

Brain galanin system genes interact with life stresses in depression-related phenotypes

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Galanin is a stress-inducible neuropeptide and cotransmitter in serotonin and norepinephrine neurons with a possible role in stress-related disorders. Here we report that variants in genes for galanin (*GAL*) and its receptors (*GALR1*, *GALR2*, *GALR3*), despite their disparate genomic loci, conferred increased risk of depression and anxiety in people who experienced childhood adversity or recent negative life events in a European white population cohort totaling 2,361 from Manchester, United Kingdom and Budapest, Hungary. Bayesian multivariate analysis revealed a greater relevance of galanin system genes in highly stressed subjects compared with subjects with moderate or low life stress. Using the same method, the effect of the galanin system genes was stronger than the effect of the well-studied *5-HTTLPR* polymorphism in the serotonin transporter gene (*SLC6A4*). Conventional multivariate analysis using general linear models demonstrated that interaction of galanin system genes with life stressors explained more variance (1.7%, $P = 0.005$) than the life stress-only model. This effect replicated in independent analysis of the Manchester and Budapest subpopulations, and in males and females. The results suggest that the galanin pathway plays an important role in the pathogenesis of depression in humans by increasing the vulnerability to early and recent psychosocial stress. Correcting abnormal galanin function in depression could prove to be a novel target for drug development. The findings further emphasize the importance of modeling environmental interaction in finding new genes for depression.

galanin receptors | mood disorders | network-based analysis | neurogenesis | transmitter coexistence

Major depressive disorder (MDD) is a common and serious disease afflicting more women than men, and a leading cause of disability worldwide, associated with much suffering and major costs for society (1, 2). Environmental psychosocial stressors are important in pathogenesis, because episodes are usually preceded by adverse life events, and early childhood experiences of physical and emotional abuse and parental neglect are important vulnerability factors (3, 4). Genetic vulnerability is significant with a heritability of about 35% (5). We remain ignorant about the brain processes that translate these genetic and environmental influences into depressive symptoms or risk. A major clue is that effective antidepressant drugs act directly or indirectly to enhance neurotransmission in serotonin (5-HT) and norepinephrine monoamine pathways, proving the monoamine hypothesis of depression (6–8). Many other candidate mechanisms have been identified in anatomical, pharmacological, and behavioral studies of stress in rodents. However, the demonstration of state- or trait-related abnormalities in human monoamine or other neural systems remains frustratingly elusive, despite modern brain-imaging methods. To determine whether the neuropeptide galanin has a role in depression, we used a unique Bayesian systems-based analysis to dissect out the influence of variation in

genes for the peptide and its receptors on the interaction between different psychosocial stressors and risk of depression.

Current drug treatment of depression is far from satisfactory; the drugs target a limited range of monoamine mechanisms, they have an appreciable side-effect burden, and response is often partial (8, 9). In the search for better antidepressants, much attention has focused on neuropeptides and their receptors, the most diverse neurotransmitter system in the brain (8, 10–21), which includes galanin. As yet, however, there is no compelling evidence of efficacy of the neuropeptide approach or that particular peptides are involved in the pathogenesis of MDD.

Galanin, a 29-aa (30 in humans) peptide (22), is widely distributed in the rodent (23, 24) and human (25–27) brain. In rat it coexists with noradrenaline (NA) in the locus coeruleus (LC) and with 5-HT in the dorsal raphe complex (28). Like other peptide cotransmitters (29), it is released when neurons fire in high-frequency bursts in response to strong behavioral and pharmacological challenge (30–32). Galanin exerts its action via three cloned receptors, GALR1, GALR2, and GALR3 (33, 34) with a broad distribution in rat (35) and primate brain (26, 36). Animal behavioral studies (31, 32, 37–41) and a single study in humans (42) suggest that galanin has a role in stress, depression-like behavior, and anxiety. In addition, there is indication from

Significance

Early and recent environmental stressors, such as maltreatment in childhood, or stressful life events in adulthood, are important risk factors for depression. Nevertheless, not all people who suffer from these will be depressed. The resilience or vulnerability to these stressors, and thus depression, is likely to reside in our genes. In the present study, we used different statistical methods to demonstrate that variations in genes for galanin and its receptors increase the risk of depression only in heavily stress-exposed subjects. The work was predicated on the finding that galanin expression is strongly stimulated by stress in animal studies. In humans, variation in galanin function would appear to be important determinants of the outcome of psychosocial stress.

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previous genetic studies on humans that the galanin system is involved in psychiatric disorders including alcoholism/addiction (43–47), panic disorder (48, 49), and chronic pain-associated depression (50). Furthermore, recent functional studies provided the first evidence that polymorphisms in a highly conserved genetic region upstream from the *GAL* gene regulates *GAL* expression in brain areas, such as the amygdala and hypothalamus, implicated in the pathogenesis of depression (51, 52).

Genetic studies have the potential to identify molecular mechanisms of MDD vulnerability (53), but even mega- and meta-analyses of large genome-wide association studies (GWAS) have not identified genetic variants associated with MDD that survive genome-wide statistical correction (54, 55). Nominally significant associations will include many false-positives. Nevertheless it is noteworthy that SNPs in the gene for galanin (*GAL*) were among the top 10 genes whose variation was associated with MDD in a recent GWAS (55). One way to improve sensitivity is to take a system-based approach: if galanin is mechanistically involved in depression, genetic variation in the peptide and its receptors should exert similar influences, despite the fact the genes are located on entirely different chromosomes without linkage disequilibrium (LD) and with a low probability of randomly similar effects. Others have argued that improved sensitivity will come from deeper phenotyping (56) and characterization of environmental factors (3, 4, 57), because neither genetic nor environmental factors can be identified in isolation, if they modify each other's action to a high degree. Combining these two approaches, and in view of its preclinical properties, we predicted that variation in galanin genes would strongly interact with environmental stress in determining depression vulnerability. However, including more phenotypic and environmental variables exacerbates the problem of false-positives from multiple comparisons. Consequently, analyses of gene–environment interactions involving multiple phenotypes face a similar burden as GWAS in terms of correction for multiple testing. Furthermore, the conditional nature of such interactions frequently leads to separate analysis of multiple subpopulations (i.e., to even more statistical tests). To cope with multiple hypothesis testing, we applied a Bayesian systems-based approach both at structural and parametric levels, which allows multiple correlated outcomes. This approach supported the joint exploration of the underlying mechanism at genotype, haplotype, and diplotype levels in different depression-related phenotypes, and we validated the results by conventional multivariate analysis using independent subsamples.

Results

Genetic Association and Gene × Environment Interaction Analysis with Linear and Logistic Regression. Table 1 summarizes the demographic and phenotypic characteristic of the studied population. To show the genetic effects alone or in interaction with environmental factors, the effects of single SNPs (*SI Methods*, Figs. S1–S4, and Table S1) and their combination (haplotypes, HT) (Table S2) were studied. First, we carried out a traditional linear and logistic regression analysis using additive genotypic and diplotypic models for the selected variables. (For power calculations see *SI Methods* and Table S3.) Of the 12 SNPs studied, 7 statistically associated with one or more of the three clinical phenotypes (Fig. 1). Furthermore, all but one (*GAL* rs3136541) of the seven acted through interaction with either childhood adversity or recent life events. Two of the six *GALR1* SNPs interacted with recent life events (rs1893829, rs1162010) and two with childhood adversity (rs5375, rs11665337) to influence phenotypes. Three *GALR1* haplotypes (HT2:GAGTAG, HT6:GAGTGA, HT12:GGTCGG) interacted with childhood adversity and one with recent life events (HT10:AAGCAG). The single SNP representing *GALR2* (rs8836) interacted with life events, whereas a *GALR3* SNP (rs2285179) and the main haplotype (HT1:GA) interacted with childhood adversity. These

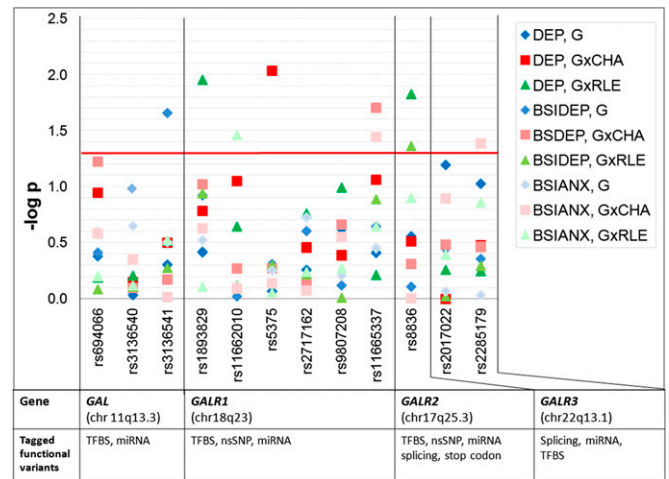


Fig. 1. Summary figure of the SNP association and SNP × environment interaction results. This figure shows the $-\log P$ values (vertical axis) of the genetic main effects (G), gene and childhood adversity interaction (G×CHA), and gene and recent negative life events interaction (G×RLE) analysis (additive genetic model in PLINK v1.07, <http://pngu.mgh.harvard.edu/purcell/plink>). The red line represents $P = 0.05$ nominal significance level; above that line significant results can be seen. Outcome variables were lifetime depression (DEP), current depression scores (BSIDE), and current anxiety scores (BSIANX). Age and sex were covariate in all analysis. Horizontal axis lists the investigated SNPs, the genes and their chromosomal positions, and the tagged functional variants based on in silico functional analysis (see also Table S7). TFBS, transcription factor-binding site; splicing, SNPs that are located at 2 base pairs of intron–exon junction region; miRNA, miRNA binding site activity; nsSNP, SNPs in protein-coding regions that can cause amino acid change; stop codon, SNPs that may lead to premature termination of peptides (nonsense), which would disable the protein function.

nominally significant findings can be seen in Tables S4 and S5, which summarize all of the regression results. The results suggested to us that *GALR1* and probably *GALR3* modulate neurodevelopmental processes relevant to the effects of childhood adversity, whereas *GALR2* might modulate neuroplastic changes connected with stress responses to recent life events. Despite their interest and the corroboration that functionally related, genomically distant genes show similar gene-by-environment (G×E) interactions, these nominally significant effects did not survive Bonferroni correction for multiple testing. To reach an optimal correction for multiple-hypotheses testing concerning the numerous potential dependencies between multiple predictors and phenotypes, we applied a systems-based approach in the second phase using the Bayesian model averaging framework (58–60). This approach allowed the principled and detailed investigation of G×E interactions as model properties. The analysis consisted of a joint multivariate analysis of *GAL*, *GALR1*, *GALR2*, and *GALR3* genes on all three phenotypes—reported lifetime depression, current depression, and anxiety—both in the Bayesian and conventional (traditional regression) statistical framework.

