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Social isolation induces schizophrenia-like behavior potentially associated with HINT1, NMDA receptor 1, and dopamine receptor 2

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Both genetic factors and early life adversity play major roles in the etiology of schizophrenia. Our previous studies indicated that social isolation (SI) during early postnatal development leads to several lasting abnormal behavioral and pathophysiological features resembling the core symptoms of some human neuropsychiatric disorders in mice. The glutamate and dopamine hypotheses are tightly linked to the development of schizophrenia. The cross-talk between glutamate *N*-methyl-D-aspartate acid receptors and dopamine receptors is associated with histidine triad nucleotide binding protein 1 (HINT1), which is correlated with diverse psychiatric disorders. We examined the effects of SI on schizophrenia-like behavior and used enzyme-linked immunosorbent assays to investigate the expression levels of HINT1, the NR1 subunit of *N*-methyl-D-aspartate acid receptor, and dopamine type 2 receptor (D2R) in C57 mice. We found that SI leads to a series of schizophrenia-related deficits, such as social withdrawal, anxiety disorder, cognitive impairments, and sensorimotor gating disturbances. These abnormal phenotypes paralleled changes of HINT1, NR1, and D2R. SI may be considered a robust model of the effects of early life stress on the schizophrenia-related behaviors in mice. Potential interactions among HINT1, NR1, and D2R may underlie the behavioral deficits induced by SI. *NeuroReport* 28:462–469 Copyright © 2017 The Author(s). Published by Wolters Kluwer Health, Inc.

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Introduction

Schizophrenia is a common and severe mental illness and is a leading cause of adult disease burden [1]. It has a lifetime prevalence of about 0.7% and a peak age of onset in the early 20s in men and 3 or 4 years later in women [2]. The disorder is characterized by psychotic symptoms, including positive symptoms, such as delusions and hallucinations, negative symptoms such as social withdrawal and amotivation, emotional symptoms such as depression and anxiety, aggressive behavior, and cognitive impairment [3].

Although the exact molecular mechanisms underlying schizophrenia psychosis still remain unclear, dopamine and glutamate dysfunction are conventionally believed to be involved in this process. Several reports with indirect evidence have led to the proposal of the dopamine hypothesis of schizophrenia. Post-mortem studies continue to identify abnormalities in both presynaptic and postsynaptic dopaminergic systems in schizophrenia [4]. In the past, animal experiments have enabled testing and refinement of major aspects of the dopaminergic hypothesis of schizophrenia [5]. The involvement of glutamatergic mechanisms in schizophrenia has been hypothesized for many years. The glutamate hypothesis of schizophrenia originally stated that there is a simple deficit in glutamatergic neurotransmission in this disease [6]. Glutamate models involving *N*-methyl-D-aspartate acid receptor (NMDAR) blockade appear to be better able to account for the range and nature of some aspects of schizophrenia [7]. Until now, strong evidence implicated dysfunctions in dopaminergic neurotransmission in the genesis of psychotic symptoms and abnormalities in glutamate signaling were believed to account for the negative and cognitive symptoms of schizophrenia.

Early-life adversity is an established risk factor for schizophrenia and is modeled using stress protocols such as postweaning social isolation (SI). SI leads to robust behavioral, neurobiological, and neurochemical deficits resembling several core symptoms in patients with schizophrenia. It is therefore considered an animal model of schizophrenia [8].

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Histidine triad nucleotide binding protein 1 (HINT1) is a highly conserved 14 kDa protein belonging to the histidine triad superfamily, the members of which contain the HisXHisXHisXX motif (where X is a hydrophobic amino acid), for which histidine triad proteins are named [9]. Three HINT proteins have been described in humans: HINT1, HINT2, and HINT3. HINT1 is expressed widely in the central nervous system and is associated with several neuropsychiatric disorders, such as schizophrenia [10,11], nicotine addiction, depression, anxiety, bipolar disorder, and neuromyotonia [12].

We have reported that HINT1 is involved in the behavioral abnormalities induced by SI and that it plays distinct roles in different encephalic regions [13]. To improve our understanding of the neuropathology and molecular mechanisms underlying schizophrenia, we further examined the effects of SI on schizophrenia-like behaviors and the expression of HINT1, the NR1 subunit of NMDAR, and dopamine type 2 receptor (D2R) in mice.

