RBFOX1 and working memory: from genome to transcriptome revealed post-transcriptional mechanism separate from ADHD

Yuanxian Zhong, Na Zhang, Feng Zhao, Suhua Chang, Wei Chen, Qingjiu Cao, Li Sun, Yufeng Wang, Zhiyuan Gong, Lin Lu, Dong Liu, Li Yang

PII: S2667-1743(22)00099-4
DOI: https://doi.org/10.1016/j.bpsgos.2022.08.006
Reference: BPSGOS 167

To appear in: Biological Psychiatry Global Open Science

Received Date: 27 May 2022
Revised Date: 9 August 2022
Accepted Date: 12 August 2022

Please cite this article as: Zhong Y., Zhang N., Zhao F., Chang S., Chen W., Cao Q., Sun L., Wang Y., Gong Z., Lu L., Liu D. & Yang L., RBFOX1 and working memory: from genome to transcriptome revealed post-transcriptional mechanism separate from ADHD, Biological Psychiatry Global Open Science (2022), doi: https://doi.org/10.1016/j.bpsgos.2022.08.006.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2022 Published by Elsevier Inc. on behalf of Society of Biological Psychiatry.
**RBFOX1** and working memory: from genome to transcriptome revealed post-transcriptional mechanism separate from ADHD

Yuanxin Zhong\(^1,6\), Na Zhang\(^2,3,6,\#\), Feng Zhao\(^2,6,\#\), Suhua Chang\(^1\), Wei Chen\(^1\), Qingjiu Cao\(^1\), Li Sun\(^1\), Yufeng Wang\(^1\), Zhiyuan Gong\(^3\), Lin Lu\(^1,4,\,*\), Dong Liu\(^2,\,*\), Li Yang\(^1,\,*\)

1 Peking University Sixth Hospital, Peking University Institute of Mental Health, NHC Key Laboratory of Mental Health (Peking University), National Clinical Research Center for Mental Disorders (Peking University Sixth Hospital), Beijing, 100191, China.
2 Department of Biology, School of Life Science, Southern University of Science and Technology, Shenzhen, 518055, China
3 Department of Biological Science, National University of Singapore, Singapore
4 Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100191, China; International Data Group/McGovern Institute for Brain Research at Peking University, Peking University, Beijing 100191, China.

\#These authors contribute equally.
*Correspondence:
Li Yang
Peking University Sixth Hospital, Huayuan Bei Road 51, Haidian District, Beijing, 100191, China. Tel: +86-13651220707. E-mail: yangli_pkuimh@bjmu.edu.cn
and
Dong Liu
Department of Biology, School of Life Sciences, Southern University of Science and Technology, Shenzhen, 518055, China. Tel: 86-755-88018487. E-mail: liud@sustech.edu.cn
and
Lin Lu
Peking University Sixth Hospital, Huayuan Bei Road 51, Haidian District, Beijing, 100191, China. E-mail: linlu@bjmu.edu.cn

Running title: **RBFOX1** and working memory.
Abstract

BACKGROUND: Many psychiatric disorders share working memory (WM) impairment phenotype, yet the genetic causes remain unclear. Here, we generate the genetic profiles of WM deficits using attention-deficit/hyperactivity disorder (ADHD) samples and validate the results in the zebrafish models.

METHODS: We have employed two relatively large ADHD cohorts, 799 and 776 samples, respectively. WM impairment was characterized by the Rey Complex Figure Test. Firstly, association analyses were conducted at single variant, gene-based, and gene-set levels. Deeper insights into the biological mechanism were gained from further functional exploration by bioinformatic analyses and zebrafish models.

RESULTS: Genomic analyses have identified and replicated a locus with rs75885813 as the index SNP to be significantly associated with WM defects but not with ADHD. Functional feature exploration found these SNPs may regulate the expression level of RBFOX1 through the chromatin interaction. Further pathway enrichment analysis of potential associated SNPs revealed the involvement of post-transcription regulation that affects mRNA stability and/or alternative splicing. Zebrafish with functionally knocked-down or genome-edited rbfox1 exhibited WM impairment but no hyperactivity. Transcriptome profiling of rbfox1 defective zebrafish indicated that alternative exon usages of snap25a and exon-skipping of grin1 genes separately yet partially lead to reduced WM learning of larval zebrafish.
CONCLUSION: The locus with rs75885813 of RBFOX1 was identified to be associated with working memory. Rbfox1 regulates synaptic and long-term potentiation related genes to adjust WM at the post-transcriptional level.

Keywords: working memory, genetic basis, RBFOX1, zebrafish, transcription regulation, Transcriptome profile
**Introduction**

Working memory (WM) is the ability to maintain and manipulate information in the brain/CNS to guide goal-directed behaviors, requiring gene expression control. WM impairments are a common trait of many psychiatric disorders, especially attention deficit/hyperactivity disorder (ADHD) (1). With an estimated prevalence of 5.9% in school-age children and core symptoms of inattention, hyperactivity and impulsivity, ADHD children usually exhibit impairments in working memory (2). WM defects are the leading cause of academic failure in ADHD patients (3), yet the underlying genetic and neurobiological bases are still not fully understood.

