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Malika El Yacoubi, Claire Altersitz, Violaine Latapie, Elari Rizkallah, Sébastien Arthaud, et al.. Two polygenic mouse models of major depressive disorders identify TMEM161B as a potential biomarker of disease in humans. *Neuropsychopharmacology*, 2024, 10.1038/s41386-024-01811-8 . inserm-04444848

HAL Id: inserm-04444848

<https://inserm.hal.science/inserm-04444848v1>

Submitted on 7 Feb 2024

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Two polygenic mouse models of major depressive disorders identify *TMEM161B* as a potential biomarker of disease in humans

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Running title: *TMEM161B* as a biomarker of depression

Key words: Animal model, Depression, Exome sequencing, Biomarker, TMEM161B

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Abstract

Treatments are only partially effective in major depressive disorders (MDD) but no biomarker exists to predict symptom improvement in patients. Animal models are essential tools in the development of antidepressant medications, but while recent genetic studies have demonstrated the polygenic contribution to MDD, current models are limited to either mimic the effect of a single gene or environmental factor. We developed in the past a model of depressive-like behaviors in mice (H/Rouen), using selective breeding based on behavioral reaction after an acute mild stress in the tail suspension test. Here, we propose a new mouse model of depression (H-TST) generated from a more complex genetic background and based on the same selection process. We first demonstrated that H/Rouen and H-TST mice had similar phenotypes and were more sensitive to glutamate-related antidepressant medications than selective serotonin reuptake inhibitors. We then conducted an exome sequencing on the two mouse models and showed that they had damaging variants in 174 identical genes, which have also been associated with MDD in humans. Among these genes, we showed a higher expression level of *Tmem161b* in brain and blood of our two mouse models. Changes in *TMEM161B* expression level was also observed in blood of MDD patients when compared with controls, and after 8-week treatment with duloxetine, mainly in good responders to treatment.

Altogether, our results introduce H/Rouen and H-TST as the two first polygenic animal models of MDD and demonstrate their ability to identify biomarkers of the disease and to develop rapid and effective antidepressant medications.

Introduction

With a lifetime prevalence of 13%, major depressive disorders (MDD) are the most common forms of mental illnesses and constitute a major public health concern [1]. According to the World Health Organization, it is the leading cause of disability worldwide and a major contributor to the overall global burden of disease [2]. Although many antidepressant medications are available on the market, most of them target the monoamine neurotransmitter systems [3]. Therefore, two thirds of patients are forced to switch medications at least once, and one third keeps resisting treatment even after four different medication courses [4]. This problem reflects the lack of knowledge on the cause of MDD and the absence of biomarkers in the diagnosis and the selection of appropriate treatment. Twin studies estimated a genetic heritability for depression around 40% [5] and recent genome-wide association studies (GWAS) demonstrated that this heritability was due to the additive effect of many genetic variants, each having a low effect on the phenotype [6,7]. In addition, numerous studies reported the effect of stressful life events to trigger depression in human [8]. Although many animal models have been developed to decipher the pathophysiology of MDD, they either mimicked the effect of a single gene or a single environmental factor [9]. We developed in the past a model of depressive-like behaviors, using selective breeding of CD-1 outbred mice, based on behavioral reaction after an acute mild stress [10,11]. Helplessness being a cardinal symptom of depression, we used the immobility time during the tail suspension test (TST), a standard test to measure the antidepressant efficacy, to select helpless animals (H/Rouen), *i.e.*, those showing a near absence of mobility in the test, and non-helpless ones (NH/Rouen), which displayed a persistence in the escapement behavior until the end of the test. We thus observed that these behaviors were stable through generations [10] and were associated with other

depressive-like phenotypes, including anhedonia, anxiety and sleep alterations [10-13]. A pitfall of this polygenic model was to determine which genetic variants were responsible of the depressive-like behaviors from those having selected through the genetic drift. To answer this question, we generated here a complex genetic background using eight inbred mouse strains and conducted a bidirectional selective breeding using the immobility time in the TST to generate a new helpless (H-TST) and a new non-helpless (NH-TST) mouse line. We first demonstrated that H/Rouen and H-TST mice had similar phenotypes and were more sensitive to glutamate-related treatments than selective serotonin reuptake inhibitors. We then conducted an exome sequencing comparing helpless and non-helpless animals in the two genetic backgrounds to identify genes carrying selective variants associated with the helpless phenotype. We compared these genes with those identified through GWAS on MDD in humans and identified two genes that could be used as biomarker of the disease and treatment response in humans.

Methods and Materials

Human data

The two human cohorts analyzed here have already been described elsewhere [14,15]. The mRNA expression datasets (GSE98793 and GSE146446) were downloaded from the gene expression omnibus (GEO) data repository (<https://www.ncbi.nlm.nih.gov/geo/>).

GSE98793 provided gene expression data in whole blood samples of 128 individuals with MDD and 64 unaffected controls [15]. These samples have been collected through the GlaxoSmithKline–High-Throughput Disease-specific target Identification Program (GSK-HiTDiP), using Affymetrix Human Genome U133 Plus 2.0 Arrays (Affymetrix,

Santa Clara, CA. U.S.A.). Few clinical data were available on these samples, which was designed primarily as a genetic association study. Patients were evaluated using the semi-structured Schedule for Clinical Assessment in Neuropsychiatry (SCAN [16]), administered by trained research assistants. Patients were included if they had a diagnosis of recurrent MDD, *i.e.*, at least two episodes of depression satisfying DSM-IV or ICD10 criteria. No information on current medication status was available.

GSE146446 provided gene expression data collected in 237 patients in a major depressive episode (MDE) randomized in a double-blind clinical trial testing duloxetine at 60 mg daily versus placebo for eight weeks [14]. Expression data were generated using Illumina HumanHT-12 V4.0 expression beadchips (Illumina Inc., San Diego, CA, U.S.A.). To avoid ethnic-dependent gene expression variations, patients with a non-European origin have been excluded, resulting in a total of 218 subjects (103 treated with duloxetine and 115 receiving placebo) with gene expression data collected at inclusion and after eight weeks.

All patients and controls signed an informed consent for their research participation and the studies were approved by independent ethic review boards.

Animals

See supplementary methods and materials and supplementary Figure S1 for details about mouse lines, selection breeding and behavioral tests. All experiments were approved by the local ethics committee CEEA-UCBL 55 (BH 2009-03) and Celyne CEEA-42 (Agreement APAFIS #17130) for the use of laboratory animals, and in accordance with the regulations of the European Community Council (2010/63/EU).

Pharmacological treatments

Solutions were prepared daily. Doses always refer to the free bases. Acute treatments were performed on adult mice (10 to 14-week-old). They received an acute intraperitoneal injection with saline, 20 mg/kg fluoxetine HCl (Interchim, Montluçon, France), 30 mg/kg ketamine (Virbac, Carros, France), or 3 mg/kg LY341495 (Bio-Techne, Minneapolis, MN, U.S.A.). All mice were tested in the TST 30 minutes after injection. Chronic treatments were performed in three-month-old mice treated daily, at 10 a.m., with fluoxetine HCl (10 mg/kg i.p.) or saline for three weeks. On day 22, H-TST mice were tested (between 1 and 4 p.m.) in the TST.

DNA extraction and exome sequencing

Genomic DNA was isolated from two male prefrontal cortices from helpless and non-helpless animals of TST and Rouen strains using DNeasy Tissue kit (QIAGEN, Hilden, Germany), at generations S18 and S43, respectively, and following manufacturer's instructions. After quality control, exons were captured using the SureSelect^{XT} Target Enrichment System kit for Illumina Multiplexed Sequencing (Agilent Technologies, Santa Clara, CA, U.S.A.). Whole-exome sequencing was conducted on HighSeq2500 (Illumina Inc., San Diego, CA, U.S.A.) with paired-end 100 bp reads. Data processing consisted in using CASAVA 1.8.2 (Illumina Inc.) and Bowtie v.2.0.2 [17] for demultiplexing and aligning sequences to mouse reference genome (GRCm38:mm10), respectively. Variant calling was performed using Genome Analysis ToolKit (GATK) v.0.1.16 [18] best practice recommendations and annotation was made with snpEff v.3.2a [19]. Only variants passing quality filter of VariantRecalibration tool of GATK, with a depth of read higher than 10 and a genotype quality score higher than 20 were kept. Damaging variants included codon insertion or deletion, missense

variants, gain or loss of a stop codon, loss of a start codon, frameshifts and change in splice-site acceptor or donor. A variant was considered associated to the helpless phenotype when the genotypes of the two helpless animals were identical, the two non-helpless animals were identical, and the genotypes of the helpless and non-helpless animals were different.

RNA extraction and gene expression analysis

Total RNA was extracted from 20 mg of prefrontal cortex (PFC) using QIAzol Lysis Reagent and RNeasy Lipid Tissue Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. PFC samples were defined as sagittal brain sections of 2 mm of thickness in the frontal cortical region directly posterior to the olfactory bulb. Both left and right sides of the PFC have been collected. For blood samples, total RNA was extracted from blood clots after centrifugation-based serum separation using miRNeasy kit (Qiagen). Five hundred nanograms of total RNA was then reverse transcribed using the High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.), according to the manufacturer's protocol.

Quantitative PCR (qPCR) reactions were performed for *Gria1* and *Bdnf* using Fast SYBR Green Master Mix and the Applied Biosystems™ 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, California, USA). Melting-curve analysis was performed to check the specificity of the amplification. All primers were designed using NCBI Primer-BLAST and selected to generate amplicons of 100–200 bp length and are available on request. Quantification of *Lrn5* and *Tmem161b* was performed using Mm00724566_m1 and Mm01352348_m1 TaqMan assays, respectively, and the Applied Biosystems™ TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific). The mouse actin beta gene (*Actb*) was used as housekeeping gene and

cDNA from a mouse brainstem was used as a reference sample (calibrator) for all experiments. Twice more cDNA was used for blood samples than for PFC due to the *Tmem161b* low expression level in blood. Relative quantification was performed according to the $2^{-\Delta\Delta C_T}$ method [20].

In situ hybridization

Fresh frozen coronal cryostat sections (14 μ m) of PFC were prepared and hybridized with 35 S-radiolabeled antisense riboprobes against *Gria1*. The sections were exposed to Kodak MR Film in room temperature for 7-21 days prior to development of films, according to a previously published protocol [21]. Densitometric measurements were obtained from autoradiograms using the NIH ImageJ 1.40 software (National Institute of Mental Health, Bethesda, MD, USA). All optical density values were normalized. For each target analyzed, the average of the NH-TST group was normalized to 100% and results from each H-TST group are presented as percentage of the NH-TST group.