Materials and methods

Animals

C57 BL/6J mice (postnatal day 21) were obtained from the Animal Care and Use Committee of Xi'an Jiaotong University. The cages contained sawdust bedding changed once per week. Each mouse was weighed during the cage changes. Mice were otherwise undisturbed until behavioral testing commenced 5 weeks later. Mice had free access to water and food, and were maintained on a 12 h light–dark cycle (lights on at 7:00 a.m.) in controlled humidity (45 ± 15%). All experimental procedures were approved by the Animal Care and Use Committee of Xi'an Jiaotong University.

SI paradigm

At weaning (postnatal day 21), pups were separated from their mothers and reared either in SI (one mouse/cage) or in groups (three or four mice/cage) for 5 weeks (until postnatal day 64). This experimental protocol lasted until the animals were used for behavioral and biochemical analyses. The SI and group-housed (GH) groups each had two subgroups. One subgroup was subjected to the nest-building test, the open-field test (OFT), the T-maze test (TMT), and prepulse inhibition (PPI) testing. The other subgroup was subjected to the elevated plus maze test (EPMT) and the Morris water-maze test (MWMT). The mice were killed after the PPI and MWMT.

OFT

Mice were placed individually into an open-field chamber (45 × 45 × 45 cm) under a dark light (25 lx) and were allowed to explore the arena for 1 h. Data were recorded using a video tracking system (SMART 3.0; Panlab SL, Barcelona, Spain).

NBT

Each animal was transferred to a clean cage containing fresh thin bedding. Two piles of eight layers of soft cotton square paper (5 × 5 cm) were placed in a corner ~1 h before the dark phase. Cages remained on their original racks in the colony room throughout the experiment. The next morning, five individuals who did not participate in this test evaluated the mice's nest-building and compared the ability to nest between the SI and GH groups. We used the average of the five ratings. The rating scale was as follows: 0, paper was not touched; 1, the paper is scattered on the cage everywhere, but there are no obvious bite marks; 2, the paper is mostly in one corner of the cage, but there are no obvious bite marks; 3, the paper is mostly in one corner of the cage and there are bite marks, but there is no visible nest; and 4, the paper is mostly bitten and forms a nest.

EPMT

The apparatus was composed of two open arms (25 × 5 cm) and two closed arms (25 × 5 × 20 cm) emerging from a cross-center platform (5 × 5 cm). The open and closed arms were cross shaped. The entire apparatus was elevated to 50 cm above the floor level. At the start of the test, the mouse was placed on the cross-center platform facing an open arm. The activity of the mouse was recorded by a video camera above the apparatus for 6 min.

TMT

The T-maze apparatus had three identical arms (start arm: 10 × 47 cm; goal arms: 10 × 35 cm). Each mouse was placed at the end of the start arm facing the center and allowed to freely explore the goal arms 10 times. The pattern of entries into the goal arm was visually scored. The mouse was then removed from the apparatus and the apparatus was cleaned using 30% alcohol. Alternation behavior was defined as consecutive entries into the two arms without repeated entries, as on overlapping triplet sets. Percent of spontaneous alternation equaled the number of alternations divided by the total number of entries multiplied by 100.

MWMT

The apparatus consisted of a stainless-steel circular pool (124 cm in diameter, 62.5 cm in height), a platform (34 cm in height, 12 cm in diameter, placed 1 cm below the surface), and the recording system. The pool water temperature was controlled at (22 ± 1°C). The camera was mounted about 1 m directly above the pool to record the animal's motion trajectory. The test was divided into three phases: adaptation, spatial learning training, and a subsequent probe test. The protocol is also described in a previous study [14]. The swimming routes and the numbers of platform site crosses were recorded for analysis.