Bayesian Network-Based Bayesian Multilevel Analysis of Relevance. Bayesian network-based Bayesian multilevel analysis (BN-BMLA) was carried out using a method that allows a detailed investigation of the relevance of factors with respect to multiple dependent variables such as phenotype descriptors (61). The resulting scores are posterior probabilities of relevance (Pr) ranging from 0 to 1. This method involves Bayesian model averaging over possible models reflecting relationships between variables, thus handling the multiple hypothesis testing problem optimally by taking into consideration the potential interdependencies of the predictors (for detailed description of the BN-BMLA method, see *SI Methods*).

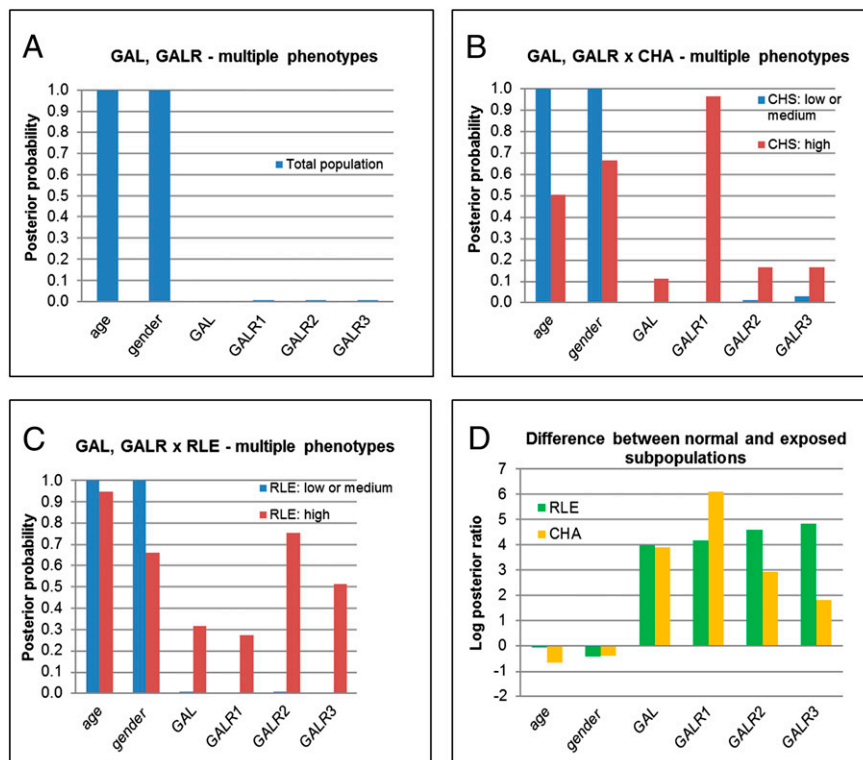


Fig. 2. Comparison of posterior probabilities of relevance for the total population and for subpopulations under the influence of different environmental factors. Subpopulations were created by dividing the original sample into two groups based on childhood adversity (CHA) and recent negative life events (RLE). The posteriors range from 0 to 1 and are estimated with respect to all three phenotypes (reported lifetime depression, current depression, and anxiety). A high posterior probability indicates that the corresponding factor is highly relevant. *GAL*, *GALR1*, and *GALR3* represent corresponding diplotypes, whereas *GALR2* denotes the single related SNP. Age and sex were included as cofactors. (A) The posterior probability of relevance of factors for the total population, not taking into account life stressors. Age and sex are highly relevant, but none of the genetic factors are relevant. (B) The posterior probability of relevance of factors for patients with low-medium CHA versus patients with high CHA. None of the genetic factors are relevant in the low-medium CHA group. In contrast, in case of patients with high CHA there is at least one highly relevant genetic factor, the *GALR1* with a high posterior probability (Pr = 0.96). Furthermore, the corresponding log posterior ratio is high (6.09), which means that there is a strong difference between the relevance of *GALR1* in the two subpopulations. (C) A comparison of the posterior probability of relevance of factors in case of patients with low or medium RLE versus patients with high RLE. In the case of the former subpopulation, none of the genetic factors are relevant, contrary to the high RLE group, where several factors are found to be relevant. The results indicate that *GALR2* is the most relevant factor in case of high RLE having a relatively high posterior probability for relevance (Pr = 0.75). *GALR3* has the second largest probability score, although it is only moderately relevant (Pr = 0.51). Furthermore, *GAL* and *GALR1* are even less relevant, and can be considered as weak results. (D) To compare the Bayesian posteriors across exposures we calculated log posterior ratios. The high (>3) log posterior ratios indicate in case of every genetic factor that there is a substantial difference in terms of posterior probability of relevance between those who experienced high life stresses and who did not. In contrast, the effects of age and sex factors do not differ substantially between those who experienced high life stresses and who did not.

In the total population, excluding life stressors, the galanin pathway genes showed minimal relevance (Fig. 2A). This finding is supported by the moderate/weak genetic main effects in the initial regression analysis. Next, we performed separate analyses in subpopulations defined by childhood adversity categories: low or medium (0–6) versus high (≥ 7) on the short version of the Childhood Trauma Questionnaire or by the number of recent negative life events: low or medium (0–2) versus high (≥ 3). In people with exposure to high childhood adversity, the *GALR1* diplotypes were highly relevant (Pr = 0.96) with respect to multiple phenotypes, but it was nonrelevant (Pr = 0.002) in the low/medium childhood adversity group (Fig. 2B). To compare the Bayesian posteriors across exposures, we calculated log posterior ratios. The striking magnitude of the difference is confirmed by the sixfold log posterior ratio. In contrast, *GALR1* showed little relevance to the effect of exposure to recent negative life events; the *GALR1* diplotypes had a relatively low posterior probability (Pr = 0.27) in the high negative life-events group, and a negligible posterior probability (Pr = 0.004) in those with low/medium exposure. (Fig. 2C). The single SNP rs8836 related to *GALR2* had high relevance (Pr = 0.75) to multiple

phenotypes in the high negative life-event group but had no relevance in the low/medium life-events group. This substantial difference was also indicated by the high log posterior ratio of 4 (Fig. 2D). Although the other galanin pathway genes have only moderate or low probability of relevance in the high life-stressor groups, the log posterior ratios (>3) indicate that for each genetic factor there is a substantial difference in terms of posterior probability of relevance between those who were highly exposed to environmental life stressors and those who were not (Fig. 2D and Table S6).

As an interesting comparison, the same Bayesian analysis of relevance as here used for the galanin system was carried out in the present cohorts for the well-known *5-HTTLPR* polymorphism. Note that from the statistical point of view this comparison can be seen as a benchmark and from the systems biological point of view as a comparison with an experimentally validated reference. Our results show that the *5-HTTLPR* polymorphism is moderately relevant (Pr = 0.55, log posterior ratio 4.56) in those who experienced a high level of recent negative life events, and minimally relevant in those who experienced a high level of childhood adversities (Pr = 0.04; log posterior ratio 1.67) (Fig. S5).

In addition, further testing the relevance of the *5-HTTLPR* and the galanin system genes in one model in those who experienced high level of recent negative life events, the relevance of the galanin system genes remained stable, whereas the relevance of the *5-HTTLPR* modestly decreased (from $Pr = 0.55$ to $Pr = 0.34$). This result suggests that the effect of the *5-HTTLPR* may be partially mediated by the galanin system but not vice versa. These results corroborate previous findings and suggest that the galanin system probably has similar or stronger effect on stress-induced depressive symptoms compared with the *5-HTTLPR* functional polymorphism.

Galanin Pathway Level Analysis. To assess the overall contribution of galanin genes to variation in risk of our depression-related phenotypes, two general linear models were constructed: a “Reduced” model containing only environmental factors (childhood adversity and recent negative life events), and a “Full” model containing environmental factors, genetic factors (*GAL*, *GALRI*, *GALR2*, *GALR3*), and their interactions. Table 2 shows residual variances for the phenotypes, namely reported lifetime depression, current depression, and anxiety separately, and also for the multivariate case (i.e., combining the variance across all phenotypes). The results indicate that the Full model explains more variance, resulting in less residual or unexplained variance in every case than the life stress-only model. In the overall multivariate case the difference is 0.017, which means that the investigated genetic variants and their interactions with life stressors contribute 1.7% to the total variance. In our study, the difference between the Full and Reduced models in the multivariate comparison was significant ($F = 1.838$, $F_{critical} = 1.759$, $\alpha < 0.005$). This effect was significant separately for the population recruited in Budapest ($F = 1.632$, $F_{critical} = 1.452$, $\alpha < 0.05$) and in Manchester ($F = 1.531$, $F_{critical} = 1.448$, $\alpha < 0.05$), and in the combined sample separately both in males ($F = 1.645$, $F_{critical} = 1.459$, $\alpha < 0.05$) and in females ($F = 2.108$, $F_{critical} = 2.039$, $\alpha < 0.0005$) with similar magnitude of effect size (3.9% vs. 3%, respectively). Conducting the comparison of the models for the phenotypes individually in the combined sample showed that the difference between the models was most significant in case of the current depression phenotype ($F = 2.174$, $F_{critical} = 2.031$, $\alpha < 0.0005$).

In Silico Functional Analysis and Comparison with Psychiatric Genetic Consortium GWAS Results. Finally, in silico functional prediction was carried out using the SNP Function Prediction (FuncPred) tool (<http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm>). This process revealed that two of our investigated SNPs have functional effects. Namely, rs11662010 (near to the 5' end of the *GALRI* gene) modifies a transcription factor binding site, and rs8836 (downstream to the *GALR2* gene in strong LD with it) has miRNA binding activity. In addition, our 12 haplotype tag SNPs captured an additional 23 potentially functional variants within the galanin system (Fig. 1), suggesting that the genetic regions covered by our haplotype-tagging SNPs have functional consequences on the gene transcription and translation, thus may reflect real functional differences (Table S7). In addition, the Psychiatric Genetic Consortium's latest mega-analysis showed several nominally significant associations and trends between MDD and the *GAL*, and *GALRI* genes (Table S7), further supporting our results.

