Journal of Integrative Neuroscience

J. Integr. Neurosci. **2026**; 25(11): 53224
https://doi.org/10.31083/JIN53224

Original Research

Effects of Metformin on Behaviors in *nde1*$^{-/-}$ Zebrafish

Shenglan Gou[1], Qi Zhang[1,*], Jia Lin[1], Yinglan Zhang[1], Ting Liu[1], Qiang Li[1,*]

[1]Translational Medical Center for Development and Disease, Shanghai Key Laboratory of Birth Defect Prevention and Control, NHC Key Laboratory of Neonatal Diseases, Institute of Pediatrics, Children's Hospital of Fudan University, National Children's Medical Center, 201102 Shanghai, China
*Correspondence: qiqi_zhang@fudan.edu.cn (Qi Zhang); liq@fudan.edu.cn (Qiang Li)

Academic Editors: George Panagis and Bettina Platt
Submitted: 23 April 2026 Revised: 30 June 2026 Accepted: 20 July 2026 Published: 19 November 2026

Abstract

Background: Behavioral impairments, including altered locomotor activity, anxiety-like behavior, and social deficits, are common features of various neurodevelopmental disorders. This study investigated the effects of metformin hydrochloride on behavioral abnormalities and inflammatory-marker expression in *nde1*$^{-/-}$ zebrafish, a model associated with behavioral impairments. We hypothesized that metformin hydrochloride might alleviate selected behavioral abnormalities and that these effects could be associated with changes in inflammatory-marker expression. **Methods:** Wild-type and *nde1*$^{-/-}$ zebrafish were treated with metformin hydrochloride. Locomotor activity, social preference and shoaling behavior were evaluated using automated video-tracking assays, and brain inflammatory-marker mRNA expression, including tumor necrosis factor-alpha (*TNF-α*), interleukin-1 beta (*IL-1β*), interleukin-6 (*IL-6*), RELA proto-oncogene, NF-kB subunit (*RELA*) were quantified by quantitative real-time PCR. **Results:** Metformin hydrochloride treatment increased locomotor activity and improved shoaling behavior in *nde1*$^{-/-}$ zebrafish, whereas social preference and anxiety-like thigmotaxis were not significantly rescued. At the molecular level, metformin hydrochloride reduced *TNF-α* mRNA expression, while *IL-1β* and *IL-6* mRNA expression levels were non-significant numerical increased and *RELA* expression was not significantly altered, suggesting selective changes in inflammatory-marker expression. **Conclusions:** These results suggest that metformin hydrochloride improves specific behavioral impairments in *nde1*$^{-/-}$ zebrafish, and are associated with changes in selected inflammatory-marker mRNA levels, suggesting potential relevance for further investigation in neurodevelopmental disorders with behavioral deficits.

Keywords: metformin; zebrafish; neurodevelopmental disorders; behavior, animal

1. Introduction

1.1 Behavioral Impairments in Neurodevelopmental Disorders May Be Associated With Neuroimmune Dysregulation

Behavioral impairments, including social-communication difficulties, altered activity, and anxiety-related symptoms, occur across neurodevelopmental disorders and can substantially reduce quality of life. These phenotypes arise from interactions between genetic susceptibility and environmental exposures, with inflammation-related effects on developing neural circuits proposed as one contributing mechanism [1,2]. Neuroinflammation, including altered microglial function and cytokine signaling, has therefore received increasing attention in studies of neurodevelopmental and psychiatric conditions [3,4]. The relationship is likely bidirectional: inflammatory processes may contribute to disease development or progression, whereas chronic neural dysfunction may itself promote inflammatory responses and further impair cognition and behavior [5].

Autism spectrum disorder (ASD) is characterized by persistent difficulties in social communication and interaction, together with restricted or repetitive patterns of behavior, interests, or activities [6,7]. Postmortem studies have reported microglial activation in several brain regions of individuals with ASD [8,9], and innate immune signaling may influence synaptogenesis, neuronal proliferation, and neural-circuit maturation during early development [10]. Dysregulated immune activity may also promote oxidative stress and tissue injury, potentially contributing to persistent central nervous system dysfunction [11]. ASD management is multimodal, and non-pharmacological interventions remain central. Evidence-based approaches include behavioral, developmental, naturalistic developmental behavioral, parent-mediated, psychosocial, and technology-assisted interventions aimed at improving social communication, language, adaptive behavior, and daily functioning [12]. Pharmacological treatment is generally directed toward co-occurring or associated symptoms, including sleep disturbance, attention-deficit/hyperactivity disorder symptoms, anxiety, depression, and irritability, rather than the core social-communication features of ASD [13]. Dietary interventions and immunomodulatory compounds, including luteolin, have also been investigated, although evidence varies by approach [14,15]. These limitations support continued investigation of mechanism-informed adjunctive strategies.

1.2 NDE1 Deficiency Is Associated With Cortical Malformations and Behavioral Abnormalities

Nuclear distribution element 1 (*NDE1*) is a microtubule-associated regulator of cytoplasmic dynein

Copyright: © 2026 The Author(s). Published by IMR Press.
This is an open access article under the CC BY 4.0 license.

Publisher's Note: IMR Press stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

that contributes to neural progenitor proliferation, neuronal migration, intracellular transport, and brain morphogenesis [16]. The human *NDE1* gene is located at chromosome 16p13.11, a region susceptible to copy-number variation. Pathogenic *NDE1* variants have been linked particularly to severe microcephaly and lissencephaly [16,17,18]. In mouse models, loss of *Nde1* causes neural progenitor cell-cycle arrest and reduced cortical size [19,20]. Previous work in zebrafish showed that *nde1* deficiency is accompanied by neuronal apoptosis, altered brain morphology, increased expression of inflammatory markers, and abnormalities in locomotor, repetitive, and social-related behaviors [21]. *NDE1* and its paralog nuclear distribution protein nudE-like 1 (NDEL1) regulate dynein-dependent transport and interact with proteins involved in neurodevelopment, although their precise and potentially distinct molecular functions remain incompletely resolved [22,23]. *nde1* knockdown can also disrupt ciliogenesis in zebrafish Kupffer's vesicle, further illustrating its broad developmental role [24]. These observations support the use of *nde1*$^{-/-}$ zebrafish to examine behavioral and molecular consequences of *NDE1* deficiency and to screen candidate interventions.

1.3 Zebrafish Provide a Complementary Model for Studying Neurodevelopmental Disorders

Zebrafish offer several advantages for studying neurodevelopmental disorders. Approximately 84% of human disease-associated genes and 62% of reported autism-risk genes have conserved zebrafish homologs [25,26]. Although mouse and rat models remain predominant in ASD and other neurodevelopmental research, their longer experimental timelines, higher maintenance requirements, and more complex procedures can limit rapid developmental and pharmacological screening. They are also useful for screening therapeutic compounds and evaluating developmental toxicity and behavioral phenotypes [27]. Major zebrafish brain regions, including the telencephalon, mesencephalon, and cerebellum, show functional conservation with mammalian structures involved in sensory, motor, cognitive, and social processes [28]. The zebrafish lateral pallium is commonly considered functionally analogous to the mammalian hippocampal formation, and zebrafish possess conserved gamma-aminobutyric acid (GABA)ergic, glutamatergic, dopaminergic, and serotonergic systems. Neurodevelopmental toxicity can be detected through behavioral changes, including altered locomotor activity and thigmotaxis [29,30]. External fertilization and embryonic transparency facilitate observation of axonal growth, neuronal migration, and apoptosis [31], while clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) and transcription activator-like effector nuclease (TALEN)-based manipulation, high fecundity, and aqueous drug exposure support large-scale genetic and pharmacological studies [32,33].

1.4 Neuroinflammatory Signaling May Contribute to Behavioral Abnormalities in Neurodevelopmental Disorders

Neuroinflammation is characterized by changes in glial activity and inflammatory mediators, including tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6), and has been proposed as one contributor to neurodevelopmental dysfunction [34]. Animal studies indicate that peripheral immune activation can induce inflammatory responses in the central nervous system and lead to behavioral abnormalities [2]. Inflammatory signaling may also disrupt neurotransmitter homeostasis; serotonin, for example, participates in both immune regulation and neuroplasticity [35]. Activated glial cells can generate pro-inflammatory mediators and reactive oxygen species, potentially impairing neurotrophic support, synaptic plasticity, and neuronal viability [4]. These observations provide a rationale for investigating compounds with anti-inflammatory or immunometabolic properties as potential modulators of selected behavioral phenotypes.

1.5 Rationale for Investigating Metformin in ASD-Related Behavioral Phenotypes

Metformin is a first-line treatment for type 2 diabetes that primarily reduces hepatic glucose production and improves insulin sensitivity, in part through adenosine monophosphate (AMP)-activated protein kinase (AMPK)-dependent and AMPK-independent mechanisms [36,37]. Beyond glucose regulation, metformin has anti-inflammatory and immunometabolic effects [36,38]. Its ability to enter the central nervous system has prompted investigation of its actions in neurons and glial cells [39].