PPI

Acoustic startle and PPI were measured using a startle chamber (Med Associates Inc., Georgia, USA). Test sessions

began upon placing the mouse in a clear Plexiglas (Med Associates Inc., Georgia, USA) holding cylinder for a 5 min acclimation period. A 65 dB broadband background noise was presented during acclimation and throughout the test session. During the test session, subjects were subjected to startle trials (40 ms: 120 dB sound pulse), prepulse trials (20 ms: 68, 71, or 77 dB prepulse sound), or prepulse + startle trials (20 ms: prepulse sound, followed 100 ms later by a 40 ms 120 dB sound pulse). There were three different prepulse intensities (3, 6, and 12 dB above background). Each trial type was presented 10 times with a variable interval (range: 12–30 s) between each presentation. Five pulse-alone trials began and ended the sessions. These trials were not included in the analysis.

PPI was calculated as 100 − [(startle response for prepulse + startle trials/startle response for startle − alone trials) × 100].

The amplitude of the response to the startle pulse was calculated from the averages of the first five startle responses evoked by the respective 120 dB presentations flanking the remaining sequence.

Reverse transcriptase PCR

The prefrontal cortex (PFC), nucleus accumbens (NAc), and hippocampus (HIP) from control or SI mice were dissected and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, California, USA). Total mRNA was extracted from cells according to the instructions of the TRIzol kit (Invitrogen). cDNA was then obtained using reverse transcription (Roche, Shanghai, China). The reactions were performed in a 20 μl volume. Each reaction contained 1 μl mRNA and 2 μl random hexamer primer. The reactions were incubated at 65°C for 15 min. We then added 4 μl (8 mM $MgCl_2$) protector RNase inhibitor, 0.5 μl deoxynucleotide mix, and 2 μl transcriptor reverse transcriptase. The mixture was then incubated at 25°C for 10 min (one cycle), 55°C for 30 min (one cycle), and 85°C for 5 min (one cycle). The reaction was then cooled at 4°C. The primers for HINT1, NR1, D2R, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were synthesized by Takara (Dalian, Japan). The sequences of the primers are as follows:

(1) HINT1
   (a) F: 5′-GGCAAGATCATCCGCAAAG-3′
   (b) R: 5′-TCATCTGCTACAGAAATCTGGGATA-3′
(2) NMDAR1
   (a) F: 5′-AGCGAGGAGGAGGAGGAG-3′
   (b) R: 5′-GGTTGGCAGTGTAGGAAGC -3′
(3) D2R
   (a) F: 5′-ATCTACATCGTTCTCCGCAAG-3′
   (b) R: 5′-TCTCCGCCTGTTCACTGG-3′
(4) GAPDH:
   (a) F: 5′-ATGGTGAAGGTCGGTGTGAACG-3′
   (b) R: 5′-CGCTCCTGGAAGATGGTGATGG-3′

Reactions were performed in 10 μl SYBR Green PCR Master Mix (Takara), 0.5 μl forward primer, 0.5 μl reverse primer, and 4 μl nuclease-free water. The reaction sequence was 95°C for 30 s (one cycle), 95°C for 5 s, 60°C for 34 s (39 cycles), 95°C for 15 s (one cycle), and cooling at 4°C. Quantity One software (CFX Manager; Bio-Rad, California, USA) was used for quantification analysis.

ELISA

PFC, NAc, and HIP samples were obtained from the second subset of mice. Brain issues were first weighed, then added normal saline according to a certain proportion (1 mg: 25 μl), homogenized by Soniprep (BioSpec Products, Inc., Bartlesville, USA), and supernatants were extracted after centrifugation. NMDAR1 and D2R levels were measured using a mouse enzyme-linked immuno-sorbent assay (ELISA) Kit (Shanghai Hushang Biotechnology, Shanghai, China) following standard procedures described in a previous study [15]. First, the prepared sample and the standard were placed in separate plate wells and incubated for 30 min at 37°C. Second, the plate was washed four times with wash solution, horseradish peroxidase conjugate reagent was added, and the solution was incubated for 30 min at 37°C. Finally, after the plate was washed four more times, we added chromogen solutions A and B. After 15 min of incubation at 37°C, the reaction was stopped using a stop solution. The optical densities of the samples were determined at 450 nm using a Metertech microplate reader (BioTek Instruments, Winooski, Vermont, USA) after the reader was zeroed using a well containing the blank solution. The concentration of each sample was then extrapolated from a standard curve. Each measurement was performed in duplicate. The variations between duplicate values were less than 5%.

