Deficiency of nde1 in zebrafish induces brain inflammatory responses and autism-like behavior

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Highlights
nde1<sup>−/−</sup> zebrafish display autism-like behavior features
nde1 deficiency results in immune responses in the brain
Minocycline treatment inhibits immune responses in the adult nde1<sup>−/−</sup> brain
Minocycline rescued the impaired social behavior and locomotor activity
Deficiency of nde1 in zebrafish induces brain inflammatory responses and autism-like behavior

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SUMMARY
The cytoskeletal protein NDE1 plays an important role in chromosome segregation, neural precursor differentiation, and neuronal migration. Clinical studies have shown that NDE1 deficiency is associated with several neuropsychiatric disorders including autism. Here, we generated nde1 homologous deficiency zebrafish (nde1−/−) to elucidate the cellular molecular mechanisms behind it. nde1−/− exhibit increased neurological apoptotic responses at early infancy, enlarged ventricles, and shrank valvula cerebelli in adult brain tissue. Behavioral analysis revealed that nde1−/− displayed autism-like behavior traits such as increased locomotor activity and repetitive stereotype behaviors and impaired social and kin recognition behaviors. Furthermore, nde1 mRNA injection rescued apoptosis in early development, and minocycline treatment rescued impaired social behavior and overactive motor activity by inhibiting inflammatory cytokines. In this study, we revealed that nde1 homozygous deletion leads to abnormal neurological development with autism-related behavioral phenotypes and that inflammatory responses in the brain are an important molecular basis behind it.

INTRODUCTION
NDE1 (NudE Nuclear Distribution E homologue1) is located within 16p13.11, a hotspot chromosome segment associated with neuropsychiatric disorders (Ramalingam et al., 2011). Several clinical studies have shown that copy number deletion and duplication variants in the NDE1 region are associated with neuropsychiatric disorders (Bakircioglu et al., 2011; Borlot et al., 2017; Kirov et al., 2009; Ramalingam et al., 2011; Ullmann et al., 2007; Williams et al., 2010). Among which, deletions of NDE1 are strongly associated with intellectual disability (ID), autism, epilepsy, microcephaly, and so on. In 2010, Reinhard et al. detected deletion mutations of the NDE1 region in three patients with ID (Ullmann et al., 2007). In 2011, Ramalingam et al. reported four patients containing deletion mutations in the NDE1 region with clinical features including autism (2/4), epilepsy (3/4), and developmental delay (4/4) (Ramalingam et al., 2011). In the same year, Bakircioglu et al. identified severe microcephaly with ID in the off-springs of three families with NDE1 deletions (Bakircioglu et al., 2011). In 2013 and 2017, three cases of microcephaly that resulted from NDE1 deletions were reported (Paciorkowski et al., 2013; Tan et al., 2017).

NDE1 encodes a cytoskeletal protein located in the centrosome that can act as part of the LIS1-NDE1-cytoplasmic kinesin complex (Hennah et al., 2007). This complex is responsible for transporting vesicles and other entities from the axon tip to the neuronal cell body, involved in the proliferation and differentiation of neuronal precursors, neuronal migration, and neurite outgrowth (Hennah et al., 2007; Lancot et al., 2013). Specifically, NDE1 involved in mitosis cell-cycle-linked oscillatory movement of the nucleus of radial glia progenitor (RGP) cells, and Nde1 knockout in mice results in abnormal cortical development (Doobin et al., 2016; Houlihan and Feng 2014). Knockdown of nde1 in zebrafish by MO technique leads to a delay in cell-cycle re-entry that correlates with ciliary length (Feng and Walsh 2004; Kim et al., 2011). However, the cellular-molecular mechanisms associated with neuropsychiatric disorders remain incompletely elucidated.

The cortical development and overall structure of the zebrafish brain is similar to that of mammals. For instance, neuronal epithelial cells of zebrafish proliferate to constitute the cortex through the interkinetic nuclear migration, similar to human (Leung et al., 2011). In addition, the immune system of zebrafish has almost all the lymphoid organs and immune cell types of mammals, the main innate immune signaling pathways such as TLR and NLR signaling pathways, and cytokines are conserved in zebrafish and mammals (Gomes and Mostowy 2020; Trede et al., 2004). A range of zebrafish motor behavior traits, such as...
spontaneous motor behavior, shoaling behavior, social behavior, repetitive stereotyped behavior, environmental adaptation, and aggressive behavior can serve as indicators of anxiety, depressive, pressure, or conflict symptoms (Geng and Peterson 2019; Kabashi et al., 2011). Hence, zebrafish are great models for pathogenesis research of neuropsychiatric disorders.

RESULTS
nde1 expression analysis in zebrafish during development

To investigate the expression pattern of nde1 during development, we first analyzed the expression levels of nde1 in zebrafish embryos or brain tissues at different time points. The results showed that nde1 expression peaked at around 24 hpf-36 hpf (hours postfertilization) during early embryonic development and then decreased to one-fifth of 24 hpf expression levels at five dpf (days postfertilization) (Figure 1A). From five

Figure 1. nde1 Expression analysis and generation of nde1 deficiency zebrafish

(A) Relative expression of nde1 in zebrafish embryos during early development. Data are represented as mean ± SEM.