Discussion

Galanin is, as revealed in animal experiments, a highly “dynamic” neuropeptide, frequently showing a robust up-regulation of expression in response to stress, both under physiological and extreme conditions. We tested the hypothesis that the genetic effects of the galanin system in the development of depression and anxiety would be greatest in those exposed to the most life stress. In the present study, genetic variants of *GALRI* significantly interacted with childhood adversity, suggesting it also has a role in neuronal damage and wiring during neuronal development. The interaction of *GALRI* SNPs and childhood adversity in the regression analysis was confirmed by the Bayesian multivariate analysis of relevance. Moreover, *GALR2* rs8836 significantly moderated the effect of recent negative life events, also confirmed by the Bayesian analysis. In addition, *GALR3* showed a moderate relevance in interaction with recent negative life events in our study. Finally, high log posterior ratios indicated that *GAL* gene effect was more relevant in the highly stressed population compared with the low or moderately stressed subjects. These results indicate that the galanin pathway has a role in the development of depression in humans but only in persons exposed to high levels of childhood adversity or recent

Table 1. Demographic and phenotypic characteristic of the sample

Demographics	Combined	Budapest	Manchester
Sex			
Female	1,641 (70%)	702 (69%)	939 (70%)
Male	720 (30%)	313 (31%)	407 (30%)
Age (mean \pm SEM)	32.8 \pm 0.2	31.1 \pm 0.3	34.0 \pm 0.3
Personal psychiatric history			
Reported depression	974 (41%)	217 (21%)	757 (56%)
Recurrent episodes	690 (71%)	118 (54%)	572 (76%)
Ever treated with antidepressant	637 (65%)	70 (32%)	567 (75%)
Reported suicide attempt	285 (12%)	48 (5%)	237 (18%)
Reported anxiety disorder	641 (27%)	202 (20%)	439 (33%)
Reported substance use disorder	130 (6%)	24 (2%)	106 (8%)
Family psychiatric history			
Reported depression in immediate blood relatives	632 (27%)	135 (13%)	497(37%)
Symptom scores (range 0–4)			
BSI depression (mean \pm SEM)	0.85 \pm 0.02	0.56 \pm 0.02	1.08 \pm 0.03
BSI anxiety (mean \pm SEM)	0.88 \pm 0.02	0.69 \pm 0.02	1.02 \pm 0.03
Adversities			
Recent negative life events (mean \pm SEM)	1.22 \pm 0.03	1.08 \pm 0.04	1.3 \pm 0.04
Childhood adversity (mean \pm SEM)	3.3 \pm 0.07	2.8 \pm 0.09	3.7 \pm 0.1

BSI, Brief Symptom Inventory.

negative life events, and that the different receptors have different roles in mediating the effects of different stressors.

The paradigmatic example of a candidate gene interacting with recent negative life events and childhood adversity is the serotonin transporter gene (*SLC6A4*). This gene has a functional polymorphism in the promoter region (*5-HTTLPR*) (62), whose risk variant is associated with a 50% reduction in serotonin transporter protein and predisposition to depression after negative life events (63–65), although there are negative studies. In our study we used this gene as a benchmark and reference for the Bayesian analysis to allow the comparison of posterior probabilities of relevance. The results of Bayesian analysis supported the relevance of *5-HTTLPR* in stress-related depression, but the galanin system had a stronger effect. Indeed, the investigated genetic variants in the galanin pathway and their interactions with life stressors explained 1.7% of the total variance in the depression-related phenotypes. This is a large proportion in comparison with the 0.6% explained variance by the whole-genome polygenic risk score seen in a recent GWAS mega-analysis for MDD (54). According to the Psychiatric Genetic Consortium suggestion, at least 100,000 MDD cases (plus controls) would be required to achieve GWAS-significant findings for MDD (54). However, our results further emphasize that using subjects with high life stresses, because MDD is a stress-related disorder, could potentially decrease the required number of cases to 5,500–35,000 (Table S3). According to the differential sensitivity hypothesis (57), some risk genotype-by-stress interactions also involve increased sensitivity to beneficial environments, such as social supports.

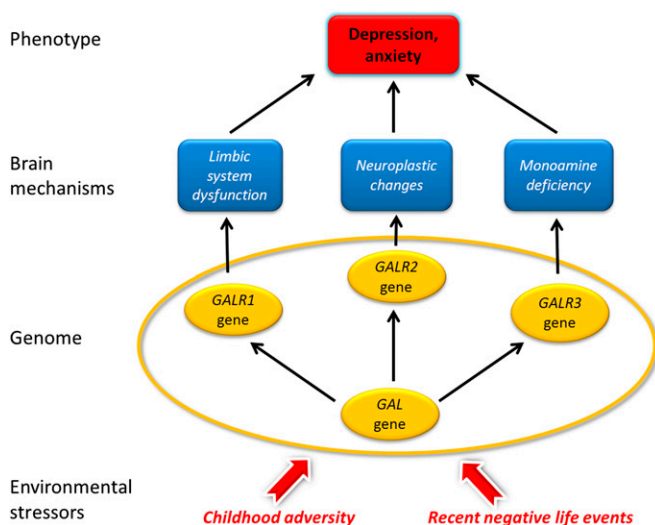


Fig. 3. Galanin mechanisms hypothetically involved in MDD in humans. Galanin, a neuropeptide, and its receptors are colocalized in some monoaminergic neurons in the brain. The galanin system is highly sensitive to experimental and naturalistic stressors. Stress-induced activation of the galanin system represents the first phase in the development of depression. Recent analysis of human brain has shown that the Gi protein-coupled GALR3 (and not GALR1 as in rodents) is the main galanin receptor in NA-LC and probably 5-HT dorsal raphe nucleus cells, and that the Gi protein-coupled GALR1 is the main receptor in the forebrain. Antidepressive effects may be achieved by (i) GALR3 antagonists (71), by reinstating normal monoamine turnover in the brainstem, and by (ii) GALR1 antagonists in the forebrain by normalization of limbic system activity, or by (iii) agonists at GALR2, a Gq protein-coupled receptor, promoting neuroprotection. The present genetic analysis suggests that GALR1 risk variants may compromise galanin signaling during childhood, whereas GALR2 signaling may be influenced by recent negative life events. In addition, all four galanin system genes have relevant roles in the development of depression-related phenotypes in those persons who were highly exposed to life stressors.

However, no protective effects of galanin-related genotypes were seen in the low-stress groups in our study.

Potential mechanisms that may explain the galanin system effect in the development of depression are summarized in Fig. 3. The *GAL* gene is widely expressed in the human brain [e.g., LC, forebrain, amygdala, and hypothalamus (26, 51)], but its involvement in the development of depression is not well understood. Although previous studies indicated that it might have a sex- or estrogen-dependent effect (49), in our study the galanin system genetic variants significantly influenced the depression-related phenotypes both in males and in females, with similar magnitude of effect size, providing evidence that the excessive stress effect is not mediated by sex.

The monoamine neurotransmitters, NA, 5-HT, and dopamine, have been implicated in the mechanism of action of antidepressants and thus the pathogenesis of MDD for more than half a century, and also shown to interact in intricate ways in the development and treatment of this serious disease (6, 7, 66). Some of the effects of galanin may fit into this framework. Involvement of the galanin system in regulation of mood-related behavior in animals has focused on several brain sites, via different mechanisms. For example, in rat galanin may have a prodepressive role via modulating 5-HT_{1A} receptors in the forebrain (37, 67) or, when released from soma and dendrites in the LC, via inhibitory GALR1 autoreceptors (68, 69). The same receptor mediates inhibition of pyramidal neurons in the ventral hippocampus (70). Thus, galanin may cooperate with its cotransmitter norepinephrine, both at the LC cell body autoreceptor level and postsynaptically in the hippocampus. However, it is important to note that recent studies demonstrated that in humans GALR3 receptors are more prevalent in the brainstem compared with GALR1, whereas GALR1 is widely expressed in the human forebrain (26). In the 5-HT neuron-rich rat dorsal raphe nucleus/periaqueductal gray, Lu et al. (39) have suggested that mood is controlled through a balance between signaling via prodepressive GALR1/3 (71, 72) and antidepressive GALR2 receptors (38, 39). In the ventral tegmental area galanin inhibits dopamine neurons, inducing depression-like behavior (40).

Accumulating evidence suggests that hippocampal atrophy and loss of dendritic spine synapses are associated with depressive symptoms (73–75), whereas recovery of MDD patients involves normalization of the hippocampal volume (76), possibly related to enhancement of functional synapses (77–79). Interestingly, galanin has been reported to act as a neuroprotective factor for hippocampal neurons (80–82) via GalR2 (83). Moreover, it is now established that adult neural stem/progenitor cells generate new neurons in, for example, the hippocampal granule cell layer (84, 85). Subsequently, the proliferation and integration of neuronal stem cells in this brain region have emerged as a focus in attempts to understand mechanisms underlying stress, depression, and the effects of antidepressants (86–88). In the hippocampus, galanin's trophic and proliferative effects via GALR2/3 receptors, on neuronal stem cells in the subgranular zone in the dentate gyrus (89–91), may be involved and could mediate some of the effects of genetic variation that we have observed. The latter idea has gained more weight in view of the recent report that, in humans, a large subpopulation of hippocampal neurons, constituting one-third of the neurons, is subject to exchange (92), substantiating the first report of adult neurogenesis in humans (93). Thus, in adults 700 neurons are added in the hippocampus each day, and around one-third of the hippocampal neurons constantly renew, involving most neurons in the dentate gyrus (92). Interestingly, galanin was more abundant in mouse embryonic stem cells compared with any other examined tissues (94), and in human stem cells galanin was in the top 50 overexpressed genes (95). Furthermore, galanin receptors can also act through cAMP formation (96), and thus the cyclic AMP-responsive element binding (CREB) signaling pathway (97–99),

which is an important modulator of the brain-derived neurotrophic factor (BDNF) production. BDNF mediates activity-dependent neuroplasticity in the hippocampus and cortex, which is critical to the adaptation of environmental stress and also contribute to antidepressant effects (77, 100, 101). It is interesting to note that our previous study demonstrated that genetic variation in the *CREBI-BDNF-NTRK2* pathway also interacts with childhood adversity to increase risk of depression (102).

There are some limitations of our study. For example, we used self-reported questionnaires to measure lifetime depression, depressive and anxiety symptoms, and negative life events that, although proven and widely used, might be influenced by recall bias. Therefore, we validated them in a subpopulation of 142 during face-to-face interviews showing good reliability (102, 103). In addition, we did not control for the timing of depression and life events. It has been demonstrated that childhood adversity has a long-term effect on the pathogenesis of depression (104), and the questionnaire we used to measure recent (last year) negative life events builds on items with long-term contextual threat (105). Finally, our nominally significant G×E interaction results did not survive traditional correction for multiple testing, which was expected in case of weak genetic effects. However, our Bayesian network-based approach accommodates multiple interdependent outcome variables and predictors (i.e., system genes and life stresses), minimizes the loss of power, and quantitatively characterizes the dependency structure of galanin G×E interactions. Results were also confirmed by conventional multivariate analysis using general linear models and comparatively evaluated against the *5-HTTLPR* as reference. Thus, development of probabilistic graphical model-based methods using Bayesian statistical framework may be essential for detecting G×E interactions in modestly heritable disorders.

In conclusion, the present results indicate that the galanin system plays a significant role in the pathogenesis of depression, almost entirely by modulating the vulnerability to early and recent psychosocial stress. The results validate the galanin system as an illness-related target for novel antidepressant drug development.

In addition, our results support suggestions that G×E interactions may significantly contribute to the “missing heritability” in genome-wide case-control studies that lack environmental measures because of their large scale.