Statistics

Data are presented as mean ± SEM. We used one-way analysis of variance to analyze all data, except for the latency times in the MWMT, which were analyzed using repeated-measures tests. P value of less than 0.05 was considered to be significant.

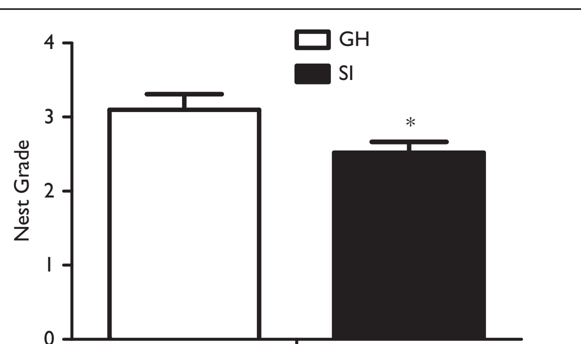
Results

SI elicited schizophrenia-like behavior. It significantly affected performance on the nest-building test, anxiety, spatial memory, and PPI, but had no effect on total locomotion or startle amplitude.

Effects of SI on nest-building

Nest-building behavior is a reflection of social behavior. Deficits in nest-building are considered to represent a negative phenotype of schizophrenia. GH mice were successful in building nests, whereas SI mice had poor nest-building ability (Fig. 1, $F = 5.260$, $P = 0.034$).

Fig. 1



Nest-building test. SI mice have deficits in nest building ($F = 5.260$, $P = 0.034$). GH, group-housed; SI, social isolation. $^*P < 0.05$.

Effects of SI on the OFT and the EPMT

In the OFT, SI mice covered significantly shorter distances than GH mice in the center zone (Fig. 2, $F = 12.717$, $P = 0.03$). In addition, the numbers of entries and time in the unprotected open arm of the EPMT showed no significant difference between the SI and GH mice (Fig. 3). SI induced anxiety-like behavior in mice according to the distances in the center zone.

Effects of SI on spatial working memory

Spatial working memory deficits were evaluated using the TMT and the MWMT. The natural tendency of mice to alternate their arm entries is used to evaluate working memory. In the T maze, SI mice showed a significant reduction in alternation (Fig. 4a, $F = 34.564$, $P = 0.000$). In the third probe test of the MWMT, the SI mice made markedly fewer crossings over the platform area (Fig. 4b–d, $F = 7.940$, $P = 0.014$). This suggests a spatial working memory deficit in SI mice.

Effects of SI on PPI

PPI of the acoustic startle reflex is a measure of sensorimotor gating. SI mice were significantly impaired in PPI, although they had normal startle reflex amplitudes (Fig. 5, $F = 4.626$, $P = 0.049$).

Effects of SI on HINT1, NMDAR1, and D2R mRNA and protein levels in the PFC, NAc, and HIP

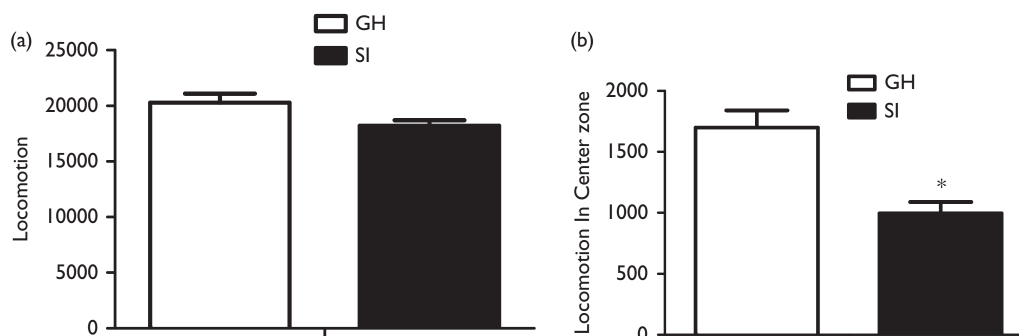
To investigate the possible causes underlying neurochemical alterations in the brain in schizophrenia, we evaluated the transcription and translation levels of HINT1, the NR1 subunit of NMDAR, and D2R using RT-PCR and ELISA. Both HINT1 mRNA and protein levels were reduced in the PFC and NAc, whereas they were increased in the HIP following SI (Fig. 6a, PFC: $F = 11.141$, $P = 0.015$; NAc: $F = 6.344$, $P = 0.045$; HIP: $F = 7.3$, $P = 0.035$; Fig. 6b, PFC: $F = 20.148$, $P = 0.002$; NAc: $F = 18.918$, $P = 0.002$; HIP: $F = 9.761$, $P = 0.014$). In SI mice, mRNA levels of the NR1 subunit of NMDAR were consistent with the decreased protein levels (Fig. 7a, $F = 6.771$, $P = 0.026$; Fig. 7b, $F = 7.036$, $P = 0.038$) in the PFC. Interestingly, SI elicited upregulation of D2R mRNA and protein levels in the NAc (Fig. 8a, $F = 5.688$, $P = 0.044$; Fig. 8b, $F = 6.092$, $P = 0.043$).

