Increased hippocampal Disrupted-In-Schizophrenia 1 expression in mice exposed prenatally to lead*

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Abstract
Disrupted-In-Schizophrenia 1 is a susceptibility gene for schizophrenia and other psychiatric disorders. Developmental lead exposure can cause neurological disorders similar to hyperactivity disorder, dyslexia and schizophrenia. In the present study, we examined the impact of developmental lead exposure, administered in vitro and in vivo, on hippocampal Disrupted-In-Schizophrenia 1 expression. Our results show that in cultured hippocampal neurons, in vitro exposure to 0.1–10 µM lead, inhibited neurite growth and increased Disrupted-In-Schizophrenia 1 mRNA and protein expression dose-dependently. In addition, blood lead levels in mice were increased with increasing mouse maternal lead (0.01–1 mM) exposure. Hippocampal neurons from these mice showed a concomitant increase in Disrupted-In-Schizophrenia 1 mRNA and protein expression. Overall our findings suggest that in vivo and in vitro lead exposure increases Disrupted-In-Schizophrenia 1 expression in hippocampal neurons dose-dependently, and consequently may influence synapse formation in newborn neurons.

Key Words
lead exposure; Disrupted-In-Schizophrenia 1; hippocampus; neuron; neurotoxicity; synapse; neural regeneration

Research Highlights
(1) Our study examined neurotoxicity induced following lead exposure from the aspect of a predisposing factor for psychiatric disease.
(2) Our study is the first to determine the influence of lead exposure on Disrupted-In-Schizophrenia 1 expression in hippocampal neurons.
(3) Our results indicate that lead exposure dose-dependently increases Disrupted-In-Schizophrenia 1 expression and consequently may influence synaptogenesis in newborn neurons.

Abbreviations
DISC1, disrupted-In-Schizophrenia 1; MAP-2, microtubule-associated protein 2; APP, amyloid precursor protein
INTRODUCTION

Decades of research has shown that lead (Pb) exposure affects most organ systems including the liver⁴¹, kidneys⁴² and reproductive systems⁴³. In the nervous system, lead exposure can lead to a variety of neurological disorders. In children and adolescents, blood lead levels (≥100 µg/L) are significantly associated with lower IQs⁴⁴, learning deficits⁴⁵, antisocial behavior⁴⁶, mental retardation⁴⁷, attention deficit hyperactivity disorder and schizophrenia⁴⁸. Moreover, the effects remain for a long period after children are removed from lead contact and can persist into adulthood. Neuronal exposure to lead causes oxidative stress⁴⁹, excitotoxicity⁵⁰, DNA damage⁵¹, and at higher levels, can result in neuronal death⁵². However, the mechanisms underlying lead-induced neurotoxicity remain poorly understood.

Disrupted-In-Schizophrenia 1 (DISC1) is a susceptibility gene for major psychiatric disorders, including schizophrenia⁵³, bipolar disorder and recurrent major depression⁵⁴. DISC1 regulates multiple aspects of embryonic and adult neurogenesis. During adult neurogenesis, DISC1 controls the timing of neural progenitor cell cycle exit, as well as multiple processes involved in development of newborn neurons⁵⁵-⁵⁶. DISC1 knockdown in newborn dentate granule cells of the adult hippocampus leads to soma hypertrophy, accelerated axon and dendrite development, neuronal mispositioning due to an over-extended migration, enhanced intrinsic excitability and accelerated synapse formation of new neurons⁵⁷. These findings indicate that DISC1, a schizophrenia susceptibility gene, serves as a key regulator for controlling the tempo of neuronal development and thereby regulates the process of new neuron integration in the adult brain.

The hippocampus is a brain region important in the acquisition and consolidation of certain forms of learning and memory, especially those involved in place and spatial learning⁵⁸. The disruption of hippocampal function by a variety of methods, produces deficits in behavioral paradigms that assess these forms of learning and memory⁵⁹-⁶⁰. It has been shown that the direct injection of lead into the hippocampus of normal adult rats, produces a deficit in the acquisition of learning in the water maze task⁶¹. Thus, the hippocampus is susceptible to lead effects if a sufficient dosage of lead reaches the brain. Because of the overlapping physiological and pathophysiological function of lead and DISC1 in the hippocampus, we sought to determine whether DISC1 is mechanistically involved in lead neurotoxicity. Therefore, we examined the effects of in vivo and in vitro lead exposure on hippocampal DISC1 mRNA and protein levels.