To date, studies of the etiology of ADHD have identified only a few variants of candidate genes, largely due to the heterogeneous nature of clinical phenotypes (4). Endophenotypes, on the other hand, may play important roles in unveiling the psychopathological processes of most psychiatric disorders. In this sense, WM defect is a reliable and promising endophenotype that could combine various pathways, including neurotransmission systems, ion channels, transcription regulators, and neurodevelopmental genes (5-9). In neurotransmission system, presynaptic components, such as calcineurin, Dystrobrevin Binding Protein 1 (DTNBP1), dysbindin, Glutamate Decarboxylase 1 (GAD1) and Synaptosome Associated Protein 25 (SNAP25) (10-15); postsynaptic proteins, such as AKT Serine/Threonine Kinase 1 (AKT1), Glutamate Ionotropice Receptor NMDA Type Subunit 1 (GRIN1), Glutamate Ionotropice Receptor NMDA Type Subunit 2B (GRIN2B), Nitric Oxide Synthase 1
and Neuregulin 1 (NRG1) (16-20), their interacting factors, such as D-Amino Acid Oxidase (DAO), and Olfactomedin 3 (OLFM3) (21-23), and some transcriptional regulators, such as Pro-Apoptotic WT1 Regulator (PAWR), T-Box Transcription Factor 1 (TBX1), Zinc Finger Protein 804A (ZNF804A), DGCR8 Microprocessor Complex Subunit (DGCR8), and Cytoplasmic FMR1 Interacting Protein 1 (CYFIP1) (24, 25) are all associated with WM. Such a research strategy to pick up candidate genes related to WM defects has shed light on understanding that defects in the neurotransmission system contribute significantly to WM deficiency.

Visual working memory is crucial in processing visual information. Its deficiencies are linked to general dysfunction in cognition (26), and presented as one of the important symptoms in several psychiatric disorders (27, 28). Zebrafish, a kind of vertebrate, has high similarity in physiological structure, growth and development process with human beings, and has shown high conservatism in evolution, making it a hot model organism in the field of biomedical research in recent years (29, 30). Although the central nervous system of zebrafish is different from that of mammals, several key brain regions of zebrafish are homologous with that of mammals (31). The behavioral patterns of zebrafish and mammals are also quite similar. Zebrafish can show high-level behavior and neural integration, including memory, conditioned reflex and social behavior (32, 33). In this study, we performed genomic analyses of visual spatial WM in a relatively large number of ADHD children and validated the genetic variants significantly associated with WM abnormality. We further tested the affected gene in zebrafish,
taking advantage of this highly tractable model system (29, 30).


Methods and Materials

Subjects: discovery and replication cohorts

Two consecutive ADHD samples were recruited from our child psychiatric clinics, which included 1040 cases in discovery cohort and 1192 cases in replication cohort. Both cohorts were medication-free and aged between 6 and 16 years old. All cases met DSM-IV ADHD diagnostic criteria based on a semi-structured interview by senior child and adolescent psychiatrists using the Clinical Diagnostic Interview Scale (CDIS). The other inclusion criteria were as follows: full-scale intelligence quotient (FSIQ) ≥ 70, and both biological parents were of Han descent. Those comorbidities with major neurological or psychiatric disorders, such as schizophrenia, bipolar disorder, major depression disorder, pervasive development disorder and epilepsy were excluded. Individuals without WM measures (n = 200 and 342 respectively), age (n = 0 and 2 respectively) or IQ (n = 27 and 37 respectively) were excluded from genetic analyses (see the pipeline in Fig. S1). Majority of the cases were medication-naïve. If the child had been medicated, a wash-out period for at least 1 month was necessary before the recruitment. This work was approved by our Institute Review Board. Written informed consent was obtained from parents.

Visuospatial working memory task: Rey complex figure test Delayed component (REYD)

The subject was instructed to observe a complex figure designed by Rey (38), then was asked to draw the figure from memory onto a blank sheet of paper after 30s. After a 20-
min delay, the subject was asked to recall and draw the figure from memory again. The
test was scored according to the structure (0–6) and detail accuracy (0–36). The delayed
structure and detail scores were the primary variables of interest in this study for their
higher cognitive load. We conducted principal component analyses (PCAs) to extract
common features from delayed structure and detail accuracy to produce an index of
REY complex figure test Delayed component (REYD), which assess delayed working
memory.

Genomic and bioinformatics analyses of working memory in ADHD patients

DNA extraction and genotyping

Genomic DNA was extracted directly from peripheral blood sample of each subject.
For the first cohort, all participants were genotyped in our ADHD GWAS project using
Affymetrix 6.0 array. For the second one, genotyping was performed by Illumina
Infinium PsychArray. Genotypes were called by GENOME STUDIO calling algorithm
with the human reference genome (hg19). The same quality control steps were
performed. Individuals with per-individual autosomal heterozygosity S.D larger than
the mean, gender inconsistent with site reports, a per-individual call rate < 95%, and
the lower call rate in a pair of individuals with proportion identity by descent (IBD)
PI_HAT >0.185 were excluded (39). Furthermore, the relatedness between two cohorts
was checked (PI_HAT < 0.05). Then, the variants were filtered based on per-SNP call
rate < 95%, deviation from Hardy-Weinberg equilibrium with P < 0.001 or a minor
allele frequency (MAF) < 1% (39). After quality control, 1026 samples with 644166
SNPs and 1147 samples with 284176 SNPs remained for the two cohorts respectively (the pipeline shown in Fig. S12). We imputed non-genotyped SNPs of two cohorts using IMPUTE2, with 2186 phased haplotypes from the full 1000 Genomes Project Integrated Phase 3 Release (40) as the reference panel. We removed imputed SNPs with a squared correlation with the true genotypes $r^2 < 0.9$ or with minor allele frequency $< 0.01$. Finally, 6552994 and 5468003 SNPs were included for two cohorts respectively after imputation.

