GLRB allelic variation associated with agoraphobic cognitions, increased startle response and fear network activation: A potential neurogenetic pathway to panic disorder

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Abstract

The molecular genetics of panic disorder (PD) with and without agoraphobia (AG) are still largely unknown and progress is hampered by small sample sizes. We therefore performed a genome-wide association study with a dimensional, PD/AG - related anxiety phenotype based on the Agoraphobia Cognition Questionnaire (ACQ) in a sample of 1,370 healthy German volunteers of the CRC TRR58 MEGA study wave 1. A genome-wide significant association was found between ACQ and single non-coding nucleotide variants of the *GLRB* gene (rs78726293, $p=3.3x10^{-8}$; rs191260602, $p=3.9x10^{-8}$). We followed up on this finding in a larger dimensional ACQ sample (N=2,547) and in independent samples with a dichotomous AG phenotype based on the Symptoms Checklist (SCL-90; N=3,845) and a case-control sample with the categorical phenotype PD/AG ($N_{combined}$ =1,012) obtaining highly significant p-values also for *GLRB* single nucleotide variants rs17035816 ($p=3.8x10^{-4}$) and rs7688285 ($p=7.6x10^{-5}$). *GLRB* gene expression was found to be modulated by rs7688285 in brain tissue as well as cell culture. Analyses of intermediate PD/AG phenotypes demonstrated increased startle reflex and increased fear network as well as general sensory activation by *GLRB* risk gene variants rs78726293, rs191260602, rs17035816 and rs7688285. Partial *Glrb* knockout-mice demonstrated an agoraphobic phenotype. In conjunction with the clinical observation that rare coding *GLRB* gene mutations are associated with the neurological disorder hyperekplexia characterized by a generalized startle reaction and agoraphobic behavior, our data provide evidence that non-coding, though functional *GLRB* gene polymorphisms may predispose to PD by increasing startle response and agoraphobic cognitions.

Introduction

Panic disorder (PD) with a life-time prevalence of 2-3 percent, causing a huge burden of disease [1], is characterized by sudden panic attacks, anticipatory anxiety of the next panic attack and frequently accompanied by agoraphobia (AG) [2]. AG is a clinical condition characterized by abnormal open space behavior [3] and, importantly, distorted cognitive processes [4] thus reflecting a dimensional phenotype which can be assessed with the Agoraphobic Cognitions Questionnaire (ACQ) [5]. Family studies reveal familial aggregation in PD and AG [6, 7] and twin studies estimated heritabilities of about 38% and 48%, with a genetic correlation of 0.83 between both disorders [8].

Linkage and candidate gene association studies of PD/AG were mostly negative or inconsistent [9-12] due to phenotypic diversity, genetic heterogeneity and underpowered sample sizes. Of the candidate genes, only the catechol-O-methyltransferase (*COMT*) [13-15] gene, the neuropeptide S receptor gene (*NPSR1*) [16] and the monoamine oxidase A (*MAOA*) gene [17-19] have been implicated in susceptibility to PD by several independent studies and meta-analyses within the European population [11, 12, 20, 21].

Two genome-wide association studies (GWAS) on PD/AG [22-25] have been published. In contrast to the more advanced GWAS e.g. in schizophrenia [26], GWAS in PD are characterized by small sample sizes (hundreds versus thousands). However, the *TMEM132D* gene identified in a GWAS was confirmed in the European population in an independent meta-analysis [11]. An alternative approach utilized were GWAS studies on dimensional traits (neuroticism and phobic anxiety) [27-29], which supported a locus on chromosome 1, but so far showed inconclusive results with regard to individual genes. Recently, an approach studying anxiety disorders combined and quantitative phenotypic scores was applied providing genome-wide evidence for a non-coding RNA locus on chromosome 3q12.3 and the *CAMKMT* (calmodulin-lysine N-methyltransferase) gene on chromosome 2p21 [30, 31].

Assuming a dimension from agoraphobic cognitions to full PD/AG, we (1) conducted a GWAS on a dimensional anxiety phenotype (ACQ) in a sample of 1,370 healthy German volunteers to generate hypotheses for further investigations. We then (2) evaluated the *GLRB* locus in a larger dimensional ACQ sample, comprising 2,547 healthy volunteers. Next, we validated our findings (3) in a Dutch control sample (N=3,845) with a dichotomous

measure of AG symptoms [SCL-90] and (4) assessed the relevance of the association for the categorical phenotype PD/AG by analyzing 506 case-control pairs. To probe molecular consequences of *GLRB* genetic variation, we (5) measured mRNA expression *in vitro* and *post mortem*. As single nucleotide mutations in *GLRB* underlie hyperekplexia 2 (OMIN #614619), characterized by exaggerated startle response, we examined the effect of the identified *GLRB* risk polymorphisms on startle habituation (6), potentiation (7), and generalization (8). This was complemented by (9) fMRI analysis of fear network and general sensory activation. Finally (10), we performed an analysis of agoraphobic behavior in mice with a partial *Glrb* knock-out.

Materials and Methods

GLRB locus, agoraphobic cognitions and PD/AG

Samples

To identify loci associated with agoraphobic traits as defined by the ACQ [5](German version [32]), we examined 1,370 healthy German volunteers by conducting a GWAS and then evaluated the genome-wide significant locus in a larger sample comprising 2,547 German healthy controls (MEGA study waves 1 and 2 [33, 34]). We validated the locus in 3,845 independent Dutch participants of the Nijmegen Biomedical Study (NBS [35]) for a dichotomous SCL-90 [36, 37] (Dutch version [38]) based agoraphobic cognitions phenotype and at the categorical level by comparing 506 PD patients (Panic-Net study waves 1 and 2 [16, 19, 21, 39, 40]) with 506 matched controls from MEGA study waves 1 and 2. In all but the NBS studies, PD/AG patients and probands with severe psychiatric, neurological, or somatic disorders as well as drug and alcohol abuse were excluded. Only individuals with written informed consent were enrolled, which complied with the Declaration of Helsinki and was approved by the respective local Ethics Committee. For a demographic overview see supplementary table 1.

Genotyping

Participants of the MEGA wave 1 sample were genotyped on Illumina's Human-Hap550v3 BeadChips using the Infinium II assay (Illumina, San Diego, CA, USA) at the Department of Genomics, Life & Brain Center, University of Bonn, Germany. Quality control procedures were performed as described previously [41] with slightly modified exclusion criteria (SNPs and subjects with call rates (CR) ≤99%; minor allele frequency (MAF) ≤1%; failing Hardy-Weinberg equilibrium (HWE) ($p<10^{-5}$); principal component analysis (PCA) > 4fold SD of the first three principal components. To increase genomic coverage, imputation was conducted using MACH v1.0.18.c/ MINIMACH v2013-07-17 [42, 43] and the 1000 Genomes reference data set [44]. Post-imputation QC includes: SNPs and subjects CR≥95%, MAF≥1%, failing HWE ($p\geq10^{-5}$), imputation quality score (IQS) <0.3, population stratification, gender and unreported relatedness check. The GWAS was free from genomic inflation in QQ-plots (λ=1.000933) as shown in supplementary figure 1.

NBS was genotyped using the Illumina Human Omniexpress-12 and -24 chip. The pre-imputation QC steps applied to the NBS cohort include: SNP MAF>1%, HWE>10^{-4}, SNP yield and individual CR>95%. Imputation was done using IMPUTE v2.3.0 following the BBMRI-NL pipeline (http://www.bbmriwiki.nl/wiki/Impute2Pipeline) using the 1000 genomes phase 1 v3 and GoNL reference panels combined. Post-imputation QC comprised a gender check, unreported relatedness between participants and population stratification.