RESULTS

In vitro study

Chronic lead exposure inhibited growth of cultured hippocampal neurons

Microtubule-associated protein 2 (MAP-2) is a cytoskeletal protein, commonly used as a neuronal marker⁶². MAP-2 immunofluorescence in cultured hippocampal neurons, showed that compared with control, lead-exposed (0.1–10 µM) primary neurons grew poorly, often with abnormal nuclei or soma and inhibited neurite growth (Figure 1).

Chronic lead exposure increased DISC1 protein expression in cultured hippocampal neurons

DISC1 western blot analysis showed that the addition of lead acetate (0.1–10 µM) to primary hippocampal neurons, induced a dose-dependent increase in DISC1 protein level (Figure 2A), with an approximately 3-fold increase in DISC1 expression following 10 µM of lead acetate exposure (Figure 2B). Compared with the control group, DISC1 expression levels were significantly increased following 1 µM and 10 µM of lead acetate exposure (P < 0.05).

Chronic lead exposure increased DISC1 mRNA expression in cultured hippocampal neurons

Quantitative real-time PCR confirmed our observed increase in DISC1 protein expression, with an increase in
DISC1 mRNA levels detected following lead acetate treatment. Similar to our protein expression data, the relative mRNA expression ratio of DISC1/β-actin in the 1 μM and 10 μM lead acetate groups were statistically increased compared with the control group (P < 0.05; Figure 2C).

In vivo study

Quantitative analysis of experimental animals

Our in vivo study used 40 pregnant mice which were randomly assigned to four groups. They were given water containing lead acetate, either low-dose lead (0.01 mM), moderate-dose lead (0.1 mM) or high-dose lead (1 mM), until the end of the experiment. The control group was given no lead acetate. Mice at postnatal day 21 (P21) were used for experiments.

Blood lead levels of P21 mice

The blood lead levels (mean ± SD) for each group were 12.6 ± 7.3 μg/L in controls, 68.8 ± 26.1 μg/L in low-dose lead group, 381.9 ± 40.7 μg/L in moderate-dose lead group and 1 649.0 ± 140.8 μg/L in high-dose lead group. These results demonstrate that the blood lead levels are related to the degree of lead exposure (n = 5, P < 0.05). Overall, developmental lead exposure resulted in a significant increase in blood lead level in both moderate and high lead acetate mice.

Chronic lead exposure increased hippocampal DISC1 protein expression in weanling mice

Western blot analysis revealed an increase in DISC1 protein expression in lead-treated (moderate- and high-dose) mice compared with controls (Figure 3A). Relative to control hippocampal tissues, DISC1 protein expression levels were significantly increased in the moderate and high-dose lead groups (P < 0.05; Figure 3B). There was a slight, albeit non-significant, increase observed in the low-dose lead group also.

Chronic lead exposure increased hippocampal DISC1 mRNA expression in weanling mice

Quantitative real-time PCR showed that the relative mRNA expression ratio of DISC1/β-actin in the low-dose, moderate-dose and high-dose lead groups were all statistically increased, compared with the control group (P < 0.05; Figure 3C). The mRNA transcription of DISC1 started to increase with the lowest (0.01 mM) lead acetate exposure. Immunohistochemical examination of DISC1 staining in
the hippocampus also identified increased expression of DISC1 protein after lead exposure in vivo (Figure 4). Both control and lead-exposed groups expressed positive granules, stained brown yellow, however, compared with the control group, following chronic lead exposure, DISC1 staining in the hippocampus was darker with an increase in the number of positive granules.