(B) Relative expression of nde1 in zebrafish brain during five dpf to six mpf.

(C–G) Whole mount in situ hybridization of nde1 during early development. Scale bar: 0.2 mm.

(D) Amplified images of boxed area in (D).

(H) Diagram of target site on second exon of zebrafish nde1 genomic DNA, representative sequencing results and predicted ORF sequence of nde1+/+ and nde1−/−; red letter indicates N20 sequence; blue letter indicates NGG.

(I) Reduced expression of nde1 mRNA in the brain of 6 mpf nde1−/− zebrafish. ***p < 0.001. Data are represented as mean ± SEM.

spontaneous motor behavior, shoaling behavior, social behavior, repetitive stereotyped behavior, environmental adaptation, and aggressive behavior can serve as indicators of anxiety, depressive, pressure, or conflict symptoms (Geng and Peterson 2019; Kabashi et al., 2011). Hence, zebrafish are great models for pathogenesis research of neuropsychiatric disorders.
In situ hybridization analysis on early development embryos was consistent with expression quantification results. At 12 hpf, *nde1* was broadly expressed in the brain primordium and somites (Figure 1C). At 24 hpf, *nde1* was abundantly expressed in the nervous system, especially in the midbrain, hindbrain, and spinal cord (Figure 1D, arrows); magnified images in Figure 1D’ displayed the widespread expression of *nde1* in the hindbrain and spinal cord. At 36 hpf and 48 hpf, *nde1* expression in the midbrain and hindbrain persisted. From 36 hpf, *nde1* expression in the spinal cord was diminished and eventually disappeared at 48 hpf. At three dpf, *nde1* was only minimally expressed in the mid-hindbrain boundary (Figures 1E–1G).

**Generation of *nde1*-deficient zebrafish**

We generated *nde1* knockout zebrafish using the CRISPR/Cas9 system. 20 nt *nde1* gene-specific guide RNA was designed to target the second exon. Genotyping of target PCR products after two generations of heterozygotes cross-confirmed that targeting allele carries a 4bp (TATG) deletion (Figures 1H and S1). The deletion resulted in a frameshift mutation and generates an early stop codon in exon 2 (Figure 1H). RT-qPCR analysis confirmed that *nde1* mRNA expression was significantly reduced in *nde1*−/− zebrafish (Figure 1I). These results indicate that we have successfully generated an *nde1*-deficient zebrafish transgenic line.

**Morphological analysis and apoptosis analysis of *nde1*−/−**

To further investigate the effect of *nde1* knockout on zebrafish embryonic development, we performed morphological analysis and apoptosis analysis of *nde1*−/−. During *nde1*−/− development, no significant cosmetic malformations were observed (data not shown), whereas at 30hpf, the *nde1*−/− nervous system, including telencephalon, midbrain, rhombomere, and spine, exhibited increased apoptosis (Figure 2A). Statistical analysis also showed a significant increase in apoptotic bodies in *nde1*−/− (Figure 2B).

Subsequently, we performed anatomical analysis for adult *nde1*+/+ and *nde1*−/− zebrafish; the brain size and weight both showed no significant differences (Figure S2). Further analysis with HE-stained paraffin sections revealed the brains of adult *nde1*−/− exhibited relatively loosened structure, enlarged ventricles (Figure 2C, black arrow), and reduced valvula cerebelli (Figure 2C, red arrow). Figure S3 showed sections of three additional independent experiments. These results suggest that *nde1* knockout leads to increased apoptosis in early development and abnormal brain structure.

**Locomotor activities of *nde1*−/− zebrafish at five dpf, two mpf, and three mpf**

Neuropsychiatric disorders are mainly manifested by abnormal behavior phenotype. To determine whether the loss of function of *nde1* affects the locomotor activities during development, we performed the behavioral analysis at 5dpf (early childhood), 2mpf (young adulthood), and 3mpf (adulthood).

Figure 3A shows the experimental timing settings of analysis of 5dpf zebrafish locomotor activities. Firstly, locomotor activities of 25 min adaptation period (AP) and then 30 min activity stabilization period (ASP) were recorded. Then, we set up two light-dark switch cycles (5 min 100 lx for brightness and 5 min 0 lx for dark for one cycle) to detect the response of zebrafish under light variation. Results showed that the average swimming distances per minute of *nde1*−/− is significantly higher than that of *nde1*+/+ throughout the whole experiment (Figures 3B and 3C). Under the two light-dark switch cycles, *nde1*−/− zebrafish displayed the same light-dark responses as the wild type (Figure 3B). We also analyzed the activity frequency of ASP at three intensity scales, 0–40 as low intensity, 40–80 as medium intensity, and 80–120 as high intensity. *nde1*−/− shows decreased low intensity activities and increased medium and high intensity activities (Figure 3D).