Statistical analyses

Statistical analyses were conducted with R v.3.6.3 and GraphPad Prism v.10. All variables normally distributed were expressed as mean \pm standard deviation (SD) and comparisons between conditions were examined by t-tests or one-way ANOVA. Not normally distributed variables were expressed as median and interquartile range (IQR) and between-group comparisons were analysed by Mann-Whitney test. We named combined Mann Whitney test when TST and Rouen mice were pooled in the test to increase the statistical power. Network and pathway analyses were performed using the STRING v.11 database [22]. Network was defined using a minimum interaction

score of 0.4 and with active interaction sources as text-mining, co-expression, experiments, and database.

Results

H-TST mice displayed depressive-like behaviors similar to those observed in H/Rouen

To generate founders for the selective breeding with a complex genetic background, we performed an eight-way cross involving inbred mouse strains A/J, ABP/Le, BALB/cJ, C57BL/6J, C3H/HeJ, CBA/H, DBA/2J, and SWXL-4. We then used the TST to select animals with the longest and the shortest immobility times and bred them across generations. After 10 generations, we observed an 80-fold difference in immobility time between H-TST and NH-TST, in both males and females (Figure 1A), as previously observed with H/Rouen and NH/Rouen strains [10]. In order to check whether the immobility time measured in the TST resulted from the helplessness and not from a decreased motor activity, we showed there was no correlation between the time of immobility recorded in the TST and the total distance travelled in an open field during 45 min neither for H-TST (Pearson's correlation, $r_{H-TST}=0.21$, $p_{H-TST}=0.31$; Figure 1B) nor for NH-TST ($r_{NH-TST}=-0.03$, $p_{NH-TST}=0.88$; Figure 1C). We weighed the mice during the tests to ensure that the reduced mobility observed in the H-TST mice was not the result of greater difficulty in moving. Significant lower weights were reported for both males and females H-TST when compared to NH-TST (one-way ANOVA, $F_{males}(1, 74)=63.48$, $p_{males}<0.0001$; $F_{females}(1, 64)=11.68$, $p_{females}<0.001$; Supplementary Figure S2).

We then tested H-TST and NH-TST animals for depressive-like behaviors. First, using the forced swim test (FST), we showed that helpless males and females displayed a higher immobility time (Mann Whitney test, $U_m=14$, $p_m<10^{-4}$ and $U_f=0$, $p_f<10^{-4}$ in males

and females respectively) when compared with non-helpless animals (Figure 1D). Second, anhedonia and anxiety are two symptoms frequently reported in patients with MDD and we previously showed that H/Rouen mice displayed anhedonia-like and an increased anxiety-like behaviors in anxiogenic conditions. We assessed anhedonia using the two-bottle sucrose preference test in the TST line and showed a 43% (Mann Whitney test, $U=23$, $p=0.02$) and 53% (Mann Whitney test, $U=26$, $p=0.01$) decrease in the consumption of sucrose for H-TST when compared with NH-TST, in males and females respectively (Figure 1E). We then assessed the anxiety level using a light/dark box and an elevated plus maze. H-TST males and females spent less time in the anxiogenic brightly lit compartment of the light/dark box (Mann Whitney test, $U_m=21$, $p_m=0.004$ and $U_f=30$, $p_f=0.05$ in males and females respectively; Figure 1F) and less time in the anxiogenic open arms of the elevated plus maze (Mann Whitney test, $U_m=17$, $p_m=0.006$ and $U_f=10$, $p_f=0.0004$ in males and females respectively) than their NH-TST counterparts (Figure 1G). These results showed that H-TST mice displayed depressive-like behaviors with anhedonia-like and anxiety-like behaviors.

Sleep disturbances are often observed in depressed patients and we previously reported sleep disturbance in H/Rouen when compared with NH/Rouen [10,12,13]. We thus carried out polysomnographic recordings in H-TST and NH-TST mice. We showed that H-TST mice were more often in wakefulness phases than NH-TST mice (Mann Whitney test, $U=10$, $p=0.001$; Figure 1H). Moreover, they spent more time in paradoxical sleep, *i.e.*, rapid eye movement phase of sleep (Mann Whitney test, $U=21$, $p=0.0002$; Figure 1I), and less time in slow wave sleep (Mann Whitney test, $U=5$, $p=0.006$; Figure 1J) than non-helpless animals.

The brain derived neurotrophic factor (BDNF) levels has been proposed as a biomarker for MDD in humans [23] and its decrease expression level might result from

stress-dependent alteration of brain structure and function, mainly in hippocampus and PFC [24]. We thus quantified the *Bdnf* expression level in these two regions in H-TST and NH-TST and showed a significant decrease in *Bdnf* expression level in PFC (Mann Whitney test, $U=2$, $p=0.03$; Supplementary Figure S3A), but not in hippocampus (Mann Whitney test, $U=6$, $p=0.22$; Supplementary Figure S3B). Interestingly, the same decrease *Bdnf* expression levels in PFC was observed also in H/Rouen when compared with NH/Rouen (Mann Whitney test, $U=2$, $p=0.03$; Supplementary Figure S3A), whereas no significant difference was measured in hippocampus (Mann Whitney test, $U=3$, $p=0.06$; Supplementary Figure S3B).

H-TST mice displayed an increased mobility in response to antidepressant treatments

One of the three validity criteria to define animal models for psychiatric disorders is the predictive validity, *i.e.*, the ability to rescue the phenotype with psychotropic drugs. In previous studies, we showed that H/Rouen mice poorly responded to an acute injection of a selective serotonin reuptake inhibitor, *i.e.*, fluoxetine, at a dose of 20 mg/kg, but showed an increased mobility after three-weeks treatment with a daily administration of 10 mg/kg fluoxetine [10]. We thus measured the effect of acute and chronic injections of fluoxetine in H-TST animals. Acute injections slightly decreased the immobility time only in males (Mann Whitney test, $U=78.5$, $p=0.04$) but not in females (Mann Whitney test, $U=88.5$, $p=0.31$; Figure 2A). As previously observed for H/Rouen, chronic administration of 10 mg/kg fluoxetine significantly decreased the immobility time displayed by H-TST mice of both genders (Mann Whitney test, $U_m=15$, $p_m<0.05$ and $U_f=19$, $p_f=0.03$ in males and females, respectively; Figure 2B), showing that depressive-like behaviors in our models decreased after chronic injection of a high dose of fluoxetine.

As the phenotypic rescue after the administration of a selective serotonin reuptake inhibitor was modest, we assessed the effect of a rapid-acting antidepressant, by an acute injection of 30 mg/kg ketamine in H-TST male mice. We observed a slight decrease of immobility time in the TST (Mann Whitney test, $U=37$, $p=0.002$; Supplementary Figure S4). In addition, we tested LY341495, a selective orthosteric antagonist for the group II metabotropic glutamate receptors (mGluR2/3), which was known to increase the mobility time during the TST in the chronic unpredictable mild stress model of depression [25] and to share some neural mechanisms with ketamine in the exertion of antidepressant-like effects [26,27]. We then observed a dramatic decrease in the immobility time of males and females of H-TST (Mann Whitney test, $U_m=13$, $p_m<0.0001$ and $U_f=11$, $p_f<0.0001$ in males and females, respectively; Figure 2C) and H/Rouen mice (Mann Whitney test, $U_m=0$, $p_m<0.0001$ and $U_f=2$, $p_f<0.0001$ in males and females, respectively; Figure 2D). These observations showed that the depressive-like behaviors of our mouse models were more efficiently rescued by glutamate-related treatments than using selective serotonin reuptake inhibitors.

H-TST showed impairment in glutamatergic system.

We have demonstrated that the immobility time in TST were more efficiently rescued by glutamate-related treatments both in H-TST and H-Rouen. To explore the molecular mechanisms underlying the behavior observed in our models, we quantified the glutamate ionotropic receptor α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) type subunit 1 (*Gria1*) gene by real-time PCR in PFC and hippocampus of H-TST and NH-TST males as well as in H/Rouen and NH/Rouen males. We observed a dramatic decrease in the expression level of this gene for the two models both in PFC (Mann Whitney test, $U_{TST}=0$, $p_{TST}=0.008$, and $U_{Rouen}=0$, $p_{Rouen}=0.008$ for TST and

Rouen, respectively; Figure 3A) and hippocampus (Mann Whitney test, $U_{TST}=0$, $p_{TST}=0.008$, and $U_{Rouen}=0$, $p_{Rouen}=0.008$ for TST and Rouen, respectively; Figure 3B). This downregulation of *Gria1* was confirmed using *in situ* hybridization in H-TST and NH-TST mice (Mann Whitney test, $U=6$, $p=0.03$; Figure 3C).

Genes associated with helplessness are identical between Rouen and TST models

The selective breeding of extreme phenotype issued from two different genetic backgrounds resulted in two consistent mouse models for depression, *i.e.*, H/Rouen and H-TST. To determine whether these behaviors were genetically driven by genetic variants in the same genes, we conducted an exome sequencing on two helpless and non-helpless animals of each model (Figure 4). The Rouen and TST lines showed similar variability of the genomes between helpless and non-helpless animals for both damaging variants (Fisher's exact test, $p=1$) and other variants (Fisher's exact test, $p=1$; Figure 4A and 4B). In total, we identified 4,023 damaging variants in 1,688 genes in H-TST when compared with NH-TST (Supplementary Table S1), and 2,360 damaging variants in 1,091 genes in H/Rouen when compared with NH/Rouen (Supplementary Table S2). We then identified common genes with damaging variants in H/Rouen and H-TST, when compared with their non-helpless counterparts NH/Rouen and NH-TST, respectively. We identified 278 genes mutated both in H/Rouen and in H-TST (Figure 4C). Ten thousand random sampling of 1,688 and 1,091 genes, in the mouse genome, corresponding to the number of genes with damaging variants in H-TST and H/Rouen respectively, never reached 278 common genes ($p<10^{-5}$). This showed a highly significant enrichment in common genes with damaging variants in these two mouse lines.

Genes with damaging variants in H/Rouen and H-TST have been associated with major depression in human and demonstrate that these mouse lines are polygenic models of MDD

We identified common genes and variations segregating with the selected phenotype in our two mouse models. We thus determine whether the same genes were associated with MDD in humans. For each gene with a damaging variant in both H/Rouen and H-TST, we looked for the most associated p-values reported in one of the largest GWAS conducted in MDD to date [6]. It has been shown in humans that MDD is a polygenic disorder, and the individual risk increases with the number of associated common variants [7]. We thus varied the p-value threshold of human studies in a range from 5×10^{-8} to 0.5 and showed for each threshold that the number of genes in common between our two mouse models and those associated with MDD was significantly higher than expected by chance ($p < 10^{-5}$) (Figure 4D). This suggests that our mouse models are consistent polygenic models of human MDD. The maximum number of genes mutated in both H/Rouen and H-TST and associated with MDD was observed for a p-value threshold of 0.3 in the human meta-analysis, with 174 genes.

In order to identify what were the molecular mechanisms that were commonly affected in our mouse models and in MDD, we performed a biological pathway analysis on these 174 genes. Although, there was no significant gene ontology (GO) pathway enrichment either in biological processes or in molecular functions, we identified an enrichment in protein-protein interactions ($p=0.007$) and a major protein interacting network clustering 69 out of the 174 genes (Figure 4E). This network was enriched in genes involved in the regulation of intracellular signal transduction (GO1902531,

FDR=0.03) and more specifically in the regulation of small GTPase mediated signal transduction (GO0051056, FDR=0.0003).

