Loss-of-function of the *NOMOI* gene causes neuropsychiatric disorder-related phenotypes

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Abstract

Background: Clinical genome-wide analysis identified NOMO1 in human chromosome 16p13.11 as a candidate gene associated with neuropsychiatric disorders such as autism, schizophrenia and epilepsy. However, the important contributions underlying NOMO1 deficiency resulting in neuropsychiatric disorders is not understand, and the molecular and pathogenesis mechanisms of nomo1 gene are unclear. Therefore, it is necessary to construct animal models to systematically study the effects of nomo1 deficiency on neuropsychiatric system and explore pathogenic molecular mechanism of diseases.

Methods: We developed a viable vertebrate model of loss-of-function of nomo1 using CRISPR/Cas9 and studied the characterization of nomo1 mutant zebrafish. Phenotypic research was performed in developing nomo1 mutant zebrafish, including morphological measurements, behavioral tests, and functional mechanism analyses.

Results: The nomo1 loss-of-function zebrafish model accurately recapitulated key neuropsychiatric disorders traits. The mutant zebrafish showed decreased locomotion in the larval stage (7 dpf), increased spontaneous movement in infancy (15 dpf and 30 dpf), and social defects and repetitive behaviors in adolescence (2 mpf). More importantly, we demonstrated that these behavioral phenotypes stem from abnormal brain structure and neurotransmitter metabolism. Transcriptome analysis provided insights for studying the functional mechanism of nomo1 pathogenesis. Further results revealed that the neuroactive drug PTZ recovered the decreased locomotion phenotype in larval zebrafish, which provides functional basis for the exploration of drug sensitivity and intervention in behavioral phenotypes of nomo1 mutant zebrafish.

Conclusion: This study firstly reveal the functional evidence that loss-of-function of nomo1 elicits neuropsychiatric disorders, and emphasize the relationship between nomo1 deficiency and neuropsychiatric disorders from the perspectives of behavioral phenotypes, brain development, and neurotransmitter metabolism.

Limitation: The behavioral phenotype intervention of neuroactive drug in nomo1−/− zebrafish can be directly translated to the behavior of human associated diseases need further study.

Key Words: Nom01, CRISPR/Cas9, Zebrafish, Neuropsychiatric disorders, Social behavior, Neurotransmitter, Functional mechanism
Background

Clinical genetic evidence supported the viewpoint that chromosome 16p13.11 is a hotspot associated with various neuropsychiatric disorders such as epilepsy, autism spectrum disorder (ASD), schizophrenia, and attention deficit hyperactivity disorder (ADHD) [1-4]. Nodal modulator 1 (NOMO1), a negative regulator of the Nodal signaling pathway, is located in chromosome 16p13.11. In 2015, Tassano et al. reported a 13-year-old Italian male patient with epilepsy, mental retardation, developmental disorders, and dysmorphic features who had paternally inherited interstitial deleted copy number variations (CNVs) that included NOMO1 using comparative genomic hybridization (CGH) analysis [5]. In 2016, Brownstein et al. reported evidence of a psychosis patient with duplicated CNVs and found that the interval contains the NOMO1 gene [6]. Clinical studies suggest that NOMO1 may play a role in neuropsychiatric disorders; however, the important contributions underlying NOMO1 deficiency resulting in neuropsychiatric disorders is unclear.

Nodals, which are part of the transforming growth factor (TGF)-β superfamily, are essential for formation of the neuroectoderm and mesoderm during the development of early embryos. NOMO1, TMEM147 and nicalin form protein complexes that inhibit the nodal signaling pathway in the early development of zebrafish [7-9]. Researchers have found that NOMO1 is a candidate gene associated with glioma, early-onset colorectal cancer and facial asymmetry [10-12]. In 2018, Cao et al. constructed a nomol knockout zebrafish model that exhibited hypoplasia and dysmorphism symptoms with a phenotype similar to that of chondrodysplasia in humans [13]. By determining the expression pattern of nomol in zebrafish using whole-mount in situ hybridization (WISH), the nomol gene was highly expressed in the anterior mesendoderm and endoderm during early embryo development and abundant in the brain in larvae zebrafish [9, 13]. These studies revealed the implications of nomol in development of the central nervous system, whereas the biological mechanisms underlying nomol remain largely unknown.

Zebrafish have been accepted and applied to functional mechanistic studies of neuropsychiatric disorders as vertebrate model organisms. Researchers have established corresponding detection and analytical methods to measure the characteristic behavior phenotypes including locomotion, thigmotaxis, social behavior and aggressive behavior in zebrafish [14, 15]. The CRISPR/Cas9 technique was widely used for gene editing, and a number of transgenic zebrafish models have been developed [13, 16-18]. Notably, zebrafish models exhibit phenotypes similar to those observed in human diseases. FMRI is a gene related to autism and fragile X syndrome,
and an fmr1 mutant zebrafish model exhibited disease-related phenotypes [19]. Knockout of the shank3b gene resulted in autism-like behaviors and an enlarged ventricles size in zebrafish, which is retrospect the Phelan-McDermid syndrome (PMS) patients that frequently reported in human [16, 20].

Here, we provide in vivo evidence that loss-of-function of nomol causes neuropsychiatric disorders. Nomol deficiency conspicuously influenced behavioral phenotypes, brain development and neurotransmitter metabolism in zebrafish, which replicating key characteristics of the human diseases. These results conclusively demonstrate nomol in the phenotypes of neuropsychiatric disorders and highlight the critical role of nomol in brain development.

Methods

Zebrafish breeding and the generation of nomol mutant zebrafish

Wild-type (WT) zebrafish Tuebingen (TU) strain were provided by the zebrafish facility of the Translational Medical Center for Development and Disease, Shanghai Key Laboratory of Birth Defect, Institute of Pediatrics, Children’s Hospital of Fudan University. The zebrafish were raised in a circulating water system with a water temperature of 28.5°C with 14 h of light and 10 h of darkness per day (8:00-22:00, light). Zebrafish breeding, feeding and spawning were strictly in accordance with the Zebrafish Book (http://zfin.org/zf_info/zfbook/zfbk.html).