At two mpf and three mpf, locomotor activities were analyzed under light condition for 30 min, with AP for the first 10 min and ASP for the last 20 min. Results were consistent with that of five dpf; the average swimming distances per minute of *nde1*−/− were significantly higher than that of *nde1*+/+ (Figures 3E–3G, comparison of 20 min ASP of locomotor activity of 3 mpf; see Figure S4).

When the swimming trajectories of zebrafish were analyzed by the software, we noticed that repetitive behaviors of two mpf and three mpf *nde1*−/− zebrafish, including back and forth, big circling, and small circling, exhibited a significantly higher frequency than *nde1*+/+ (Figures 3H–3J).
Social behavior analysis of $nde1^{-/-}$ zebrafish at 2 mpf and 3 mpf

Zebrafish usually swim in clusters, and single fish tend to join groups of zebrafish of the same species (Spence et al., 2008). This characteristic of zebrafish can be used as a metric for its social activity. In order to explore the impact of $nde1$ knockout on social behavior of $nde1^{-/-}$, we further analyzed the shoaling behavior, social preference, and kin preference. Each analysis lasts for 30 min, with AP for the first 10 min and ASP for the last 20 min.

In shoaling behavior analysis, the interindividual distance between populations of $nde1^{-/-}$ zebrafish at two mpf and three mpf was significantly higher than that of wild type, indicating that the population homogeneity of $nde1^{-/-}$ was impaired (Figures 4A–4B). In the analysis of social preference behavior, two mpf and three mpf $nde1^{-/-}$ zebrafish both spend significantly shorter time and distance in the social region (area 1).
compared with nde1+/- . It indicates that for nde1 knockout zebrafish, the group tendency is relatively weak in both adolescence and adulthood (Figures 4C and 4D).

In the kin recognition and preference test, nde1+/- adult zebrafish (n = 28) spent more time at the kin region (area 1, kin group was conspecific and same color) and spent less time and distance at the non-kin region (area 2, non-kin group was red zebrafish) (Figures 4E and 4F). This suggests that nde1+/- are inclined to recognize similar groups and exhibit low sociality.

Transcriptome analysis of nde1 knockout zebrafish

Morphological, anatomical, and behavioral analyses suggest that nde1 deletion mutations result in morphological and functional abnormalities in the nervous system. To further explore the underneath molecular mechanism, we performed transcriptome profiling and analyzed global gene expression patterns of the 2mpf zebrafish brain. Sample clustering analysis showed good homogeneity within both the control groups (nde1+/+) and experimental groups (nde1-/-) (Figure S5A).

As was shown in volcano plot of the gene expression profiles, nde1 deficiency resulted in approximately 850 DE-Gs (p-adjust < 0.05 and \(|\log2FC| \geq 1.0\), of which 484 genes were upregulated and 366 genes were downregulated (Figure S5A) (Table S1, Detailed information of DE-Gs, related to Figure S5A). GO clustering analysis showed that DE-Gs were mainly enriched in biological process such as regulation of RNA metabolic and biosynthetic (Figure S5B). KEGG clustering analysis revealed that differential genes were mainly enriched in immune disease and inflammation-related pathways, such as antigen processing and presentation, Legionellosis, and inflammatory bowel disease (Figure S5B). The KEGG pathway enrichment string map (Figure S6) showed genes that differ significantly in the enrichment pathway.

Analysis of enriched KEGG pathways and key node genes revealed significant increase in the expression of the inflammatory signaling molecule NF-kb and apoeb, marker gene of the main immune cells of brain, microglia. Of note, microglia are a primary source of pro-inflammatory cytokines in the brain (Miller et al. 2009), which prompt us to analyze the expression levels of inflammatory cytokines in brain. Results showed that interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), IL-1β was significantly promoted, whereas IL-4 and IL-12 were unaffected in nde1-/- brain (Figure 5C). These results suggest that nde1 mainly affects the expression levels of immune and inflammation-related pathways, especially inflammatory cytokines.

Analysis of mitosis, apoptosis, and microglia-development-related gene expression levels in early embryonic development

nde1 is involved in the mitotic process of nerve cells, and former results in this study showed nde1 deficiency results in excessive apoptosis in nervous system. To investigate the molecular mechanisms behind this, we first analyzed the expression levels of genes related to mitosis and apoptosis in early embryonic development. Results showed that at 24 hpf, DNA replator pcam, geminin DNA replication inhibitor gmmn, and bicdc2, which recruited the dynein to the nuclear envelope during early G2, were all upregulated in nde1-/- embryos (Figure 6A).

We then analyzed the expression of the pro-apoptotic genes pidd1 and bbc3 and genes that inhibit apoptosis, bcl2a, and bcl2l at 30 hpf. The results were consistent with the phenotype, pidd1 and bbc3 were promoted, and bcl2a and bcl2l were inhibited in nde1-/- embryos (Figure 6B). After 60 hpf, macrophages in the brain began transforming into microglia and abruptly started expressing apoeb (Herbomel et al. 2001). Quantitative analysis results showed nde1-/- embryos express significantly higher expression level of apoeb at four dpf (Figure 6C).
Figure 4. Social behavior analysis of nde1−/− zebrafish

(A) Schematic of shoaling test.