Discussion

Here, we show that SI mice have a wide range of schizophrenia-related deficits, including not only aggressive behavior, which has been described in our previous study [10], but also social withdrawal, anxiety, impairment of cognition, and sensorimotor gating disturbances. Therefore, SI may be considered a robust model for early life stress as it evokes schizophrenia-related behavior in mice. As predicted, SI induced not only robust schizophrenia-liked symptoms but also led to neurochemical deficits related to schizophrenia.

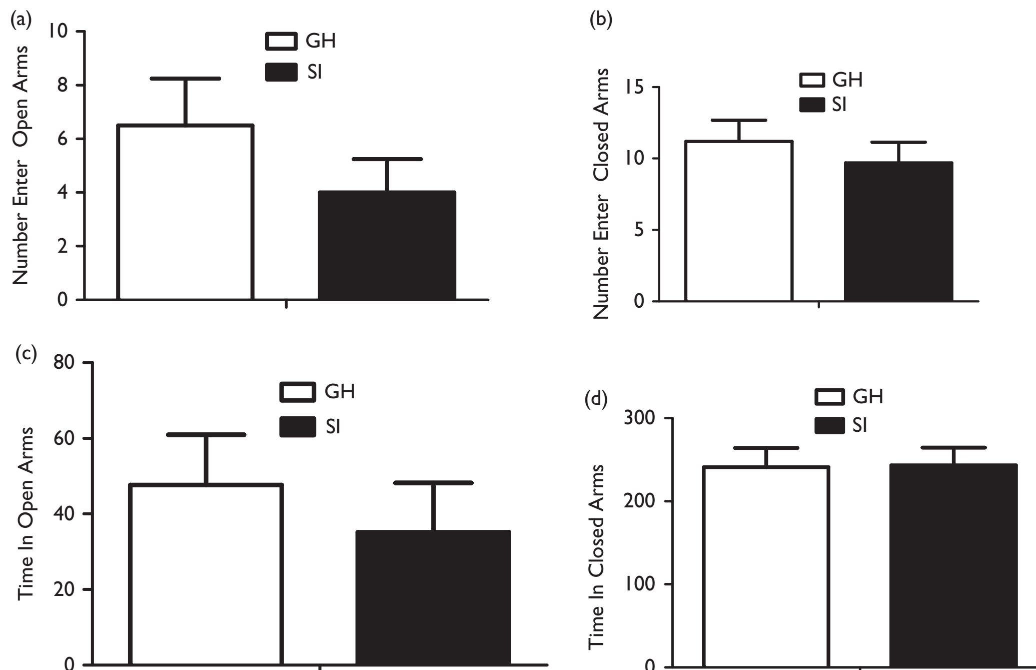
We observed that HINT1 gene and protein expression levels were decreased in the PFC and increased in the HIP in SI mice, consistent with our previous study [13]. The mRNA and protein levels of the NR1 subunit of NMDAR were downregulated in the PFC following SI,