**Genetic Single-marker analyses**

We performed association analyses on single SNP for REYD using PLINK version 1.9 (41). By multi-dimensional scaling (MDS), no substantial population stratification was found. As WM is correlated with age, sex, and IQ (42, 43), we included them as parts of the covariates. Linear regression models were used with age, sex, IQ and the top 10 eigenvectors from the genetic principal component analysis as covariates for pruned SNPs ($r^2 = 0.2$) by EIGENSOFT 4.2 (44). When we performed phenotypic association analyses for ADHD (i.e. ADHD diagnosis and inattention, impulsivity, and hyperactivity measures based on CDIS), all covariates mentioned above were included except IQ. Bonferroni correction was used and $P < 5e^{-8}$ was regarded as whole-genome-wide significance. Significant loci were tested in the replication sample ($P_{corrected} < 0.05$). Meta-analyses were also implemented by PLINK (41). All reported $P$-values were two-sided.
Gene-based and Pathway enrichment analyses

We further conducted gene-based analyses for both REYD and ADHD by MAGMA (45). Then we performed pathway enrichment analyses using SNPs with $P < 10^{-4}$ from the meta-analyses as implemented in MAGMA (45) with a 35 Kb upstream and 10Kb downstream window around genes as the default setting. A total of 10185 Gene Ontology (GO) and 186 KEGG gene sets (obtained from MSigDB(46)) were included. Competitive $p$ values were computed and interpreted. MAGMA's built-in Bonferroni correction was used for multiple testing corrections.

Polygenic Risk Score

Then we performed polygenic analyses to detect shared genetic basis between working memory and ADHD symptoms. Based on the association analysis on the REYD of the discovery sample, we used PRSice (47) to perform the polygenic risk score analyses to select the most precise threshold for the $p$ value that predicted ADHD symptoms in both discovery and validation cohorts respectively, with step increased $p$-value thresholds $(0.000001, 0.00001, 0.0001, 0.001, 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, \text{and } 1)$. An empirical multiple testing correction implemented in PRSice was applied, which is based on a permutation procedure. The significance of the regression results was corrected by a permutation test with 10,000 replicates and alpha = 0.001 as suggested was used (47). We also explored whether genetic components of glutamate receptor (gluR) or long-term potentiation (LTP)-related pathways shared between working memory and ADHD symptoms. We included 236 and 75 genes that are
belonged to gluR and LTP-related pathways according to MSigDB (46) and performed the same PRS analyses as mentioned above.

Regulatory feature analyses and network construction

We obtained the regulatory features of the significant SNPs from rSNPBase3.0 (48) and HaploReg v4.1 (49), two user-friendly graphic interface web tools which integrate comprehensive information related to genomic regulation. We also searched the expression quantitative loci in the Genotype-Tissue Expression (GTEx) (50). The expression plot was generated using THE HUMAN PROTEIN ATLAS. Using STRING (51), the interacting genes network were constructed for RBFOX1 and those enriched genes from pathway analyses.

Functional analyses of rbfox1 in zebrafish

Zebrafish husbandry and care

Zebrafish were raised at 28 °C with a density of 8-10 fish/L and experimented with the established standards (52). The wild-type (WT) zebrafish used in this study was Oregon AB strain/line. All zebrafish experiments were conducted according to the guidelines approved by the Institutional Animal Care and Use Committee of Southern University of Science and Technology (SUSTech).

Zebrafish loss-of-function studies

Loss-of-function (knockdown or knockout) experiments conducted in present study
were achieved by Morpholino antisense oligo technique (Gene Tools Inc., Corvallis, OR), four guide RNAs/Cas9 technique, or Cas9 ribonucleoproteins (RNPs) technique. To obtain mutants of zebrafish rbfox1 to evaluate if the gene affects VSM, we employed synthetic crRNA:tracrRNA duplex guide RNAs and Cas9 (Hoshijima et al., 2019). Two target sequences in RRM of Rbfox1 were chosen and each created indels (found in each copy of the target locus of rbfox1) in over 80% injected embryos (Supplementary Fig. S4). Since most injected F0 embryos could be treated as true null mutants (Hoshijima et al., 2019), we used 5dpf injected larval zebrafish, and Morpholino anti-sense oligo-injected morphant to conduct behavior tests. In case of snap25a, four gRNAs (Supplementary Table S5, http://crispor.tefor.net/) were co-injected with Cas9 nuclease, and the resulted F0 fish larvae were called knock-out fish or snap25a 4gRNA fish.

Genotyping

Genomic DNA was extracted from 24 hpf zebrafish embryo(s) using TIANamp Genomic DNA Kit (TIANGEN). The aimed region(s) of the gene locus was amplified by gene-specific PCR primers, and the expected DNA fragment was purified by QIAquick Gel Extraction Kit (QIAGEN). The purified PCR product(s) was cloned for sequencing purpose (Sangon Biotech). The cloning primers sequence were listed in Supplementary Table S6.

Quantitative real-time PCR
Total RNA was extracted from 50 wild-type or mutant larvae at 5 dpf after fixing them in TRIzol reagent (Invitrogen). A total of 1 μg RNA was reverse-transcribed to the first strand of cDNA with the random primer using a cDNA synthesis kit (Promega). qRT-PCR primers for snap25a, grin1a, grin1b and β-actin gene were listed in Supplementary Table S7. qRT-PCR was performed in an ABI 7500 Real-Time PCR instrument with the SYBR green detection system, and results were normalized with β-actin expression using ΔΔCt method.