(B) Significantly increased inter-fish distance of 2 mpf (n = 10) and 3 mpf (n = 10) nde1−/− zebrafish.

(C) Schematic and representative heatmap of social preference test; area1: social area, area2: nonsocial area.
The phenotypes of enlarged ventricles and reduced cerebellar valves of NDE1-deficient zebrafish compared with NDE1+/− zebrafish showed variable degrees of shrank cerebral hemispheres and ventriculomegaly (Abdel-Hamid et al., 2019). The open field, bellum (Akshoomoff et al., 2004; Stigler et al., 2011), CT scans of patients with NDE1 deficiency results in increased locomotor activity that does not habituate over time. In tests of shoaling, social preference, and kin preference during youth and adulthood, NDE1+/− zebrafish showed significantly reduced preference for groups and exhibited abnormal social novelty (Figure 4). In the open field, NDE1+/− showed significantly increased repetitive behaviors such as back and forth (large circles and small circles) (Figure 3). Social deficits, repetitive behaviors, and hyperactivity are among the main clinical features of autism (Lai et al., 2014). These behaviors in NDE1 knockout zebrafish can be interpreted as autism-like behaviors. In addition, brain MRI imaging of autistic patients showed reduced cerebellum (Akshoomoff et al., 2004; Stigler et al., 2011), CT scans of patients with NDE1 double allele variants showed variable degrees of shrank cerebral hemispheres and ventriculomegaly (Abdel-Hamid et al., 2019). The phenotypes of enlarged ventricles and reduced cerebellar valves of NDE1+/− zebrafish were consistent with autism patients (Figure 2). This suggests that NDE1 knockout zebrafish can be used as a model for etiological studies of neuropsychiatric disorders such as autism caused by NDE1 variants.

**Rescue of mitotic disorders and excessive apoptosis by NDE1 mRNA injection**

Furthermore, we performed rescue experiments of NDE1 mRNA restoration in NDE1 knockout embryos. The results showed that at 24 hpf, upregulated bicd2, gmn, and pcna were inhibited in mutant embryos with NDE1 mRNA supplementation, and gmn and pcna were restored to normal expression levels (Figure 6A), whereas at 30 hpf, pidd1 and bbc3, bc12a, and bc12l expression levels were also back to normal (Figure 6B); apoptotic bodies in the nervous system of NDE1-rescue embryos were significantly reduced than NDE1+/− (Figures 6D and 6E). At four dpf, the expression level of apoeb was significantly decreased compared with the NDE1+/− but still significantly higher than the wild type (Figure 6C).

**Rescue of behavior deficits and brain inflammation of NDE1+/− zebrafish by minocycline**

Minocycline (MC) is a tetracycline-derived antibiotic that could penetrate the blood-brain barrier and inhibits microglia activation. Previous research demonstrate that MC attenuates inflammation associated with microglial activation, decreases cytokines expression such as IL-1β, and inhibits nuclear factor NF-kb activation (Henry et al., 2008; Si et al., 2004).

In order to determine whether MC could inhibit the brain inflammatory response and rescue autism-like behavior in NDE1-deficient zebrafish, we performed series of analysis. After MC immersion treatment for two weeks, expression of NF-kb, IL-6, TNF-α, and IL-1β in 3.5 mpf NDE1+/− zebrafish brains were restored to normal levels (Figure 7A).

Then we analyzed the behavior performance of four experimental groups of zebrafish. The locomotor activities of NDE1−/− were significantly higher than that of NDE1+/−, which was consistent with former result. After MC treatment, average swimming distances per minute of NDE1−/− zebrafish showed no significant difference compared with control group NDE1+/− (Figures 7B and 7C).

In shoaling behavior analysis, MC treated NDE1−/− zebrafish showed rescued population homogeneity, the inter-individual distance between populations displayed no significantly difference from the control (Figure 7D). Repaired group tendency confirmed also by the analysis of social preference behavior, the total time and distances of MC-treated NDE1−/− zebrafish swam in the social area did not differ from wild type (Figure 7E). These results indicate that MC treatment downregulated the overexpression of inflammatory cytokines in NDE1−/− zebrafish and rescued their impaired social behavior and locomotor activity.