Among the 174 genes, two (*Lrfn5* and *Tmem161b*) have been reported in humans with SNPs (*rs61990288* and *rs3099439*, respectively) that reached the genome-wide significant threshold of 5×10^{-8} in a meta-analysis comparing individuals with MDD and controls. Interestingly, we looked for these two SNPs into the Genotype-Tissue Expression (GTEx) database (<https://www.gtexportal.org/>) and observed they were expression quantitative trait loci (eQTL). The at-risk allele *rs61990288* G was associated with an increased *LRFN5* expression level in cerebellar hemisphere ($p=5.7 \times 10^{-16}$). The *rs3099439* C allele, which was located in the first intron of *TMEM161B*, was associated with an increased expression level of the long non-coding RNA *TMEM161B-AS1* in cerebral cortex ($p=5.1 \times 10^{-15}$) and nucleus accumbens ($p=9.6 \times 10^{-14}$). Note, this long non-coding RNA overlaps the first exon of *TMEM161B*. However, whether it modulates its expression level has yet to be determined.

Tmem161b is differentially expressed between helpless and non-helpless animals

To assess whether the genetic damaging variants identified in H/Rouen and H-TST affected the *Lrfn5* and *Tmem161b* expression levels, we performed a real-time PCR on PFC, one of the regions that are most consistently impaired in MDD [28-30] and the one showing differences in *Bdnf* and *Gria1* expression levels in our models. No significant difference was observed for *Lrfn5* between helpless and non-helpless animals, neither for H/Rouen, nor for H-TST (Figure 5A, combined Mann Whitney test, $U=1292$, $p=0.82$). In contrast, we observed a significant increase in both mouse lines for *Tmem161b* (Figure 5B, combined Mann Whitney test, $U=847$, $p=0.001$). To assess

whether the *TMEM161B* expression level may be used as a MDD biomarker in human, we then checked the *Tmem161b* expression level in mouse blood. Thus, we showed a significant increase of *Tmem161b* expression level in helpless animals when compared to non-helpless ones (Supplementary Figure S5A, combined Mann Whitney test, $U=89.5$, $p=0.01$). However, no significant correlation was observed between PFC and blood expression level (Supplementary Figure S5B, Pearson's correlation, $r=0.27$, $p=0.10$). We also quantified the *Tmem161b* expression level in PFC of H-TST and H/Rouen lines after an acute injection of 20 mg/kg fluoxetine or 3 mg/kg LY341495 but did not show a significant difference when compared with vehicle injection (Supplementary Figure S5C and S5D).

TMEM161B as a marker of treatment response in human with MDD

As *Tmem161b* expression level was increased in the PFC and blood of our models, we assess whether *TMEM161B* might be used as a marker of MDD or treatment response. We thus used available data in the Gene Expression Omnibus database (<https://www.ncbi.nlm.nih.gov/geo/>). As a biomarker must be easily measured on bedside, we focused on transcriptome studies from blood samples. We identified two studies conducted on more than one hundred individuals. GSE98793 reported the gene expression level in 128 patients with MDD and 64 controls. GSE146446, corresponding to a clinical trial on 237 individuals with a major depressive episode, reported gene expression levels at baseline and after eight weeks of treatment either with duloxetine or placebo. We thus compared the *TMEM161B* expression level between patients and controls using the GSE98793 data. Intriguingly, we observed a significant lower level of *TMEM161B* in patients with MDD when compared to unaffected individuals (Student t-test, $t=2.29$, $df=190$, $p=0.02$; Figure 5C). We then

assessed whether *TMEM161B* expression level might change with antidepressant treatment using the GSE146446 data. Interestingly, no difference in *TMEM161B* expression level was observed after 8-week treatment with placebo (Student t-test, $t=0.52$, $df=99$, $p=0.60$; Figure 5D). In contrast, a significant lower *TMEM161B* expression level was observed after 8-week treatment with duloxetine (Student t-test, $t=2.71$, $df=89$, $p=0.008$). Note, when distinguishing between responders and non-responders, we observed a significant lower expression level only in responders (Student t-test, $t=2.35$, $df=69$, $p=0.02$; Figure 5D) and not in non-responders (Student t-test, $t=1.47$, $df=19$, $p=0.16$; Figure 5D), suggesting the expression level of *TMEM161B* may be used as a marker of treatment response in MDD.

Discussion

One of the major issues when developing animal models of MDD is the great difficulty in modeling the polygenic nature of the illness. In this study, we used selective breeding to generate a new animal model with depressive-like behaviors and demonstrated that this model was associated with several genetic variations in MDD-associated genes in humans. In contrast to transgenic models or virally mediated genes delivery to the brain, selective breeding-based models are the only ones that mimic the genetic complexity encountered in humans [9]. One limit of selective breeding is to determine which are the genetic variants that are associated with the phenotype from those that result from the genetic hitchhiking, *i.e.*, the genetic drift resulting from linkage disequilibrium between near alleles. The strength of our model lies in having generated in parallel two selective breeding-based models with two different genetic backgrounds and to have shown these models have in average 21% of their damaging variants in identical genes. This is consistent with the polygenic origin of MDD, for which a

cumulative effect of genetic variants seems necessary to increase the risk of developing the illness [7]. Note, we showed here that two third of genes with damaging variants in both models, *i.e.*, H/Rouen and H-TST, have been associated with MDD in the largest meta-analysis conducted in humans to date [6], suggesting that H/Rouen and H-TST are relevant polygenic models for MDD.

Although genes commonly affected in both H/Rouen and H-TST, as well as in individuals with MDD, did not reveal an enrichment in a specific gene ontology pathway, we noticed that 40% of them clustered in a protein-protein interaction network with 26% of genes known to be involved in the regulation of intracellular signal transduction. This observation is consistent with the fact that antidepressants elicit their intracellular effects through canonical second messenger systems [31]. This has been shown not only for traditional monoaminergic antidepressants, but also for mood stabilizers and electroconvulsive therapy [32] and more recently for the rapid-acting treatments, such as ketamine. The efficiency of the latter in treatment-resistant depression effects on glutamate through the mechanistic target of rapamycin (mTOR) signaling pathway [33]. Interestingly, we reported here damaging variants in *Mtor*, *Telo2* and *Prr5l*, three MDD-associated genes of this system, suggesting H/Rouen and H-TST may model, more specifically, treatment-resistant depression. Supporting this hypothesis, we observed only a slight phenotypic rescue in our models when using a high dose of a selective serotonin reuptake inhibitor, which is the first line of pharmacological treatment for MDD. In contrast, both H/Rouen and H-TST showed a dramatic improvement in depressive-like behaviors when we targeted the glutamatergic system, as alternatively proposed for treatment-resistant depressions [34]. These results were also consistent with the dramatic decrease of the *Gria1* expression level that we observed in PFC and hippocampus of H-TST and

H/Rouen when compared with their non-helpless counterparts. This suggests that H/Rouen and H-TST might be particularly relevant tools to study treatment-resistant depression and test new pharmacological targets to manage this highly disabling disease.

Among the genes with genetic variants identified in both H/Rouen and H-TST, the most associated with MDD was *Lrfn5*. This gene has been associated in three independent studies [35-37] and one meta-analysis [6] and reached the genome-wide significant threshold in the latter. *Lrfn5* encodes a protein that belongs to the leucine-rich repeat and fibronectin type III domain-containing proteins (LRFNs), also called synaptic adhesion-like molecules (SALMs) that consist of five members (SALM1-5) [38-40]. The LRFN family is mainly expressed in the brain [40], where it mediates cell-cell adhesion and promote neurite outgrowth [41]. Note, only LRFN3 and LRFN5 have a synaptogenic activity, inducing excitatory and inhibitory presynaptic differentiation in contacting axons [42]. *LRFN5* has recently been implicated in severe progressive autism [43,44] and familial schizophrenia [45]. In addition, it has been associated with depressive symptoms in older adults [46] as well as in chronic pain-associated depression [47]. These results suggest that brain development impairment resulting from dysregulation of *LRFN5* might result in various psychiatric conditions either depending on the effect size of the associated variant or on the direction of the effect. More recently, a higher level of the LRFN5 protein has been reported in patients with MDD [48]. Note, a lower level was observed in patients who received antidepressant treatment when compared to the drug-naïve group, suggesting that the LRFN5 level might decrease in treated MDD patients [48]. Although we did not observe variations in RNA levels in our models, these results strengthened the interest of our models in detecting biomarkers for MDD in humans.

The second gene with damaging variants in H/Rouen and H-TST that reached the genome-wide significant threshold in humans with MDD was *Tmem161b*. This brain expressed gene encodes a transmembrane protein that regulates Sonic Hedgehog signaling in the developing mammalian central nervous system and regulates cerebral cortical gyration [49]. This gene has been first reported in the large European population of 23andMe in which cases were included with self-reports of clinical diagnosis or treatment for depression [36]. This association has then been confirmed in two other large studies [35,37], as well as in the meta-analysis of the three studies [6]. This MDD-associated SNP has also been associated with life-time alcohol dependence and putamen activation during reward processing in alcohol dependence [50]. Interestingly in a social isolation-based mouse model of depression, *Tmem161b* exhibits a decreased methylation level of histone H3 at lysine 9 and 27 [51], suggesting the dysregulation of this gene consistently results in depressive-like behaviors in various animal models. Note, we showed a significant increase of *Tmem161b* mRNA level in blood and brain from both H-TST and H/Rouen models. In humans, *TMEM161B* level was significantly lower in MDD patients treated with antidepressant when compared to controls and we showed its expression level significantly decreased after 8-week treatment with duloxetine mainly in patients who had improvement in clinical symptoms. As reported for LRFN5 [48], our results suggest that *TMEM161B* level might be higher in drug-naïve patients with MDD. The lower expression level that we observed when comparing MDD patients and controls may then be explained by the treatment received by patients in this cohort. While needed to be replicated on an independent cohort, these results suggest that *TMEM161B* could be a good biomarker for MDD in humans as well as to predict treatment response.

One limit of our study is that we have restricted our analyses to damaging variants identified in coding regions of genes. We cannot exclude that changes in gene expression observed between helpless and non-helpless animals in TST and Rouen lines may result from genetic variants located in non-coding regions. This should be studied in the future using whole genome sequencing approaches.

Altogether, our results demonstrate that H-TST and H/Rouen are the two first polygenic animal models for treatment-resistant MDD and support their relevance in the identification of biomarkers of MDD or antidepressant treatment response. The lower variance and the more marked phenotypes that we observed for H-TST in the behavioral tests suggest this new model, resulting from a more complex genetic background, will be of particular interest for screening new therapies for the development of rapid-acting antidepressant medications in the future.