Transcriptome sequencing analysis

Total RNA was separately prepared from 50 larvae (5dpf) of WT or rbfox1 morphant and gone through the DNBSEQ platform, with the NCBI accession number, GCF_000002035.6_GRCz11 as the zebrafish genome reference. The sequencing data was filtered with SOAPnuke (v1.5.2), then the clean reads were mapped to genome using HISAT2 (v2.0.4). After aligning the clean reads to genes via Bowtie2 (v2.2.5), BGI created a database to include all annotated coding transcripts with actual number of reads/counts calculated by RSEM (v1.2.12). Based on Hypergeometric test and corrected by Q value with a rigorous threshold (Q value ≤ 0.05), respectively, the GO and KEGG enrichment analysis of differential gene expressions and significant levels of terms and pathways were obtained.

Zebrafish behavioral tests: working memory

Working memory can be represented by habituation in zebrafish. Short-term
habituation could be analyzed through repeated acoustic or visual stimulations. Free swimming 5 dpf zebrafish larvae were dispersed in a 96-well plate to sit in the observation chamber (DanioVision, Noldus). Response velocity to acoustic/vibrational or dark flash stimuli was detected and calculated by using EthoVision XT13 video-tracking software (Noldus). For acoustic habituation assay, 10 stimuli with the acoustic intensity of 90 dB were delivered with 1 sec inter-stimulus interval (ISI) as baseline, followed by 20 stimuli with 1 sec ISI. Acoustic habituation was indicated as: % acoustic habituation= \[1-(\text{velocity of stimuli 21-30)/ (response velocity of baseline})\] *100. For visual habituation assay, 4-block training protocol was performed without any break between blocks. Each block consisted 120 dark flash with a 15 sec ISI, and each dark flash lasted for 1 sec. Habituation was indicated as: % Visual Habituation= \([1-\text{Block4/Block1}] *100\) (Supplementary Fig. S7).

Statistical analyses of zebrafish experiments

GraphPad Prism version 7.00 was used for statistics analysis of zebrafish experiments. For two-group comparison, one-tailed t test with 95% confidence interval was used. For three or more groups comparison, One-way ANOVA with 95% confidence interval was used.
Results

Genome-wide association of working memory and the relationship with ADHD symptoms at the single-variant and polygenic levels

We first performed a genome-wide analyses of visuospatial WM in ADHD children. A total of 799 and 776 cases, were ultimately qualified for the discovery and replication stages, respectively (MDS plots are available in Supplementary Material). The demographic description, Intelligence Quotients, and cognitive phenotype data for the discovery and replication samples are presented in Supplementary Table S1.

We used delayed component (REYD) which was the first PC axis score accounting for 94.1% and 91.1% variance in the discovery and validation cohorts respectively, in the following analyses. In the discovery stage we identified five significant SNPs in whole genome-wide association analysis, among which rs75885813 was ranked on top ($P_{corrected} = 3.83 \times 10^{-9}$). The other four SNPs were in high linkage disequilibrium with rs75885813 ($r^2 > 0.9$, Table 1). Quantile-quantile (QQ) plot for SNP associations is presented in Supplementary Fig. S2. All five SNPs were located on 16p13.3 (Chr16:7120001-7160000, hg19) within the RBFOX1 gene (Fig. 1). They reached statistical significance in both the replication stage ($P_{corrected} < 0.05$) and meta-analyses ($P_{corrected} < 5 \times 10^{-9}$). However, associations between the RBFOX1 SNPs and core symptoms of ADHD (i.e., inattention, hyperactivity, impulsivity, and overall symptoms) were not statistically significant ($P_{corrected} > 0.05$, data available upon request), nor was the association for Polygenic Risk Score (PRSs) weighted by WM for ADHD patients.
Associated variants may affect transcriptional regulation

Given the strong association between rs75885813 (i.e., an intron variant of RBFOX1) and REYD, we further examined the regulatory features of this variant, as well as its interaction networks and co-expression genes. The chromatin state analyses showed that rs75885813 is in functional regulatory regions in various tissues indicated by the enhancer-specific H3K4me1 and promotor-specific H3K4m3 markers. It also alters the regulatory motifs of some transcription factors in the brain (Supplementary Table S3).

The Hi-C data suggested that a chromatin loop can form between the genomic region (16:7120001-7160000, hg19) where rs75885813 is located and the promoter of RBFOX1 (16:6040001-6080000, hg19), also suggesting a possible regulatory effect (Supplementary Fig. S3) on RBFOX1 transcriptional regulation. We further constructed a network to include its interacting partners and co-expressing genes. As shown in Supplementary Fig. S4, the functional protein interaction analyses indicated that most of them (8/11) are RNA-binding proteins and may play roles in RNA alternative splicing events.

Gene-based and pathway enrichment analyses

No gene achieved significance after multiple corrections for neither REYD nor ADHD.

We investigated subthreshold variants from the REYD association analysis using a pathway enrichment test. After removing gene sets that contained less than two genes
defined in the GO analyses, 773 gene sets were included. Sixteen GO pathways reached significance ($P_{\text{corrected}} < 0.05$, Fig. 2 and Table 2). All of these pathways are involved in post-transcription regulation, among which the mRNA metabolic process was ranked on top. The relationships of these significantly enriched pathways are presented in Supplementary Fig. S5. The extended interaction gene network showed the potentially interacted genes, many of which are overlapped with the risk genes for psychiatric disorders (Supplementary Fig. S6).