**DISCUSSION**

Increased locomotor activity is one of the behaviors that characterize anxiety in human (Maphanga et al., 2020). NDE1 deficiency results in increasement in locomotor activity that does not habituate over time. In tests of shoaling, social preference, and kin preference during youth and adulthood, NDE1−/− zebrafish showed significantly reduced preference for groups and exhibited abnormal social novelty (Figure 4). In the open field, NDE1−/− showed significantly increased repetitive behaviors such as back and forth (large circles and small circles) (Figure 3). Social deficits, repetitive behaviors, and hyperactivity are among the main clinical features of autism (Lai et al., 2014). These behaviors in NDE1 knockout zebrafish can be interpreted as autism-like behaviors. In addition, brain MRI imaging of autistic patients showed reduced cerebellum (Akshoomoff et al., 2004; Stigler et al., 2011), CT scans of patients with NDE1 double allele variants showed variable degrees of shrank cerebral hemispheres and ventriculomegaly (Abdel-Hamid et al., 2019). The phenotypes of enlarged ventricles and reduced cerebellar valves of NDE1−/− zebrafish were consistent with autism patients (Figure 2). This suggests that NDE1 knockout zebrafish can be used as a model for etiological studies of neuropsychiatric disorders such as autism caused by NDE1 variants.
NDE1/LIS1/dynein complex essentially organizes the mitotic spindle to control nuclear migration, cell-cycle progression, and mitosis (Bakircioglu et al., 2011; Soto-Perez et al., 2020). The depletion of NDE1 results in the failure of microtubules to focus on kinetochores, followed by impaired chromosome segregation (Vergnolle and Taylor 2007). In this study, \( \text{nde1} \) knockout zebrafish exhibit abnormal high expression of mitosis-related genes such as \( \text{bicd2}, \text{gmn} \), and \( \text{pcna} \) during early embryonic development (Figure 6A), as well as increased expression of genes that promote apoptosis such as \( \text{pidd1}, \text{bbc3} \) and decreased expression of genes that inhibit apoptosis such as \( \text{bcl2a}, \text{bcl2l} \) (Figure 6B). In mice, \( \text{Nde1} \) mutants have been found to cause DNA damage, leading to the activation of \( \text{p53} \)-dependent apoptosis and affecting the differentiation of neural progenitor cells (Pei et al., 2014). In the brains of autistic patients, increased apoptosis was detected in both the frontal cortex and cerebellum with downregulation of \( \text{Bcl2} \) (Araghi-Ni-\kern0pt knam and Fatemi, 2003; Malik et al., 2011; Sheikh et al., 2010). We hypothesize that \( \text{nde1} \) mutations may trigger mitotic catastrophe, a mechanism that senses mitotic failure and subsequently drives cells into apoptotic or necrotic steps (Vitale et al., 2011). This speculation was partially supported by the \( \text{nde1} \) mRNA rescue assay, in which the number of apoptotic cells in the nervous system was dramatically reduced after mitosis-related gene expression was restored to normal levels (Figure 6).

There is growing evidence that the immune system plays an important role in brain development and individual life behaviors. Inflammatory cytokines affect brain development, and most neurodevelopmental disorders such as autism, cognitive impairment, cerebral palsy, epilepsy, and schizophrenia are associated with early inflammation (Jiang et al., 2018). Some autistic patients exhibited enhanced neuroinflammatory responses with elevated expression of IL-6, HSP70, TGF-\( \alpha2 \), Caspase 7, and INF-\( \gamma \) (El-Ansary and Al-Ayadhi 2012; Wei et al., 2011). In our study, \( \text{nde1}^{-/-} \) zebrafish expressed high levels of...
microglia marker gene apoeb in both early developmental and adult brains (Figures 5C and 6C). Transcriptomic analysis of adult nde1-/- brains showed the affected pathway was enriched in immune processes, with high levels of expression of pro-inflammatory cytokine IL-6, TNF-α, IL-1β, and immune signaling molecule NF-kb (Figures 5B and 5C). Introduction of nde1 mRNA only partially rescued the upregulated apoeb at 4 dpf, which could be that nde1 mRNA was depleted during development, resulting in incomplete restoration of apoeb to normal levels. Previous studies showed that IL-1β and TNF-α induce neuronal cell death in vivo (Fricker et al., 2018; Matelski et al., 2021). Here in our study, apoptosis occurs earlier in development than the inflammatory response; the inflammatory response could be induced by apoptosis or other reasons.

Taking advantage of the characteristics of minocycline crossing the blood-brain barrier to inhibit microglia activation and inflammatory cytokines, we initially explored the association between brain inflammatory response and autistic-like behavior. The results revealed that the expression of inflammatory factors IL-6, TNF-α, IL-1β, and NF-kb was restored to normal (Figure 7A). More importantly, impaired social behaviors and locomotor activity were also restored to wild-type levels (Figure 7B). Recent studies generated several inferences about how cytokines and microglia are involved in the mechanisms of CNS development and function. Cytokines in the CNS may negatively regulate activity-dependent synaptic pruning by interacting with MHC1; microglia are also involved in functional neuronal pruning and in the regulation of synaptic