Experimental studies indicate that metformin can influence cellular energy metabolism, oxidative stress, apoptosis, nuclear factor kappa B (NF-κB)-related signaling, and inflammatory cytokine production [37,38,39,40]. It has also been studied in autoimmune, inflammatory, oncological, and endocrine conditions [41,42]. In autistic children and adolescents receiving atypical antipsychotics, metformin has an established role in managing treatment-associated weight gain [43]. These observations do not establish efficacy for core ASD symptoms, but they provide clinical experience with metformin in an ASD population and support investigation of its broader neurobehavioral effects.

Current ASD research increasingly emphasizes individualized, multimodal intervention. Non-pharmacological approaches remain the foundation of care, whereas medications are generally used for co-occurring symptoms [12,13]. Within this context, metformin has attracted interest as a repurposed, mechanism-informed candidate because metabolic dysfunction, oxidative stress, inflammatory signaling, and altered mechanistic target of rapamycin (mTOR)-related pathways are relevant to several neurodevelopmental models. In mice, metformin has been reported

to attenuate selected autism-related behaviors in high-fat-diet and maternal-separation paradigms and to reduce anxiety- and depressive-like behaviors in an inflammation-induced model [44,45,46]. A randomized trial also reported reduced irritability when metformin was used as an adjunctive treatment in children with ASD [47], whereas a trial in children with fragile X syndrome did not meet its primary endpoint but identified improvements in hyperactivity and sleep-related secondary outcomes [48]. Collectively, these findings support further investigation of metformin in selected behavioral domains while underscoring that its role in ASD remains experimental.

1.6 Study Objectives

This study evaluated the effects of metformin hydrochloride on locomotor activity, thigmotaxis, social preference, shoaling behavior, and brain inflammatory-marker mRNA expression in WT and *nde1*$^{-/-}$ zebrafish. We hypothesized that metformin hydrochloride would modify selected behavioral abnormalities in *nde1*$^{-/-}$ zebrafish and that these changes might be associated with altered inflammatory-marker expression. The study was designed as an exploratory assessment of a neuroactive small molecule in a genetically defined zebrafish model.

2. Materials and Methods

2.1 Animals

Wild-type Tuebingen zebrafish (TU; WT controls) and *nde1*$^{-/-}$ zebrafish were supplied by the zebrafish facility of the Translational Medical Center for Development and Disease, Shanghai Key Laboratory of Birth Defect Prevention and Control, Institute of Pediatrics, Children's Hospital of Fudan University. Fish were maintained in system water at 28.5 °C under a 14 hours light/10 hours dark cycle (lights on, 08:00–22:00). Breeding, feeding, and spawning were conducted in accordance with the Zebrafish Book guidelines [49]. The study was carried out in accordance with the ARRIVE guidelines (**Supplementary Material**).

2.2 Behavioral Testing

Behavioral experiments were conducted on weekdays between 09:00 and 16:30 in a room maintained at 28 °C. Behavior was recorded from above using a fixed camera (Hikvision color camera with a RICOM 2.8–12 mm manual varifocal lens, Hikvision Digital Technology, Hangzhou, China) and quantified with ZebraLab software (3.22, ViewPoint Behavior Technology, Lyon, France). Lighting and tank-background conditions were kept constant. The assays were conducted in the following order: social preference, shoaling, and locomotion/thigmotaxis. Within each assay, groups were tested in a fixed sequence (WT, *nde1*$^{-/-}$, WT with metformin, and *nde1*$^{-/-}$ with metformin). Fish were randomly selected from within each group for each assay. Because the same experimental cohort was used across assays, individual fish could be tested in more than one assay; a minimum interval of 29 h was maintained between assay sessions. Fish were gently transferred and allowed to acclimate for 10 min before recording. The general behavioral protocol followed our previous work [50].

For locomotor and thigmotaxis testing, each 11.5-week post-fertilization (wpf) zebrafish was placed individually in a 30 × 30 × 30 cm tank containing 5 cm of system water (Fig. 1A). Each trial lasted 30 min: the first 10 min served as acclimation, and the subsequent 20 min were recorded. Locomotor distance was quantified in 30-s intervals [21]. For thigmotaxis analysis, the arena was divided into a central zone and a wall-adjacent peripheral zone of equal area. The center-zone time ratio was calculated as the time spent in the central zone divided by the total recording time, and the center-zone distance ratio was calculated as the distance traveled in the central zone divided by the total distance traveled in the arena. The complementary edge-zone ratios were calculated analogously. Lower center-zone ratios (or higher edge-zone ratios) were interpreted as greater thigmotaxis.

The social preference test was conducted in a transparent mating tank with internal dimensions of 21 × 10 × 7.5 cm. A focal zebrafish was placed in one compartment, and six conspecific zebrafish were placed in the adjacent stimulus compartment, allowing visual interaction (Fig. 2A). The focal-fish compartment was divided into two equal sectors. The social zone (conspecific sector) was defined as the half adjacent to the conspecific group. The social-zone time ratio was calculated as the time spent in the social zone divided by the total test time, and the social-zone distance ratio was calculated as the distance traveled in the social zone divided by the total distance traveled within the focal-fish compartment.

The shoaling test was performed in an opaque white 30 × 30 × 30 cm tank containing 5 cm of system water. Groups of six zebrafish randomly selected from each group were allowed to swim freely and recorded for 20 min (n = 12 trials per group) (Fig. 3A). Shoaling behavior was quantified in 30-s intervals as the shoal-level mean pairwise inter-fish distance; lower values indicate greater group cohesion.

2.3 Drug Preparation and Experimental Procedures

The metformin hydrochloride exposure concentration was selected on the basis of previous zebrafish studies and preliminary dose planning. A previous study administered 50 μM metformin for 90 days to investigate cardiac regeneration through autophagy induction [51]. During preliminary planning, concentrations of 50, 25, and 8.3 μM were considered. According to the conversion used during experimental planning, 8.3 μM corresponded to approximately 1.075 mg/L metformin-base equivalents. To establish a lower exploratory exposure level, this concentration was reduced by one order of magnitude to approximately 0.1075 mg/L; the final nominal exposure concentration was therefore set at 107.5 μg/L metformin-base equiv-

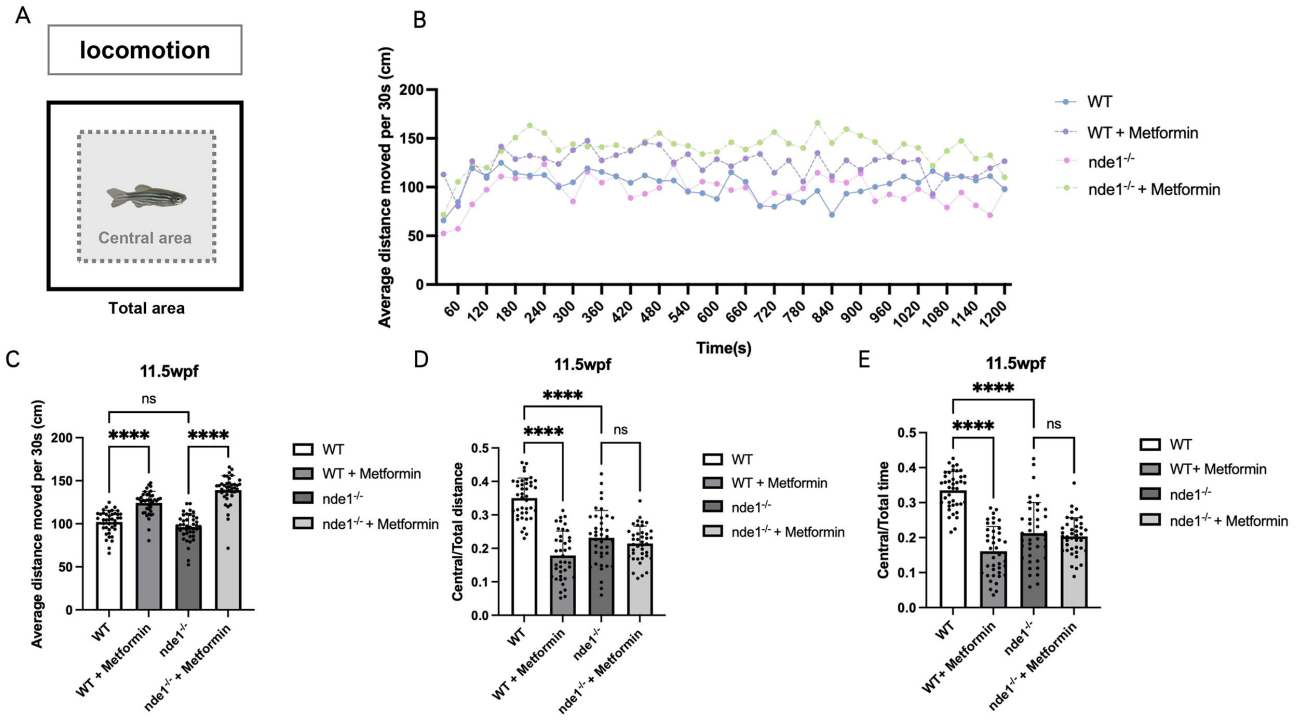


Fig. 1. Effects of metformin hydrochloride on locomotor activity and thigmotaxis. (A) Schematic of the locomotor assay. The central zone is shown in gray, and the outlined square represents the full arena. After 10 min of acclimation, behavior was recorded for 20 min. (B) Mean distance traveled in consecutive 30-s intervals. (C) Mean distance traveled per 30-s interval. (D,E) Center-zone distance ratio (D) and time ratio (E). n = 12 fish per group. Data are presented as mean ± standard deviation (SD); individual points in (C–E) represent group means for consecutive 30-s intervals. ns, not significant; ****$p < 0.0001$.

