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The Neuronal Transporter Gene *SLC6A15* Confers Risk to Major Depression

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SUMMARY

Major depression (MD) is one of the most prevalent psychiatric disorders and a leading cause of loss in work productivity. A combination of genetic and environmental risk factors probably contributes to MD. We present data from a genome-wide association study revealing a neuron-specific neutral amino acid transporter (*SLC6A15*) as a susceptibility gene for MD. Risk allele carrier status in humans and chronic stress in mice were associated with a down-regulation of the expression of this gene in the hippocampus, a brain region implicated in the pathophysiology of MD. The same polymorphisms also showed associations with alterations in hippocampal volume and neuronal integrity. Thus, decreased *SLC6A15* expression, due to genetic or environmental factors, might alter neuronal circuits related to the susceptibility for MD. Our convergent data from human genetics, expression studies, brain imaging, and animal models suggest a pathophysiological mechanism for MD that may be accessible to drug targeting.

INTRODUCTION

Major depression (MD) is a common psychiatric disorder with a lifetime prevalence rate of 15%–17% (95% confidence interval [CI]) (Ebmeier et al., 2006). It is not only a potentially fatal disease with about 2% of patients committing suicide (Bostwick and Pankratz, 2000) but also one of the leading causes worldwide for loss in work productivity (Ebmeier et al., 2006; Ustün et al., 2004). Current treatments are indispensable but their clinical efficacy is still unsatisfactory, as reflected by high rates of treatment resistance and side effects (Fava and Rush, 2006). Identification of mechanisms causing depression is pertinent for discovery of better antidepressants. The heritability of this disorder has been estimated to range from 34%–42% (95% CI) (Ebmeier et al., 2006) and several attempts to identify susceptibility genes by linkage and candidate gene approaches have been undertaken. In candidate gene studies, *BDNF*, *SLC6A4*, *ACE*, *P2RX7*, *TPH2*, *PDE9A*, *PDE11A*, *DISC1*, and *GRIK3* have been reported to be associated with the disease (Levinson, 2006). Only a few of these initial reports have been confirmed by subsequent studies or in meta-analyses. In the last years, the first genome-wide association (GWA) case-control studies in MD were published. None reported genome-wide significant results, and their top hits were difficult to replicate (Lewis et al., 2010; Muglia et al., 2010; Rietschel et al., 2010; Shi et al., 2011; Sullivan

et al., 2009; Wray et al., 2010). Phenotypic diversity and genetic heterogeneity as well as a considerable environmental contribution inherent to MD have been considered to represent major obstacles for the identification of causative variants.

Here we present results of a GWA case-control study in a stringently selected sample of MD inpatients of a tertiary clinic in Munich, Germany, and matched controls devoid of any lifetime psychiatric diagnoses (n = 353/366) recruited for the Munich Antidepressant Response Signature (MARS) study (Hennings et al., 2009; Ising et al., 2009). We performed replication of the results of the GWAS in six additional independent samples of German, Dutch, United Kingdom (UK), and African American origin (Binder et al., 2008; Choy et al., 2009; Hofman et al., 2007; Lewis et al., 2010; Muglia et al., 2010; Rietschel et al., 2010). The herein reported association results are based on an overall sample size of 15,089 unrelated individuals.

To further characterize the functional relevance of the identified locus, we analyzed genotypic influences of associated SNPs on premortem human hippocampus and lymphoblastoid cell line expression profiles. We also employed in vivo high-resolution structural magnetic resonance imaging (MRI) and proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) with a focus on the hippocampal formation. We selected this brain region based on our gene expression results and because decreased neuronal integrity in this brain region had previously been identified as a risk factor for major depression (Frodl et al., 2002). Moreover, we investigated a possible role of the candidate locus in mediating stress vulnerability by interrogating its hippocampal expression in a well-established mouse model of chronic social stress (Schmidt et al., 2007) as chronic stress represents an established risk factor for MD (Wang, 2005).

RESULTS

SNPs on 12q21.31 Are Associated with MD

We performed a GWA study in a sample of 353 unipolar depressed German inpatients from the MARS study (Hennings et al., 2009) and 366 screened controls using Illumina 100k and 300k Beadchips (Manhattan plot, see Figure S2 available online). After applying stringent quality-control criteria (see Experimental Procedures), 365,676 SNPs entered association analysis. Neither genomic controls nor Eigenstrat showed evidence for population stratification in this sample (Figure S1). The common SNP rs1545843 (MAF = 0.41 in controls) on chr12q21.31 showed experiment-wide significance in a recessive mode of inheritance (AA versus AG+GG) after applying the permutation-based minimum p method for multiple comparison correction over all tested SNPs and genetic models (Table 1, Figure 1B, and Figure S2; n = 353/366, nominal p = 5.53e-08; OR = 2.84 [95% CI 1.92–4.21]). Seven additional common SNPs in linkage disequilibrium (LD) with rs1545843 located in a region spanning about 450 kb gave nominal p values smaller than 5.0e-04 applying the recessive model (Table 1, Figure 1B, and Figure S2). The pairwise r^2 values ranged from 0.40 to > 0.99 in controls (Figure 2A and Figure S2A), suggesting that all eight SNPs might tag the same underlying causative variant. In fact, rs1545843 and rs1031681 can be used as tagging SNPs for the associated variants within this locus in Europeans and fall into two separate bins, with an interbin r squared of 0.67.

We then genotyped the genome-wide significant SNP (rs1545843) of the GWA study together with seven to nineteen SNPs in LD within this locus in five independent samples. These comprised three German case-control samples, including two samples for which GWA data have been published (Muglia et al., 2010; Rietschel et al., 2010). The German samples consist of patients with recurrent MD and matched controls screened for the absence of lifetime anxiety and mood disorders recruited in Southern Germany (n = 920/1024) (Muglia et al., 2010), patients with major depression and controls recruited around the German city of Bonn (n = 292/1155), as well as patients and controls recruited as a follow-up of the discovery sample (n = 300/236). In addition, the association was tested in a sample from the Netherlands. In the Erasmus Rucphen Family (ERF) study subsample (n = 1160) (Choy et al., 2009), symptoms of depression during the past week were assessed using the Center for Epidemiologic Studies Depression Scale (CES-D) and the depression subscale of the Hospital Anxiety and Depression Scale (HADS-D). To create a proxy for case/control status, we compared the individuals rating in the upper depression scale quartile (CES-D $\geq$ 16.0: cases, indicative of a depressive disorder [Luijendijk et al., 2008]) with those rating in the lower quartile (CES-D $\leq$ 3: controls). Finally, we tested for association of the identified locus in a cross-sectional study of African-American subjects with significant levels of trauma recruited in the waiting rooms of an urban public hospital in Atlanta (n = 991) (Binder et al., 2008). Depression was rated by using the quantitative Beck Depression Inventory (BDI). In contrast to populations of European descent these SNPs displayed much less LD among each other (Figure 2B). For this study, we also created a proxy for case-control status. As BDI scores higher than 16 are equated to clinically relevant symptoms of current MD (Viinamäki et al., 2004), we divided the sample at this cutoff for a case-control analysis.

Table 1 shows the results of the association in all six samples for rs1545843 as well as two SNPs in moderate LD with it, rs1031681 and rs7975057. Testing the recessive model of rs1545843, we observed nominally significant association in four of the five replication samples, with the same direction of the effect in all samples. A meta-analysis conducted across all samples resulted in a genome-wide significant association with a p value of 2.34e-08 (4.37e-08 corrected for three tested genetic models) for the recessive model of rs1545843 (see Table 1). Homozygote carriers of the A-allele of this SNP had a 1.42-fold-higher risk to suffer from depression and depressive symptoms compared to carriers of the two other genotypes.

