A recurrent SHANK1 mutation implicated in autism spectrum disorder causes autistic-like core behaviors in mice via downregulation of mGluR1-IP3R1-calcium signaling

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The genetic etiology and underlying mechanism of autism spectrum disorder (ASD) remain elusive. SHANK family genes (SHANK1/2/3) are well known ASD-related genes. However, little is known about how SHANK missense mutations contribute to ASD. Here, we aimed to clarify the molecular mechanism of and the multilevel neuropathological features induced by Shank1 mutations in knock-in (KI) mice. In this study, by sequencing the SHANK1 gene in a cohort of 615 ASD patients and 503 controls, we identified an ASD-specific recurrent missense mutation, c.2621 G > A (p.R874H). This mutation demonstrated strong pathogenic potential in in vitro experiments, and we generated the corresponding Shank1 R882H-KI mice. Shank1 R882H-KI mice displayed core symptoms of ASD, namely, social disability and repetitive behaviors, without confounding comorbidities of abnormal motor function and heightened anxiety. Brain structural changes in the frontal cortex, hippocampus and cerebellar cortex were observed in Shank1 R882H-KI mice via structural magnetic resonance imaging. These key brain regions also showed severe and consistent downregulation of mGluR1-IP3R1-calcium signaling, which subsequently affected the release of intracellular calcium. Corresponding cellular structural and functional changes were present in Shank1 R882H-KI mice, including decreased spine size, reduced spine density, abnormal morphology of postsynaptic densities, and impaired hippocampal long-term potentiation and basal excitatory transmission. These findings demonstrate the causative role of SHANK1 in ASD and elucidate the underlying biological mechanism of core symptoms of ASD. We also provide a reliable model of ASD with core symptoms for future studies, such as biomarker identification and therapeutic intervention studies.

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INTRODUCTION

Autism spectrum disorder (ASD) is a lifelong neurodevelopmental disorder characterized by two core symptoms: deficits in social interaction and communication and the presence of restricted interests and repetitive behaviors [1]. The genetic etiology and underlying mechanism remain elusive, even though the heritability of ASD is as high as 70–90% [2, 3]. As major scaffolding proteins, SHANK proteins (SHANK1/2/3) individually play important roles at postsynaptic densities (PSDs) of glutamatergic synapses, where numerous pathways associated with ASD-risk genes converge [4–6]. SHANK family genes (SHANK1/2/3) are well-known ASD-related genes with multiple types of molecular defects [7], since the first report of SHANK3 mutations in ASD patients was published in 2007 [8]. Earlier research found that deletions of SHANK genes account for a large percentage of SHANK-related ASD cases [9]. In the last decade, with the development of whole-exome sequencing (WES) and whole-genome sequencing (WGS), approximately 50% of SHANK-related ASD cases have been attributed to missense mutations in SHANK genes (https://www.sfari.org/). However, the mechanisms of ASD induced by SHANK missense mutations are poorly understood. To date, most Shank-related mouse models have been produced through disruption of entire Shank genes, which might not mimic the pathophysiology caused by missense mutations identified in ASD patients due to the possible gain-of-function effects produced by missense mutations or the potential genetic compensation response caused by gene knockout (KO). Although two knock-in (KI) mouse models of Shank3 missense mutations have been reported thus far, autistic-like core behaviors are absent or mild in these mice [10, 11]. The limited research on two lines of

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Shank3-KI mice seems inconsistent with the importance of the SHANK family in ASD and the predominance of missense mutations in ASD-related SHANK variants. Therefore, further study of SHANK missense mutations in KI mice might aid in deciphering the heterogeneity of ASD, especially the essential mechanism related to the core symptoms.

The role of SHANK1 in ASD has been largely ignored. SHANK1 has been thought to be a low-risk gene for ASD based on findings that a Shank1-KO mouse model does not display apparent repetitive behaviors or robust social deficits [12–15] and that overexpression of Shank1 does not alter synaptic density [16–19]. However, the genetic compensation response triggered by gene KO may ablate the expected phenotypes in KO animal models [20, 21]. The important roles of Shank1 in synaptogenesis and synapse maturation also cannot be ignored, as overexpression of Shank1 induces spine maturation as well as spine head enlargement [16], and knockdown of Shank1 in vitro or KO of Shank1 in mice results in decreased synaptic density [12, 17]. In addition, in both humans and rodent animals, Shank1 expression is present exclusively in the brain and is particularly enriched in the cortex, hippocampus and cerebellum, which is spatiotemporally different from the expression of Shank2 and Shank3 [22–26] (see also the HPA RNA-seq normal tissue dataset and the Human Brain Transcriptome database). The above evidence indicates that SHANK1 is not redundant. Notably, among SHANK1 variant-related ASD cases, SHANK1 missense mutations account for more than 80% of the cases (Supplementary Table 1) [18, 19, 27–29]. However, a SHANK1 missense mutation KI mouse model has not yet been reported. Thus, generation and characterization of an ASD-related SHANK1 missense mutation KI mouse model would help to better elucidate the pathological role of SHANK1 in ASD.