alents, corresponding to 137.8 µg/L metformin hydrochloride. A metformin hydrochloride stock solution was prepared in ddH_2O and serially diluted in exposure medium to achieve this nominal concentration. All mass concentrations are expressed as metformin-base equivalents.

Potential toxicity was also considered. Chronic exposure to 1 µg/L metformin for four months has been reported to induce neurotoxic responses in 5-month-post-fertilization zebrafish [52], and embryonic exposure from 4 to 96 h post-fertilization produced concentration-dependent developmental toxicity [53]. These studies involved substantially different life stages and exposure durations and were used as toxicity-related cautions rather than as directly equivalent exposure models.

A total of 48 zebrafish (24 WT and 24 *nde1*$^{-/-}$) were used. Sex was not determined or used as a stratification factor because this exploratory study was not powered for sex-specific analysis. Within each genotype, fish were randomly allocated to untreated or metformin-treated groups (n = 12 per group) by simple randomization. The individual fish was defined as the experimental unit for individual behavioral assays, whereas each six-fish video trial served as the experimental unit in the shoaling assay (quantified in consecutive 30-s intervals). The four groups were WT, *nde1*$^{-/-}$, WT with metformin hydrochloride (CAS 1115-70-4, Sangon Biotech, Shanghai, China, purity ≥98%), and *nde1*$^{-/-}$ with metformin hydrochloride. The behavioral sample size was based on previous experience with the model [21]. Each group was maintained in a 1-L tank containing 600 mL of drug-free or drug-containing system water. Each day, 150 mL of water was replaced and the appropriate volume of stock solution was added to restore the nominal concentration. Exposure continued from 6 to 11.5 wpf, spanning juvenile development to near sexual maturity, after which behavioral testing commenced.

2.4 RNA Isolation and Quantitative Real-Time PCR

Following completion of all behavioral assays, zebrafish designated for molecular analysis were euthanized on ice, and whole brains were dissected under a microscope (M205C, Leica Microsystems GmbH, Wetzlar, Germany). Fish were maintained under hypothermic conditions until loss of orientation and cessation of opercular movement, consistent with the AVMA Guidelines for the Euthanasia of Animals 2020 (https://www.avma.org/sites/default/files/2020-02/Guidelines-on-Euthanasia-2020.pdf). Brain tissue collection was then performed immediately while the specimens remained continuously exposed to ice-cold conditions during the procedure. Whole brain tissues were carefully dissected under a microscope and subsequently

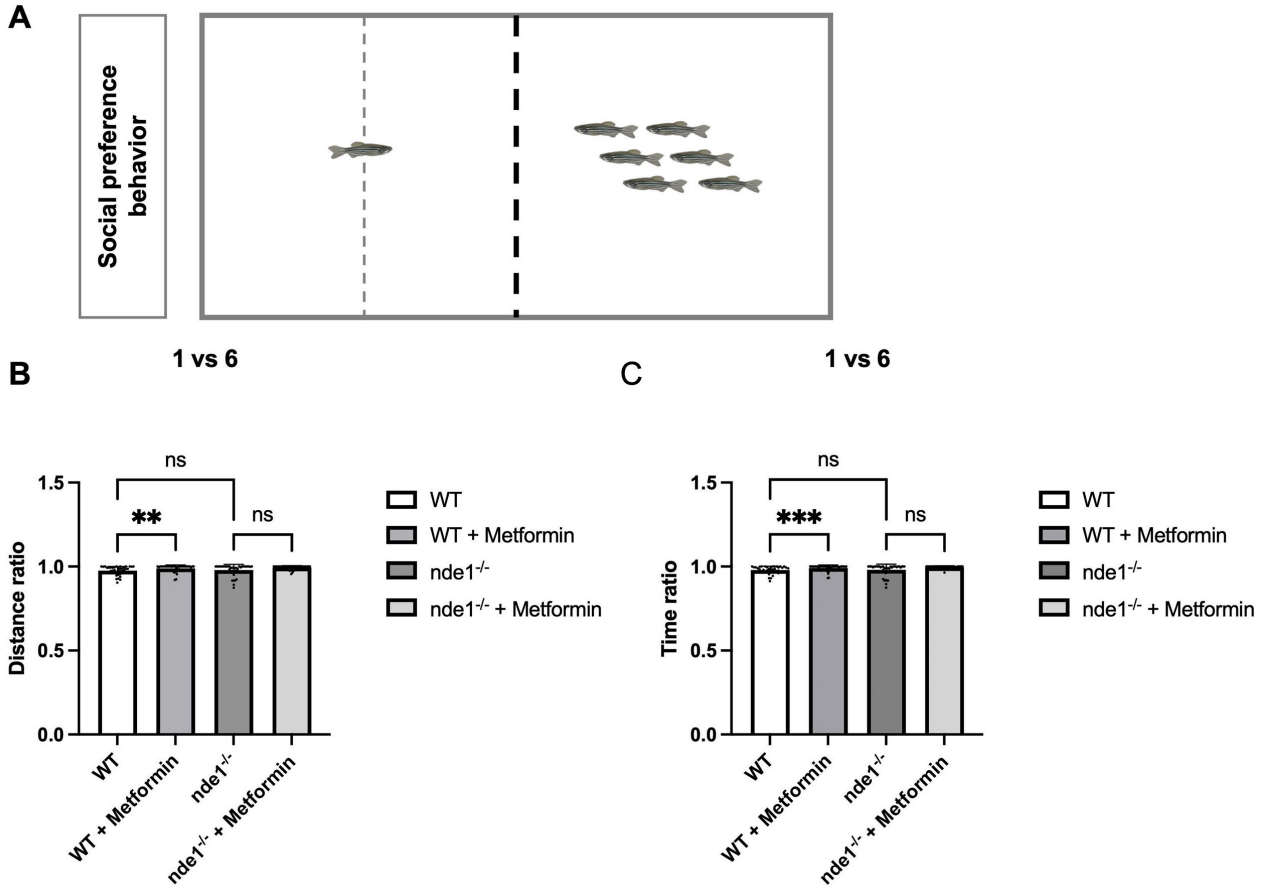


Fig. 2. Effects of metformin hydrochloride on social preference. (A) Schematic of the one-versus-six social preference assay. The black dashed line indicates the transparent partition between compartments, and the gray dashed line marks the boundary of the social zone. (B,C) Social-zone distance ratio (B) and time ratio (C). n = 12 fish per group. Data are presented as mean ± SD with individual observations. ns, not significant; $^{**}p < 0.01$; $^{***}p < 0.001$.

collected for downstream RNA analysis. Total RNA was extracted from brain tissue collected at 11.5 wpf using TRIzol reagent (Y1222, Tiangen, Beijing, China). One microgram of RNA was reverse-transcribed using the PrimeScript RT reagent kit (R047A, PrimeScript™ RT reagent Kit with gDNA Eraser, Takara, Kusatsu, Japan). Quantitative real-time PCR (RT-qPCR) was performed with TB Green® Premix Ex Taq™ (Tli RnaseH plus, RR420A, Takara) on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) under the following cycling conditions: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s, and a melting-curve analysis from 65 °C to 95 °C (5 s per step). Three independent biological replicates (pooled brain samples) were analyzed per group, and each cDNA sample was assayed in technical triplicate. Technical replicates were averaged before analysis. *β-actin* was used as the reference gene, and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are provided in Table 1.

2.5 Statistical Analysis

No predefined inclusion or exclusion criteria were applied. No animals or data points were excluded; all 48 fish that completed the study were included in the behavioral analyses. Investigators were aware of genotype and treatment allocation during animal handling, but behavioral recordings and molecular samples were assigned randomized numerical codes for blinded quantification. Codes were revealed only after all measurements had been completed. Standardized procedures and inclusion of all recorded observations were used to reduce observer bias.

