Genetic and functional analysis reveals $TENM4$ contributes to schizophrenia

**Highlights**
- Possible pathogenic rare missense mutations in $TENM4$ gene contribute to SCZ
- Aberrant expression of Ten-m leads to behavioral disturbances related to SCZ symptoms
- Ten-m affects stimulation, metabolic process, neurogenesis, and ATPase activity
Genetic and functional analysis reveals TENM4 contributes to schizophrenia

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SUMMARY

TENM4, encoding a member of the teneurin protein family, is a risk gene shared by many types of mental diseases and is implicated in neuronal plasticity and signaling. However, the role and the mechanisms of TENM4 in schizophrenia (SCZ) remain unclear. We identified possible pathogenic mutations in the TENM4 gene through target sequencing of TENM4 in 68 SCZ families. We further demonstrated that aberrant expression of Ten-m leads to lower learning ability, sleep reduction, and increased aggressiveness in animal models. RNA sequencing showed that aberrant expression of Ten-m was related to stimulus perception and metabolic process, and Gene Ontology enrichment terms were neurogenesis and ATPase activity. This study provides strong evidence that TENM4 contributes to SCZ, and its functional mutations might be responsible for the impaired neural circuits and behaviors observed in SCZ.

INTRODUCTION

Schizophrenia (SCZ) is a severe psychiatric disorder with high heritability, genetic heterogeneity, and polygenic effect (Kavanagh et al., 2015). It has been reported that SCZ shares the same risk genes and pathways as common mental diseases, including bipolar disorder, autism, depression, Alzheimer disease, mental retardation, and epilepsy (Sullivan et al., 2012). Previous studies have confirmed that TENM4 is associated with bipolar disorder (Psychiatric GWAS Consortium Bipolar Disorder Working Group, 2011) (Ikeda et al., 2018) (Ivorra et al., 2014) (Heinrich et al., 2013), autism (Iossifov et al., 2014), and essential tremor (Hor et al., 2015) (Yan et al., 2020) (Chao et al., 2016). In most genome-wide association studies (GWAS) of SCZ, the common single-nucleotide polymorphisms (SNPs) in TENM4 did not reach genome-wide significance. However, a case-control replication study showed that an SNP (rs12290811) located in TENM4 was associated with SCZ (Ivorra et al., 2014). A whole-exome sequencing study in an SCZ family also identified one missense mutation co-segregated with the SCZ in TENM4 (Kue et al., 2018). A genome-wide meta-analysis with individuals of East Asian or European ancestry identified 176 loci associated with SCZ, including TENM4 (Lam et al., 2019). These studies partly provided some genetic evidence for TENM4 as an important candidate gene for SCZ.

TENM4, also known as ODZ4, is a member of the teneurin protein family, which comprises large transmembrane proteins (Baumgartner et al., 1994). The TENM4 gene is highly conserved among species (Tucker and Chiquet-Ehrismann, 2006). The temporal and spatial expression of teneurin family proteins in Drosophila (Baumgartner et al., 1994), zebrafish (Feng et al., 2002), birds (Tucker et al., 2000), and mice (Zhou et al., 2003) showed that Tenm4 protein was highly expressed in the nervous system, particularly in the brain. In Drosophila, a study showed that Ten-m acts as a synaptic cooperative matching molecule between the axons of olfactory receptor neurons (ORNs) and the dendrites of projecting neurons (PNs) in the olfactory system, and Ten-m could also guide synapses to achieve specific matches by homophilic interactions to ensure proper matching between neurons, indicating Ten-m plays an important role in neuron orientation (Hong et al., 2012). In addition, Ten-m was required for the organization and target selection of synapses in neurons (Mosca et al., 2012). In mice, a mutation in Tenm4 suppressed oligodendrocyte differentiation and myelination of small-diameter axons (Suzuki et al., 2012). Knockdown expression of Tenm4 in Neuro-2a cell lines decreased the length of individual neurites, while overexpression of Tenm4 promoted cellular protrusion formation and neurite outgrowth (Suzuki et al., 2014). These studies indicated that aberrant expression of Ten-m might lead to mis-matched neural circuits.

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Based on these previously reported findings, we hypothesized that TENM4 plays an important role in SCZ development and contributes to SCZ-related clinical symptoms, such as learning disability, sleep disorders, and violent behavior. Patients with SCZ often show violence, social withdrawal, learning and memory declines, and sleep disorders (Dowd and Barch, 2012; Cohrs, 2008; Patel et al., 2014). To verify this hypothesis, we performed targeted sequencing of TENM4 in 68 SCZ families to investigate the genetic contribution of TENM4 on SCZ. Then, to identify the role of TENM4 in SCZ, we examined the phenotypic characteristics by knockdown and overexpression of Ten-m in Drosophila and evaluated the effects of Ten-m on learning ability, sleep behavior, and aggressive behaviors. We also used mRNA sequencing to reveal the potential affected signaling pathways and molecular mechanisms of TENM4.

RESULTS

Rare missense mutations in TENM4 are co-segregated with disease status in SCZ families

To determine whether rare mutations in TENM4 are the candidate causal variants of SCZ, we initially performed whole TENM4 exome sequencing in 351 subjects from 68 SCZ families. A total of 36 mutations in TENM4 were identified using a step-by-step depth, quality filtering method. Then, 17 noncoding and synonymous variants were filtered to remove, and 19 nonsynonymous variants were kept. The 19 variants were annotated by ANNOVAR software (2019Oct24) with dbnsfp35c, gnomad211_exome, avsnp150, and intervar_20180118 databases to evaluate the function and allele frequency in the population. Given the pattern of dominant inheritance in the families, only rare heterozygous variants (allele frequency<1%) shared by the patients but not found in the normal controls in the same family were considered to be candidate mutations. At last, we found 5 variants were co-segregated with SCZ in their families, which were p.S1745P in Family_1, p.Q2735E and p.N965S in Family_2, p.V396I in Family_3, and p.T1849A in Family_4 (Table 1 and Figure 1A). In addition, all the five missense mutations were predicted to be deleterious to the TENM4 protein function by no less than three software.