In the present study, we first sequenced the SHANK1 gene in a cohort of 613 ASD patients and 507 controls and found six ASD-specific missense mutations. A novel recurrent missense mutation, c.2621 G > A (p.R874H), experimentally showed the strongest pathogenic potential and was then chosen to generate a corresponding Shank1 R882H-KI mouse model. A series of abnormalities ranging from molecular and neuronal abnormalities to brain and behavioral abnormalities were characterized in Shank1 R882H-KI mice. Our studies clarify the molecular mechanism of and the multilevel neuropathological features induced by the Shank1 R882H mutation in KI mice, which display the typical core symptoms of human ASD.

METHODS
Details are provided in Supplementary Methods, including study participants, mutation screening of SHANK1 in ASD, in vitro functional characterization of mutations, as well as behavioral assays, neuroimaging, synapse morphology and molecular dissection of the constructed Shank1 R882H-KI mice.

RESULTS
Identification of six ASD-specific missense mutations in SHANK1
Following sequencing of the coding regions and splice sites of SHANK1 in 615 ASD patients and 503 healthy controls, six missense mutations were identified only in ASD cases, which had extremely low frequencies in 1000 Genomes Project, ExAC, gnomAD, and CMDB human population datasets (Fig. 1A, Table 1). c.2621 G > A (p.R874H) and c.5417 C > T (p.P1806L) were two recurrent missense mutations that have not been reported previously. c.2621 G > A (p.R874H) was identified in two unrelated ASD individuals: one was de novo, and the other was inherited from his mother, who had moderate depression and mild anxiety (Supplementary Table 2). c.5417 C > T (p.P1806L) was detected in two unrelated ASD individuals: one was de novo, and the other was inherited from his mother, who lacked psychiatric symptoms (Supplementary Table 2). For the ASD488P and ASD837P trios, paternity was confirmed by STR analysis (Supplementary Table 3). One ASD individual simultaneously carried two missense mutations, c.1835C>T (p.A612V) and c.2621 G>A (p.R874H), which were both inherited from his mother, who had moderate depression and mild anxiety (Supplementary Table 2). Another ASD individual simultaneously carried two missense mutations, c.5776 G>A (p.D1926N) and c.6110 G>A (p.G2037D), which were respectively inherited from his father, who had mild depression and anxiety, and his mother, who lacked psychiatric symptoms (Supplementary Table 2). c.6076 G>A (p.G2026R) was detected in one ASD individual, which was inherited from his mother (Supplementary Table 2). In total, A612V, R874H, P1806L and G2037D were first identified in patients with ASD, while D1926N and G2026R had been previously observed [18, 19]. All SHANK1 variants identified here were heterozygous. No other types of variants, such as nonsense, frameshift or splicing-site mutations, were identified in the present cohort.

The locations of these mutations are illustrated in the structural diagram of the SHANK1 protein (Fig. 1B). Based on multiple alignments among species (Fig. 1B) and scores from PhyloP and PhastCons (Table 1), the variants A612V, R874H and G2026R are evolutionarily highly conserved. To investigate the potential pathogenic effects of these variants, we performed in silico analyses using SIFT, PolyPhen-2, MutationTaster2, CADD and DANN tools. The vast majority of these bioinformatic tools predicted that three mutations, R874H, D1926N and G2037D, were deleterious; these mutations were classified as high-risk variants (Table 1). In particular, the variant R874H was predicted to be pathogenic by all bioinformatic tools, indicating that it had the highest probability of being deleterious.

Impaired maturation of dendritic spines in neurons overexpressing SHANK1 mutations in vitro
Shank1 is localized at postsynaptic sites, and overexpression of Shank1 has been proven to promote spine maturation [16]. To further assess the pathogenic effects of these mutations, we performed morphological analysis of neurons in vitro. Constructs containing wild-type (WT) or mutant Shank1 were cotransfected with GFP into cultured hippocampal neurons derived from rats, and then the spine neck length, spine head width and spine density were quantified (Fig. 2A). Compared with neurons overexpressing WT Shank1, neurons overexpressing Shank1 mutations except G2037D showed decreases in spine length (Fig. 2A, b, c). Neurons overexpressing R874H, P1806L, D1926N or G2037D displayed significant reductions in spine width (Fig. 2A, d), of which those overexpressing R874H showed the most dramatic reductions in spine size (Fig. 2A, e). Spine density was not affected by any Shank1 mutation (Supplementary Fig. 1A). There were no significant differences in Shank1 protein levels between these mutations and WT (P = 0.1426; Supplementary Fig. 1B). In summary, R874H, P1806L, and D1926N presented consistent effects on spine length and spine width. Given the high conservation and the finding that the highest score of bioinformatic pathogenicity prediction was for R874H, R874H was selected for further study in in vivo experiments to understand the mechanism of ASD induced by SHANK1.