Statistical analyses and data visualization were performed using GraphPad Prism 10.0 (GraphPad Software, LLC, Boston, MA, USA). Data are presented as mean ± standard deviation (SD). Normality was assessed using the Shapiro–Wilk test, and homogeneity of variance was evaluated using Levene's test. Behavioral time-course data, for which the same fish contributed measurements across consecutive time intervals, were analyzed using repeated-measures analysis of variance (ANOVA), with time as the within-subject factor and experimental group

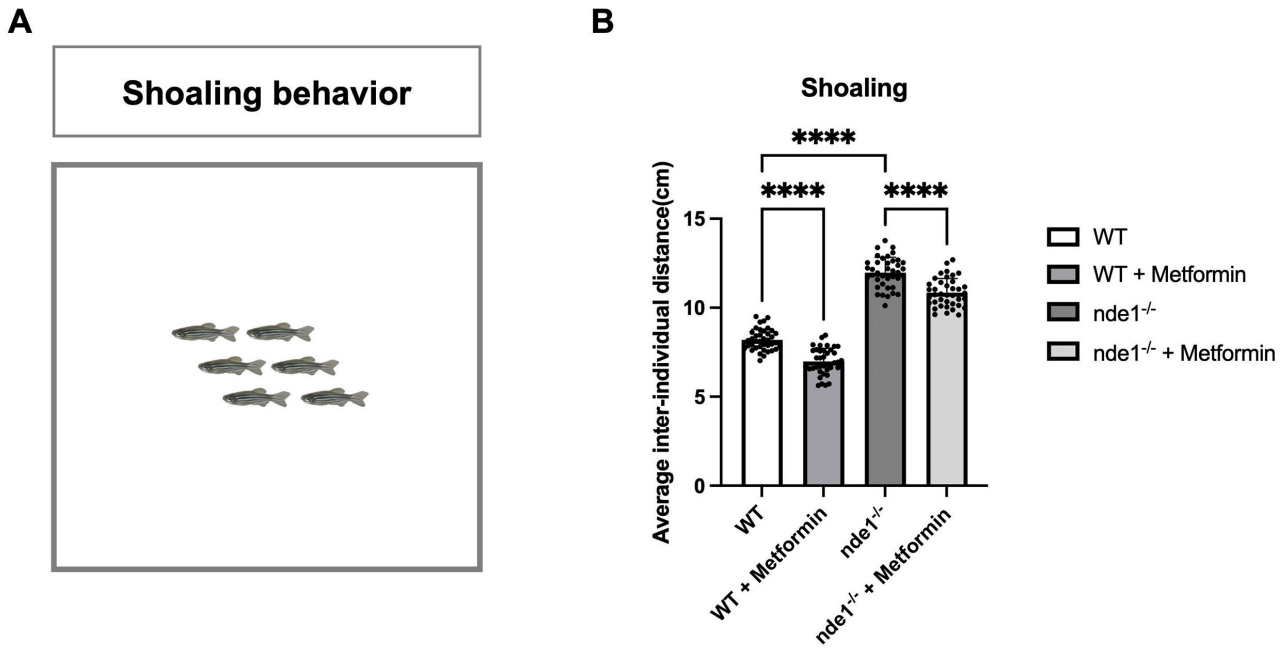


Fig. 3. Effects of metformin hydrochloride on shoaling behavior. (A) Schematic of the shoaling assay. (B) Mean pairwise inter-fish distance across the four experimental groups. $n = 12$ fish per group. Data are presented as mean ± SD; individual points in (B) represent group means for consecutive 30-s intervals. ****$p < 0.0001$.

Table 1. Primer sequences used for RT-qPCR.

Gene name	Forward primer sequence (5′ to 3′)	Reverse primer sequence (5′ to 3′)	Product size (bp)	Reference or accession number
β-actin	AGAGCTATGAGCTGCCTGACG	CCGCAAGATTCCATACCCA	106	NM_131031.2
TNF-α	ATCGCATTTCACAAGGCAATTT	TGGTCATCTCTCCAGTCTAAGG	97	NM_212859.2
IL-1β	CCAATCCACAGAGTTTGGAGAA	AGTGTAAGACGGCACTGAATC	103	NM_212844.2
IL-6	GATCTGTACAAGGACGTGAAGAC	TTGAGGAGAGGAGTGCTGAT	113	NM_001261449.1
NF-κB p65 (*RELA*)	CGGCCCACTGTAGTTGTG	TGCGTTTCCGTTATAAGTGTG	113	XM_005170796.4

TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1 beta; IL-6, interleukin-6; RELA, RELA proto-oncogene, NF-κB subunit.

as the between-subject factor. When occasional time-interval values were missing, a repeated-measures mixed-effects model was applied. Endpoint behavioral variables and molecular data were analyzed by one-way ANOVA with Tukey's multiple-comparisons test when parametric assumptions were met. Nonparametric data were analyzed using the Kruskal–Wallis test followed by Dunn's multiple-comparisons test.

For RT-qPCR, the biological replicate was the unit of statistical analysis ($n = 3$ per group). Technical triplicates were averaged and were not treated as independent observations. Because the molecular sample size was limited, these analyses were considered exploratory.

All available test statistics and degrees of freedom are reported. A two-sided p-value < 0.05 was considered statistically significant. Significance levels are denoted as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. Summary plots display individual observations together with the mean ± SD; the unit represented by each point is specified in the corresponding figure legend.

3. Results

3.1 Metformin Hydrochloride Increases Locomotor Activity in Both Genotypes but Does Not Alter Thigmotaxis in nde1$^{-/-}$ Zebrafish

Locomotor activity was quantified as the mean distance traveled per 30-s interval during the 20-min recording period (Fig. 1A,B). Repeated-measures analysis showed a main effect of time ($F(13.27, 583.8) = 3.90$, $p < 0.0001$) and a subject-level group effect of $F(3, 44) = 2.423$ ($p = 0.0784$), while analysis of group means across the 40 consecutive 30-s intervals identified a significant difference among groups ($F(3, 156) = 71.33$, $p < 0.0001$). Baseline locomotor activity did not differ significantly between untreated WT and *nde1*$^{-/-}$ zebrafish ($p > 0.05$). Metformin hydrochloride significantly increased locomotor activity in

both WT and *nde1*$^{-/-}$ zebrafish compared with their respective untreated groups (both $p < 0.0001$; Fig. 1B,C).

Thigmotaxis was assessed using center-zone distance and time ratios. Subject-level mixed-effects analysis yielded group effects of $F(3, 44) = 1.85$ ($p = 0.1506$) for the distance ratio and $F(3, 44) = 1.90$ ($p = 0.1433$) for the time ratio, whereas significant overall differences across the 40 consecutive 30-s interval group means were detected for both the distance ratio ($F(3, 156) = 48.68$, $p < 0.0001$) and time ratio ($F(3, 156) = 48.81$, $p < 0.0001$). Untreated *nde1*$^{-/-}$ zebrafish spent a smaller proportion of time and distance in the center than untreated WT fish ($p < 0.0001$), consistent with greater thigmotaxis. Metformin treatment further reduced center-zone ratios in WT fish ($p < 0.0001$), indicating increased thigmotaxis, but produced no significant change in *nde1*$^{-/-}$ zebrafish ($p > 0.05$; Fig. 1D,E). Thus, metformin increased locomotor activity in both genotypes but altered thigmotaxis only in WT fish.

3.2 Metformin Hydrochloride Affects Social Preference in WT but Not nde1$^{-/-}$ *Zebrafish*

Social preference was quantified as the distance and time ratios within the social zone adjacent to six conspecifics (Fig. 2A). Because these data were non-normally distributed, the Kruskal–Wallis test was used. Significant group differences were detected for both the distance ratio ($H(3) = 22.48$, $p < 0.0001$) and time ratio ($H(3) = 24.45$, $p < 0.0001$). Untreated WT and *nde1*$^{-/-}$ zebrafish did not differ significantly in either measure (both $p > 0.05$). Metformin hydrochloride increased the social-zone time ratio ($p < 0.001$) and distance ratio ($p < 0.01$) in WT fish. In contrast, neither measure changed significantly in metformin-treated *nde1*$^{-/-}$ fish (both $p > 0.05$; Fig. 2B,C).

Accordingly, metformin enhanced social preference in WT fish but did not significantly rescue social preference in *nde1*$^{-/-}$ zebrafish.

3.3 Metformin Hydrochloride Reduces Inter-Fish Distance in the Shoaling Assay

Shoaling behavior was evaluated using the mean pairwise inter-fish distance within groups of six zebrafish (Fig. 3A). Inter-fish distance differed significantly among the four experimental groups ($F(3, 44) = 18.51$, $p < 0.0001$). Untreated *nde1*$^{-/-}$ zebrafish exhibited greater inter-fish distances than untreated WT fish ($p < 0.0001$), consistent with reduced group cohesion. Metformin hydrochloride significantly reduced inter-fish distance in both genotypes relative to their respective untreated groups (both $p < 0.0001$; Fig. 3B).