For fine mapping we captured *GLRB* and its flanking regions (+40/-10 kb upstream/downstream) by 21 tagSNPs derived from dbSNP European data (http://manticore.niehs.nih.gov/snpinfo/snptag.htm). Genotyping was performed using the Sequenom MassArray® system (Sequenom, San Diego, CA, USA; for Primer sequences see supplementary table 2) and for rs17035628 using a "KASP on demand" assay (LGC Genomics, Hertfordshire, UK) as recommended by the manufacturers. After QC, the final dataset included 20 (two GWAS SNPs + 18 tagSNPs) markers with a MAF≥1%, CR≥90% and HWE (controls only) p≥0.01; rs17035590, rs17035814 and rs17035628 had to be excluded.

For details on statistical and power analysis see supplementary methods.

Functional assessment: Bioinformatic, post mortem and cell system expression analyses

To detect functional variation on expression of *GLRB* and neighbor genes, we analyzed our strongly associated variants, using the GTEx eQTL database (http://www.gtexportal.org/home).

For expression analysis of the promotor SNP rs7688285, post-mortem brain samples of 76 individuals (mean age 48.6±12.8) were obtained from the Medical Research Council (MRC) Sudden Death Brain and Tissue Bank, Edinburgh. Detailed information on the sample and mRNA quantification are described elsewhere [45]. Allele-specific changes of rs7688285 on mRNA expression were calculated by linear regression with genotype and sample RIN as independent and expression values as dependent variable according to a dominant model.

For expression analysis in a heterologous cell system, 20 bp flanking rs7688285 up- and downstream were subcloned into pGL4.23 vector for both alleles, allowing expression of firefly luciferase under the control of a minimal promotor. For details see supplementary methods.

Functional assessment: Startle Reflex

Samples

Participants of MEGA and PanicNet waves 1, who had taken part in various assessments of defensive behavior [33, 46, 47], were studied regarding the effect of *GLRB* variation on startle reflex modulation. Individuals carrying at least one *GLRB* risk allele (i.e., those highly significantly associated with either ACQ or PD, namely: rs78726293 (A allele), rs191260602 (G allele), rs17035816 (G allele) or rs7688285 (A allele) were classified as "risk allele carriers". Accordingly, of 101 healthy volunteers who participated in an emotion-potentiated startle paradigm 24, of 76 healthy volunteers who participated in a context conditioning paradigm 23, and of 115 PD/AG patients who participated in a behavioral avoidance test (BAT) 52 were risk allele carriers. For sample characteristics and genotype counts see supplementary table 3.

Paradigms

Startle reactivity was investigated in three paradigms selected to allow for hierarchical analysis of startle reactivity with increasing complexity, focusing on a) startle habituation during an emotion-potentiated startle paradigm [33], b) startle potentiation triggered by a threatening environment during the BAT [47], and c) startle generalization during context-conditioning [46]. For a detailed description on startle methodology, paradigms and statistical analyses see supplementary methods.

Functional assessment: Fear Network

Sample

Healthy volunteers from two studies (Study 1: N=72, [48]; Study 2: N=38) with identical experimental design during a cue fear conditioning paradigm were included. All participants were right-handed with normal or corrected-to-normal vision. All participants were recruited from the MEGA study wave 2. Participants were grouped into risk and no-risk allele carriers as described above (risk: N=33). For sample characteristics see supplementary table 3.

Experimental Design

Two visual stimuli served as conditioned stimuli (CSs) and three electro-tactile stimuli as US. The CS+ was always, and the CS- never was followed by the US while skin conductance responses (SCRs), fear ratings and fMRI data, were acquired (see [48, 49]).

All behavioral data (SCR, ratings) were analyzed using SPSS 22 for Windows (IBM Corp., Armonk, New York) using repeated measures analyses for CS type per experimental phase (first half acquisition, second half acquisition, extinction). Rating values after fear acquisition were corrected for pre-acquisition scores. An α-level of $p<.05$ was considered significant (unless otherwise stated), and Greenhouse-Geisser corrected degrees of freedom were used when appropriate. For a detailed description on fMRI data acquisition, preprocessing and statistical analyses see supplementary methods.

Agoraphobic behavior in heterozygous Glrb knockout mice

Animals

The *spastic* mouse [50, 51] has an insertion of a LINE1 element into intron 5, resulting in lower expression levels of the full-length glycine receptor beta (*Glrb*^{spa}). Heterozygous *Glrb*$^{+/spa}$ do not show the *spastic* phenotype. All experiments were done with adult C57BL/6J and C57BL/6J *Glrb*$^{+/spa}$ mice (Jackson Laboratories, Bar Harbor, ME, US) in accordance with European Union guidelines, as approved by our institutional animal care and utilization committee. The experiments were authorized under reference number 55.2-2531.01-95/13. For details on genotyping, membrane preparation, mRNA quantification, Western Blot and immunostaining see supplementary methods.

Open field test

Wild-type and heterozygous *Glrb*$^{+/spa}$ mice were tested individually for anxiety like behaviour. They were placed in a 48x48cm square box, illuminated with ~40lux. Animals were monitored for 10 or 30 min each and tracked with the Video Mot Software (TSE, Germany). For analysis, the box was divided into fields of interest: centre of the arena (24x24 cm) versus the periphery. The first 5 min were analysed counting entries and time spent in centre [52]. Differences between genotypes were tested using a two-way ANOVA with Bonferroni posttest or an unpaired t-test.

Results

GLRB locus, agoraphobic cognitions and PD/AG

After quality control of post-imputational data, altogether 1,370 healthy volunteers from MEGA wave 1 with information of 7,071,105 autosomal markers were available for analysis. GWAS analysis on ACQ yielded 122 markers with $p<1x10^{-5}$ (figure 1a, supplementary table 4) distributed over 22 genomic regions (supplementary table 5). On chromosome 4, genome-wide significance was reached for the imputed SNPs rs78726293 ($p=3.3x10^{-8}$; IQS= 0.58) and rs191260602 ($p=3.9x10^{-8}$; IQS= 0.68), both located in an intronic region of the glycine receptor beta (*GLRB*) gene. This locus was supported by a nearly genome-wide signal, rs115177500 ($p=8.3x10^{-8}$), upstream of *GLRB*, and by further 26 strongly associated variants ($p<1x10^{-4}$) within a window of 400 Kb around this gene (figure 1b).

Both genome-wide significant polymorphisms rs78726293 and rs191260602 and further 18 SNPs fully tagging *GLRB* were genotyped and analyzed in a larger ACQ sample (MEGA waves 1 and 2; N=2,547; table 1). The genome-wide significant SNPs, rs78726293 and rs191260602, and in addition rs17035816 were strongly associated in the larger sample ($p_{rs78726293}=4.3x10^{-4}$; $p_{rs191260602}=8.8x10^{-5}$; $p_{rs17035816}=3.8x10^{-4}$), always with the minor allele increasing ACQ sum scores (table 1).

All 20 examined SNPs were additionally analyzed for association with a SCL-90 based dichotomous agoraphobia phenotype in the Nijmegen Biomedical Study (NBS) comprising 3,845 healthy participants. The minor allele of a further SNP rs7688285, was strongly associated with increased risk for the SCL-90 based dichotomous agoraphobia phenotype ($p_{rs7688285}=4.3x10^{-4}$; table 1).

Analysis of the 20 examined SNPs for the categorical phenotype of PD/AG showed an overlap of significant results with both the dimensional ACQ and the dichotomous SCL-90 based agoraphobia phenotype for both genome-wide significant SNPs rs78726293 ($p_{rs78726293}=0.033$) and rs191260602 ($p_{rs191260602}=0.033$), as well as for rs7688285 ($p=7.6x10^{-5}$). Again, always the minor alleles conveyed genetic risk (table 1).