Fig. 2



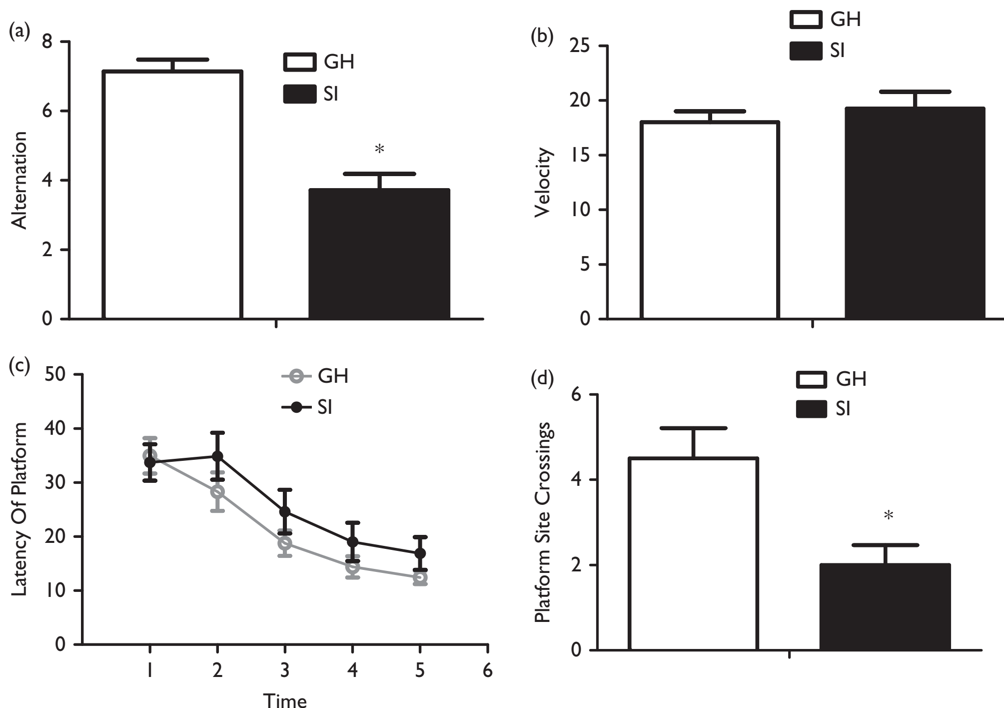
Open-field test. (a): Total distance moved in 60 min; (b): Distance moved in the center zone ($F = 12.717$, $P = 0.03$). GH, group-housed; SI, social isolation. $^*P < 0.05$.

Fig. 3



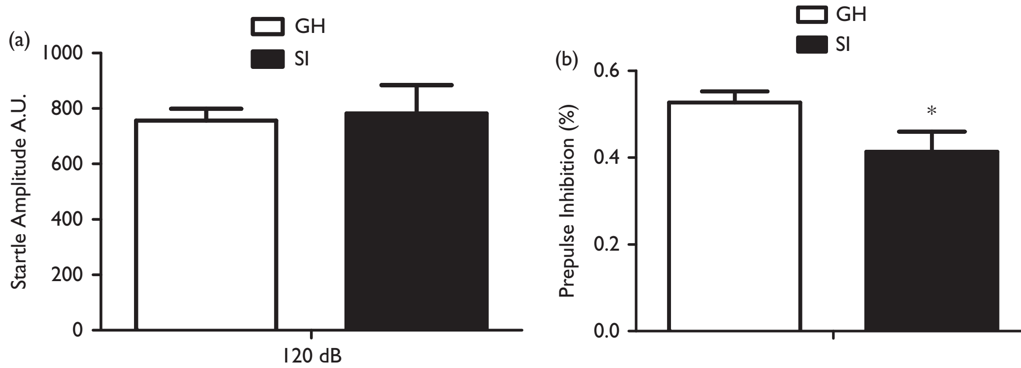
Elevated plus maze. (a): Number of entries into open arms in 6 min; (b): Number of entries into closed arms in 6 min; (c): time in open arms in 6 min; (d): Time in closed arms in 6 min. GH, group-housed; SI, social isolation.

