeJ intercross that that overlapped with a cohort used in a different study to select stress-sensitive and stress-insensitive mice (Bilkei-Gorzo *et al.* 2008). Behavioral data were normalized by square-root transformation for the zero maze and light–dark data, logarithmic transformation was performed for the startle response measurements. Normalization of data from the forced swim test and for the open time values of the light–dark test was not possible, thus the original data of these tests were directly applied in a non-parametric QTL analysis. (Figure S1)

The results of all QTL analyses are presented in Table 1. We detected two QTL on chromosome 5 (58 cM) for the parameters *time* (s) (LOD 5.74; $p < 0.001$) and *distance* (cm) (LOD 4.72; $p = 0.003$) of the light–dark trait (Fig. 1a and b). Furthermore, we found an additional locus for the *distance* (cm) parameter on chromosome 1 (57 cM; LOD 4.69; $p = 0.003$) (Fig. 1b). Analysis of the zero maze traits revealed two QTL, one on chromosome 15 (22.2 cM; LOD 4.59; $p = 0.006$) for the parameter *time* (s) (Fig. 1c) and one on chromosome 5 (61 cM; LOD 4.84; $p = 0.005$) for the *distance* (cm) parameter (Fig. 1d). One strong QTL for the *immobility time* (s) measured in the forced swim test was identified on chromosome 5 (53 cM) with a high LOD score of 14.41 ($p < 0.001$) (Fig. 1f). Analysis of the startle response data revealed no significant QTL (Fig. 1e).

We also performed QTL analyses with the sample set separated into male and female individuals to identify sex-specific loci (Table 1). We detected five male-specific QTL, two of those were associated with the *time* (s) parameter of the light–dark test on chromosome 5 (57 cM; LOD 3.69;

Table 1 Quantitative Trait Loci found with behavioral data

Trait	Sex	Chr.	Locus (cM)	LOD score	*p*-value	% var.	95% CI (cM)
Light–dark Time	All	5	58	5.74	< 0.001	5.09	49–60
Light–dark Distance	All	1	57	4.69	0.003	3.52	46–69
		5	58	4.72	0.003	3.54	49–62
O-Maze Time	All	15	22.2	4.59	0.006	3.82	8,7–25,7
O-Maze Distance	All	5	61	4.84	0.005	4.02	49–65
Startle Response	All	–	–	–	–	–	–
Forced Swim	All	5	53	14.41	<0.001	12.4	41–58
Light–dark Time	Male	5	57	3.69	0.034	6.39	44–62
		14	55.5	4.42	0.007	5.57	38.5–58.5
Light–dark Distance	Male	1	58	4.79	0.002	6.82	50–85
O-Maze Time	Male	–	–	–	–	–	–
O-Maze Distance	Male	–	–	–	–	–	–
Startle Response	Male	–	–	–	–	–	–
Forced Swim	Male	5	52	6.53	<0.001	9.82	42–59
		7	3.4	4.02	0.016	6.42	1.5–8.5
Light–dark Time	Female	–	–	–	–	–	–
Light–dark Distance	Female	–	–	–	–	—	–
O-Maze Time	Female	–	–	–	–	–	–
O-Maze Distance	Female	–	–	–	–	–	–
Startle Response	Female	–	–	–	–	–	–
Forced Swim	Female	5	40	9.1	<0.001	10.3	36–60

Chr., chromosome number; cM, centimorgan; 95% CI, the 95% confidence interval for the QTL (in cM) calculated by R/qtl;% var., % of phenotypic variance the QTL accounts for. A *p* value < 0.05 represents significant effects.

