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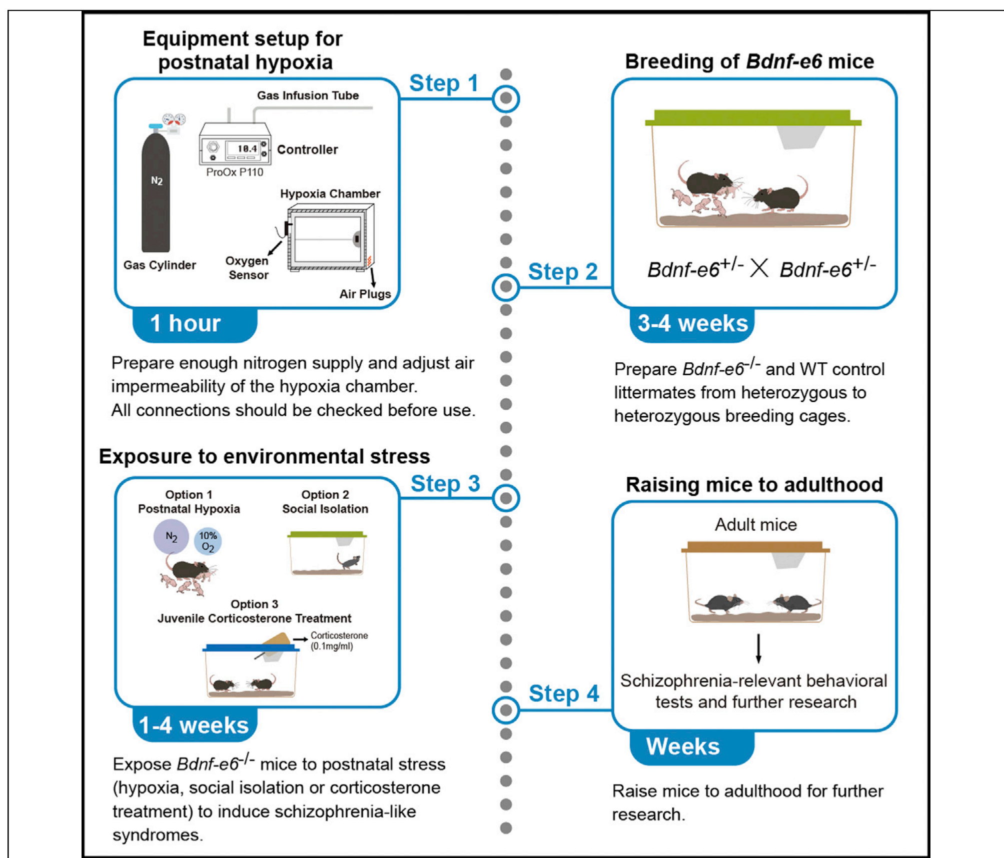
Protocol

A protocol for establishing a male G×E schizophrenia mouse model



Schizophrenia pathogenesis involves both genetic and environmental factors (G×E). Here, we present a protocol to prepare a schizophrenia rodent model with a specific G×E pair. We describe the breeding of *Bdnf*-e6−/− mice with genetic deficiency in promoter-VI-driven BDNF expression. We then detail the procedure to expose the mice to postnatal environmental stress including hypoxia, social isolation, and corticosterone. This model better represents the etiology of schizophrenia and thus may facilitate basic research and drug development for schizophrenia.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Tianyi Zhang, Shangjin Li, Fan Mei, He You, Yanghui Chen, Feng Yang, Bai Lu

bai_lu@tsinghua.edu.cn

Highlights

This protocol illustrates a new schizophrenia rodent model based on G × E interactions

Bdnf-e6 deficiency mouse breeding is described in detail

Steps to expose mice to three types of postnatal environmental stress are described in detail

This G×E schizophrenia model expresses a comprehensive set of schizophrenia phenotypes

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A protocol for establishing a male G×E schizophrenia mouse model

Tianyi Zhang,[1,5,6] Shangjin Li,[1,5] Fan Mei,[4] He You,[1] Yanghui Chen,[1] Feng Yang,[2,3] and Bai Lu[1,3,7,*]