Author contributions

M.E.Y., J.M.V. and S.J designed the study. M.E.Y. and J.M.V. generated mouse lines by selective breeding with the help of B.M.. M.E.Y. and L.B. conducted mouse behavioural analyses and pharmacological studies, with the help of S.A. for sleep exploration. C.A., V.L., E.R., A.W. and J.L. generated and analysed real-time PCR. P.S. and M.P. performed and quantified *in situ* hybridization. A.W. and J.L. generated exome data and conducted genetic analyses with S.J.. W.E.H. and S.J. conducted human genetic analyses. R.B. and G.T. generated transcriptome data after duloxetine treatment and analysed them with S.J.. S.J., M.E.Y., J.M.V. and J.L. wrote the article.

Funding

This work was supported the Agence National de la Recherche (ANR-12-SAMA-0012-01, project DEPSOM) and by the *Fondation de France* under references 00081240 and 00091264. This work also received financial support from the *Institut National pour la Santé et la Recherche Médicale* (Inserm) and the *Centre National pour la Recherche Scientifique* (CNRS). The Jamain's team is affiliated to the Paris School of Neuroscience (ENP).

Competing interests

The authors have nothing to disclose.

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Figure legends

Figure 1. H-TST mice display depressive-like behaviors. A. H-TST males and females showed a progressive decrease in mobility time over generations in contrast to NH-TST males and females which showed an increased mobility time. The extreme phenotype was reached after five generations for NH-TST and ten generations for H-TST. S0 has been estimated using the mean immobility duration of all mice (H-TST and NH-TST). **B, C.** The immobility time observed during the tail suspension test is not correlated to the motor activity measured in open field, neither for H-TST (N=24) (**B**), nor for NH-TST (N=24) (**C**). **D.** H-TST males (N=16) and females (N=12) displayed a higher immobility time during the forced swim test than NH-TST males (N=14) and females (N=8), respectively. **E.** H-TST males (N=11) and females (N=12) did not show sucrose preference in contrast to NH-TST males (N=10) and females (N=11), respectively. **F, G.** H-TST males and females displayed a higher anxiety-like behavior than NH-TST mice. H-TST males (N=11) and females (N=12) spent less time in the lit compartment of the dark-light box than NH-TST males (N=12) and females (N=10), respectively (**F**). H-TST males (N=11) and females (N=11) spent less time in the open arm of the elevated plus maze than NH-TST males (N=10) and females (N=11), respectively (**G**). **H, I, J.** H-TST females (N=10) have changes in sleep pattern when compared with NH-TST females (N=10). Vigilance states amounts were analyzed during a 48h-period (12h-light/dark cycles). They spent more time in wakefulness phases (**H**) and paradoxical sleep (**I**) and less time in slow wave sleep (**J**) than NH-TST mice. Scatter plots depict values of all males (squares) and females (circles) tested, and bars represent the median value with interquartile range. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 2. Antidepressant treatments increase the mobility of H-TST males and females in the tail suspension test. **A.** Acute injection of 20 mg/kg i.p. fluoxetine slightly increased the mobility of H-TST males (N=12) but not of females (N=12), when compared with vehicle injection in H-TST males (N=23) and females (N=19), respectively. **B.** Chronic injection of 10 mg/kg i.p. fluoxetine slightly increased the mobility of H-TST males (N=8) and females (N=9), when compared with vehicle injection in H-TST males (N=9) and females (N=10), respectively. **C.** Acute injection of 3 mg/kg i.p. LY341495 substantially increased the mobility of H-TST males (N=14) and females (N=14), when compared with vehicle injection in H-TST males (N=23) and females (N=19), respectively. **D.** Acute injection of 3 mg/kg i.p. LY341495 substantially increased the mobility of H/Rouen males (N=15) and females (N=15), when compared with vehicle injection in H/Rouen males (N=21) and females (N=26), respectively. Scatter plots depict values of all males (squares) and females (circles) tested, and bars represent the median value with interquartile range. * $p < 0.05$; **** $p < 0.0001$

Figure 3. *Gria1* expression level in the prefrontal cortex (PFC) and the hippocampus of the TST and Rouen lines. **A.** *Gria1* mRNA relative expression level was significantly lower in the PFC of helpless (grey) than in that of non-helpless (clear) mice of the TST and Rouen lines. (N=5). **B.** *Gria1* mRNA relative expression level was significantly lower in the hippocampus of helpless (grey) than in that of non-helpless (clear) mice of the TST and Rouen lines. (N=5). **C.** *In situ* hybridization with ³⁵S-radiolabeled antisense riboprobes against *Gria1* on H-TST and NH-TST male PFC coronal sections (left). The quantification of the densitometric measurement (right)

showed a significant decrease in the *Gria1* expression level in H-TST when compared with NH-TST. * $p < 0.05$; ** $p < 0.01$.

Figure 4. H-TST and H/Rouen are polygenic models of depression with genetic variants in genes associated with major depressive disorder in human.

A. Genetic variants identified by exome sequencing between H/Rouen and NH/Rouen. **B.** Genetic variants identified by exome sequencing between H-TST and NH-TST. **C.** Venn diagram displaying the overlap between genes with damaging variants in H-TST (red) and H/Rouen (blue). **D.** The histogram represents the number of genes with damaging variants shared by H/Rouen and H-TST and identified in a meta-analysis of genome-wide association studies conducted in humans with major depressive disorder [6]. The x axis shows the p -value thresholds used in the meta-analysis to determine SNPs associated with MDD in human. **E.** Protein-protein interaction networks and biological pathways identified using the 174 genes commonly identified with damaging variants in both H/Rouen and H-TST and associated with MDD in humans. **** $p < 0.0001$.

Figure 5. *LRFN5* and *TMEM161B* expression level in mouse lines and humans.

A, B. *Lfn5* (**A**) and *Tmem161b* (**B**) mRNA relative expression level in the prefrontal cortex (PFC) of helpless (grey) and non-helpless (clear) mice of the Rouen and TST lines. **C, D.** Log2 of the *TMEM161B* expression level in human blood. (**C**) Patients with major depressive disorder (cases) have been compared with controls. (**D**) *TMEM161B* expression level in patients with major depressive episode before (T0) or after 8-week treatment (T8) with duloxetine (DLX) or placebo (PLB). Scatter plots depict values of all males (squares) and females (circles) tested, and bars represent the median value

with interquartile range (**A**, **B**) or the mean value with the standard deviation (**C**, **D**).

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

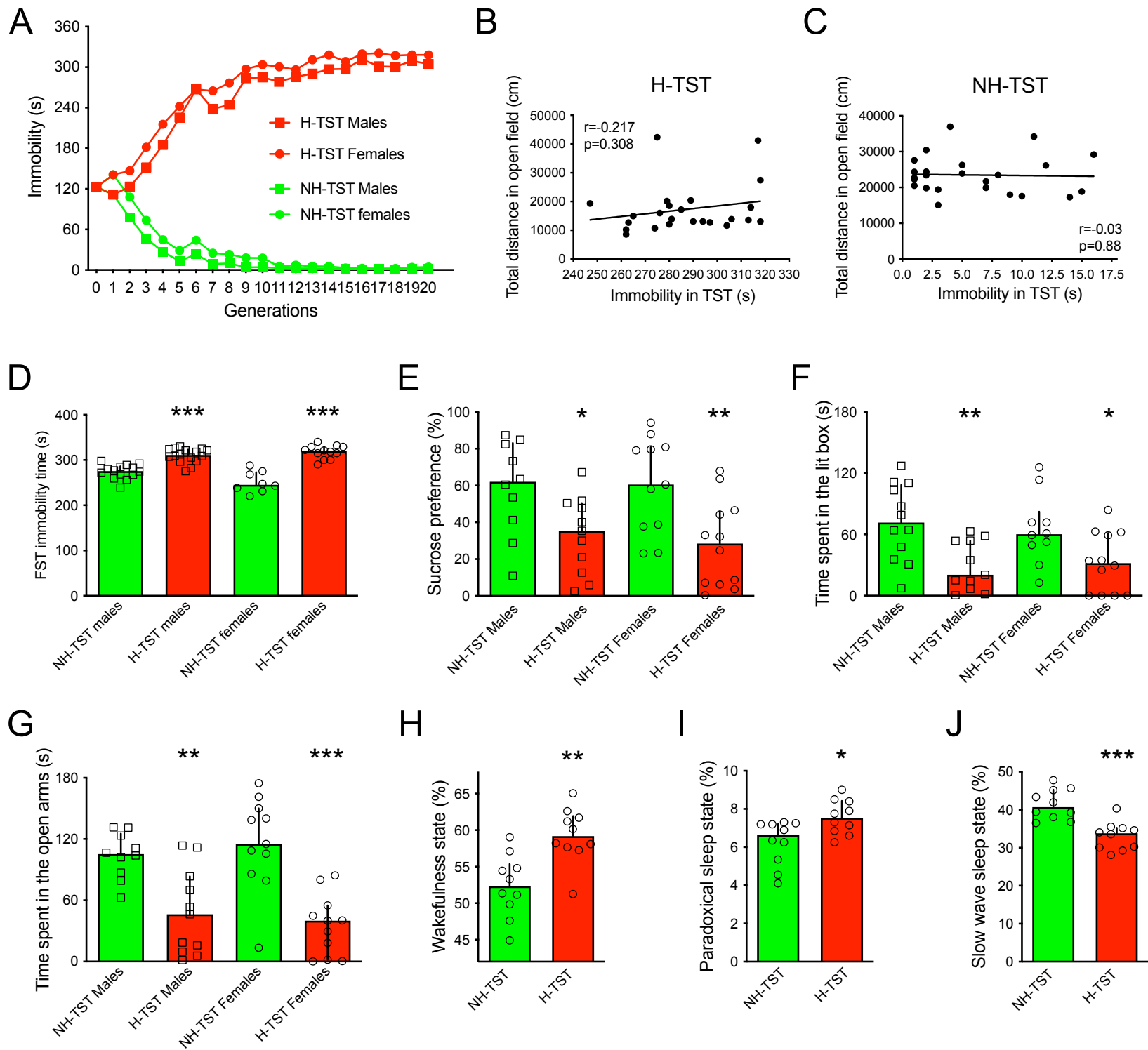
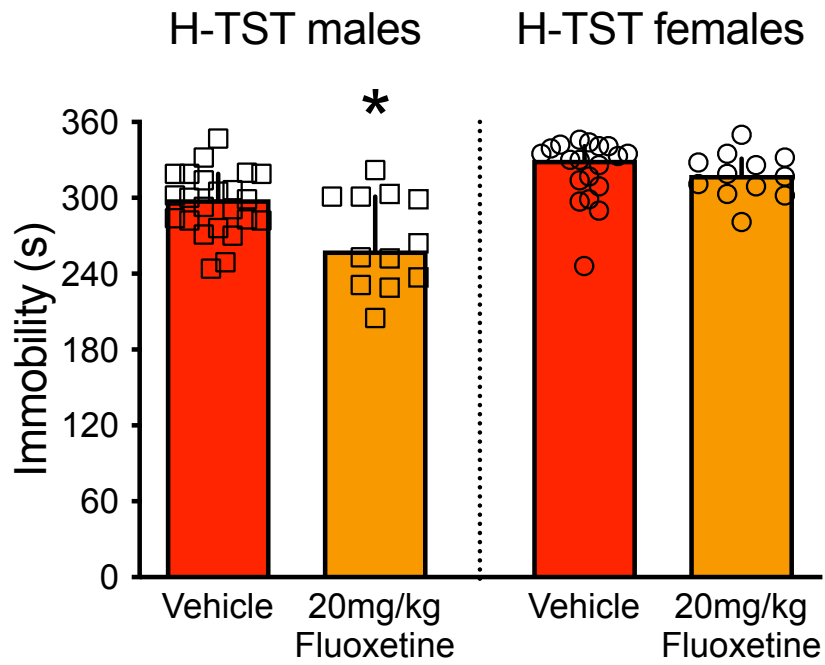
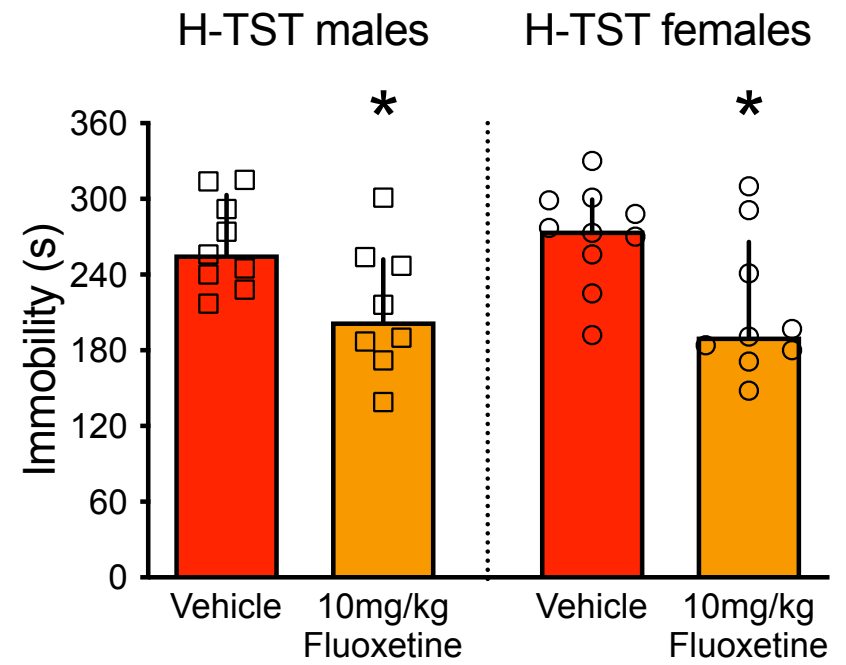