Methods

Population. Population cohorts were recruited in Budapest, Hungary and Manchester, United Kingdom in the European Union-funded NewMood study (New Molecules in Mood Disorders, Sixth Framework Program of the European Union, LSHM-CT-2004-503474) using harmonized phenotyping and genotyping methods that enabled us to carry out a mega-analysis. From the recruited $n = 2,588$ subjects, $n = 2,361$ ($n = 1,015$ from Budapest and $n = 1,346$ from Manchester) were eligible for this study who filled out the questionnaires, provided DNA, which was successfully genotyped for the galanin pathway, and have European White ethnic origin. Data of all eligible participants were included in the analysis, regardless of reported psychiatric disorders (Table 1). Details of the recruitment strategy and the population cohorts can be read in previous publications (64, 102, 103). In short, we recruited participants aged between 18–60 y from Greater Manchester, United Kingdom through general practices, advertisements, and a Web site, and from Budapest, Hungary, through general practices and advertisements. Participants returning the signed consent form and the questionnaire were then sent a genetic sampling kit, which they returned. Both studies were approved by the local ethics committees and were carried out in accordance with the Declaration of Helsinki. All participants provided written informed consent.

Phenotypic Assessment. Three stress-related phenotypic outcome variables were analyzed. Reported lifetime depression was derived from targeted questions of a self-reported questionnaire and was validated in a subpopulation during face-to-face diagnostic interviews (102). To measure current depression and anxiety we used the Brief Symptom Inventory (106) anxiety and depression subscales with additional items for depression. A short version of the Childhood Trauma Questionnaire (107) assessed the experience of emotional and physical abuse and neglect in childhood, as validated in a previous study (102). Recent stressors were assessed using a validated measure of negative life events covering intimate relationships, financial difficulties, illnesses/injuries, and social network problems (105). Further details of the phenotypic measures can be seen in *SI Methods*.

Table 2. Residual variances for the full models and the reduced models

Models	Variance			
	Reported lifetime depression	Current depression	Current anxiety	Multivariate
Total sample				
Reduced	0.215	0.727	0.710	0.551
Full	0.210	0.701	0.692	0.534
Explained variance	0.6%	2.7%*	1.8% [†]	1.7% [‡]
Budapest				
Reduced	0.154	0.418	0.464	0.345
Full	0.147	0.387	0.440	0.325
Explained variance	0.7%	3.1% [‡]	2.4%	2.1% [†]
Manchester				
Reduced	0.225	0.888	0.882	0.665
Full	0.216	0.844	0.843	0.634
Explained variance	0.9%	4.4% [†]	4.0% [†]	3.1% [†]
Total males				
Reduced	0.196	0.554	0.501	0.417
Full	0.184	0.501	0.450	0.378
Explained variance	1.2%	5.2% [†]	5.1% [‡]	3.9% [†]
Total females				
Reduced	0.227	0.803	0.798	0.609
Full	0.220	0.759	0.759	0.579
Explained variance	0.7%	4.4%*	3.9%*	3.0%*

*Significant difference in explained variance $P < 0.001$.

[†]Significant difference in explained variance $0.01 < P \leq 0.05$.

[‡]Significant difference in explained variance $0.001 \leq P \leq 0.01$.

Genetic Data. Genetic samples (buccal mucosa cells) were collected according to a validated method (108). Because there are no known functional polymorphisms within this pathway we used haplotype tagging method (www.broad.mit.edu/personal/jcbarret/haploview) to represent the selected genes and scientific literature to identify previously investigated SNPs. Our haplotype-tagged SNPs capture genetic regions that tend to inherit together ($LD r^2 > 0.8$) in populations with European ancestry [based on the Centre d'Etude du Polymorphisme Humain population data of the International HapMap Project (www.hapmap.org) Phase I. June 2005]. The selected 12 SNPs (Figs. S1–S4 and Table S1) were genotyped with the Sequenom's MassARRAY technology (Sequenom, www.sequenom.com). Genotyping was blinded with regard to phenotype and was performed under the ISO 9001:2000 requirements.

Statistical Analysis. PLINK v1.07 (<http://pngu.mgh.harvard.edu/purcell/plink>) was used to test additive genetic association using linear and logistic regression models, G \times E interactions, to impute haplotypes (Table S2), and to calculate Hardy–Weinberg equilibrium *P* values. Bayesian and non-Bayesian multivariate analyses were performed to assess the joint effect of *GAL*, *GALR1*, *GALR2*, and *GALR3* on all three phenotypes (reported lifetime depression, current depression, and anxiety). Non-Bayesian statistical analyses were performed with SPSS 21.0 for Windows (IBM). Age and sex were covariates in all analyses. All statistical testing used two-tailed *P* = 0.05 threshold. For detailed

description of the statistical methods and for power calculations, see *SI Methods*. First, we carried out statistical analysis in the total sample because of the moderate sample size, and then replicated our main findings in subpopulations according to study sites (Budapest and Manchester) and sex (female and male).

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Supporting Information

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SI Methods

Phenotypic Data. We tested three depression related phenotypes. **Lifetime depression.** Lifetime depression was measured by the self-reported NewMood Background questionnaire (1–3) and was validated in a subpopulation during face-to-face interviews using Structured Clinical Interview for DSM-IV diagnosis (4), similar to that which we have reported previously (2).

Brief Symptom Inventory depression plus additional items score. Brief Symptom Inventory (BSI) depression plus additional items score (5) was validated in a subpopulation during face-to-face interviews using the interviewer rated Montgomery Åsberg Depression Rating Scale (6). A continuous weighted score (sum of item scores divided by the number of items completed) was used in the analysis.

Brief Symptom Inventory anxiety score. BSI anxiety score (5) which was validated in a subpopulation during face-to-face interviews using interviewer rated Clinical Anxiety Scale (7). A continuous weighted score (sum of item scores divided by the number of items completed) was used in the analysis.

Life Stressors Were Measured by Two Different Scales. To investigate gene \times environment (G \times E) interactions we used confirmed environmental stressors that were consequently associated with the development of depression (8), early life stresses, and recent life stresses.

Early life stresses were scored using childhood adversity (CHA) questions, which were derived from the Childhood Trauma Questionnaire (CTQ) (9) to measure emotional and physical abuse, and emotional and physical neglect and parental loss during childhood, and were validated against CTQ in a subpopulation (2). A five-point Likert scale was used ranging from never true to very often true. The sum of CHA scores was used in the analysis.

Recent life stresses were measured by the List of Life-Threatening Experiences (10), which is a validated measure of negative life events related to intimate relationships, financial difficulties, illnesses/injuries, and network problems. The sum of life-event items reported for the last year was used in the analysis.

Genetic Data

Summary of the selected SNPs can be seen in Table S1, and for the imputed haplotypes in Table S2.

Statistical Analysis. PLINK v1.07 (<http://pngu.mgh.harvard.edu/purcell/plink>) was used to test additive genetic association using linear and logistic regression models, SNP by life-stressor interactions (age and sex were covariates in all analyses), and to calculate Hardy–Weinberg equilibrium P values. Main effects of genotype were investigated for reported lifetime depression, current depression, and current anxiety. Interactions with CHA and recent negative life events were then included in the models for the different depression related phenotypes (in these models main effects of environmental factors were also covariates). To test the diplotypic effects of *GAL*, *GALR1*, and *GALR3*, haplotypes were imputed with a frequency greater than 1% and considering phases $P(H|G) \geq 0.01$, requiring per individual per haplotype missingness <0.5 , as defined for default in PLINK v1.07 (Table S2). The computed haplotypes have been used for further analysis, as described for SNPs above. The default method in PLINK was used to calculate statistical results for the minor allele/haplotype.

Bayesian and non-Bayesian multivariate analyses were performed to assess the joint effect of *GAL*, *GALR1*, *GALR2*, and *GALR3* on all three phenotypes (reported lifetime depression,

current depression, and anxiety). We applied the Bayesian network-based Bayesian multilevel analysis of relevance (BN-BMLA) method to achieve a detailed characterization of associations corresponding to these phenotypes. Non-Bayesian multivariate statistical analyses were performed with SPSS 21.0 for Windows (IBM). All statistical testing used two-tailed $P = 0.05$ threshold.

BN-BMLA. The development of this methodology was largely influenced by the emerging role of detailed or “deep” phenotyping in genetic association studies (11). This recent trend of detailed phenotyping, particularly in psychiatrics, was motivated by several factors, such as the modest success of case/control genome-wide association studies (GWAS), clinical considerations of under- or ill-defined diseases, growing number of cohort studies with detailed epidemiologic, lifestyle, and environmental data, and increasing computational power coupled with better algorithms (12–14). Currently, such detailed phenotyping is typically used to clarify the associations found in follow-up candidate gene association studies performed after GWA. However, the analysis of the relevance of predictors with respect to this set of phenotypic, clinical, and environmental descriptors is a challenging task, as these descriptors themselves are typically strongly interdependent, whereas the effects of the predictors are frequently found to be relatively weak and contextual.

A candidate for this task, the probabilistic graphical model (PGM) class, provides a unifying framework for systems-based modeling and data analysis in computational biomedicine (15). PGMs and its subclass: Bayesian networks (BNs), were applied rapidly in genetics and in genetic association studies, partly because of its causal aspects and partly because of its systems-based foundations (16, 17). Specifically, this framework offers a principled foundation for multivariate association and interaction analysis.

Although PGMs provide an attractive theoretical foundation for the systems-based analysis of a network of phenotypic, environmental, and heterogeneous omic (e.g., genetic) variables, the complexity of the PGMs is much higher than that of other multivariate genetic association study methods. An additional challenge is the explicit modeling of the interdependency structure of the target (or outcome) variables. These targets are typically clinical variables or disease-state descriptors, which frequently show much stronger statistical dependencies than that of the predictors. We proposed a systems-based association analysis methodology in the Bayesian statistical framework to cope with this challenge, called Bayesian multilevel analysis (18, 19). A Bayesian network model $BN(G, \theta)$ consists of a directed acyclic graph structure G , representing the multivariate dependency and independence relations of the variables, and a parameterization θ defining quantitatively the dependencies. The dependency relationships of variables V (factors) are represented by the graph structure. Dependency relationships between the modeled variables are characterized by conditional probability distributions, denoted as θ (parameterization). The a posteriori probability (posterior) of a BN is defined by the Bayes rule:

$$P(G, \theta|D) \propto P(D|G, \theta)P(G, \theta),$$

which is the product of marginal likelihood $P(D|G, \theta)$ of the data D given structure G and its parameters θ , and the prior probability $P(G, \theta)$. The Bayesian framework allows the incorporation of prior knowledge on multiple levels in the forms of hard and soft structure priors represented by $P(G)$ and parameter priors $P(\theta|G)$. Finding an appropriate combination of structure and

parameter priors is still an open research question, which requires the generalization of previous univariate works used in the GAS analysis of SNPs. We used a uniform prior over structures with restricted parental set size and BDeu parameter priors with virtual sample size 1 (20–23). Assuming complete data, these priors allow the efficient analytic computation of the posterior of the structure $G|P(D)$. Based on this equation the posterior probability of strong relevance of a genetic factor X to target variables \underline{Y} $P(MBM(X,Y,G)|D)$ is defined as

$$P(MBM(X,Y,G)|D) = \sum_G P(G|D)1(MBM(X,Y,G)),$$

where $1(MBM(X,Y,G))$ is 1 if the property holds in G and 0 otherwise.