To replicate the genome-wide significant association of increased risk for depression in homozygous carriers of the A-allele of rs1545843, we performed an additional replication study with the UK cases and controls of the RADIANT study (Lewis et al., 2010) and added the WTCCC2 control cohorts. This resulted in a cohort of 1636 cases with recurrent unipolar depression and 7246 controls. An analysis using logistic regression showed significant evidence both for an effect of the AA genotype on risk in the same direction as in the other studies (OR = 1.344, 95% CI 1.080-1.672, p = 0.008) as well as for an

Table 1. Association Results of the Discovery GWAS and the First Round of Replication

SNP (Position on chr12, hg18)	Alleles[a]	MARS Study (GWAS SAMPLE)			Southern German Recurrent Depression Replication Sample			MARS Study Replication Sample			Bonn Replication Sample (West Germany)			ERF Study (Dutch Replication Sample)			EMORY Study (African-Americans)			Meta Analysis		
		n	p	OR	N	p	OR	N	p	OR	n	p	OR	n	p	OR	n	p	OR	n	p	OR
		Co	Allelic	Allelic	Co	Allelic	Allelic	Co	Allelic	Allelic	Co	Allelic	Allelic	Co	Allelic	Allelic	Co	Allelic	Allelic	Co	Allelic	Allelic
		Ca	Rec	Rec	Ca	Rec	Rec	Ca	Rec	Rec	Ca	Rec	Rec	Ca	Rec	Rec	Ca	Rec	Rec	Ca	Rec	Rec
		All	Dom	Dom	All	Dom	Dom	All	Dom	Dom	All	Dom	Dom	All	Dom	Dom	All	Dom	Dom	All	Dom	Dom
rs1545843 (83088199)																						
		366	6.0E-05	1.55	1022	3.3E-03	1.19	236	1.3E-01	1.15	1157	4.4E-01	0.99	290	6.8E-02	1.19	684	6.8E-03	1.29	**3755**	**1.9E-06**	**1.20**
	A/G	353	5.5E-08	2.85	917	1.6E-02	1.27	300	4.2E-02	1.47	292	1.4E-01	1.18	283	9.3E-03	1.62	307	2.9E-02	1.30	**2452**	**2.3E-08**	**1.42**
		719	1.8E-01	0.80	1939	1.1E-02	0.79	536	3.9E-01	0.95	1449	1.1E-01	1.19	573	4.3E-01	0.97	991	1.1E-02	0.59	**6207**	**2.9E-02**	**0.87**
rs7975057 (83288331)																						
		366	1.5E-03	1.42	998	1.4E-03	1.22	236	2.9E-02	1.27	1155	5.0E-01	1.00	290	4.1E-03	1.38	675	3.0E-02	1.20	**3720**	**4.1E-07**	**1.22**
	A/G	353	3.0E-05	2.43	898	3.2E-02	1.25	300	8.4E-03	1.82	291	1.7E-01	1.17	283	2.2E-03	1.92	299	3.6E-02	1.30	**2424**	**2.0E-07**	**1.43**
		719	1.7E-01	0.80	1896	1.6E-03	0.75	536	1.8E-01	0.84	1446	2.2E-01	1.11	573	4.9E-02	0.75	974	1.1E-01	0.80	**6144**	**1.3E-03**	**0.83**
rs1031681 (83444465)																						
		366	2.0E-03	1.41	1016	1.6E-03	1.21	236	3.3E-02	1.27	1130	3.8E-01	0.97	290	1.7E-03	1.42	681	3.2E-01	1.05	**3719**	**1.2E-05**	**1.18**
	A/G	353	2.5E-04	2.17	915	1.2E-02	1.31	300	3.0E-02	1.61	289	3.7E-01	1.05	283	1.4E-03	1.98	303	5.0E-01	1.00	**2443**	**4.7E-05**	**1.32**
		719	1.0E-01	0.77	1931	5.7E-03	0.78	536	1.1E-01	0.79	1419	2.4E-01	1.11	573	2.5E-02	0.71	984	2.1E-01	0.87	**6162**	**1.6E-03**	**0.83**

rs1545843 showed genome-wide significant association with MD in the discovery case-control GWAS (MARS) under a recessive model. This genome-wide significant association was confirmed in a subsequent meta-analysis over a total of six samples from five independent studies. rs1545843 and rs1031681 best tag the region of association with MD on chr12q.21.31 defined by eight SNPs in moderate to strong linkage disequilibrium with each other in Europeans (Figures 1 and 2). rs7975057 is shown as an example of a third SNP, which was one of the more consistently associated SNPs across all round 1 replication samples. Abbreviations: Allelic, additive allele dosage model (A versus G); Ca, cases; Co, controls; Dom, dominant model (GG versus AA+AG); hg18, human genome on UCSC build 18 (NCBI 36. 1); n, number of individuals; OR, odds ratio; p, nominal p value; Rec, recessive model (AA versus AG+GG).

[a] The allele shown in bold confers greater odds that the carrier is a case (risk allele for depression). The direction of association is consistent between samples. There is a minor-major allele switch between Europeans (minor allele: A, MAF in controls: 0.36–0.45) and African-Americans (minor allele G, MAF in controls: 0.35–0.46).

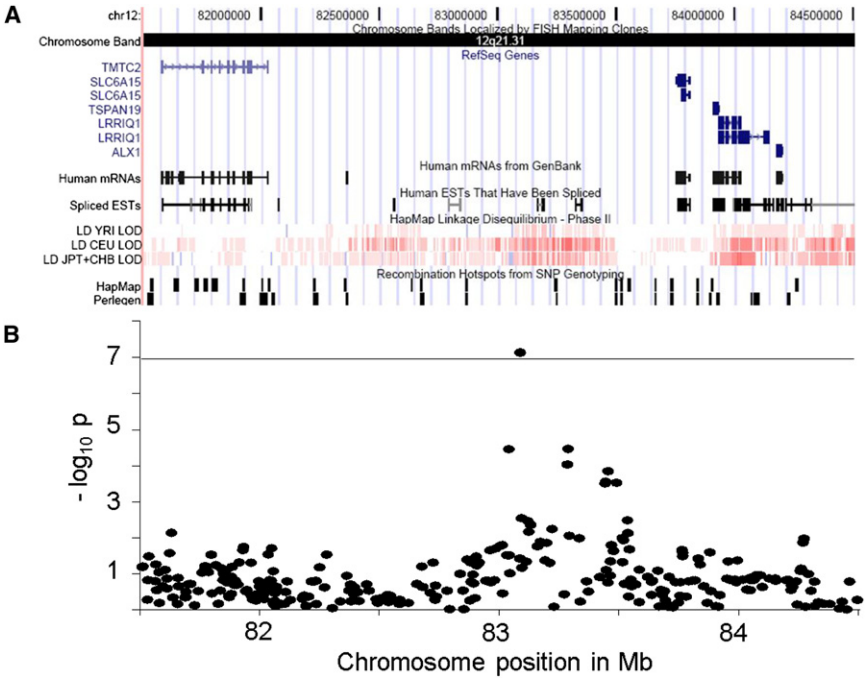

Figure 1. Genomic Context of the Associated Region on 12q21.31

(A) Relevant features of the genomic architecture of a 3 Mb region comprising the 450 kb region of association with MD according to the UCSC Genome Browser: RefSeq annotated genes (blue), human mRNAs and expressed sequence tags from GenBank (black), HapMap Linkage Disequilibrium (red: high LD, white: low LD), and hotspots of homologous recombination from SNP genotyping data provided by HapMap and Perlegen (black). The associated region did not map to any known gene (compare with Figure 2B). The flanking genes next to the region of association are *SLC6A15* (+287 kb), a solute carrier family 6 gene that codes for a sodium-dependent branched amino acid transporter with high gene expression in neurons of the brain, and *TMTC2* (−989 kb), the transmembrane and tetratricopeptide repeat containing 2 gene of unknown function (see also Table S1).

(B) The negative common logarithm ($-\log_{10}$) of the best model p values (y axis) of all tested SNPs in the shown region from genome-wide case-control association testing in the discovery sample were plotted against the SNPs' chromosome positions (x axis). The horizontal line across the figure indicates the experiment-wide significance level of the study. The dot above this line represents the $-\log_{10}$ p value of rs1545843. The corresponding Manhatten plot over all tested SNPs and chromosomes is shown in Figure S2.

interaction of sex with this effect (p = 0.0150). The RADIANT/WTCCC2 study was the only study showing such sex × genotype interaction on depression. A more detailed description of this association is given in the Supplemental Information section. Finally, when combining the corrected estimate for the genotypic effect of AA on depression in the RADIANT/WTCCC2 study with the effects in the previous studies, we arrive at an estimate of an OR = 1.398 (95% CI 1.254–1.557) with a combined two-sided p value of 1.41e-09 (Figure 3). Considering only the replication studies (thus excluding MARS), we have an estimate of 1.315 for the OR (95% CI 1.172–1.477) with a two-sided p value of 3.19e-06.