3.4 Metformin Hydrochloride Reduces Elevated TNF-α mRNA Expression in nde1$^{-/-}$ *Zebrafish*

Relative brain mRNA expression of *TNF-α*, *IL-1β*, *IL-6*, and *RELA* was quantified by RT-qPCR and normalized to *β-actin* (three biological replicates per group, each assayed in technical triplicate; Fig. 4).

One-way ANOVA identified significant overall group effects for *TNF-α* ($F(3, 8) = 11.98$, $p = 0.0025$), *IL-1β* ($F(3, 8) = 8.688$, $p = 0.0067$), and *IL-6* ($F(3, 8) = 9.665$, $p = 0.0049$). The overall effect for *RELA* did not reach statistical significance ($F(3, 8) = 3.969$, $p = 0.0528$). Untreated *nde1*$^{-/-}$ zebrafish had higher *TNF-α* mRNA expression than untreated WT fish ($p < 0.01$). Pairwise differences in *IL-1β*, *IL-6*, and *RELA* between untreated genotypes were not statistically significant ($p > 0.05$).

Metformin hydrochloride significantly reduced *TNF-α* mRNA expression in *nde1*$^{-/-}$ zebrafish ($p < 0.01$) (Fig. 4A). No significant treatment effects were detected for *IL-1β*, *IL-6*, or *RELA* (all $p > 0.05$), although *IL-1β* and *IL-6* showed numerical increases (Fig. 4B–D).

4. Discussion

4.1 Metformin Hydrochloride Modulates Behavior and Reduces Elevated TNF-α mRNA Expression Level in nde1$^{-/-}$ *Zebrafish.*

This exploratory study evaluated behavioral and inflammatory-marker responses to metformin hydrochloride in WT and *nde1*$^{-/-}$ zebrafish. Metformin increased locomotor activity in both genotypes and reduced inter-fish distance in the shoaling assay. However, it did not significantly rescue social preference or thigmotaxis in *nde1*$^{-/-}$ fish. At the molecular level, treatment reduced elevated *TNF-α* mRNA expression in *nde1*$^{-/-}$ brains but did not significantly alter *IL-1β*, *IL-6*, or *RELA*. The data therefore indicate selective and domain-dependent effects rather than a generalized correction of behavioral abnormalities or inflammatory signaling.

4.2 Differential Effects of Metformin on Locomotor and Social-Related Behavior.

Social behavior depends on distributed neural circuits [54]. In zebrafish, the dorsal and dorsolateral telencephalon participate in learning, memory, emotion, and social behavior, while microglia contribute to synapse formation, pruning, and plasticity [3]. Neuroplasticity, including synaptic plasticity, neurogenesis, and activity-dependent remodeling, is important for learning and memory [55]. Metformin has been reported to improve recognition memory and hippocampal structural plasticity in a rat model of metabolic syndrome [56]. In the present study, however, neuroplasticity was not measured directly. Metformin was associated with increased locomotor activity and reduced inter-fish distance, but it did not rescue social preference in *nde1*$^{-/-}$ zebrafish. The behavioral effect was therefore more evident for locomotion and group cohesion than for direct social preference.

Baseline locomotor activity did not differ significantly between WT and *nde1*$^{-/-}$ fish in our study, whereas previous work reported increased locomotion in *nde1*$^{-/-}$ zebrafish [21]. The discrepancy may reflect individual variability, behavioral heterogeneity, assay sensitivity, or the slightly

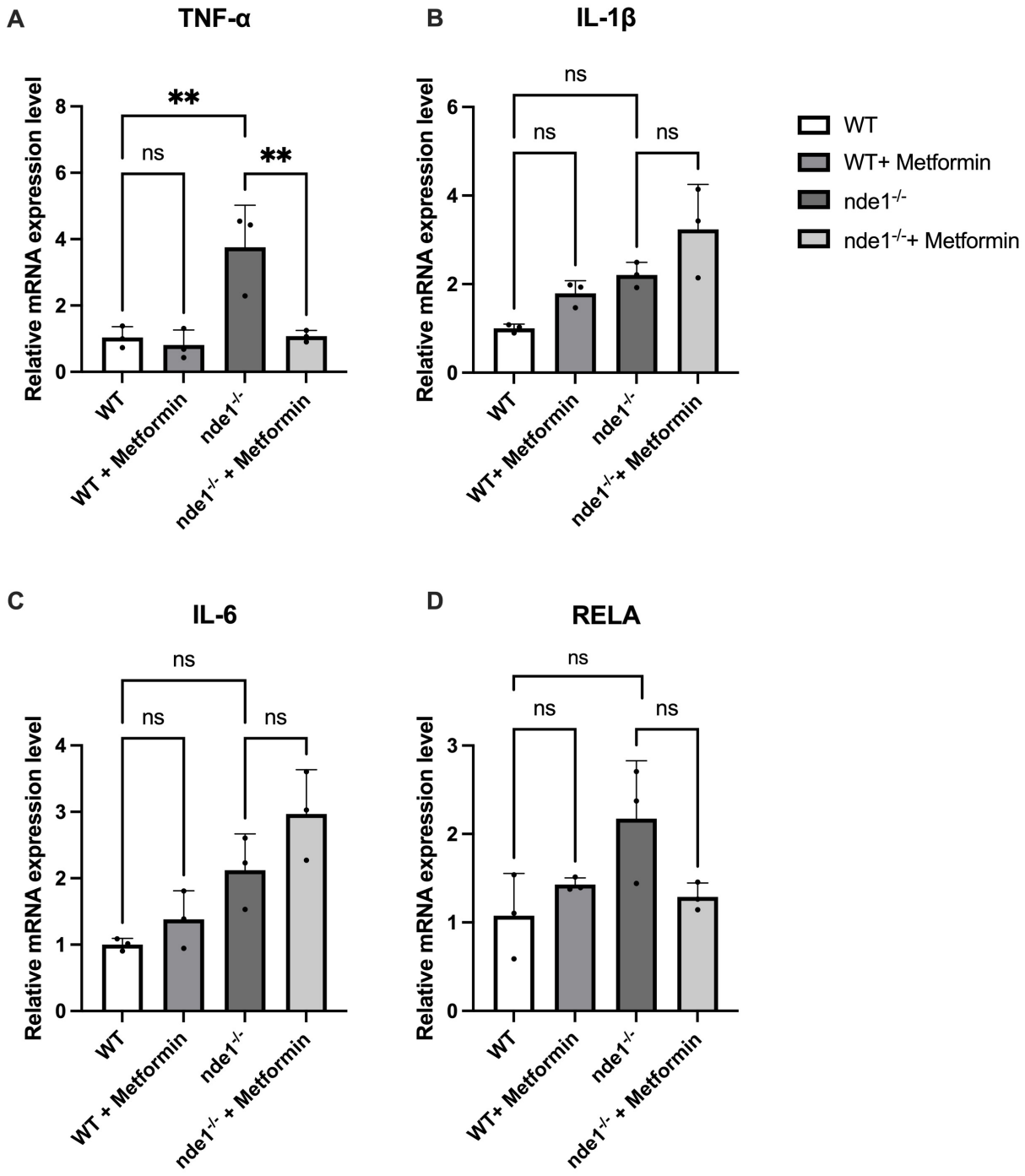


Fig. 4. Brain inflammatory-marker mRNA expression. Relative expression of *TNF-α* (A), *IL-1β* (B), *IL-6* (C), and *RELA* (D) in WT and *nde1*$^{-/-}$ zebrafish with or without metformin hydrochloride treatment. Expression was quantified by quantitative real-time PCR (RT-qPCR) and normalized to *β-actin*. $n = 3$ biological replicates per group; each sample was assayed in technical triplicate. Data are presented as mean ± SD. ns, not significant; *$p < 0.05$; **$p < 0.01$.

younger testing age in the current study (11.5 wpf versus 12 wpf previously). In contrast, the present *nde1*$^{-/-}$ group showed greater thigmotaxis and larger inter-fish distances than WT controls, indicating genotype-related differences in anxiety-like and group-cohesion measures.

Metformin increased locomotor activity in both genotypes. Consequently, changes in the shoaling assay may have been influenced partly by altered movement dynamics rather than by social motivation alone. Thigmotaxis is widely used as an indicator of anxiety-like behavior in adult zebrafish open-field paradigms [57]. Untreated *nde1*$^{-/-}$ fish

already exhibited greater thigmotaxis than WT controls. Metformin further increased thigmotaxis in WT fish but had no significant effect in *nde1*$^{-/-}$ fish, demonstrating a dissociation between locomotor and anxiety-like responses. These findings argue against interpreting increased locomotion as uniformly beneficial and emphasize the need to evaluate multiple behavioral domains.