Generation of Shank1 R882H-KI mice
KI mice with the R882H substitution (corresponding to a human R874H substitution) were generated using a CRISPR/Cas9 strategy in the C57BL/6N strain. Shank1 R882H-KI mice were viable and fertile and exhibited normal development. Both heterozygous (Het) and homozygous (Hom) R882H-KI mice (K1-Het and K1-Hom) had body weights similar to those of WT littermates (Supplementary Fig. 2A). Immunoblot quantification revealed no significant alterations in Shank1 protein levels in the PSD fractions derived from the whole brains of mutant mice (Supplementary Fig. 1C).
Shank1 R882H-KI mice displayed core behavioral features of ASD
Since the diagnostic criteria for ASD are defined behaviorally and since no biomarkers have been identified, the validity of mouse models for ASD depends strongly on their behavioral phenotypes [15]. There are two core features of ASD: persistent difficulties with social communication and social interaction, and restricted, repetitive patterns of behavior, interests, or activities. These features were assessed in Shank1 R882H-KI mice.
Spending significantly more time with the object or the stranger mouse in KI-HET and KI-HOM mice than in that with the object in Experiment II (Fig. 3A). In addition, the results of chamber time and sniffing preference, WT littermates performed normally, spending more time in the chamber containing the novel mouse (stranger #2) than in that containing the familiar mouse (stranger #1) (Fig. 3B). However, neither KI-HET nor KI-HOM mice showed differences in the time spent in the two chambers (Fig. 3B), exhibiting impairments in social novelty preference. Assessment of direct sniffing interaction with the familiar or novel mouse, another sensitive measure of social novelty preference, also supported the disability of social novelty preference in R882H-KI mice. Compared with WT mice, neither KI-HET nor KI-HOM mice showed significantly more sniffing time with stranger #2 than with stranger #1 (Fig. 3B). As a within-task control for levels of general exploratory locomotion, entries into the left and right side chambers were measured, but the entries did not differ for the three genotypes (Supplementary Fig. 2C). Collectively, the social

**Table 1. Overview of the SHANK1 variants identified in ASD subjects.**

| Variants | SNP ID | DNA change | AA alteration | Minor Allele Frequency in Human Population<sup>a</sup> | Conservation<sup>b</sup> | Functional prediction |
|----------|--------|------------|--------------|-----------------------------------------------------|------------------------|----------------------|
|          |        | c.1835C > T | p.A612V     | 1000 Genomes (0/0) 2.00E-04 (1/5008) 0 (0) | PhyloP 3.047 (30/14861) | SIFT T D D D D D |
|          | rs780734174 | c.2621 G > A | p.R874H     | ExAC 2.50E-05 (3/120164) 5.23E-05 (6/114684) 0 (0) | PhastCons 0.999 (30/14861) | PolyPhen-2 D D B B B D |
|          | rs143496044 | c.5417 C > T | p.P1806L    | gnomAD 1.77E-05 (5/282358) 7.18E-05 (18/250768) 1.66E-05 (4/241264) | CADD 0.999 (30/14861) | MutationTaster2 H D H D D D D |
|          | rs1433344069 | c.5776 G > A | p.D1926N    | CMDB<sup>c</sup> 1.56E-03 (3/120164) 0.172 (0/0) | DANN 0.989 (30/14861) | DANN T D T T T T |
|          | rs374230001 | c.6076 G > A | p.G2026R    |                  | Number of “deleterious” 4 | 5 |
|          | rs200040610 | c.6110 G > A | p.G2037D    |                  | 2 |

NA means not available. AA means amino acid.

<sup>a</sup>Allele frequencies were estimated according to the 1000 Genomes Project Phase 3, ExAC v1.0, gnomAD v2.1 and CMDB phase 1 databases.

<sup>b</sup>PhyloP assigned positive scores to sites predicted to be conserved and negative scores to sites predicted to be fast-evolving. The closer the PhastCons value was to 1, the more likely the nucleotide was to be conserved.

<sup>c</sup>Allele frequency is calculated as the number of affected individuals in the database divided by the total number of individuals in the database.

**Impaired social behavior**

Both social interaction and social novelty preference were examined with three-chamber social test and determined based on consistent results by chamber time and sniffing time [30]. In the social interaction test, mice were given the choice to interact with an inanimate object or a stranger mouse. KI-HET and KI-HOM mice presented normal social interaction, comparable to WT mice, spending significantly more time in the chamber with the stranger mouse than in that with the object in Experiment II (Fig. 3A). Simultaneously, there existed differences in the sniffing time spent with the object or the stranger mouse in KI-HET and KI-HOM mice (Fig. 3A). In addition, the results of chamber time and sniffing time in Experiment I were inconsistent (Supplementary Fig. 2A). Consequently, the social interaction of R882H-KI mice was not impaired in our experiments. In the test of social novelty preference, WT littermates performed normally, spending more time in the chamber containing the novel mouse (stranger #2) than in that containing the familiar mouse (stranger #1) (Fig. 3B). However, neither KI-HET nor KI-HOM mice showed differences in the time spent in the two chambers (Fig. 3B), exhibiting impairments in social novelty preference. Assessment of direct sniffing interaction with the familiar or novel mouse, another sensitive measure of social novelty preference, also supported the disability of social novelty preference in R882H-KI mice. Compared with WT mice, neither KI-HET nor KI-HOM mice showed significantly more sniffing time with stranger #2 than with stranger #1 (Fig. 3B). As a within-task control for levels of general exploratory locomotion, entries into the left and right side chambers were measured, but the entries did not differ for the three genotypes (Supplementary Fig. 2C). Collectively, the social
behavior of KI mice was impaired, manifesting in the aspect of social novelty preference.