A target site in exon 7 of nomol gene in zebrafish was selected, and the specific guide RNA (sgRNA) sequence used was 5’-GGGCTATGATGTCTCTGGAG-3’. The detailed CRISPR/Cas9-mediated editing were performed following standards procedures [21, 22]. Synthetic gRNA and Cas9 mRNA (concentrations of 30 ng/μL and 300 ng/μL, respectively) in a total volume of 3 μL were coinjected into WT zebrafish embryos at the single-cell stage. Genomic DNA was extracted from fifteen chimeric embryos, and genotyping samples were screened for mutation frequency by comparison with WT zebrafish samples. The primer sequences used for genotyping are shown in Table S1. The mutant chimeric zebrafish were mated with the TU strain to purify the background to obtain nomol+/− zebrafish. Male and female nomol+/− zebrafish were crossed to acquire homozygous zebrafish for behavioral experiments and phenotypic analyses.

RT-qPCR
Total RNAs have been extracted from embryos, heads and the brain tissues of different developmental stages of zebrafish using TRIzol reagent (Ambion, USA). Genomic DNA was removed by DNase I, and total RNA (1 μg) was reverse transcribed using a PrimeScript cDNA Synthesis Kit (TaKaRa, Japan). RT-qPCR was conducted with a LightCycler® 480 apparatus (Roche, Germany) and SuperReal PreMix Plus (Tiangen, China) following the manufacturers’ instructions. The fold changes in RNA levels were calculated using the ΔΔCt method. The RT-qPCR primer sequences are listed in Table S1.

WISH

The targeted DNA were cloned into the pGEM-T Easy vector, and probes were synthesized using linearized plasmid by in vitro transcription with the DIG-RNA labeling Kit (Roche, Austria). The related primer of synthetic probes are shown in Table S1. Embryos of WT and mutant zebrafish were respectively collected at different stages (12 hpf, 24 hpf and 48 hpf) and fixed in 4% paraformaldehyde at 4°C overnight. WISH was performed as previously described [17], and images were captured and processed using a Leica 6000 microscope.

Drugs

Pentylenetetrazole (PTZ) (Sigma-Aldrich; P6500, St. Louis, MO) was dissolved in ultrapure water to make a 32 mM stock solution that was frozen at -80°C. The PTZ working solution was diluted to the appropriate concentration with blue egg water prior to experiments.

Behavioral tests in mutant zebrafish

Locomotion and thigmotaxis tests

Behavioral tests of larval zebrafish were performed at 28.5°C in 24-well plates (Fig. 2G), and the inner diameter of each well was 18-mm, giving the larvae enough space to swim. The 24-well plates were then placed in a Zebrabox (ViewPoint Life Sciences, Lissieu, Calvados, Lower Normandy Region, France) that recorded videos tracking the larvae zebrafish. The experimental procedure consisted of 55 min of continuous illumination with light at an intensity of 100 lx and two 10-min light-dark transition cycles for a total time of 75 min to elicit a photo motor response (PMR) (Fig. 2A). The experiment examined not only spontaneous movement but also changes due to lighting transitions. The data were quantified with ZebraLab software (ViewPoint Behavior Technology, France), the video rate was set to 25 frames per second (fps), and the
frames were pooled into 1-min time bins. The threshold was set to 29, a suitable level to accurately detect the trajectory of larval zebrafish in motion.

Zebrafish at 15 dpf, 30 dpf and 2 mpf swam freely in the open field at 28.5°C, and the experimental time was 30 min. Zebrafish at 15 dpf were examined in a 9-cm diameter dish since the smaller size (Fig. 3A). Zebrafish at 30 dpf and 2 mpf were examined in the same experimental container used for the shoaling behavior test (Fig. 3C). The collected data were exported using ZebraLab software. We analyzes the data to detect spontaneous movement and thigmotaxis behavior.

Social and repetitive behavior tests

The individual social behavior (social preference behavior) and group social behavior (shoaling behavior) of adolescent zebrafish were assessed at 28.5°C. To examine social preference behavior, a single zebrafish was placed on one side of a standard mating tank (inner dimensions, 21 × 10 × 7.5 cm), and another six WT zebrafish were placed on the other side of the mating tank and separated from the single zebrafish by a transparent plastic plate (Fig. 4A). Region 1 was regarded as a social area, whereas Region 2 was regarded as a nonsocial area, and the experiment lasted for 30 min. Behavioral recording began after an adaptation period (1-2 min) when the zebrafish acclimated to the tank. The behavior of the zebrafish was quantified as a distribution of distances or regions adjacent to the group. The ratio of the time the zebrafish stayed in the social area and the distance spent away from the social area can directly reflect the social activity of a single adolescent zebrafish.

For the shoaling test, six zebrafish were acclimated to a novel tank (inner dimensions, 30 × 30 × 20 cm) (Fig. 4F). A camera recorded the trajectory of the experimental zebrafish over 30 min, and the indicator inter-individual distance (IID) was used to assess the average distance between each zebrafish in the shoal [16, 23].

The spontaneous movement of adolescent zebrafish was examined; the mutant zebrafish showed different types of repetitive behavior (back-and-forth motions, stereotypic movement and large circular movement) (Fig. 4H). Back-and-forth motion was defined as moving one time on one edge or adjacent edges of the tank and returning to the origin. Stereotypic movement referred to repeated movement of the zebrafish in a small area, where the maximum movement distance from the beginning to the end was less than 30 mm and continuous swimming time was greater than 5 s. Large circular movement referred to the swimming of the zebrafish in a counterclockwise or clockwise circle along the edges of the tank. After the stereotypic movement defined, the data was obtained objectively by computer program within the experimental period.
Kin recognition test

The mating tank, the specifications of which were the same as those for the tank used in the social preference test, was divided into three compartments using transit plates in order to examine kin preference behavior. Three zebrafish of the same strain and same age were placed on one side of the tank, and three red zebrafish of different strains were placed on the other side of the tank. The adolescent WT and mutant zebrafish were placed in the middle area of the tank (Fig. S3A). Region A was regarded as kin preference area, whereas Region B was regarded as non-kin preference area. Behavioral recording began after an adaptation period (10 min) when the zebrafish adapted to the tank. Videos were recorded for 30 min. The time spent/distance moved ratio for the zebrafish that stayed in the Region A was used to measure the ability of kin recognition in zebrafish.

Preparation of paraffinized sections and HE staining

The brain tissues of adult zebrafish (3 mpf) completely removed under a microscope and immersed in 4% paraformaldehyde for 24 hour. Tissues were dehydrated and transparentized with the following conditions and protocol: 70% ethanol, 30°C, 30 min; 95% ethanol, 30°C, 10 min; 95% ethanol, 30°C, 10 min; 100% ethanol (I), 30°C, 10 min; 100% ethanol (II), 30°C, 10 min; xylene (I), 30°C, 30 min; and xylene (II), 30°C, 30 min. The tissues were waxed and embedded using a paraffin embedding station (Leica EG1150H) for 3 h at 65°C. Then, a microtome (Leica RM2235) was used to produce continuous slices with a thickness of 4 μm. The slices were floated in 40°C warm water to flatten their surfaces, and then baked in a 60°C oven. HE staining was carried out by staining with hematoxylin and eosin for 5 min each. Photographs were taken with a Leica 205C microscope.