4.3 Selective Inflammatory-Marker Changes and Possible Mechanisms.

The prior study that established the *nde1*$^{-/-}$ zebrafish model reported increased expression of the microglial marker apolipoprotein Eb (*ApoEb*) during early development and in the adult brain, enrichment of immune-related transcriptomic pathways, and elevated *IL-6*, *TNF-α*, *IL-1β*, and *NF-κB* expression [21]. Apoptosis preceded the inflammatory response, suggesting that inflammation could be secondary to early cellular injury or other upstream events. In that study, minocycline normalized inflammatory-marker expression and restored impaired social and locomotor behavior. These findings support a functional association between inflammatory signaling and behavioral abnormalities in this model, although they do not identify a single causal cell type or pathway.

Minocycline and metformin may therefore have partially overlapping anti-inflammatory consequences but distinct pharmacological entry points. Minocycline is commonly used experimentally to suppress microglial activation and inflammatory cytokine production. Metformin is better characterized as an immunometabolic regulator. In other models, metformin has been linked to modulation of gut microbiota and serotonin metabolism [44], attenuation of hippocampal oxidative stress and Toll-like receptor 4 (TLR4)/TNF-α/IL-1β/NOD-like receptor family pyrin domain-containing 3 (NLRP3)-related signaling [45], and reduction of neuroinflammation, oxidative stress, and nitric-oxide-related injury [46]. Metformin has also produced AMPK-dependent effects in primary microglia [58], reduced macrophage TNF-α production [59], and promoted an M2-like macrophage phenotype through the AMPK/peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α)/peroxisome proliferator-activated receptor gamma (PPAR-γ) pathway [60].

In the present study, metformin reduced *TNF-α* mRNA expression in *nde1*$^{-/-}$ brain tissue concurrently with increased locomotor activity and reduced inter-fish distance. One possible hypothesis is that metformin attenuated a component of TNF-α-related inflammatory signaling through AMPK-associated immunometabolic regulation. This interpretation remains provisional. We did not assess AMPK activation, cytokine protein abundance, microglial number or morphology, ionized calcium-binding adaptor molecule 1 (Iba1)-like immunostaining, or cell-type-specific transcription. Accordingly, the data support an association with altered TNF-α expression, not a direct claim that metformin modulated microglial activation or that TNF-α reduction mediated the behavioral effects.

More broadly, peripheral immune activation can induce neuroinflammation and behavioral changes [61], and pilot studies or preclinical experiments have explored intravenous immunoglobulin [62], probiotics [14], and microglia-directed strategies [63]. Pro-inflammatory mediators can affect oxidative stress, neuronal survival, synaptic plasticity, and neural-network function [64,65]. Thus, reducing selected inflammatory mediators could plausibly support neural function. Nevertheless, the lack of rescue in the social-preference assay and the influence of metformin on WT behavior indicate that the behavioral findings cannot be explained solely by a generalized anti-inflammatory action.

The molecular response was also nonuniform: TNF-α decreased, whereas IL-1β and IL-6 increased numerically and *RELA* was unchanged after treatment. This pattern suggests context-dependent regulation rather than broad inflammatory suppression. Moreover, metformin altered several behavioral measures in WT fish without corresponding changes in the measured inflammatory transcripts, indicating that additional metabolic, neurotransmitter, or mTOR-related mechanisms may contribute [66,67,68]. These alternatives should be tested directly before linking any specific pathway to the observed behavior.

4.4 Limitations and Future Directions

Several limitations constrain interpretation of these findings. Only one metformin concentration was tested, precluding dose-response analysis. Thigmotaxis was the sole anxiety-related measure, and the metformin-induced increase in locomotor activity may have influenced social and shoaling readouts. The current cohort was tested at 11.5 wpf, slightly earlier than in prior work, and sex was not determined or incorporated as a biological variable. In addition, only one tank per group was used during exposure; fish were transferred to clean water for recording and each fish was treated as an independent sample, but tank-level replication is lacking. These design features may have contributed to variability and should be addressed in future studies.

The molecular analysis was exploratory and included three biological replicates per group. Measurements were limited to whole-brain mRNA expression and were not validated at the protein or cellular level. The study therefore cannot determine whether the observed TNF-α change arose from microglia, astrocytes, neurons, or peripheral immune cells, nor whether AMPK, mTOR, oxidative-stress, or neuroplasticity-related pathways were altered. Future work should incorporate larger molecular sample sizes, protein assays, cell-type-specific imaging, and pathway-level analyses.

Future studies should test multiple doses and exposure windows, include additional behavioral assays, and evalu-

ate whether behavioral effects persist after treatment withdrawal. Comparison with other anti-inflammatory or immunometabolic compounds, including minocycline, may help distinguish shared from drug-specific mechanisms. Because ASD management is individualized and multimodal, compound-based approaches should be considered as potential complementary strategies for selected symptom domains rather than as universal replacements for established behavioral and developmental interventions.

5. Conclusion

Metformin hydrochloride produced selective, domain-specific effects in *nde1*$^{-/-}$ zebrafish. Treatment increased locomotor activity and reduced inter-fish distance in the shoaling assay but did not significantly rescue social preference or thigmotaxis. Metformin also reduced elevated *TNF-α* mRNA expression without significantly altering *IL-1β*, *IL-6*, or *RELA*, indicating a selective transcriptional response rather than uniform suppression of inflammatory signaling. These exploratory findings identify metformin as a candidate for further mechanistic and dose-response investigation in genetically defined neurodevelopmental models, while emphasizing that its behavioral effects are domain-specific and cannot yet be attributed to a particular inflammatory pathway.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Author Contributions

SG: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. JL: Investigation. YZ: Methodology. TL: Methodology. QL: Writing – review & editing, Supervision, Funding acquisition, Conceptualization. QZ: Writing – review & editing, Supervision, Funding acquisition, Conceptualization. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All procedures were approved by the Research Ethics Board of Children's Hospital of Fudan University, China (Approval number: 2022-311) and conducted in accordance with the Zebrafish Book guidelines. The study was carried out in accordance with the ARRIVE guidelines.

Acknowledgment

Thanks to all the peer reviewers for their opinions and suggestions.

Funding

This study was supported by grants from the National Natural Science Foundation of China (NSFC, no. 82201310) to Qi Zhang, the National Natural Science Foundation of China (NSFC, no. 81771632 and no. 81271509) to Qiang Li, the Natural Science Foundation of Shanghai (grant No. 21ZR1410100) to Qiang Li.

Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/JIN53224.

References

[1] Trost B, Thiruvahindrapuram B, Chan AJS, Engchuan W, Higginbotham EJ, Howe JL, et al. Genomic architecture of autism from comprehensive whole-genome sequence annotation. Cell. 2022; 185: 4409–4427.e18. https://doi.org/10.1016/j.cell.2022.10.009

[2] Usui N, Kobayashi H, Shimada S. Neuroinflammation and Oxidative Stress in the Pathogenesis of Autism Spectrum Disorder. International Journal of Molecular Sciences. 2023; 24: 5487. https://doi.org/10.3390/ijms24065487

[3] Andoh M, Koyama R. Microglia regulate synaptic development and plasticity. Developmental Neurobiology. 2021; 81: 568–590. https://doi.org/10.1002/dneu.22814

[4] Block ML, Hong JS. Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. Progress in Neurobiology. 2005; 76: 77–98. https://doi.org/10.1016/j.pneurobio.2005.06.004

[5] Fourrier C, Singhal G, Baune BT. Neuroinflammation and cognition across psychiatric conditions. CNS Spectrums. 2019; 24: 4–15. https://doi.org/10.1017/S1092852918001499

[6] Kamp-Becker I. Autism spectrum disorder in ICD-11-a critical reflection of its possible impact on clinical practice and research. Molecular Psychiatry. 2024; 29: 633–638. https://doi.org/10.1038/s41380-023-02354-y

[7] Lai MC, Lombardo MV, Chakrabarti B, Baron-Cohen S. Subgrouping the autism "spectrum": reflections on DSM-5. PLoS Biology. 2013; 11: e1001544. https://doi.org/10.1371/journal.pbio.1001544

[8] Morgan JT, Chana G, Pardo CA, Achim C, Semendeferi K, Buckwalter J, et al. Microglial activation and increased microglial density observed in the dorsolateral prefrontal cortex in autism. Biological Psychiatry. 2010; 68: 368–376. https://doi.org/10.1016/j.biopsych.2010.05.024

[9] Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo CA. Neuroglial activation and neuroinflammation in the brain of patients with autism. Annals of Neurology. 2005; 57: 67–81. https://doi.org/10.1002/ana.20315

[10] Takano T. Role of Microglia in Autism: Recent Advances. Developmental Neuroscience. 2015; 37: 195–202. https://doi.org/10.1159/000398791

