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Effects of norfloxacin exposure on neurodevelopment of zebrafish (Danio rerio) embryos

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ABSTRACT

In view of the wide application of fluoroquinolones (FQs), a group of broad-spectrum synthetic antibacterial agents, and their large ingress into the environment, the toxic effects on non-target organisms caused by FQs have received great attention. In this study, we used zebrafish embryo as a model, measured the general toxic effects of norfloxacin, a commonly used FQs, and investigated the effects of norfloxacin on the neurodevelopment of zebrafish embryos. Our data showed that norfloxacin significantly inhibited the hatching rate of zebrafish embryos, and increased the mortality and malformation rate of the embryos. To discuss the developmental neurotoxicity of norfloxacin, we measured the expression of several stem cell and neuron lineage markers in the zebrafish embryos. We found that norfloxacin exposure inhibited the expression of GFAP (glial cell marker), and enhanced the expression of Sox 2 (stem cell marker) and Eno2 (mature neuron marker). By measuring the level of active Caspase 3 and the expression ratio of Bax to Bcl2, we discovered that norfloxacin induced obvious cell apoptosis in the brain of zebrafish embryos. To explore the mechanism of the developmental neurotoxic effects of norfloxacin, we applied MK-801, a non-competitive NMDA receptors antagonist, to block the actions of NMDA receptors. The results indicated that MK-801 could rescue the upregulated cell apoptosis and disrupted balance of neuro-glial differentiation induced by norfloxacin in the brain of zebrafish embryos. Our results suggest that the activation of NMDA receptors mediates the developmental neurotoxicity of norfloxacin.

1. Introduction

Quinolones are broad-spectrum synthetic antibacterial agents. The majority of quinolone antibiotics in modern use are fluoroquinolones (FQs), which contain a fluorine atom addtion to the bicyclic core structure, 4-quinolone, and are effective against both Gram-negative and Gram-positive bacteria (Gootz and Brighty, 1996; Paton and Reeves, 1988). Due to their broad antibacterial spectrum, strong antibacterial activity, low cross resistance and high cost-effectiveness, FQs are presently one of the most commonly prescribed antibacterial agents that are used in the treatment of a variety of infections in human, including genitourinary tract, respiratory tract, gastrointestinal tract, skin, bone, soft tissue and sexually transmitted infections (Crain et al., 2017; Naber and Adam, 1998). They are also extensively used in veterinary medicine and as growth promoters in livestock husbandry and aquaculture (Done et al., 2015; Vancutsem et al., 1990).

In the past several decades, huge quantities of antibiotics including FQs were produced and consumed. In general, after the consumption, a significant fraction (30–90%) of the antibiotics is excreted in the parent form, and finally reached the environment (Hanna et al., 2018). FQs are

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extensively detected in the global environment with high detection frequencies (Balakrishna et al., 2017; Hanna et al., 2018; Kafaei et al., 2018). Compared with other antibiotics, FQs have been found to be more persistent, and are more likely to accumulate in the environment (Yang et al., 2018). The antibiotic residues in the environment might potentially pose a high probability of being biologically active towards non-target organisms, and lead to the adverse effects on the ecosystems and human health besides the spread of antibiotic resistance genes (Hanna et al., 2018; Liu et al., 2017).

The effects of FQs on the central nervous system (CNS) are the second most frequently reported form of FQs toxicity after the gastrointestinal tract disturbance (Owens and Ambrose, 2005). Common and mild CNS effects of FQs include dizziness, headache and drowsiness. Other less common but more severe effects, such as anxiety, insomnia, hallucination, seizures and psychosis are also documented (Owens and Ambrose, 2005; Stahlmann, 2002). The mechanism of neurotoxicity caused by FQs is not fully understood. In a hippocampal slice model, FQs showed obvious excitatory potential in a concentration-dependent manner. The pathogenesis of this neurotoxic effects induced by FQs is hypothesized to be related to the activation of *N*-methyl-D-aspartate (NMDA) receptors (Schmuck et al., 1998).

NMDA receptors are voltage-dependent ion channels activated by the excitatory neurotransmitter glutamate and are essential to multiple aspects of brain functions, including learning and memory formation (Zhu et al., 2016). The excitatory effects caused by the overactivation of NMDA receptors could lead to the excessive stimulation of the CNS, and may increase the risk of seizures (Barker-Haliski and White, 2015). Moreover, NMDA receptors are also essential for the early neural development, and are involved in multiple processes shaping brain development (Burnashev and Szepetowski, 2015; Ewald and Cline, 2009; Uzunova et al., 2014).

The zebrafish (Danio rerio) is an in vivo vertebrate animal model that can be used to evaluate developmental neurotoxicity (Haque and Ward, 2018). The mechanisms underlying zebrafish CNS development are similar to those of mammals. The test substance can be delivered to the embryos directly through the environment (i.e. the water they inhabit), so that the developmental toxicity of the xenobiotics can be evaluated without any maternal influences. Moreover, because they are transparent at the early developmental stages, the development of the zebrafish embryos can be easily visualized (Rafferty and Quinn, 2018).

Capitalizing on these advantages of the zebrafish model, this study aimed to explore the potential developmental toxicity of FQs, especially the developmental neurotoxicity of FQs on zebrafish. We exposed the zebrafish embryos to norfloxacin (NOR), one of the most frequently used FQs, at the early developmental stages. We found that NOR induced cell apoptosis and disturbed the balance between the expression of neuron and glial cell marker genes in the developing brain of zebrafish embryos. We also showed that MK-801, a non-competitive NMDA receptor antagonist, could effectively antagonize the effects of NOR on cell apoptosis and the expression of neuron and glial cell marker genes in zebrafish embryos. Our findings suggest that the developmental neurotoxic effects of NOR on the brains of zebrafish embryos are mediated by the activation of NMDA receptors.

2. Materials and methods

2.1. Fish and embryos maintenance

Wild-type zebrafish (Danio rerio, AB strain) were obtained from China Zebrafish Resource Center (Wuhan, China), and were maintained at 28 °C under a 14-h light/10-h dark cycle (light on at 8:00 a.m.). Embryos were collected by natural spawning, and raised in fish water in Petri dishes. The embryos were staged according to Kimmel et al. (1995). We expressed the embryonic ages in hours post fertilization (hpf) and days post fertilization (dpf). Zebrafish embryos and adults were handled in compliance with Regulations for the Administration of Affairs Concerning Experimental Animals (Hubei Province, China).