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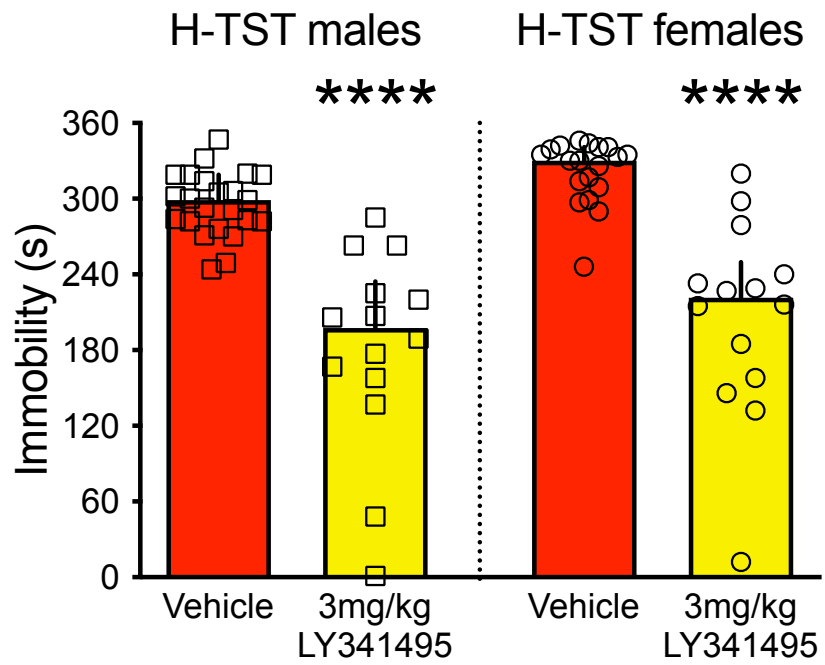
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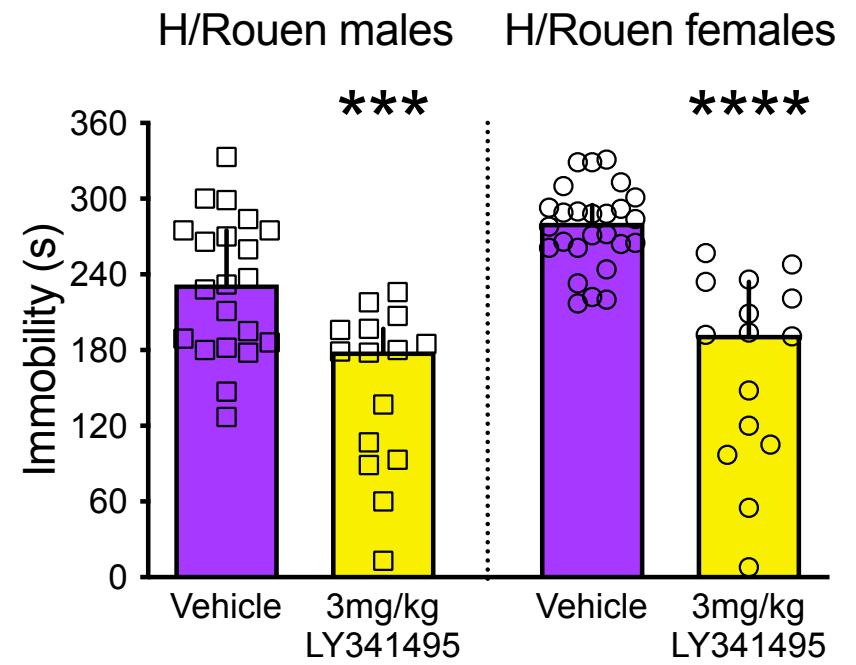


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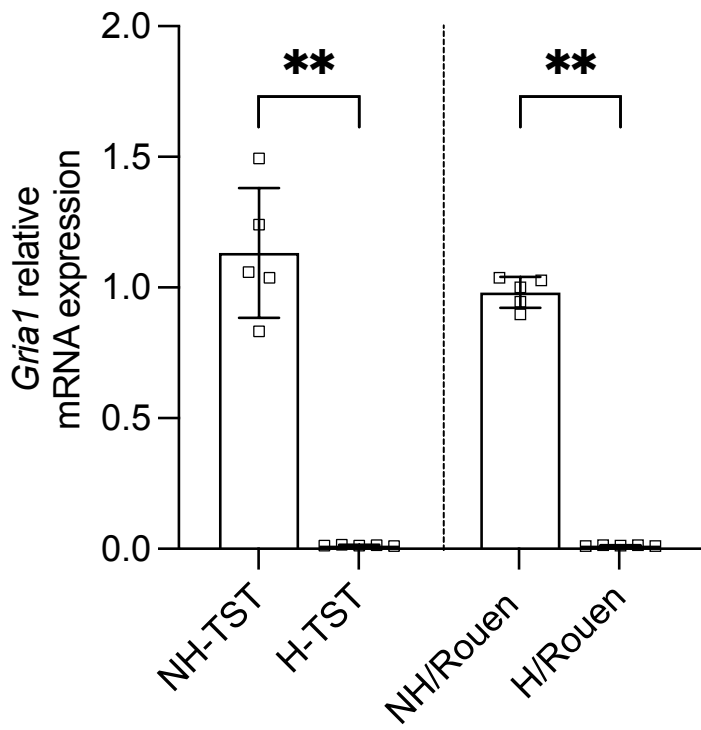
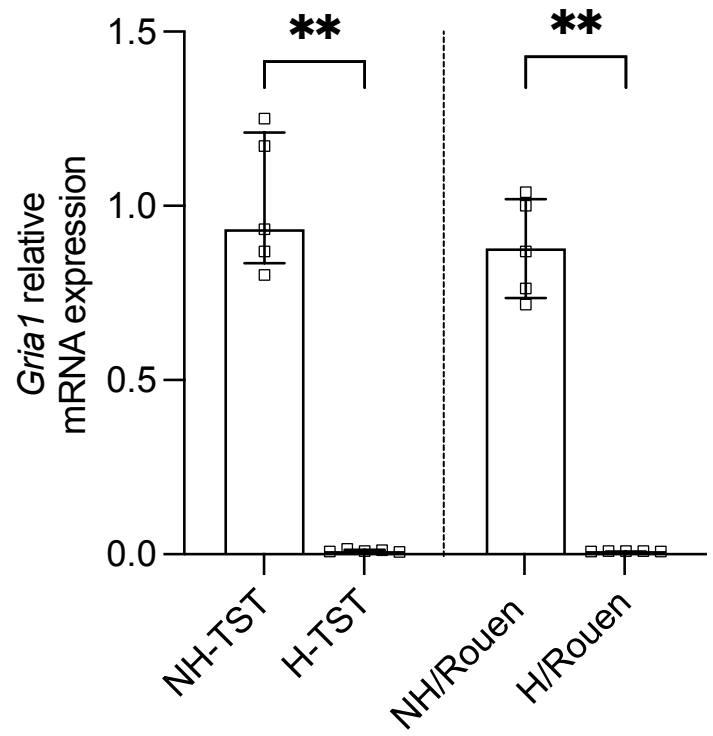
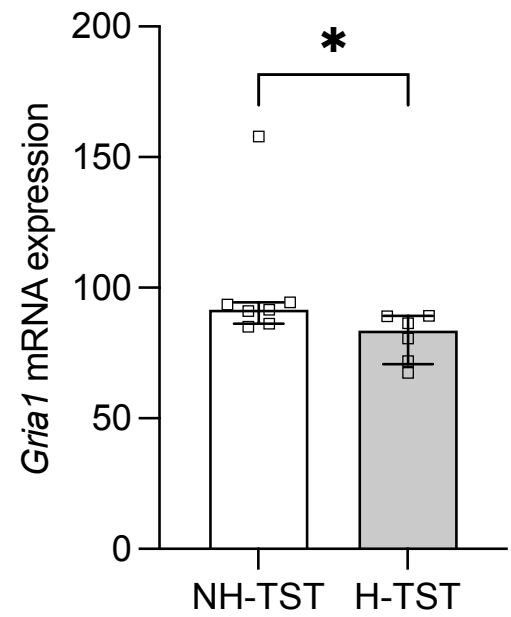
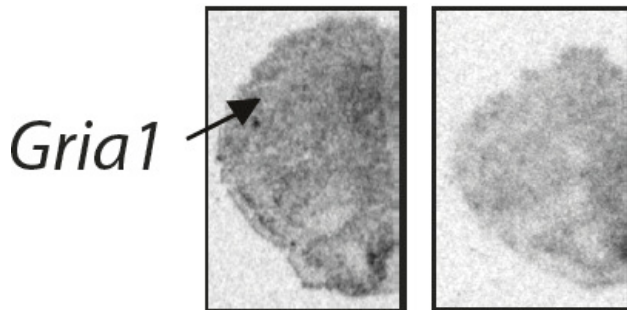
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Figure 3.