Neurotransmitter targeted metabolomics analysis

Adolescent zebrafish were frozen in liquid nitrogen and placed on ice. Under the microscope, the brains were directly removed with a surgical blade, and 10 brains were placed in an Eppendorf (EP) tube. Selective/Multiple reaction monitoring assays (SRM/MRM), which is based on method of liquid chromatography- tandem mass spectrometry (LC-MS/MS), has been used to simultaneously detect neurotransmitters in animals. LC-MS/MS can determine absolute quantitative amounts of target metabolites with strong specificity and high sensitivity and accuracy [24, 25].

Brain tissue samples were added to 1 ml of methanol/acetonitrile/ultrapure water (2:2:2, v/v/v), vortexed and ultrasonicated. After incubation at -20°C for 30 min, the precipitated proteins were centrifuged at 14,000 rcf for 4 min at 4°C. The supernatant
was removed and dried in vacuo. For spectrometric detection, 100 μL of an acetonitrile/water solution (1:1, v/v) was used to reconstitute the pellet, which was centrifuged at 14000 rcf for 4 min at 4°C, after which the supernatant was taken for analysis. Samples were separated using an Agilent 1290 Infinity LC Ultra Performance LC System. Mass spectrometry was carried out in negative ion mode using a 5500 QTRAP mass spectrometer (AB SCIEX) for analysis. Data on stability and repeatability were evaluated using cluster analysis and statistical analysis.

**HPLC**

High-precision liquid chromatography (HPLC) was also employed to examine the levels of norepinephrine, dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), and serotonin in the brain of adolescent zebrafish. The samples were suspended in iced PBS (20 μL/sample) and ground completely. Then, the samples were centrifuged at 11900 rpm at 4°C for 10 min, and the protein content of 1 μL of the supernatant from each sample was quantified. The rest of the supernatants were added to 2 μL of perchloric acid (0.2 N) and centrifuged again at 11900 rpm at 4°C for 10 min. The supernatant was collected and stored at -80°C. HPLC analysis was carried out using an Agilent 1200 HPLC system (Agilent, USA) with Antec DECADE SDC electrochemical detector (Antec, the Netherlands). The expression levels of neurotransmitters were normalized to the protein content.

**Transcriptomics**

The total RNA of each sample was extracted from the brain tissues of adolescent zebrafish using TRIzol reagent (Ambion, USA) according to the manufacturer’s instructions. RNA degradation and contamination were monitored on 1% agarose gels. RNA concentration was measured using a Qubit® RNA Assay Kit with a Qubit®2.0 fluorometer (Life Technologies, CA, USA), and RNA integrity was assessed using an RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total of 3 μg of RNA per sample was used as input for RNA sample preparation. Sequencing libraries were generated using an NEBNext® UltraTM RNA Library Prep Kit for Illumina® (New England Biolabs, USA) following the manufacturer’s recommendations, and index codes were added to attribute sequences to each sample. PCR products were then purified (AMPure XP system), and library quality was assessed on the Agilent Bioanalyzer 2100 system. Sequencing fragment data detected by a high-throughput sequencer was converted from image data into sequence data (reads) containing sequence information from each sequenced fragment and its corresponding sequencing quality by CASAVA base recognition. High-quality reads
were aligned to the zebrafish reference genome (GRCz11) using HISAT2 v2.0.5. Differentially expressed genes (DEGs) were identified using the DESeq2 R package (1.16.1), which uses statistical methods to determine differential expression from digital gene expression data using a model based on a negative binomial distribution. The resulting p-values were adjusted using Benjamini and Hochberg’s approach for controlling the false discovery rate. Genes with an adjusted p-value <0.05 according to DESeq2 were defined as DEGs. In addition, gene ontology (GO) enrichment analysis of the DEGs was implemented by the clusterProfiler R package, which corrected gene length bias. GO terms with corrected p-values <0.05 were considered significantly enriched in DEGs. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database is a resource used to understand high-level functions and utilities of biological systems, such as a cell, an organism and an ecosystem, and is especially focused on analyzing large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/). We used the clusterProfiler R package to test the statistical enrichment of DEGs in KEGG pathways.

**Statistical analysis**

The experimental data in this study were analyzed and mapped with GraphPad Prism 6.0. Values are presented as the mean ± SEM. Differences between two groups were analyzed by paired Student’s t-test and corrected Student’s t-test, and p-values <0.05 indicated a significant difference.

**Results**

The *nomol* mRNA expression level from early embryonic development to adolescence and generation of *nomol* mutant zebrafish

The gene *nomo* in zebrafish is homologous to human *NOMO1*, and they share 68% and 70% identity in cDNA and protein sequences. Cao et al. and Haffner et al. have confirmed the expression pattern of *nomol* from early embryonic development to larvae zebrafish using WISH [9, 13]. To determine the expression level of *nomol* during different developmental stages, RT-qPCR was performed. At 12 hpf-7 dpf, whole zebrafish were used since the smaller size. At 14 dpf, heads of zebrafish were used and at 1 mpf- 2 mpf, the brain tissues were studied. The expression of *nomol* mRNA in embryonic zebrafish increased before 48 hpf and decreased at 5 dpf and 7 dpf, as determined by RT-qPCR (12 hpf-7 dpf). As development progressed, *nomol* mRNA peaked a second time at 14 dpf, and the highest mRNA expression level was detected in brain tissues of 2 mpf zebrafish (Fig. 1F).
The specific gRNA of nomo1 was designed by editing exon 7, which was located before the functional structural domain FN3 in the genomic sequence of zebrafish [26] and comprised a 20-base sequence (Fig. 1A). Fig. 1B-D shows the process by which nomo1 homozygous zebrafish were generated using CRISPR/Cas9. Genomic DNA was extracted from the zebrafish tail, and the specific PCR products were sequenced, confirming that nomo1 contained a 1-base deletion (Fig. 1E), resulting in a frameshift mutation and truncated protein 10 amino acids after the mutation (Fig. 1H). The mRNA expression level of nomo1 zebrafish was decreased in mutant zebrafish at 48 hpf, 14 dpf and 2 mpf at the transcriptional level, as shown by RT-qPCR (Fig. 1G).