[1]School of Pharmaceutical Sciences, IDG/McGovern Institute for Brain Research, Tsinghua-Peking Joint Center for Life Sciences, Tsinghua University, Room B303, Beijing 100084, China

[2]China National Clinical Research Center for Neurological Diseases, Beijing Tiantan Hospital, Capital Medical University, Beijing 100070, China

[3]Advanced Innovation Center for Human Brain Protection, Capital Medical University, Beijing 100070, China

[4]Institute of Systems Biomedicine, Department of Pathology, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100191, China

[5]These authors contributed equally

[6]Technical contact: zhangty17@mails.tsinghua.edu.cn

[7]Lead contact

*Correspondence: bai_lu@tsinghua.edu.cn
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SUMMARY

Schizophrenia pathogenesis involves both genetic and environmental factors (G×E). Here, we present a protocol to prepare a schizophrenia rodent model with a specific G×E pair. We describe the breeding of *Bdnf*-e6−/− mice with genetic deficiency in promoter-VI-driven BDNF expression. We then detail the procedure to expose the mice to postnatal environmental stress including hypoxia, social isolation, and corticosterone. This model better represents the etiology of schizophrenia and thus may facilitate basic research and drug development for schizophrenia.
For complete details on the use and execution of this protocol, please refer to Chen et al. (2022).[1]

BEFORE YOU BEGIN

Current schizophrenia models established by pharmacological treatment,[2] neurodevelopmental intrusion,[3] or genetic deficiency[4] have various limitations in terms of face validity (exhibits core schizophrenia-like symptoms), construct validity (is developed based on human disease mechanisms), and predictive validity (exhibits antipsychotic effectiveness and utility in discovering new therapeutics).[5] Thus, basic research and drug development on schizophrenia have been significantly hampered by the lack of appropriate animal models.

Schizophrenia is a complex psychiatric disease that involves both genetic and environmental factors (G×E).[6] Therefore, a schizophrenia model based on G×E interaction should better replicate the etiology and pathophysiology of human schizophrenia. Brain-derived neurotrophic factor (BDNF) has long been thought to be involved in schizophrenia development.[7] Serum BDNF level in schizophrenia patients is significantly lower than that in healthy control.[8,9] However, *Bdnf* deficiency alone seems not sufficient to induce schizophrenia-like behavior in animals.[10] Hypoxia during development is one of the environmental factors relevant to the risk of schizophrenia.[11] Hypoxia related perinatal complications are associated with doubling of the risk of psychotic disorders in humans.[12] Among schizophrenia patients, hypoxia is associated with greater brain abnormalities.[13] Interestingly, there is an association between two single-nucleotide polymorphisms (SNPs) in human *Bdnf* gene and obstetric complications in relation to schizophrenia.[14] Other environmental factors of

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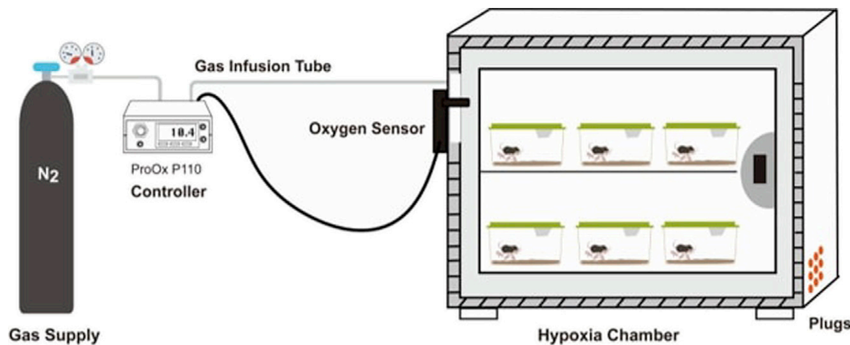

Figure 1. Connection between the N2 gas cylinder, O2 controller/monitor, and hypoxia chamber
During hypoxia treatment, air plugs should be unplugged.

schizophrenia include developmental trauma, minority group position, urban environment, social/economic status, etc.[15]

By using a mouse line with specific disruption of *Bdnf* promoter VI (*Bdnf-e6*$^{-/-}$ mouse) as the genetic factor, and elevated postnatal stress as an environmental factor (i.e., postnatal hypoxia, post-weaning social isolation, or juvenile corticosterone treatment), we have developed a new G×E model for schizophrenia.[1] This new model exhibits core schizophrenia-like endo-phenotypes and therefore may serve as a useful tool for studying pathophysiological mechanisms and testing therapeutics for schizophrenia.