**Functional analyses of rbfox1 in zebrafish**

To test our hypothesis that a down regulation of Rbfox1 might lead to WM defect, we first established a measurement to monitor habituation learning behaviors in zebrafish larvae. We found that during acoustic/tapping stimulation with a total of 30 taps, larval zebrafish gradually reduced the extent of their startle responses (Supplementary Fig. S7A-B). After exposure to a massed/continued dark flash-training period, visual habituation was also evident (Supplementary Fig. S6C-D). Mutant-like larval zebrafish (5dpf), co-injected with chemically modified crRNAs/tracrRNAs and Cas9 protein against two sites of rbfox1 at the one-cell stage in wildtype (WT) fertilized eggs (Supplementary Fig. S8), exhibited a decrease in habituation (Fig. 3A-D). The knockdown of Rbfox1 function with anti-sense morpholino oligos that blocked either rbfox1 pre-RNA splicing or mRNA translation (ATG MO) resulted in similar habituation phenotypes (Fig. 3E-F), suggesting a relationship between Rbfox1 and the WM defect in zebrafish.
Posttranscriptional regulation of WM genes

To select zebrafish WM-related genes in an unbiased way, we profiled the transcriptome of *rbfox1* Splicing MO injected larvae at 5dpf. The GO and KEGG enrichment analyses indicated that the most significantly changed pathways included the long-term potentiation (LTP), which was tightly related to memory formation, and TRP channels, which had been reported to define the hippocampal synaptic transmission and working memory (Fig. 4A). The most affected cellular components included pre- and post-synapse connections (Fig. 4B). Gene expression of proteins in postsynaptic density, the presynaptic membrane and the ionotropic glutamate receptor complex were all significantly changed. We found that *grin1a* and *grin1b* were most abundantly expressed N-methyl-D-aspartate receptor genes in 5 dpf larvae, so were *snap25a* and *snap25b* that encode synaptosome-associated proteins (Supplementary Fig. S9).

Zebrafish *grin1* mutants exhibited a reduction of habituation (53) and MK-801 (NMDA receptor antagonist) treatment severely impacted larval zebrafish’s acoustic habituation (Supplementary Fig. S10A) also support the idea that these LTP genes are mainly responsible for a normal WM. Interestingly, in an attempt to learn how *grin1a* and *grin1b* were regulated in *rbfox1* mutants/morphants, we found that post-transcriptional exon 4-skipping events in processing pre-mRNAs of both genes (Supplementary Fig. S10B-S10D). Thus, abnormal Rbfox1-mediated alternative splicing of *grin1a* and *grin1b* might in part result in the WM defect of *rbfox1* mutant or morphant.

Among well-studied neurotransmission-related genes, functional protein association analyses revealed that SNAP25 is within a few closely related RNA processing
components networked with RBFOX1 (Supplementary Fig. S4). Zebrafish Snap25a is the most abundant SNAP 5dpf larval, responsible for the presynaptic neurotransmitter release (Supplementary Fig. S9A). Zebrafish snap25a is a homolog of mouse Snap25, which is known to have two alternative splicing variants: Snap25a and Snap25b that correspond to two zebrafish splicing variants, snap25a-202 and snap25a-201 transcripts (Supplementary Fig. S9D), respectively during 24-96hpf. Only 11 amino acids are different between Snap25a-201 and -202, and these amino acids are all due to the alternative usage of exon-201/exon-202. Apparently, snap25a-201 is predominantly expressed during embryogenesis and early larval stage (Supplementary Fig. S9C).

In rbfox1 ‘mutant’ and morphant, snap25a-201 transcripts were significantly decreased \((p<0.01)\) while snap25a-202 expression was significantly increased \((p<0.001)\) (Supplementary Fig. S9B). An Rbfox1 binding motif, (U)GCAUG is found in the exon 5b of rbfox1 and the overall reduced snap25a expression level also indicate that an imbalanced expression of two splicing variants most likely changes zebrafish VSM or acoustic/vibration-mediated memory.

To obtain zebrafish snap25a mutants, we employed another gene knockout method that produces null F0 zebrafish with high probability (Wu et al., 2018). Using a mix of four sgRNAs against snap25a and Cas9 to inject the yolk of fertilized eggs, we evaluated learned memory of snap25a ‘mutant’ at 5dpf and discovered the significantly reduced acoustic/vibration and visual habituation (Fig. 5A), similar to those found in rbfox1
‘mutant’ and morphant that were responsible for the impaired short-term memory.

**Discussion**

The past few decades have witnessed studies of many candidate genes that are related to the genetic vulnerability of WM impairments. However, most picked candidate genes were limited to the hypotheses. Our genome-wide single-variant and polygenic analyses of WM defects in ADHD children identified five *RBFOX1* intronic variants/SNPs that may affect RBFOX1 level in the brain. Zebrafish loss-of-function of *rbfox1* experiments showed both visual and auditory habituation defects in larval fish. When *rbfox1* was knocked down or out, *snap25a, grin1a* and *grin1b* were found down-regulated and mis-spliced. To our knowledge, this is the first study that uncovers *RBFOX1/rbfox1* regulates WM post-transcriptionally.