2.2. Tested compounds and exposure protocols

In the general toxicity experiment, three concentrations (600, 900 and 1200 mg/L) of NOR (MB1351, Meilunbio, China) were used. The concentrations were selected based on previous studies (Zhang et al., 2016). Acetic acid (HAc) is required for the dissolution of NOR. To exclude the influence of acidity on the development of the zebrafish embryos, we set up three HAc control groups (0.07%, 0.09% and 0.13%) in which the equivalent amount of HAc was supplied as the corresponding NOR groups (600, 900, 1200 mg/L). Since there was no evident effect on the development of zebrafish embryos observed in three HAc control groups in all general toxicity experiments, only the results of the control with the highest concentration of HAc (0.13%) were shown along with that of NOR exposed groups in final results. A minimum sample size of 100 embryos per condition was used in each experiment, and each experiment was repeated as least three times. The hatching rate was assessed at 72 hpf. The numbers of dead and malformed embryos were recorded daily until 14 dpf, and the representative malformed embryos were photographed using a stereomicroscope.

2.3. Microinjection

MK-801 was delivered into individual embryos by microinjection. Microinjection is one of the most effective methods to introduce exogenous material into the embryos of zebrafish and other spices. This technology has been proved to be a simple and reliable method in a variety of fields including developmental biology (Pei and Burgess, 2019; Xu, 1999). The injections were performed using a LPP01-100 Picoliter Injector (Longer, China) and a SMZ168 stereoscopic microscope (Motic, China). Microinjection needles were pulled on a Pull-1000 Micropipette Puller (World Precision Instruments, USA) using TW100F-4 thin wall glass capillary (World Precision Instruments, USA). The needle was backloaded with 2 μL MK-801 working solution before the injection.

MK-801 (MB4775, Meilunbio, China) was resuspended in dimethyl sulfoxide (DMSO) at 3 g/L stock concentration. Immediately prior to the injection, MK-801 was diluted to the intended concentration of 300 mg/L in embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, pH 7.0). In order to monitor the injection efficiency, 0.05% phenol red (Sigma, USA) were included in the solution. About 2 nl of MK-801 solution was injected into the yolk of one-cell stage embryos. The control was injected with the equivalent amount of DMSO. After the injection, the embryos were exposed to 600 mg/L NOR till 72 hpf. A minimum sample size of 100 embryos per condition was used in each experiment.

2.4. RNA isolation and quantitative RT-PCR (qRT-PCR)

Embryos were collected after the exposure. Total RNA of the embryos was isolated with TRIzol regent (Thermo Fisher Scientific, USA). First strand DNAs (cDNAs) was synthesized using Revert Aid First Strand Synthesis cDNA Synthesis Kit (Thermo Fisher scientific, USA) according to the manufacturer's instructions. The quantitative PCR (qPCR) reactions were conducted with iTaq Universal SYBR Green Supermix (Bio-Rad, USA), and were performed using a Bio-Rad CFX96 Connect Real-Time PCR system. The qPCR thermal cycler conditions were: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 20 s at 72 °C. Data were normalized against GAPDH expression. One hundred embryos were combined to one sample. A minimum of triplicate samples was assayed each time. The primer sequences were listed in Tab S1.