In most cases, the sample size of the dataset is not sufficient with respect to the number of variables to select a single dominant model structure with posterior close to 1. Rather, there are several models with nonnegligible posteriors. A possible solution is to use Bayesian model averaging and certain structural features can be confirmed sufficiently by the data (i.e., receiving relatively high posteriors) (24–27). The BN-BMLA method performs a random walk in the space of BN structures G by applying a Markov-chain Monte Carlo (MCMC) sampling method, which inserts, deletes, and inverts edges (28). The probability to apply different directed acyclic graph operators in the proposal distribution is uniform, the length of the burn-in is 10^6 and the length of the sample collection is 5×10^6 . To check the convergence of the MCMC simulation for the estimated posteriors, we calculated the Geweke z -score and the Gelman–Rubin R -score (less than 0.1 and 1.1, respectively) and confidence intervals (less than 0.1) (18, 19).

We also performed separate analyses in subpopulations to cope with the heterogeneity of effects. The subpopulations were defined by CHA or by the number of recent life events (RLE). Based on CHA scores two groups were created, patients with low or medium (0–6) CHA ($n = 1,959$), and patients with high CHA (≥ 7 ; $n = 390$). Similarly, two groups were formed based on RLEs, patients with low or moderate (0–2) number of RLEs ($n = 2,014$), and patients with high number of RLEs (3 or more; $n = 342$). The current depression and current anxiety scores were converted into a categorical variable with three distinguished categories: low (0 = 0–0.99; depression $n = 1,594$, anxiety $n = 1,530$), moderate (1 = 1–1.99; depression $n = 419$, anxiety $n = 478$), and severe (2 = 2–4; depression $n = 344$, anxiety $n = 349$).

To facilitate the systems-based analysis, the cardinality of haplotypes was decreased by merging. In case of *GAL* and *GALR3*, the major (most frequent) haplotype served as a separate category and all other haplotypes were merged into a separate category. In the case of *GALR1*, haplotypes were dichotomously grouped using a greedy univariate heuristic: haplotypes were ordered according to the average current depression scores of related samples, then haplotypes showing a mainly protective effect were separated into one group, and the other group contained haplotypes of increased risk. Diploypes were formed according to the most probable maternal and paternal haplotypes selected by PLINK and using the haplotype merging detailed above, which lead to ternary diploype variables, [e.g., in case of two *GAL* haplotype groups: the major (0) and all other haplotypes (1), there were four possible diploypes (00), (01) (10), and (11)].

Non-Bayesian Multivariate Analysis. A non-Bayesian multivariate analysis using general linear models (GLM) was applied to test the significance of the total effect of the investigated genetic factors versus the effect of environmental factors (CHA and negative RLE) on all phenotypes (reported lifetime depression, current depression, and current anxiety). For this purpose, two GLM models were constructed: a reduced model containing only environmental factors and a full model containing both environmental and genetic factors, and their interactions. Age and sex were included as cofactors in both models. The residual variance of the reduced and the full model was compared and tested using an F -statistic.

GLM and other statistical analyses were performed with SPSS 21.0 for Windows (IBM). All statistical testing used two-tailed $P = 0.05$ threshold.

Power Calculation for Genetic Association Studies That Use Logistic and Linear Regression Analysis. For power calculation, Quanto 1.2.4 version (<http://hydra.usc.edu/gxe>) was used.

Our population consisted of $n = 2,361$ subjects with $n = 974$ cases (control/case ratio = 1.4) and minor-allele frequency ranged between 3% and 44%.

For a Disease. We used additive model with an overall disease risk = 15% in the general population (similar to that in ref. 29) and with $P = 0.05$ type I error rate: We have 19–70% power to detect genetic main effects with odds ratio (OR) = 1.2, or 86–99% power to detect genetic main effects with OR = 1.5; we have 17–53% power to detect G×E interactions with OR = 1.2, or 77–99% power to detect G×E interactions with OR = 1.5 assuming a continuous environmental effect (SD = 1.5–3.4 based on our RLE and CHA data) with the same conditions as above and an OR = 1.0 for genetic relative risk (assuming that genetic effects only relevant when environmental effect present) and an OR = 2.5 for environmental relative risk.

For a Continuous Trait. We used additive model with depression score mean = 0.85, SD = 0.97, based on our BSI depression data, which corresponds well with the BSI questionnaire validation data in adult nonpatients and psychiatric outpatients (5): We have 93% power to detect genetic main effects that explain $R^2 = 0.5\%$ of variations in a continuous variable; We have 96% power to detect gene × environment interactions that explain $R^2 = 0.5\%$ of variations in a continuous variable assuming a continuous environmental effect (SD = 1.5–3.4 based on our RLE and CHA data) with the same conditions as above and an explained variance of genetic effects $R^2 = 0\%$ (assuming that genetic effects only relevant when environmental effect present) and an explained variance of environmental effects $R^2 = 7.4$ –14% (based on our RLE and CHA data).

For a Disease in an Exposed-Only Population at GWAS Significance Level ($P \leq 5 \times 10^{-8}$). In an exposed-only population with high level of life stressors the relative risk of depression is about 2.5-times higher, thus the overall disease risk = 37.5%. Using a control/case ratio = 1, minor allele frequency between 4% and 49% (based on the HapMap project CEU+TSI populations), and assuming that the genetic relative risks are stronger with the OR = 1.2–1.5 the required number of subjects to achieve $P \leq 5 \times 10^{-8}$ significance with 90% power.

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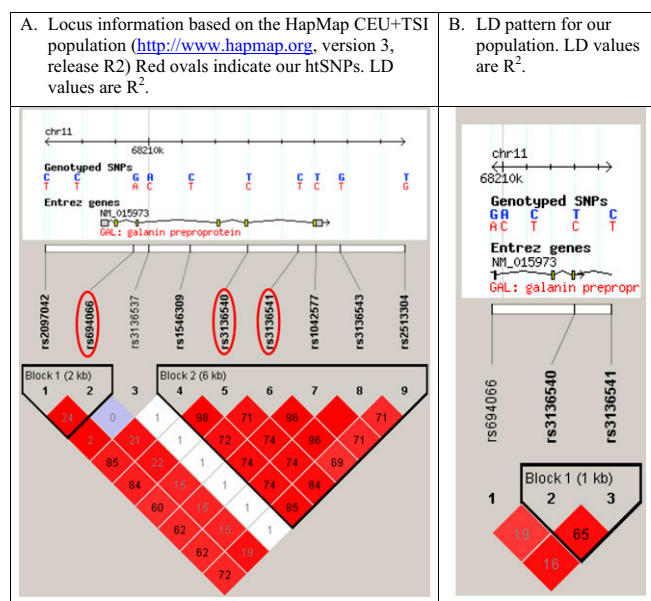


Fig. S1. (A and B) Galanin. Three SNPs within the GAL gene (chromosome 11q13.3, rs694066, rs3136540, and rs3136541) were selected.

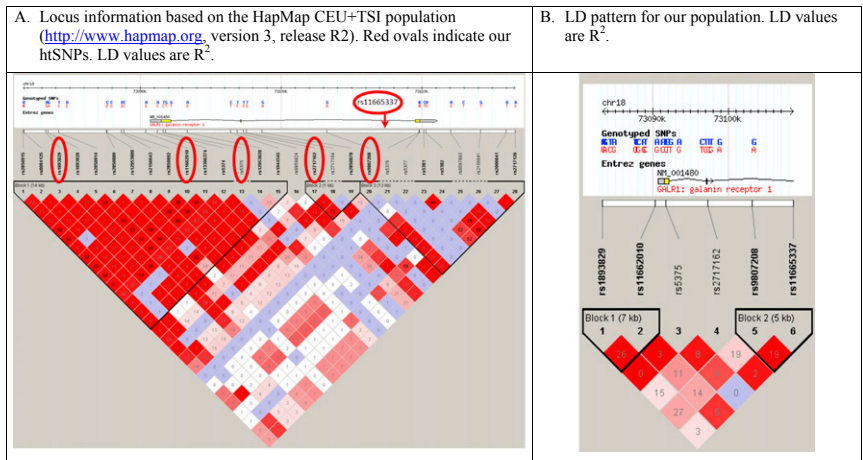


Fig. S2. (A and B) *GALR1*. Five SNPs within the *GALR1* gene (chromosome 18q23, rs11662010, rs5375, rs2717162, rs9807208, and rs11665337) plus one SNP (rs1893829) upstream from the *GALR1* gene (potential functional effect on the expression) were selected.

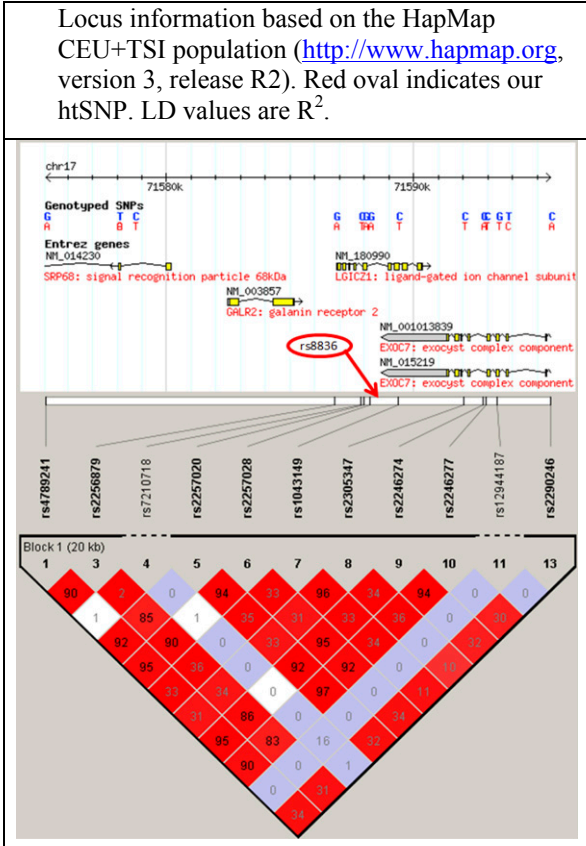


Fig. S3. *GALR2*. For *GALR2* (chromosome 17q25.3), which sits in a conserved (monomorphic) genetic region. We selected rs8836 which is downstream to the *GALR2* gene in strong linkage disequilibrium with it, and was associated with depression in chronic pain patients previously (1).