**Increased repetitive behaviors**

The marble-burying (MB) test has been proven to be a reliable method for measurement of repetitive behaviors in mouse models [31, 32]. The extent of repetitive behaviors is reflected by the number of buried marbles. In this assay, marble-burying performance significantly differed among the three genotypes (Fig. 3C). Post hoc analysis showed that more marbles were buried by both KI-HET and KI-HOM mice than by WT littermate controls. This meant that R882H mutants exhibited increased repetitive behaviors.
Normal locomotor ability and no anxiety-like behavior
Comorbid anxiety symptoms and motor abnormalities appear in approximately 42–56 and 79% of ASD cases, respectively [33]. With regard to mouse behaviors, motor abnormalities or heightened anxiety could affect performance and confound interpretations of results related to the core phenotypes [13, 34, 35]. To assess locomotor ability and anxiety-like behavior, we performed an open-field (OF), light-dark (LD) exploration and elevated plus maze (EPM) test. The R882H-KI mice showed normal locomotor activity, as measured by total distance traveled and movement time in the OF (Fig. 3D). This was consistent with the results for the numbers of entries in three-chamber tests (Supplementary Fig. 2C), the numbers of transitions between two compartments in the LD test (Supplementary Fig. 2D), and the numbers of total entries into each arm in the EPM test for three genotypes (Supplementary Fig. 2E), all of which served as within-task controls for the levels of general exploratory locomotion. Anxiety-like behaviors were not detected in mutant mice, as assessed by the central tendency in the OF test (Fig. 3E), the time spent in the light compartment in the LD test (Fig. 3F) as well as the time spent in open arms and the entries into open arms in the EPM test (Fig. 3G), compared to WT littermates. These data provide further evidence to support the findings that impaired sociability and repetitive behaviors in R882H-KI mice are intrinsic and unaffected by comorbidities such as abnormal motor function and heightened anxiety.

No learning or memory deficits
In addition to the core symptoms, a number of individuals with ASD have atypical cognitive profiles [33]. Here, in mice, recognition memory was evaluated by the novel object recognition (NOR) assay [36]. Spatial learning and memory were assessed with the Barnes maze (BM) task, which assesses a mouse's ability to learn the location of a target zone with the use of visual cues [37]. R882H-KI mice showed significant discrimination scores for the novel object to WT mice, indicating that object recognition memory were not affected in mutant mice (Fig. 3H). In BM, both mutant and WT mice were able to successfully learn the task, as evidenced by similar decreasing trends in the numbers of total errors before finding the escape box over the course of 15 trials (Fig. 3I) and supported by another parameter, latency (Supplementary Fig. 2F). On day 5, there was no difference in the number of total errors or the percentage of time spent in the target quadrant between mutant and WT mice (Fig. 3J and Supplementary Fig. 2G), indicating that mutant mice had normal short-term retention. On day 12, mutant mice displayed more errors and less quadrant time than WT mice, reflecting the trends of impairment in long-term retention, but these differences did not reach statistical significance (Fig. 3J and Supplementary Fig. 2G). Overall, no obvious impairment of spatial learning or memory was observed in mutant mice.

Brain structural changes in Shank1 R882H-KI mice focused on the frontal cortex, hippocampus and cerebellar cortex
To investigate potential neuroanatomical abnormalities underlying ASD-related core behaviors in R882H-KI mice, the brains of WT mice and KI-HOM mice were imaged using in vivo structural magnetic resonance imaging (sMRI). Voxel-based morphometry (VBM) was applied to measure gray matter volume (GMV) differences between the two groups. Significant differences in GMV were obtained in the frontal cortex (including septal area), striatum, piriform area, thalamus, hippocampus and cerebellar cortex, where KI-HOM mice revealed a notable increase compared with WT mice (Fig. 4). Of these regions, the frontal cortex, hippocampus and cerebellar cortex were regions where murine Shank1 is the most highly expressed [23–25] and were also implicated in ASD in previous neuroimaging studies on humans and mice. The discovery of structural changes in KI-HOM mice further supported the pathological effects of these regions in ASD. Therefore, these three regions were selected as key brain regions for further exploration.