[11] Pardo CA, Vargas DL, Zimmerman AW. Immunity, neuroglia and neuroinflammation in autism. International Review of Psychiatry (Abingdon, England). 2005; 17: 485–495. https://doi.org/10.1080/02646830500381930

[12] Sandbank M, Bottema-Beutel K, Crowley LaPoint S, Feldman JI, Barrett DJ, Caldwell N, et al. Autism intervention meta-

analysis of early childhood studies (Project AIM): updated systematic review and secondary analysis. BMJ (Clinical Research Ed.). 2023; 383: e076733. https://doi.org/10.1136/bmj-2023-076733

[13] Manter MA, Birtwell KB, Bath J, Friedman NDB, Keary CJ, Neumeyer AM, et al. Pharmacological treatment in autism: a proposal for guidelines on common co-occurring psychiatric symptoms. BMC Medicine. 2025; 23: 11. https://doi.org/10.1186/s12916-024-03814-0

[14] Wang X, Yang J, Zhang H, Yu J, Yao Z. Oral probiotic administration during pregnancy prevents autism-related behaviors in offspring induced by maternal immune activation via anti-inflammation in mice. Autism Research: Official Journal of the International Society for Autism Research. 2019; 12: 576–588. https://doi.org/10.1002/aur.2079

[15] Parker-Athill E, Luo D, Bailey A, Giunta B, Tian J, Shytle RD, et al. Flavonoids, a prenatal prophylaxis via targeting JAK2/STAT3 signaling to oppose IL-6/MIA associated autism. Journal of Neuroimmunology. 2009; 217: 20–27. https://doi.org/10.1016/j.jneuroim.2009.08.012

[16] Tsai MH, Ke HC, Lin WC, Nian FS, Huang CW, Cheng HY, et al. Novel lissencephaly-associated NDEL1 variant reveals distinct roles of NDE1 and NDEL1 in nucleokinesis and human cortical malformations. Acta Neuropathologica. 2024; 147: 13. https://doi.org/10.1007/s00401-023-02665-y

[17] Alkuraya FS, Cai X, Emery C, Mochida GH, Al-Dosari MS, Felie JM, et al. Human mutations in NDE1 cause extreme microcephaly with lissencephaly [corrected]. American Journal of Human Genetics. 2011; 88: 536–547. https://doi.org/10.1016/j.ajhg.2011.04.003

[18] Tan L, Bi B, Zhao P, Cai X, Wan C, Shao J, et al. Severe congenital microcephaly with 16p13.11 microdeletion combined with NDE1 mutation, a case report and literature review. BMC Medical Genetics. 2017; 18: 141. https://doi.org/10.1186/s12881-017-0501-9

[19] Doobin DJ, Kemal S, Dantas TJ, Vallee RB. Severe NDE1-mediated microcephaly results from neural progenitor cell cycle arrests at multiple specific stages. Nature Communications. 2016; 7: 12551. https://doi.org/10.1038/ncomms12551

[20] Feng Y, Walsh CA. Mitotic spindle regulation by Nde1 controls cerebral cortical size. Neuron. 2004; 44: 279–293. https://doi.org/10.1016/j.neuron.2004.09.023

[21] Zhang Q, Li T, Lin J, Zhang Y, Li F, Chen X, et al. Deficiency of *nde1* in zebrafish induces brain inflammatory responses and autism-like behavior. iscience. 2022; 25: 103876. https://doi.org/10.1016/j.isci.2022.103876

[22] Garrott SR, Gillies JP, DeSantis ME. Nde1 and Ndel1: Outstanding Mysteries in Dynein-Mediated Transport. Frontiers in Cell and Developmental Biology. 2022; 10: 871935. https://doi.org/10.3389/fcell.2022.871935

[23] Bradshaw NJ, Hayashi MAF. NDE1 and NDEL1 from genes to (mal)functions: parallel but distinct roles impacting on neurodevelopmental disorders and psychiatric illness. Cellular and Molecular Life Sciences: CMLS. 2017; 74: 1191–1210. https://doi.org/10.1007/s00018-016-2395-7

[24] Kim S, Zaghloul NA, Bubenshchikova E, Oh EC, Rankin S, Katsanis N, et al. Nde1-mediated inhibition of ciliogenesis affects cell cycle re-entry. Nature Cell Biology. 2011; 13: 351–360. https://doi.org/10.1038/ncb2183

[25] Kalueff AV, Stewart AM, Gerlai R. Zebrafish as an emerging model for studying complex brain disorders. Trends in Pharmacological Sciences. 2014; 35: 63–75. https://doi.org/10.1016/j.tips.2013.12.002

[26] Tayanloo-Beik A, Hamidpour SK, Abedi M, Shojaei H, Tavirani MR, Namazi N, et al. Zebrafish Modeling of Autism Spectrum Disorders, Current Status and Future Prospective. Frontiers in Psychiatry. 2022; 13: 911770. https://doi.org/10.3389/fpsyt.2022.911770

[27] Chahardehi AM, Arsad H, Lim V. Zebrafish as a Successful Animal Model for Screening Toxicity of Medicinal Plants. Plants (Basel, Switzerland). 2020; 9: 1345. https://doi.org/10.3390/plants9101345

[28] Tropepe V, Sive HL. Can zebrafish be used as a model to study the neurodevelopmental causes of autism? Genes, Brain, and Behavior. 2003; 2: 268–281. https://doi.org/10.1034/j.1601-183x.2003.00038.x

[29] Gu J, Hu J, Zhou D, Fan Y, He Y, Ji G, et al. Gabapentin lactam induces neurodevelopmental deficits via calcium-mitochondria-ROS cascade. Journal of Hazardous Materials. 2025; 499: 140079. https://doi.org/10.1016/j.jhazmat.2025.140079

[30] Fan R, Chen J, Gao X, Zhang Q. Neurodevelopmental toxicity of alumina nanoparticles to zebrafish larvae: Toxic effects of particle sizes and ions. Food and Chemical Toxicology: an International Journal Published for the British Industrial Biological Research Association. 2021; 157: 112587. https://doi.org/10.1016/j.fct.2021.112587

[31] Rea V, Van Raay TJ. Using Zebrafish to Model Autism Spectrum Disorder: A Comparison of ASD Risk Genes Between Zebrafish and Their Mammalian Counterparts. Frontiers in Molecular Neuroscience. 2020; 13: 575575. https://doi.org/10.3389/fnmol.2020.575575

[32] Stewart AM, Nguyen M, Wong K, Poudel MK, Kalueff AV. Developing zebrafish models of autism spectrum disorder (ASD). Progress in Neuro-psychopharmacology & Biological Psychiatry. 2014; 50: 27–36. https://doi.org/10.1016/j.pnpbp.2013.11.014

[33] Li T, Li F, Lin J, Zhang Y, Zhang Q, Sun Y, et al. Deletion of c16orf45 in zebrafish results in a low fertilization rate and increased thigmotaxis. Developmental Psychobiology. 2020; 62: 1003–1010. https://doi.org/10.1002/dev.21984

[34] Siniscalco D, Schultz S, Brigida AL, Antonucci N. Inflammation and Neuro-Immune Dysregulations in Autism Spectrum Disorders. Pharmaceuticals (Basel, Switzerland). 2018; 11: 56. https://doi.org/10.3390/ph11020056

[35] Wu H, Denna TH, Storkersen JN, Gerriets VA. Beyond a neurotransmitter: The role of serotonin in inflammation and immunity. Pharmacological Research. 2019; 140: 100–114. https://doi.org/10.1016/j.phrs.2018.06.015

[36] Bharath LP, Nikolajczyk BS. The intersection of metformin and inflammation. American Journal of Physiology. Cell Physiology. 2021; 320: C873–C879. https://doi.org/10.1152/ajpcell.00604.2020

[37] Rena G, Hardie DG, Pearson ER. The mechanisms of action of metformin. Diabetologia. 2017; 60: 1577–1585. https://doi.org/10.1007/s00125-017-4342-z

[38] Cameron AR, Morrison VL, Levin D, Mohan M, Forteath C, Beall C, et al. Anti-Inflammatory Effects of Metformin Irrespective of Diabetes Status. Circulation Research. 2016; 119: 652–665. https://doi.org/10.1161/CIRCRESAHA.116.308445

[39] Cao G, Gong T, Du Y, Wang Y, Ge T, Liu J. Mechanism of metformin regulation in central nervous system: Progression and future perspectives. Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie. 2022; 156: 113686. https://doi.org/10.1016/j.biopha.2022.113686

[40] Rotermund C, Machetanz G, Fitzgerald JC. The Therapeutic Potential of Metformin in Neurodegenerative Diseases. Frontiers in Endocrinology. 2018; 9: 400. https://doi.org/10.3389/fendo.2018.00400