Meanwhile, to confirm the relationship between TENM4 and SCZ, we re-analyzed the data from the genome-wide association analysis in a total of 50,874 SCZ cases and 83,493 controls from the Chinese and PGC2 genome-wide meta-analysis (Li et al., 2017). We analyzed 1196 SNPs in the region (chr11:78,358,876-79,156,992) and found that 39 SNPs showed nominally significant association with SCZ (p < 0.01, Figure 1B).

Ten-m affects the learning abilities in Drosophila

To investigate the potential contribution of TENM4 to SCZ, we examined the function of Ten-m in the Drosophila nervous system. We used the courtship conditioning assay, a behavioral assay that tests the courtship short-term memory (STM) to indicate learning ability (Siegel and Hall, 1979). After selection at the eclosion stage, male flies that had never mated were solely paired with mated females whose cuticles had already obtained cis-vaccenyl acetate (cVA), a male-specific and volatile pheromone, from males on mating (Everaerts et al., 2010). The males would be rejected by the mated females; then, the males formed STM, resulting in the suppression of courtship behavior when placed with other mated female flies over a period of time (Siwicki et al., 2005). The reduction of suppression indicates a lack of STM and the damage to learning ability. We used courtship indices (CIs), calculated as the percentage of courtship duration within 10 min, and learning indices (LIs), generated by the relative difference between the mean CIs of naive and trained male flies, to quantify the STM. Our data revealed that males of control genotypes (elav-GAL4, UAS-
Ten-m, and UAS-Ten-m-RNAi) exhibited courtship suppression after training compared to naive flies. Otherwise, there was no significant difference in CIs between naive and trained experimental flies (elav > UAS-Ten-m and elav > UAS-Ten-m-RNAi) with Ten-m aberrant expression in the nervous system, indicating that pan-neuronal overexpression or downexpression of Ten-m lead to lack of the learning ability to suppress courtship behavior (Figure 2A, naive vs trained; elav-GAL4, p < 0.0001; UAS-Ten-m, p < 0.0001; UAS-Ten-m-RNAi, p < 0.0001; elav > Ten-m, p = 0.820, elav > Ten-m-RNAi, p = 1.000). In addition, compared with the control groups, flies with aberrantly expressed Ten-m pan-neuronally revealed a significant reduction in LIs, which highlighted the courtship STM deficits (Figure 2B, elav-GAL4 vs elav > Ten-m, p < 0.0001, UAS-Ten-m vs elav > Ten-m, p < 0.0001; elav-GAL4 vs elav > Ten-m-RNAi, p < 0.0001; UAS-Ten-m-RNAi vs elav > Ten-m-RNAi, p < 0.0001). These results suggested that aberrant expression of Ten-m in the nervous system impairs STM.

Ten-m affects sleep behavior in Drosophila

To determine whether Ten-m regulates sleep in Drosophila, we increased or decreased Ten-m expression levels using transgenes UAS-Ten-m and UAS-Ten-m-RNAi, respectively, driven by pan-neuronal elav-GAL4. Total and
nighttime sleep amounts were decreased both in elav > Ten-m (Figures 3A and 3B nighttime: elav-GAL4 vs elav > Ten-m, p < 0.0001; UAS-Ten-m vs elav > Ten-m, p < 0.0001; UAS-Ten-m vs elav > Ten-m, p = 0.002) and elav > Ten-m-RNAi flies (Figures 3G and 3H, nighttime: elav-GAL4 vs elav > Ten-m-RNAi, p = 0.012; UAS-Ten-m-RNAi vs elav > Ten-m-RNAi, p < 0.0001; total: elav-GAL4 vs elav > Ten-m-RNAi, p = 0.019; UAS-Ten-m-RNAi vs elav > Ten-m-RNAi, p = 0.012) compared to controls. In contrast, there was no change in mutants in the daytime sleep of the mutants (Figures 3A and 3B, daytime: elav-GAL4 vs elav > Ten-m, p < 0.0001; UAS-Ten-m vs elav > Ten-m, p = 1.000; Figures 3G and 3H, elav-GAL4 vs elav > Ten-m-RNAi, p = 0.853; UAS-Ten-m-RNAi vs elav > Ten-m-RNAi, p = 0.986) which might be due to a floor effect, that is, daytime sleep of the controls was minimal. Further analysis revealed that the decreased night and overall sleep in elav > Ten-m flies was due to the reduced sleep bout duration but not sleep bout number (Figure 3C, elav-GAL4 vs elav > Ten-m, p = 0.905; UAS-Ten-m vs elav > Ten-m, p = 0.154; Figure 3D, elav-GAL4 vs elav > Ten-m, p = 0.905; UAS-Ten-m vs elav > Ten-m, p = 0.121; Figure 3E, p < 0.0001; Figure 3F, elav-GAL4 vs elav > Ten-m, p = 0.005; UAS-Ten-m vs elav > Ten-m, p < 0.0001). These results indicated that over-expressing Ten-m in all neurons would impair sleep behavior in Drosophila by reducing night sleep bout duration. The night and total sleep bout numbers were significantly increased in elav > Ten-m-RNAi flies compared to the control groups (Figures 3I and 3J, p < 0.0001). Meanwhile, the night and total sleep bout durations of elav > Ten-m-RNAi groups were shorter than those of the control groups (Figure 3K, elav-GAL4 vs elav > Ten-m-RNAi, p < 0.0001; UAS-Ten-m-RNAi vs elav > Ten-m-RNAi, p = 0.001; Figure 3L, p < 0.0001), suggesting the lack of Ten-m impaired sleep maintenance and caused sleep fragmentation in Drosophila. Overall, both Ten-m overexpression and downexpression impaired sleep maintenance and caused fragmented sleep.