Synaptic structural and functional anomalies in Shank1 R882H-KI mice
At the cellular level, we examined spine morphology and spine density in hippocampal CA1 pyramidal neurons by Golgi staining. Similar to the morphological changes in vitro, reduced spine width and length were observed in mutant mice. A significantly shorter spine length was found in R882H mutant mice than in WT littermates, which was evident by the shift of the cumulative probability to the left (0.82 ± 0.03 μm and 0.81 ± 0.02 μm in HET and HOM, respectively, versus 0.89 ± 0.02 μm in WT; Fig. 2B, a, b). R882H-KI mice showed a pronounced reduction in spine width, with a dramatic shift to the upper left quadrant in cumulative probability plots (0.47 ± 0.01 μm and 0.48 ± 0.01 μm in HET and HOM, respectively, versus 0.62 ± 0.01 μm in WT, Fig. 2B, a, c). In terms of spine density, R882H-KI mutants displayed declines of 12.2% and 31.4% in HET and HOM mice, respectively, compared with their WT littermates (from 8.78 ± 0.25 in WT to 7.71 ± 0.29 in HET and 6.02 ± 0.20 in HOM per 10 μm; Fig. 2B, d), which was different from the result of no change in cultured neurons in vitro. We compared the ultrastructure of glutamatergic synapses in hippocampal CA1 neurons between WT and KI-HOM mice using electron microscopy (Fig. 2C). PSD length was increased by 17% in KI-HOM mice (from 0.35 ± 0.01 μm in WT to 0.41 ± 0.02 μm in HOM; Fig. 2C, a, b). A reduction of 10% was observed in the thickness of the PSDs of KI-HOM mice relative to controls (from 0.048 ± 0.001 μm in WT to 0.043 ± 0.001 μm in HOM; Fig. 2C, a, c).
We performed electrophysiological recordings of the hippocampus to assess synaptic function. Extracellular recordings at SC-CA1 synapses showed that long-term potential (LTP) induced by theta burst stimulation (TBS) of the SCs was severely impaired in KI-HOM mice from the induction period to the maintenance period; the amplitude of field excitatory postsynaptic potentials (fEPSPs) was 25.1% lower in KI-HOM mice than in WT mice (Fig. 2D, a). Basal synaptic transmission at CA1 pyramidal neurons was assessed by mEPSCs using whole-cell patch-clamp recordings. A significantly decrease of mEPSCs in the frequency, but not the amplitude, was observed in KI-HOM mice compared with controls (Fig. 2D, b), indicating weakened basal excitatory transmission.
**Fig. 3** Shank1 R882H-KI mice exhibit ASD-like core behaviors. R882H-KI mice exhibited impaired social behavior in the three-chamber social test in Experiment II (n = 14–16 for each genotype) (A, B). A Social interaction measured. S #1, a stranger mouse placed in one chamber. O, a novel object (inverted wire cup) placed in another chamber. B Social novelty preference measured. S #1, an already-investigated familiar mouse. S #2, a novel unfamiliar mouse. C R882H-KI mice manifest increased repetitive behaviors, as shown by burying of more marbles in the MB test (n = 20–25 for each genotype). D R882H-KI mice showed normal exploratory locomotion in the OF test (n = 16 for each genotype). No anxiety-like behavior was exhibited by R882H-KI mice with face validity, as shown by the center distance ratio in the OF test (E), the time in the light compartment in the LD exploration (F) (n = 16 for each genotype), and the time and entries in open arms in the EPM test (G) (n = 16–17 for each genotype). H No deficits in recognition memory in R882H-KI mice, assessed by NOR (n = 13–14 for each genotype). I Normal performance of spatial learning in the BM (n = 13 for each genotype), measured by the number of errors made before reaching the target hole (genotype, \( P = 0.1921 \), trial, \( P < 0.0001 \), interaction, \( P = 0.7528 \)). J No deficits in short-term (Day 5) or long-term (Day 12) spatial memory were observed in R882H-KI mice, as indicated by the number of errors made before reaching the target hole. Paired Student’s t-test for A, B. One-way ANOVA for C–H. J. Repeated-measures ANOVA for I. All data are presented as the mean ± SE. ns, no significance; *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \) for the groups compared with WT.

**Fig. 4** Prominent changes in GMV focusing in frontal, hippocampal and cerebellar regions of Shank1 R882H-KI HOM mice. Structural MRI with T2-weighted images revealed the GMVs of multiple brain regions significantly increased in Shank1 R882H-KI HOM mice compared with WT mice. Of these regions, the frontal cortex, hippocampus and cerebellar cortex showed dramatically increased GMVs in frontal cortex (FC), hippocampus (HP) and cerebellar cortex (CBC) in Shank1 R882H-KI HOM mice. Piriform area has the frontal and temporal components. Septal area is the part of the frontal lobe. Voxel-based morphometry (VBM) analysis of brain coronal sections on an averaged MRI template by comparing Shank1 R882H-KI HOM mice (n = 8) with WT mice (n = 9) (\( P < 0.05 \), clusters >200 voxels, uncorrected, then followed by FDR correction with significance of 0.05).

**Dysfunction of mGluR1-IP3R1-calcium signaling in Shank1 R882H-KI mice**

Downregulation of mGluR1-IP3R1 signaling. Shank proteins are described as the “master regulators” of glutamatergic synapses, as they function as core components of the PSD to modulate synaptic structure and function. We tried to illuminate the molecular basis responsible for the aberrant synaptic structure and function observed in R882H-KI mice. First, we performed quantitative proteomic analysis of PSDs from the hippocampus through LC-MS/MS. In total, 2081 proteins were identified, of which 472 proteins (22.7%) were differentially expressed in KI-HOM versus WT mice. The glutamatergic synapse was the most significant term among the 9 enriched terms in KEGG pathway analysis (Supplementary Fig. 3B). In this enriched pathway, there were 14 proteins located in postsynapses, which were ionotropic glutamate receptors (NMDAR subunits GluN1/N2A and AMPAR subunits GluA1/A2/A4), metabotropic glutamate receptor (mGluR1), all members of the Homer family (Homer1/2/3), PSD-95, IP3R, extracellular-signal-regulated kinase (ERK) 1/2 and calcineurin (Ppp3ca/3cb) (Supplementary Table 4). All of them have direct or indirect interactions with SHANK1. In RNA-Seq analysis of mRNAs in the hippocampus in KI-HOM and WT mice, 23718 genes were identified. No differences were found for any of the aforementioned molecules, and only 14 genes showed significant changes (Supplementary Table 5). However, the functions of these genes are unknown, or the genes are not directly related to ASD. No terms were enriched in KEGG pathway analysis (Supplementary Fig. 3A).