Functional assessment: Bioinformatic, post mortem and cell system expression analyses

None of the four strongly associated SNPs could be classified as an expression quantitative trait locus (eQTL) in the GTEx database.

Genotype-specific differences of the promoter region risk variant rs7688285on mRNA expression levels were found in the midbrain (N=50; AA=0/AG=12/GG=38), where the minor, risk (A)-allele increased the mean expression of *GLRB* (*beta*=0.498; *p*=0.013) significantly (supplementary figure 2A). Neither in forebrain (N=59; AA=1/AG=16/GG=42; *p*=0.421) nor in the amygdalae (N=56; AA=2/AG=14/GG=40; *p*=0.487), rs7688285 affected mRNA expression.

In line with data of human tissue, normalized luciferase activity was significantly increased for the (A)-allele of rs7688285 compared to the (G)-allele (0.185±0.09 vs. 0.155±0.09, *p*=0.029, N=7; supplementary figure 2B).

Functional assessment: Startle Reflex

Startle habituation (figure 2A) during an emotion-potentiated startle paradigm was significant in a healthy sample of no-risk allele carriers (N=77; $t(76)=5.12$, $p<0.004$), but not in risk allele carriers (N=24; $t(23)=1.15$, $p>0.60$) indicating impaired *startle habituation* in risk allele carriers (figure 2A).

During a Behavioral Avoidance Test (figure 2B), PD/AG patients carrying a *GLRB* risk allele (N=52) exhibit increased startle responsivity during threat as reflected in stronger increases in *startle potentiation* from last minute of anticipation to first minute of exposure than no-risk allele carriers (N=63; Group x Time $F(1,113)=5.22$, $p<0.05$; $\eta^2_p=0.044$).

In a context conditioning paradigm (figure 2C) healthy risk-allele carriers (N=23) exhibited as well overall stronger *startle potentiation* during acquisition compared to no-risk allele carriers (N=63; $F(1,74)=4.39$, $p=0.040$, $\eta^2_p=0.056$). The high risk group revealed potentiated startle in both the threat (CTX+) and the safety context (CTX-) without difference between the two ($t(22)=0.11$, $p=0.915$), in contrast to the no-risk group that featured potentiated startle in the CTX+ only ($t(52)=2.75$, $p=0.008$). Accordingly, startle potentiation in the CTX- was significantly higher for risk-allele carriers compared to the no-risk group ($t(74)=2.50$, $p=0.015$). Again pointing to impaired startle habituation, we found that startle responses in CTX- declined across acquisition in the no-risk group (Acquisition1 vs. Acquisition2: $t(52)=2.79$, $p=0.007$), but not in the risk group ($t(22)=0.24$, $p=0.811$). Finally, we observed no group differences during extinction, but the high risk group showed a stronger

sensitization of startle magnitudes in CTX+ during test, i.e. spontaneous recovery (Context x Group interaction: $F(1,74)=5.77$, $p=.019$, $\eta^2_p=0.072$). The resulting significantly increased startle responses in CTX+ in the risk compared to the no-risk group ($t(74)=2.78$, $p=0.007$) further emphasizes increased responsivity of the startle system in the risk group.

Functional assessment: Fear Network

During late cue conditioning, *GLRB* risk-allele carriers showed significantly stronger CS+-reactivity in several regions of interest of the fear network (thalamus, putamen/pallidum at $p_{(FEW\text{-}corrected)}$; figure 3A, B and supplementary table 6) as well as stronger CS+-reactivity in skin conductance responses (SCRs) [stimulus x group interaction: $F(1,105)=3.56$, one-sided $p=0.031$, $\eta^2=0.03$, figure 3F].

In addition, *GLRB* risk-allele carriers showed significantly stronger general CS-unspecific activation in left pre- and post-central gyrus (figure 3D), the bilateral pallidum (figure 3E) and putamen as well as the right thalamus during both early and late acquisition in absence of any main effects of group on SCRs [$F(1,105)=3.56$, $p=0.17$, $\eta^2=0.02$]. In addition, during early acquisition *GLRB* risk carriers also displayed generally stronger activation in the left amygdala (figure 3C) than no-risk allele carriers as well as stronger bilateral insula activation during late acquisition (supplementary table 6).

Agoraphobic behavior in heterozygous Glrb Knockout mice

In heterozygous $Glrb^{+/spa}$ mice, decreased *Glrb* expression levels were observed both at the mRNA and protein level, in particular in thalamus and hippocampus (supplementary figure 3). Interestingly, these regions were found to be differentially activated as a function of *GLRB* genotype in the human fear conditioning paradigm (see above). Heterozygous $Glrb^{+/spa}$ mice showed a significantly enhanced agoraphobic behavior demonstrated by less time spent in the center of the open field (*+/+* 0.52±0.05 min; *+/spa* 0.4±0.03 min). Both groups of animals, however, did not differ in distance traveled and number of entries into the field (figure 4).

Discussion

Beyond classical diagnostic phenotypes, the specification of functional dimensions of behavior as done in the Research Domain Criteria (RDoC) approach [53] aims at obtaining insights into the biological basis of mental illness. In this context, negative valence and arousal systems [54] are relevant to PD/AG. The development of PD/AG in early adulthood is preceded by increased scores on dimensional anxiety phenotypes with negative valence such as anxiety sensitivity [55] and agoraphobic cognitions [56] which are thus relevant proxies for PD/AG.

To start with, we therefore performed a hypothesis-generating GWAS using the ACQ in a German cohort (MEGA wave 1) which suggested that allelic variation in *GLRB* on chromosome 4q31-34 is associated with quantitative ACQ scores. Results from a larger sample (MEGA waves 1 and 2) and two independent sample (NBS, PanicNet waves 1 and 2) supported this finding and extended it to a dichotomous agoraphobia phenotype as well as the categorical phenotype PD/AG. This chromosomal locus (4q31-34) had previously been proposed as genome-wide linkage locus for anxiety disorders [57]. In the linkage scan, the most significant marker (D4S413) is located a mere 384 kb from rs7688285 in *GLRB*. On the molecular level, we found the risk allele to go along with altered *GLRB* expression *in vitro* and *post mortem*.

In the next step we asked which neural mechanisms are linked to the behavioral phenotypes, along the RDoC idea. The inhibitory glycine system is more ancient than the GABA system and thus more prevalent in evolutionary older brain regions from the spinal cord to the midbrain [58]. The effects of glycine are mediated by heteromeric receptors formed by $Gly_{alpha}1\text{-}4$ and Gly_{beta} subunits [59]. Single point mutations in the *GLRB* gene have recently been shown to lead to hyperekplexia 2 (OMIN #614619), a rare neurological disorder with Mendelian heritability [60, 61]. These patients suffer from exaggerated startle responses to unexpected noise or tactile stimuli. On the translational level, the phenotype is confirmed by the *spastic* mouse (featuring a substantial reduction of *Glrb*), which is characterized by an increased startle reaction [62]. While, in humans, startle symptoms may diminish with age, (agora-) phobic behavior may become a more prominent clinical feature [63, 64].