![Image](image_url)

**Figure 4** Disrupted-In-Schizophrenia 1 (DISC1) expression in the hippocampus of weanling mice exposed prenatally and lactationally to lead (immunohistochemical staining with light microscopy, x 400). (A) Control group; (B) 1 mM lead acetate group. Hippocampal neurons positive for DISC1 are stained brown-yellow. Compared with the control group, lead-exposed groups show an increase in the number of DISC1-positive neurons and a darker staining color, indicating increased DISC1 expression.

### DISCUSSION

Lead has well characterized effects on the nervous system, leading to cognitive, behavioral, and physical impairments\[28\]. It has been reported that the effects of lead exposure on damaging or disrupting the developing central nervous system are associated with schizophrenia and related disorders\[8, 24\]. Genetic association and linkage studies have shown that the DISC1 gene is implicated in schizophrenia in independent populations\[29, 26\]. Therefore, we hypothesized that DISC1 may be involved in lead-induced developmental neurotoxicity. Consistent with our hypothesis, we found that lead-up-regulated DISC1 expression in primary hippocampal neurons in a dose-dependent manner. In addition, the observed increase in DISC1 expression occurred at the transcriptional level, with DISC1 mRNA also increased dose-dependently in response to lead acetate treatment. Lead-induced neurotoxicity was shown using MAP-2 as a neuronal marker. The full-length DISC1 protein contains 854 amino acids, with multiple coiled coil motifs, and is located in the nucleus, cytoplasm and mitochondria\[27\]. It is a multifunctional, neurodevelopmentally regulated scaffold protein that is involved in neurite outgrowth, neural positioning, and hippocampal and cortical development, through its interaction with other proteins\[29\]. It has been reported that DISC1 mRNA levels are increased in the hippocampus of schizophrenia patients\[29\]. Similarly, our study shows an increase in hippocampal DISC1 expression in weanling mice exposed to lead. Increased DISC1 expression in hippocampal tissue was observed after lead exposure in vivo. Consistent with our in vitro results, DISC1 mRNA also increased dose-dependently in response to lead acetate treatment. These results indicate that the high concentration of DISC1 in the intracellular space may be important in postnatal hippocampal neurogenesis. Thus, in our study both in vitro and in vivo experiments show that lead exposure increases expression of DISC1 mRNA and protein, with a correlation between DISC1 expression and dose-dependent lead exposure.

At present, we do not know the mechanistic link between lead exposure and increased neuronal DISC1 expression. It is feasible that lead exposure increases expression of DISC1 mRNA and protein via increased amyloid precursor protein (APP) mRNA expression. It has been reported that lead exposure in rodents during the postnatal period results in a transient increase in APP mRNA during the first month\[30\]. Recently it was shown that DISC1 acts downstream of APP to regulate cortical precursor cell migration\[31\]. Namba and colleagues reported that NMDA receptor (NMDAR) antagonists lead to a decrease in DISC1 expression\[32\]. The effect of lead exposure during development on NMDAR subunit expression has been documented in rodent models, and show that exposure to environmentally relevant levels of lead is effective in producing marked changes in NMDAR subunit gene expression in the developing as well as the mature brain\[33\].

Several studies suggest DISC1 serves as a key intrinsic modulator of AKT signaling, in regulating the morphogenesis and tempo of dendritic development of new neurons in the adult brain\[34\]. Furthermore, it has been reported that the phosphoinositide 3-kinase/AKT pathway participates in the lead-induced death of primary cultured rat hippocampal neurons\[12\]. Moreover, AKT signaling has been implicated in the morphogenesis and dendritic development of postmitotic dentate granule cells in the adult hippocampus\[34\]. Thus, we hypothesize that DISC1 may be involved in lead-induced developmental neurotoxicity mediated via the AKT signaling pathway.

In conclusion, our findings demonstrate that lead exposure, both in vitro and in vivo, induces transcriptional and translational upregulation of DISC1, implicating a critical role for DISC1 expression in lead-induced neuronal neurotoxicity. Further studies are needed to investigate if DISC1 regulates the death of primary cultured hippocampal neurons via AKT, a key serine/threonine specific kinase with multiple signaling properties that is already
implicated in lead toxicity.