Next, we validated the above 14 differentially expressed proteins in KI-HOM mice and WT littermates by immunoblotting (Fig. 5A). PSD
fractions from the frontal cortex, hippocampus and cerebellar cortex, whose GMVs were significantly altered in KI-HOM mice, were chosen for analyses. Among the 6 detected membrane receptors, only mGluR1 was downregulated at the protein level, while NMDAR and AMPAR subunits (GluN1/N2A and GluA1/GluA2/GluA4) remained unchanged in the three brain regions (Fig. 5A, a and c). mGluR1 was downregulated by up to nearly 50% (−26% in the frontal cortex, −42.6% in the hippocampus and −29.4% in the cerebellar cortex). The consistent downregulation of mGluR1 in three regions provides robust evidence of dysfunction of metabotropic glutamate receptors.
mGluR1 is anchored to the PSD through its interaction with Homer, which directly binds to Shanks. The protein levels of Homer1, Homer2, and Homer3 were highest in the hippocampus, frontal cortex and cerebellar cortex, respectively (Fig. 5B and Supplementary Fig. 3C). Interestingly, we found that the protein levels of Homer1, Homer2 and Homer3 showed the strongest down-regulation in the specific brain regions with the highest expression levels among the three tested brain regions. A decrease of 25% was observed for Homer1 in the hippocampus, a decrease of 30.6% was observed for Homer2 in the frontal cortex, and a decrease of 34.4% was observed for Homer3 in the cerebellar cortex (Fig. 5A, a, b). The comprehensive and severe down-regulation of Homer protein subtypes expression in mutant mice indicates that the effect of Shank1/R882H on Homer proteins is general and subtype-specific.

IP3R1, tethered to Shanks by Homer, is an important downstream target of mGluR1. Significant reductions of up to 50% in IP3R1 levels were observed in the three brain regions (−30.8% in the frontal cortex, −35% in the hippocampus and −50.0% in the cerebellar cortex) (Fig. 5A, a, d). Phosphorylated ERK1/2, a downstream effector of mGluR1 and IP3R1, showed reductions of 24.6% in the frontal cortex, 35.4% in the hippocampus and 37.0% in the cerebellar cortex (Fig. 5A, a, d). Calcineurin is a calcium-dependent phosphatase that plays a critical role in synaptic plasticity. Ppp3ca, a subunit of calcineurin, showed reductions of 23.3% in the frontal cortex and 13.6% in the cerebellar cortex, while another subunit of calcineurin, Ppp3cb, showed reductions of 33.3% in the hippocampus and 17.3% in the cerebellar cortex (Fig. 5A, a, d). There were no changes in PSD-95 protein levels in the three brain regions (Fig. 5A, c, a).

**Imperfection of intracellular release of calcium.** IP3R1 is an intracellular calcium channel that mediates intracellular calcium release from ER calcium storage by binding with and being activated by IP3 generated through mGluR1 [38]. Since mGluR1 and IP3R1 levels were diminished in R882H-KI mice, we speculated that the amount of calcium released from the ER might have been lessened, affecting the concentration of neuronal cytosolic calcium [39]. To test this hypothesis, we measured the calcium transients in the hippocampus stimulated by DHPG, a specific group I metabotropic receptor agonist. DHPG-induced calcium transients in R882H-KI mice were significantly lower (by more than 20%) than those in WT mice (50 µM and 100 µM, Fig. 5C, a, b), indicating impaired mGluR1/IP3R1-mediated intracellular release of calcium in KI mice. Next, we tested whether glutamate-induced intracellular calcium concentration ([Ca^{2+}]i) is affected by aberrant mGluR1/IP3R1 signaling in KI mice. The concentration of glutamate was within the range used for physiological stimulation (from low micromolar concentrations up to 1 mM) to simulate the physiological state during neurotransmission [40]. Effective responses to glutamate were obtained in hippocampal cells from both KI mice and WT controls. The ([Ca^{2+}]i), was elevated markedly at 10 min, and the elevation lasted for 30 min (Fig. 5C, c). However, the ([Ca^{2+}]i), was always significantly lower in KI mice than in WT mice (approximately 20% lower), indicating that glutamate-induced ([Ca^{2+}]i), was disturbed in mutant mice.

Molecular investigation shows that the Shank1 R882H substitution specifically affects mGluR1/IP3R1-calcium signaling, leading to a decrease in the intracellular calcium concentration. Combined with the corresponding cellular and brain imaging changes in the same regions, these findings provide reasonable mechanistic explanations for the behavioral abnormalities observed in Shank1/ R882H-KI mice.