Based on the startle phenotype of hyperekplectic patients and the observation that startle reactivity is inherited [65, 66], we investigated functional intermediate phenotypes with a focus on startle response as possible immediate functional consequences of *GLRB* genetic variation. Three different samples provide converging evidence that *GLRB* risk SNPs result in slower habituation, stronger potentiation and generalization of startle, although subtler as compared to the – potentially more deleterious –mutations in *GLRB* causing hyperekplexia. These genetic modulations of defensive reactivity of brain stem reflexes during potential threat, which is mediated at a subcortical level [56, 67], did not go along with concordant panic or anxiety ratings. Accordingly, at the level of brain function – and possibly mediated by *GLRB* expression changes in midbrain as suggested by our post-mortem data – *GLRB* risk alleles were associated not only with increased activation of the fear network, but with a generally stronger activation of sensory networks including the thalamus and postcentral gyrus as well as the motor network (precentral gyrus, pallidum and putamen) suggesting a general higher reactivity independent of the presence of an aversive stimulus.

Being a very basic mechanism, the observed increased startle responses likely suggest a link between the Arousal system and the Negative valence system causing an increased likelihood to shy away from threats, i.e. increased defensive reactivity [53, 54]. Consistent with our findings in humans and extending the known phenotype of the *spastic* mouse, we showed that partial *Glrb* knock-out mice exhibited avoidance of a novel open space, a behavior we recently confirmed to be related to agoraphobic fear in humans [3]. At the mechanistic level, it is somewhat puzzling that the phenotype-associated rs7688285 A allele in fact was not found with decreased, but instead increased *GLRB* expression in a heterologous cell system as well as in post mortem samples. However, an increase in *GLRB* expression does not necessarily result in enhanced functional pentameric glycine receptors, which are composed of both GlyRbeta and GlyRalpha subunits. An enhanced expression of GlyRbeta might e.g. result in an upregulation of the 2alpha/3beta variant, to the disadvantage of the functionally different homomeric GlyR alpha receptor [68], and hence at the functional level may well have similar consequences as the mouse knockout phenotype. As the subunit composition of the glycine receptor changes during the life span [69, 70], this may be particularly relevant during neural development. As such divergences between mouse knockout and human genetic variants are a common though not well understood

phenomenon in psychiatric genetics (e.g. for the *5HTT* and *NPSR1* genes), mouse models for specific human genetic variants, in our case of *GRLB*, are definitively needed.

There are a few limitations of our study. First, this is a study in rather homogenous proband and patient samples from Germany and the neighboring Netherlands. Thus, the generalizability to other populations remains to be elucidated. Second, the sample sizes are comparably small, especially for the PD/AG sample. Hence, further replication studies and metaanalyses [30] e.g. in the context of a psychiatric genomics consortium framework are paramount. Third, the associated polymorphisms differed between samples. This may, however simply be due to the different phenotypes studied with greater power of the dimensional sample for rare polymorphisms with bigger effect sizes and greater power of the dichotomous sample for the more common polymorphisms. Nevertheless, the ultimate phenotype PD/AG demonstrated significant associations for both types of polymorphisms. Fourth, the human intermediate phenotype paradigms were not specifically designed to examine *GLRB* gene effects on startle responses. The proximity of the startle reaction to the hyperekplexia phenotype in our opinion, however, is close enough to overcome this shortcoming. Fifth, the definition of the risk population in the intermediate phenotype paradigms is based on a combination of significant alleles, as sample sizes precluded gene-based analyses as well as investigating the effects of individual rare variants on their own. Reverse-phenotyping studies which are designed to specifically test a potential influence of specific *GLRB* variants on intermediate phenotypes in larger samples are needed. Deep sequencing of the *GLRB* gene for rare variants in larger samples of patients as compared to healthy probands with calculation of a polyallelic risk score will help to clarify this issue [71].

In summary, our findings provide evidence that *GLRB* allelic variation may contribute not only to the rare severe neurological disorder hyperekplexia, but also to the risk of the comparably milder categorical anxiety disorder PD/AG by increasing startle response and, as a result, agoraphobic cognitions. Our data point to the startle reflex being one pathomechanism in PD/AG. Within the RDoC matrix, this places *GLRB* in the Arousal gene list, while adding PD/AG to the clinical entities linked to this domain. As GLRB can be subjected to pharmacological interventions, its modulation may comprise a novel therapeutic option in PD/AG.

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Conflict of interest

Over the last 3 years V Arolt has been a member of the advisory boards and/or gave presentations for the following companies: Astra-Zeneca, Eli Lilly, Janssen-Organon, Lundbeck, Otsuka, Servier, and Trommsdorff. He also received sponsorships for symposia and educational activities from Astra-Zeneca, Jansen-Organon, Lundbeck and Servier. C Büchel received speaker´s honoraria from Janssen. K Domschke has received a honorarium for a scientific talk from Hexal. T Kircher received fees for educational programs from Janssen, Eli Lilly, Servier, Lundbeck, Bristol Myers Squibb, Pfizer and Astra-Zeneca. P Pauli and A Mühlberger are shareholders of a commercial company that develops virtual environment research systems for empirical studies in the field of psychology, psychiatry, and psychotherapy. A Ströhle received research funding from Lundbeck, and speaker honoraria from AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, Eli Lilly & Co, Lundbeck, Pfizer, Wyeth and UCB. He was a consultant for Actelion. Educational grants were given by the Boehringer Ingelheim Fonds, the Eli Lilly International Foundation, Janssen-Cilag, Pfizer and Eli Lilly & Co. H-U Wittchen has served as a general consultant (non-product related) for Pfizer, Lundbeck, Organon, Servier and EssexPharma and has received grant funding for his institution from Sanofi Aventis, Pfizer, Lundbeck, Novatis, Essex Pharma, Servier and Wyeth. These cooperations have no relevance to the work that is covered in the manuscript. The other authors declare no conflict of interest.

Supplementary information is available at Molecular Psychiatry´s website (http://www.nature.com/mp). Supplementary methods and results, supplementary figures and tables give complimentary information on sample demographics, strategies used for genetic and expression analysis, startle and imaging experiments as well as mouse behavioral analysis.

Figure legends

Figure 1: Genome-wide association study (GWAS, N=1,370. (**A**) Manhattan plot: Horizontal lines show threshold for genome-wide significance ($p<5\times10^{-8}$ in orange) and nominal association ($p\leq0.05$ in yellow). (**B**) Regional plot depicting genome-wide significant *GLRB* markers on chromosome 4, centered in a genomic region of 2 MB. Results are shown as $-\log_{10}$ (*p*-value) for genotyped and imputed SNPs. The SNP showing strongest association is shown in purple. The color of the remaining markers reflects r^2 of the strongest associated SNP. The recombination rate is plotted in blue.

Figure 2: Startle reflex reactivity (means and standard error as a function of *GLRB* genotype with a risk genotype, comprising carriers of at least one risk allele of either rs78726293 (A allele), rs7688285 (A allele), rs191260602 (G allele), or rs17035816 (G allele). Depicted are (**A**) startle habituation during an emotion-potentiated startle paradigm (risk: N=24; no-risk: N=77), (**B**) startle potentiation in PD/AG patients during a behavioral avoidance test (risk: N=52; no-risk: N=63), and (**C**) startle generalization in contextual fear conditioning, its extinction and spontaneous recovery reflected in startle potentiation relative to baseline responses (risk: N=23; no-risk: N=53). * p<0.05; ** p<0.01; *** p<0.001.

Figure 3: Fear Network Activation. Significant group differences at a significance threshold of $pFWE_{SVC}$ between *GLRB* risk- allele carriers and no-risk allele carriers for CS discrimination in thalamic (**A**) and striatal regions (**B**) during the second half of fear acquisition. Group differences during early acquisition (corresponding activation pattern in late acquisition is not shown) in general CS-unspecific neural activation in the amygdala (**C**) pre-/postcentral areas (**D**) as well as striatal (putamen) areas **(E)** and their respective peak-voxel parameter estimates (for illustrative purposes)**.** Corresponding group differences in SCR CS-discrimination between *GLRB* non-risk and risk-allele carriers **(F).** Rc: range-corrected, Error-bars represent s.e.m. Visualization threshold is set to p<0.01 for illustrative purposes only.