Ten-m affects the aggressive behavior in Drosophila

We examined the aggressive behavior of Ten-m and Ten-m-RNAi flies using different GAL4 drivers. The latency to initiate fighting was significantly reduced (Figure 4A p < 0.0001; Figure 4B, elav-GAL4 vs elav > Ten-m-RNAi, p = 0.009; UAS-Ten-m-RNAi vs elav > Ten-m-RNAi, p < 0.0001), and the frequency was increased (Figure 4A, p < 0.0001; Figure 4B, elav-GAL4 vs elav > Ten-m-RNAi, p = 0.832; UAS-Ten-m-RNAi vs elav > Ten-m-RNAi, p = 0.001) in the flies with aberrantly expressed Ten-m pan-neuronally compared to the control groups. Male-male aggression is promoted by cVA in Drosophila via Or67d and Or65a receptors expressed by ORNs (Wang and Anderson, 2010). ORNs form synapses with PNs and local interneurons (LN)s in the antennal lobe (Das et al., 2008). Mz699-GAL4 is expressed in 90% of all ventral PNs, and 80% of these Mz699-PNs have been identified as GABAergic neurons, which are related to aggression (Lai et al., 2008; Alekseyenko et al., 2019), and Mz19 PNs are inherently connected to Or67d ORNs (Hong et al., 2012). We examined the aggressive behavior of UAS-Ten-m and UAS-Ten-m-RNAi transgenic flies using Mz699-GAL4, Mz19-GAL4, and LN1-GAL4, and latency and frequency changed sharply between the experimental and the control groups (Figures 4A and 4B, p < 0.05, **p < 0.01, ***p < 0.001; n.s., no significance.). These results reveal that aberrant expression of Ten-m in Drosophila would lead flies to become more aggressive.

RNA sequencing reveals the potential molecular mechanism

Through RNA sequencing of brain tissues in Drosophila, differentially expressed genes (DEGs) under the conditions of Ten-m overexpression or downexpression were compared (Figure 5A), and the functional classification of these DEGs was carried out. A total of 164 significant DEGs were identified between elav > Ten-m-RNAi and elav-GAL4 groups, including 83 upregulated genes and 81 downregulated genes.
Among DEGs in the elav > Ten-m-RNAi vs elav-GAL4, Gene Ontology (GO) enrichment analysis was related to stimulation, such as biological process (BP) terms linked to response to light stimulus and detection of external stimulus (Figure 5C, Table S5). We also revealed that 130 genes were differentially expressed in elav > Ten-m vs elav-GAL4 groups, and 85 genes were upregulated, and 45 genes were downregulated (Figure 5D, Table S2). In the elav > Ten-m vs elav-GAL4, the significantly enriched GO terms were those involved in metabolic processes such as the nucleoside monophosphate metabolic process (Figure 5E, Table S4). As shown in the Venn diagram (Figure 5F), we found there were 55 overlapped DEGs with both overexpressed and decreased expression of Ten-m. The clustering heatmap analysis of 55 overlapped significant DEGs indicated a contrasting gene expression profile between experimental groups. elav > Ten-m-RNAi and elav > Ten-m had a very similar profile of gene expression, revealing the same high and low expression trends while showing a different profile against elav-GAL4 (Figure 5G). GO enrichment analysis of overlapped significant DEGs showed that the only BP term was neurogenesis, and the only molecular function (MF) term was ATPase activity (Figure 5H, Table S6).

**DISCUSSION**

To date, hundreds of genes related to SCZ genes have been identified from GWAS, exome sequencing studies, transcriptomic studies, and epigenetic studies according to the SCZ database (SZDB, http://www.szdb.org/SZDB/index.html) (Sanders and Gill, 2007; Khavari and Cairns, 2020; Xu et al., 2011; Chen et al., 2015; Farrell et al., 2015). Previous studies have also provided amount of evidence for the polygenic architecture of SCZ (Smeland et al., 2020). Many common genetic variants and rare functional mutations make up the polygenic architecture of SCZ (Henriksen et al., 2017). In SCZ, common and rare genetic variants are associated with disorders of...
Figure 5. RNA sequencing of brain tissues with aberrant expression of Ten-m in Drosophila
(A) FPKM of controls (elav-GAL4) and experimental flies (elav > Ten-m and elav > Ten-m-RNAi). Data are represented as mean ± SD. The Kruskal-Wallis test was used for comparison. Compared to the control groups, the experimental groups had increased or decreased the expression of Ten-m.
(B) Volcano plots of DEGs from elav > Ten-m-RNAi vs elav-GAL4 groups. Red denotes high expression while blue denotes low expression. In total, 164 significant DEGs were identified, including 83 upregulated genes and 81 downregulated genes.
(C) GO enrichment analysis of DEGs in the elav > Ten-m-RNAi group. The DEGs were enriched in stimulation-associated pathways, including light stimulus and detection of external stimulus.
(D) Volcano plots of DEGs from elav > Ten-m vs elav-GAL4 groups. Red denotes high expression while blue denotes low expression. In total, 130 significant DEGs were identified, including 85 upregulated genes and 45 downregulated genes.
(E) GO enrichment analysis of DEGs in the elav > Ten-m group. The DEGs were enriched in metabolism-associated pathways, such as the nucleoside monophosphate metabolic process.
(F) Venn diagram showing the intersection of significant DEGs observed in elav > Ten-m and elav > Ten-m-RNAi groups. There were 55 overlapped DEGs for both decreased and overexpressed Ten-m.
(G) Heatmap of overlapped significant DEGs. Hierarchical clustering was performed to highlight the DEGs, indicating a contrasting gene expression profile between experimental groups. Red denotes high expression while blue denotes low expression.
(H) GO enrichment analysis of overlapped significant DEGs in elav > Ten-m and elav > Ten-m-RNAi groups. The only BP term was neurogenesis, and the only MF term was ATPase activity.
biological processes related to neurodevelopment, neuroexcitability, synaptic function, and the immune system (Jikkola-Vuoti et al., 2019; Boyajyan et al., 2015; Jiang et al., 2020). In this study, we focused on rare functional mutations with minor allele frequency (MAF) < 0.01 in the candidate gene, TENM4. We identified five rare functional mutations of TENM4 gene in SCZ families, suggesting TENM4 contributes to SCZ. Interestingly, common SNPs in TENM4 only presented a nominally significant association with SCZ and did not reach the level of genome-wide significance, which might be because most mutations identified in TENM4 were missense mutations. Unlike the loss of function mutations, most missense mutations have a reduced effect on gene function, and some mutations might even not affect gene function. It is not easily to distinguish these mutations from the true functional missense mutations, but the GWAS results revealed that there might still be a genetic influence of TENM4 on SCZ. We have not performed the sequencing in patients with sporadic SCZ because the limited sample size would affect the results a lot, and the very big sample size may find the association between rare functional mutations.