**DISCUSSION**

In the present study, the recurrent missense mutation c.2621 G > A (p.R874H) of SHANK1 was identified in ASD cases for the first time, and the underlying neurological and molecular mechanisms were illuminated. This variant was located at a highly conserved region near the PRO domain of Shank1. The R882H-KI mice demonstrated core symptoms of ASD, namely, social disability and repetitive behaviors, without anxiety-like behavior, locomotor abnormalities, or learning and memory deficits. We found structural changes in the frontal cortex, hippocampus and cerebellar cortex in KI mice via sMRI, which were also brain regions with highly expressed murine Shank1. This finding was consistent with the corresponding cellular and brain imaging changes in the same regions, thus providing reasonable mechanistic explanations for the behavioral abnormalities observed in Shank1/ R882H-KI mice.
The KI mouse model of Shank1 R882H displays two ASD core symptoms with no confounding comorbidities and precisely demonstrates the causative relationship of the Shank1 gene with ASD. In contrast, Shank1-KO mice display mainly ASD-nonspecific symptoms with no typical or consistent core symptoms and can thus hardly be used to draw the certain conclusions described above [12–15]. Previous studies have found that ASD-associated social and repetitive behaviors are influenced by abnormal locomotion or anxiety-like behavior in mice. For example, low social interaction or social novelty preference could be partially attributed to low exploratory activity and/or high anxiety-like behavior in mice, thus rendering social assay data meaningless [13, 34]. Enhanced repetitive performance in marble-burying could also be affected by exploratory activity [31, 35]. A series of behavioral tests showed that R882H-KI mice are characterized by ASD core symptoms with no confounding comorbidities of heightened anxiety and abnormal locomotion. Among all of the summarized ASD-related KI mice (Supplementary Table 6), our established Shank1 R882H model, together with the Slc6a4 G56A model, manifests ASD core symptoms without confounding comorbidities. However, it should be noted that the increased repetitive behaviors in the Slc6a4 G56A model are totally different from those in the Shank1 R882H model. Specifically, restricted and repetitive behaviors (RRBs) in ASD fall into two categories: higher-order and lower-order RRBs [41–44]. Of these, only higher-order RRBs are unique to ASD, whereas lower-order RRBs are observed in many developmental disorders [45, 46]. Shank1 R882H mice exhibited increased repetitive behaviors in the marble-burying task, which serves as a test for higher-order RRBs in mouse models [31, 47], while Slc6a4 G56A mice showed weakly increased lower-order RRBs without exhibiting abnormal higher-order RRBs [48] (Supplementary Table 6). Therefore, Shank1 R882H mice are an appropriate model for pathological or therapeutic studies of ASD core symptoms.

This study is the first to emphasize that mGluR1-IP3R1-calcium signaling is dramatically downregulated in ASD and is especially related to ASD core symptoms. SHANK indirectly interacts with glutamate receptors via different proteins at defined functional domains: AMPARs via GRIP at the SH3 domain of Shank1, NMDARs via the PSD55/GKAP complex at the PDZ domain and mGlus via Homer at the PRO domain. Specifically, we determine that mGluR1-IP3R1 signaling is comprehensively and significantly dampened in the brain regions of frontal cortex, hippocampus and cerebellar cortex in Shank1 R882H-KI mice. These three brain regions are the regions in which Shank1 is highly expressed, and structural alterations in these regions were also highlighted by sMRI scanning. Unlike Shank1 R882H-KI mice, Shank-KO mice demonstrate the involvement of ionotropic glutamate receptors (NMDARs and AMPARs) in the pathogenesis of ASD, and the roles of metabotropic glutamate receptors (mGluRs) in ASD in these mice seem ambiguous or unimportant. No changes in glutamate receptors have been observed in Shank1-KO mice [12], opposite changes in NMDAR function have been observed in Shank2-KO mice [49, 50] and downregulations of NMDARs and AMPARs have been observed in Shank3-KO mice [25, 51–53]. Recently, mGlus and mGluR5-mediated signaling were found to be altered in Shank3-KO mice [54, 55]. The differences in molecular changes between Shank1-KI and Shank-KO mice would lead to different pathological processes and totally different outcomes. Here, the Shank1 R882H-KI mouse model precisely illuminates the relationship between ASD core symptoms and the corresponding molecular changes. Our study sheds light on the irreplaceable importance of KI mouse models bearing ASD-related missense mutations for investigating and better understanding the underlying mechanism of ASD with clinical heterogeneity.

The significant downregulation of mGluR1-IP3R1-calcium signaling is highlighted to clarify the molecular mechanisms underlying the core symptoms of ASD. The fact that mGluR1 is the only altered glutamate receptor in R882H-KI mice indicates its unique and fundamental role in the pathogenesis of ASD. It has been demonstrated that group I mGluRs increase neuronal excitability [56–58], and mice deficient in mGluR1 display impaired synaptic plasticity, spatial learning deficits and severe motor coordination deficits [59, 60]. Functionally, activation of mGluR1 upon glutamate binding leads to the production of IP3, which binds to IP3R1 on the ER and subsequently triggers calcium release from the ER, thereby contributing to calcium signaling in neurons [61]. A wide range of neuronal processes, such as synaptic plasticity, neuronal excitability and neural circuit regulation, are under the control of cytosolic calcium signals [62] through the activation of multiple downstream signaling cascades, such as ERK signaling [63–65] and calcineurin signaling [66]. In our study, the levels of cytosolic calcium, downstream p-ERK1/2 (representing ERK1/2 activity) and Ppp3ca/Ppp3cb (catalytic subunit of calcineurin) were decreased in multiple brain regions. Therefore, downregulation of mGluR1-IP3R1-calcium signaling could explain the impaired synaptic plasticity and reduced basal excitatory transmission in Shank1 R882H-KI mice and finally lead to the core behavioral disability of ASD. Moreover, a number of human genetics studies have identified multiple rare variants of HOMER1 and IP3R1, the core genes in the mGluR-IP3R pathway, in ASD patients [67–70], supporting the importance of this pathway in the development of ASD.