[41] Ursini F, Russo E, Pellino G, D'Angelo S, Chiaravalloti A, De Sarro G, et al. Metformin and Autoimmunity: A "New Deal" of an Old Drug. Frontiers in Immunology. 2018; 9: 1236. https://doi.org/10.3389/fimmu.2018.01236

[42] Pernicova I, Korbonits M. Metformin–mode of action and clinical implications for diabetes and cancer. Nature Reviews. Endocrinology. 2014; 10: 143–156. https://doi.org/10.1038/nrendo.2013.256
[43] Anagnostou E, Aman MG, Handen BL, Sanders KB, Shui A, Hollway JA, et al. Metformin for Treatment of Overweight Induced by Atypical Antipsychotic Medication in Young People With Autism Spectrum Disorder: A Randomized Clinical Trial. JAMA Psychiatry. 2016; 73: 928–937. https://doi.org/10.1001/jamapsychiatry.2016.1232
[44] Deng W, Ke H, Wang S, Li Z, Li S, Lv P, et al. Metformin Alleviates Autistic-Like Behaviors Elicited by High-Fat Diet Consumption and Modulates the Crosstalk Between Serotonin and Gut Microbiota in Mice. Behavioural Neurology. 2022; 2022: 6711160. https://doi.org/10.1155/2022/6711160
[45] Dehkordi ZV, Ghadiri M, Amini-Khoei H. Metformin Partially Lessens Autism-Related Behaviors in Mice Subjected to Maternal Separation Stress by Combating Hippocampal Neuroinflammation and Oxidative Stress Imbalance. Journal of Biochemical and Molecular Toxicology. 2026; 40: e70930. https://doi.org/10.1002/jbt.70930
[46] Kakhki FSH, Asghari A, Bardaghi Z, Anaeigoudari A, Beheshti F, Salmani H, et al. The Antidiabetic Drug Metformin Attenuated Depressive and Anxiety-like Behaviors and Oxidative Stress in the Brain in a Rodent Model of Inflammation Induced by Lipopolysaccharide in Male Rats. Endocrine, Metabolic & Immune Disorders Drug Targets. 2024; 24: 1525–1537. https://doi.org/10.2174/0118715303275039231228065050
[47] Bazrafshan Z, Mohammadi P, Hasanzadeh A, Sanjari Moghaddam M, Kabiri M, Sanjari Moghaddam H, et al. Metformin efficacy and safety as an adjunctive treatment for irritability in children with autism spectrum disorder: A randomized, double-blind, placebo-controlled trial. Journal of Psychopharmacology (Oxford, England). 2025; 39: 214–222. https://doi.org/10.1177/02698811241303593
[48] Zhu Y, Li D, Hu C, Tian Y, Lu P, Hagerman RJ, et al. Effects of Metformin on children with Fragile X Syndrome: a randomized, double-blind, placebo-controlled trial. Molecular Autism. 2025; 16: 57. https://doi.org/10.1186/s13229-025-00691-z
[49] Westerfield M. The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio). 5th edition. University of Oregon Press: Eugene. 2007.
[50] Zhang Q, Gou S, Lin J, Zhang Y, Li Q. The ameliorative effects of honeysuckle extract and its major component luteolin on autism-like behaviors in the NDE1 deficiency model. Journal of Holistic Integrative Pharmacy. 2025; 6: 195–203. https://doi.org/10.1016/j.jhip.2025.06.007
[51] Xie F, Xu S, Lu Y, Wong KF, Sun L, Hasan KMM, et al. Metformin accelerates zebrafish heart regeneration by inducing autophagy. NPJ Regenerative Medicine. 2021; 6: 62. https://doi.org/10.1038/s41536-021-00172-w
[52] Elizalde-Velázquez GA, Gómez-Oliván LM, García-Medina S, Rosales-Pérez KE, Orozco-Hernández JM, Islas-Flores H, et al. Chronic exposure to realistic concentrations of metformin prompts a neurotoxic response in Danio rerio adults. The Science of the Total Environment. 2022; 849: 157888. https://doi.org/10.1016/j.scitotenv.2022.157888
[53] Elizalde-Velázquez GA, Gómez-Oliván LM, García-Medina S, Islas-Flores H, Hernández-Navarro MD, Galar-Martínez M. Antidiabetic drug metformin disrupts the embryogenesis in zebrafish through an oxidative stress mechanism. Chemosphere. 2021; 285: 131213. https://doi.org/10.1016/j.chemosphere.2021.131213
[54] Sato M, Nakai N, Fujima S, Choe KY, Takumi T. Social circuits and their dysfunction in autism spectrum disorder. Molecular Psychiatry. 2023; 28: 3194–3206. https://doi.org/10.1038/s41380-023-02201-0
[55] Teles MC, Cardoso SD, Oliveira RF. Social Plasticity Relies on Different Neuroplasticity Mechanisms across the Brain Social Decision-Making Network in Zebrafish. Frontiers in Behavioral Neuroscience. 2016; 10: 16. https://doi.org/10.3389/fnbeh.2016.00016
[56] Muñoz-Arenas G, Pulido G, Treviño S, Vázquez-Roque R, Flores G, Moran C, et al. Effects of metformin on recognition memory and hippocampal neuroplasticity in rats with metabolic syndrome. Synapse (New York, N.Y.). 2020; 74: e22153. https://doi.org/10.1002/syn.22153
[57] Stewart A, Gaikwad S, Kyzar E, Green J, Roth A, Kalueff AV. Modeling anxiety using adult zebrafish: a conceptual review. Neuropharmacology. 2012; 62: 135–143. https://doi.org/10.1016/j.neuropharm.2011.07.037
[58] Labuzek K, Liber S, Gabryel B, Adamczyk J, Okopień B. Metformin increases phagocytosis and acidifies lysosomal/endosomal compartments in AMPK-dependent manner in rat primary microglia. Naunyn-Schmiedeberg's Archives of Pharmacology. 2010; 381: 171–186. https://doi.org/10.1007/s00210-009-0477-x
[59] Hyun B, Shin S, Lee A, Lee S, Song Y, Ha NJ, et al. Metformin Down-regulates TNF-α Secretion via Suppression of Scavenger Receptors in Macrophages. Immune Network. 2013; 13: 123–132. https://doi.org/10.4110/in.2013.13.4.123
[60] Zhou Z, Luo G, Li C, Zhang P, Chen W, Li X, et al. Metformin induces M2 polarization via AMPK/PGC-1α/PPAR-γ pathway to improve peripheral nerve regeneration. American Journal of Translational Research. 2023; 15: 3778–3792.
[61] Bullmore E. The art of medicine: Inflamed depression. Lancet (London, England). 2018; 392: 1189–1190. https://doi.org/10.1016/S0140-6736(18)32356-0
[62] Melamed IR, Heffron M, Testori A, Lipe K. A pilot study of high-dose intravenous immunoglobulin 5% for autism: Impact on autism spectrum and markers of neuroinflammation. Autism Research: Official Journal of the International Society for Autism Research. 2018; 11: 421–433. https://doi.org/10.1002/aur.1906
[63] Thomas SD, Abdalla S, Eissa N, Akour A, Jha NK, Ojha S, et al. Targeting Microglia in Neuroinflammation: H3 Receptor Antagonists as a Novel Therapeutic Approach for Alzheimer's Disease, Parkinson's Disease, and Autism Spectrum Disorder. Pharmaceuticals (Basel, Switzerland). 2024; 17: 831. https://doi.org/10.3390/ph17070831
[64] Pavlov VA, Tracey KJ. Neural circuitry and immunity. Immunologic Research. 2015; 63: 38–57. https://doi.org/10.1007/s12026-015-8718-1
[65] Lecca D, Jung YJ, Scerba MT, Hwang I, Kim YK, Kim S, et al. Role of chronic neuroinflammation in neuroplasticity and cognitive function: A hypothesis. Alzheimer's & Dementia: the Journal of the Alzheimer's Association. 2022; 18: 2327–2340. https://doi.org/10.1002/alz.12610
[66] Saisho Y. Metformin and Inflammation: Its Potential Beyond Glucose-lowering Effect. Endocrine, Metabolic & Immune Disorders Drug Targets. 2015; 15: 196–205. https://doi.org/10.2174/1871530315666150316124019
[67] Liu D, Nanclares C, Simbriger K, Fang K, Lorsung E, Le N, et al. Autistic-like behavior and cerebellar dysfunction in Bmal1 mutant mice ameliorated by mTORC1 inhibition. Molecular Psychiatry. 2023; 28: 3727–3738. https://doi.org/10.1038/s41380-022-01499-6
[68] Wang L, Cai Y, Fan X. Metformin Administration During Early Postnatal Life Rescues Autistic-Like Behaviors in the BTBR T+ Itpr3tf/J Mouse Model of Autism. Frontiers in Behavioral Neuroscience. 2018; 12: 290. https://doi.org/10.3389/fnbeh.2018.00290