**Morphological analysis of nomo1−/− zebrafish**

After zebrafish passed three generations, the morphological measurement of developing nomo1−/− zebrafish were performed (Fig. S1). We calculated the mortality of the early embryos (24 hpf) and found that the death rate of the mutant zebrafish was higher than that of WT zebrafish (Fig. S1F). Mutant 24 hpf zebrafish embryos exhibited the following morphological changes: developmental retardation, tail bending, pericardium edema and developmental malformation. However, these morphological differences in the phenotype of nomo1−/− zebrafish gradually become less noticeable over the process of development (Fig. S1A-D). We statistically analyzed the body length of zebrafish at different developmental stages and found no significant difference (Fig. S1E).

*Nomo1* deficiency significantly affected the locomotion of 7 dpf zebrafish under different illumination intensities

To determine whether the loss of nomo1 would modulate the behavior of larval zebrafish, we examined the locomotor activity of 7 dpf zebrafish and their reactions to light-dark transitions. After 20 min of adaptation time, the locomotion and thigmotaxis behavior in larval zebrafish were analyzed (Fig. 2A). We demonstrated the swimming trend of WT and mutant zebrafish in 24-well plates (Fig. 2B). The horizontal axis is the swimming time, and the vertical axis is the distance moved, which can be used to intuitively demonstrate locomotion of the zebrafish over the experimental time. Analysis of the average distance moved per minute from minutes 21 to 60 (L0) under light conditions (Fig. 2C) showed that the locomotion of nomo1−/− zebrafish was significantly reduced compared with that of nomo1+/+ zebrafish, confirming the specificity of the phenotype.

Similarly, the mutant zebrafish demonstrated statistically decreased locomotor activity under the two light-dark cycles, and WT and mutant zebrafish showed a light-sensitive reaction in every light-dark cycle (Fig. 2D-E). Although the WT and mutant zebrafish
showed photosensitivity in the two light-dark cycles, the mutant zebrafish had a more intense response to lighting changes in the first light-dark cycle than WT zebrafish, whereas there were no statistically significant difference in photosensitivity in the second light-dark cycle (Fig. 2F).

In this study, we measured the thigmotaxis of WT and mutant zebrafish under light conditions using the percentage of time spent/distance moved in the inner zone. The nomo1 mutant zebrafish demonstrated an edge-preference under continuous illumination, indicating increased thigmotaxis behavior (Fig. 2H).

**Infant mutant zebrafish showed increased locomotion**

We also analyzed the spontaneous movement of zebrafish during development and recorded the trajectory of single zebrafish over 30 min. The different size of the container used for infant zebrafish; and the trajectory of the zebrafish are shown in Fig. 3A and 3C. The diagram shows a trend in the swimming behavior of WT and mutant developmental zebrafish during the experimental period (Fig. 3B, D, and E). The spontaneous movement of infant zebrafish (15 dpf and 30 dpf) significantly enhanced. The average distance moved per minute was no different between nomo1/− zebrafish and WT zebrafish at 2 mpf (Fig. 3F).

**Adolescent nomo1/− zebrafish exhibited autism-like behaviors**

The mutant adolescent zebrafish showed different types of repetitive behaviors; these behaviors were back-and-forth motion (Video. S1), stereotypic movement (Video. S2) and large circular movement (Video. S3). The number of different types of repetitive behaviors were counted. Our result showed that the mutant zebrafish exhibited more such behaviors than the WT and demonstrated a repetitive behavior-related pattern. We found that the back-and-forth motion significantly increased in mutant zebrafish compared to WT zebrafish (Fig. 4I). However, the number of stereotypic movements and large circular movements were not significantly different between nomo1/− and WT zebrafish (Fig. 4I).

The advanced social behaviors of adolescent zebrafish, that is, social preference behavior (Video. S4-S5) and shoaling behavior (Video. S6-S7), were assessed in this study. The nomo1+/+ zebrafish swim along the social area throughout the experiment, whereas the nomo1/− zebrafish swim in a dispersed and random manner. The ratio of time spent/distance moved in the social area was statistically significantly lower for nomo1/− zebrafish than for nomo1+/+ zebrafish (Fig. 4D-E). We use one of the videos to demonstrate the trajectory of the zebrafish. The heat map in Fig. 4B-C was obtained by analyzing the trajectory of the zebrafish in the mating tank.
Generally, the WT zebrafish swim together in an open-field test, which reflects the social nature of the species. Therefore, the shoaling behavior was detected to assess the social skills of WT and nomo1-/- zebras [27]. The nomo1-/- zebrafish showed a higher IID with their companions than the nomo1+/- zebrafish, indicating that the nomo1-/- zebrafish have a weaker clustering ability (Fig. 4G).

To investigate the cognitive ability of mutant zebrafish, the kin recognition test of adult zebrafish was performed. The WT zebrafish (Video. S8) and nomo1-/- zebrafish (Video. S9) demonstrated same tendencies in kin preference region and non-kin preference region. The time spent/distance moved ratio of the mutant was the same as that determined for nomo1+/- zebrafish (Fig. S3B-C).

**Loss-of-function of nomo1 affected the midbrain and hindbrain during zebrafish development**

To identify the behavioral differences, we conducted functional mechanism analysis. At early stages, abundant nomo1 mRNA is transcribed in the endoderm and anterior mesendoderm [9]. In our study, changes in the patterning of the endoderm marker sox17 and mesoderm marker hgg1 were detected in nomo1-deficient mutants. Loss-of-function of nomo1 resulted in the differential expression pattern of hgg1 (Fig. 5A-B) (50% of cases, n=20). In our results, nomo1 mutant embryos display an increased number of sox17-positive cells (Fig. 5C-D, arrows) (62% of cases, n=18).

Considering that nomo1 is important for the development of the early mesendoderm, we examined a variety of key neural development-related genes in WT and mutant zebrafish. The results showed that the expression levels of the genes (HuC, neurog1, islet1, egr2b and foxb1a) in nomo1 mutant zebrafish had no detectable differences compared with the levels in WT zebrafish at 24 hpf (Fig. 5E), whereas the mRNA expression levels of the genes decreased significantly in the zebrafish at 48 hpf (Fig. 5F). We used RT-qPCR to detect the differences in expression levels in zebrafish, and then, semi-quantitative analysis was performed by WISH to verify the expression pattern. The nomo1-/- zebrafish exhibited significant differences in brain development compared with the WT, which was characterized by a smaller volume of the midbrain and hindbrain at 48 hpf (Fig. 5G-L, arrow).