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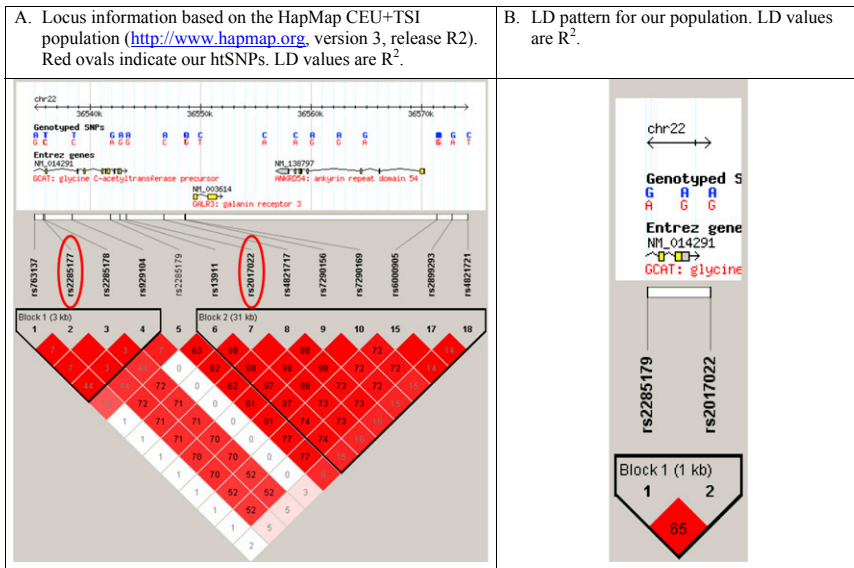
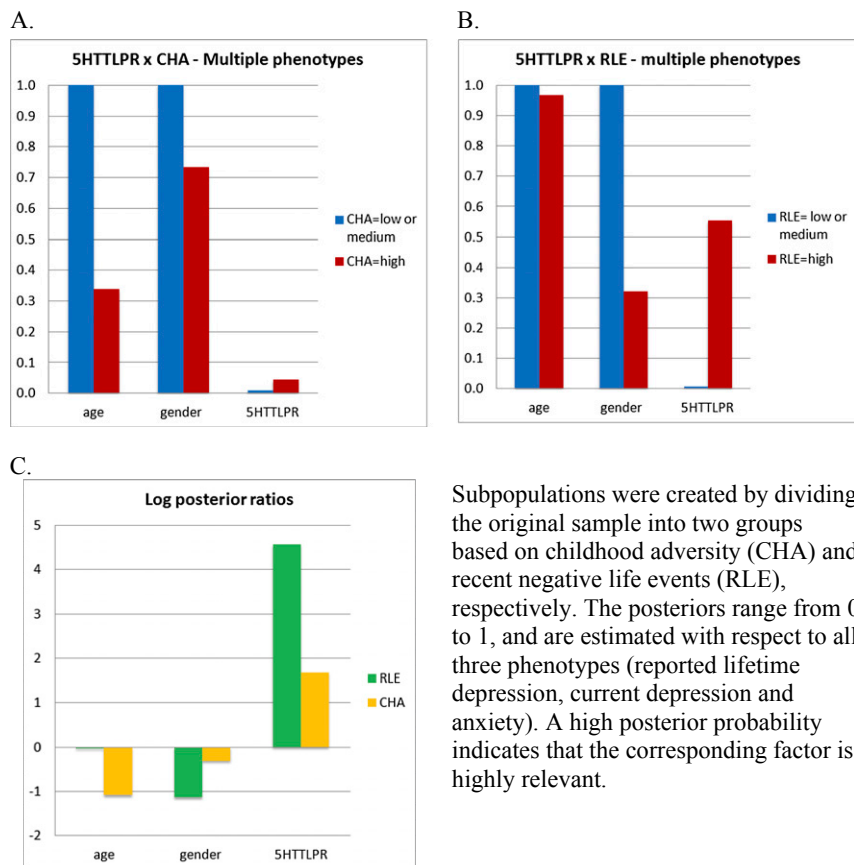


Fig. S4. (A and B) *GALR3*. Because the genetic region which contains the *GALR3* gene is not polymorphic we selected 2 SNPs upstream to this gene (chromosome 22q13.1, rs2017022 and rs2285179) that are in strong linkage disequilibrium (LD) with the *GALR3* gene and have potential functional effect on the expression of *GALR3*.



Subpopulations were created by dividing the original sample into two groups based on childhood adversity (CHA) and recent negative life events (RLE), respectively. The posteriors range from 0 to 1, and are estimated with respect to all three phenotypes (reported lifetime depression, current depression and anxiety). A high posterior probability indicates that the corresponding factor is highly relevant.

Fig. S5. The comparison of posterior probabilities of relevance of the *5HTTLPR* polymorphism for subpopulations under the influence of different environmental factors. (A) This panel shows the posterior probability of relevance of *5HTTLPR* for patients with low-medium CHA versus patients with high CHA. *5HTTLPR* is not relevant in the low-medium CHA group or in subjects with high CHA ($Pr = 0.04$). In contrast, age and sex, which were included as cofactors, are highly relevant, especially in low-medium CHA patients. For comparison with GAL system genes see Fig. 1B. (B) This panel compares the posterior probability of relevance (Pr) of *5HTTLPR* in case of patients with low or medium RLE versus patients with high RLE. In case of the former subpopulation the *5HTTLPR* is nonrelevant, contrary to the high RLE group, where it is moderately relevant ($Pr = 0.55$). Traditional regression analysis supported that the S allele was this risk variant for all three investigated phenotype (for S allele \times RLE interaction: lifetime depression: OR = 1.366 $CI_{0.95} = 0.964-1.937$ $P = 0.080$; current depression: $\beta = 0.151$ SE = 0.076 $P = 0.048$; current anxiety: $\beta = 0.083$ SE = 0.074 $P = 0.260$). For comparison with GAL system genes see Fig. 1C. (C) The high (>3) log posterior ratio indicates that there is a substantial difference in terms of posterior probability of relevance in case of *5HTTLPR* between those who experienced high recent negative life stress and who did not. In addition, *5HTTLPR* is more relevant in those who experienced high CHA, but the log posterior ratio has not reached substantial level. For comparison with GAL system genes see Fig. 1D.

Table S1. Selected SNPs for the galanin pathway

CHR	SNP	A1	A2	MAF	HWE p	P _{diff} p	MAF
							CEU+TSI
GAL (Chr 11)	rs694066	A	G	0.08	0.89	0.67	0.09
	rs3136540	T	C	0.26	0.33	0.86	0.29
	rs3136541	C	T	0.34	0.84	0.052	0.33
GALR2 (Chr 17)	rs8836	G	C	0.41	0.39	0.15	0.42*
GALR1 (Chr 18)	rs1893829	A	G	0.24	0.31	0.09	0.21
	rs11662010	G	A	0.45	0.93	1.00	0.49
	rs5375	T	G	0.03	1.00	0.22	0.04
	rs2717162	C	T	0.24	0.07	0.91	0.23
	rs9807208	G	A	0.32	0.70	0.88	0.32
GALR3 (Chr 22)	rs11665337	A	G	0.08	0.32	0.35	0.06
	rs2017022	A	G	0.34	0.55	0.42	0.34
	rs2285179	G	A	0.44	0.93	0.62	0.45

A1, minor allele; A2, major allele; Chr, chromosome number; HWE p, Hardy-Weinberg equilibrium P value in the combined population; MAF, minor allele frequency in the combined population; MAF CEU+TSI, minor allele frequency in the HapMap project CEU+TSI populations; P_{diff} p, genotypic difference between the Budapest and Manchester cohorts (P value) using additive genetic model and logistic regression.

*MAF data for the HapMap CEPH population, the ancestral allele is C but in this population this is the minor allele, as well. In our population the minor allele is G.

Table S2. Imputed haplotypes for the galanin pathway and their frequencies for the combined population

Locus	Haplo type	Frequency	Order	Haplotype grouping*
GAL	GCT	0.66	HT1	0
GAL	GTC	0.18	HT2	1
GAL	GCC	0.08	HT3	1
GAL	ATC	0.07	HT4	1
GALR1	GGGTAG	0.39	HT1	0
GALR1	GAGTAG	0.18	HT2	0
GALR1	AAGCGG	0.10	HT3	1
GALR1	GAGCAG	0.04	HT4	0
GALR1	AAGTGG	0.04	HT5	1
GALR1	GAGTGA	0.04	HT6	1
GALR1	AAGTGA	0.04	HT7	0
GALR1	AAGTAG	0.04	HT8	1
GALR1	GAGCGG	0.03	HT9	0
GALR1	AAGCAG	0.03	HT10	1
GALR1	GGGTGG	0.02	HT11	1
GALR1	GGTCGG	0.02	HT12	0
GALR3	GA	0.56	HT1	0
GALR3	AG	0.34	HT2	1
GALR3	GG	0.10	HT3	1

The order of the alleles in the haplotypes corresponds to the order of SNPs in Table S1. HT order is according to the haplotype frequency in the combined population.

*Haplotype grouping shows the haplotype groups used in the systems based analysis. For haplotype grouping strategy see *Statistical Analysis*.

Table S3. Required sample size for GWAS significant association with major depressive disorder (MDD) using an exposed only design (all subjects have high level of life stresses)

Genetic relative risk (OR)	Allele frequency 4%			Allele frequency 49%		
	No. cases	No. controls	No. total	No. cases	No. controls	No. total
1.2	34,916	34,916	69,832	5,477	5,477	10,954
1.3	16,779	16,779	33,558	2,656	2,656	5,312
1.4	10,170	10,170	20,340	1,624	1,624	3,248
1.5	6,991	6,991	13,982	1,125	1,125	2,250

Table S4. Genotypic association results investigating the main effects of galanin genes and their interaction with life stressors on the three outcome variables separately