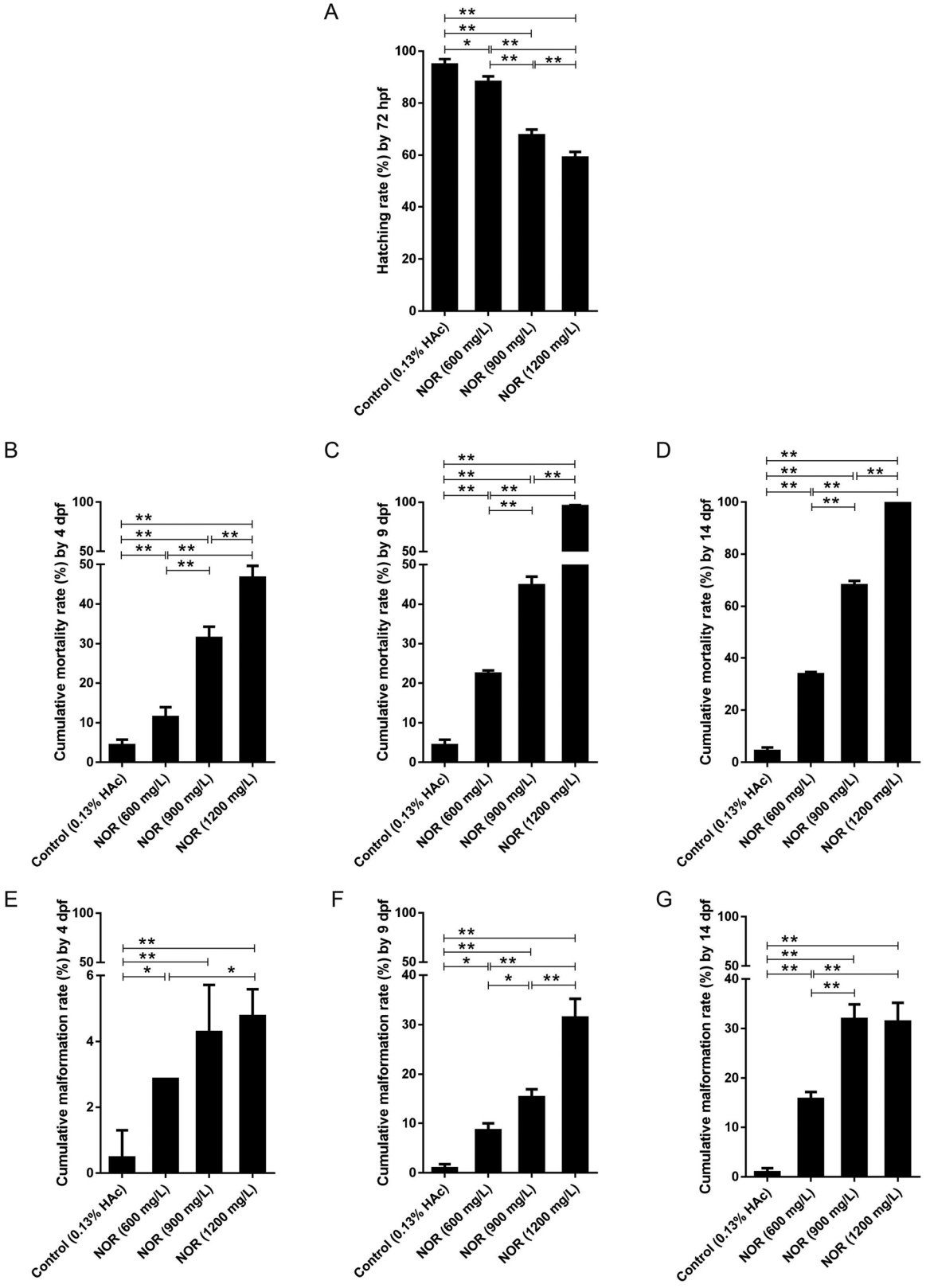


Fig. 1. General toxicity of NOR.

The zebrafish embryos were exposed to three concentrations of NOR (600 mg/L, 900 mg/L and 1200 mg/L) till 14 dpf, and the control were supplemented with 0.13% HAc. (A) Compared with the control, the hatching rates of three NOR-exposed groups were markedly decreased ($p < 0.01$). The effects of NOR on hatching rates were concentration-dependent ($p < 0.01$). (B–D) The cumulative mortality rate of three NOR treated groups were significantly higher than that of the control at all three examination time points (B, 4 dpf, C, 7dpf and D, 14dpf) ($p < 0.01$). (E–G) The cumulative malformation rates of NOR exposed groups were remarkably higher than that of the control ($p < 0.01$). The effects of NOR on the mortality and malformation rates were concentration and time-dependent ($p < 0.01$).

2.5. In situ hybridization

Whole mount in situ hybridization (ISH) was performed according to the protocol described by Wilkinson (Wilkinson, 1992) with modifications. Briefly, the embryos were fixed overnight (O/N) in 4% paraformaldehyde (PFA) at 4 °C, pretreated with proteinase K (Sigma, USA), refixed in 4% PFA, prehybridized for 1 h and then hybridized O/N with digoxigenin-labelled probes at 70 °C in a hybridization buffer. After hybridization, tissues were washed in high-stringency conditions and preblocked in antibody blocking solution, and then incubated with preabsorbed antibody O/N at 4 °C. Color development was performed in BM Purple AP Substrate Precipitating Solution (Roche, USA). The tissues were photographed by Zeiss Axio Zoom V16 stereo microscope.

To prepare the RNA probes, the linear DNA, which was generated by PCR reaction, was used as the template for in vitro transcription. The primers used for the PCR reaction, were designed corresponding to the cDNA of interest genes (Sox2, GFAP and Eno2), and the sequence of T7 RNA polymerase promoter was added to 5′ of the reverse primers for the antisense probes and the forward primers for the sense probes. The primer sequences are listed in Table S1. To generate the RNA probes, in vitro transcription was performed by using the Riboprobe system T7 (Promega, USA) according to the manufacturer's instructions.

2.6. Immunohistochemistry

Immunostaining was conducted on whole embryos. After the exposure, embryos were fixed O/N in 4% PFA at 4 °C. Non-specific staining was blocked by incubating with 4% normal goat serum in 0.5% PBS-T (0.5% TritonX-100 in PBS) for 2 h at room temperature. Following blocking, embryos were incubated with the primary antibody (activated Caspase 3, 1:400, Cell Signaling Technology, USA) at 4 °C for one days. Subsequently they were incubated O/N at 4 °C with Alexa Fluor conjugated secondary antibody (1:300, Life Technologies, USA). Images were captured using Zeiss Axio Zoom V16 fluorescent stereo microscope.

2.7. Statistical analysis

For general toxicity data, hatching rate was analyzed by one-way ANOVA, and the rates of mortality and malformation were analyzed using two-way repeated measures ANOVA with dose and time as the variables. We also ran linear aggression analysis to test the dose and time dependent effects of NFX on the rates of mortality and malformation. For gene expressions measured by qPCR, factorial ANOVA with time and exposure as fixed factors was used. Gene expressions measured by in situ hybridization and immunostaining was quantified by software Image-pro plus, and the quantified data were statistically analyzed by *t*-test for the expression of genes measured only at one time point and factorial ANOVA for genes measured at two time points. In rescue experiments, the expressions of Sox2, GFAP and Eno2 measured at one time point were analyzed by one-way ANOVA. Post-hoc comparisons (Tukey) were carried out after ANOVA analysis, when necessary. The results were presented as mean ± SD (qPCR, n = 3; ISH and immunostaining, n = 6). In all comparisons, $p < 0.05$ was used as the criterion for statistical significance.

3. Results

3.1. General toxicity of NOR

To explore the impact of NOR on the hatching rate, mortality rate and malformation rate of zebrafish embryos, the embryos were exposed to NOR with three different concentrations (600 mg/L, 900 mg/L and 1200 mg/L) from 2 hpf to 72 hpf. We observed and recorded the cumulative hatching rate by 72 hpf for each group of embryos. In the control group, the cumulative hatching rates of zebrafish embryos at 72 hpf were 94.76%, and only few embryos died without hatching. Compared with the control, the hatching rates of three NOR-exposed groups decreased by 7.0% (600 mg/L), 28.6% (900 mg/L) and 37.7% (1200 mg/L) [$F(3,8) = 178.03$, $p < 0.01$]. To examine the dose-dependent effects of NOR, linear aggression analysis was also calculated for hatching rates. The results showed that the inhibitory effects of NOR on the hatching rate were concentration-dependent - the higher the concentration of NOR was, the lower the hatching rate was [$F(1,10) = 70.01$, $p < 0.01$; $R = 0.935$] (Fig. 1A, Tab. S2).