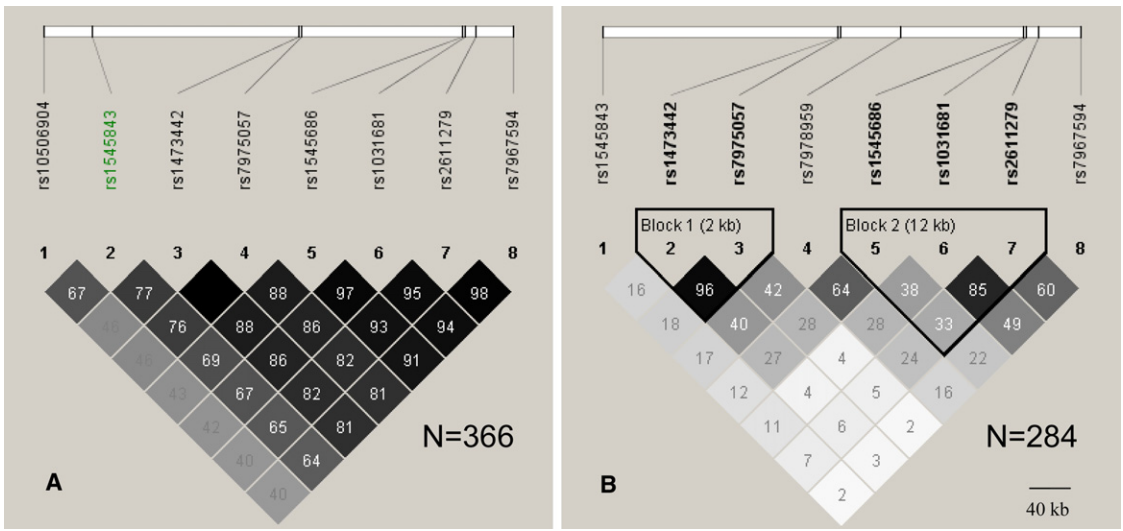

Figure 2. LD Structure of the Eight SNPs Associated with MD on 12q21.31

Presented in (A) German controls of the GWAS in MD (n = 366) and (B) in the African-American control sample (BDI < 14, n = 284). Pairwise r-squared values multiplied with 100 are shown for each SNP pair. rs1545843 (SNP 2), which reached experiment-wide significance in the GWAS, is in moderate LD with the other seven associated SNPs in Europeans but in low LD in African-Americans (SNP 1).

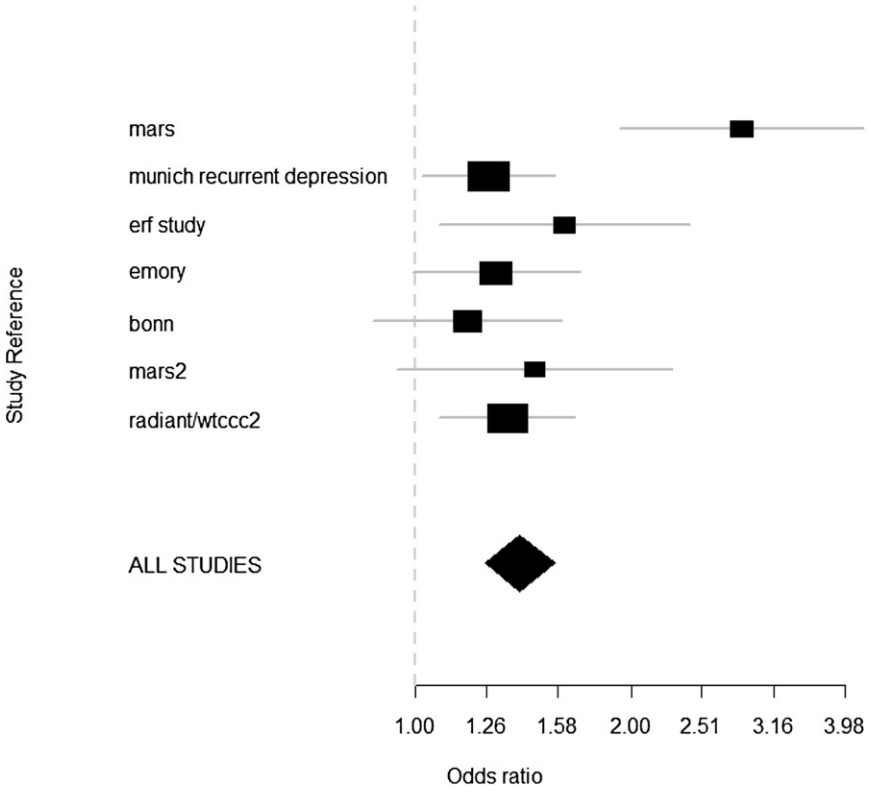

Figure 3. Forest Plot of the Combined Meta-Analysis over Six Independent Studies

rs1545843 remained genome-wide significantly associated with MD in the meta-analysis after replication round 1 under the recessive model (AA versus AG+GG, see Table 1). This association was further replicated in the RANDIANT/WTCCC2 sample. The combined meta-analysis p value over a total of seven samples was 1.41e-09. MARS: The Munich antidepressant response signature study, the German GWAS discovery case-control MD sample (n = 353/366). Munich recurrent depression: the Southern German recurrent depression and control replication sample (n = 917/1022). ERF: the Dutch Erasmus Rucphen Family study MD case-control subsample (n = 283/290). Emory: the African-American MD case-control subsample from Emory University in Atlanta (n = 307/684). Bonn: West German MD case-control replication sample (n = 292/1157) (Rietschel et al., 2010). MARS2: additional MD cases and controls from the MARS study that were recruited after the GWAS (n = 300/236). RADIANT/WTCCC2: UK cases and controls of the RADIANT study and additional controls from the WTCCC2 cohorts (n = 1636/7246).

While the association with major depression and depressive symptoms thus was consistent in samples across different ethnicities, this did not hold true for incident late-life depression. The association did not replicate in the Rotterdam study (n = 3512) (Hofman et al., 2007), where subjects older than 55 years of age and free of dementia were screened with the CES-D at baseline and two follow-up time points (Luijendijk et al., 2008). A case-control analysis was performed by comparing subjects who developed depressive disorders and depressive syndromes at follow up time points (n = 438) with individuals without clinically relevant depressive symptoms (n = 3074) (mean age ± standard deviation [SD] of cases: 72.7 ± 7.4 years and controls: 73.9 ± 8.3 years). None of the investigated SNPs reached significant association. The average age of 72 years at which the index depressive episode in the Rotterdam sample was diagnosed is substantially older than the average age in the combined German discovery sample and recurrent depression sample (50.4 ± 13.9 years), the Dutch ERF sample (48.7 ± 15.0 years), and the African-American sample (39.3 ± 13.7 years). In fact, in the other samples significant associations with rs1545843 were only observed when individuals $\leq$ 55 years were selected but not in the older age group. A series of studies indicate that late-life depression is pathophysiologically distinct from earlier onset MD being more strongly related to vascular disease and future cognitive impairment (Alexopoulos, 2006).

In summary, as shown in the forest plot in Figure 3, the initial GWAS revealed a SNP (rs1545843) on chr12q21.31 to be associated with MD with experiment-wide significance. This could be confirmed in a meta-analysis across six additional independent samples, including one sample of African-American heritage, with the recessive model of rs1545843 reaching genome-wide significance.