GAL genotypic				Reported lifetime depression				Current depression			Current anxiety		
SNP	A1	Test		OR	L95	U95	<i>P</i>	β	SE	<i>P</i>	β	SE	<i>P</i>
SNP1	rs694066	A	ADD	1.10	0.88	1.36	0.4	0.05	0.05	0.4	0.06	0.05	0.3
SNP2	rs3136540	T	ADD	1.01	0.88	1.16	0.9	0.06	0.03	0.1	0.04	0.03	0.2
SNP3	rs3136541	C	ADD	1.05	0.92	1.20	0.5	0.08	0.03	0.02	0.03	0.03	0.3
SNP1	rs694066	A	ADD×CHA	0.95	0.88	1.01	0.1	-0.03	0.01	0.06	-0.02	0.01	0.3
SNP2	rs3136540	T	ADD×CHA	1.01	0.96	1.06	0.7	0.003	0.01	0.8	0.01	0.01	0.4
SNP3	rs3136541	C	ADD×CHA	0.98	0.93	1.02	0.3	-0.004	0.01	0.7	0.0004	0.01	1.0
SNP1	rs694066	A	ADD×RLE	0.96	0.81	1.14	0.6	-0.01	0.04	0.8	0.02	0.04	0.6
SNP2	rs3136540	T	ADD×RLE	0.97	0.87	1.09	0.6	-0.01	0.03	0.8	-0.01	0.03	0.8
SNP3	rs3136541	C	ADD×RLE	0.94	0.84	1.06	0.3	-0.02	0.03	0.5	-0.03	0.03	0.3
SNP1	rs1893829	A	ADD	0.93	0.80	1.09	0.4	0.06	0.04	0.1	0.04	0.04	0.3
SNP2	rs11662010	G	ADD	0.99	0.87	1.11	0.8	0.002	0.03	0.9	0.01	0.03	0.8
SNP3	rs5375	T	ADD	1.04	0.71	1.51	0.8	-0.07	0.09	0.5	-0.05	0.09	0.6
SNP4	rs2717162	C	ADD	0.96	0.83	1.11	0.5	-0.04	0.04	0.2	-0.05	0.04	0.2
SNP5	rs9807208	G	ADD	0.92	0.81	1.05	0.2	-0.01	0.03	0.8	-0.02	0.03	0.6
SNP6	rs11665337	A	ADD	0.91	0.72	1.13	0.4	-0.07	0.06	0.2	-0.05	0.05	0.4
SNP1	rs1893829	A	ADD×CHA	1.04	0.98	1.10	0.2	0.02	0.01	0.1	0.01	0.01	0.2
SNP2	rs11662010	G	ADD×CHA	0.96	0.93	1.01	0.09	-0.01	0.01	0.5	-0.002	0.01	0.8
SNP3	rs5375	T	ADD×CHA	0.87	0.79	0.97	0.009	-0.01	0.02	0.5	-0.01	0.02	0.7
SNP4	rs2717162	C	ADD×CHA	0.98	0.93	1.03	0.3	0.003	0.01	0.7	0.00	0.01	0.8
SNP5	rs9807208	G	ADD×CHA	1.02	0.97	1.07	0.4	0.01	0.01	0.2	0.01	0.01	0.3
SNP6	rs11665337	A	ADD×CHA	1.09	0.99	1.19	0.09	0.04	0.02	0.02	0.04	0.02	0.04
SNP1	rs1893829	A	ADD×RLE	1.18	1.04	1.34	0.01	0.05	0.03	0.1	0.01	0.03	0.8
SNP2	rs11662010	G	ADD×RLE	0.94	0.86	1.04	0.2	0.01	0.02	0.8	0.05	0.02	0.03
SNP3	rs5375	T	ADD×RLE	0.90	0.64	1.25	0.5	-0.05	0.08	0.5	0.01	0.08	0.9
SNP4	rs2717162	C	ADD×RLE	1.09	0.96	1.23	0.2	0.01	0.03	0.6	-0.02	0.03	0.6
SNP5	rs9807208	G	ADD×RLE	1.10	0.98	1.23	0.1	-0.001	0.03	1.0	-0.02	0.03	0.5
SNP6	rs11665337	A	ADD×RLE	1.06	0.86	1.30	0.6	-0.07	0.05	0.1	-0.06	0.05	0.2
SNP1	rs8836	G	ADD	1.07	0.95	1.21	0.3	0.01	0.03	0.8	0.001	0.03	1.0
SNP1	rs8836	G	ADD×CHA	1.02	0.98	1.07	0.3	-0.01	0.01	0.5	0.000	0.01	1.0
SNP1	rs8836	G	ADD×RLE	1.13	1.02	1.25	0.02	0.05	0.02	0.04	0.03	0.02	0.1
SNP1	rs2017022	A	ADD	1.13	0.99	1.28	0.06	0.03	0.03	0.4	0.01	0.03	0.9
SNP2	rs2285179	G	ADD	1.12	0.98	1.27	0.09	0.03	0.03	0.4	0.003	0.03	0.9
SNP1	rs2017022	A	ADD×CHA	1.00	0.96	1.05	1.0	-0.01	0.01	0.3	-0.01	0.01	0.1
SNP2	rs2285179	G	ADD×CHA	0.98	0.94	1.02	0.3	-0.01	0.01	0.3	-0.02	0.01	0.04
SNP1	rs2017022	A	ADD×RLE	0.97	0.88	1.07	0.6	-0.001	0.02	1.0	0.02	0.02	0.4
SNP2	rs2285179	G	ADD×RLE	1.03	0.93	1.15	0.6	0.02	0.02	0.5	0.04	0.02	0.1

Linear and logistic regression models were used in PLINK (<http://pngu.mgh.harvard.edu/purcell/plink/>), age and sex were covariants in all models. None of the significant findings survived Bonferroni correction for multiple testing. A1, minor allele/haplotype (1=yes, 2=no); ADD, additive model, CHA: childhood adversity; HT, haplotype; RLE, recent negative life events. Bold: $P \leq 0.05$; Italic: $0.05 < P < 0.1$. The order of the alleles in the haplotypes corresponds to the order of SNPs in Table 1.

Table S5. Diplotypic association results investigating the main effects of galanin genes and their interaction with life stressors on the three outcome variables separately

GAL diplotypic				Reported lifetime depression				Current depression			Current anxiety		
Haplotype	A1	Test		OR	L95	U95	<i>P</i>	β	SE	<i>P</i>	β	SE	<i>P</i>
HT1	GCT	2	ADD	1.05	0.93	1.20	0.4	0.08	0.03	0.01	0.04	0.03	0.2
HT2	GTC	1	ADD	0.96	0.81	1.12	0.6	<i>0.07</i>	<i>0.04</i>	<i>0.09</i>	0.03	0.04	0.5
HT3	GCC	1	ADD	1.17	0.92	1.48	0.2	0.09	0.06	0.1	0.02	0.06	0.7
HT4	ATC	1	ADD	1.09	0.86	1.37	0.5	0.02	0.06	0.8	0.05	0.06	0.4
HT1	GCT	2	ADD×CHA	0.99	0.95	1.04	0.7	0.00	0.01	0.9	0.01	0.01	0.4
HT2	GTC	1	ADD×CHA	1.02	0.96	1.08	0.5	0.01	0.01	0.2	0.01	0.01	0.2
HT3	GCC	1	ADD×CHA	0.98	0.90	1.05	0.5	0.01	0.02	0.4	0.02	0.01	0.2
HT4	ATC	1	ADD×CHA	0.97	0.90	1.05	0.5	-0.03	0.02	0.1	-0.01	0.02	0.5
HT1	GCT	2	ADD×RLE	0.95	0.86	1.06	0.4	-0.01	0.02	0.6	-0.02	0.02	0.4
HT2	GTC	1	ADD×RLE	1.00	0.88	1.14	1.0	0.02	0.03	0.5	-0.01	0.03	0.7
HT3	GCC	1	ADD×RLE	0.91	0.76	1.10	0.3	-0.04	0.04	0.4	-0.05	0.04	0.3
HT4	ATC	1	ADD×RLE	0.94	0.79	1.12	0.5	-0.04	0.04	0.4	0.01	0.04	0.8
HT1	GGGTAG	1	ADD	0.97	0.84	1.11	0.7	0.01	0.03	0.8	0.03	0.03	0.3
HT2	GAGTAG	1	ADD	1.09	0.91	1.29	0.3	0.01	0.04	0.8	0.01	0.04	0.8
HT3	AAGCGG	1	ADD	0.93	0.75	1.15	0.5	0.03	0.05	0.5	0.004	0.05	0.9
HT4	GAGCAG	1	ADD	1.19	0.85	1.67	0.3	-0.17	0.08	0.05	-0.11	0.08	0.2
HT5	AAGTGG	1	ADD	1.12	0.79	1.57	0.5	0.20	0.09	0.02	0.22	0.08	0.01
HT6	GAGTGA	1	ADD	1.00	0.69	1.46	1.0	-0.08	0.09	0.4	-0.10	0.09	0.3
HT7	AAGTGA	1	ADD	<i>0.71</i>	<i>0.49</i>	<i>1.03</i>	<i>0.07</i>	-0.07	0.09	0.4	-0.10	0.08	0.2
HT8	AAGTAG	1	ADD	1.31	0.89	1.91	0.2	0.15	0.10	0.1	0.11	0.09	0.2
HT9	GAGCGG	1	ADD	0.81	0.55	1.21	0.3	-0.09	0.10	0.4	-0.12	0.09	0.2
HT10	AAGCAG	1	ADD	1.00	0.60	1.67	1.0	-0.06	0.13	0.7	-0.06	0.12	0.6
HT11	GGGTGG	1	ADD	0.82	0.49	1.39	0.5	-0.03	0.13	0.8	-0.07	0.12	0.6
HT12	GGTCGG	1	ADD	1.09	0.71	1.66	0.7	-0.01	0.11	0.9	0.02	0.10	0.8
HT1	GGGTAG	1	ADD×CHA	1.00	0.96	1.05	0.9	-0.01	0.01	0.5	-0.003	0.01	0.8
HT2	GAGTAG	1	ADD×CHA	0.98	0.93	1.04	0.5	-0.02	0.01	0.05	-0.03	0.01	0.009
HT3	AAGCGG	1	ADD×CHA	1.03	0.96	1.12	0.4	0.02	0.01	0.2	0.01	0.01	0.5
HT4	GAGCAG	1	ADD×CHA	1.05	0.92	1.19	0.5	0.02	0.02	0.3	0.04	0.02	0.1
HT5	AAGTGG	1	ADD×CHA	1.08	0.95	1.23	0.2	0.00	0.02	0.8	0.03	0.02	0.1
HT6	GAGTGA	1	ADD×CHA	<i>1.17</i>	<i>0.99</i>	<i>1.39</i>	<i>0.07</i>	0.07	0.03	0.005	0.07	0.02	0.003
HT7	AAGTGA	1	ADD×CHA	1.02	0.89	1.17	0.8	0.01	0.03	0.8	-0.005	0.03	0.9
HT8	AAGTAG	1	ADD×CHA	1.09	0.93	1.28	0.3	0.04	0.03	0.2	0.04	0.03	0.2
HT9	GAGCGG	1	ADD×CHA	0.94	0.84	1.05	0.3	-0.05	<i>0.02</i>	<i>0.06</i>	-0.02	0.02	0.4
HT10	AAGCAG	1	ADD×CHA	0.96	0.82	1.12	0.6	0.05	0.04	0.2	0.02	0.03	0.5
HT11	GGGTGG	1	ADD×CHA	0.91	0.79	1.07	0.2	0.00	0.03	0.9	-0.05	0.03	0.1
HT12	GGTCGG	1	ADD×CHA	0.88	0.78	0.98	0.02	-0.05	0.03	0.05	-0.04	0.03	0.2
HT1	GGGTAG	1	ADD×RLE	0.94	0.84	1.05	0.3	0.01	0.03	0.8	<i>0.05</i>	<i>0.03</i>	<i>0.07</i>
HT2	GAGTAG	1	ADD×RLE	0.93	0.80	1.07	0.3	-0.04	0.03	0.3	-0.05	0.03	0.1
HT3	AAGCGG	1	ADD×RLE	1.11	0.94	1.31	0.2	0.03	0.04	0.5	-0.005	0.04	0.9
HT4	GAGCAG	1	ADD×RLE	1.06	0.80	1.41	0.7	-0.05	0.07	0.4	-0.06	0.06	0.4
HT5	AAGTGG	1	ADD×RLE	1.22	0.90	1.64	0.2	0.00	0.07	1.0	0.04	0.06	0.5
HT6	GAGTGA	1	ADD×RLE	1.22	0.86	1.73	0.3	-0.08	0.08	0.3	-0.13	<i>0.08</i>	<i>0.08</i>
HT7	AAGTGA	1	ADD×RLE	1.00	0.70	1.45	1.0	-0.07	0.08	0.4	-0.08	0.08	0.4
HT8	AAGTAG	1	ADD×RLE	1.02	0.75	1.39	0.9	0.05	0.07	0.5	-0.004	0.07	1.0
HT9	GAGCGG	1	ADD×RLE	0.95	0.66	1.36	0.8	0.04	0.09	0.6	-0.02	0.08	0.8
HT10	AAGCAG	1	ADD×RLE	1.38	0.83	2.27	0.2	0.29	0.10	0.004	0.21	0.10	0.03
HT11	GGGTGG	1	ADD×RLE	1.00	0.69	1.46	1.0	-0.04	0.08	0.6	-0.07	0.08	0.4
HT12	GGTCGG	1	ADD×RLE	0.86	0.60	1.22	0.4	-0.05	0.08	0.5	-0.01	0.08	0.9
HT1	GA	2	ADD	1.11	0.98	1.26	0.1	0.03	0.03	0.4	0.001	0.03	1.0
HT2	AG	1	ADD	1.10	0.96	1.27	0.2	0.02	0.03	0.5	0.002	0.03	1.0
HT3	GG	1	ADD	1.04	0.85	1.29	0.7	0.02	0.05	0.8	0.002	0.05	1.0
HT1	GA	2	ADD×CHA	0.98	0.93	1.02	0.3	-0.01	0.01	0.3	-0.02	0.01	0.02
HT2	AG	1	ADD×CHA	0.99	0.94	1.04	0.6	-0.01	0.01	0.2	-0.02	<i>0.01</i>	<i>0.08</i>
HT3	GG	1	ADD×CHA	0.96	0.89	1.04	0.4	0.00	0.02	0.9	-0.01	0.02	0.4
HT1	GA	2	ADD×RLE	1.04	0.93	1.15	0.5	0.02	0.02	0.5	0.04	0.02	0.1
HT2	AG	1	ADD×RLE	1.00	0.89	1.12	1.0	0.01	0.03	0.8	0.03	0.02	0.2
HT3	GG	1	ADD×RLE	1.10	0.92	1.31	0.3	0.03	0.04	0.5	0.02	0.04	0.5