Our pathway enrichment analysis has also directed to transcription regulation that linked to WM in accordance with previous candidate gene, gene-set, and polygenic studies (24, 25). Some of the regulators are important for the synthesis of proteins required during neurobiological process of WM and mRNA metabolic process. If the expression of more *RBFOX1/rbfox1*-regulated LTP/synaptic genes is found to be controlled at the pre-RNA splicing and mRNA stability stages, the fine and quick regulation of neural activities such as habituation, startle response and dependent transcription could be economically and efficiently achieved.
RBFOX1/rbfox1 is a pleiotropic gene that has been associated with seven specific psychiatric disorders (54). The most significant SNP, rs75885813 SNP was previously associated with Alzheimer’s disease (AD) related phenotypes in AD patients whose characteristic traits include WM deficits (55, 56). The association between RBFOX1 rs7193263 and major depression is also evident in a GWAS ($p = 9.73\text{e}{-9}$) (57). RBFOX1 and WM were never directly associated, yet in a genome-wide gene expression study, Rbfox1 was linked to RNA processing after memory retrieval (58). However, in the present study, no association is found between hyperactivity, an ADHD core symptom, and RBFOX1/rbfox1 or snap25a, either in the population genetic analyses or animal experiments. We noticed that the association of RBFOX1 was mainly driven by major depression (MD) sample (GWAS p-value: $9.73\text{e}{-9}$) among 7 psychiatric disorders (57). The $p$-value for the association of ADHD was only 0.0065 (59). Thus, the effect of RBFOX1 on WM is potentially not only confined to ADHD. The present study further implies that RBFOX1 may be the common susceptibility gene of psychiatric disorders via regulating WM. More association studies of RBFOX1-regulated neurotransmission system genes and WM may be needed to strengthen our view.

In the functional protein association networks of RBFOX1, SNAP25 attracted our attention. A previous study indicated that SNAP25 is related to WM deficits in ADHD patients (14). In our study, we found a reduction of snap25a and snap25b expression in zebrafish rbfox1 morphant, which showed WM defect but no hyperactivity. The decrease of snap25a expression was more pronounced than that of snap25b. Zebrafish
*snap25a* is homologous to mouse *Snap25*, which is known to have two alternative splicing variants: *Snap25a* (exon 5a, expressed late, corresponding to zebrafish *snap25a*-202) and *Snap25b* (exon 5b, expressed early, corresponding to zebrafish *snap25a*-201). Mouse *Snap25a* and *Snap25b* are expressed during embryonic and early postnatal development respectively. Snap25 differentially affects interactions with other soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) and SNARE-interacting proteins (60, 61). The *Rbfox1*−/− mouse brain exhibited normal mRNA level, but decreased *Snap25b* transcripts and increased *Snap25a* transcripts (62), consistent with our findings of a decreased *snap25a*-201/25a-202 ratio (Fig. 5B). It is worth exploring whether reversing the *snap25a*-201/25a-202 ratio or simply increasing *snap25a*-201 mRNA can rescue the WM defect in *rbfox1* mutants in the future study.

However, there were some limitations in this study. First, due to the limited sample sizes, the current study is underpowered to detect other WM-related variants and unveil its genetic structure more comprehensively. Enlarging the sample size and cooperation with other groups could strengthen the statistical power.

In summary, our present findings have revealed possible causal pathways of post-transcription regulators that trigger WM deficits and alternative splicing events, mediated by Rbfox1, control SNARE and LTP genes to affect WM.
Acknowledgements

This work was supported by grants from the Major State Basic Research Development Program of China (973 Program, 2014CB846100), National Key R&D Program of China (2016YFC1306103 and 2018YFA0801006) and National Natural Science Foundation of China (grant numbers: 81671358, 31771618, 81761128035, 81873803).
Disclosures
The authors report no biomedical financial interests or potential conflicts of interest.

Contributions
We thank all the patients for participating in this study. YXZ and LY conceived the study. YXZ conducted the population genetic analysis and wrote the first draft of the manuscript. NZ and FZ generated the zebrafish models and performed the statistical analyses of the animal experiments. WC and SHC helped with the data analyses. WC and LS helped with data collection. WFY helped with data collection and provided intellectual input. DL conducted the animal experiments. DL, LY, and LL provided intellectual input, edited the manuscript, and approved the final version.

Corresponding authors
Correspondence to Li Yang.

Data availability
The summary statistics for the GWAS analyses will be publicly available after publication of this study. Data that support the findings of this study are available from the corresponding author upon request from qualified researchers for reasonable, non-commercial research purposes. A data application and an agreement may be required.
References


(2008): Knock-in mice lacking the PDZ-ligand motif of mGluR7a show impaired
PKC-dependent autoinhibition of glutamate release, spatial working memory deficits,
and increased susceptibility to pentylenetetrazol. *The Journal of neuroscience: the

9. Eagle AL, Gajewski PA, Robison AJ (2016): Role of hippocampal activity-

(2007): Allelic variation in GAD1 (GAD67) is associated with schizophrenia and

Association of the DTNBP1 genotype with cognition and personality traits in healthy

Cognitive dysfunction and glutamate reuptake: effect of EAAT2 polymorphism in

13. Jentsch JD, Trantham-Davidson H, Jairl C, Tinsley M, Cannon TD, Lavin A
(2009): Dysbindin modulates prefrontal cortical glutamatergic circuits and working
memory function in mice. *Neuropsychopharmacology: official publication of the
American College of Neuropsychopharmacology*. 34:2601-2608.

(SNARE) genes and their interactions contribute to the susceptibility and working
memory of attention-deficit/hyperactivity disorder in males. *Progress in neuro-


42. Eve M Valera, Ph.D., Ariel Brown, Ph.D., Joseph Biederman, M.D., Stephen V. Faraone, Ph.D., Nikos Makris, M.D., Ph.D., Michael C. Monuteaux, Sc.D., et


50. GTEx Consortium (2013): The Genotype-Tissue Expression (GTEx) project.


Figure 1. Regional plot of significant locus for genome-wide association of REYD.

The grey horizontal line represents the threshold for genome-wide significant association ($P = 5 \times 10^{-8}$). rs75885813, $P_{corrected} = 3.83e-9$, was the top-ranked SNP. The other four SNPs significant were in high linkage disequilibrium with rs75885813 ($r^2 > 0.8$). All significant SNPs were located within RBFOX1.