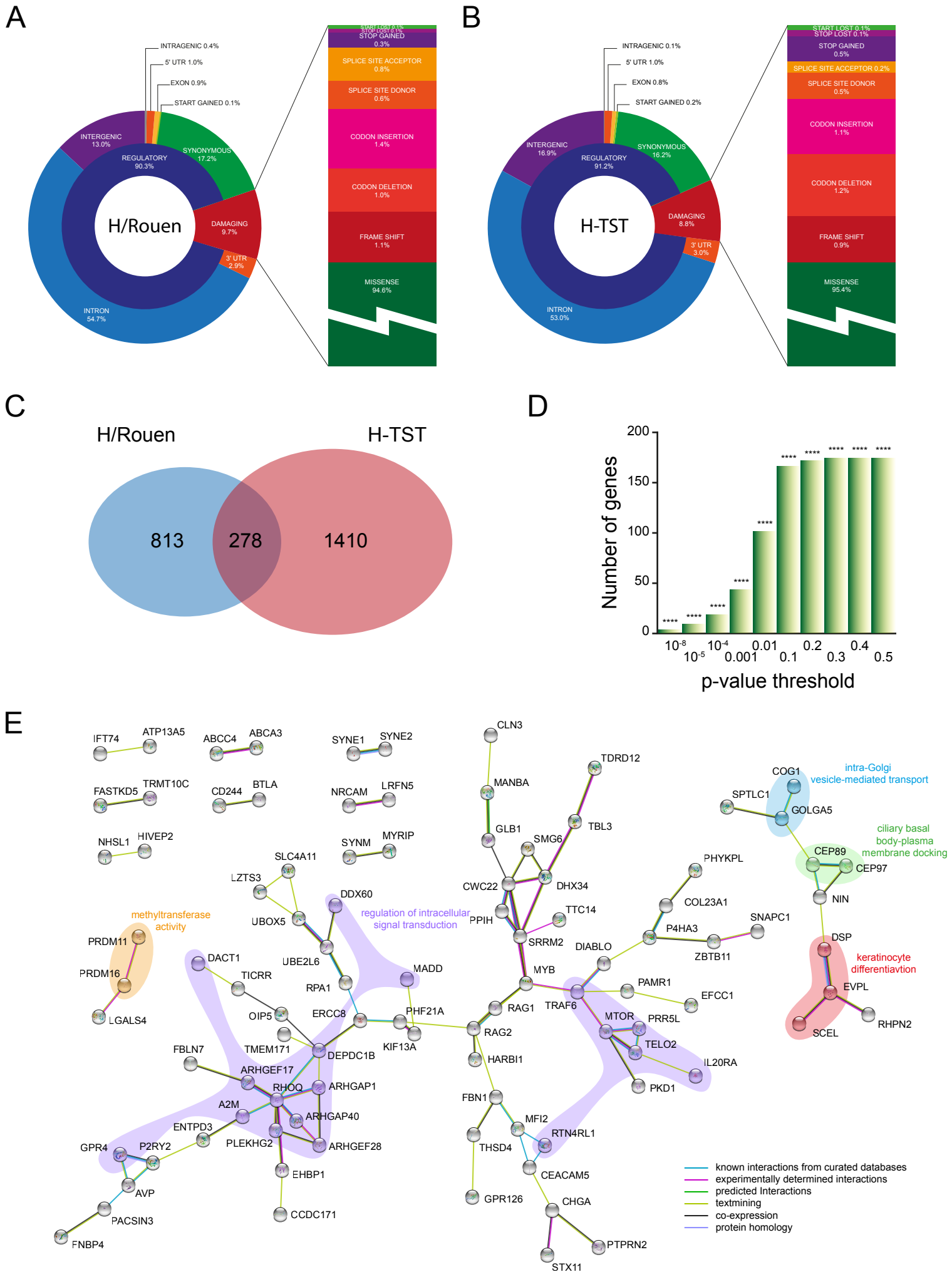


Figure 4.

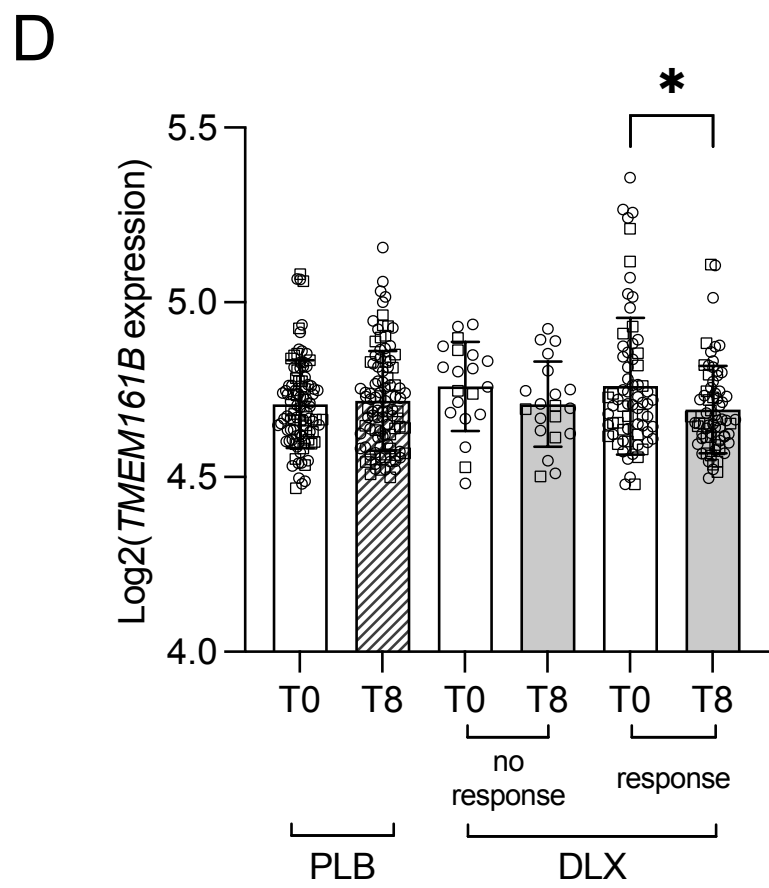
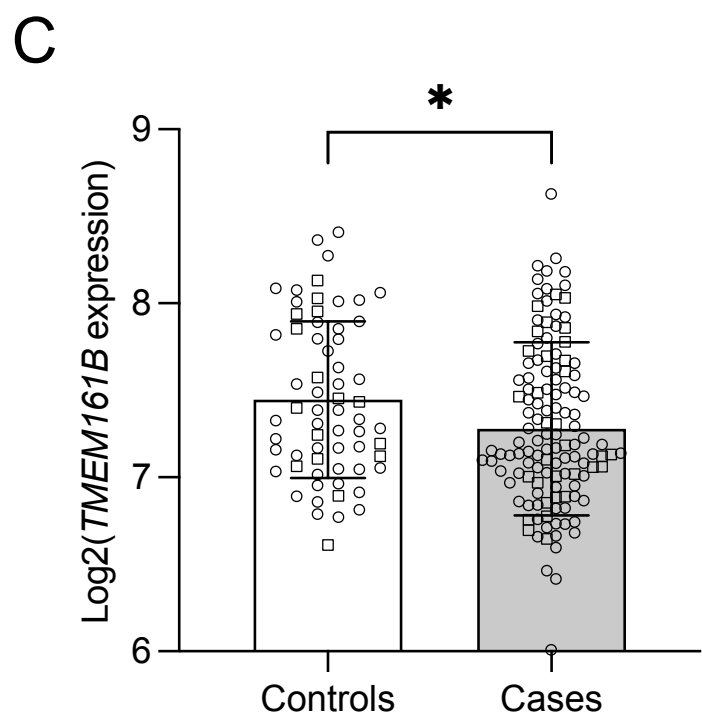
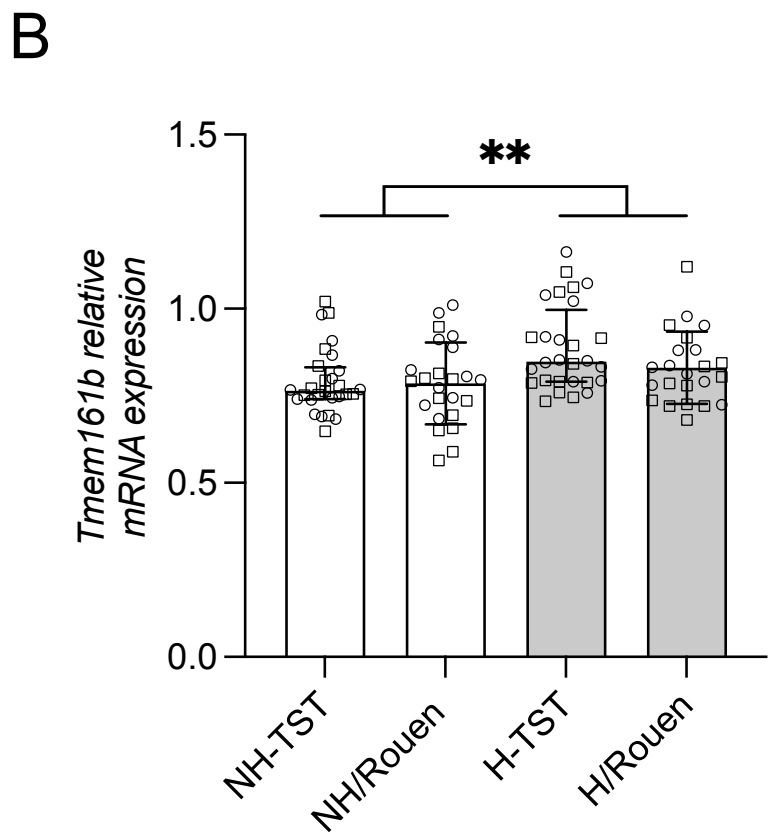
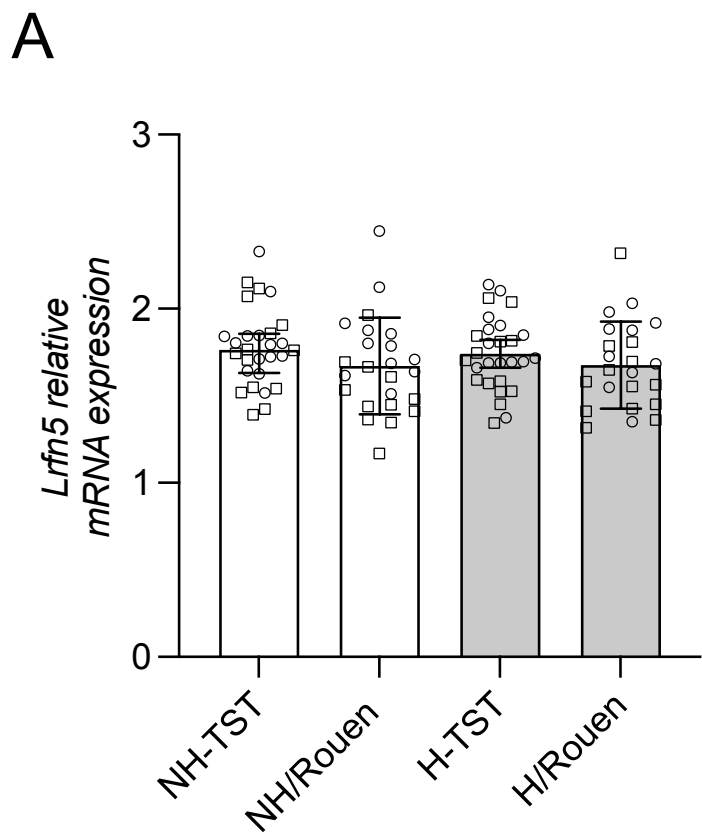


Figure 5.