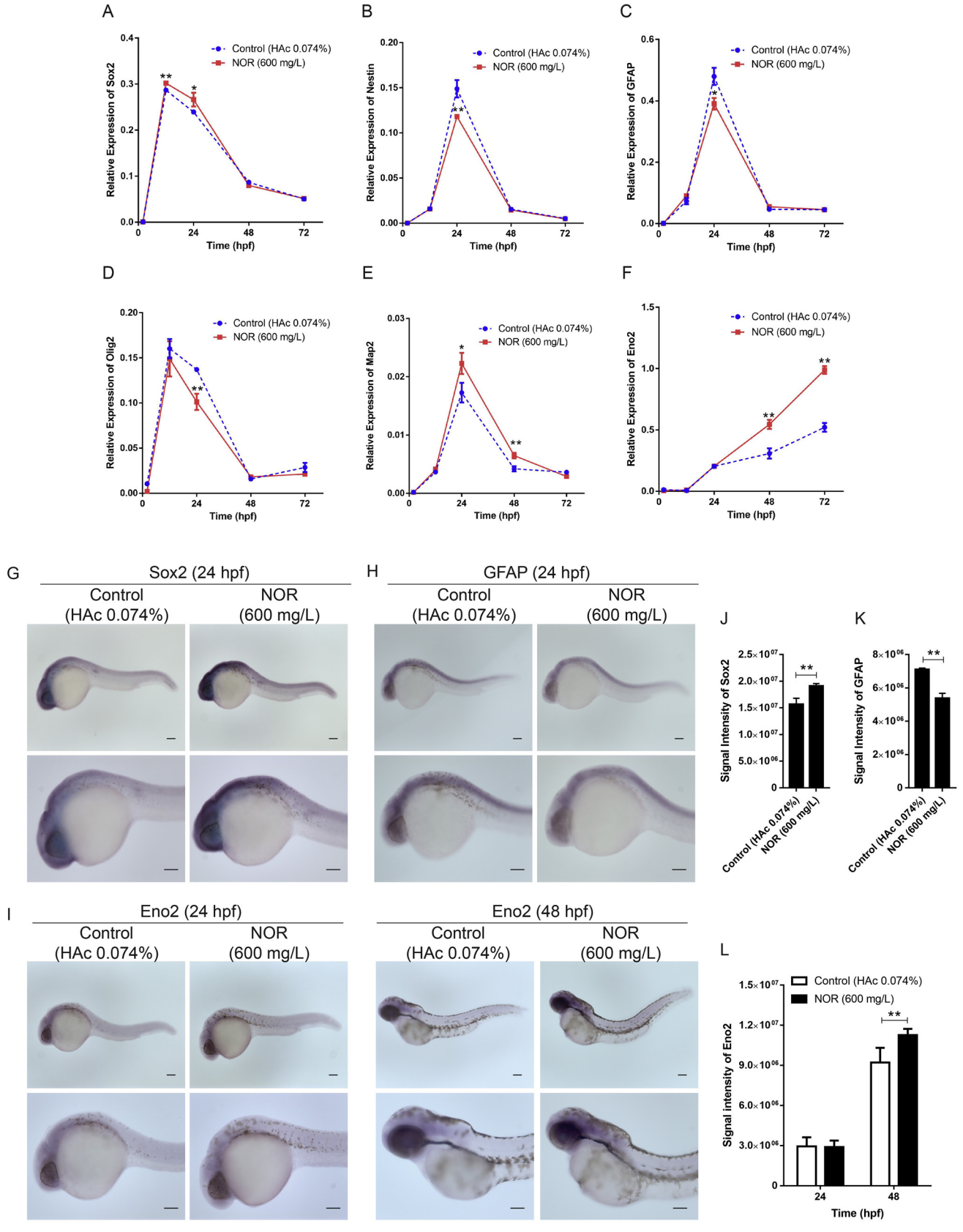
The cumulative mortality rate and malformation rate at 4 dpf, 9 dpf and 14 dpf were recorded. Dead and malformed embryos were counted daily under the stereomicroscope. The cumulative mortality rate [$F(3,8) = 2522.82$, $p < 0.01$] (Fig. 1B–D, Tab. S3) and malformation rate [$F(3,8) = 130.36$, $p < 0.01$] (Fig. 1E–G, Tab. S4) of three NOR-exposed groups were significantly higher than that of the control group at all three examination time points (4 dpf, 7 dpf and 14 dpf). The cumulative mortality rate of the control groups was 4.3% at 4 dpf, and no embryo died after 4 days. On the contrary, in three NOR exposed groups dead embryos were continuously observed throughout the course of the exposure (Fig. 1B–D, Tab. S3). In three NOR treated groups, the cumulative mortality rate reached 11.4% (600 mg/L), 31.4% (900 mg/L) and 46.7% (1200 mg/L) by 4 dpf (Fig. 1B, Tab. S3), and increased to 33.8% (600 mg/L), 68.1% (900 mg/L) and 100% (1200 mg/L) by 14 dpf (Fig. 1D, Tab. S3).

Although the average malformation rates of all three NOR treated groups were below 5% at 4 dpf (600 mg/L 2.9%; 900 mg/L 4.3%; 1200 mg/L 4.8%), they were still significantly higher than that of the control (0.5%) [$F(3,8) = 130.36$, $p < 0.01$] (Fig. 1E, Fig. S1, Tab. S4). The cumulative malformation rates of NOR treated embryos were increased to 8.6% (600 mg/L), 15.2% (900 mg/L), and 31.4% (1200 mg/L) at 9 dpf, and reached 15.7% (600 mg/L) and 31.9% (900 mg/L) at 14 dpf (Fig. 1F–G, Fig. S1, Tab. S4). Since almost all embryos in 1200 mg/L NOR group died at 9 dpf, the malformation rate of this group was not recorded after 9 dpf. The mortality rates and malformation rates were also tested with linear regression analysis to examine the dose and time dependent effects of NOR. Similar to the effects on the hatching rates, the effects of NOR on the mortality and malformation rates were also concentration and time dependent. With the increasing of NOR concentration and the extension of NOR exposure time, the mortality rates [$F(3,8)_{dose} = 208.20$, $p < 0.01$; $R = 0.994$] [$F(3,8)_{time} = 486.07$, $p < 0.01$; $R = 0.997$] and malformation rates [$F(3,8)_{dose} = 67.78$, $p < 0.01$; $R = 0.981$] [$F(3,8)_{time} = 241.32$, $p < 0.01$; $R = 0.995$] of embryos were increased significantly (Fig. 1B–G, Tab. S3-S4). There were also significant time × dose interactions [$F(6,16)_{mortality} = 223.60$, $p < 0.01$] [$F(6,16)_{malformation} = 59.19$, $p < 0.01$], which suggested that the dose-dependent effects of NOR on mortality rates and malformation rates were also affected by the exposure time to NOR. The dose-dependent effects of NOR became more obvious with the extension of the exposure time; this might be due to the cumulative effects of NOR.