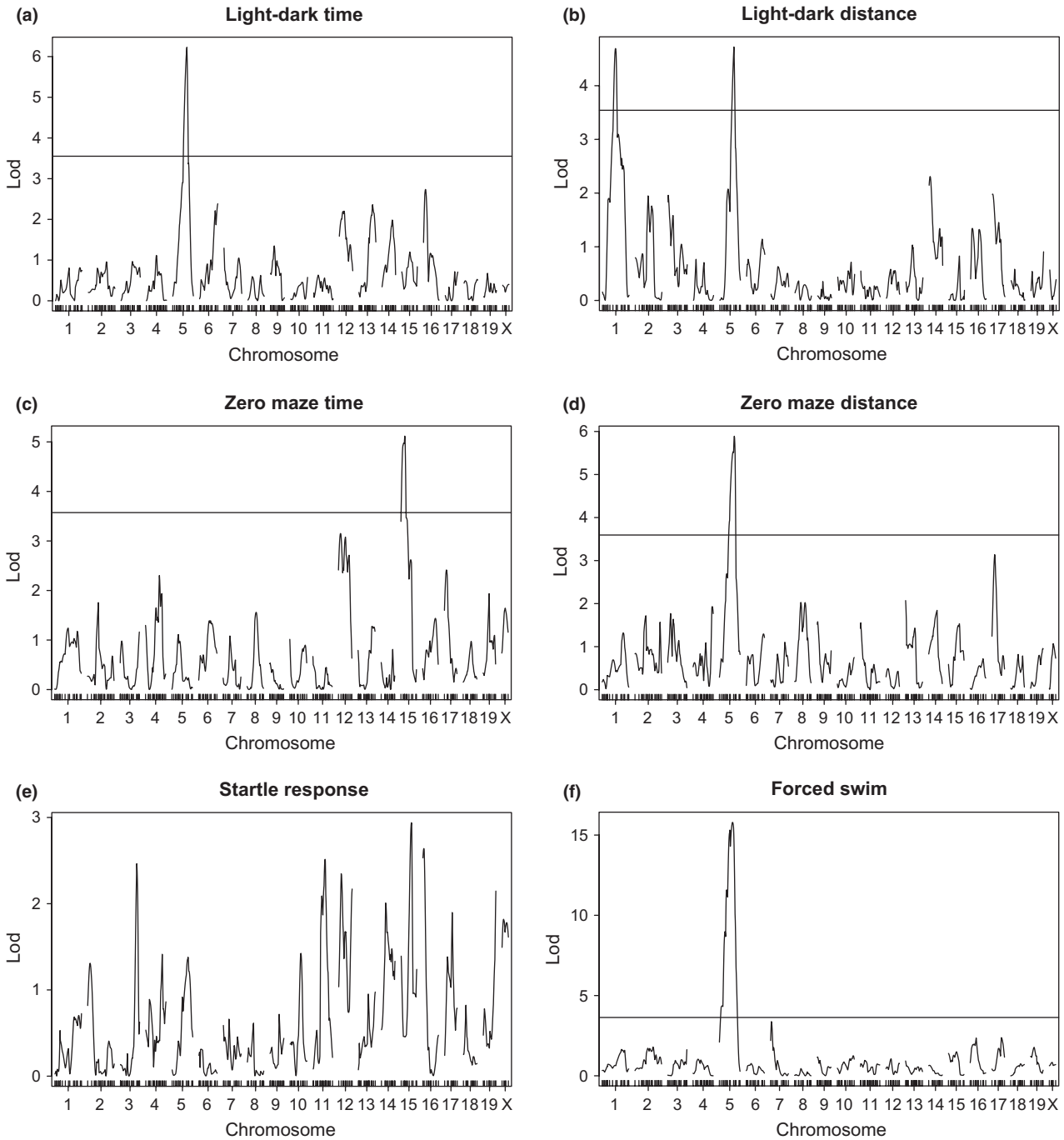

Fig. 1 Quantitative Trait Loci (QTL) analyses of 6 behavioral measurements. Graphs present LOD plots for (a) time in the open area and (b) distance in the open area in the light–dark test, for (c) time in the open area and (d) distance in the open area in the zero maze test, for (e) the startle amplitude in the startle response test and for (f) the immobility time in the forced swim test. The microsatellite markers are shown on the *x*-axis sorted by their position in the genome and the LOD-scores are indicated on the *y*-axis. The horizontal line in each plot indicates the significance threshold with $p < 0.05$.