To study whether the abnormalities in the brains of larval zebrafish persist during the developmental stage, we performed HE staining on brain slices of adult zebrafish (Fig. 5M-P). HE staining of the whole brains of adult male zebrafish showed that the nomo1-deficient zebrafish had abnormal brain structure development, and the midbrain and
hindbrain regions of these zebrafish contained enlarged interstitial spaces. However, staining for forebrain-related markers, such as fezf2, and dopaminergic neurons did not reveal differences between WT and nomol−/− larvae, indicating that nomol deficiency had almost no influence on forebrain and dopaminergic neuron development (Fig. S2). Based on the above-described microscopic morphology at the overall cellular level, we concluded that nomol regulated early neural developmental patterning in vivo, strongly influencing the development of the midbrain and hindbrain.

Expression levels of neurotransmitter and metabolite were changed in brain of adolescent nomol−/− zebrafish

In order to reveal the mechanism underlying the autism-like behaviors, we examined neurotransmitters and metabolites in adolescent zebrafish brains. Here, we focused on the following 13 neurotransmitters and their metabolites using LC-MS/MS method: r-amino-butyric acid, levodopa, DA, epinephrine, 5-HIAA, serotonin, 3-methoxytyramine, acetylcholine, histamine, normetanephrine, tyramine, glutamate, and glutamine. Fig. 6A shows the cluster relationships of each metabolite between nomol+/+ and nomol−/− zebrafish, and the tree structure at the left shows the clustering relationship of each group. Hierarchical clustering results showed that a total of ten neurotransmitters and their metabolites were increased in nomol−/− zebrafish compared with that of nomol+/+ zebrafish. Detailed quantities of the different metabolites are shown in Fig. 6B, and the expression of γ-aminobutyric acid, levodopa, epinephrine, serotonin, 3-methoxytyramine, histamine, normetanephrine, tyramine, glutamate, and glutamine was significantly higher in nomol−/− zebrafish than in WT zebrafish.

Another method for detecting neurotransmitters, HPLC analysis, showed the same trend. In mutant zebrafish, the levels of serotonin and norepinephrine were obviously increased (Fig. 6C). Although dopamine and its metabolites DOPAC showed an upward trend, there was no statistically significant difference (Fig. 6C).

Transcriptome analysis shows that the WT and mutant zebrafish have different expression patterns at the genetic level

To further study the relationship between neuropsychiatric disorder-related phenotypes and nomol deficiency at the genetic level, the adolescent zebrafish was selected for transcriptome sequencing analysis. A volcano map visually shows the distributions of DEGs for each of the comparative combinations. A total of 292 genes were downregulated, 254 genes were upregulated, and the expression of 546 genes was changed at the transcriptional level in mutant zebrafish compared to WT zebrafish (Fig. 7A). To understand the functions and roles of DEGs, we performed GO annotation and KEGG pathway analyses. The GO annotation classification chart demonstrated the
functions of DEGs in biological processes, cellular components and molecular functions (Fig. 7B). Through the classification and statistical analysis of KEGG signaling pathways, nomo1 was shown to participate in biological processes of metabolism, cellular function, intestinal immunity, and pathogen infection (Fig. 7C).

Functional analysis of the DEGs revealed that neuropsychiatric disease-related genes nos2a and hbbal were upregulated, whereas rnpsl and assl were downregulated in mutant zebrafish compared to their expression in WT zebrafish. Moreover, transcriptome analysis showed that glutamate transporters (slc39a8 and slc43a2b) and immune-associated genes (cfap100, slc15a2 and entpd8) were downregulated in mutant zebrafish compared to their expression in WT zebrafish. RT-qPCR was used to verify the mRNA expression levels of DEGs and confirmed that the difference in gene expression was consistent with the sequencing results (Fig. 7D).

The decreased locomotion in larval zebrafish was recovered by neuroactive drug PTZ

In order to explore drug effects that change neurological disease-related phenotypes, we applied the neuroactive drug PTZ, an antagonist of the $\gamma$-aminobutyrate (GABA) receptor, and measured the locomotor activity of larval zebrafish. Here, we utilized 8 mM PTZ treatment to explore the effect in mutant zebrafish. The experiment lasted for 60 min and was carried out under continuous illumination. The zebrafish was maintained in a 24-well plate at 28°C room temperature. The movement trend of zebrafish (nomo1+/+, nomo1−/−, nomo1+/++PTZ, and nomo1−/−+PTZ) in the experimental period is shown in Fig. 2I. Under the influence of PTZ, WT zebrafish exhibited increased locomotion, which is similar to the results of previous research [14]. The locomotor activity of mutant zebrafish treated with PTZ increased significantly compared with that of nomo1−/− zebrafish, and there was a statistically significant difference in locomotor activity under continuous light conditions (Fig. 2J).

Discussion

Here, we focused on the developmental and behavioral function of zebrafish caused by nomo1 deficiency at different developmental stages. In 2018, Cao et al. reported, for the first time, a nomo1 knockout vertebrate model that was associated with bone formation and cartilage development. In contrast to our research, they selected exon 19 to edit and established a mutant line with an 85-base deletion, resulting in a frameshift mutation in exon 20. Their study proved loss-of-function of nomo1 in zebrafish reduced the expression of nomo1 mRNA in mature zebrafish, which may related to the shear site coinciding with the mutation point [13]. They observed early-stage bone
developmental defects in larvae before 7 dpf, which might be one of reasons for the behavioral disorders in larvae and infant zebrafish. Behavioral feature influenced by many factors, especially in context of the neuropsychiatric system and motor ability. Although it is difficult to estimate the effect of behavioral disorder from spontaneous movement, the social-level results make sense, emphasizing the significance of neuropsychiatric systems. The locomotion of adolescent mutant zebrafish did not change compared with that of WT zebrafish; however, the specific movement pattern did change. Based on the abundance of nomol in the whole brain, we consider that the overall fiber morphology of the brain might have changed after the mutation, in turn influencing the nervous system to affect the behavioral phenotype. Thus, our findings showed that loss-of-function of nomol had an important influence on the development of the nervous system in zebrafish. In this study, we provide strong validation via functional evidence and suggest the importance of nomol in the etiology of neuropsychiatric disorders.