Supplementary Information for

Two polygenic mouse models of major depressive disorders identify *TMEM161B* as a potential biomarker of disease in humans

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Supplementary Methods and Materials

Supplemental Figure S1

Supplemental Figure S2

Supplemental Figure S3

Supplemental Figure S4

Supplemental Figure S5

Supplementary Methods and Materials

Animals

Mice were selectively bred in animal facilities (Faculté de Médecine et de Pharmacie, Lyon 1 University, France) for high or low spontaneous mobility in the TST. The mice used as founders had a broad genetic background and came from an eight-way cross of A/J, ABP/Le, BALB/cJ, C57BL/6J, C3H/HeJ, CBA/H, DBA/2J, and SWXL-4 mice strains obtained from the Institute of Transgénose (Orléans, France). These mice were chosen for their genetic heterogeneity to maximize the polymorphism into the initial segregating pool of breeders. They were kept on a 7 a.m.-7 p.m. light cycle with food and water ad libitum. For breeding, male and female mice were housed together in pairs. When not under experimentation, they were kept in same-sex groups. Testing was performed between 9 a.m. and 5 p.m..

Selection and breeding

The bidirectional selective breeding was initiated with a starting population issued from the F3 population obtained after the eight-way cross (Supplementary Figure S1). It was constituted with 36 breeding pairs that gave birth to 456 male and 436 female mice. These mice were then selected for helplessness in the TST (see below). Helplessness was estimated by counting bouts of immobility while the mouse was suspended by the tail. During the test, mice which climbed up their tail were excluded from the experiment. We selected the two extreme 10% of all tested mice, corresponding to an immobility score higher than 189s for males and 222s for females of the 'helpless' (H-TST) group, and an immobility score lower than 44s for males and 64s for females of the 'non-helpless' (NH-TST) group. For generations S1 to S12, each mouse was tested 3 times (at weekly intervals) to assess its helplessness or non-helplessness. The helpless group comprised mating pairs of females and males having the highest

immobility in the test (H-TST). The non-helpless group was made up of mating pairs of females and males having the lowest immobility in the test (NH-TST). Pups were weaned at 21 ± 2 days, and animals were subjected to the first TST at age 42-50 days (middle adolescence) to assess their helplessness and to select new pairs to obtain the next generation. For the following generations, we systematically selected the two extreme 10% of all tested mice (Supplementary Figure S1). Two groups (H-TST, NH-TST) of 17-31 breeding pairs were then established from S2 to S6 and 6-23 breeding pairs from S7 onwards. If not indicated otherwise, behavioral assays were conducted on 10 to 14-week-old mice from generations S6 to S20.

Tail Suspension Test

The TST was performed with a computerized device. The mice, averaging 7-8 weeks of age, were suspended by the tail with adhesive tape to a hook connected to a strain gauge. This gauge transmitted movements to a computer calculating total duration of immobility during a 6-min test. Mice that climbed up their tail during the test session were excluded from the study.

Forced Swim Test

For the FST, the mice were an average of 13 to 14 weeks old. They were plunged individually into a vertical Plexiglas cylinder (25 cm high; 10 cm in diameter) filled with 9 cm deep water (21-23°C) for 6-min. They were then removed and allowed to dry. Immobility (i.e. making only minimal movements to keep the head above water or floating) was measured by an observer blind to the condition of the mouse.

Automated locomotor activity test

Exploratory motor activity was monitored in a 50 x 50-cm arena of an open-field during 45 minutes. The mice, aged 12 weeks, were placed for the test in a dimly lit, silent room. A video camera was placed in the ceiling overlooking the testing area and monitoring four open field cages. The test was performed using a video image processor (Videotrack, View Point, Lyon, France). The total distance travelled by the mice was recorded.

Sucrose preference test

Hedonic behavior was assessed using the two-bottle sucrose preference test [1]. The mice were 14 to 16 weeks old. Consumption of a 2% sucrose solution in one bottle and of water in a second bottle was measured (over a 3-day period) after a 6-day period of adaptation with switches between water and sucrose solution as only available fluid. Results are expressed as the percentage of sucrose solution consumed out of the total fluid drunk.

Light/dark box

Anxiety was first assessed using the light/dark box test [1]. This test is based on the innate aversion of rodents to illuminated areas. The light/dark box consisted in two compartments (20 cm large x 20 cm long x 30 cm high) connected by a 6 cm wide by 6 cm height opening located centrally at floor level. The time spent into the 200 lux-lit white (anxiogenic) compartment was recorded during the 5-min testing session using the video-tracking device (Viewpoint). The mice were 16 weeks old.

Elevated Plus Maze

Anxiety was also assessed in the elevated plus maze. This maze consisted of a plastic Greek cross placed 50 cm above the floor. The arms were 40 cm long and 6 cm wide. Two opposite arms were surrounded by walls (15 cm high, closed arms), while the two other arms were devoid of such walls (open arms). The mice, aged 13 weeks, were placed for the test in a dimly lit and quiet room. The time spent in the open (anxiogenic) arms over a 5-min period was recorded by the video-tracking device (Viewpoint).

Sleep and Wakefulness Analysis

Electrodes for polygraphic sleep monitoring were implanted under general anesthesia as described in detail elsewhere [2]. After a one-week recovery period, female mice (10 to 12- week-old) were placed in a Plexiglas barrel (20 cm in diameter) containing wood chips, food and water ad libitum, in a recording chamber for seven days. They were connected to a cable plugged in a rotating connector (Bilaney, Plastics One, Germany) to allow free movements. Unipolar EEG and bipolar EMG signals were amplified (MCP+, Alpha-Omega Engineering, Israel), digitized at 250 Hz, collected with a CED interface using Spike2 software (Cambridge Electronic Design, UK) and analyzed off-line using custom scripts written under Spike2 software. Vigilance states were scored using a 5 s window frame according to standard criteria. After three days of habituation to their cable and new environment, polysomnographic recordings were performed for four days, with the two last used for analyses.

Supplementary Figures

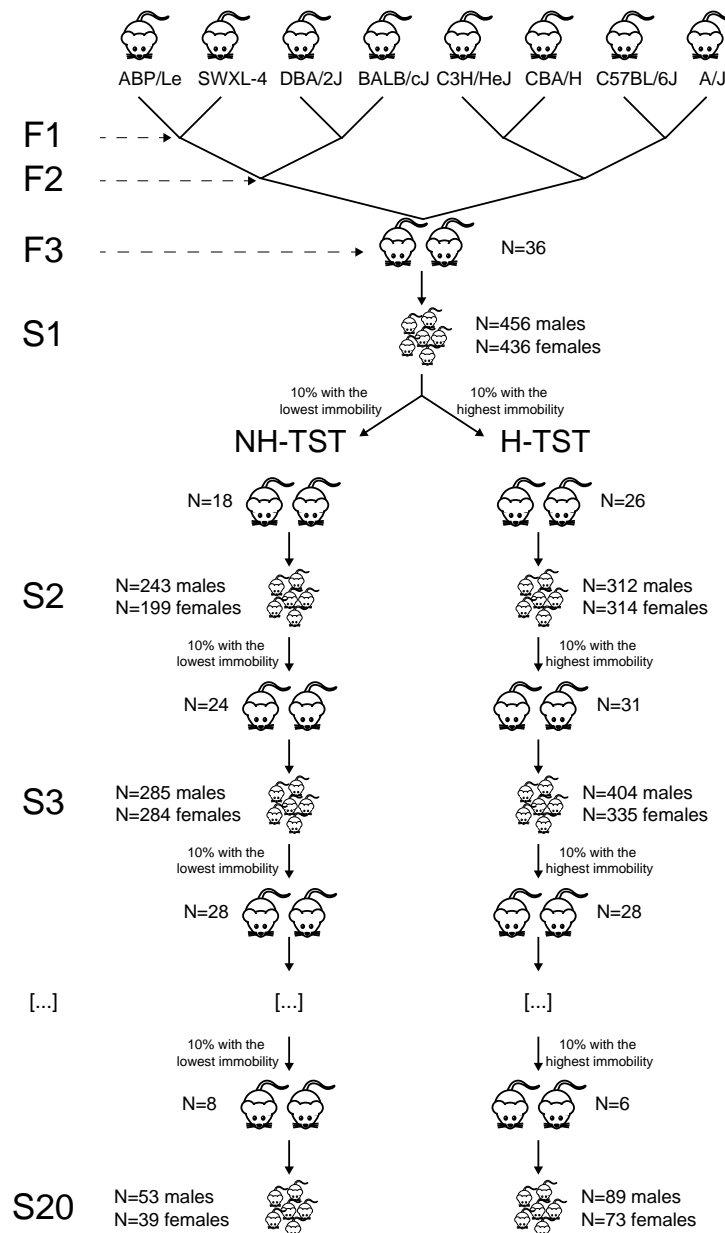


Figure S1. Schematic diagram of the bidirectional selective breeding. Founders were obtained at the 3rd generation (F3) after an eight-way cross involving mouse strains A/J, ABP/Le, BALB/cJ, C57BL/6J, C3H/HeJ, CBA/H, DBA/2J, and SWXL-4. Offspring was then tested with the tail suspension test and 10% of the extreme phenotypes, i.e. high or low immobility time, constituted the helpless (H-TST) or non-helpless (NH-TST) groups, respectively. In these groups males and females were mated to produce the next generation which were selected for the highest (H-TST) or the lowest (NH-TST) immobility score until the generation S20.