$p = 0.034$) and chromosome 14 (55.5 cM; LOD 4.42; $p = 0.007$). Further we found two QTL for the *immobility time* (s) value of the forced swim test on chromosome 5 (52 cM; LOD 6.53; $p < 0.001$) and chromosome 7 (3.4 cM; LOD 4.02; $p = 0.016$). The fifth male-specific QTL was identified with the *distance* (cm) parameter of the light–dark test on chromosome 1 (58 cM; LOD 4.79; $p = 0.002$). Within the female mice, we identified only one QTL for the

immobility time (s) in the forced swim test on chromosome 5 (40 cM; LOD 9.1; $p < 0.001$).

Selection of candidate genes

We found that all QTLs on chromosome 5 were located within the same chromosomal region (28–67 cM) with a common core interval (49–58 cM). Therefore, it is possible that a gene in this chromosomal region contributes to the genetic differences in stress responsivity across paradigms (Fig. 2).

To search for candidate genes in this genomic region, we selected the two QTLs exhibiting the best *p*-values: the time measurement from the light–dark test (49–60 cM) and the immobility measurement in the Porsolt forced swim test (41–58 cM). The QTL interval of forced swim test contains 478 annotated genes and the core QTL of anxiety-related tests contains 321 annotated genes.

For prioritization of these genes, we utilized the FunSimMat software with the 'susceptibility to unipolar depression' phenotype (no. 607478) as reference phenotype for the forced swim test and 'anxiety' phenotype (no. 607834) as reference for anxiety tests. Both references were obtained from the 'online Mendelian inheritance in man' (OMIM) database (http://www.ncbi.nlm.nih.gov/omim). These phenotypes matched best with the anxiety and depression-related behavior. Of the 321 annotated genes for anxiety tests, we obtained 670 UniProt codes and of the 478 genes for forced swim test we identified 941 UniProt codes, which were both used for the prioritization software. After prioritization, we selected the genes whose similarity scores were above our defined threshold as possible candidates for further studies. For the anxiety tests, we detected 12 genes and for the forced swim test 24 genes with similarity to the corresponding reference genes (Table 2 and 3).

Most interestingly, we identified the gene enolase phosphatase 1 (*Enoph1*) in both prioritization lists. The *Enoph1* protein is part of the methionine salvage pathway, which is a ubiquitous pathway for the reconstitution of methionine. *Enoph1* catalyzes the transformation of 1-phospho 2,3-dioxomethiopentane to 1,2-dihydroxy 3-oxomethiopentene (Pirkov *et al.* 2008). We selected this gene as the most interesting one, since it opens the opportunity to reveal a common gene for anxiety as well as depression-related behavior.

Analysis of candidate gene

First, we compared the expression of *Enoph1* mRNA in different brain regions between the strains. We found significantly higher expression levels of *Enoph1* in cingulate cortex ($p < 0.01$), hippocampus ($p < 0.01$) and hypothalamus ($p < 0.05$) of C3H/HeJ mice compared to C57BL/6J mice. In the amygdala, we found no differences in *Enoph1* expression between the parental strains (Fig. 3a).

Enoph1 immunoreactivity is largely homogenous throughout the brain with only moderate, albeit significant ($F_{5,29} = 22.58$; $p < 0.001$) differences between brain areas. Thus, Enoph1 protein levels were approximately 25% lower in the hippocampus and amygdala compared to cortex, thalamus or hypothalamus. As a next step, we investigated Enoph1 protein levels in stressed and unstressed animals of both parental strains. Two-way ANOVA revealed a significant effect for stress ($F1,20 = 10.45$; $p < 0.01$) and genotype ($F1,20 = 6.241$; $p < 0.05$). The interaction between stress and genotype just failed to reach the level of significance ($F1,20 = 4.328$; $p = 0.0506$). In good agreement with the mRNA data, expression of the 27–28 kDa Enoph1 protein was significantly lower in control (unstressed) C57BL/6J animals compared to C3H/HeJ mice. Stress exposure significantly increased Enoph1 levels in C57BL/6J, but not in C3H/HeJ animals, therefore the difference between the genotypes disappeared after stress (Fig. 3b and c). Please