Genomic Context of the Associated Region on 12q21.31

The associated SNP lies within a region of SNPs in moderate LD that span a gene desert of about 450 kb in size on 12q21.31 mapping neither to any annotated gene nor to predicted human mRNAs with the exception of some small human expressed sequence tags (EST, Figure 1A and Table S1). The closest RefSeq annotated gene is *SLC6A15* (NM_182767), which ends 287 kb further distal to the region of association. It belongs to the solute carrier 6 (SLC6) gene family and codes for a sodium-dependent branched-chain amino acid transporter (Bröer, 2006). *SLC6A15* gene expression is highest in the human brain as well as the brain of other vertebrate species (UniGene, 2009; Allen Brain Atlas, 2008). In rodents and humans, *SLC6A15* expression has been shown to be restricted to neurons with strong expression in many brain regions including the hippocampus (Farmer et al., 2000; Masson et al., 1996). Other genes distal to *SLC6A15* are *TSPAN19*, *LRRIQ1*, and *ALX1* (Figure 1A). Their function is largely unknown and their expression levels are low in the vertebrate brain (UniGene, 2009). The nearest gene on the proximal side, transmembrane and tetratricopeptide repeat containing 2 gene (*TMTC2*, NM_152588), ends 989 kb from the region of association. It is expressed in a variety of tissues including the brain, but its function is also unknown. According

A

rs1545843 association with hippocampal *in cis* gene expression (N=137)

mRNA	P (AA vs. AG+GG)
SLC6A15 FL	0.00043*
ALX1	0.031
SLC6A15 S	0.084
TSPAN19	0.58
SLC6A15 FL/S	0.72
TMTC2	0.45
LRRIQ1	0.20

(* Bonferroni-corrected p<0.05)

B

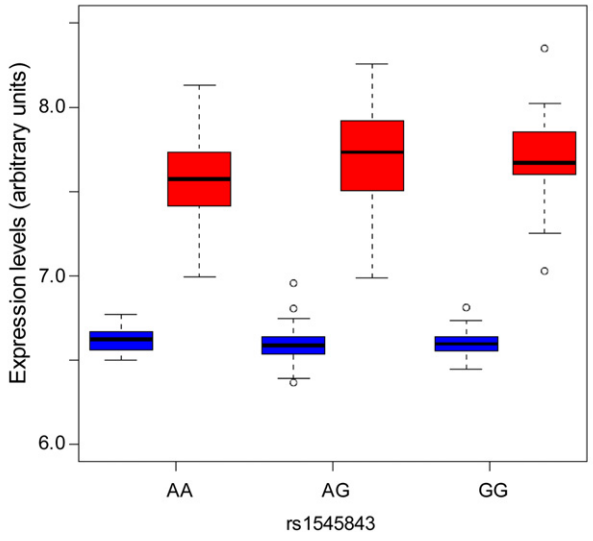

Figure 4. *SLC6A15* mRNA Expression per rs1545843 Genotype Group

Measured in premortem human hippocampus from individuals of European descent with temporal lobe epilepsy.

(A) The MD risk genotype (AA) is associated with reduced full-length (FL, red boxes in B) *SLC6A15* mRNA expression levels compared to the nonrisk genotypes (AG+GG). None of the other genes flanking the region of association with MD showed experiment-wide significant rs1545843 genotype-specific alterations in expression levels. *SLC6A15* S: short mRNA isoform of *SLC6A15*.

(B) Box plot diagrams of FL (red) and S (blue) *SLC6A15* mRNA expression levels in human hippocampus. On the x axis the three genotype groups of rs1545843 are plotted against normalized *SLC6A15* mRNA levels on the y axis (group means: solid horizontal lines). Blue box-plots depict the expression levels of the short *SLC6A15* isoform (S) and red plots expression levels of the full-length (FL) *SLC6A15* transcript. For results of an analogous eQTL analysis in lymphoblastoid cell lines of HapMap individuals see supplemental Figure S3.

to HapMap and Perlegen (Myers et al., 2005) genotyping data, several hotspots of homologous recombination are predicted between the associated region and the flanking genes (Figure 1A), making it unlikely that the underlying functional variant might directly hit a classical promoter region or the open reading frame of a known gene. However, long-range regulatory effects have been described (Kleinjan and van Heyningen, 2005). To address this issue, we analyzed genome-wide gene expression data sets of human hippocampus and lymphoblastoid cell lines (Stranger et al., 2005).

Gene Expression Studies Reveal *SLC6A15* as Putative Candidate Gene within the 12q21.31 Locus

We analyzed genome-wide Illumina expression array data on the locus associated with MD on 12q21.31 in a premortem human hippocampus expression study from individuals with temporal lobe epilepsy of European descent and gene expression from EBV-transformed lymphoblastoid cell lines of the 210 unrelated HapMap individuals of different human populations (CEU, CHB, JPT, YRB) (Stranger et al., 2005). Previous studies reported that the median distance between SNPs and genes whose mRNA expression is significantly regulated by them is approximately 30 kb, ranging up to a maximum of 1 Mb (Myers et al., 2007). We therefore assessed all five RefSeq annotated genes within 1.5 Mb proximal to and distal of rs1545843 on 12q21.31 (Figure 1A and Table S1, *TMTC2*, *SLC6A15*, *TSPAN19*, *LRRIQ1*, *ALX1*). Expression levels of all seven available probes (three for *SLC6A15*) were related to genotypes of two of the SNPs associated with MD which best tag the overall associated SNPs on 12q21.31 for European populations, rs1545843 and rs1031681 (Table 1). We tested the allelic and both alternative recessive-dominant genetic models of rs1545843 and rs1031681 and each probe and applied Bonferroni correction for the number of performed statistical tests. Both SNPs showed association only with the hippocampal expression of the full-length mRNA isoform of *SLC6A15* reaching experiment-wide significance under a recessive model of inheritance (AA versus AG+GG: rs1545843: p = 4.3e-04, corrected p = 1.8e-02, and rs1031681: p = 1.4e-04, corrected p = 6.6e-03, n = 137). Risk genotype carrier status was associated with less *SLC6A15* transcript (Figures 4A and 4B). These associations were supported by data from lymphoblastoid cell lines from the HapMap individuals where expression of the full-length *SLC6A15* transcript was lower in carriers of the depression risk genotypes (Figure S3) and in an expression data set from peripheral blood monocytes (Heinzen et al., 2008) but not in a frontal cortex expression study (Myers et al., 2007), probably due to the lower expression of this gene in this brain region. Thus, gene expression experiments, including hippocampus expression, point toward an effect of the associated locus on *SCL6A15* expression via long-range regulatory mechanisms (Kleinjan and van Heyningen, 2005).

Presumed Function of *SLC6A15*

SLC6A15 belongs to the solute carrier 6 (SLC6) gene family, which also includes the monoamine and gamma-amino butyric acid (GABA) transporters and codes for a sodium-dependent branched-chain amino acid transporter (Bröer, 2006). Experimental data from *SLC6A15* knockout mice indicate a moderate contribution of *SLC6A15* to total proline and leucine transport into cortical synaptosomes of about 15% (Drgonova et al., 2007). Proline, the amino acid with the highest affinity for *SLC6A15*, and leucine may act as precursors for glutamate synthesis (Broer et al., 2006), and this transporter could thus be involved in the regulation of glutamate transmission (Tapiero et al., 2002).

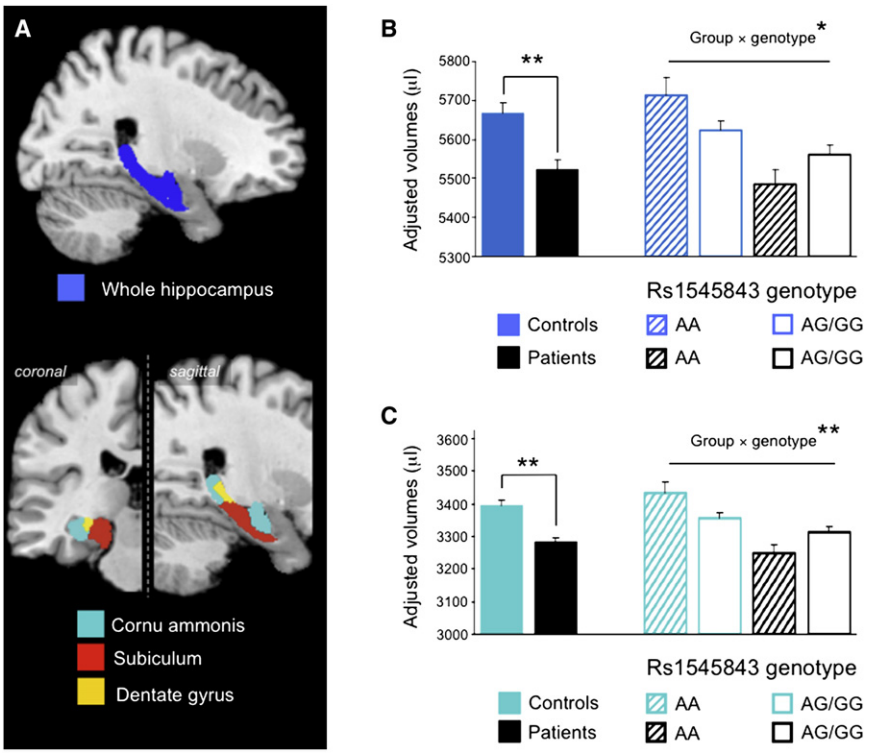

Figure 5. NMR Imaging: Genotype-by-Diagnosis Interaction Effects on Hippocampal Volume

(A) Based on cytoarchitectonic probability maps, automated volumetry of gray matter (GM) of the total hippocampus (cornu ammonis, subiculum and dentate gyrus) and respective subregions was performed in 390 subjects after optimized segmentation and coregistration. The resulting maximum probability maps projected on a standard brain template in atlas space are shown.