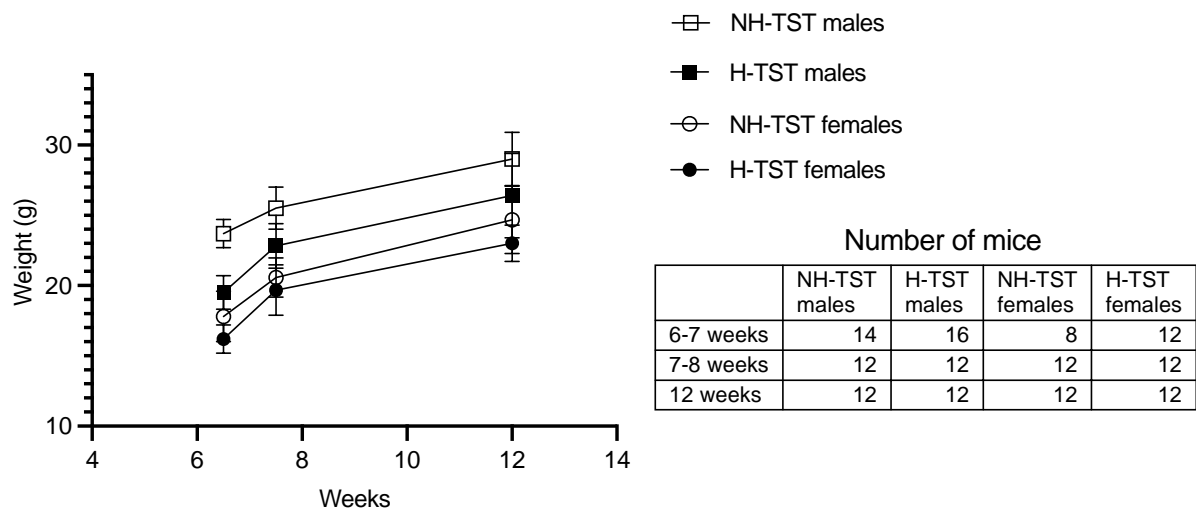


Figure S2. Weights of NH-TST (clear) and H-TST (black) mice over the weeks. Several independent groups of males (squares) and females (circles) were weighted before TST (6-8 weeks of age) or before automated locomotor activity (12 weeks of age). The number of animals weighed before each experiment is specified in the table. Symbols show mean \pm standard deviation (SD).

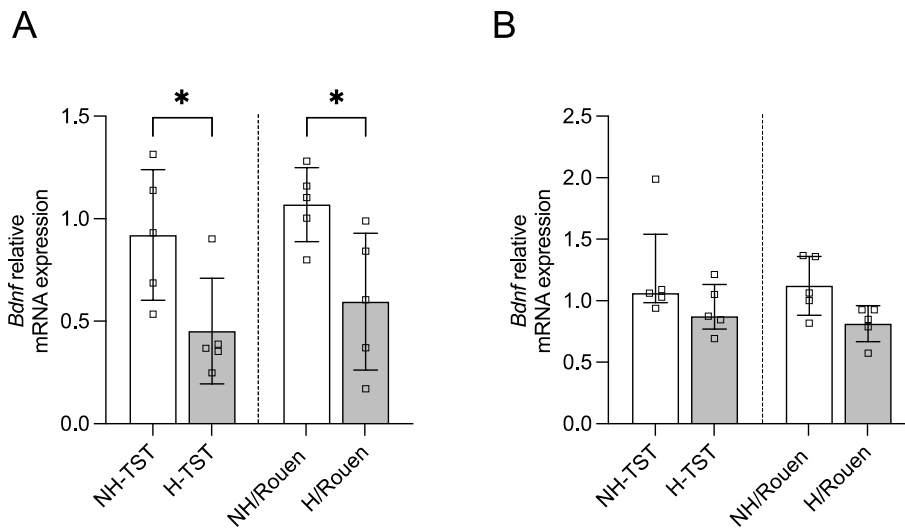


Figure S3. *Bdnf* expression level in the prefrontal cortex (PFC) (A) and the hippocampus (B) of TST and Rouen lines. A. *Bdnf* mRNA relative expression level was significantly lower in the PFC of helpless (grey) than in that of non-helpless (clear) mice of the TST and Rouen lines (N=5 per group). **B.** *Bdnf* mRNA relative expression level was not significantly different in the hippocampus of helpless (grey) and non-helpless (clear) mice of the TST and Rouen lines (N=5 per group). * $p < 0.05$.

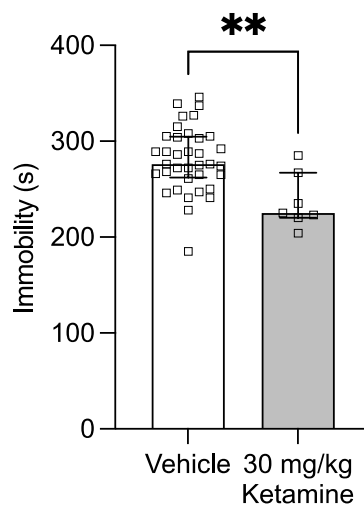


Figure S4. Ketamine treatment increases the mobility of H-TST males in the tail suspension test. Acute injection of 30 mg/kg i.p. ketamine slightly increased the mobility of H-TST males (N=7), when compared with vehicle injection in H-TST males (N=36). Scatter plots depict values of all mice tested, and bars represent the median value with interquartile range. * $p < 0.05$.

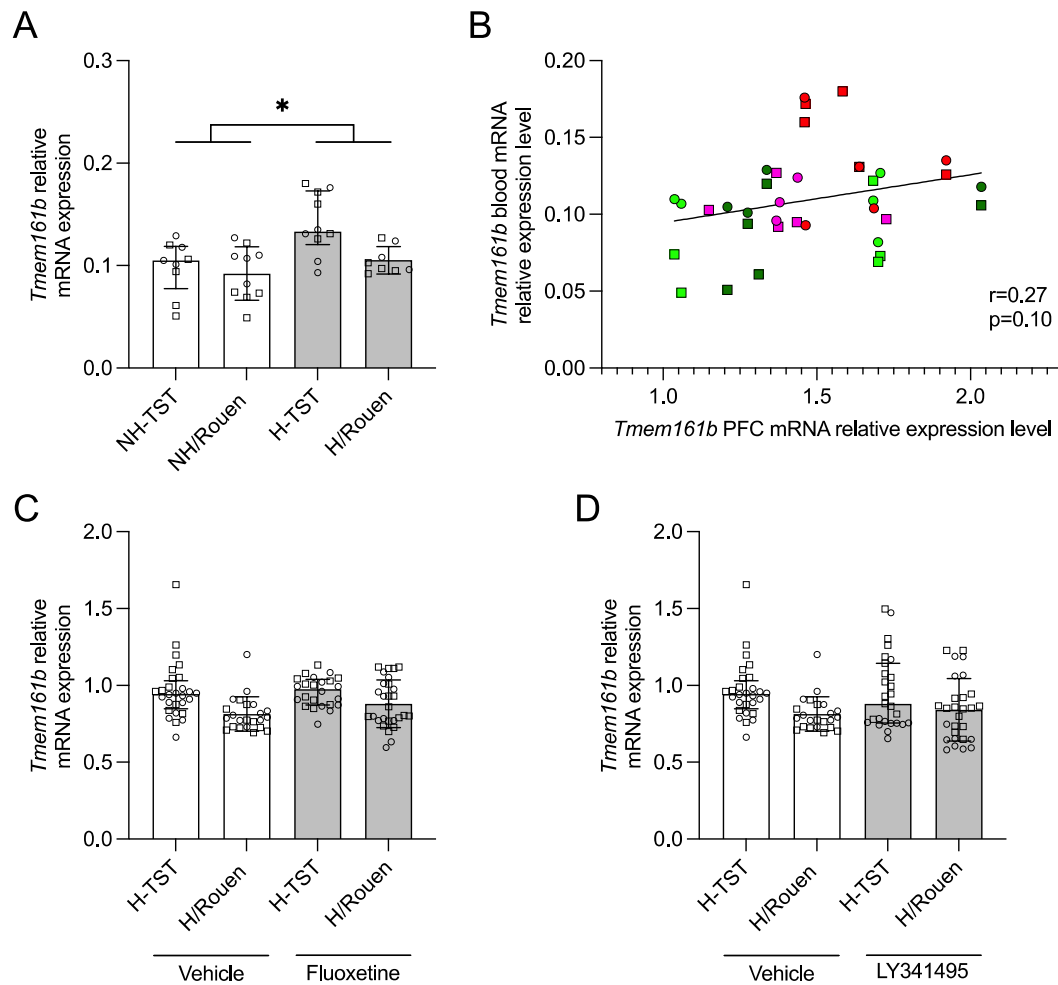


Figure S5. *Tmem161b* expression level in blood and brain of TST and Rouen lines. **A.** *Tmem161b* mRNA relative expression level was significantly higher in the blood of helpless (grey) than in that of the non-helpless (clear) mice of the TST (N_{NH-TST}=9, N_{NH-TST}=10) and Rouen lines (N_{NH/Rouen}=10, N_{H/Rouen}=8). **B.** Pearson's correlation between blood and prefrontal cortex (PFC) *Tmem161b* expression level. Males (N=20) and females (N=17) are displayed with squares and circles, respectively. NH-TST (dark green), NH/Rouen (light green), H-TST (red) and H/Rouen (purple) are depicted in colors. **C.** Acute injection of 20 mg/kg i.p. fluoxetine (grey) did not affect the PFC *Tmem161b* expression level neither in H-TST (N_{males}=12, N_{females}=12) nor in H/Rouen (N_{males}=16, N_{females}=12) mice, when compared with vehicle injection (clear) in H-TST (N_{males}=13, N_{females}=14) and H/Rouen (N_{males}=10, N_{females}=12), respectively. **D.** Acute injection of 3 mg/kg i.p. LY341495 (grey) did not affect the PFC *Tmem161b* expression level neither in H-TST (N_{males}=14, N_{females}=13) nor in H/Rouen (N_{males}=13, N_{females}=15)

mice, when compared with vehicle injection (clear) in H-TST ($N_{\text{males}}=13$, $N_{\text{females}}=14$) and H/Rouen ($N_{\text{males}}=10$, $N_{\text{females}}=12$), respectively.

References

- 1 El Yacoubi M, Rappeneau V, Champion E, Malleret G, Vaugeois JM. The H/Rouen mouse model displays depression-like and anxiety-like behaviors. *Behav Brain Res.* 2013;256:43-50.
- 2 Arthaud S, Varin C, Gay N, Libourel PA, Chauveau F, Fort P, et al. Paradoxical (REM) sleep deprivation in mice using the small-platforms-over-water method: polysomnographic analyses and melanin-concentrating hormone and hypocretin/orexin neuronal activation before, during and after deprivation. *J Sleep Res.* 2015;24(3):309-19.