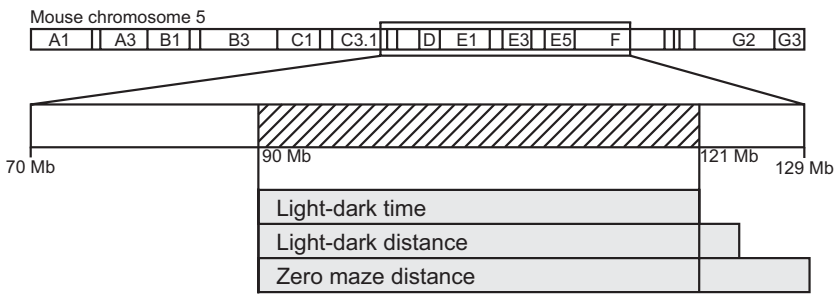

Fig. 2 Narrowing of the Quantitative Trait Loci (QTL) interval on chromosome 5. The white bar shows the magnification of the region on chromosome 5 where most of the QTL intervals were found. The light gray bars represent the found QTL intervals on chromosome 5 with the corresponding trait. The dark gray bar indicates a syntenic region (73.9–105 Mb), homolog to a human region linked to agoraphobia and panic disorders (human chr. 4 52.7–89 Mb). The intervals were stacked on top of each other to get their common intersection representing the narrowed QTL interval (shaded area).

Table 2 Prioritization of candidate genes for the anxiety QTL

Genome Coordinates (strand)	Symbol	Name	BP simRel	BP Lin	rfunSim
101108503–101148735 (–)	Hpse	heparanase	0.96	1.00	0.73
115592990–115608188 (–)	Mlec	malectin	0.96	1.00	0.68
115461146–115548799 (+)	Sppl3	signal peptide peptidase 3	0.75	0.75	0.53
101083742–101104159 (–)	Coq2	coenzyme Q2 homolog, prenyltransferase	0.60	0.61	0.42
115729675–115746981 (+)	Coq5	coenzyme Q5 homolog, methyltransferase	0.56	0.57	0.40
114881043–114894068 (–)	Mmab	methylmalonic aciduria type B homolog	0.55	0.56	0.40
114222757–114228297 (+)	Iscu	IscU iron-sulfur cluster scaffold homolog	0.52	0.53	0.52
100469013–100497781 (+)	Enoph1	enolase-phosphatase 1	0.48	0.53	0.43
110425540–110439430 (+)	Zfp932	zinc finger protein 932	0.33	0.37	0.50
110103564–110120060 (–)	5430403G16Rik	RIKEN cDNA 5430403G16 gene	0.33	0.37	0.50
110352419–110372955 (+)	Gm15446	predicted gene 15446	0.33	0.37	0.50
105242366–105246371 (–)	Zfp951	zinc finger protein 951	0.33	0.37	0.50

BP simRel, biological process similarity with simRel measure; BP Lin, biological process similarity with Lin's measure; rfunSim, calculation of biological process and molecular function scores.