Linear and logistic regression models were used in PLINK (<http://pngu.mgh.harvard.edu/purcell/plink/>), age and sex were covariants in all models. None of the significant findings survived Bonferroni correction for multiple testing. A1, minor allele/haplotype (1=yes, 2=no); ADD, additive model; CHA, childhood adversity; HT, haplotype; RLE, recent negative life events. Bold: $P \leq 0.05$; Italic: $0.05 < P < 0.1$. The order of the alleles in the haplotypes corresponds to the order of SNPs in Table 1. HT order is according to the haplotype frequency in the combined population.

Table S6. The posterior probabilities of relevance for subpopulations under the influence of different environmental factors

Gene	Recent negative life events			Childhood adversity		
	Low or medium	High	Log posterior ratio	Low or medium	High	Log posterior ratio
<i>GAL</i>	0.006	0.32	3.98	0.002	0.11	3.88
<i>GALR1</i>	0.004	0.27	4.15	0.002	0.96	6.09
<i>GALR2</i>	0.008	0.75	4.60	0.009	0.16	2.94
<i>GALR3</i>	0.004	0.51	4.83	0.03	0.16	1.80

Low or medium: indicate low or medium level exposure to recent negative life events or to childhood adversity. High: indicate high level exposure to recent negative life events or to childhood adversity. Log posterior ratio is the difference between the posteriors of subpopulations. Log posterior ratios >3 indicate substantial difference in terms of posterior probability of relevance between groups, namely relevant effect in the high exposure group only.

Table S7. Galanin system related genetic association results from the Psychiatric Genetic Consortium (PGC) mega-analysis for MDD and potential functional relevance of genetic variants in the tagged genetic regions

SNP	a1	a2	OR	SE	P	Functional prediction	Regulatory potential
GAL (chr.11)							
rs2156464*	A	G	1.07	0.03	0.022		0.09
rs7935394	A	G	0.96	0.02	0.09		
rs4930593	A	C	1.04	0.02	0.09		
rs6591348	T	C	0.98	0.04	0.62		0.08
rs2513298	T	C	2.26	0.55	0.14		
rs2510387	A	G	0.95	0.03	0.045		
rs2513297	T	C	1.06	0.03	0.048		
rs2187331	T	C	0.95	0.03	0.046		
rs7342173	T	C	0.14	2.67	0.47	TFBS	0.08
rs2097042	T	C	0.97	0.02	0.22	TFBS	
rs694066	A	G	0.97	0.04	0.42		0.20
rs3136537	A	C	1.04	0.06	0.53		
rs1546309	T	C	0.98	0.02	0.45		
rs3136540	T	C	1.02	0.02	0.47		
rs3136541	T	C	0.96	0.02	0.087		
rs1042577	T	C	1.04	0.02	0.11	miRNA	0.18
rs3136543	T	G	0.96	0.02	0.11		
rs2513304	T	G	1.01	0.02	0.78		
GALR1 (chr.18)							
rs2850915	A	G	1.05	0.02	0.023		
rs8084125	T	C	1.02	0.03	0.56		
rs1893829	A	G	0.95	0.03	0.048		
rs1893828	A	G	1.05	0.03	0.046		
rs2850914	T	C	0.95	0.02	0.023		
rs2850889	T	C	0.96	0.02	0.07	TFBS	
rs12953809	T	C	1.06	0.04	0.13	TFBS	
rs2156643	A	G	0.96	0.02	0.07	TFBS	
rs2850892	T	C	0.96	0.02	0.07	TFBS	
rs11662010	A	G	0.96	0.02	0.06	TFBS	0.25
rs13306374	A	C	0.96	0.02	0.06		
rs5374	T	C	0.96	0.02	0.10		0.52
rs5375	T	G	0.92	0.07	0.20		0.52
rs12953828	A	G	0.93	0.04	0.09		
rs1944545	T	C	0.99	0.04	0.78		
rs9959924	T	C	1.06	0.04	0.21		0.17
rs2717162	T	C	1.03	0.03	0.30		
rs2717164	T	G	1.27	0.09	0.012		
rs2850878	A	G	1.01	0.03	0.71		
rs9807208	A	G	1.06	0.02	0.009		0.07
rs11665337[†]							
rs5376	A	G	0.57	0.59	0.34	nsSNP	0.17
rs5377	T	C	0.86	0.33	0.64	nsSNP	0.22
rs5381	T	C	0.98	0.04	0.56	miRNA	
rs5382	A	G	1.02	0.04	0.61	miRNA	
rs8097893	A	G	1.08	0.05	0.12		0.15
rs2156641	T	G	1.23	0.06	0.001		0.28
rs2000841	A	T	1.04	0.03	0.16		0.14
rs2717128	A	G	1.05	0.03	0.15		0.11
GALR2 (chr.17)							
rs4789241	A	G	1.00	0.02	0.64		
rs2256879	A	G	1.00	0.02	0.70	TFBS	0.28
rs7210718	T	C	0.96	0.11	0.70	TFBS	0.29
rs2257020	A	G	1.01	0.02	0.71	nsSNP	0.28
rs2257028	A	G	1.01	0.02	0.67		0.12
rs8836[†]						miRNA	0.20
rs1043149	T	C	1.01	0.03	0.85	Splicing, miRNA, stop codon	0.21
rs2305347	T	C	1.01	0.03	0.81		0.20
rs2246274	A	G	1.01	0.03	0.75		0.18
rs2246277	T	C	1.01	0.02	0.75		0.02
rs2290246	A	C	1.00	0.03	0.93	TFBS	0.03

Table S7. Cont.

SNP	a1	a2	OR	SE	P	Functional prediction	Regulatory potential
GALR3 (chr.22)							
rs763137	A	G	0.97	0.03	0.32		
rs2285177	T	C	0.99	0.02	0.55		0.06
rs2285178	T	C	0.99	0.02	0.54	Splicing	0.16
rs929104	T	C	0.98	0.05	0.51		
rs2285179	A	G	1.00	0.02	0.95		
rs13911	A	G	1.00	0.02	0.99	miRNA	0.30
rs2017022	A	G	1.00	0.02	1.00		
rs4821717	A	C	1.00	0.02	0.98	TFBS	
rs7290156	T	C	1.00	0.02	0.97	TFBS	0.12
rs7290169	A	G	1.00	0.02	1.00	TFBS	0.06
rs6000905	A	G	1.00	0.02	0.70	TFBS	
rs2899293	A	G	0.99	0.02	0.69	TFBS	
rs4821721	T	C	1.02	0.04	0.58	TFBS	

Yellow rows indicate our haplotype tags. Gray rows indicate additional SNPs between the nominally significant SNP in the *GAL* gene (1) and our tagged region. White rows are SNPs in linkage with our tagging SNPs (see below). Bold and italic fonts indicate nominally significant association in the PGC mega-analysis; Italic: trend-association in the PGC mega-analysis (2). Functional prediction: The National Institute of Health Sciences tool [<http://snpinfo.nih.gov/snpinfo/snpfunc.htm>] (3) was used to investigate the possible functional relevance of the tagged genetic regions. Our genotyped SNPs were fed into the program and it automatically added all other nearby SNPs that are in LD ≥ 0.8 and present in the CEU+TSI populations based on the HapMap project (www.hapmap.org). Next the algorithm determined the potential functional effects of the selected SNPs on protein structure, gene regulation, splicing and miRNA binding and listed it in a table format. Regulatory Potential: Regulatory potential score [ESPERR Regulatory Potential (7 Species)] downloaded from UCSC genome bioinformatics web site (<http://genome.ucsc.edu>). Conservation, Vertebrate Multiz Alignment and Conservation score (17 Species) downloaded from UCSC genome bioinformatics web site (<http://genome.ucsc.edu>). miRNA, miRNA binding site activity; nsSNP, SNPs in protein-coding regions that can cause amino acid change; Splicing, SNPs that are located at two base pairs of the intron-exon junction region; Stop codon, SNPs that may lead to premature termination of peptides (non-sense), which would disable the protein function. TFBS: transcription factor-binding site.

*rs2156464: The best SNP for the *GAL* gene which was in the top 10 genes in the previous PGC analysis for MDD (1).
[†]SNPs for which there are no data in the PGC analysis.

1. Wray NR, et al. (2012) Genome-wide association study of major depressive disorder: New results, meta-analysis, and lessons learned. *Mol Psychiatry* 17(1):36–48.
2. Ripke S, et al.; Major Depressive Disorder Working Group of the Psychiatric GWAS Consortium (2013) A mega-analysis of genome-wide association studies for major depressive disorder. *Mol Psychiatry* 18(4):497–511.
3. Xu Z, Taylor JA (2009) SNPinfo: Integrating GWAS and candidate gene information into functional SNP selection for genetic association studies. *Nucleic Acids Res* 37(Web Server issue): W600–W605.