(B) Results of the left total hippocampal GM: bars show adjusted mean values and one standard error of the mean for the main effect of diagnosis and the rs1545843 genotype (AA versus AG/GG) × diagnosis interaction effect. Lowest mean volumes were seen for patients with the AA genotype.

(C) Corresponding depiction for the left cornu ammonis (*nominal $p < 0.05$, **Bonferroni corrected $p < 0.05$.). Results of other subregions and of right hemisphere are reported in Table S2.

Effects of Risk Genotypes on Hippocampal Volume and Neurochemistry

Due to the expression profile of *SLC6A15* and its presumed role in neuronal amino acid transport and glutamate synthesis (Bröer et al., 2006) and due to reported hippocampal volume changes in MD (Frodl et al., 2002; Videbech and Ravnkilde, 2004), we investigated both volumetric and ^{1}H-NMR-spectroscopy (^{1}H-NMR) markers of hippocampal integrity and signaling in subsamples of the Southern German discovery and replication samples (for sample see Supplemental Experimental Procedures).

We confirmed bilateral hippocampal volume reductions in recurrent depression ($F_{5,381} > 15.128$, $p < 1.2e\text{-}04$, $n = 204$, Table S2) and found a rs1545843 genotype × diagnosis interaction effect on both left and right total hippocampal volumes (left: group: case-control, genotypes AA versus AG/GG: $F_{5,381} = 5.861$, $p = 0.016$, right: $F_{5,381} = 5.686$, $p = 0.018$). Subregional analysis within the hippocampal formation revealed strongest effects for the bilateral cornu ammonis (CA) (left: group: case-control, genotypes AA versus AG/GG: $F_{5,381} = 9.512$, $p = 0.002$, $p_{corr} < 0.05$, right: $F_{5,381} = 5.686$, $p = 0.011$, $n = 204$ cases and 186 controls, Table S2). For rs1081681, which is highly correlated with rs1545843 in the MR morphology sample ($r = 0.819$), diagnosis × genotype interaction effects were even stronger with a similar emphasis on the left hemisphere and the CA region (Figure 5 and Table S2). No genotype or diagnosis × genotype effects were observed for either polymorphism for the dentate gyrus and the subiculum of the hippocampus and the control region (precentral gyrus). Hippocampal morphology is a heritable trait ($h^2 = 0.4$) (Sullivan et al., 2001); nonetheless, it is subject to stronger environmental influences compared to other brain regions (Glahn et al., 2007), and interactions between recurrent depression and specific genetic predispositions as indicated by our results may thus promote hippocampal atrophy, which has been repeatedly reported for MD (Videbech and Ravnkilde, 2004).

These analyses were complemented by analyzing ^{1}H-NMR markers of hippocampal integrity, including N-acetyl aspartate (NAA). While NAA serves mainly as a marker of neuronal viability, it is also regarded as a reservoir for glutamate (Benarroch, 2008). To investigate genotype effects of left hippocampal neurochemistry, we focused on healthy, nonmedicated control subjects ($n = 81$) as mood state and medication might influence hippocampal neurochemistry. Multivariate analysis detected a significant genotype effect of rs1031681 on hippocampal metabolites (Wilks' lambda: 0.683, $F_{2,75} = 2.976$, $p = 0.002$) with univariate comparisons pointing toward NAA ($F_{2,75} = 6.143$, $p = 0.003$, $p_{corr} < 0.05$). More specifically, A-risk-allele-carriers of rs1031681 showed lower levels of hippocampal NAA and glutamate/glutamine (Glx), indicating impaired neuronal integrity and Glx signaling already in healthy carriers (NAA: $F_{1,76} = 5.575$, $p = 0.021$; Glx: $F_{1,76} = 5.752$, $p = 0.019$; Cr: $F_{1,76} = 4.009$, $p = 0.049$, Figure S4B). For NAA, a similar effect was detected for A-carriers of rs1545843 ($F_{2,75} = 5.333$, $p = 0.024$).

The imaging data thus suggest that risk allele carrier status is associated with a decrease in hippocampal neuronal integrity already in healthy controls and that patients with recurrent major depression and the risk genotype experience an exacerbated reduction in hippocampal volume.

Evidence for a Role of *SLC6A15* in Stress Vulnerability

Epidemiological studies on MD report a 2- to 3-fold risk increase for individuals exposed to chronic stress (Wang, 2005), and twin

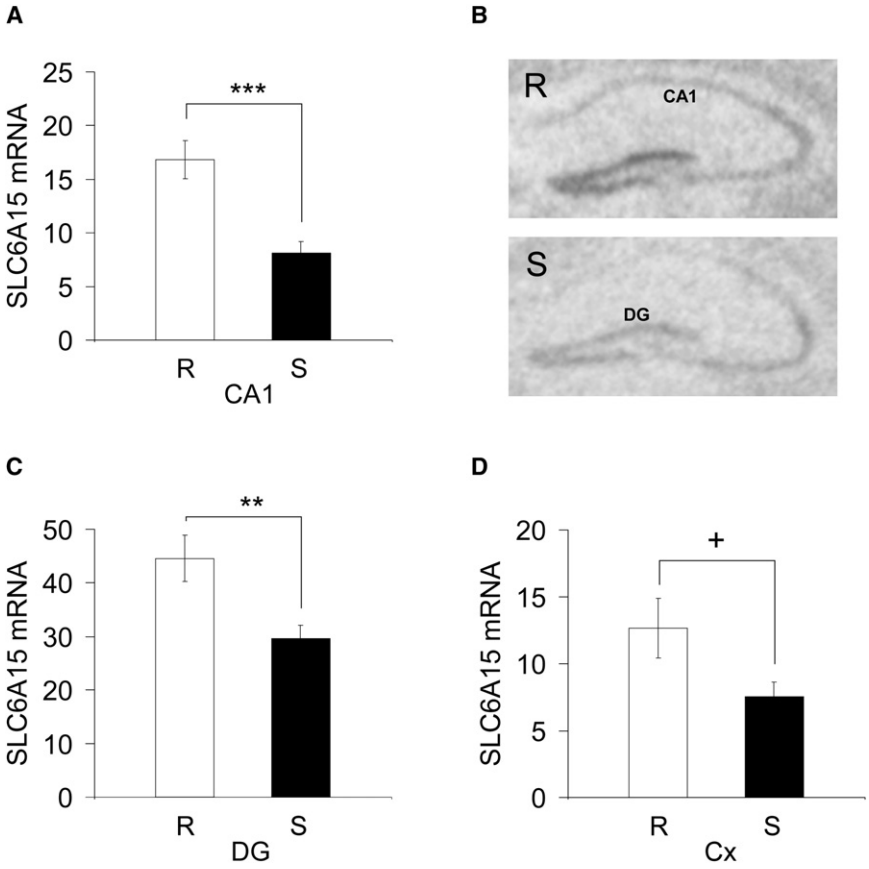

Figure 6. Reduced Hippocampal *SLC6A15* mRNA Expression in Stress-Susceptible Mice

(A) The significant reduction in *SLC6A15* mRNA levels in the CA1 hippocampal region between stress-resilient (R) and -susceptible (S) mice detected by microarray analysis could be confirmed by in situ hybridization (n = 9/9, −2.1-fold reduction).

(B) Two representative autoradiographs of hippocampal slices from one animal per group are shown.