TENM4 is known to be involved in mental illness and cognition, but there has been no behavioral analysis of TENM4 in animal models. Thus, we used the Drosophila model to demonstrate the potential mechanism of SCZ caused by TENM4 mutations. We observed reduced STM with abnormal neuronal expression of Ten-m. Intellectual disability and learning disabilities are prevalent in patients with SCZ. SCZ is also associated with a substantial cognitive impairment that affects working memory, processing speed, attention, and verbal memory (Dickinson et al., 2007). It is also difficult for patients with SCZ to learn from positive feedback (Dowd and Barch, 2012). Patients with SCZ often reveal social withdrawal, and the possibility of violence is increased (Lamberti, 2007). Sleep and circadian rhythm disorders occur in many psychiatric patients, including SCZ (Wulff et al., 2010). Furthermore, sleep disturbances occur frequently in patients with SCZ (Cohrs, 2008), including shorter duration, sleep fragmentation, insomnia, or other sleeping disorders (Monti and Monti, 2004). We found that the aberrant expression of Ten-m could cause sleep reduction and increased aggressiveness, which exhibit an SCZ-like phenotype. Thus, we inferred that TENM4 is a promising candidate gene, which might be related to some SCZ symptoms in SCZ, such as high aggression levels, sleep disorders, and learning ability related to cognitive deficits.

Overexpression and downexpression of Ten-m both caused night-sleep reduction in Drosophila, but the effect was not the same. Overexpression of Ten-m caused reduced sleep duration, and both overexpression and downexpression of Ten-m caused sleep fragmentation. We speculated that these effects were caused by different mechanisms, which need to be studied. The GWAS of actigraphic sleep phenotypes indicated that wake after sleep onset is associated with TENM4 (Spada et al., 2016).

SCZ is related to severe abnormal mutations in genes essential for synaptic plasticity (Gulsuner et al., 2020). Neural circuits of the human brain are composed of approximately 100 million neurons intercommunicated by synapses which are eventually responsible for behavior and brain function (Li et al., 2016). Ten-m affects the synapses morphological structure in Drosophila, suggesting that the aberrant expression of TENM4 may cause changes in synapse morphological structure which could lead to SCZ.

RNA sequencing showed that the effect of Ten-m on stimulation and the metabolic process might cause behavioral abnormalities. The sensitivity to cVA in males plays an important role in learning, memory, and aggressive behavior, while the sensory perception of light stimulation affects sleep in Drosophila. In addition, the imbalance of the nucleoside pathway and metabolism significantly affects the neuromodulatory function significantly (Ipati et al., 2011). The GO enrichment analysis of overlapped significant DEGs revealed neurogenesis and ATPase activity. Moreover, hippocampal neurogenesis and synaptic functions are crucial for learning and memory (Nabavi et al., 2014). Aberrant subcortical neurogenesis often occurs in SCZ (Hoffmann et al., 2019). The abnormal regulation of energy metabolism leads to changes in the synthesis and release of ATP. Studies have shown that ATP is the main stimulant of microglial and can be used as a signal to mediate the growth of microglial and the communication of nerve cells, which would affect the function of neurons and synapses (Davalos et al., 2005). Thus, the molecular mechanism of TENM4 on SCZ might be mainly through affecting neurogenesis and regulating ATPase activity.

Limitations of the study

There are still some limitations in our study. For example, we need a big sample size of sporadic SCZ patients to perform the target sequencing to find the association between rare functional mutations. More functional experiments should be set to find more direct pathological evidence in SCZ. The molecular mechanism of TENM4 on SCZ remains unclear.
STAR METHODS
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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103063.

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AUTHOR CONTRIBUTIONS
D.Z., Y.P., G.H., and L.H. designed the study and interpreted the results. X.L., D.C., L.W., F.Y., and X.C. collected the samples and performed the primary experiments. X.Y. and H.D. conducted targeted sequencing, and D.Z. performed statistical analysis. X.Y. and M.L. performed the animal experiments. X.Y. and D.Z. performed the RNA-seq and GO analysis. D.Z., Y.P., X.Y., and M.L. verified the underlying data. X.Y. and M.L. drafted the manuscript, and all authors contributed to the final version of the paper.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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REFERENCES

Alekseyenko, O.V., Chan, Y.B., Okaty, B.W., Chang, Y., Dymecki, S.M., and Kravitz, E.A. (2019). Serotonergic modulation of aggression in Drosophila involves GABAergic and cholinergic opposing pathways. Curr. Biol. 29, 2145–2156 e5.

Anders, S., Pyl, P.T., and Huber, W. (2015). HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics 31, 166–169.

Baumgartner, S., Martin, D., Hagios, C., and Chiquest-Ehrismann, R. (1994). Tenm, a Drosophila gene related to tenascin, is a new pair-rule gene. EMBO J. 13, 3728–3740.

Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120.

Boyajyan, A., Zakaryan, R., Atshemyan, S., Chavushyan, A., and Mirkhchyan, G. (2015). Schizophrenia-associated risk and protective variants of c-Fos encoding gene. Recent Adv. DNA Gene Seq. 9, 51–57.

Chao, Y.X., Lin Ng, E.Y., Tio, M., Kumar, P., Ten, L., Au, W.L., Yih, Y., and Tan, E.K. (2016). Essential tremor linked TENM4 mutation found in healthy Chinese individuals. Parkinsonism Relat. Disord. 31, 139–140.

Chen, J., Cao, F., Liu, L., Wang, L., and Chen, X. (2015). Genetic studies of schizophrenia: an update. Neurosci. Bull. 31, 87–96.

Chen, S., Lee, A.Y., Bowens, N.M., Huber, R., and Kravitz, E.A. (2002). Fighting fruit flies: a model......
system for the study of aggression. Proc. Natl. Acad. Sci. U S A 99, 5664–5668.

Cohrs, S. (2008). Sleep disturbances in patients with schizophrenia: impact and effect of antipsychotics. CNS Drugs 22, 939–962.

Das, A., Sen, S., Lichtneckert, R., Okada, R., Ito, K., Rodrigues, V., and Reichert, H. (2008). Drosophila olfactory local interneurons and projection neurons derive from a common neuralblast lineage specified by the empty spiracles gene. Neuron Dev. 3, 13.