Institutional permissions

All procedures in our study were carried out according to regulatory standards of Institutional Animal Care and Use Committee (IACUC). The experimental design was approved and executed under the supervision of laboratory animal resources center at Tsinghua University. Any use of this protocol should acquire permission from the authority of researchers' home institution.

Equipment setup for postnatal hypoxia

Timing: days

1. Set up for high pressure nitrogen cylinder.
   a. Be sure to tire high-pressure N_2 gas cylinders safely on cylinder racks.

   Note: Use nitrogen (N_2) to create low oxygen (O_2) hypoxia environment.

   Note: The use of high-pressure gas cylinder should comply with the requirements of the experimental environment.

   b. Elicit postnatal hypoxia stress by a consecutive 6-day exposure to 10% oxygen.

   Note: Substitute 10% atmospheric O_2 by N_2. Prepare several full N_2 gas cylinders for continuous N_2 supply.

   Note: We suggest that experimenters figure out how many N_2 gas cylinders will be used to maintain 6 days of hypoxia environment in the hypoxia chamber before experiments.

2. Set up for hypoxia chamber and oxygen concentration controller/monitor.
   a. Connect the high-pressure N_2 gas cylinder and the O_2 concentration controller/monitor (BioSpherix ProOx P110) to the hypoxia chamber (BioSpherix A-30274-P) (Figure 1).

Note: The hypoxia chamber and O_2 concentration controller/monitor can be substituted by other brands or customized equipment.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Mouse: postnatal *Bdnf-e6*$^{-/-}$ male mice (C57/BL6)	Chen et al., 2022.[1]	N/A
Chemicals, peptides, and recombinant proteins		
One Step Mouse Genotyping Kit	Vazyme	PD101-01
Corticosterone	Sigma-Aldrich	Cat# 235135
Ethanol	General Reagent	G73537B
Oligonucleotides		
Bdnf-e6 WT allele; genotyping forward primer (5′→3′):	Sangon Biotech	AATCGAAGCTCAACCGAAGA
Bdnf-e6 WT allele; genotyping reverse primer (5′→3′):	Sangon Biotech	TTTTTCTCTCACACTGAAGGGATT
Bdnf-e6 mutant allele; genotyping forward primer (5′→3′):	Sangon Biotech	AATCGAAGCTCAACCGAAGA
Bdnf-e6 mutant allele; genotyping reverse primer (5′→3′):	Sangon Biotech	TCCAGCTCGACCAGGATG
Other		
Hypoxia chamber	BioSpherix A-30274-P	https://biospherix.com/animal-products/
Oxygen concentration monitor/controller	BioSpherix ProOx P110	https://biospherix.com/animal-products/
Xeye Startle Reflex System	Beijing Macroambition S&T Development Co., Ltd	http://www.bjtmhy.com/
Sociability interaction test apparatus	Custom-made	N/A
Open field test apparatus	Custom-made	N/A
Novel object recognition apparatus	Custom-made	N/A
Software and algorithms		
EthoVision XT	Noldus Information Technology	RRID: SCR_000441
Xeye Startle	Beijing Macroambition S&T Development Co., Ltd	http://www.bjtmhy.com/
Graphpad Prism 8.0	GraphPad software	https://www.graphpad.com

STEP-BY-STEP METHOD DETAILS

Bdnf-e6 heterozygous breeding cage establishment

Timing: 10–16 weeks

This section describes procedure to establish *Bdnf-e6* mice breeding cage and obtain *Bdnf-e6*$^{-/-}$ pups for environmental stress exposure.