Figure 4: Agoraphobic behavior in *Glrb*$^{+/spa}$ mice. **(A)** Open field test of +/+ and +/spa animals; shown are the first 5 min of the open field test. Walking pattern of representative +/+ and +/spa animals. **(B)** Time spent in the open center, distance travelled and entries, *$p<0.05$. Note that there are no differences in the distance traveled and in the number of entries between +/+ and +/spa mice. Number of animals analyzed were N=18 for +/spa and N=13 for +/+ mice.

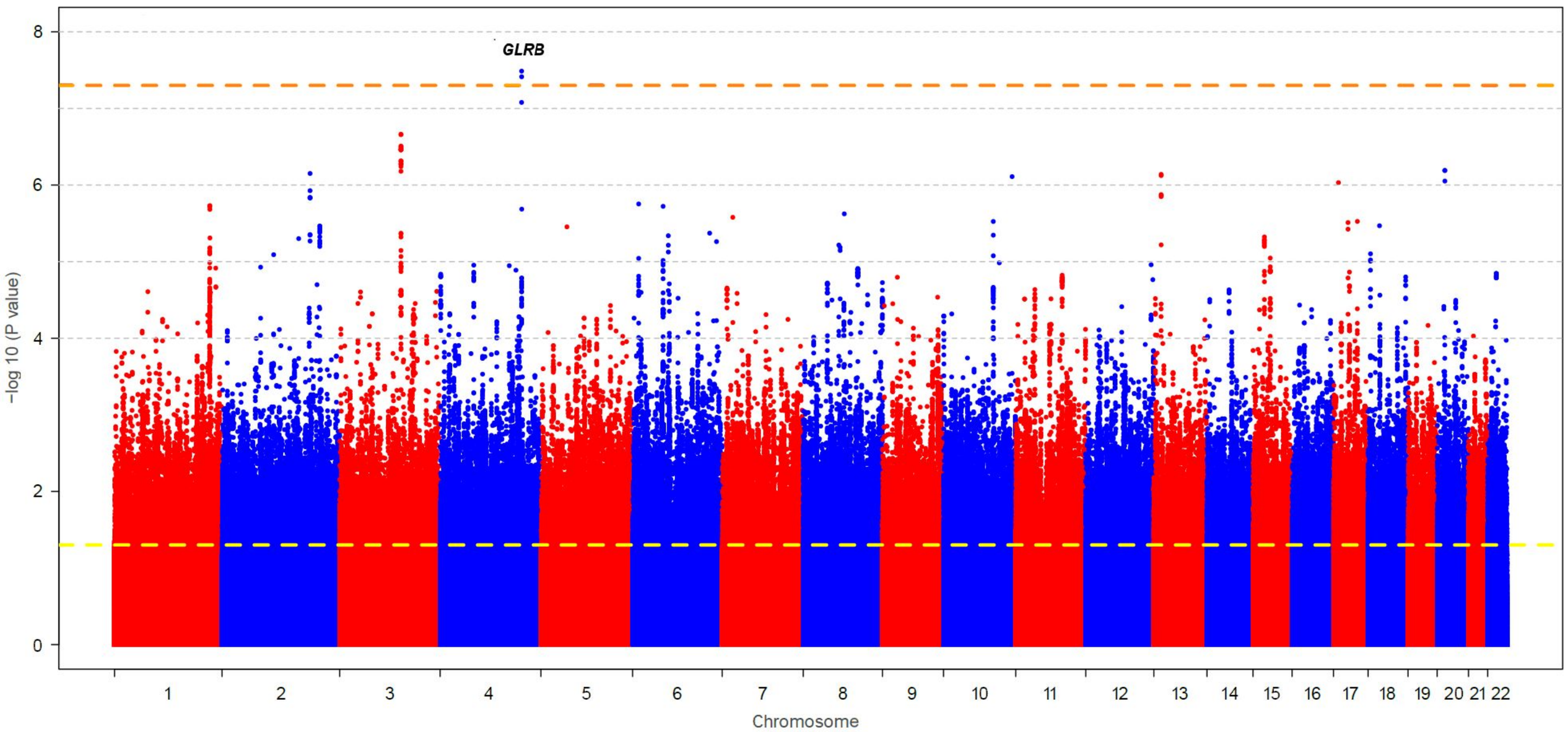
Tables

Table 1: *GLRB* variants*, agoraphobic cognitions and PD/AG*

In the ACQ sample, linear regressions for the dimensional phenotype ACQ were carried out assuming additive risk of the minor allele. Association results for the first and the second validation sample are reported as minor allele frequencies for controls and "cases" (SCL-90 agoraphobia score ≥ 1, or panic disorder patients, respectively) along with respective association *p*-values. Chromosomal positions correspond to the GRCh38 annotation. Bold indicates nominal $p<0.05$. All *p*-values were rounded to the third decimal.

		Allele	ACQ Sample (N=2,547)		SCL-90 Sample (N=3,845)			Case/Control Sample (N=506_{each})		
SNP_ID	Chr:Bp	m/M	Beta	*P*-value	SCL-90 = 0	SCL-90 ≥ 1	*P*-value	Controls	Cases	*P*-value
rs7664666	4:157038807	T/G	0.01	0.602	0.032	0.040	0.140	0.065	0.057	0.567
rs13139693	4:157044110	T/C	0.02	**0.039**	0.091	0.084	0.414	0.178	0.176	0.983
rs7688285	4:157047466	A/G	0.01	0.546	0.118	0.146	**$4.3x10^{-4}$**	0.225	0.344	**$7.6x10^{-5}$**
rs2343747	4:157047961	G/C	0.00	0.617	0.278	0.261	0.161	0.536	0.460	0.100
rs7689138	4:157066577	T/G	-0.01	0.591	0.102	0.105	0.757	0.184	0.168	0.502
rs6812324	4:157070440	C/T	0.01	0.452	0.197	0.189	0.494	0.391	0.338	0.201
rs11100093	4:157072890	A/T	0.01	0.530	0.156	0.145	0.269	0.296	0.265	0.322
rs4690879	4:157074560	C/T	0.00	0.726	0.207	0.203	0.767	0.397	0.344	0.248
rs78726293	4:157079880	A/T	0.12	**$4.3x10^{-4}$**	0.019	0.012	0.052	0.016	0.038	**0.033**
rs6852066	4:157081744	T/C	0.00	0.851	0.207	0.195	0.270	0.405	0.354	0.123
rs2880774	4:157106830	T/C	0.00	0.811	0.151	0.136	0.163	0.310	0.255	0.071
rs7655209	4:157130233	A/G	0.01	0.361	0.172	0.172	0.936	0.281	0.320	0.242
rs191260602	4:157140489	G/A	0.13	**$8.8x10^{-5}$**	0.018	0.012	0.072	0.016	0.038	**0.033**
rs17035763	4:157145432	A/G	0.00	0.864	0.15	0.136	0.159	0.312	0.241	**0.048**
rs7662298	4:157166613	G/A	0.04	**0.007**	0.076	0.081	0.403	0.132	0.162	0.180
rs17035816	4:157167312	G/A	0.06	**$3.8x10^{-4}$**	0.052	0.051	0.896	0.081	0.097	0.383
rs17035818	4:157168560	G/C	-0.01	0.613	0.051	0.048	0.534	0.075	0.063	0.450
rs17035820	4:157169837	T/A	0.01	0.283	0.177	0.18	0.838	0.281	0.336	0.112
rs1129304	4:157171270	A/T	0.01	0.221	0.320	0.308	0.355	0.591	0.573	0.930
rs17035827	4:157172585	G/A	0.02	0.055	0.102	0.101	0.954	0.162	0.213	**0.047**