Davalos, D., Grutzendler, J., Yang, G., Kim, J.V., Zuo, Y., Jung, S., Littman, D.R., Dustin, M.L., and Gan, W.B. (2005). ATP mediates rapid microglial response to local brain injury in vivo. Nat. Neurosci. 8, 752–758.

Dickinson, D., Ramsey, M.E., and Gold, J.M. (2007). Overlooking the obvious: a meta-analytic comparison of digit symbol coding tasks and other cognitive measures in schizophrenia. Arch. Gen. Psychiatry 64, 532–542.

Dowd, E.C., and Barch, D.M. (2012). Pavlovian reward prediction and receipt in schizophrenia: relationship to anhedonia. PLoS One 7, e35622.

Everaerts, C., Farine, J.P., Cobb, M., and Ferveur, J.F. (2010). Drosophila cuticular hydrocarbons system for the study of aggression. Proc. Natl. Acad. Sci. U S A 99, 5664–5668.

Hof, H., Francescato, L., Bartesaghi, L., Ortega-Cubero, S., Kousi, M., Lorenzo-Betancor, O., Jimenez-Jimenez, F.J., Gironell, A., Clarimon, J., Drechsel, O., et al. (2015). Missense mutations in TENM4, a regulator of axon guidance and central myelination, cause essential tremor. Hum. Mol. Genet. 24, 5677–5686.

Ikeda, M., Takahashi, A., Kamatani, Y., Okahisa, Y., Kunugi, H., Mori, N., Sasaki, T., Ohmori, T., Okamoto, Y., Kawasaki, H., et al. (2018). A genome-wide association study identifies two novel susceptibility loci and trans population convergence. Mol. Psychiatry 23, 639–647.

Iossifov, I., O’roak, B.J., Sanders, S.J., Ronemus, M., Krupicka, H., Huddleston, J., Stankiewicz, P., H.A., Wetherspoon, K.T., Vives, L., Patterson, K.E., et al. (2014). The contribution of de novo coding mutations to autism spectrum disorder. Nature 515, 216–221.

Ipata, R.L., Camici, M., Micheli, V., and Tozz, M.G. (2011). Metabolic network of nucleosides in the brain. Curr. Top Med. Chem. 11, 999–922.

Iivara, J.L., Rivero, O., Costas, J., Iniesta, R., Arrojo, M., Ramos-Rios, R., Carracedo, A., Palomo, T., Rodriguez-Jimenez, R., Cervilla, J., et al. (2016). Replication of previous genome-wide association studies of psychiatric diseases in a large schizophrenia case-control sample from Spain. Schizophr. Res. 159, 107–113.

Jiang, S., Zhou, D., Wang, Y.Y., Jia, P., Wan, C., Li, X., He, G., Cao, D., Jiang, X., Kendler, K.S., et al. (2020). Identification of de novo mutations in prenatal neurodevelopment-associated genes in schizophrenia in two Han Chinese patient-sibling family-based cohorts. Transl. Psychiatry 10, 307.

Kavanagh, D.H., Tansey, K.E., O’Donovan, M.C., and Owen, M.J. (2015). Schizophrenia genetics: emerging themes for a complex disorder. Mol. Psychiatry 20, 72–76.

Kelemen, K., Vrontou, E., Kruytner, S., Yu, J.Y., Kurtovic-Kozaric, A., and Dickson, B.J. (2012). Dopamine neurons modulate pheromone responses in Drosophila courtship learning. Nature 489, 145–149.

Khavari, B., and Cairns, M.J. (2020). Epigenomic dysregulation in schizophrenia: in search of disease etiology and biomarkers. Cells 9, 1837.

Kim, D., Peresta, O., Trapnell, C., Pimentel, H., Kelley, R., and Levin, J.Z. (2015). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14, R36.

Lai, S.L., Awaski, T., Ito, K., and Lee, T. (2008). Clonal analysis of Drosophila antennal lobe neurons: diverse neuronal architectures in the lateral neuronblast lineage. Development 135, 2883–2893.

Lam, M., Chen, C.Y., Li, Z., Martin, A.R., Bryois, J., Ma, X., Gasspar, H., Ikeda, M., Benjamin, B.M., Brown, B.C., et al. (2019). Comparative genetic architectures of schizophrenia in East Asian and European populations. Nat. Genet. 51, 1670–1678.

Lamberti, J.S. (2007). Understanding and preventing criminal recidivism among adults with psychiatric disorders. Psychiatr. Serv. 58, 773–781.

Langmead, B., and Saltzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357–359.

Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25, 1754–1760.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., and Genome Project Data Processing. S. (2009). The sequence alignment/map format and SAMtools. Bioinformatics 25, 2078–2079.

Li, J., Wilkinson, B., Clementel, V.A., Hou, J., O’dell, T.J., and Coba, M.P. (2016). Long-term potentiation modulates synaptic phosphorylation networks and reshapes the postsynaptic interactome. Sci. Signal. 9, r88.

Li, Z., Chen, J., Yu, H., He, L., Xu, Y., Zhang, D., Yi, Q., Li, C., Li, X., Shen, J., et al. (2017). Genome-wide association analysis identifies 30 new susceptibility loci for schizophrenia. Nat. Genet. 49, 1576–1583.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550.

McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M., and Depristo, M.A. (2010). The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 20, 1297–1303.

Monti, J.M., and Monti, D. (2004). Sleep in schizophrenia patients and the effects of antipsychotic drugs. Sleep Med. Rev. 8, 133–148.

Mosca, T.J., Hong, W., Dani, V.S., Favaloro, V., and Loo, L. (2012). Trans-synaptic Teneurin signalling in neuromuscular synapse organization and target choice. Nature 484, 237–241.

Nabavi, S., Fox, R., Proulx, C.D., Lin, J.Y., Tien, R.Y., and Malinow, R. (2014). Engineering a memory with LTD and LTP. Nature 511, 348–352.

Patel, K.R., Cherian, J., Gohil, K., and Atkinson, D. (2014). Schizophrenia: overview and treatment options. P T 39, 638–645.

Psychiatric GWAS Consortium Bipolar Disorder Working Group (2011). Large-scale genome-wide association analysis of bipolar disorder identifies a new susceptibility locus near ODZ4. Nat. Genet. 43, 977–983.

Robinson, J.T., Thorvaldsdottir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., and Mesirov, J.P. (2011). Integrative genomics viewer. Nat. Biotechnol. 29, 24–26.