3.2. The effects of NOR on the expression of stem cell (SC), neuron and glial cell marker genes during zebrafish embryonic development

The developmental stages before hatching are critical for the neurodevelopment of zebrafish embryos. To explore the effects of NOR on the early embryonic neurodevelopment of zebrafish, the expression of several SC, neuron and glial cell marker genes was measured during the treatment of NOR (from 2 hpf to 72 hpf). In the above-described experiments, all three NOR concentrations showed obvious general toxicity to zebrafish embryos, therefore in this part we chose the lowest dose (600 mg/L) to examine the effects of NOR on the neurodevelopment of zebrafish embryos. We collected embryos at several developmental stages after NOR treatment, and extracted total RNA from the whole embryos for the quantitative RT-PCR experiments.

In both control and NOR treated embryos, among all examined



(caption on next page)

Fig. 2. The effects of NOR on the expression of SC, neuron and glial cell marker genes during zebrafish embryonic development.
The zebrafish embryos were exposed to NOR at the concentration of 600 mg/L till 72 hpf, and the control were supplemented with the equivalent amount of HAc (0.074%).
(A–F) In both control and NOR treated embryos, five genes showed pulse expression patterns (Sox2, Nestin, GFAP, Olig2 and MAP2) (A–E), while only the expression of Eno2 increased gradually (F). (A) The expression of Sox2 was upregulated by NOR at 12 hpf ($p < 0.01$) and 24 hpf ($p < 0.05$). The transcription of Nestin ($p < 0.01$) (B), GFAP ($p < 0.05$) (C) and Olig2 ($p < 0.01$) (D) were downregulated by NOR at 24 hpf. NOR exposure resulted the up-regulation of the expression of MAP2 (E) at 24 hpf ($p < 0.05$) and 48 hpf ($p < 0.01$) and the transcription of Eno2 (F) at 48 hpf and 72 hpf ($p < 0.01$).
(G and J) Sox2 was expressed in the brain and spinal cord in both control and NOR exposed embryos at 24 hpf, while its expression was significantly up-regulated upon NOR treatment in the brain area ($p < 0.01$). (H and K) GFAP signal was distributed in the brain and spinal cord at 24 hpf, whereas its transcription was obviously inhibited by NOR in both areas ($p < 0.01$). (I and L) Eno2 was expressed weakly in the brain of zebrafish embryos at 24 hpf with no visible difference between the control and NOR treated embryos, while NOR exposure increased Eno2 signal strength in the brain at 48 hpf ($p < 0.01$). Scale bar = 100 μm.

genes, five genes showed similar pulse expression pattern with the peak at 12 hpf (Sox2 and Olig2) [$F(4,20)_{Sox2} = 4019.00$, $p < 0.01$; $F(4,20)_{Olig2} = 503.36$, $p < 0.01$] or 24 hpf (Nestin, GFAP and MAP2) [$F(4,20)_{Nestin} = 1891.65$, $p < 0.01$; $F(4,20)_{GFAP} = 1434.06$, $p < 0.01$; $F(4,20)_{MAP2} = 397.88$, $p < 0.01$], while only the expression of Eno2 increased gradually from 2 hpf to 72 hpf [$F(4,20)_{Eno2} = 1059.87$, $p < 0.01$]. Compared with the control, the expression of Sox2, a SC marker, was upregulated by NOR exposure at 12 hpf [$F(1,4) = 96.60$, $p < 0.01$] and 24 hpf [$F(1,4) = 9.80$, $p < 0.05$] (Fig. 2A, Tab. S5). NOR treated embryos showed a lower peak expression of Nestin, a neural progenitor marker, than the control embryos at 24 hpf [$F(1,4) = 29.97$, $p < 0.01$] (Fig. 2B, Tab. S5). The transcription of two glial cell markers, GFAP (astrocyte marker) and Olig2 (oligodendrocyte marker), were also downregulated by NOR treatment at 24 hpf [$F(1,4)_{GFAP} = 20.77$, $p < 0.05$; $F(1,4)_{Olig2} = 46.15$, $p < 0.01$] (Fig. 2C and 2D, Tab. S4). On the contrary, NOR exposure resulted the up-regulation of the expression of MAP2, an early neuron marker, at 24 hpf [$F(1,4) = 8.81$, $p < 0.05$] and 48 hpf [$F(1,4) = 35.61$, $p < 0.01$] (Fig. 2E, Tab. S5). Similar to MAP2, the transcription of Eno2, a mature neuron marker, was also up-regulated by NOR but at later stages (48 hpf and 72 hpf) [$F(1,4)_{48\ hpf} = 53.20$, $F(1,4)_{72\ hpf} = 285.04$, $p < 0.01$] (Fig. 2F, Tab. S5). There were also significant time × exposure interactions for all the genes measured [$F(4,20)_{Sox2} = 11.27$, $p < 0.01$] [$F(4,20)_{Nestin} = 27.68$, $p < 0.01$] [$F(4,20)_{GFAP} = 21.27$, $p < 0.01$] [$F(4,20)_{Olig2} = 5.56$, $p < 0.01$] [$F(4,20)_{MAP2} = 6.27$, $p < 0.01$] [$F(4,20)_{Eno2} = 117.37$, $p < 0.01$], which indicated that the effects of NOR on the expression of marker genes were influenced by the exposure time. More specially, NOR induced the obvious up or down-regulation of the expression level of marker genes only on specific time points that stated above, and the possible reason for the distinct changes in the expression patterns of marker genes upon the treatment of NOR might be the differential sensitivity of marker genes to NOR at different developmental stages. All above gene expression results suggest that NOR interferes with the expression of SC cell, neuron and glial cell marker genes, and may disrupt the differentiation balance between neuron and glial lineages in early embryonic neurodevelopment that the glial cell differentiation was down-regulated, while the neuron differentiation was up-regulated.