A


GLRB
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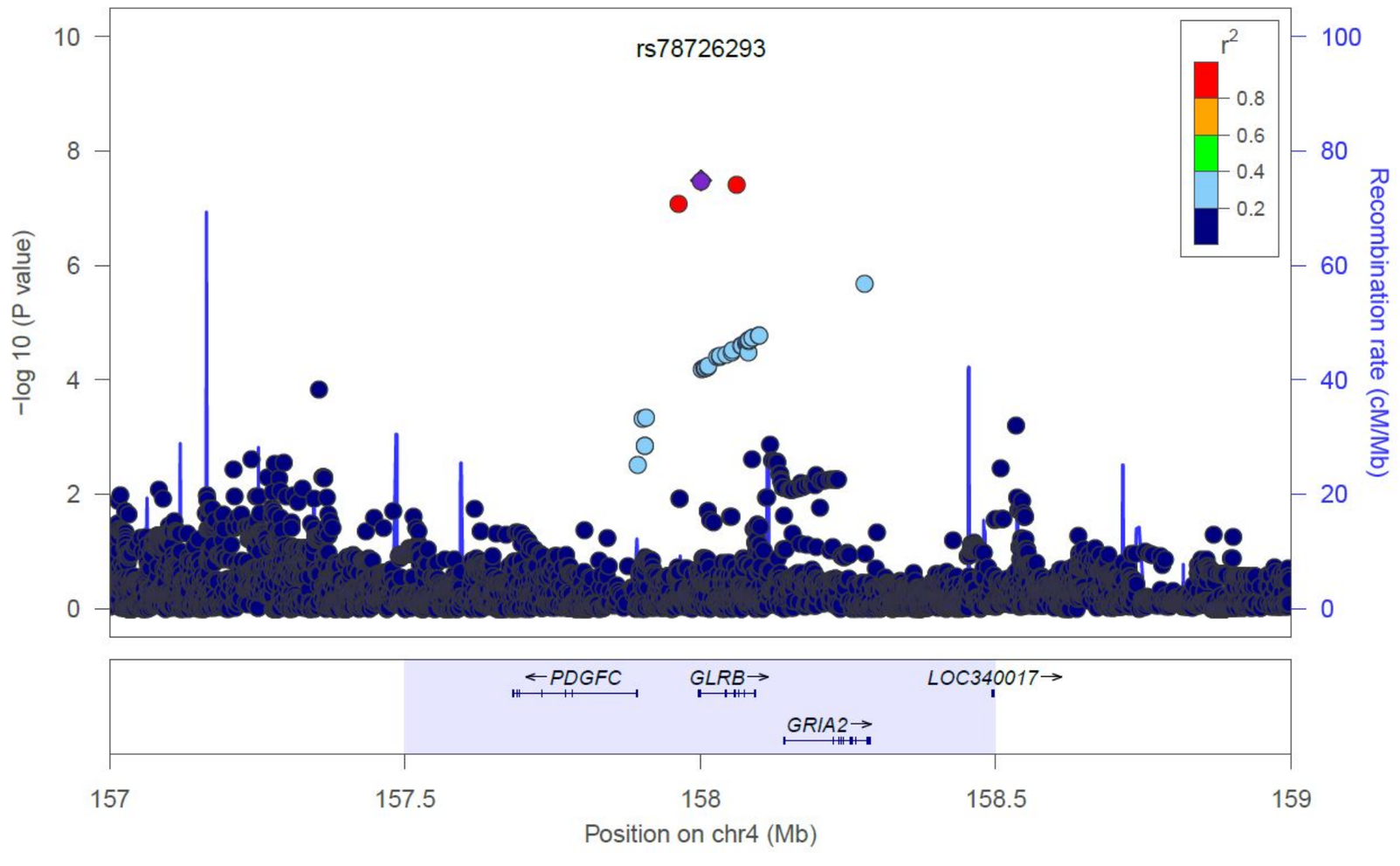