Sanders, J., and Gill, M. (2007). Unravelling the genome: a review of molecular genetic research in schizophrenia. Ir. J. Med. Sci. 176, 5–9.

Siegel, R.W., and Hall, J.C. (1979). Conditioned emotional responses in Drosophila courtship learning. Nature 284, 1837.
Swicki, K.K., Riccio, P., Ladewski, L., Marcillac, F., Dartevelle, L., Cross, S.A., and Ferveur, J.F. (2005). The role of cuticular pheromones in courtship conditioning of Drosophila males. Learn Mem. 12, 636–645.

Smeland, O.B., Frei, O., Dale, A.M., and Andreassen, O.A. (2020). The polygenic architecture of schizophrenia - rethinking pathogenesis and nosology. Nat. Rev. Neurol. 16, 366–379.

Song, Q., Feng, G., Huang, Z., Chen, X., Chen, Z., and Ping, Y. (2017). Aberrant axonal arborization of PDF neurons induced by abeta42-mediated JNK activation underlies sleep disturbance in an Alzheimer’s model. Mol. Neurobiol. 63, 636–645.

Spada, J., Scholz, M., Kirsten, H., Hensch, T., Horn, K., Jawinski, P., Ulke, C., Burkhardt, R., Wirkner, K., Loeffler, M., et al. (2016). Genome-wide association analysis of actigraphic sleep phenotypes in the LIFE Adult Study. J. Sleep Res. 25, 690–701.

Sullivan, P.F., Daly, M.J., and O’donovan, M. (2012). Genetic architectures of psychiatric disorders: the emerging picture and its implications. Nat. Rev. Genet. 13, 537–551.

Suzuki, N., Suzuki, K., Kosaki, K., Doyle, A.D., De Vega, S., Yoshizaki, K., Akazawa, C., and Hirase, T. (2012). Teneurin-4 is a novel regulator of oligodendrocyte differentiation and myelination of small-diameter axons in the CNS. J. Neurosci. 32, 11586–11599.

Suzuki, N., Numakawa, T., Chou, J., De Vega, S., Mizuniwa, C., Sekimoto, K., Adachi, N., Kunugi, H., and Akazawa, C. (2014). Teneurin-4 promotes cellular protrusion formation and neurite outgrowth through focal adhesion kinase signaling. FASEB J. 28, 1386–1397.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L., and Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat. Protoc. 7, 562–578.

Tucker, R.P., and Chiquet-Ehrismann, R. (2006). Teneurins: a conserved family of transmembrane proteins involved in intercellular signaling during development. Dev. Biol. 290, 237–245.

Tucker, R.P., Martin, D., Kos, R., and Chiquet-Ehrismann, R. (2000). The expression of teneurin-4 in the avian embryo. Mech. Dev. 98, 187–191.

Ukkola-Vuoti, L., Torniainen-Holm, M., Ortega-Sanz, A., Sinha, V., Tsuilo-Henriksson, A., Paunio, T., Lonnqvist, J., Suvisaari, J., and Hennah, W. (2019). Gene expression changes related to immune processes associate with cognitive endophenotypes of schizophrenia. Prog. Neuropsychopharmacol. Biol. Psychiatry 88, 159–167.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., and Rozen, S.G. (2012). Primer3–new capabilities and interfaces. Nucleic Acids Res. 40, e115.

Wang, K., Li, M., and Hakonarson, H. (2010). ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 38, e164.

Wang, L., and Anderson, D.J. (2010). Identification of an aggression-promoting pheromone and its receptor neurons in Drosophila. Nature 463, 227–231.

Wu, H., Zhou, D.Z., Berki, D., Geirg, A., Zou, W.B., Sun, X.T., Hu, L.H., Zhao, Z.H., Zhao, A.J., He, L., et al. (2017). No significant enrichment of rare functionally defective CPA1 variants in a large Chinese idiopathic chronic pancreatitis cohort. Hum. Mutat. 38, 1599–1599.

Wulff, K., Gatti, S., Wettstein, J.G., and Foster, R.G. (2010). Sleep and circadian rhythm disruption in psychiatric and neurodegenerative disease. Nat. Rev. Neurosci. 11, 589–599.

Xu, B., Roos, J.L., Dexheimer, P., Boone, B., Plummer, B., Levy, S., Gogos, J.A., and Karayiorgou, M. (2011). Exome sequencing supports a de novo mutational paradigm for schizophrenia. Nat. Genet. 43, 864–868.

Xue, C.B., Xu, Z.H., Zhu, J., Wu, Y., Zhuang, X.H., Chen, Q.L., Wu, C.R., Hu, J.T., Zhou, H.S., Xie, W.H., et al. (2018). Exome sequencing identifies TENM4 as a novel candidate gene for schizophrenia in the SCZD2 locus at 11q14-21. Front. Genet. 9, 725.

Yan, Y.P., Xu, C.Y., Gu, L.Y., Zhang, B., Shen, T., Gao, T., Tian, J., Pu, J.L., Yin, X.Z., Zhang, B.R., and Zhao, G.H. (2020). Genetic testing of FUS, HTR2A, and TENM4 genes in Chinese patients with essential tremor. CNS Neurosci. Ther. 26, 837–841.

Zhou, C., Yao, Y., and Rao, Y. (2008). A subset of octopaminergic neurons are important for Drosophila aggression. Nat. Neurosci. 11, 1059–1067.