This step prepares *Bdnf-e6*$^{-/-}$ and wildtype (WT) control littermates (at least 5 vs 5) for subsequent exposure to environmental stress and behavioral tests. We recommend establishing at least 5 heterozygous to heterozygous breeding cages.

1. Propagation of *Bdnf-e6*$^{+/-}$ mice.
   a. Breed mice carrying *Bdnf-e6* mutant allele with WT mice.
   b. Extract offspring DNA: Cut offspring tail tip tissue at postnatal days 14–21. Use One Step Mouse Genotyping Kit (Vazyme, PD101-01) for DNA isolation and genotyping.
      i. Collect tail tissue to a sterile 1.5 mL centrifugation tube, add 100–200 μL lysis buffer (2% proteinase K) and incubate at 55°C for 8–12 h. Be sure that tail tissue is fully immersed in lysis buffer.
      ii. Centrifugate 1.5 mL tube at 12,000 g for 2 min. Transfer supernatant to 0.25 mL PCR tube and heat at 95°C for 10 min to inactivate the proteinase K in lysis buffer. The lysate will be ready to use for genotyping PCR after 95°C heating.

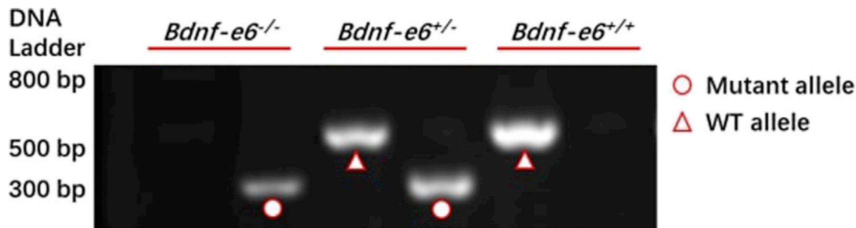

Figure 2. Genotypes of Bdnf-e6 mice revealed by agarose gel electrophoresis

iii. Offspring genotyping: Primers for genotyping are listed in the key resources table. The conditions for PCR are set as follows. Agarose gel electrophoresis of PCR product shows genotypes. Homozygous= ∼ 367 bp, Heterozygous= ∼ 566 bp and 367 bp, WT=∼ 566 bp (Figure 2).

c. Obtain at least 5 male and 10 female *Bdnf-e6*$^{+/-}$ mice for breeding.

PCR reaction master mix

Reagent	Amount
DNA template	1 μL
DNA Polymerase (2×)	5 μL
Forward Primer (100 nM)	0.1 μL
Reverse Primer (100 nM)	0.1 μL
ddH_2O	3.8 μL

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	5 min	1
Denaturation	94°C	30 s	10 cycles
Annealing	59°C	30 s	
Extension	72°C	45 s	
Denaturation	94°C	30 s	25 cycles
Annealing	58°C	30 s	
Extension	72°C	45 s	
Final extension	72°C	10 min	1
Hold	4°C	∞	

2. Establish 5–10 heterozygous to heterozygous *Bdnf-e6* breeding cages.
   a. Co-house male and female *Bdnf-e6* heterozygous mice in one cage. We recommend pairing 1 male with 2 female mice together to get sufficient pups from each breeding cage.
3. Watch for female pregnancy and birth of pups.
   a. Inspect breeding cages daily. Normally female mice will be pregnant after 2–6 weeks of breeding cage establishment. If female mice are not pregnant after 8 weeks, replace the male mouse with a new one. Pups will be born after approximately 3 weeks of pregnancy.

⚠ CRITICAL: *Bdnf-e6* mRNA is highly enriched in placenta tissue. However, whether lack of *Bdnf-e6* in the placenta would affect fetal development has not been investigated. Therefore, we recommend experimenters not to use homozygotes for breeding purposes.

Exposure to environmental stress

Option 1: Postnatal hypoxia

Timing: 1 week (for step 4)

This section describes procedure for postnatal hypoxia environmental stress exposure.