Fig. 4



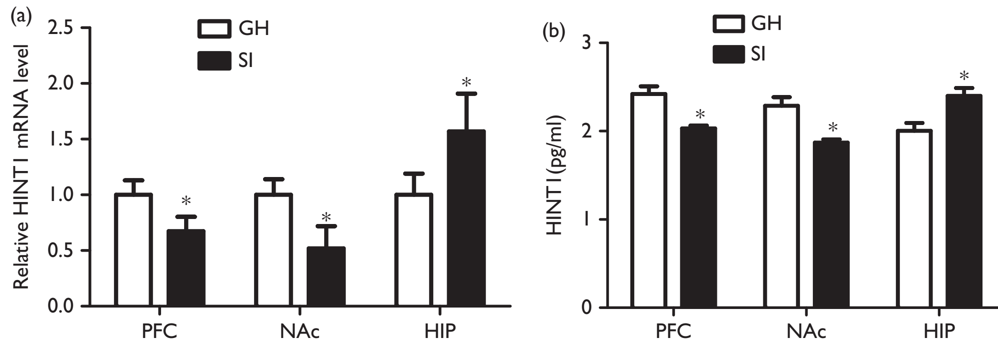
Cognition tests. (a): Spontaneous alternations on the T maze. ($F=34.564$, $P=0.000$) (b–d). Morris water-maze test. (b): Swimming velocity on the pretest day. (c): Latency to platform during the spatial learning training phase. (d): Platform site crossings on the probe test day ($F=7.940$, $P=0.014$). GH, group-housed; SI, social isolation. *$P < 0.05$.

Fig. 5



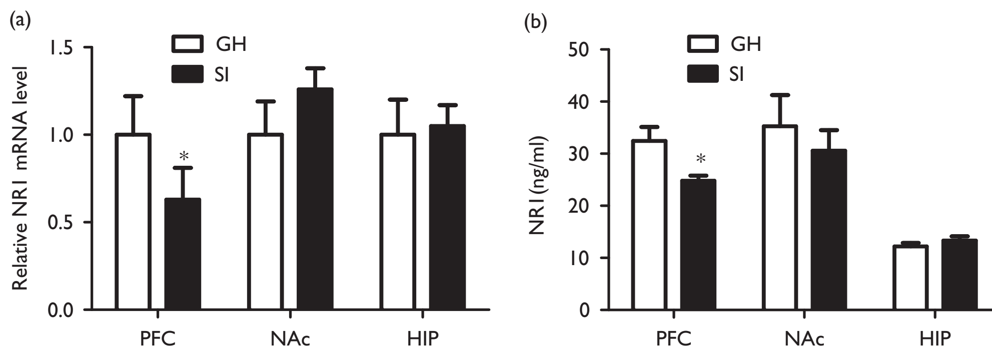
Prepulse inhibition test. (a): Average absolute startle response (120 dB); (b): Relative PPI% ($F = 4.626$, $P = 0.049$). GH, group-housed; PPI, prepulse inhibition; SI, social isolation. $^{*}P < 0.05$.

Fig. 6



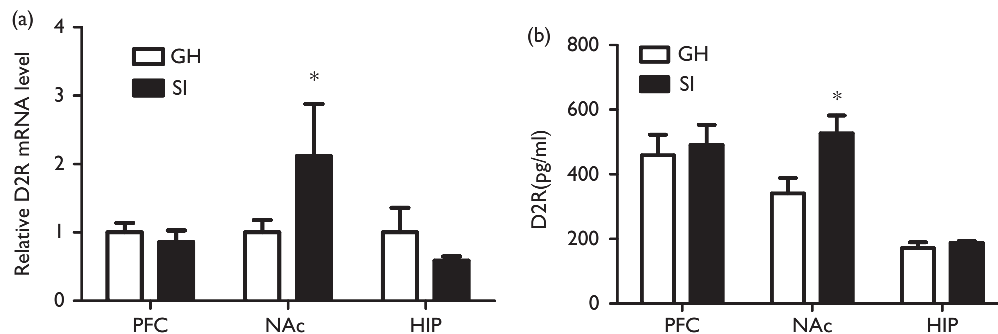
Expression of HINT1. (a): Relative HINT1 mRNA levels (PFC: $F = 11.141$, $P = 0.015$; NAc: $F = 6.344$, $P = 0.045$; HIP: $F = 7.3$, $P = 0.035$); (b): relative HINT1 protein levels (PFC: $F = 20.148$, $P = 0.002$; NAc: $F = 18.918$, $P = 0.002$; HIP: $F = 9.761$, $P = 0.014$). GH, group-housed; HINT1, histidine triad nucleotide binding protein 1; HIP, hippocampus; NAc, nucleus accumbens; PFC, prefrontal cortex; SI, social isolation. $^{*}P < 0.05$.