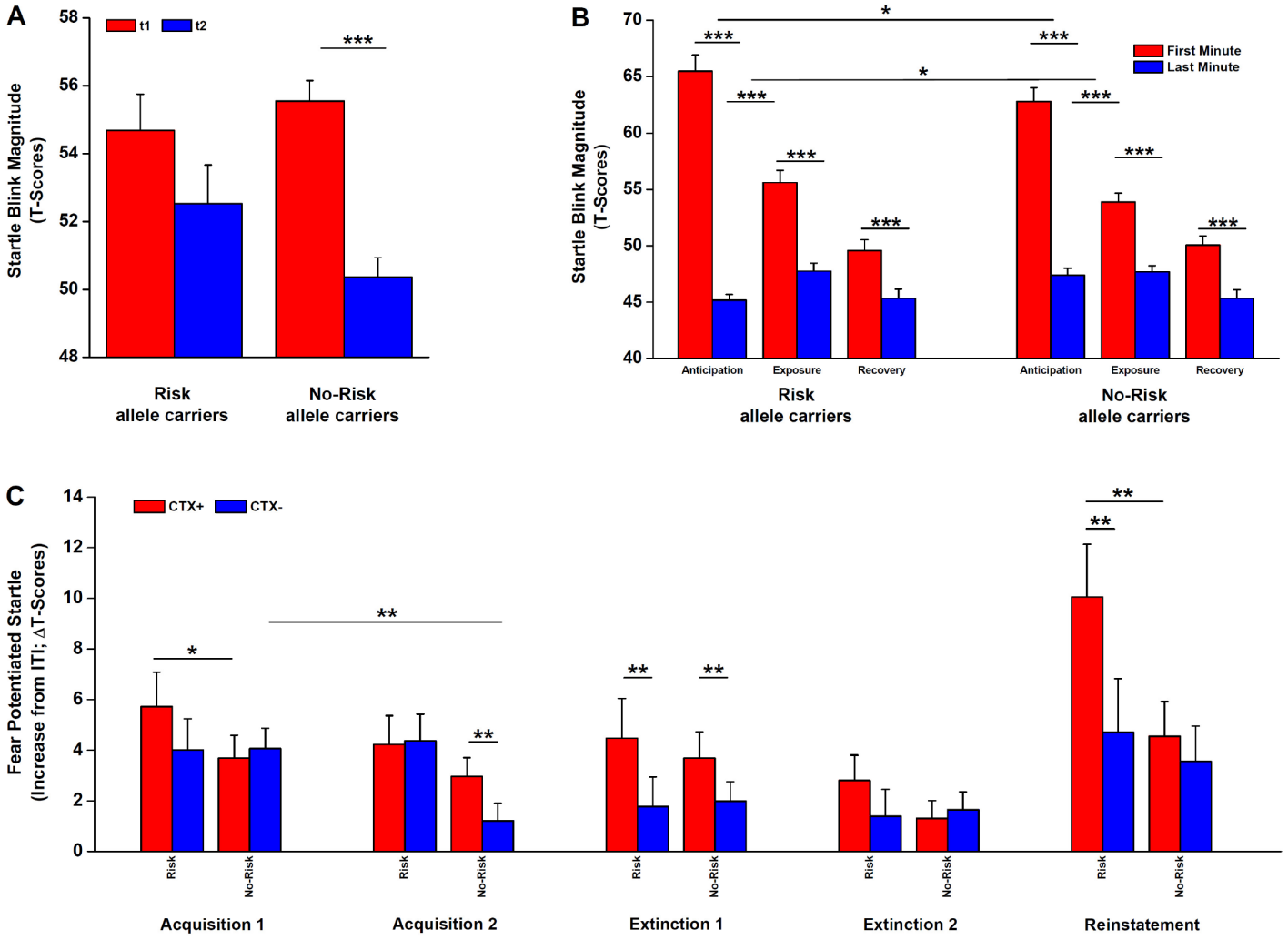
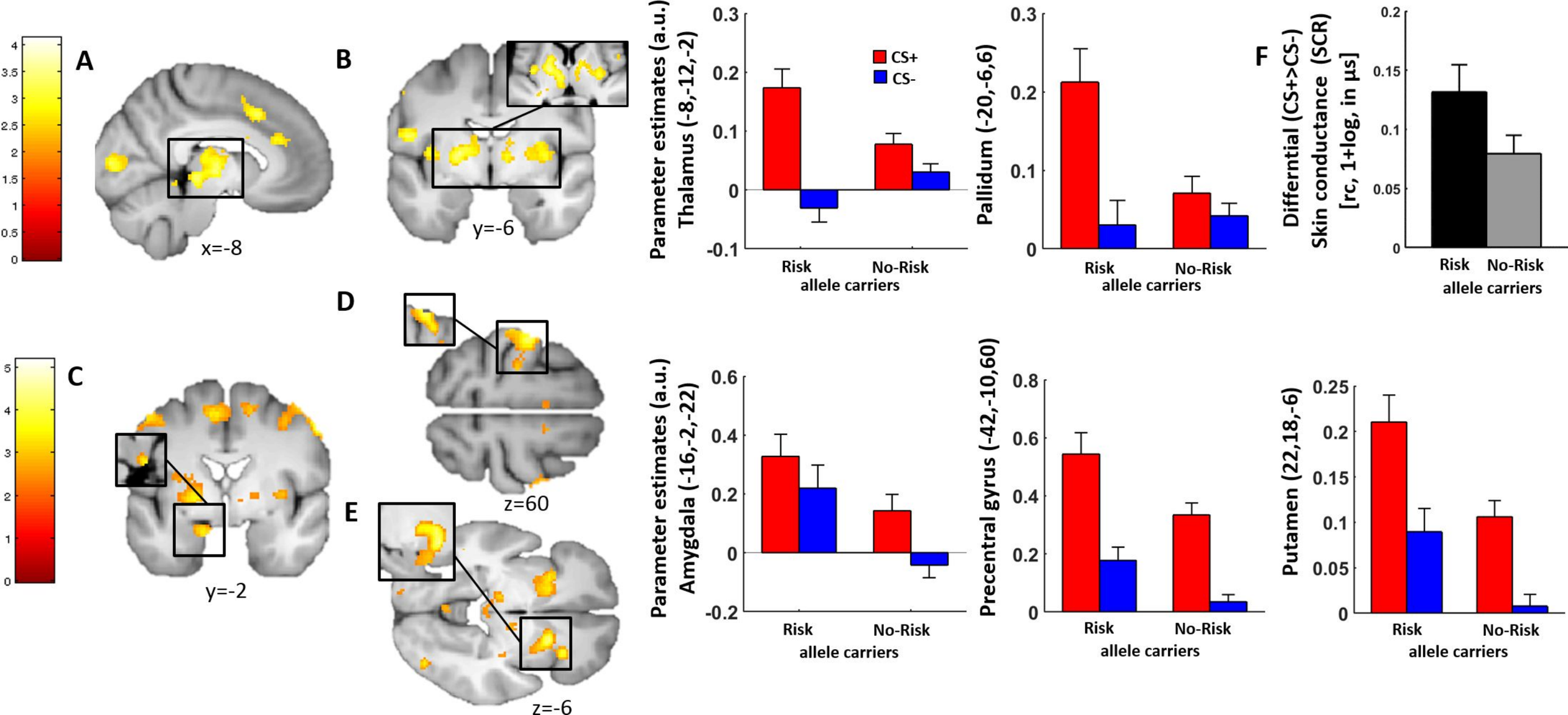
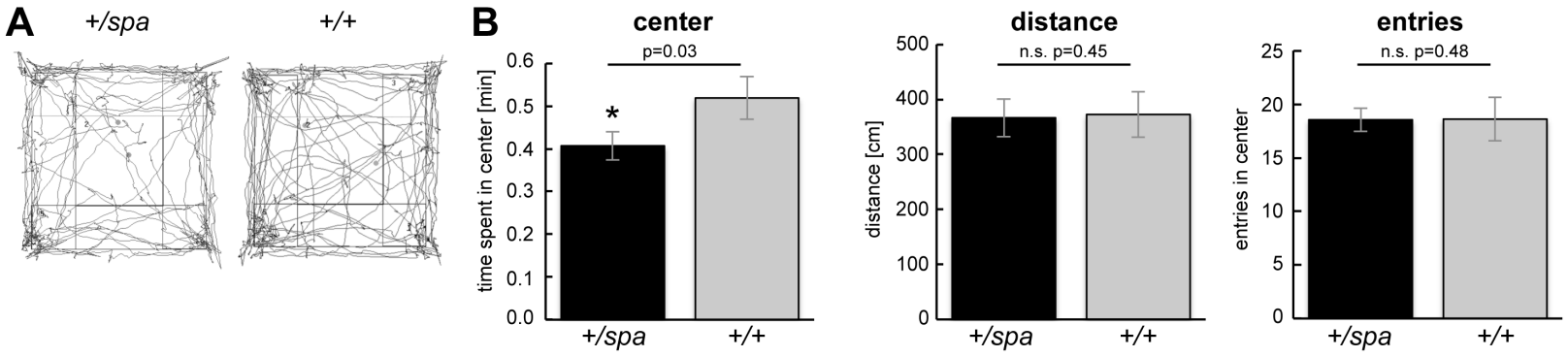
Chromosome