4. Expose female parents and offspring to hypoxia environment from postnatal day 4 to day 10.
   a. Remove male mice from the breeding cages. Place offspring with their female parents (in the original breeding cage, do not change bedding) into hypoxia chamber.
   b. Maintain hypoxia chamber at 10% O_2. Monitor remnant N_2 in cylinder from time to time via pressure gauge. Replace with a full N_2 cylinder when the cylinder inner-pressure is at low level.
5. Separate offspring from their female parents to new home cages (raise male and female offspring separately) and genotype offspring at postnatal day 21–27.
   a. Raise offspring with their female parents in the original home cages to postnatal day 21–27 under normoxia environment. Separate male and female offspring to new home cages at postnatal day 21–27 (after weaning).
   b. Genotyping protocol is the same as steps 1b and 1c. Tag offspring with a serial number through toes truncation or ear tags.
6. Group house *Bdnf-e6* homozygous male mice to adulthood for behavioral tests and further research.

⚠ CRITICAL: Replace the N_2 cylinder in time before used up. Because the hypoxia chamber obtains O_2 through outside air, the hypoxia chamber is not 100% air sealed. When the N_2 cylinder is empty, the O_2 level in the chamber will bounce back to normoxia level (21% O_2). Normoxia period during postnatal hypoxia stress may lead to inconsistent or failure of schizophrenia modeling.

⚠ CRITICAL: Must remove male parent before exposing breeding cage into hypoxia environment. Male mice might eat pups under environmental stress.

Option 2: Postweaning social isolation

Timing: 4 weeks (for step 7)

This section describes procedure for postweaning social isolation as an environmental stress.

7. Raise offspring with their parents in the original home cage under the normoxia environment by postnatal day 20.
8. Place *Bdnf-e6*$^{-/-}$ male offspring to each individual home cage from postnatal day 21–49. After isolation, these mice are ready for behavioral tests and further research.

Option 3: Juvenile corticosterone treatment

Timing: 3 weeks (for step 9)

This section describes procedure for juvenile corticosterone treatment as an environmental stress.

9. Raise offspring with their parents in the normoxia environment to postnatal day 20. Separate male and female offspring from their parents to new home cages on postnatal day 21, respectively.
10. From postnatal day 42–63, treat *Bdnf-e6*$^{-/-}$ male mice with corticosterone through drinking water (0.1 mg/mL). After corticosterone treatment, these mice are ready for behavioral tests and further research.
    a. Dissolve corticosterone powder with 100% ethanol. Subsequently, aliquot and stock the solution in -20°C.
    b. Add corticosterone into mice drinking water to achieve the final concentration of 0.1 mg/mL corticosterone and 1% ethanol. Control group mice are only exposed to 1% ethanol.