Table 3 Prioritization of candidate genes for the depression QTL

Genome Coordinates (strand)	Symbol	Name	BP simRel	BP Lin	rfunSim
77342274–77380603 (–)	Ppat	phosphoribosyl pyrophosphate amidotransferase	0.65	0.66	0.46
104418781–104450938 (–)	Hsd17b11	hydroxysteroid (17-beta) dehydrogenase 11	0.64	1.00	0.58
104384459–104406429 (–)	Hsd17b13	hydroxysteroid (17-beta) dehydrogenase 13	0.64	1.00	0.58
114453712–114475458 (+)	Dao	D-amino acid oxidase	0.59	0.83	0.52
91360222–91450386 (+)	Mthfd2 l	methylenetetrahydrofolate dehydrogenase 2-like	0.56	0.86	0.44
83707170–83784220 (–)	Tecrl	trans-2,3-enoyl-CoA reductase-like	0.52	0.62	0.64
112814468–112821233 (–)	Asphd2	aspartate beta-hydroxylase domain containing 2	0.51	0.60	0.60
115560308–115569355 (–)	Acads	acyl-Coenzyme A dehydrogenase, short chain	0.50	0.69	0.51
77302684–77334539 (–)	Aasdh	aminoadipate-semialdehyde dehydrogenase	0.50	0.69	0.50
114596544–114700770 (+)	Acacb	acetyl-Coenzyme A carboxylase beta	0.49	0.51	0.40
100469013–100497781 (+)	Enoph1	enolase-phosphatase 1	0.49	0.66	0.41
97811243–97821349 (–)	Naa11	N(alpha)-acetyltransferase 11	0.40	1.00	0.29
107860568–107864058 (+)	Lpcat2b	lysophosphatidylcholine acyltransferase 2B	0.40	1.00	0.33
108741628–108778374 (+)	Pigg	phosphatidylinositol glycan anchor biosynthesis, class G	0.40	1.00	0.32
87494952–87521580 (–)	Ugt2b36	UDP glucuronosyltransferase 2 family, polypeptide B36	0.40	1.00	0.29
87553985–87569343 (–)	Ugt2b5	UDP glucuronosyltransferase 2 family, polypeptide B5	0.40	1.00	0.29
87429885–87440699 (+)	Ugt2b35	UDP glucuronosyltransferase 2 family, polypeptide B35	0.40	1.00	0.29
87753997–87766220 (–)	Ugt2a3	UDP glucuronosyltransferase 2 family, polypeptide A3	0.40	1.00	0.29
87318792–87335962 (–)	Ugt2b34	UDP glucuronosyltransferase 2 family, polypeptide B34	0.40	1.00	0.29
87345663–87355555 (–)	Ugt2b1	UDP glucuronosyltransferase 2 family, polypeptide B1	0.40	1.00	0.29
87669518–87683829 (–)	Ugt2b37	UDP glucuronosyltransferase 2 family, polypeptide B37	0.40	1.00	0.29
87838967–87853228 (–)	Ugt2b38	UDP glucuronosyltransferase 2 family, polypeptide B38	0.40	1.00	0.29
114573935–114578227 (–)	Alkbh2	alkB, alkylation repair homolog 2	0.36	0.47	0.51
118231041–118408849 (+)	Nos1	nitric oxide synthase 1, neuronal	0.36	0.46	0.55

BP simRel, biological process similarity with simRel measure; BP Lin, biological process similarity with Lin's measure; rfunSim, calculation of biological process and molecular function scores.

note that C57BL/6J animals also expressed a 22–23 kDa protein cross-reacting with our antibody.

Enoph1 contains two non-synonymous SNPs in exon 2 (rs13460000) and exon 3 (rs13460001) that result in a functionally relevant amino acid exchange. We therefore also conducted a SNP analysis of the two parental strains. Both SNPs co-segregated in the parental strains. All C57BL/6J mice carried a guanine in the first and a cytosine in the second position (Fig. 4), while the C3H/HeJ strain carried an adenine in the first and a thymine in the second position