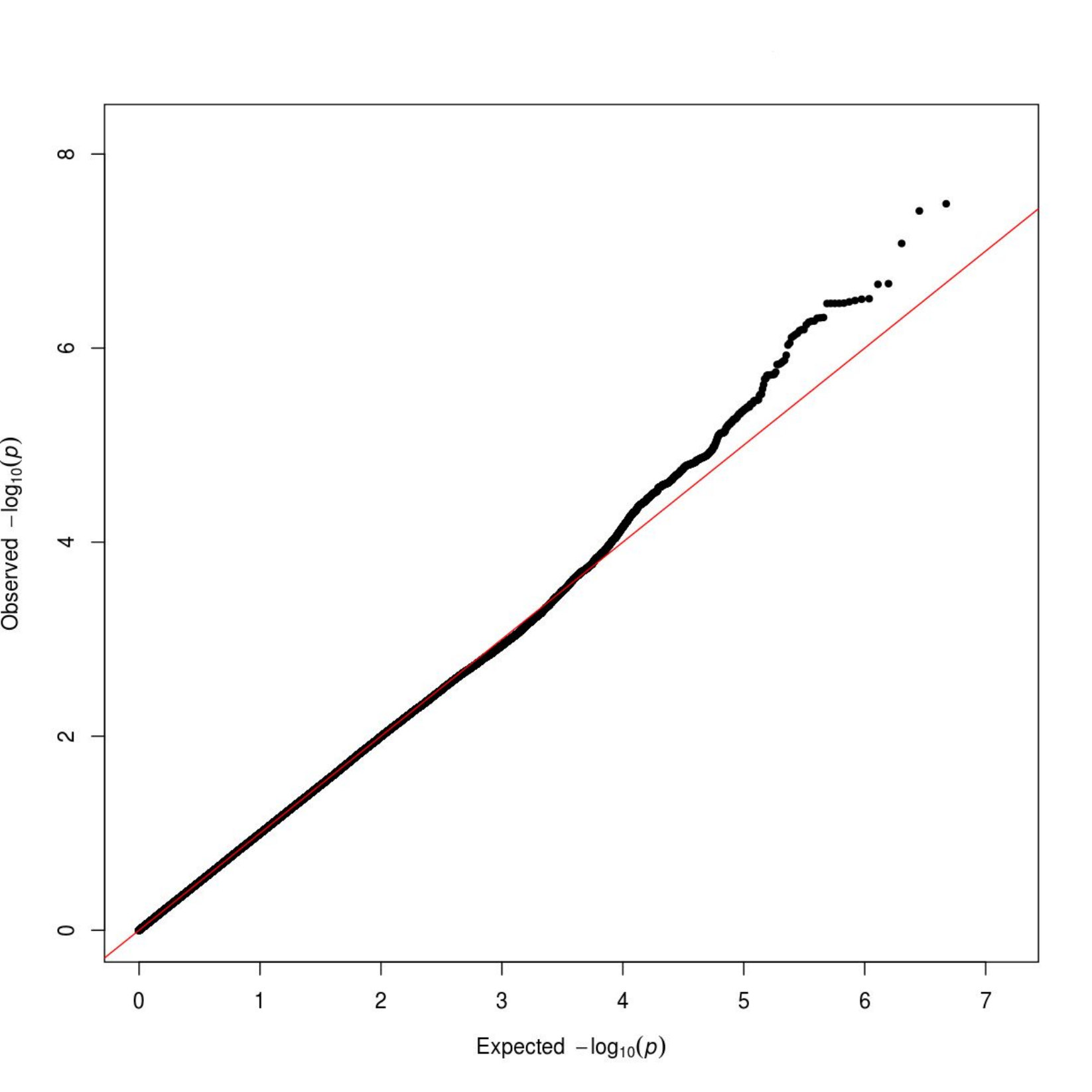
B

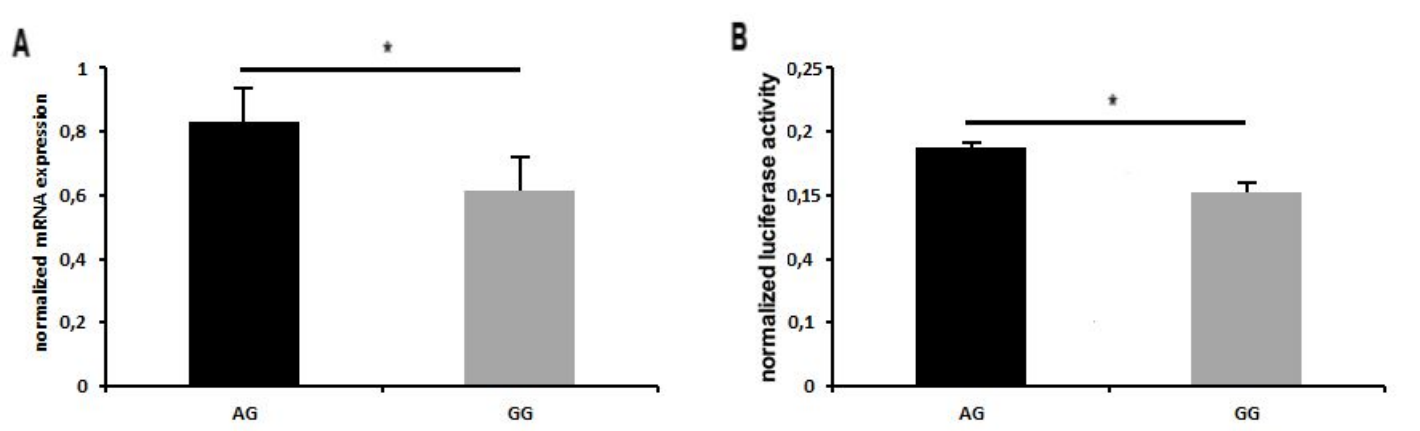

rs78726293
r2
0.8
0.6
0.4
0.2
−log 10 (P value)
Recombination rate (cM/Mb)
PDGFC
GLRB
LOC340017
GRIA2
157
157.5
158
158.5
159
Position on chr4 (Mb)

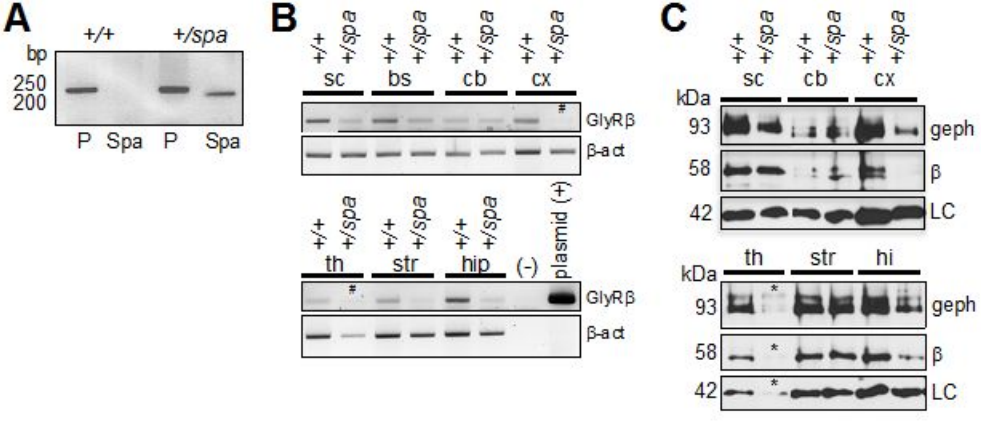

A
t1
t2
Startle Blink Magnitude (T-Scores)
58
56
54
52
50
48
***
Risk allele carriers
No-Risk allele carriers
B
First Minute
Last Minute
Startle Blink Magnitude (T-Scores)
70
65
60
55
50
45
40
*
***
Anticipation
Exposure
Recovery
Risk allele carriers
No-Risk allele carriers
C
CTX+
CTX-
Fear Potentiated Startle (Increase from ITI; ΔT-Scores)
14
12
10
8
6
4
2
0
*
**
Risk
No-Risk
Acquisition 1
Acquisition 2
Extinction 1
Extinction 2
Reinstatement


A
x=-8
B
y=-6
C
y=-2
D
z=60
E
z=-6
F
CS+
CS-
Parameter estimates (a.u.)
Thalamus (-8,-12,-2)
Pallidum (-20,-6,6)
Amygdala (-16,-2,-22)
Precentral gyrus (-42,-10,60)
Differntial (CS+>CS-) Skin conductance (SCR) [rc, 1+log, in µs]
Putamen (22,18,-6)
Risk
No-Risk
allele carriers


A
+/spa
+/+
B
center
p=0.03
*
time spent in center [min]
0.6
0.5
0.4
0.3
0.2
0.1
0.0
+/spa
+/+
distance
n.s. p=0.45
distance [cm]
500
400
300
200
100
0
+/spa
+/+
entries
n.s. p=0.48
entries in center
25
20
15
10
5
0
+/spa
+/+


Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$
0
2
4
6
8
0
1
2
3
4
5
6
7


A
normalized mRNA expression
1
0,8
0,6
0,4
0,2
0
*
AG
GG
B
normalized luciferase activity
0,25
0,2
0,15
0,4
0,1
0
*
AG
GG


A
+/+
+/spa
bp
250
200
P
Spa
P
Spa
B
+/+
+/spa
sc
bs
cb
cx
GlyRβ
β-act
th
str
hip
(-)
plasmid (+)
C
kDa
93
58
42
geph
β
LC
hi