**Figure 6. nde1 mRNA rescue mitosis, apoptosis, and immune-related phenotype**
(A–C). Relative expression level of bicd2, gmn, pcna, pid1, bbc3, bcl2a, and bcl2l1, apoeb in nde1+/+/, nde1-/-, and nde1 mRNA rescued zebrafish. (D) AO staining of nde1+/+/, nde1-/-, and nde1 mRNA rescued zebrafish at 28 hpf. White dotted oval indicates telencephalon, midbrain, rhombomere, and spine, respectively. Scale bar: 0.2 mm. (E) Statistics of apoptotic bodies after AO staining. *p < 0.05, **p < 0.01, ***p < 0.001. Data are presented as mean ± SEM.
plasticity in cognitive processes such as learning and memory (Miller et al. 2009). Another theory states that cytokines may influence human behavior by activating the hypothalamic-pituitary-adrenal axis and stimulating the secretion of pro-adrenocorticotropic hormones, arginine vasopressin, and corticosterone. In the context of overactive microglia responses, excessive amounts of pro-inflammatory cytokines have been shown to induce excitotoxicity and neuronal death (Jiang et al., 2018).

Limitations of the study
Our work demonstrates nde1 deletion mutation results in autistic behavioral phenotype similar to that of human in zebrafish, and increased immune response and apoptosis in brain are molecular basis behind the autism-like behavior. The MC rescue experiment indicates immune response inhibition across the blood-brain barrier could act as a candidate therapeautic pathway in nde1-deficiency-resulted autism. However, the pathogenic mechanisms behind inflammatory responses lead to behavioral abnormalities, and how the nde1 mutation leads to inflammatory responses in the brain need further research.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
All procedures are approved by the institutional animal care committee of Children’s Hospital of Fudan University, China.

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AUTHORS CONTRIBUTIONS
Conceptualization: QZ, TTL, QL. Methodology: QZ, TTL, JL, YLZ. Investigation: QZ, JL, YLZ, FL. Visualization: QZ, JL, XDC. Supervision: XW, QL. Writing—Original Draft: QZ, TTL, QL. Writing—Review & Editing: QZ, QL.

DECLARATION OF INTERESTS
Authors declare that they have no competing interests.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| Plasmid: pCas9 | Addgene | Cat: 42876 |
| Plasmid: pcs108-nde1 ORF | This paper | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| Minocycline | Beyotime | Cat: ST1528 |
| Anti-dig-AP, Fab fragments | Roche | Cat: 1109274910 |
| Dig RNA labeling mix | Roche | Cat: 11277073910 |
| Paraformaldehyde | Solarbio | Cat: P1112 |
| TB Green Premix Ex Taq | TaKaRa | Cat: RR820A |
| Trizol | Ambion | Cat: 15596-026 |
| Paraflin | Solarbio | Cat: YA0012 |
| **Critical commercial assays** | | |
| AO staining kit | Solarbio | Cat: CA1143 |
| HE staining kit | Solarbio | Cat: G1102-3 |
| mMESSAGE mMACHINE SP6 kit | Ambion | Cat: AM1304 |
| PrimeScript™ RT reagent kit | TaKaRa | Cat: RR047A |
| Quick spin columns | Roche | Cat: 11274015001 |
| **Deposited data** | | |
| SRA RNA-seq data | This paper | PRJNA776712 |
| **Experimental models: Organisms/strains** | | |
| TU strain | Our lab | N/A |
| Nde1-KO | This paper | N/A |
| **Software and algorithms** | | |
| GraphPad prism | Prism | https://www.graphpad.com/scientific-software/prism |
| Adobe illustrator | Adobe | https://www.adobe.com/cn/products/illustrator.html |
| Zebralab software | ViewPoint Life Sciences | N/A |
| **Oligonucleotides** | | |
| Primers for nde1 ORF amplification, see Table S2 | This paper | N/A |
| Primers for qPCR, see Table S3 | This paper | N/A |

RESOURCE AVAILABILITY

Lead contact

Further information and requests about this study should be directed to and will be fulfilled by the lead contact, Qiang Li (liq@fudan.edu.cn).

Material availability

This study did not generate new unique reagents. All requests for resources and reagents should be directed to the lead contact and will be made available on request after completion of a Materials Transfer Agreement.
Data and code availability
Sequencing data reported in this paper has been deposited to SRA (PRJNA776712) and is publicly available.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENT MODEL AND SUBJECT DETAILS

Animals
2- and 3-month-old Wild type (Tu strain), and nde1−/− zebrafish were raised in a circulating water system at a constant temperature of 28.5°C, and 14/10 h light/dark cycle. Embryos were collected by natural spawning after 1 h and incubated for 7 days in blue water. Then, the larvae were fed in the circulating water system. All animal experiments were approved by the institutional animal care committee of Children’s Hospital of Fudan University (Ethical approval number: 2013-098).