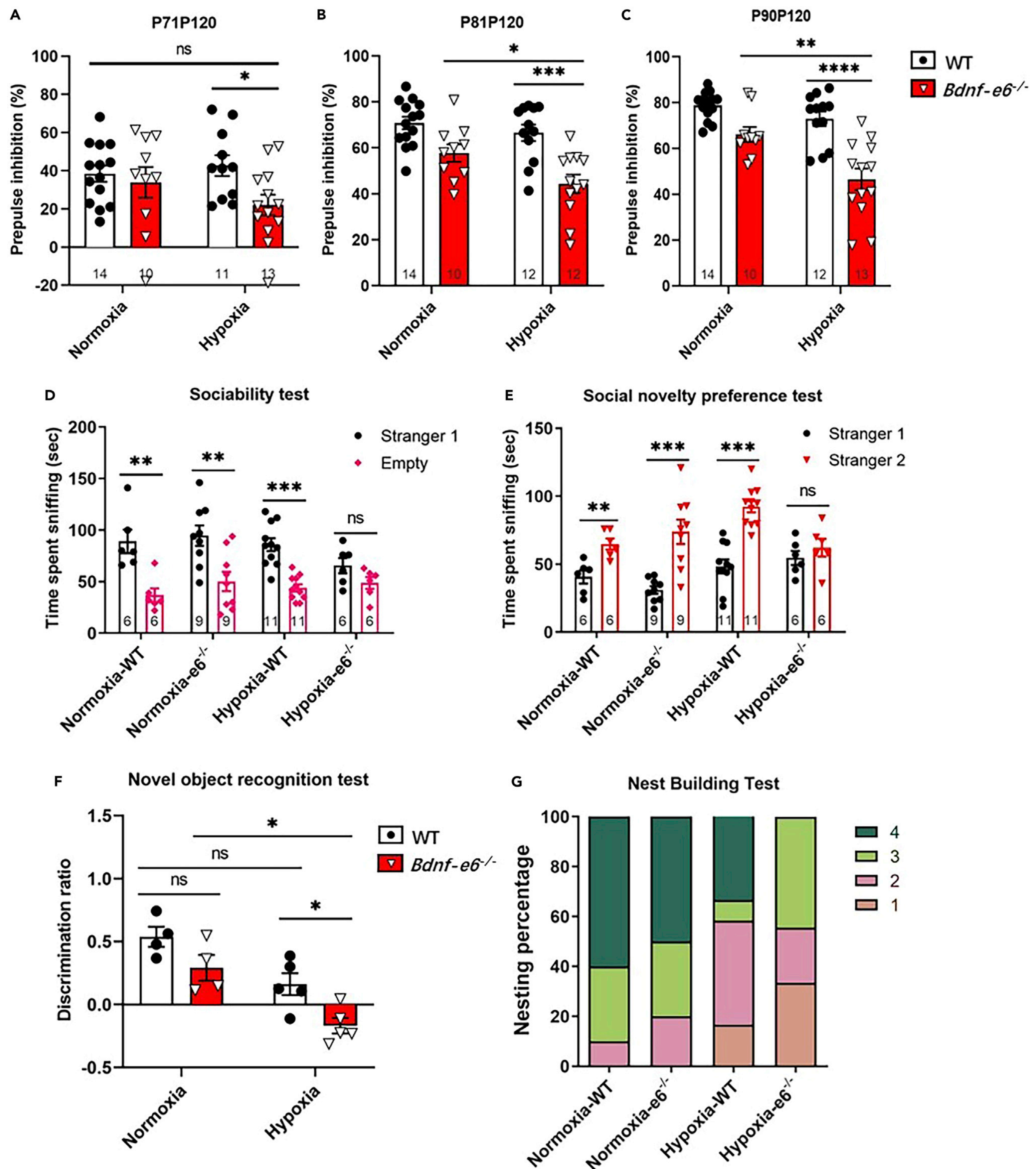

Figure 3. Expected results of schizophrenia-relevant behavioral tests of adult WT or *Bdnf-e6*$^{-/-}$ mice subjected to postnatal hypoxia stress

(A–C) Pre-pulse inhibition test results. PPI ratios at 71, 81, and 90 dB are demonstrated. Note that all PPI ratios of *Bdnf-e6*$^{-/-}$ mice subjected to postnatal hypoxia are significantly decreased compared with WT mice and *Bdnf-e6*$^{-/-}$ mice raised in normoxia environment.

(D and E) Social Interaction test results. Note that postnatal hypoxia stress resulted in significant social affiliation (D) and social memory deficiency (E) in *Bdnf-e6*$^{-/-}$ mice, but not WT mice.

Figure 3. *Continued*

(F) Novel object recognition test results. Note that *Bdnf-e6*$^{-/-}$ mice subjected to postnatal hypoxia exhibit significant recognition memory deficiency compared with WT mice and *Bdnf-e6*$^{-/-}$ mice raised in normoxia environment.

(G) Nest building test results. Note that *Bdnf-e6*$^{-/-}$ mice subjected to postnatal hypoxia exhibit lower nesting score compared with control groups. Data are shown as mean ± SEM. ns, no significant change. * indicates significant difference between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. The number of animals used for each test was shown in the bar graph. Original data have been published in Chen et al.[1]

c. Fully cover drinking water bottle by silver paper and change every 3 days to prevent degradation of corticosterone.

Note: Maintain the mice in a 12/12-h light/dark cycle, 22°C–26°C with sterile pellet food and water ad libitum under standard conditions.