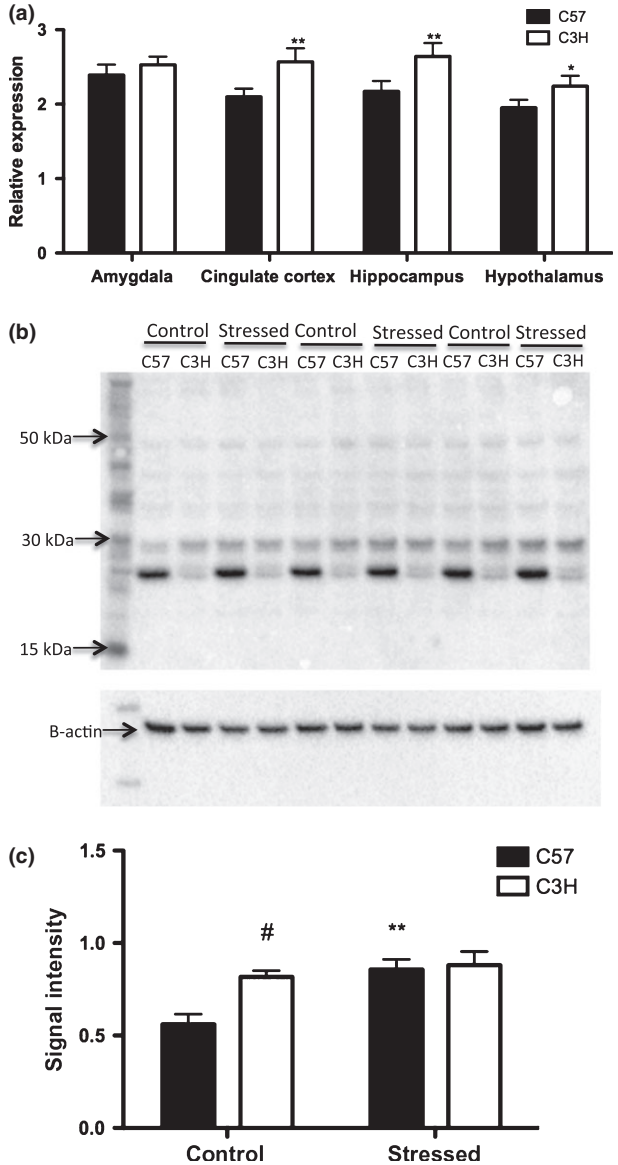

Fig. 3 (a) Expression of Enoph1. The relative expression levels were calculated using the $2^{-\Delta C(t)}$ method with the TATA box binding protein as the reference gene. The relative expression levels are shown on the *y*-axis and the different brain regions were presented on the *x*-axis. Bars indicate the mean of 10 individuals ± SEM, $*p < 0.05$; $**p < 0.01$; $n = 10$; C57 = C57BL/6J; C3H = C3H/HeJ. (b) Representative image of a western blot using brain homogenates from stressed and unstressed animals of both strains. (c) Densitometric analysis of Enoph1 signal intensities normalized to β-actin. Bars indicate the mean of six individuals ± SEM, $\#p < 0.05$; significant difference between C57BL/6J and C3H/HeJ control mice. $**p < 0.01$ significant difference between unstressed control and stressed C57BL/6J mice. Both two-way ANOVA followed by Bonferroni test. C57 = C57BL/6J; C3H = C3H/HeJ.

(Fig. 4). Thus, the *Enoph1* protein of C57BL/6J mice carried a valine in the position 56, where C3H/HeJ had an isoleucine. The second SNP results in a proline at position

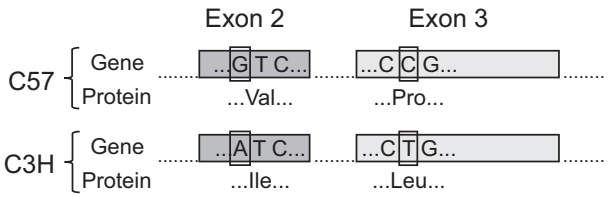

Fig. 4 Single nucleotide polymorphisms (SNPs) of the Enoph1 gene. Comparisons of the genomic sequences of C57BL/6J (C57) and C3H/HeJ (C3H) mice are shown. The two SNPs are marked in the coding triplets at the end of exon 2 and beginning of exon 3. The resulting non-synonymous amino-acid substitutions in the EP protein are shown beneath each triplet. SNP rs13460000 causes an amino acid change of isoleucine (Ile) to valine (Val) in position 56 of the protein; SNP rs13460001 causes an amino acid change of leucine (Leu) to proline (Pro) in position 74 of the protein.

74 in the C57BL/6J strain and a leucine in the C3H/HeJ strain.

The Enoph1 protein is part of the methionine salvage pathway, a ubiquitous pathway for the reconstitution of methionine, which is also connected to polyamine synthesis, where SAM serves as a substrate for polyamine synthesis. We therefore investigated the levels of SAM and polyamines by HPLC analysis (Fig. 5). SAM and spermine levels did not differ in brain tissues of parental mice. However, spermidine levels were significantly ($p < 0.05$) elevated in the C3H/HeJ strain.