Fig. 7



Expression of NR1. (a): Relative NR1 mRNA levels ($F = 6.771$, $P = 0.026$); (b): relative NR1 protein levels ($F = 7.036$, $P = 0.038$). GH, group-housed; HIP, hippocampus; NAc, nucleus accumbens; PFC, prefrontal cortex; SI, social isolation. $^{*}P < 0.05$.

Fig. 8



Expression of D2R. (a): Relative D2R mRNA levels ($F = 5.688$, $P = 0.044$); (b): relative D2R protein levels ($F = 6.092$, $P = 0.043$). D2R, dopamine type 2 receptor; GH, group-housed; HIP, hippocampus; NAc, nucleus accumbens; PFC, prefrontal cortex; SI, social isolation. $^{*}P < 0.05$.

similar to the levels of HINT1. D2R mRNA and protein levels were markedly increased after SI in the NAc. We observed low NR1 expression in the PFC and high D2R expression in the NAc, which is consistent with the prevailing hypothesis [16]. Several lines of evidence indicated that mesolimbic DA projections from the ventral tegmental area to the NAc, which is a part of the limbic system, are involved in the development of delusions, hallucinations, and aggression in schizophrenia [6,17]. Hypofunction of NMDAR in the PFC may weaken the inhibition of GABA interneurons, which connect to the mesolimbic dopamine pathway. This may lead to excessive dopaminergic activity in mesolimbic areas [18]. These phenomena suggest that there is a potential relationship between HINT1, NR1, and D2R in the pathogenesis of schizophrenia.

The HINT1 gene is located in the *5q31.2* genetic locus, which is a region associated with schizophrenia [19]. Fine mapping of chromosome *5q22–33* indicates that the SPEC2/PDZ-GEF2/ACSL6 haplotype is associated with schizophrenia. Interestingly, the HINT1 gene is located in this region [20]. In a microarray study, HINT1 gene expression was shown to be decreased in the dorsolateral prefrontal cortex in patients with schizophrenia [21]. Since then, a number of post-mortem and patients studies have suggested the presence of an association between HINT1 and schizophrenia [10,11,22]. However, Barbier *et al.* [23] reported that HINT1 knockout mice have abnormal postsynaptic DA transmission, which indicates that the absence of HINT1 leads to abnormal DA transmission associated with schizophrenia. Recent studies have shown that HINT1 is involved in the reciprocal regulation between G-protein-coupled receptors (GPCRs) and NMDARs [24]. Peptide-mapping experiments have shown that HINT1 interacts with the NR1 unit of the NMDAR, which contains the C1 (C-terminus) segment presented in the cell membrane [14]. Meanwhile, some research suggests that HINT1 can enhance μ-opioid receptors or dampen cannabinoid 1 receptors through the activity of GPCRs with NR1 subunits [14,24]. HINT1 may thus serve as a scaffold for signaling proteins that work together to couple GPCR activity with that of glutamate NMDARs. These findings suggest that there seems to be an association that has not yet been elucidated among HINT1, NR1, and D2R. Because of the cross-sectional nature of the present study, we cannot arrive at definitive conclusions on the direction of these effects. However, it seems more logical to assume that HINT1 interacts with NR1 and D2R in the course of schizophrenia. The interaction among the three proteins is only now being investigated and will become a new field of study in the future.

Conclusion

Here, we report that mice under SI rearing conditions have a series of schizophrenia-related deficits, such as social withdrawal, anxiety disorder, cognitive impairments, sensorimotor gating disturbances, and aggressive behavior, as indicated in our previous studies [10]. These abnormalities coincide with changes in HINT1, NR1, and D2R in the PFC, NAc, and HIP. The crosstalk among HINT1, NR1, and D2R may underlie schizophrenia-related deficits, but further mechanisms should be explored. The potential relationship between these proteins might provide a new direction for therapeutics for schizophrenia.

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Conflicts of interest

There are no conflicts of interest.

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