Zhou, X.H., Brandau, O., Feng, K., Oohashi, T., Ninomiya, Y., Rauch, U., and Fassler, R. (2003). The murine Ten-m/Odz genes show distinct but overlapping expression patterns during development and in adult brain. Gene Expr. Patterns 3, 397–405.
STAR★METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Biological samples** |        |            |
| Human blood | SJTU BIO-X psychiatric sample bank | http://www.bio-x.cn/ |
| **Deposited data** |        |            |
| Raw data of Target sequencing | This paper | Mendeley Data, V2, https://doi.org/10.17632/6w5zm6n68h.2. |
| mRNA sequencing analyzed data | This paper | Mendeley Data, V1, https://doi.org/10.17632/hy3bh2z8x8.1 |
| **Experimental models: Organisms/strains** |        |            |
| D. melanogaster: elav-GAL4 | Bloomington Drosophila Stock Center | flybase_FBst0000458 |
| D. melanogaster: Mz19-GAL4 | Bloomington Drosophila Stock Center | flybase_FBal0155865 |
| D. melanogaster: Mz699-GAL4 | Bloomington Drosophila Stock Center | flybase_FBal0066073 |
| D. melanogaster: UAS-Ten-m | Bloomington Drosophila Stock Center | flybase_FBst0041569 |
| D. melanogaster: UAS-Ten-m-RNAi | Bloomington Drosophila Stock Center | flybase_FBtp0052660 |
| D. melanogaster: w,Cyo/sco,MKRS/Tb | Kyoto Stock Center | flybase_FBst0305835 |
| D. melanogaster: LN1-GAL4 | Kyoto Stock Center | flybase_FBst0302813 |
| **Oligonucleotides** |        |            |
| Primers for TENM4, see supplemental information | This paper | N/A |
| Such as: GACCCGTGTGT GGAATGGA |
| **Software and algorithms** |        |            |
| Burrows-Wheeler Aligner (BWA) | Li and Durbin, 2009 | http://bio-bwa.sourceforge.net/ |
| Samtools | Li et al. (2009) | http://samtools.sourceforge.net/ |
| Genome Analysis Toolkit (GATK) | McKenna et al. (2010) | http://www.broadinstitute.org/gsa/wiki/index.php/The_Genome_Analysis_Toolkit |
| ANNOVAR | Wang et al. (2010) | https://annovar.openbioinformatics.org/en/latest/ |
| IGV | Robinson et al. (2011) | http://www.igv.org/ |
| Trimmomatic | Bolger et al. (2014) | http://www.usadellab.org/cms/index.php?page=trimmomatic |
| TopHat2 | Kim et al. (2013) | http://ccb.jhu.edu/software/tophat/manual.shtml |
| Cufflinks | Trapnell et al. (2012) | http://cole-trapnell-lab.github.io/cufflinks/ |
| Bowtie2 | Langmead and Salzberg (2012) | http://bowtie-bio.sourceforge.net/bowtie2/index.shtml |
| HTSeq | Anders et al. (2015) | https://htseq.readthedocs.io/en/master/ |
| DESeq2 | Love et al. (2014) | http://bioconductor.org/packages/release/bioc/html/DESeq2.html |

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Daizhan Zhou (zhoudaizhan@sjtu.edu.cn).
Materials availability
Materials used or generated in this study will be available upon reasonable request, and a material transfer agreement may be required.

Data and code availability
The target sequencing data of TENM4 and the RNA sequencing data have been deposited at Mendeley Data and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Patients and samples
In this study, all individuals with SCZ were interviewed by at least two independent psychiatrists. Diagnoses were made strictly according to the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV criteria, and patients with other neurological, mental, or psychiatric disorders, or with history of drug use were excluded. All participants provided written informed consent. Approval for our study was received from the Ethics Committee of Human Genetic Resources of Shanghai Jiao Tong University and local hospitals.

For the family-based sequencing study, 68 SCZ families with no less than 3 patients each were selected, and 351 samples were collected for targeted sequencing. There are 183 males and 168 females, and the average age was 39. All blood samples were collected during 1998-2005 in Shanghai, Shandong, Hebei, Anhui, Gansu, Heilongjiang and Guangxi provinces in China.

Fly lines
The fly lines used can be found in Table S1. Other genotypes were obtained by fly crossing. All procedures were approved by the Ethics Committee of Animal Research of Shanghai Jiao Tong University.

The larvae and adults were kept on a standard sugar-yeast-cornmeal medium under a 12-hour light/dark (LD) cycle at 25°C with 60% humidity. A list of fly lines used can be found in Table S1. Two genotypes of fly stocks were obtained by crossing other genotypes, elav>Ten-m and elav>Ten-m-RNAi. We used balanced lethal genotype flies: w;Cyo/sco;MKRS/Tb. The genotype includes four types of lethal alleles, which cause the death of the organism that carries them. To obtain elav>Ten-m flies, firstly, UAS-Ten-m flies were crossed with w;Cyo/sco;MKRS/Tb flies. We selected presenting Cyo and MKRS phenotypes of male flies from the offspring to cross with female elav;+;+ flies, then selected elav;Cyo/+;UAS-Ten-m/+ males. Simultaneously, female elav;+;+ flies were crossed with w;Cyo/sco;MKRS/Tb flies, and we selected presenting Cyo and MKRS phenotypes of male flies from the offspring to cross with female elav;+;+ flies, then selected elav;Cyo/+;MKRS/+ females to cross with the elav;Cyo/+;UAS-Ten-m/+ males. Finally, we obtained elav>Ten-m flies. The same methods were used to obtain elav>Ten-m-RNAi flies. Other fly genotypes were obtained by crossing parental flies. More than five generations with the w1118 strain outcrossing were performed to standardize the background.

In crossing experiment, we used 20 mature females and 10 mature males. In courtship conditioning assay, we used 10 males and 10 females which were selected at the eclosion stage. In aggression assay, we used only males which were kept separately for 7 days after eclosion stage. In sleep and activity assay, we used only virgin females which were collected immediately after eclosion stage.

METHOD DETAILS

Targeted sequencing of TENM4
Primer3 was used to design 70 target-specific primer pairs for all exons and exon-intron boundaries of TENM4 (Untergasser et al., 2012). The primers were synthesized with common adapter sequences at their 5’ ends as previously described (Forshew et al., 2012). All 70 primer pairs were divided into 6 multiplex...
primer pools. The library construction was performed essentially as previously described, including pre-amplification of the tagged gene amplicons, generation of a barcoded DNA library, quantification and clean-up (Forshew et al., 2012). The final DNA libraries were determined by Qubit 2100 (Thermo Scientific, USA) and Bioanalyzer (Illumina, USA) and then sequenced on the Illumina HiSeq X-Ten System (Illumina, USA) (Wu et al., 2017).