Note: By pre-pulse inhibition (PPI) test, we have confirmed that exposure of *Bdnf-e6*$^{-/-}$ male mice to postweaning social isolation or chronic treatment of corticosterone at juvenile can induce schizophrenia-like phenotype.[1] Similarly, we suggest experimenters to examine the PPI deficiency of this G×E model to confirm the development of schizophrenia. However, full syndrome of schizophrenia-relevant behavioral phenotypes (e.g., social novelty, social interaction, novel object recognition, and nest building deficiency) induced by postweaning social isolation, or juvenile corticosterone treatment, or other types of environmental stress combined with *Bdnf-e6*$^{-/-}$ genotype has not been firmly established. Considering the validated full syndrome of schizophrenia behavioral phenotype and strong clinical relevance, postnatal hypoxia is more appropriate to be used as the environmental stress if the research is meant to investigate the neural pathology and comprehensive behavioral abnormalities of schizophrenia. On the other hand, juvenile corticosterone treatment can be used for high-throughput testing (e.g., drug testing) to avoid the tedious process.

EXPECTED OUTCOMES

After being subjected to environmental stress, we expect *Bdnf-e6*$^{-/-}$ mice to exhibit core schizophrenia-relevant behavioral phenotypes, including PPI deficiency, social affiliation deficiency, social memory deficiency, recognition memory deficiency, and nest-building deficiency at adulthood (Figure 3). However, as we have described in Chen et al.,[1] we did not observe hyperlocomotion induced by postnatal hypoxia. Mice subjected to postnatal hypoxia exhibit decreasing body weight compared with littermates raised in normoxia from 3–5 age weeks, but back to normal in adulthood (9 age weeks) (Figure 4). However, the schizophrenia-relevant phenotypes are maintained throughout adulthood.

LIMITATIONS

The menstrual cycle of female mice significantly affects the performance of behavioral tests. For instance, PPI is reduced in luteal women compared to follicular women, whereas PPI of follicular women equals to that of men.[16] Accordingly, PPI of pre-menopausal female mice is significantly lower compared to age-matched male mice, but do not differ between post-menopausal females and old males.[17] Thus, we only use male mice to establish this G×E schizophrenia model. Whether female *Bdnf-e6*$^{-/-}$ mice subjected to environmental stress exhibit schizophrenia-like endophenotypes has not been examined. Besides, whether other types of environmental stress or different time window would elicit the same effect has not been examined. Although we have demonstrated that only *Bdnf-e6*$^{-/-}$, but not *Bdnf-e1*$^{-/-}$, *Bdnf-e2*$^{-/-}$, *Bdnf-e4*$^{-/-}$ mice, underwent postnatal hypoxia exhibit schizophrenia-like endo-phenotypes,[1] the effect of other schizophrenia-risk genetic factors combined with environmental stress has not been investigated. Also, the genetic background of *Bdnf-e6*$^{-/-}$ mice used in this protocol is C57/BL6, whether other genetic background strain affects the development of schizophrenia has not been examined.