Figure 2. Bubble diagram of the pathway analysis of potential associated variants for REY complex figure test Delayed component (REYD). The $p$ value increased from top to bottom. All the pathways displayed in the figure were significant ($P_{corrected} < 0.05$). The color represents $p$ value, while the size represents the number of associated genes enriched in the particular pathway. REYD: REY complex figure test Delayed component.

Figure 3. rbfox1 is required for visual and acoustic habituation in larval zebrafish. (A) Schematic diagram of visual habituation. (B) Mean percentage of habituation during the visual habituation phase in the rbfox1 mutant. (C) Schematic diagram of acoustic habituation. (D) Mean percentage of habituation during the acoustic habituation phase in the rbfox1 mutant. (E) Mean percentage of habituation during the visual habituation phase in the rbfox1 MO injected fish. (F) Mean percentage of habituation during the acoustic habituation phase in the rbfox1 MO injected fish. The data are expressed as mean ± SE. $n = 48$ larval fish per group. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ (paired t-test).
Figure 4. Zebrafish *rbfox1* regulates both presynaptic and postsynaptic functions. (A) KEGG pathway enrichment of DEGs in *rbfox1* morphant. (B) GO enrichment of DEGs in *rbfox1* morphant. All zebrafish larvae were collected at 5 dpf, and then whole transcriptome deep sequencing was performed.

Figure 5. Posttranscriptional regulation of *snap25a* by Rbfox1. (A) Acoustic and visual habituation defects in *snap25a*-4sg mutant, tests performed on 5 dpf, n ≥48. (B) Changes of *snap25a* isoform ratios (exon5 alternative inclusion) in *rbfox1* mutant. The data are expressed as mean ± SE. n = 48 larval fish per group. ***p < 0.001, **p < 0.01, *p < 0.05 (unpaired t-test).
Table 1. Identified significant SNPs from genome-wide analysis of REYD in discovery and replication samples and meta-analyses.

<table>
<thead>
<tr>
<th>CHR</th>
<th>SNP</th>
<th>Pos</th>
<th>A1</th>
<th>LD with rs75885813</th>
<th>BETA discovery</th>
<th>SE discovery</th>
<th>STAT discovery</th>
<th>P discovery</th>
<th>BETA replication</th>
<th>SE replication</th>
<th>STAT replication</th>
<th>P replication</th>
<th>BETA</th>
<th>SE</th>
<th>STAT</th>
<th>P meta-analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>rs75885813</td>
<td>7141263</td>
<td>T</td>
<td>-</td>
<td>-0.669</td>
<td>0.112</td>
<td>-5.96</td>
<td>3.83e-09</td>
<td>0.130</td>
<td>0.12</td>
<td>5.90</td>
<td>0.034</td>
<td>0.034</td>
<td>6.04e-09</td>
<td>3.67e-09</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>rs114891671</td>
<td>7142203</td>
<td>C</td>
<td>0.98</td>
<td>-0.669</td>
<td>0.113</td>
<td>-5.90</td>
<td>5.56e-09</td>
<td>0.135</td>
<td>0.12</td>
<td>5.90</td>
<td>0.0094</td>
<td>0.0094</td>
<td>6.04e-10</td>
<td>6.04e-10</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>rs115712598</td>
<td>7142236</td>
<td>T</td>
<td>0.98</td>
<td>-0.669</td>
<td>0.113</td>
<td>-5.90</td>
<td>5.56e-09</td>
<td>0.135</td>
<td>0.12</td>
<td>5.90</td>
<td>0.0094</td>
<td>0.0094</td>
<td>6.04e-10</td>
<td>6.04e-10</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>rs116183707</td>
<td>7142287</td>
<td>A</td>
<td>0.98</td>
<td>-0.669</td>
<td>0.113</td>
<td>-5.90</td>
<td>5.56e-09</td>
<td>0.135</td>
<td>0.12</td>
<td>5.90</td>
<td>0.0094</td>
<td>0.0094</td>
<td>6.04e-10</td>
<td>6.04e-10</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>rs7205995</td>
<td>7140331</td>
<td>G</td>
<td>0.98</td>
<td>-0.667</td>
<td>0.113</td>
<td>-5.88</td>
<td>6.05e-09</td>
<td>0.135</td>
<td>0.12</td>
<td>5.90</td>
<td>0.0097</td>
<td>0.0097</td>
<td>6.69e-10</td>
<td>6.69e-10</td>
<td></td>
</tr>
</tbody>
</table>

Five whole-genome significant SNPs were identified which are in high linkage disequilibrium ($r^2 > 0.8$). SE, standard error; STAT, coefficient t-statistic.
Table 2. Enriched biological process by potential associated variants with working memory. The pathway enrichment analyses were conducted with SNPs with P < e^{-4} from the association analyses as implemented in MAGMA. GOBP, gene ontology biological processes; GOCC, gene ontology cellular components; GOMF, gene ontology molecular functions; NGENES/total, the number of genes enriched in the pathway and the total number of genes of the pathway; GENE list, the genes enriched in the pathway; BETA STD, beta standard; SE, standard error.