(C and D) *SLC6A15* mRNA was also significantly reduced in the dentate gyrus (DG, −1.5-fold) and by trend reduced in the visual cortex (Cx, −1.7-fold). $^{+}p < 0.06$; $^{**}p < 0.01$; $^{***}p < 0.001$. See also Figure S5 for description of the mouse model and Table S3 for microarray results. Data are presented as mean volumes ± standard errors of the mean (SEM).

studies clearly point to an increased susceptibility for MD as a result of a combination of environmental and genetic risk factors (Kendler et al., 2002). To further validate a role for *SLC6A15* in MD, we used microarray gene-expression data from the hippocampus of mice subjected to chronic stress according to a recently developed and extensively validated mouse paradigm of chronic social stress in which susceptible animals show behavioral, endocrine, and molecular changes reminiscent of a depression-like phenotype (Schmidt et al., 2007, 2010) (Figure S5). We selected the six most susceptible and the six most resilient individuals from a formerly stressed group of 120 mice. Pooled mRNA samples of laser-assisted microdissections from the CA subregion 1 (CA1) of the hippocampus from both experimental groups (Supplemental Experimental Procedures) were analyzed on genome-wide Illumina BeadChips. Expression data for the probes specific for the genes in the associated region, *TMTC2*, *SLC6A15*, *LRRIQ1*, and *ALX1*, were compared between the two groups. *SLC6A15* mRNA levels were reduced 1.9-fold in the CA1 region in stress-susceptible versus stress-resilient mice. Expression levels of the other genes did not exceed background noise in the CA1 region and are thus probably not expressed at higher levels in this brain region (Table S3). This further supports *SLC6A15* as the gene of interest within this locus. The reduction of *SLC6A15* expression in CA1 could be validated by in situ hybridization in nine stress-susceptible versus nine stress-resilient mice (Figures 6A and 6B). Moreover, a significant reduction in *SLC6A15* expression could also be observed in the dentate gyrus of stress susceptible animals (Figures 6C and 6D). The demonstrated downregulation of *SLC6A15* expression in stress-susceptible mice, most prominent in the CA1 region of the hippocampus, suggests *SLC6A15* to play a role in long-term effects of chronic stress on neuronal circuits and is in accordance with the human MD risk genotype-dependent effects, assayed by in vivo volumetry, which were also strongest in the CA subregion of the hippocampus.

DISCUSSION

We performed a GWA study, with replication of the top hit and genome-wide significant association in the meta-analysis across a total of 4,088 patients and 11,001 controls, including one sample from a different ethnic background. Together with gene expression data, neuroimaging correlates and evidence from a mouse model of chronic stress our results point toward *SLC6A15*, a neuronal amino acid transporter, as a candidate gene in the pathophysiology of major depression.

Even though the direction of the association of rs1545843 with depression and depressive symptoms was the same in all samples with nongeriatric depression, the effect sizes were heterogeneous, with a much larger effect in the discovery sample (OR = 2.8 for the recessive model) as compared to the other samples with odds ratios ranging from 1.18 to 1.61. The strong association and low p value in the discovery sample is probably due to the "winners' curse," but this phenomenon has also been observed for other, now established, disease loci. For example, the association of a SNP in the *FGFR2* gene with breast cancer was much stronger in the rather small discovery sample than in any of the subsequent replication

samples. However, the direction of the association was consistent and reached a p value of 2e-76 in close to 30,000 cases and controls (Easton et al., 2007). This indicates that heterogeneous effect sizes with overestimation of the effect in a small discovery sample may still be in agreement with a true signal. In addition to the genome-wide significance (Dudbridge and Gusnanto, 2008) observed in our study, replication of the effect in samples of different ethnicities, European and African-American, might be a further indicator for a true effect.

In addition to replication in independent samples, the functional relevance of the associated locus is supported by results of gene expression analyses in premortem human hippocampus and EBV-transformed lymphoblastoid cell lines of the HapMap individuals (Stranger et al., 2005) and peripheral blood monocytes (Heinzen et al., 2008). While there is a strong indication of the regulatory relevance of the region associated with MD for *SLC6A15* expression, we cannot exclude that these variants might also influence the expression of six unspliced brain ESTs and four spliced ESTs described in nonbrain tissue that have been mapped to the region of association but were not probed by the used Illumina chip (Figure 1A and supplemental text, Table S1). Additional nonannotated transcripts, as described in the ENCODE pilot project in regions of the genome previously thought to be transcriptionally silent (Birney et al., 2007), might also be functionally relevant for this association.

The imaging genomics results provide evidence that the associated SNPs and related functional effects on *SLC6A15* expression might be of relevance for the integrity of brain neurocircuits shown to be important in MD (Frodl et al., 2002). We found lower total hippocampal volumes, particularly of the cornu ammonis, in risk genotype carriers of the patient—but not the control—group, indicating a higher vulnerability to the well-documented effects of recurrent depressive episodes on hippocampal volume (Frodl et al., 2002; Videbech and Ravnkilde, 2004). Further support for the detrimental effects of the risk allele on neuronal integrity in this brain region came from ^{1}H-NMR spectroscopy. We noted that healthy risk allele carriers exhibited lower hippocampal NAA compared to non-risk allele carriers. Reduced hippocampal NAA has been reported for different psychiatric disorders and was also decreased in currently depressed unipolar patients in this study (Figure S4b). In animal models, hippocampal NAA can be decreased by chronic stress (Czéh et al., 2001; Li et al., 2008). Thus, a genetic predisposition toward lower hippocampal NAA, similar to a condition induced by chronic stress experiments, may impair an individual's resilience to stress which is a risk factor for MD (Wang, 2005).

While the genetic association data pointed most strongly to rs1545843, gene expression and imaging data association were strong with both tag-SNPs of the locus, rs1545843 and rs1031681. In healthy subjects, genotype effects on hippocampal neurochemistry were more prominent for rs1031681 compared with rs1545843, both in terms of effects on NAA and Glx and in terms of robustness toward multiple test correction. This is an indication that both SNPs tag the likely underlying functional variants that still remains to be identified. To this aim, deep-sequencing analyses are currently underway.

Together with the demonstrated downregulation of *SLC6A15* expression in stress-susceptible mice, human gene expression and imaging data support a role for hippocampal *SLC6A15* function in stress sensitivity and the pathophysiology of MD. This would be in line with a proposed role of the *SLC6A15* transporters in neuronal metabolism and the provision of substrates for neurotransmitters, and specifically glutamate synthesis (Bröer et al., 2006). Thus, decreased hippocampal NAA and by extension glutamate neurotransmission (Benarroch, 2008), related to genetic factors, may limit excitatory signaling capacity with secondary effects on stress response regulation and hippocampal function in general (Herman et al., 2003).

In conclusion, the above presented results from human genetics, gene expression, volumetric imaging, spectroscopy, and a mouse model of chronic stress all support the notion that lower *SLC6A15* expression, especially in the hippocampus, could increase an individual's stress susceptibility by altering neuronal integrity and excitatory neurotransmission in this brain region. Recently, the prokaryotic leucine transporter homolog ($LeuT_{aa}$) of *SLC6A15* has been crystallized from Aquifex aeolicus and was shown to bind tricyclic antidepressant drugs that can directly block leucine transport by closing the molecular gate for the substrate in a noncompetitive manner (Zhou et al., 2007). Due to the high degree of phylogenetic conservation of the antidepressant binding site, these drugs probably also bind to the human transporter. Because *SLC6A15* appears amenable to drug targeting, our results may lead to the discovery of a novel class of antidepressant drugs.

EXPERIMENTAL PROCEDURES

MARS (GWAS) Sample

Three hundred and fifty-three unipolar depressive inpatients (155 males, 198 females) were recruited for the Munich Antidepressant Response Signature (MARS) project (Hennings et al., 2009; Ising et al., 2009) at the Max Planck Institute of Psychiatry (MPIP) in Munich, Germany. The mean age (±SD) was 49.5 ± 14.3 (males: 48.4 ± 13.4, females: 50.4 ± 15.0) years. See Hennings et al. (2009) and Ising et al. (2009) for more details on patient recruitment. Briefly, patients were included in the study within 1–3 days of admission to the hospital and diagnosis was ascertained according to the Diagnostic and Statistical Manual of Mental Disorders (DSM) IV criteria. Patients fulfilling the criteria for at least a moderate depressive episode (HAM-D $\geq$ 14 on the 21-item Hamilton Depression Rating Scale) entered the analysis. Patients suffered from a first depressive episode (36.8%) or from recurrent depressive disorder (63.2%). All included patients were of European descent and 88.7% were of German origin. Three hundred and sixty-six control subjects were matched to the patient sample for age, gender, and ethnicity from a randomly selected Munich-based community sample and underwent a strict screening procedure for the absence of psychiatric and severe somatic disease (Heck et al., 2009). The overall inclusion rate of all contacted probands was 50.3%. These subjects thus represent a group of individuals from the general population who have never been mentally ill. Age, gender, and ethnicity did not differ from the patient sample. This study has been approved by the ethics committee of the Ludwig-Maximilians-University (LMU) in Munich and written informed consent was obtained from all subjects.