Discussion

Anxiety and depression are heterogeneous and complex diseases with a high rate of comorbidity (Hettema 2008; Mosing *et al.* 2009). Several studies have revealed polymorphisms in a number of genes including brain derived neurotrophic factor, D-amino acid oxidase activator, catechol-O-methyl transferase (*COMT*), serotonin transporter (*5HTT*) and others, which may contribute to the etiology of depression (Craddock and Forty 2006; Kato 2007). For anxiety disorders, it has been more difficult to pinpoint specific susceptibility genes (Hovatta and Barlow 2008), but twin studies indicated that shared genetic risk factors largely account for the comorbidity of these diseases (Hettema 2008). In this study, we identified three candidate genes on chromosome 5 associated with emotional behaviors in mice that are relevant to anxiety and depression.

We confirmed several genetic regions identified in previous studies of anxiety and activity in mice. Thus, the interval identified on chromosome 1 ranging from 46 to 69 cM for the light–dark *distance* parameter is a well-known activity QTL, which was revealed by several previous studies (Kelly *et al.* 2003; Turri *et al.* 2004; Gill and Boyle 2005; Singer *et al.* 2005). This QTL seems to be sex dependent, because we detected this locus only in male mice. Another QTL was

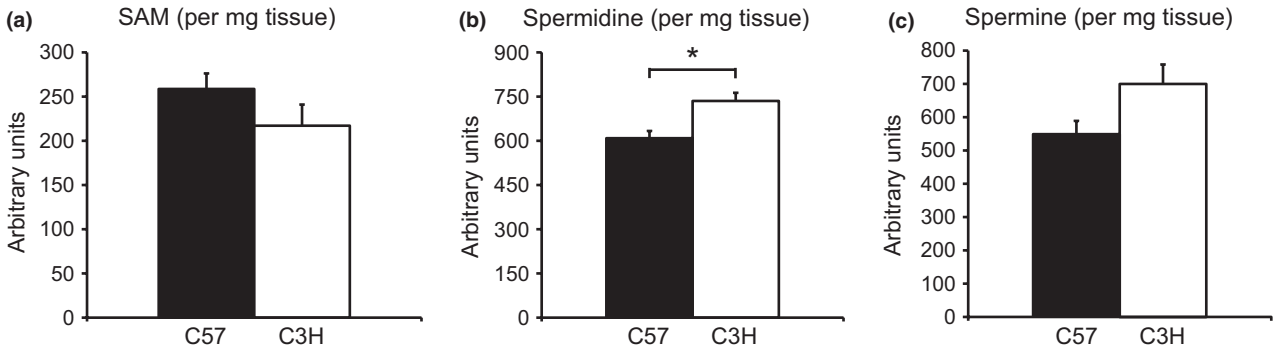

Fig. 5 S-adenosylmethionine (SAM) and polyamine assays of C57BL/6J (C57) and C3H/HeJ (C3H) brain tissues. SAM (a) was measured in brain homogenates by high-performance liquid chromatography (HPLC) analysis. spermidine (b) and spermine (c) levels were assessed by HPLC after derivatization with benzoyl chloride. Measurements were done in arbitrary units per mg tissue. Spermidine differences are statistically significant (*$p < 0.05$). Bars represent mean ± SEM; $n = 5$.

detected for the zero maze *time* value on chromosome 15. Similar results were obtained by Turri *et al.* (2001), who also found a QTL at this position for anxiety-related behaviors in the open-field test, the light–dark test and in the elevated plus maze.