Our results provide several important findings. We highlight the essential role of nomol in neuropsychiatric disorder-related characteristic behavior during development. Patients with neuropsychiatric disorders usually presented various behavioral phenotypes, including inattention, hyperactivity and increased impulsivity in ADHD patients [28] and spontaneous recurrent seizures accompanied by distinct neurobiological alterations in epilepsy patients [29]. The core symptoms of ASD patients demonstrated social defects and repetitive behaviors, associated with cognitive and learning disabilities [30, 31]. Examination of zebrafish in the adolescent period showed that the nomol mutant zebrafish exhibit autism-like characteristic behavior. Similar phenotypes have been observed in other transgenic mutant zebrafish models of shank3b [16], dyrk1a [32] and fmr1 [19], which are candidate genes associated with ASD. This finding indicated that nomol is an important pathogenic gene for ASD. However, the neuropsychiatric disorders manifested in a diverse manner and presented comorbidities in patients [33, 34]. We found that developing nomol mutant zebrafish employed decreased/increased locomotion, which showed that the mutant zebrafish not only demonstrated autism-like behavior, but also ADHD or epilepsy related phenotypes. This may be one of the reasons for comorbidities in neuropsychiatric diseases. However, whether the behavioral phenotype observed in animal models can be directly associated with a specific neurodevelopmental disease is worth exploring. Neuropsychiatric disorders have strong heterogeneity, and different gene mutations can lead to a specific disease. A single gene mutation can cause comorbidity in ASD and other neuropsychiatric diseases. Therefore, studies on the functions of different genes, different signaling pathways and different mutation models are warranted to determine the pathogenesis of the diseases. Moreover, additional behavioral measures including
comorbidities such as facial expressions, seizures, and cognitive impairment are needed to fully assess neuropsychiatric diseases-related behavior.

Our results also conclusively demonstrated that loss of function of nomol influenced the development of the midbrain and hindbrain. Haffner et al indicated that transiently decreased expression of nomol affects the development of the mesendoderm and hatching glands [9]. Specifically, our study demonstrated abnormal expression levels of mesendoderm and endodermal markers in steadily inherited embryonic mutant zebrafish. The potential neural induction properties of the mesendoderm indicate that the mesendoderm is necessary for brain development [35, 36]. Meanwhile, we detected abnormalities in brain developmental markers during the early neural development of mutant larvae accompanied by loosening of the tissue structure in the midbrain and hindbrain of adult mutant zebrafish. ASD patients exhibit reduced brain parenchyma in magnetic resonance imaging (MRI) results [37]. Thus, our findings demonstrate a logical consistency between brain structural abnormalities in the anatomy and behavioral phenotypes related to neuropsychiatric diseases.

Several studies have illustrated a potential functional mechanism in midbrain and hindbrain development and behavioral abnormalities, showing abnormal gene expression [38-40]. These aberrantly expressed genes are likely to be involved in different signaling pathways. Wnt signaling pathway, which is known to be required for normal development of the vertebrate midbrain and hindbrain [41], may be one of the possible pathway. We conducted RT-qPCR and WISH using two markers of the Wnt pathway, and no difference was observed between the WT and mutant zebrafish (data not shown). Therefore, the pathogenic mechanism of nomol deficiency may not be related to the Wnt signaling pathway, functioning instead through other signaling pathways. Moreover, researchers have proved that nomol affects body axis development [9], which may be the cause for the increased mortality and varying degrees of developmental malformations observed in larval zebrafish in this study. Notably, the delayed neurodevelopment in terms of the appearance phenotype and body length in mutant zebrafish became less noticeable over the course of development. The mechanism underlying this effect is currently unknown, but nomol may affect morphological development at specific stage that have a more pronounced effect on the nervous system across developmental stages.

Behavioral phenotypes are associated with complex pathogenesis which typically characterized by alterations in activity of neurotransmitters. Genetic association studies verified the key role of DA in the etiology of ADHD [42]. Evidence supports a novel and important link between schizophrenia, γ-aminobutyrate (GABA) and glutamate alterations [43]. In our study, neurotransmitter metabolism analysis also provide
evidence of the relationship between nomo1 deficiency and social defects. Studies have confirmed that there are many neurotransmitter system abnormalities in children or animal models with neuropsychiatric disorders, involving mainly glutamatergic, GABAergic, dopaminergic, serotonergic and so on [44-46]. The high level accumulation of the norepinephrine system was shown by HPLC, and LC-MS/MS detected an increase of its metabolite, normetanephrine. Targeted metabolomics showed a significant increase in the levels of levodopa, a precursor of DA. However, the metabolic level of DA and its metabolite DOPAC had not changed in mutant zebrafish compared to WT zebrafish, as determined by HPLC. Moreover, 5-HT was shown to accumulate at a high rate by both of these methods. Clinical studies have demonstrated elevated levels of 5-HT in the peripheral blood of children with ASD [47], and some researchers have found differences in 5-HT distribution in different regions of the brains of ASD patients [48]. Therefore, it is worth studying the cause or consequence of neurotransmitter alterations and abnormal behavior phenotype.

Transcriptome sequencing analyses have identified multiple DEGs associated with neuropsychiatric disorders. For example, the association of RNPS1 and Nos2 with neurodevelopmental disorders in patients has been verified [49, 50]. These genes may represent important clues regarding the social defects observed following nomo1 mutation. Furthermore, transcriptome analysis revealed a significant decrease in the expression levels of two genes encoding glutamate transporters, namely, Slc39a8 and Slc43a2b. Lack of glutamate transporter protein leads to accumulation of glutamate in the extracellular fluid, which phenomenon could lead to the increase in glutamate metabolic levels revealed by neurotransmitter system analysis. The accumulation of glutamate increases the neuron discharge frequency, causing epileptic sensitivity to eventually increase and persist. Interestingly, in a mouse model, it has been supported that glutamatergic dysfunction is associated with schizophrenia [45]. The results suggest that systematic studies on biomolecules ranging from nomo1 to glutamate provide important clues to reveal the molecular mechanism of nomo1 deficiency and its effect on neuropsychiatric disorders. Finally, GO analysis of cfap100, entpd8 and slc15a2 revealed the involvement of DEGs in antigen presentation and assembly of the major histocompatibility complex protein complex, and the pathways involved in these immune responses had been proved to be associated with ASD [51]. Although our study was limited, it provided new directions for studying neuropsychiatric diseases associated with nomo1 gene loss and offered ideas for further research on these diseases.

Neurotransmitter system analysis demonstrated that nomo1 is associated with GABAergic. GABA is an inhibitory neurotransmitter of the central nervous system, and PTZ is an antagonist of the GABA receptor. Therefore, we attempted to intervene partial
behavioral phenotype in the *nomol* mutant zebrafish using PTZ. PTZ increased the spontaneous movement of larval zebrafish under light conditions [14]. In addition, Mussulini *et al.* found that an increase in PTZ concentration increased body movement and seizure-like behavior, which were observed following treatment with 15 mM PTZ in adult zebrafish [52]. In this study, we inferred that mutant zebrafish remained sensitive to neuroactive drugs and that PTZ partially restored the behavioral phenotypes of larval zebrafish. Neuropsychiatric disorders include a wide range of behavioral phenotypes, whereas the therapeutic effects of targeted small-molecule compounds obviously require further research. This study provides a powerful functional basis for the future exploration of drug sensitivity and intervention in *nomol* mutant zebrafish and may provide additional insight into research performed in this direction in the future.