METHOD DETAILS

Synthesis of Digoxigenin-labeled RNA probe and whole mount in situ hybridization
Primers with homologous arm were designed based on zebrafish nde1 ORF sequence (GenBank Accession No: NM_001030203.1) and sequence of plasmid pcs108 (our lab), primer sequence were shown in Table S2. nde1 ORF was obtained by RT-PCR from cDNA libraries and then inserted into pcs108 through seamless clone follow protocols of One Step Cloning Kit (C112, Vazyme, China). Then Digoxigenin-labeled antisense RNA probes were transcribed with T7 RNA polymerase (Thermo, USA), in the presence of Digoxigenin dNTP mix (Roche, Switzerland) from the linearized plasmids. The newly synthesized RNA probes were then purified by Quick Spin Columns (Roche, Basel, Switzerland) and stored at −80°C.

Whole mount in situ hybridization was carried out as described in published paper (Thisse and Thisse, 2008). Imaging was performed using a Nikon MODEL C-DSS230 (DIC images).

Generation of nde1−/− zebrafish using CRISPR/Cas9
N20 sequence (5’-gatgagtcgagactatgagg-3’) were designed with ChopChop (http://chopchop.cbu.uib.no). 50 ng/μL of Cas9 RNA and 150 ng/μL of guide RNA were co-injected into one-cell Tu strain zebrafish embryos. The F1 progenies were screened out the germ line mutation with sequence analysis of the genomic DNA. The identified heterozygote mutant zebrafish were mated with a Tu zebrafish to purify background and produce F2 progenies. The F2 heterozygote progenies were self-crossed to produce F3 progenies. The nde1−/− zebrafish were screened from F3 progenies.

Acridine orange (AO) staining
The AO staining was performed to study the apoptosis of zebrafish embryos. Briefly, the embryos were washed with 1x AO stain buffer (Solarbio, CA1143) for 5 min at room temperature. Then, the embryos were stained with Acridine orange (2 mg L−1 in AO stain buffer) at 4°C for 30 min in the dark. After the staining, the embryos were washed 3 times with PBS for 5 min each time. Stained embryos were photographed under Fluorescence stereo microscope (Leica, MA205). Apoptosis is characterized by apoptotic bodies which were stained as bright green apoptotic bodies. Quantitative statistics of apoptotic bodies of nervous system were counted using a computer algorithm (ImageJ, National Institutes of Health, Bethesda, MD, USA). Counts were verified by manual counting.

Brain weighing
Brain tissues of 3 mpf zebrafish were collected in centrifuge tubes with 60 brains per tube and weighed, n = 5. Simple comparisons between nde1+/+ and nde1−/− zebrafish were determined by Student’s t test.

Paraffin sections
The intact brain tissues of 3mpf zebrafish were collected and fixed in 4% paraformaldehyde for 24 h at 4°C. Fixed brain tissues were dehydrated with 50%, 70%, 80%, 90%, 95%, 100%, and 100% ethanol each for 7 min.
After dehydration, the samples were soaked in xylene I and xylene II each for 5-6 min. Then incubated in melted paraffin at 60°C for 1 h on an NHP-1 hot plate. The brain tissues were incubated 3 times in melted paraffin and cooled until paraffin were set. Finally, Embedded brain tissues were sliced with a paraffin slicer (Leica, RM2235) with 4 μm thickness.

Hematoxylin-eosin (HE) staining
Paraaffin-embedded brain tissue sections were dewaxed in xylene, hydrated with an alcohol gradient and stained in hematoxylin solution for 1 min. The slices were then decolorized in acid alcohol (0.4% HCL in EtOH) for 30 s. After decolorized, the slices were washed in running tap water, and stained with eosin for 6 min. The slices were observed under microscope (Leica, MA205) after sealed with neutral resin seal.

Transcriptome resequencing analysis (RNA-seq)
Total RNA was extracted from 2 mpf brain of nde1 and nde1 zebrafish using TRIzol® Reagent according the manufacturer’s instructions (Ambion). cDNA libraries were constructed according to the manufacturer’s standard protocol (Illumina, Inc.). The Oligo (dT) isolated mRNA was fragmented, and cDNA was synthesized using the mRNA fragments as templates. Then the synthesized cDNA was subjected to end-repair, phosphorylation and ‘A’ base addition according to Illumina’s library construction protocol. During the QC steps, the Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System were used in quantification and qualification of the sample libraries. Then the libraries were sequenced using the Illumina HiSeq 4000 platform. We filtered low-quality, adaptor-polluted, and high content of unknown base (N) reads to get clean reads. Clean reads were mapped to zebrafish genome sequence (version: GRCz11) as reference, and the matching rate ranged from 90.3% to 91.54%.

To identify DEGs between two different samples, the expression level of each transcript was calculated according to the fragments per kilobase of exon per million mapped reads (FRKM) method. DEGs were detected via DESeq2 (parameters: Fold Change ≥ 2.00 and Adjusted p ≤ 0.05). GO functional enrichment and KEGG pathway analysis were carried out by Gotools and KOBAS. (parameters: Bonferroni-corrected P-value ≤ 0.05).