The Southern German Recurrent Depression Replication Sample

This sample included 920 patients (302 males, 618 females) suffering from recurrent major depression (Lucae et al., 2006; Muglia et al., 2010) as well as 1024 controls matched to the patient sample for age, gender, and ethnicity.

The MARS Replication Sample

This sample included an additional 300 unipolar depressed patients and 236 controls, recruited according to the same protocol as the MARS discovery sample but not genotyped on the initial Illumina platforms.

The Bonn Replication Sample

This sample included patients with a DSM-IV diagnosis of major depression who were recruited from consecutive admissions to the Department of Psychiatry of the University of Bonn, Germany as described in Rietschel et al. (2010). Of the 604 individuals described in this publication, only the 292 without a family history of an axis I disorder other than major depression were used in this analysis. Population-based controls were recruited as described in Rietschel et al. (2010).

The Erasmus Rucphen Family Study Subsample

This subsample included 1160 participants from the Erasmus Rucphen Family (ERF) study, part of the Genetic Research in Isolated Population (GRIP) program (Aulchenko et al., 2004). The Center for Epidemiologic Studies Depression Rating Scale (CES-D) (Radloff, 1977; Zigmond and Snaith, 1983) (Spinhoven et al., 1997; Weissman et al., 1977) was used to define depression using a cutoff of CES-D $\geq$ 16 as indicative of a depressive disorder (Luijendijk et al., 2008).

The African-American Replication Sample

This sample included 972 African-Americans (356 males, 616 females) all screened with the Beck Depression Inventory (BDI) (Beck et al., 1961; Viinamäki et al., 2004). Study design, ascertainment, and rating protocols have been described elsewhere in more detail (Binder et al., 2008). A BDI score of 16 or greater was considered indicative of current depression.

The Rotterdam Study Subsample

This subsample included 7983 participants from the Rotterdam Study, a prospective cohort study from 1990 conducted in the Netherlands. All inhabitants aged 55 and over were eligible (Hofman et al., 2007). Depression was ascertained using the CES-D, a semistructured interview with the Present State Examination (PSE) by a clinician, and GP records and specialist letters.

The UK Replication Sample (RADIANT)

This sample included 1636 patients with a diagnosis of recurrent major depression (except for 20 with first episode) recruited within the Depression Case Control (DeCC) study, the Depression Network (DeNET) affected siblings linkage study, and the Genome-Based Therapeutics in Depression (GENDEP) study (Lewis et al., 2010). The matched screened controls described in Lewis et al. (2010) (n = 1594) and the publicly available controls from the Wellcome Trust Case Control Consortium 2 (n = 5652) were used for this analysis.

A more detailed description of the study samples can be found in the Supplemental Experimental Procedures.

SNP Genotyping

Genome-wide SNP genotyping for the MARS discovery sample was performed on Sentrix Human-1 (100k) and HumanHap300 (317k) Genotyping BeadChips (Illumina, San Diego, USA) according to the manufacturer's standard protocols. On the Illumina Human-1 Genotyping BeadChip about 109,000 exon-centric SNPs can be investigated. Nearly 25,000 of the loci are located in transcripts and more than 73,000 loci are within 10 kb of coding sequences. The Illumina HumanHap300 Genotyping BeadChip comprises about 317,000 SNPs. The average call rate achieved was higher than 99%, with samples below 98% being either retyped or excluded from the study. Genotyping of the German replication samples (except MARS replication) and the African-American replication sample was performed on a MALDI-TOF mass-spectrometer (MassArray system, Sequenom, San Diego, USA) employing the manufacturer's AssayDesigner software for primer selection, multiplexing, and assay design, and the homogeneous mass-extension (hMe) process for producing primer extension products. MALDI-TOF SNP genotyping was performed at the Genome Analysis Center (GAC) facility of the Helmholtz Zentrum Munich, Germany. All primer sequences used are available upon request. The individual-wise mean call rate over all plates and these SNPs was above 98%. Genotypes of all SNPs were in HWE ($p < 0.05$). To exclude genotyping errors in the German studies, we regenotyped the two tagging SNPs (rs1545843 and rs1031681) in more than 95% and 80% of individuals in the MARS discovery GWAS and the German recurrent depressive replication sample, respectively, using the MALDI-TOF platform. We obtained a genotype concordance rate with the genotypes produced by the Illumina assays of > 99.9%. In the UK studies, all subjects had been genotyped on the Illumina 610k-Quad Beadchips. In the ERF study 1000 individuals were genotyped with Illumina 300k, 100 individuals with Illumina 370k arrays, and 200 individulas with the Affymetrix 250k array. The Rotterdam study samples were genotyped by using the Illumina 550k arrays and the additional MARS samples using the Illumina 610k array.

Imputation of Genotypes

The imputation of genotypes from ERF and Rotterdam study was performed using the Maximum Likelihood Method as implemented in the MACH software v 1.0.16. Release 22 HAPMAP CEU population was used as reference. This effort yielded a total of 2,500,000 SNPs. Only SNPs with call rates > 98%, MAF > 1%, and HWE p values > 1e-06 were used for imputations. Mean r^2 after imputations was 0.97 for the 19 SNPs tested within the 450 kb region on 12q21.31. For the MARS replication sample, the genotypes of rs1031681 were imputed using Impute v2.1.0 and the HapMap CEU as a reference population. rs1545843 failed QC in the controls of the UK sample with call rates < 98% and a p value for differential missingness < 1e-08. Its genotypes were therefore imputed in both cases and controls using BEAGLE 3.1 (Browning and Browning, 2009) on HapMap 3.

Power Calculation

Power calculations were performed using the Genetic Power Calculator (Purcell et al., 2003) (http://pngu.mgh.harvard.edu/~purcell/gpc). Given a prevalence of unipolar depression of 16% (Kessler et al., 2003), a marker in LD (D' = 1) with a risk allele R and an alternative protective allele N under an allelic log-additive, dominant or recessive model and 80% power in our discovery genome-wide study at a significance level α equal to 1.4×10^{-7} (= 0.05/365,676 SNPs), we would be able to detect an effect with an OR of 2.2 or larger.

Genomic Controls

Genomic controls (Devlin et al., 2001) for the case-control phenotype were calculated with R-2.5.0 (http://cran.r-project.org) on a genome-wide level in the MARS GWAS sample. In addition, population stratification was tested with EIGENSTRAT implemented in EIGENSOFT (Price et al., 2006) (http://genepath.med.harvard.edu/~reich/EIGENSTRAT.htm). Neither the genomic control method (λ = 1.023, see Figure S1) nor EIGENSTRAT analysis gave any indication for population stratification.

Linkage Disequilibrium

The LD pattern and haplotype block delineation were determined by applying Haploview 4.0 (http://www.broad.mit.edu/mpg/haploview) (Barrett et al., 2005). Blocks were defined using the confidence interval method described by Gabriel et al. (Gabriel et al., 2002). Pairwise LD measures (r^2 and D') were calculated in the 366 healthy controls of the GWAS sample and in 284 controls of the African-American sample for the eight most associated SNPs on chr12.21.31 (see Figure 2). German controls were also compared to the HapMap CEU population (CEPH sample consisting of Utah residents with ancestry from northern and western Europe, n = 60, http://www.hapmap.org) (Frazer et al., 2007). No deviation in LD could be observed in this comparison (data not shown).