Neuropsychiatric disorders are widespread, and their manifestations are diverse. Much genes are involved in the pathogenesis of these diseases. It is necessary for us to analyze important genes, and *nomol* is such gene. Taken together, the data presented here conclusively implicate that *nomol* deficiency causes autism-like behaviors and highlight its critical role in brain development. The *nomol* zebrafish model in this study further provides the clues of comorbidity associated with neuropsychiatric disorders and neuroactive drug screenings in precision medicine in future.

**Limitations**

This study demonstrated that loss-of-function of *nomol* elicits neuropsychiatric disorders, however, more evidence on molecular pathogenesis mechanisms of *nomol* deficiency is needed. Moreover, the intervention of neuroactive drug on the behavior phenotype and functional mechanism of mutant zebrafish needs detailed and extensive evidence.

**Conclusions**

For the first time, we generated a *nomol* loss-of-function zebrafish model that recapitulate key features of neuropsychiatric disorder-related behavioral phenotypes, especially autism-like behaviors in adolescent zebrafish. The enlarged interstitial spaces of midbrain and hindbrain in *nomol*/*−−* zebrafish suggest the molecular mechanism underlying *nomol* deficiency in brain development. The increased levels of neurotransmitters in the brain of *nomol*/*−−* zebrafish and transcriptome analysis at the genetic level also provide further evidence supporting the potential role of *nomol* in neuropsychiatric disorders. The *nomol* mutant zebrafish provides a valuable model for
future research on the molecular pathogenesis of nomol-related disorders and drug screening.

**Abbreviations**

ASD: Autism spectrum disorder, ADHD: Attention deficit hyperactivity disorder, CNVs: Copy number variations, WISH: Whole-mount in situ hybridization, PTZ: Pentylenetetrazole, dpf: Days postfertilization, mpf: Months postfertilization, SgRNA: Specific guide RNA, RT-qPCR: Real-time quantitative polymerase chain reaction, LC-MS/MS: liquid chromatography-tandem mass spectrometry, HPLC: High-precision liquid chromatography, HE staining: Hematoxylin-eosin staining, DEGs: Differentially expressed genes, WT: Wild-type, PMS: Phelan-McDermid syndrome.

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**Availability of data and materials**

The datasets in the current study are available from the corresponding author on reasonable request.

**Authors’ contributions**

This study was designed by QL, XW and FL. FL, JL, TTL and JJ performed the experiments. FL dedicated to the writing of the manuscript, with extensive editing by QL. YLZ, XYL and YXJ designed CRISPR/Cas9 mutagenesis of nomol loci in zebrafish and provided homozygous identification assistance. QZ provided technical assistance in transcriptomics and HPLC analyses. All authors read and approved the final manuscript.
Ethics approval

All procedures are approved by the institutional animal care committee of Children’s Hospital of Fudan University, China.

Competing interests

The authors declare no conflict of interest.

Consent for publication

Not applicable.

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Figure legends
Fig. 1. The expression level of nomo1 mRNA during development and generation of hereditary nomo1 mutant zebrafish. (A) Structure of the zebrafish nomo1 gene. Exon 7 was the target site in zebrafish. (B) The principle of the CRISPR/Cas9 technique. (C) Microinjection of zebrafish at the single-cell phase. (D) Acquisition of stable inherited homozygous zebrafish. (E) Sequencing results for nomo1 in WT, heterozygous and homozygous zebrafish. The mutation induced by CRISPR/Cas9 (1-base deletion) is shown in nomo1 mutant sequences. (F) The relative levels of nomo1 transcription during developmental stages. (G) Reduced expression of nomo1 mRNA in the whole embryo at 48 hpf, heads at 14 dpf and brain tissues at 2 mpf of nomo1−/− zebrafish analyzed by RT-qPCR. (H) Translation of the amino acids predicting termination codon. Data are shown as the mean ± SEM.

Fig. 2. The locomotion of 7 dpf zebrafish with or without PTZ, and the thigmotaxis behavior in WT and mutant zebrafish. (A) Light/dark test of larval nomo1+/+ and nomo1−/− zebrafish at 7 dpf. The experiment lasted for 75 min and consisted of 40 min of light (L0) and two 5-min light/dark cycles (L1-D1 and L2-D2). (B) Trend in the total distances swum by larval zebrafish during the experimental period. (C) The average distance moved within each 1-min bin under continuous illumination is shown (N = 48 for each genotype). (D-E) The average distance moved per minute under two light/dark cycles is exhibited. * indicates a comparison with the WT group under the same lighting conditions. # indicates the same zebrafish under different illumination conditions. (F) The ratio of the distance moved by larval zebrafish during each light/dark cycle. (G) The testing apparatus in a 24-well plate. The inner and outer zones were delineated as shown above. The ratio of distance moved/time spent in the inner zone indicates thigmotaxis behavior. (H) The ratio of distance moved/time spent in the inner zone under light conditions (L0 period) in 7 dpf zebrafish larvae. (I-J) The impaired locomotion of nomo1−/− larval zebrafish can be recovered by PTZ (N = 36 for each genotype). (I) The trend in the total distance swum by 7 dpf larval zebrafish during 60 min of continuous illumination. (J) The average distance moved within each 1-min bin under continuous illumination is plotted. Data are shown as the mean ± SEM, *p < 0.05, **p < 0.01, ****p < 0.0001, ####p < 0.0001.