Among the series of molecular changes triggered by R874H, changes in HOMER proteins are impressive. SHANK1 contains multiple domains, and HOMER interacts with SHANK1 at the PRO domain, which has been largely overlooked compared with other massively studied domains, such as the SPN, ANK and PDZ domains [18, 50–52, 71, 72]. However, the majority of SHANK1 variants reported so far are located in or near the PRO domain, and R874H is also located in a highly conserved region near the PRO domain (Supplementary Fig. 4), suggesting the critical role of the PRO domain in ASD. In our study, Homer proteins (Homer1/2-3) showed subtype-specific reductions in different brain regions. Each of the focused brain regions in KI mice showed significant impairment of a Homer subtype. The most strongly downregulated Homer subtype in a specific brain region in KI mice was usually the most highly expressed Homer in that region in WT mice. As Shank and Homer are major determinants of the sizes of dendritic spines and PSDs through forming a high-order polymerized complex [16, 73], the abnormal structures of dendritic spines and PSDs in the Shank1 R882H-KI mouse model could be explained by decreases in Homer levels. Our findings indicate that comprehensive and severe aberrations in Homer proteins in key brain regions of Shank1 R882H-KI mice lead to subsequent changes in other interacting proteins, profoundly affect the structure and function of postsynaptic neurons, and thereby contribute to the emergence of core symptoms of ASD.

Neuroimaging studies provide important insights into the neurological basis for ASD and the results of these studies can be leveraged to aid ASD diagnosis [74–76]. sMRI scans in Shank1 R882H-KI mice revealed that the main ASD core symptom-related brain regions are the frontal cortex, hippocampus and cerebellar cortex, emphasizing that the neurological changes in these regions are critical for ASD and connecting the pathological process from genes to behavior. These three regions are the most consistently affected regions both in ASD patients [77–83] and among twenty-six different ASD mouse models [84]. The observed correlations between structural impairments of these three brain regions and social disability or repetitive behaviors in ASD patients or in different ASD mouse models are also concordant with our findings. On the one hand, the GMV in frontal or cerebellar cortex has been found to be positively correlated with the severity of core symptoms in some autistic patients [82, 85]. On the other hand, the hippocampal or cerebellar GMV shows positive correlation with repetitive behaviors in BTBR mice [86]. Increased
hippocampal GMV is more identified in mouse models with poor social performance than those with normal/mixed social activities [84]. These findings provide strong evidence for the involvement of the three brain regions in core autistic behaviors observed in our R882H-KI mouse model. Moreover, the consistent molecular changes in these regions further support their important roles in the pathogenesis of ASD. Interestingly, altered volumes were also found in the striatum, thalamus and piriform area, consistent with previous reports that abnormalities of the above regions were implicated in the clinical core profiles of ASD. In individuals with ASD, increased GMV in the striatum and thalamus were commonly observed from multiple independent cohorts [82, 87–92]. Volume changes in the striatum were repeatedly reported to be associated positively with repetitive behaviors in ASD [82, 87, 89, 92]. Multiple lines of research have reported thalamic abnormalities are related to both social impairments and repetitive behaviors, although the relationship remains to be further determined [93–95]. Despite lack of evidence in structural relation, the piriform area has been recently functionally associated with ASD-related social behaviors [96, 97]. Notably, all the regions identified by sMRI, except piriform area, are key components of the circuits mediating the expression of social and repetitive behaviors [97–99], further indicating their close relation to ASD.

Taken together, our findings emphasize the absolute necessity of KI mouse models carrying the possible etiological human mutations to understand the pathogenesis of ASD. Shank1 R882H-KI mice, which exhibit two core symptoms of ASD without confounding comorbidities, provide a reliable model of ASD for future diverse studies, such as biomarker identification and therapeutic intervention studies. Identification of the role of mGluR1-IP3R1-calcium signaling in ASD reveals the molecular mechanism underlying the process from abnormal cellular structure and function to autistic-like core behaviors in Shank1 R882H mice, which might serve as potential targets for pharmaceutical intervention.

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AUTHOR CONTRIBUTIONS
YQ, XHG, and HYW conceived and designed the study. YQ performed experiments in genetics, cells and mice. YSD collected DNA samples and clinical information of ASD patients. YQ and YYL carried out the mutation screenings. YQ and XHG performed experiments in cultured neurons. YQ and LQC performed electrophysiological recordings. YQ, YL, and JL performed the analyses of electron microscopy. WX, YL, and XYZ finished neuroimaging scans. YLW technically helped with immunoblotting. YQ and XHG interpreted the data. JFF, XYZ, FZ, LJ, and ZLQ contributed intellectually to the interpretation of partial results. YQ drafted the manuscript and XHG and HYW revised it. All authors reviewed and approved the final version.

COMPETING INTERESTS
The authors declare no competing interests.

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