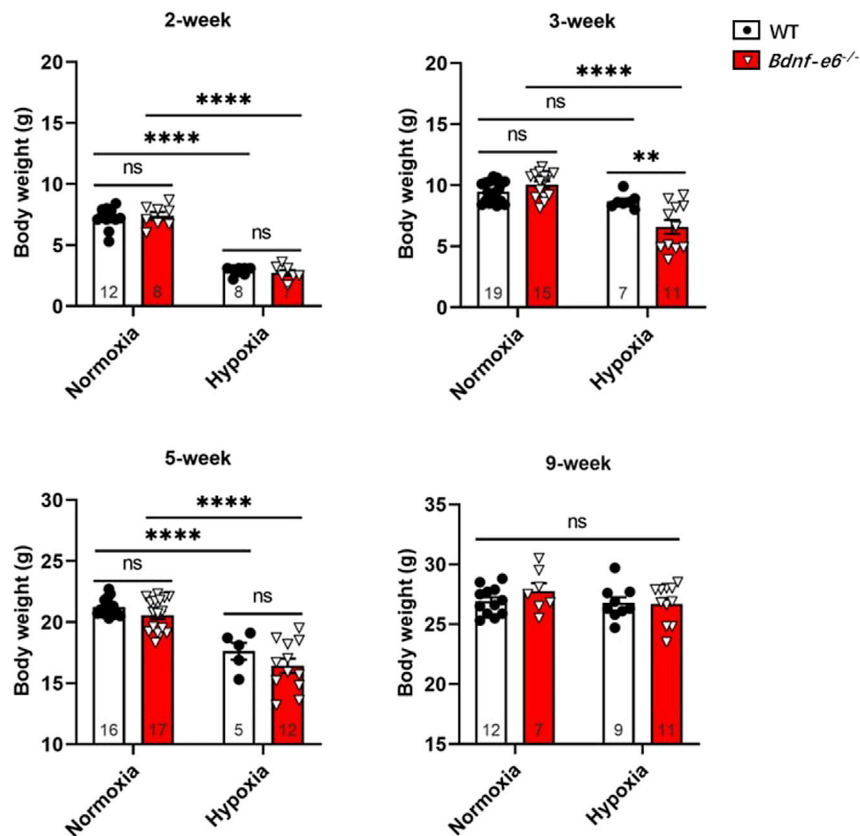

Figure 4. Body weight of WT and *Bdnf-e6*$^{-/-}$ mice subjected to normoixa or hypoxia postnatal stress
Note that both genotypes subjected to postnatal hypoxia resulted in lower body weight during adolescence but return to normal body weight after adulthood. Data are shown as mean ± SEM. ns, no significant change. * indicates significant difference between groups. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Original data have been published in Chen et al.[1]

TROUBLESHOOTING

Problem 1

Nitrogen gas is used up too quickly (e.g., less than a day) (step 1).

Potential solution

Lower the air pressure between the gas cylinder and O_2 controller. Normally we adjust the air pressure to 10–15 Mpa.

Problem 2

Pups are dead or eaten during or after postnatal hypoxia (step 4).

Potential solution

Male mice may eat pups, especially under environmental stress. Thus, experimenter should move the male parental mice from the breeding cage after pups are born, and only place the female parents and offspring into the hypoxia chamber. Besides, adjust O2 concentration to 10% level. Oxygen concentration below 10% might induce fatal effect on the offspring.

Problem 3

Fail to induce schizophrenia-like behavioral abnormality after environmental stress (steps 4–6).

Potential solution

First, we recommend postnatal hypoxia as the primary environmental factor. Second, we suggest that the experimenter follow the time window (postnatal day 4–10) and O_2 concentration (10%) rigorously. O_2 concentration in the hypoxia chamber during the whole hypoxia period should be monitored from time to time. O_2 concentration usually bounces back towards normoxia level (i.e., 20%) when the pressure in N_2 gas cylinder is at a low level.

Problem 4

Great variation of PPI test results (expected outcomes).

Potential solution

Ensure that the acclimation and habituation phases have been performed properly. Mice should be placed into the open-air cage gently to avoid any acute stress before test. Besides, considering startle amplitude is reflected by gravity alteration induced by the bounce, the test mouse should be restricted, rather than tightly constrained or free-moving within the open-air cage. In our case, 25–30 g B6J mice restricted in a 8×3×3 cm open-air cage consistently results in consistent PPI results. In addition, we suggest using the same pseudo-PPI order for all mice; otherwise mice might exhibit slightly but significantly different PPI. Detailed methods for schizophrenia-relevant behavioral tests are described in Chen et al.[1]

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact: Bai Lu. E-mail: bai_lu@tsinghua.edu.cn.

Materials availability

This study did not generate new unique reagents. The mouse line used in this study is from Chen et al.[1] and will be available upon request.

Data and code availability

Source data for figures in the paper is available at Chen et al.[1]

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

B.L., T.Z., and S.L. wrote the paper. Y.C., S.L., and T.Z. performed and analyzed the experiments. F.M., H.Y., and F.Y. contributed intellectually to the manuscript.

DECLARATION OF INTERESTS

B.L. and Y.C. are co-inventors of a pending patent application on a medicinal product for treating schizophrenia. B.L. is a co-founder and scientific advisor for 4B Technologies (Suzhou) Co. Ltd., and BioFront, Ltd., biotech companies that develop medicines for various diseases.

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