To confirm the above qPCR results and obtain spatial expression information, we conducted in situ hybridization on Sox2, GFAP and Eno2, which respectively represent SC, glial cell and neuron cell lineages. We selected the time points at which we found significant difference between the control and the NOR treated group in above qRT-PCR results as the detection time points of in situ hybridization experiments. We also used Image-Pro Plus to analyze the intensity of in situ hybridization signals. At 24 hpf, Sox2 was strongly expressed in the brain and moderately in the spinal cord in both control and NOR exposed embryos, while its expression was significantly up-regulated in the brain area upon NOR treatment [$t(10) = -7.614$, $p < 0.01$] (Fig. 2G and J, Tab. S6). GFAP showed a similar spatial expression pattern as Sox2 in control embryos at 24 hpf that its signal concentrated in the brain and spinal cord, whereas its transcription was obviously inhibited by NOR exposure in both brain and spinal cord [$t(10) = 15.23$, $p < 0.01$] (Fig. 2H and K, Tab. S6). Eno2 was expressed weakly in the brains of zebrafish embryos at 24 hpf with no visible difference between the untreated and NOR treated embryos, while NOR exposure increased the intensity of Eno2 signal in the brain of the embryos at 48 hpf [$F(1,10) = 22.44$, $p < 0.01$] (Fig. 2I and L, Tab. S6). There was a significant time × exposure interaction for Eno2 expression [$F(1,20) = 15.93$, $p < 0.01$]], which implied again that the effects of NOR on the expression of mature neuron marker Eno2 were affected by the exposure time that the effects of NOR became more remarkable as the exposure time was extended. Therefore, our in situ data are consistent with the above qPCR results.

3.3. NOR induced apoptosis in the brain of zebrafish embryos during the early embryonic development

The proper rate of apoptosis is pivotal to ensure the normal neural differentiation and neurodevelopment (Pinto-Teixeira et al., 2016; Yamaguchi and Miura, 2015). To further investigate the effects of NOR on zebrafish embryonic neurodevelopment, and to explore the underlying mechanisms, we examined the apoptotic status of the embryos during the treatment of NOR. We detected the activated Caspase 3, which marks the apoptotic cell, by fluorescent immunostaining, and measured the expression of a pro-apoptotic gene Bax and an anti-apoptotic gene Bcl2 by quantitative RT-PCR. We also used Image-Pro Plus to analyze the fluorescent signal intensity of activated Caspase 3.

There was no obvious apoptotic signal in both groups of zebrafish embryos at 12 hpf (data not shown). The apoptotic cells were detected in the developing CNS (forebrain, midbrain and hindbrain) of the control embryos at 24 hpf, and NOR exposure induced the dispersive increasing of the apoptotic signal intensity in the whole brain area [$F(1,10) = 110.93$, $p < 0.01$] (Fig. 3A and B, Tab S7). At 48 hpf, the apoptotic signals in both groups were more concentrated in the posterior cerebral region, whereas the signal intensity in the hindbrain region of NOR exposed group was markedly higher than that in the control embryos [$F(1,10) = 26.44$, $p < 0.01$] (Fig. 3A and B, Tab S7). The expression ratio of Bax to Bcl2 also showed a pulse pattern from 2 hpf to 72 hpf [$F(4,20) = 5419.61$, $p < 0.01$], which reached the peak at 12 hpf in two groups. In NOR exposed embryos, the peak was obviously higher than that of the control at 12 hpf [$F(1,4) = 173.55$, $p < 0.01$] (Fig. 3C, Tab S7). There were marked time × exposure interactions for both activated Caspase 3 level and the expression ratio of Bax to Bcl2 [$F(1,20)_{activatied\ Caspase3} = 14.87$, $p < 0.01$; $F(4,20)_{Bax/Bcl2} = 164.57$, $p < 0.01$]. This interaction suggested that the NOR induction of activated Caspase 3 was more evident at 24 hpf than at 48 hpf; NOR caused the change in the expression ratio of Bax to Bcl2 only at one time point, 12hpf. Bax and Bcl2 are upstream of Caspase 3 in the apoptotic pathway that might explain the changes in the expression of Bax and Bcl2 induced by NOR were earlier than that in the activation of Caspase 3. Our findings indicate that NOR can significantly induce apoptosis in the brain of zebrafish embryos at the early stages of embryonic development.