**MATERIALS AND METHODS**

**Design**
A randomized, controlled animal study and comparative observation of *in vitro* cytology.

**Time and setting**
The experiments were performed in the Laboratory of Biochemistry and Molecular Biology, China Medical University, China in December 2011.

**Materials**
A total of 60 adult mice (male:female = 1:2) were used for the *in vivo* study. Five newborn mice (18-day-old Kunming) were used for preparing hippocampal neurons. All mice were provided by the Animal Research Center of China Medical University (license No. SCXK (Liao) 2003-0009). All procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China.[35]

**Methods**

**Culture and treatment of primary hippocampal neurons**
Mouse hippocampal neurons, obtained from 18-day-old Kunming mice embryos, were prepared as described previously.[36] Cells were maintained in Neurobasal medium (Invitrogen, Carlsbad, CA, USA) containing 2% B27 supplement, 0.5 mM glutamine, 100 units/mL penicillin and 100 mg/mL streptomycin, in a humidified atmosphere of 5% CO₂, 95% air at 37°C. To inhibit further proliferation of non-neuronal cells, 10 µM cytosine arabinoside (Invitrogen) was added to the medium at 48 hours. Half the medium was replaced with freshly prepared medium, of the same composition, every 4 days. After 7 days of incubation, lead acetate (Sigma, St. Louis, MO, USA) was added to the medium at 48 hours. Half the medium was replaced with freshly prepared medium, of the same composition, every 4 days. After 7 days of incubation, lead acetate (Sigma, St. Louis, MO, USA) was added to the medium at the appropriate concentration (10, 1, 0.1 and 0 µM) for 14 days. Neuronal growth was recorded during the entire incubation period (21 days).[24]

**MAP-2 immunofluorescence staining in hippocampal neurons**
After 21 days of incubation, hippocampal neurons were fixed with cold methanol at −20°C for 30 hours, permeabilized with acetone at −20°C for 6 minutes and blocked in 5% bovine serum albumin in PBS. Hippocampal neurons were then incubated overnight at 4°C in a humidity chamber with primary rabbit anti-MAP-2 polyclonal antibody (1:100; Abcam, Cambridge, MA, USA). After washing, a TRITC-conjugated goat anti-rabbit IgG (1:2 000; Keygentec, Nanjing, Jiangsu Province, China) was applied for 1 hour. Following PBS washes, DAPI (1:1 000; Beyotime, Beijing, China) was incubated for 15 minutes. Coverslips were mounted onto slides for observation, and cells were examined using a LEICA LSM510 confocal microscope (Heidelberg, Germany).

**In vivo lead exposure**
Mice were housed in a ratio of 2:1 of female: male. Mice with vaginal plugs were identified (checked daily), and then housed separately and considered to be pregnant (gestational day 0). Forty pregnant female mice were housed singly with free access to food and water in temperature-controlled conditions with a 12 hour light/dark cycle. Mice were randomly and equally divided into four groups and given water containing 0 mM (control), 0.01 mM (low), 0.1 mM (moderate) and 1 mM (high) lead acetate (lead acetate, 99–100%, Sigma).[37] Each group of mice received the same treatment throughout the study, ensuring offspring were exposed to lead throughout gestation until P21. Postnatal lead exposure was through the dam’s milk. P21 was chosen as the endpoint, as at this time, the mice were considered to be adult.

**Determination of lead concentrations in blood samples**
Blood lead levels were analyzed at weaning to evaluate the accumulated lead content at the end of the experimental lead exposure period. Blood samples were collected from the fossa orbitalis of the pups on P21. The blood samples (200 µL) were mixed with 0.1 N nitric acid (2.5 mL) and incubated overnight. Next, 10% trichloroacetic acid (200 µL) was added by vortexing for 10 seconds, followed by centrifugation for 15 minutes at 3 000 r/min at room temperature. The supernatants were collected for further analysis. Blood lead levels were measured using a Thermo Elemental M6 series atomic absorption spectrophotometer (Thermo Elemental, Franklin, MA, USA).