RNA isolation, real-time PCR (qPCR)
Total RNA was extracted from whole embryos (30-50 embryos per sample) or adult zebrafish brain tissues (5-6 brains per sample) using TRIzol reagent (Ambion, USA). A PrimeScript™ RT Reagent Kit (RR047A, TaKaRa, Japan) was used to synthesize complementary deoxyribonucleic acid (cDNA) from 1 μg of total RNA stocks according to the manufacturer’s protocol. Real-time PCR was performed using TB Green Premix Ex Taq (RR820A, TaKaRa, Japan) on a LightCycler® 480 apparatus (Roche, Germany), according to the manufacturers’ instructions. The delta delta CT method was used to calculate the expression levels. The primer sequences used in Real-time PCR are shown in Table S3.

Capped nde1 mRNA synthesis
Capped nde1 mRNA was synthesized in vitro from linearized pcs108-nde1 ORF using the mMESSAGE mMACHINE SP6 Kit (Ambion, USA). Before injection, mRNA was purified by Quick Spin Columns (Roche, Switzerland) and diluted in RNase-free water.

Microinjection
All the samples were injected into the blastodisc at the 1 to 2-cell stage.

Behavioral experiments
All behavioral tests were performed in our behavioral assessment room, with ambient temperature at 28.5 °C. Behavioral experiments were carried out between 9:00 am and 4:00 pm. Zebrafish’s behaviors were quantified by the Zebralab software (ViewPoint Life Sciences, Lissieu, Calvados, Lower Normandy Region, France). Detailed procedures of each test are summarized as follows.

Larval activity
The behavior tests of 5dpf larvae were carried out in 24-well plates (the inner diameter of each well was 18 mm) (Li et al., 2017; Li et al., 2015). Then put the 24-well plates into a Zebrabox apparatus (ViewPoint Life Sciences, Lissieu, Calvados, Lower Normandy Region, France) to record 75 min video of zebrafish...
larvae activities. The zebrafish larvae were first given a 55 min continuous illumination at 110 Lx, followed by two light/dark cycles (5 min illumination followed by 5 min dark). The Zebralab software was used to quantify the zebrafish larvae locomotor activities with recorded videos. The videos were taken at the rate of 25 frames per second, and were pooled into 1 min time bins. The distance swum by the larvae in the whole well was extracted to analyze locomotor activities.

**Open-field locomotor activity test**
For open-field test, fish was tested at 2 mpf and 3 mpf. Each tank was 30 × 30 × 30 cm and filled with 5 cm-height water from circulating water system. The walls were made of opaque white partitions, and a camera was suspended above the tank. Each fish was put into the tank alone, and was allowed to swim freely. Testing sessions was recorded for 30 min. Afterwards, recordings were analyzed with our tracking software, including locomotor activities (distance traveled) and repetitive behavior. Repetitive behavior includes back and forth, small circles, and big circles. Definition of the back and forth is that the zebrafish repeatedly swim back and forth on one side of the tank at least once. The small circles defined that the zebrafish swims a circle from the beginning to the end less than 30 mm, and the swimming time is greater than 5s. The large circles mean that the zebrafish swim counterclockwise or clockwise along the edge of the tank for more than one circle. All data were analyzed after video recording for 10 min.

**Shoaling test**
Six 2 mpf or 3 mpf zebrafish of the same sex were placed in a tank, and allowed to swim freely. Zebrafish were recorded for 30 min (Liu et al., 2018). The shoaling assessment was performed by calculating the average distance between any two zebrafish, and indicated the social behaviors of zebrafish. All data were analyzed after video recording for 10 min.

**Social preference test**
Social preference testing was performed in a transparent mating tank (inner dimensions 21 × 10 × 7.5 cm). A transparent separator was used to separate the tank into two same size halves. The tested zebrafish (2mpf or 3mpf) were put on one side of the tank with a group of six conspecific zebrafish on another side (Figure 4C). Their behavior was recorded for 30 min. For the data analysis, the time ratio and distance ratio that zebrafish spent in the zone nearest to their companion (social zone) were calculated. All data were analyzed after video recording for 10 min.

**Kin preference test**
The mating tank used in the kin preference test was the same as those in the social preference test. Two transparent separators divided the tank into three compartments. The tested zebrafish (3 mpf) were put on Middle compartment of the tank, with three kin zebrafish placed on the left and three non-kin (red color) zebrafish placed on the right (Figure 4E). Their behavior was recorded for 30 min the time ratio and distance ratio that zebrafish spent in the kin sector or non-kin sector were calculated as represented kin preference. All data were analyzed after video recording for 10 min.

**Minocycline treatment**
3 mpf WT and nde1 deficiency zebrafish were treated with 10 μM minocycline for 4 weeks. Fresh minocycline water was replaced every day. Zebrafish were placed in a low light environment for minocycline is easily decomposed by light.

**QUANTIFICATION AND STATISTICAL ANALYSIS**
Statistical analysis and mapping in this study were performed using GraphPad Prism 6.0. Data are shown as mean ± SEM. Simple comparisons between nde1+/+ and nde1+/- zebrafish were determined by Student’s t test or Mann-Whitney test. Values at *p < 0.05 were considered that there is a statistically significant.