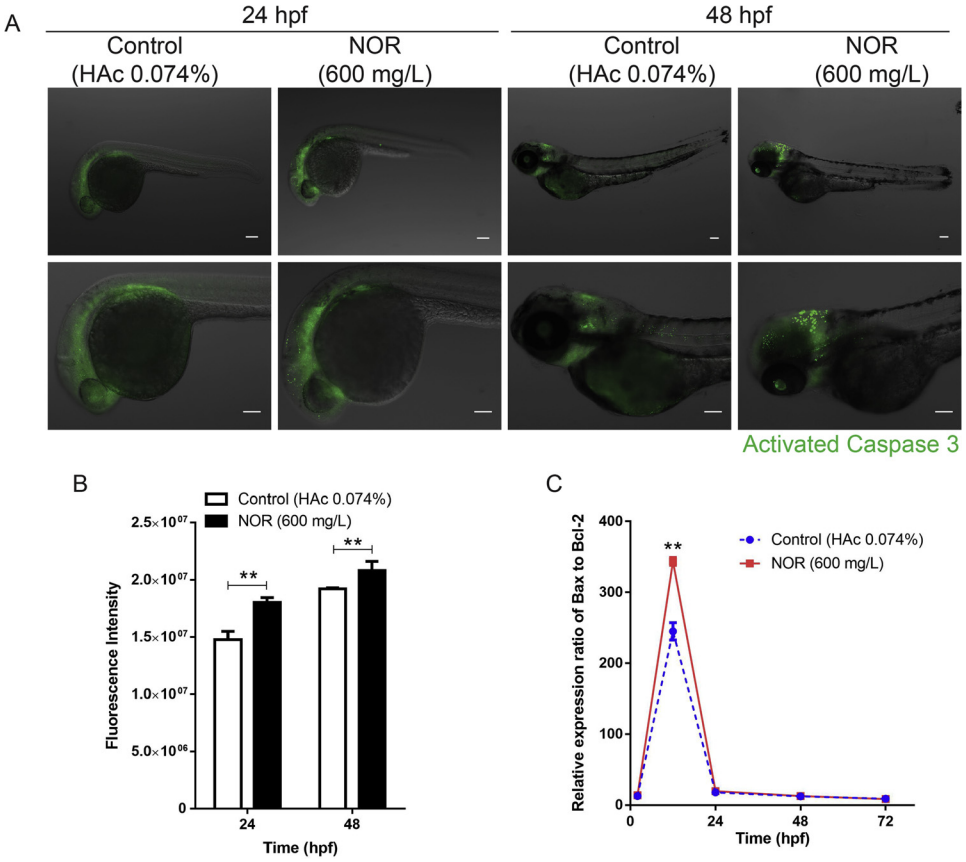


Fig. 3. NOR induced apoptosis in the brain of zebrafish embryos during the early embryonic development.
The zebrafish embryos were exposed to NOR at the concentration of 600 mg/L till 72 hpf, and the control were supplemented with the equivalent amount of HAc (0.074%).
(A and B) The activated Caspase 3 were detected in the forebrain, midbrain and hindbrain of the control and NOR exposed embryos at 24 hpf and 48 hpf, and NOR exposure increased the intensity of the apoptotic signals in the whole brain area ($p < 0.01$). (C) The expression ratio of Bax to Bcl2 showed a pulse pattern, which reached the peak at 12 hpf in both control and NOR treated embryos, and in NOR exposed embryos, the peak was remarkably higher than that in the control ($p < 0.01$). Scale bar = 100 μm.

3.4. MK-801 rescued NOR-induced apoptosis and abnormal expression of SC and neuron lineage markers in the brain of zebrafish embryos

The previous studies suggested that the neurotoxicity of NOR in adult animals is related to the activation of NMDA receptors. To explore the underlying mechanism of NOR-induced apoptosis and abnormal expression of SC and neuron lineage markers in the brain of zebrafish embryos, we chose a non-competitive NMDA receptor antagonist, MK-801, also known as Dizocilpine, to block the activation of NMDA receptor. MK-801 (300 mg/L, 2 nl) was microinjected into zebrafish embryos at one-cell stage, and then the injected embryos were exposed to NOR (600 mg/L) till 72 hpf. The control was microinjected with the equivalent amount of DMSO. Embryos were collected after the treatment for the detection of activated Caspase 3 (24 hpf and 48 hpf) and the measurement of the expression of Bax and Bcl2 (2–72 hpf).

Consistent with the above results, NOR induced an evident increase in apoptosis in the brain of zebrafish embryos at 24 hpf and 48 hpf [$F(3,20)_{24\ hpf} = 77.83$, $p < 0.01$ NOR vs Control; $F(3,20)_{48\ hpf} = 73.06$, $p < 0.01$ NOR vs Control], while MK-801 alone also somewhat enhanced the level of activated Caspase 3 in embryos brains at 24 hpf [$F(3,20) = 77.83$, $p < 0.01$ MK-801 vs Control]. When the embryos were co-exposed to NOR and MK-801, the intensity of the apoptotic signals in the brain of the embryos was similar to that in the control [$F(3,20)_{24\ hpf} = 77.83$, $F(3,20)_{48\ hpf} = 73.06$, $p > 0.05$ NOR + MK801 vs Control] (Fig. 4A and B, Tab. S8). These results suggested that MK-801 blocked the apoptosis inducing effects of NOR on the brain of zebrafish embryos. We also examined the rescue effects of MK-801 on the changes in the expression of Bax and Bcl2 induced by NOR. Compared with the control, the expression ratio of Bax to Bcl-2 was significantly up-regulated by NOR or MK-801 alone at 12 hpf [$F(3,8) = 30.96$, $p < 0.01$ NOR or MK801 vs Control], whereas this ratio showed no significant difference between the control and NOR + MK-801 co-exposed groups [$F(3,8) = 30.96$, $p > 0.05$ NOR + MK801 vs Control] (Fig. 4C, Tab. S8). These qPCR data confirmed the rescue effects of MK-801 on NOR-induced apoptosis in the zebrafish embryos suggested by the results of activated Caspase 3 immunostaining. There were time × exposure interactions for above apoptotic data [$F(3,40)_{activated\ Caspase\ 3} = 29.76$, $p < 0.01$; $F(12,40)_{Bax/Bcl2} = 30.13$, $p < 0.01$] in rescue experiments. More specially, the exposure time mainly affected the individual action of MK-801 on the level of activated Caspase 3 and the expression ratio of Bax to Bcl2, while it barely influenced the rescue effects of MK-801 on cell apoptosis in NOR exposed embryos.

The expression of SC, neuron and glial cell markers were measured by in situ hybridization after MK-801 rescue experiments. Similar to the above results, the expression of SC marker Sox2 was remarkably induced by NOR at 24 hpf especially in the brain [$F(3,20) = 44.43$, $p < 0.01$ NOR vs Control], while after the co-exposure to NOR and MK-801, the transcription level of Sox2 showed no evident difference from that of the control [$F(3,20) = 44.43$, $p > 0.05$ NOR + MK801 vs Control] (Fig. 5A and D, Tab. S9). The NOR inhibited the expression of glial cell marker GFAP in both brain and spinal cord of the zebrafish embryos [$F(3,20) = 33.03$, $p < 0.01$ NOR vs Control], whereas this inhibitory effect was entirely blocked by MK-801, and the expression level of GFAP went up to the same level as that of the control embryos [$F(3,20) = 33.03$, $p > 0.05$ NOR + MK801 vs Control] (Fig. 5B and E, Tab. S9). Similarly, the upregulated expression of mature neuron marker Eno2 in the brains [$F(3,20) = 36.54$, $p < 0.01$ NOR vs Control] was also reversed by co-exposure the embryos to NOR and MK-801 [$F(3,20) = 36.54$, $p > 0.05$ NOR + MK801 vs Control] (Fig. 5C and F, Tab. S9). Therefore, the abnormal expression of SC, glial cell and neuron markers induced by NOR was rescued by MK-801 in zebrafish embryos.