**Quantitative real-time PCR of DISC1 mRNA expression in hippocampal neurons and tissues**
DISC1 mRNA levels in cultured hippocampal neurons and hippocampal tissue from P21 mice, were determined by quantitative real-time PCR. Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer’s instructions. In a 20 µL of final volume, 5 µg of RNA was used for first strand cDNA synthesis, with Oligo dT (provided in the kit). The cDNA mixture (4 µL) was used to amplify mRNA for DISC1 and β-actin (loading control) by quantitative
real-time PCR. Quantitative real-time PCR was performed using SYBR Premix Ex TaqII (Takara, Tokyo, Japan). All reactions were conducted in triplicate. The primers used for DISC1 were 5′-TGC TGG AAG CCA AGA TGC TG-3′ for the sense, and 5′-CCT GGA GCT GAG TGC CAA CA-3′ for the antisense strands, producing a 140 bp product. Primers used for β-actin were 5′-CCC ATC TAC GAG GCT TAT-3′ for the sense, and 5′-TGT CAC GCA CGA TTT CC-3′ for the antisense strands, producing a 145 bp product. PCR conditions were 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 34 seconds. Reactions were processed and analyzed on an ABI 7500 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). Analysis on the ABI 7500 Real-Time PCR System was also performed using the manufacturers recommended protocol.

Western blot analysis of DISC1 protein expression in hippocampal neurons and tissues

Cultured neurons and hippocampal tissues were harvested in ice-cold PBS and then lysed in 100 µL lysis buffer. Protein content was determined by BCA assay (Bio-Rad Laboratories, Hercules, CA, USA). Protein was separated on a 10% polyacrylamide gel, transferred onto polyvinylidene difluoride membranes and blocked with 5% nonfat milk in Tris-buffered saline with Tween 20. Membranes were incubated with primary antibodies (rabbit anti-DISC1 polyclonal antibody, 1:1 000, Abcam; rabbit anti-β-actin polyclonal antibody, 1:1 000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After washing, membranes were incubated with a peroxidase-conjugated goat anti-rabbit IgG (1:2 000; Keygentec) for 1.5 hours at room temperature. Enhanced chemiluminescence (Amersham Biosciences, Freiburg, Germany) was used for detection of protein bands, specifically using the western blot detection kit with enhanced chemiluminescence. Absorbance of DISC1 bands was normalized to the corresponding β-actin signals using Scion Image (IBM, Armonk, NY, USA).

Immunohistochemical staining of DISC1 in the hippocampus

Three pups from each group were selected for histological analysis. The whole brain was dissected and placed in 4% paraformaldehyde in PBS, for 2 hours at 4°C, and then in 30% sucrose in PBS, for cryoprotection, at 4°C overnight. Paraffin-embedded hippocampi were sectioned (5 µm) using a microtome. Sections were collected onto poly-L-lysine-coated slides and allowed to air dry. Tissue sections were treated by microwave in 10 mM citrate buffer (pH 6) for 3 minutes, followed by blocking of non-specific binding by incubation in PBS containing 3% normal goat serum. Sections were then incubated overnight at 4°C with rabbit anti-mouse DISC1 polyclonal antibody (1:100; Abcam). After washing, the sections were incubated with biotinylated goat anti-rabbit IgG (1:100; Santa Cruz) for 1 hour at room temperature. For negative controls, the primary antibody was omitted. Sections were examined using a light microscope (Olympus, Tokyo, Japan).

Statistical analysis

All data were derived from three independent experiments and are presented as mean ± SD. Statistical analysis for comparison of mean values was performed by one-way analysis of variance followed by Dunnett’s post test, using SPSS 13.0 (SPSS, Chicago, IL, USA). P < 0.05 was considered statistically significant.

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Author contributions: Liguang Sun designed and guided the study. Yuanyuan You contributed to statistical analysis and wrote the manuscript. All authors conducted the experiments.

Conflicts of interest: None declared.

Ethical approval: The study was approved by the Animal Ethical Committee of China Medical University.

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