The targeted next-generation sequencing data were filtered by quality analysis and PCR primers and splices, and the paired 150 bp-long reads were mapped to hg19 human reference genome using the Burrows-Wheeler Aligner (BWA) software (Li and Durbin, 2009). Samtools was used for sequence sorting, gene location labeling, and format transformation (Li et al., 2009). Genome Analysis Toolkit (GATK) was used to identify mutation, filter and select comment to generate a VCF file (McKenna et al., 2010). All variants were annotated by ANNOVAR software (2019Oct24) (Wang et al., 2010), and the potential effects on the protein function were predicted using 9 software (SIFT, PolyPhen2, LRT, MutationTaster, MutationAssessor, FATHMM, PROVEAN, MetaSVM, and MetaLR). All functional mutations were visually confirmed one-by-one using IGV software (v2.8.6) (Robinson et al., 2011).

**Courtship conditioning assay**

A courtship conditioning assay was used to test courtship behavior and learning memory in *Drosophila*. Males and females were selected at the eclosion stage and raised in *Drosophila* standard culture medium. Male flies were kept separately and females were kept in groups of 10-15 flies. Male flies were divided into naive group and trained group. Male flies in trained groups aged up to 4-7 days after eclosion were solely paired with two mated females 1h for training, then the mated females were removed for half an hour before the test. Individual males from the control or trained groups were placed into the test chamber, 15 mm in diameter by 7 mm deep, and paired with two mated females. The period of experiments lasted for 10 minutes and were recorded by a video camera. The courtship index (CI) was calculated as the percentage of male courtship duration within 10 minutes. The learning index (LI) was calculated using the mean CIs, LI = (CI naive – CI trained)/CI naive (Keleman et al., 2012). As the distributions of LIs are different between groups, LIs were used for the permutation test with 100,000 random permutations to compare between groups, and the data were processed with R software. One-tail testing against the null hypothesis was used in the comparison. The Mann–Whitney U test was used for CIs.

**Aggression assay**

Pairs of male flies were introduced into a fighting chamber, which was 13 mm in diameter by 7 mm in height. We used 20 mm x 20 mm glass slides to cover the chamber. Fly behaviors were observed and recorded for 15 minutes (Zhou et al., 2008). The aggressive behaviors of male flies were evaluated in two aspects: latency and frequency. Latency is the duration between putting experimental flies into the chambers and the start of the first agonistic encounter while frequency is the number of aggressive behaviors of male flies, such as lunges, within 15 minutes. (Chen et al., 2002).

**Sleep and activity assay**

All experimental flies were virgin females collected immediately after eclosion stage; 15 flies of each genotype were required for parallel experiments. The sleep and activity of the experimental flies were monitored by the *Drosophila* Activity Monitor (DAM) system (TriKinetics, USA). The selected flies were raised in standard food vials until they were monitored, then they were moved into 65 mm x 5 mm glass tubes (Wuhan Yihong, China) containing solely 5% sucrose (Sangon Biotech, China) and 2% agar (Sangon Biotech, China). Subsequently, glass tubes with experimental flies were placed in the DAM system which was set in 12-hour LD conditions for the test. LD conditions were light-on at 9 am and light-off at 9 pm. Data collection started after the flies were acclimated to the environment for 3 to 5 days (Song et al., 2017). Data records of experimental activities were binned in one-minute periods in LD for three days. Flies were regarded to be sleeping when they were inactive for at least 5 minutes. Flies were implied to have died when their whole period of activity was less than 10 minutes, and the data were excluded. Flies’ sleep duration and parameters related to sleep behavior were analyzed by R software. Conventional sleep plots were portrayed by calculating the average amount of sleep duration within 30 minutes of experimental flies.
RNA extraction and sequencing

The flies were placed in 1 mL TRIzol (Invitrogen, USA), and we used forceps to rapidly break down the brains of the adult flies rapidly. Almost 20 heads of each genotype were saved in a 1.5 mL microcentrifuge tube with 200 μL of TRIzol. There were 3-4 replicates for each genotype. RNA extraction and purification were performed using TRIzol. The RNA was eluted with nuclease-free water, then a NanoDrop 2000 (Thermo Scientific, USA) was used to evaluate of RNA purity and concentration.

RNA-sequencing was performed on 3-4 replicates of RNA samples from each of elav-GAL4, elav>Ten-m-RNAi, and elav>Ten-m groups. The sample libraries were prepared according to the standard process of the TruSeq RNA Sample Prep Kit v2 (Illumina, USA). The final libraries were determined by Qubit 2100 (ThermoScientific, USA) and Bioanalyzer (Illumina, USA) and paired-end sequencing was performed on an Illumina HiSeq X-Ten System (Illumina, USA), with a read length of 2 × 150 bp.

Adapters and low-quality reads were removed from the raw data using Trimmomatic (v0.36) (Bolger et al., 2014). TopHat (v2.1.1) was used to align the RNA-seq reads to a reference genome (BDGP5.25) with the short-read aligner Bowtie2 (v2.3.4) (Kim et al., 2013) (Langmead and Salzberg, 2012). Cufflinks (v2.2.1) was used to analyze the BAM files, including assembling the transcripts from the aligned reads and estimating their abundances using a Fragments Per Kilobase of transcript per Million mapped reads (FPKM)-based algorithm (Trapnell et al., 2012). HTSeq (v0.10) was used to generate the read count files for each gene (Anders et al., 2015). Then, DESeq2 (v1.36.0) was used to identify DEGs, which were selected with log2 (fold change) >1 or log2 (fold change) <-1 and with statistical significance (adjusted p-value <0.05) (Love et al., 2014). GO and KEGG pathway enrichment analysis of DEGs was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) functional annotation tool (v6.8).

QUANTIFICATION AND STATISTICAL ANALYSIS

In courtship conditioning assay, the courtship index (CI) was calculated as the percentage of male courtship duration within 10 minutes. The learning index (LI) was calculated using the mean CIs, LI = [CI naive – CI trained]/CI naive (Keleman et al., 2012). As the distributions of LIs are different between groups, LIs were used for the permutation test with 100,000 random permutations to compare between groups, and the data were processed with R software. One-tail testing against the null hypothesis was used in the comparison. The Mann–Whitney U test was used for CIs.

In aggression assay and sleep assay, all behavior data analyses were performed using Sigma Plot (v14.0) and SPSS (v22.0). For multiple comparisons, one-way ANOVA followed by post hoc Tukey were used, except for the comparison of nonparametrically distributed data where the Kruskal–Wallis test was used.