Association Testing

Genome-wide case-control analyses were conducted by applying the WG-Permer software (http://www.mpipsykl.mpg.de/wg-permer/). For post-hoc analyses, applications in R-2.5.0 (http://cran.r-project.org) and SPSS for Windows (releases 16, SPSS, Chicago, IL, USA) were used. SNPs with genotype distributions deviating from HWE at a significance level of 10^{-5} or 0.05 with a call rate below 98% or 95% in the GWAS or German replication sample, respectively, and SNPs with a MAF below 5% were excluded from statistical analysis. Autosomal SNPs were tested for association with unipolar depressive disorder in a case-control design using Chi-square test statistics under allelic and both alternative recessive-dominant modes of inheritance. The level of significance was set to 5% (family-wise error rate). Nominal p values were corrected for multiple comparisons by the permutation-based minimum

p method proposed by Westfall and Young (Westfall and Young, 1993; Westfall et al., 2001) under 10^4 permutations over the three performed genetic models and all SNPs tested per study. Empirical and nominal p values for all reported associations did not deviate from each other. Moreover, sample demographic statistics and post-hoc tests on age, gender, and German origin, life events, recurrence of MD, age at onset, number of previous depressive episodes, first-degree family history of MD, and lifetime attempted suicide status were performed by logistic regression analysis and ANCOVA. P values including these covariates did not differ from those of the Chi square test statistics for all reported associations. Thus, none of these additional covariates showed a significant effect on the reported associations. In the RADIANT study from the UK, sex was coded as a factorial covariate for the analysis presented in the main text. The validity of the p values and the distribution of the estimates were verified using Monte-Carlo (permutation and bootstrap) methods. Below we give the odds ratios (OR) without sex as a factorial covariate and the ORs in a gender stratified analysis: OR of all RADIANT cases and RADIANT plus WTCCC2 controls, sex not included as covariate: 1.082 (95% C.I. 0.951; 1.231), n = 1636 cases and 7261 controls with a p = 0.274. OR of only male cases and male controls: 1.344 (95% C.I. 1.080; 1.672), n = 485 cases and 3465 controls with a p = 0.00797. OR of only female cases and female controls: 0.959 (95% C.I. 0.816; 1.127), n = 1151 cases, 3781 controls with a p = 0.615.

Meta-Analysis

Meta-analyses were conducted using the R library *rmeta* applying a fixed effect model. In the first meta-analysis, three genetic models were tested, the two opposite carrier models and an allelic model resulting in a number of 2.02 effective tests as estimated from 10,000 permutations. In the second meta-analysis (combining the results of the first meta-analysis with the data from the RADIANT/WTCCC2 sample), only the recessive model for rs1545843 was tested. The adjustment for the two tests performed in RADIANT/WTCCC2 was done by adjusting the standard error of the estimate accordingly.

Genotype-Specific mRNA Levels (eQTLs)

We used two independent genome-wide SNP/mRNA expression data sets for SNP-eQTL analyses on 12q21.31. The first data set was from premortem human hippocampus of 137 individuals involved in the Epilepsy Surgery Program at Bonn University, Germany. Methods related to the hippocampal eQTL experiment are detailed in the Supplemental Experimental Procedures. The second was the publicly available GENEVAR (GENe Expression VARiation) data set of EPV-transformed lymphocytes from the 210 unrelated HapMap individuals (http://www.sanger.ac.uk/humgen/genevar/) (Stranger et al., 2005, 2007).

Experimental Design and Statistical Analysis

In both data sets, we selected all RefSeq annotated genes (Pruitt et al., 2005) located within 1.5 megabase on both sides of the genome-wide significant SNP of the GWAS (rs1545843, total sequence of 3 Mb). The five following genes intersect with the defined genomic region (hybridization probes in brackets, see also Table S1): *TMTC2* (GI_22749210-S), *SLC6A15* (GI_33354280-A, GI_21361692-I, GI_33354280-I), *TSPAN19* (GI_37541880-S), *LRRIQ1* (hmm2373-S), and *ALX1* (GI_5901917-S). For the GENEVAR data set a residual expression variable for each probe was built by regression analysis to correct for ethnicity. We tested an allelic and both alternative recessive-dominant genetic models for rs1545843 and rs1031681 for each of the probes (n = 7) by performing ANOVA under 10^6 permutations using the WG-Permer software. p values were corrected for multiple comparisons by the Bonferroni procedure. Subsequently, we repeated this analysis by including data of all available non-RefSeq (EST) gene probes (n = +6: Hs.365699-S, Hs.506230-S, GI_41149683-S, Hs.208111-S, GI_41149726-S, hmm21473-S, Table S1) for ESTs from GenBank in the same genomic window. Data of four ESTs were excluded from the analysis, because their probes did not map completely or uniquely to any target EST sequence of the current GenBank database (GI_37541937-S, hmm21470-S, GI_37541941-S, hmm21472-S). Target sequences of all probes included in expression analyses mapped uniquely and completely to the human genome and are all devoid of known common variations denominated by dbSNP build 129.

Regional Volumetry

Structural MRI Sample Acquisition and Quality Control

Structural MRI with high-resolution T1-weighted images adequate for morphometry was available for 204 patients with recurrent unipolar depression and 186 control subjects. MRI was acquired at the MPI of Psychiatry in the context of the acquisition of the Munich recurrent unipolar depression replication samples. A detailed description of study participant selection and image processing is available in the Supplemental Experimental Procedures.

Automated Regional Volumetry

In brief, image preprocessing was performed as for voxel-based morphometry to gain gray matter (GM) maps with preserved local volume in stereotactic space. Histologically validated cytoarchitectonic probability maps (Amunts et al., 2005) were used to create regional volumetry masks for the left and right hippocampus and subregions cornu ammonis (CA: CA1–3), subiculum (SUB), and dentate gyrus (DG). The sum of all modulated GM voxels within the regional masks was calculated using in-house software programmed in IDL (http://www.creaso.com).

Statistical Analysis

Analysis of covariance (ANCOVA) was performed for left and right total hippocampal GM volume and each three subregions with two-level factors group (patients, controls), genotype (rs1545843 AA versus AG/GG, equally for rs1081681), and gender, covarying for age, squared age, total GM volume, and sequence type. Levene's tests for equality of error variances was explored and found nonsignificant for all tests (Figure S4). p values were compared with a Bonferroni-corrected threshold to adjust for 18 tests (two SNPs, nine volumetric measurements [including motor cortex as control region]: 0.05/18 = 0.0028). Both nominal and corrected p values are indicated in Figure 5 and Table S2.

Methods for ^{1}H-NMR Spectroscopy

These are discussed in the Supplemental Experimental Procedures.

Animal Housing

Male CD1 mice were used for all experiments. Animals were 28 days old at the day of arrival and were kept on a 12L:12D cycle. Food and water was provided ad libitum. The experiments were carried out in accordance with European Communities Council Directive 86/609/EEC. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

Chronic Stress Paradigm

The chronic social stress procedure was performed as described previously (Schmidt et al., 2007; Sterlemann et al., 2008) (see Figure S5 and Supplemental Experimental Procedures). In this paradigm, mice are exposed to a highly unstable social and hierarchical situation during their adolescence and early adulthood. After the 7 week stress procedure all animals were single housed for 5 weeks and then sacrificed under basal conditions.

Tissue Dissection and Expression Profiling

Frozen brains were sectioned at the level of the dorsal hippocampus and the subregions CA1 and dentate gyrus were laser-microdissected using a laser capture microscope (P.A.L.M. Microlaser Technologies, Bernried, Germany). Extracted RNA was quality checked on the Agilent 2100 Bioanalyser, subjected to two rounds of linear amplification and hybridized to Illumina MouseRef-8 v1.0 Expression BeadChips according to the manufacturer's protocol (see also Supplemental Experimental Procedures). The data discussed in this publication have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE112211.

Gene Expression Analysis in Stress-Susceptible versus Stress-Resilient Mice

We chose the same procedure to select genes adjacent to the region of association for validation in the described mouse experiment as we applied in the human expression analysis. Expression differences were checked

for *SLC6A15* (NM_175328.1; scl0003791.1), *TMTC2* (NM_025775.1; scl066807.1_5-S), *ALX1* (NM_009423.2; scl022032.1), and *LRRIQ1* (XM_137221.4). Differentially expressed genes were validated by in situ hybridization as described previously (Schmidt et al., 2007). The antisense cRNA hybridization probe of *SLC6A15* was 487 base pairs long (left primer: TGCCGTGAGCTTTGTTTATG; right primer: CAGTGTTGGGGAACCACTTT covering exons 11 to 13 of the gene). The slides were exposed to Kodak Biomax MR films (Eastman Kodak Co., Rochester, NY) and developed. Autoradiographs were digitized and relative expression was determined by computer-assisted optical densitometry (Scion Image, Scion Corporation). The software package SPSS version 16 was used for statistical analysis. Group comparisons were performed using the two-tailed paired t test to determine statistical significance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Data are presented as mean ± SEM.

ACCESSION NUMBERS

The mouse model data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE112211.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, three tables, Supplemental Experimental Procedures, and detailed author contributions and can be found with this article online at doi:10.1016/j.neuron.2011.04.005.

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