Most interestingly, we identified a new QTL interval on chromosome 5 with high LOD scores in several anxiety and depression-related traits (light–dark-, zero-maze-, and forced swim tests). This region on mouse chromosome 5 is homologous with a region on the human chromosome 4, which is associated with agoraphobia and panic disorder (Kaabi *et al.* 2006). Although several approaches have been developed for the identification of candidate genes in QTL, this task remains a major challenge if the number of genes in a QTL is large. For the selection of possible candidate genes we applied the FunSimMat tool, developed Schlicker and Albrecht (2010). This innovative method has recently been described to enhance the selection of possible candidate genes on the basis of similarity in biomedical ontologies and is based on comparisons of gene ontologies (GOs). These ontologies grew rapidly in coverage, formality and integration in the last years making them suitable for the implementation in bioinformatics approaches (Pesquita *et al.* 2009). GOs of candidate genes are compared to those of a known disease gene and the software generates a list of genes prioritized in their functional similarity to the disease gene (Schlicker and Albrecht 2010). This approach offers an enormous potential for the detection of candidate genes.

The software compared all three subtypes of GOs and used four different algorithms for similarity measures. The selection of appropriate scores and values were done according to suggestions of the software developer A. Schlicker (personal communication) and all genes above the relevance threshold were considered as candidate genes. The *TPH2* gene was selected as the reference gene in our analysis, since it is correlated with unipolar depression in the OMIM database that is comparable to the depression-related trait with our strongest QTL. With the prioritization we identified *Enoph1* in both QTL intervals studied. A locus in close vicinity to ENOPH1 (4q21.21) was associated with bipolar disorder in a genome-wide association study in the Japanese population (Hattori *et al.* 2009).

There are several lines of evidence that further support Enoph1 as a strong candidate gene in this QTL. First, it was recently associated with the anxiety phenotype of high and low anxiety-related behavior mouse lines (HAB and LAB) (Ditzen *et al.* 2010). Second, the gene is differentially expressed in the parental strains in many brain regions involved in stress responses, such as the cingulate cortex, the hippocampus, and the hypothalamus. Third, in the parental strains we identified two co-segregating SNPs, which cause amino acid exchanges and affect protein function. The first SNP, which is located at the end of exon 2 (rs13460000), encodes Val56 in C57BL/6J mice and Ile56 in C3H/HeJ mice. The second SNP at the beginning of exon 3 (rs13460001) of the Enoph1 gene is translated into Pro74 in the C57BL/6J strain and Leu74 in the C3H/HeJ strain. Ditzen *et al.* (2006); Ditzen *et al.* (2010) also demonstrated that the amino acid exchanges led to different activities of the protein. The more active form is presented in the less stress-sensitive C3H/HeJ mice. This is in complete agreement with the high-activity form being present in LAB mice and the low-activity form in HAB mice (Ditzen *et al.* 2010). In good agreement with the gene expression data, we found significantly higher Enoph1 protein levels in animals from the C3H/HeJ strain. Importantly, stress enhanced Enoph1 levels in the brain, particularly in the low-expressing C57BL/6J strain. Thus, stressed C57BL/6J mice have similar Enoph1 levels as unstressed C3H/HeJ animals. Forth, we found significantly lower spermidine levels in the brains of C57BL/6J mice, which is in agreement with the reduced *Enoph1* activity in this strain. *Enoph1* is an enzyme of the methionine salvage pathway, which is the source for SAM (Pirkov *et al.* 2008). SAM in turn is required for the synthesis of

polyamines. Spermidine, like other polyamines, has been associated with stress and anxiety in human (Bonini *et al.* 2011) and animal studies (Gomes *et al.* 2010). SAM exhibits antidepressive effects in patients (Mischoulon and Fava 2002). It is surprising that only spermidine, but not spermine levels differed between the two strains, because SAM is thought to be involved in the biosynthesis of both polyamines. Clearly, some other unknown factors seem to regulate the levels of these polyamines.

Polyamine levels also seem to be regulated by stress and might play a role in depression. Thus, spermidine levels were reduced in a rat model of depression (Genedani *et al.* 2001), but other studies detected no changes or even increased spermidine levels with a restraint stress model in mice (Hayashi *et al.* 2004; Lee *et al.* 2006) and in human suicide victims suffering from depression (Chen *et al.* 2010). These disparate results show that, despite the fact that all these studies confirm the connection of polyamines with stress and especially with depression, their exact role in this context and how they are regulated remains to be elucidated.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. Distribution of animal behavior in F2 mice.

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