Fig. 3. The locomotion of infant and adolescent WT and nomo1−/− zebrafish. (A) Container of 15 dpf zebrafish in the open field experiment. (C) Container of 30 dpf and 2 mpf zebrafish in the open field experiment. (B, D, and E) The locomotion of WT and mutant zebrafish over the total 30 min experimental period (N = 16 for each genotype in different developmental stages). (F) The average distance moved within each 1-min bin under continuous illumination is plotted. Data are shown as the mean ± SEM, *p < 0.05.
Fig. 4. Adolescent *nomo1*−/− zebrafish displayed social deficiency and repetitive behavior. (A-E) The social preference experiment with adolescent zebrafish. (A) Schematic diagram of the individual social behavior experiment. A heat map (B-C) demonstrate that *nomo1*−/− zebrafish spent significantly less time in the social area than WT zebrafish. The ratio of distance from the social area (D) and time spent in the social area (E) was significantly lower for *nomo1*−/− zebrafish than for WT zebrafish (N = 20 for each group). Schematic of the shoaling test (F-G) in which the IID exhibited by *nomo1*−/− zebrafish was significantly higher than that of WT zebrafish (N = 8 for each group). (H-I) Schematic of different representative behaviors shown by adolescent *nomo1*−/− zebrafish: including “back-and-forth” motions, “large circular movement” and “stereotypic movement”. *Nomol*−/− zebrafish demonstrated a significantly higher proportion of back-and-forth motions than *nomo1*+/+ zebrafish (N = 16 for each group). Data are shown as the mean ± SEM, *p < 0.05.

Fig. 5. Loss-of-function of *nomo1* affected neurodevelopment in larval zebrafish and the results of brain slice analysis of adult male zebrafish. (A-D) The sites of *hgg1* and *sox17* expression in WT and mutant zebrafish at 12 hpf determined using WISH. (E-F) The expression of neurological genes (*HuC, neurogl1, islet1, egr2b* and *foxb1a*) in *nomo1*−/− zebrafish at 24 hpf and 48 hpf by RT-qPCR (N = 6×10 for each group). Data are shown as the mean ± SEM. *p < 0.05, **p < 0.01. (G-L) Mutant zebrafish at 48 hpf were analyzed by WISH to detect *foxb1a* (G-H), *neurogl1* (I-J) and *islet1* (K-L) expression. The loss-of-function of *nomo1* reduced the midbrain and hindbrain volume in zebrafish at 48 hpf (arrows in H, J, L; scale bar: 100 μm). (M-P) The midbrain and hindbrain structures of adult WT and mutant zebrafish (scale bar: 200 μm).

Fig. 6. Differences of the expression of neurotransmitters and metabolites between the brain tissues of WT and *nomo1*−/− adolescent zebrafish. Thirteen neurotransmitters and metabolites were analyzed using the LC-MS/MS, and four neurotransmitters were analyzed using HPLC. (A) Cluster analysis between *nomo1*+/+ and *nomo1*−/− zebrafish using SRM/MRM demonstrated neurotransmitters and metabolites tended to be upregulated in mutant zebrafish. (B) Statistical analysis of the SRM/MRM data. The vertical axis denotes the normalized levels of neurotransmitters and metabolites (N=3×8 groups each of WT and mutant zebrafish). (C) Statistical analysis of the HPLC data. The vertical axis denotes the levels of 4 neurotransmitters and metabolites in zebrafish brain tissues (N=8×7 each group). Data are presented as the mean±SEM, *p < 0.05, **p < 0.01, ***p < 0.001.
Fig. 7. Transcriptome sequencing analysis of nomo1 mutant zebrafish. (A) Volcano map of DEGs between WT and nomo1/− zebrafish. The abscissa and ordinate indicate the fold change in expression of DEGs (log2FoldChange) and the significance level of the DEGs (-log10padj) between WT and mutant zebrafish, respectively. The upregulated genes are indicated by a red dot, and the downregulated genes are indicated by a green dot. The significant level of enrichment (padj) was set as the multihypothesis test corrected p-value (p-value< 0.05). (B) Chart showing differential expression and GO annotation classification. The horizontal coordinate is the GO term, and the vertical coordinate is the significance level of GO term enrichment. The higher the value is, the more significant it is. Different colors indicate the three GO subclasses: BP (biological process), CC (cellular component) and MF (molecular function). (C) KEGG pathway analysis of DEGs. Advanced bubble chart showing the enrichment of DEGs in signaling pathways. The vertical axis indicates the pathway, and horizontal axis indicates the gene ratio (gene ratio is the ratio of the number of DEGs to the total number of DEGs annotated to the KEGG pathway). The size and color of the bubble represent the number of DEGs enriched in a pathway and the enrichment significance, respectively. (D) Representative experimental validation of DEGs by RT-qPCR analysis. Gene expression is presented as the mean ± SEM, and a t-test was performed to compare gene expression between WT and nomo1/− zebrafish (*p < 0.05, **p < 0.01, N = 4×10 groups each for WT and mutant zebrafish).

Additional files:

Fig. S1. Morphological analysis of WT and nomo1 mutant zebrafish during developmental stages. (A-D) The morphology of WT and nomo1 mutant zebrafish at 24 hpf (A), 48 hpf (B), 14 dpf (C) and 2 mpf (D). (E) The body length of nomo1+/+ and nomo1/− zebrafish at 48 hpf, 14 dpf and 2 mpf (N= 16 for each group). (F) Abnormal morphology of nomo1+/+ and nomo1/− zebrafish at 24 dpf: developmental delay, tail bending, pericardium edema and developmental malformation (nomo1+/+, N = 20; nomo1/−, N = 18). Data are shown as the mean ± SEM.

Fig. S2. The forebrain marker expression and DA levels were no different between WT and mutant zebrafish. (A) Expression of neurological genes in nomo1/− mutant zebrafish at 48 hpf determined by RT-qPCR. Data are shown as the mean ± SEM. (B-E’) Mutant zebrafish at 48 hpf were analyzed by WISH to detect the site of related gene expression. Scale bar: 100 μm.

Fig. S3. There is no difference in kin recognition behavior among WT and nomo1/− zebrafish. (A) Schematic diagram of the kin recognition experiment. (B-C) The ratio of distance moved/time spent in the kin preference region in nomo1/− zebrafish was not
different from that determined for WT zebrafish (N = 15 for each group). Data are presented as mean ± SEM.

Table. S1. The primer sequences are used in this study.

Video. S1. *Nomol*⁻/⁻ zebrafish swimming in repetitive pattern of back-and-forth motions in the open field.

Video. S2. *Nomol*⁻/⁻ zebrafish swimming in repetitive pattern of stereotypic movement in the open field.

Video. S3. *Nomol*⁻/⁻ zebrafish swimming in repetitive pattern of large circular movement in the open field.

Video. S4. The social preference behavior of WT zebrafish in mating tank.

Video. S5. The social preference behavior of *nomol*⁻/⁻ zebrafish in mating tank.

Video. S6. The shoaling behavior of WT zebrafish in the open field.

Video. S7. The shoaling behavior of *nomol*⁻/⁻ zebrafish in the open field.

Video. S8. The kin preference behavior of WT zebrafish in mating tank.

Video. S9. The kin preference behavior of *nomol*⁻/⁻ zebrafish in mating tank.