<table>
<thead>
<tr>
<th>FULL NAME</th>
<th>NGENES/TOTAL</th>
<th>GENE LIST</th>
<th>BETA</th>
<th>BETA STD</th>
<th>SE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0016071 GOBP MRNA METABOLIC PROCESS</td>
<td>5/879</td>
<td>RBFOX1, SMG6, TNFSF13, FXR2, RAVER1</td>
<td>0.697</td>
<td>0.144</td>
<td>0.0995</td>
<td>6.963E-10</td>
</tr>
<tr>
<td>GO:1903311 GOBP REGULATION OF MRNA METABOLIC PROCESS</td>
<td>3/334</td>
<td>RBFOX1, TNFSF13, FXR2</td>
<td>1.511</td>
<td>0.244</td>
<td>0.235</td>
<td>7.520E-09</td>
</tr>
<tr>
<td>GO:0098791 GOCC GOLGI APPARATUS SUBCOMPARTMENT</td>
<td>2/887</td>
<td>RBFOX1, CNGB1</td>
<td>1.454</td>
<td>0.193</td>
<td>0.230</td>
<td>1.189E-08</td>
</tr>
<tr>
<td>GO:0003729 GOMF MRNA BINDING</td>
<td>3/538</td>
<td>RBFOX1, EIF4A1, FXR2</td>
<td>1.416</td>
<td>0.229</td>
<td>0.231</td>
<td>2.538E-06</td>
</tr>
<tr>
<td>GO:0006397 GOBP MRNA PROCESSING</td>
<td>3/543</td>
<td>RBFOX1, FXR2, RAVER1</td>
<td>1.350</td>
<td>0.218</td>
<td>0.222</td>
<td>2.917E-08</td>
</tr>
<tr>
<td>GO:0000375 GOBP RNA SPlicing VIA TRANSESTERIFICATION REACTIONS</td>
<td>3/384</td>
<td>RBFOX1, FXR2, RAVER1</td>
<td>1.350</td>
<td>0.218</td>
<td>0.222</td>
<td>2.917E-08</td>
</tr>
<tr>
<td>GO:0051236 GOBP ESTABLISHMENT OF RNA LOCALIZATION</td>
<td>3/200</td>
<td>NUP205, RBFOX1, SMG6</td>
<td>0.590</td>
<td>0.0952</td>
<td>0.0979</td>
<td>3.882E-08</td>
</tr>
<tr>
<td>GO:0006403 GOBP RNA LOCALIZATION</td>
<td>3/233</td>
<td>NUP205, RBFOX1, SMG6</td>
<td>0.590</td>
<td>0.0952</td>
<td>0.0979</td>
<td>3.882E-08</td>
</tr>
<tr>
<td>GO:0000381 GOBP REGULATION OF ALTERNATIVE MRNA SPlicing VIA SPliceosome</td>
<td>2/57</td>
<td>RBFOX1, FXR2</td>
<td>1.370</td>
<td>0.181</td>
<td>0.228</td>
<td>4.009E-08</td>
</tr>
<tr>
<td>GO:0050684 GOBP REGULATION OF MRNA PROCESSING</td>
<td>2/139</td>
<td>RBFOX1, FXR2</td>
<td>1.370</td>
<td>0.181</td>
<td>0.228</td>
<td>4.009E-08</td>
</tr>
<tr>
<td>GO:0048024 GOBP REGULATION OF MRNA SPlicing VIA SPliceosome</td>
<td>2/100</td>
<td>RBFOX1, FXR2</td>
<td>1.370</td>
<td>0.181</td>
<td>0.228</td>
<td>4.009E-08</td>
</tr>
<tr>
<td>GO:0043484 GOBP REGULATION OF RNA SPlicing</td>
<td>2/144</td>
<td>RBFOX1, FXR2</td>
<td>1.370</td>
<td>0.181</td>
<td>0.228</td>
<td>4.009E-08</td>
</tr>
<tr>
<td>GO:0035770 GOCC RIBONUCLEOPROTEIN GRANULE</td>
<td>2/244</td>
<td>RBFOX1, FXR2</td>
<td>1.370</td>
<td>0.181</td>
<td>0.228</td>
<td>4.009E-08</td>
</tr>
<tr>
<td>GO:0015931 GOBP NUCLEOBASE CONTAINING COMPOUND TRANSPORT</td>
<td>4/252</td>
<td>NUP205, SLC28A1, RBFOX1, SMG6</td>
<td>0.511</td>
<td>0.0948</td>
<td>0.0946</td>
<td>4.537E-07</td>
</tr>
<tr>
<td>GO:0003723 GOMF RNA BINDING</td>
<td>9/1938</td>
<td>RPN1, C7orf50, RALYL, RPP25L, RBFOX1, SMG6, EIF4A1, FXR2, RAVER1</td>
<td>0.491</td>
<td>0.134</td>
<td>0.0940</td>
<td>8.876E-07</td>
</tr>
<tr>
<td>GO:0008380 GOBP RNA SPlicing</td>
<td>5/478</td>
<td>C2orf49, RBFOX1, USB1, FXR2, RAVER1</td>
<td>1.091</td>
<td>0.225</td>
<td>0.219</td>
<td>2.360E-06</td>
</tr>
</tbody>
</table>
Pathway enrichment

- mRNA metabolic process
- Regulation of mRNA metabolic process
- Golgi apparatus subcompartment
- mRNA binding
- RNA splicing via transesterification reactions
- mRNA processing
- RNA localization
- Establishment of RNA localization
- Ribonucleoprotein granule
- Regulation of RNA splicing
- Regulation of mRNA splicing via spliceosome
- Regulation of mRNA processing
- Regulation of alternative mRNA splicing via spliceosome
- Alternative mRNA splicing via spliceosome
- Nucleobase containing compound transport
- RNA binding

Gene number
- 2
- 4
- 6
- 8

$-\log_{10}(P\text{value})$

- 9
- 8
- 7
- 6