4. Discussion

In the present study, we used zebrafish embryo as a model to

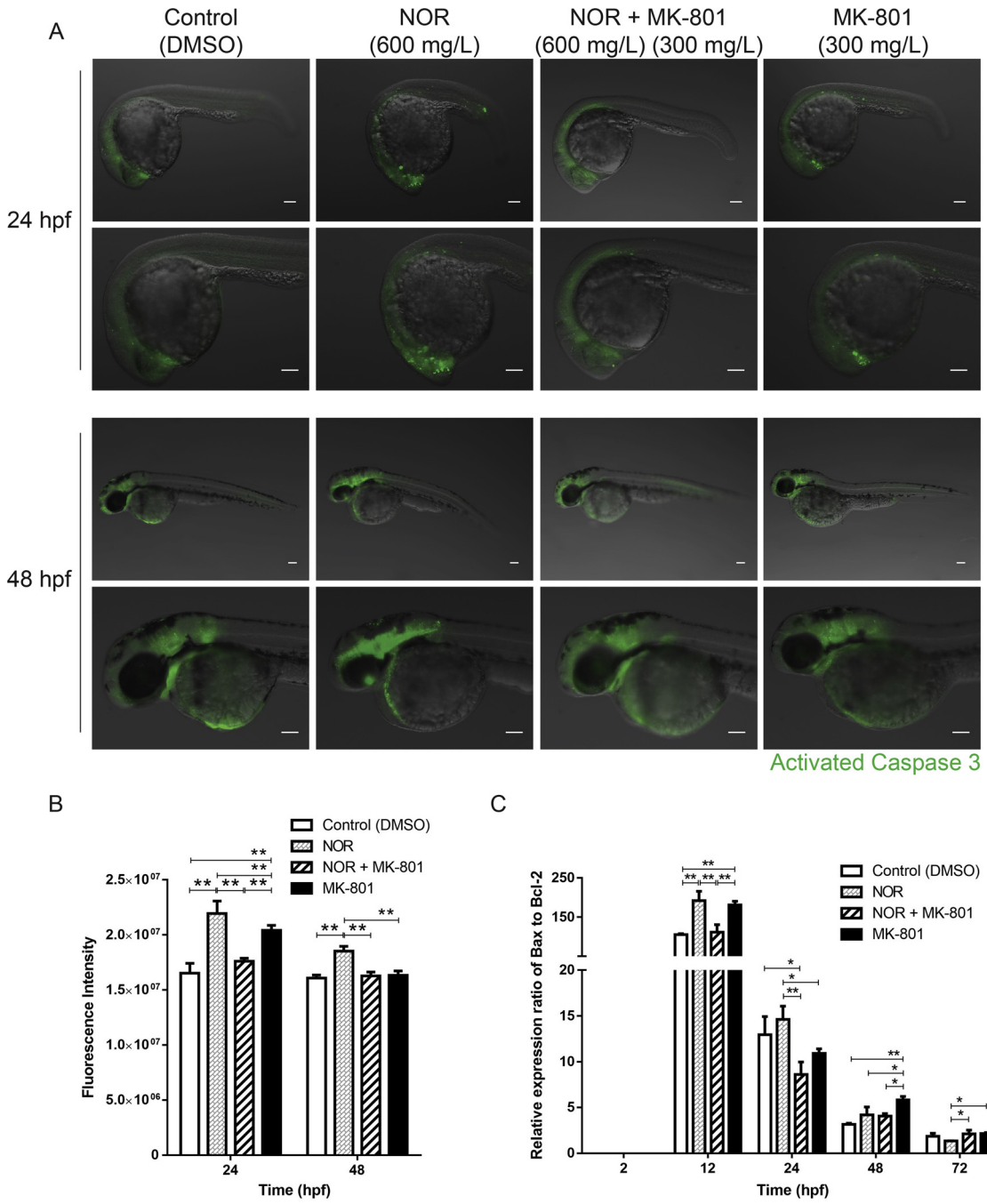


Fig. 4. MK-801 rescued NOR-induced apoptosis in the brain of zebrafish embryos.
(A and B) NOR induced an obvious increasing in apoptosis in the brain of zebrafish embryos at 24 hpf and 48 hpf ($p < 0.01$), while MK-801 alone also increased the level of activated Caspase 3 in the brain of the embryos at 24 hpf ($p < 0.01$). The intensity of apoptosis signals in the brain of the embryos co-exposed to NOR and MK-801 was similar to that in the control ($p > 0.05$). (C) The expression ratio of Bax to Bcl2 was significantly up-regulated by NOR or MK-801 alone at 12 hpf ($p < 0.01$), while MK-801 completely blocked the upregulation of the expression ratio of Bax to Bcl2 induced by NOR at 12 hpf ($p < 0.01$), and this blocking effects lasted till 24 hpf ($p < 0.01$). Scale bar = 100 μm.

evaluate the general toxicity and developmental neurotoxicity of NOR. NOR significantly inhibited the hatching rate of zebrafish embryos, and increased the mortality rate and malformation rate of the embryos. We found that NOR also induced obvious cell apoptosis in the brain of the embryos, and disrupted the balance between the neurodifferentiation and glial differentiation during the early embryonic development of zebrafish. We discovered that blocking NMDA receptors could rescue NOR-induced cell apoptosis and abnormal neurodifferentiation in the brain of the embryos. Our results suggest that the activation of NMDA receptor probably mediates the developmental neurotoxicity of NOR.

Apoptosis has been recognized as an important event in the normal development of the nervous system, where it appears to be fundamental for the control of the final number of neurons and glia cells. The cells that are transient functional in the developing brain are selectively deleted. During the early embryogenesis, approximately 50% of the neural cells die through apoptosis (Dekkers et al., 2013). The pattern and timing of cell death during development are also strictly scheduled and tightly regulated. This death is required for morphogenetic processes involved in neurodevelopment, such as neural tube closure. Abnormal downregulation and upregulation of apoptosis in the

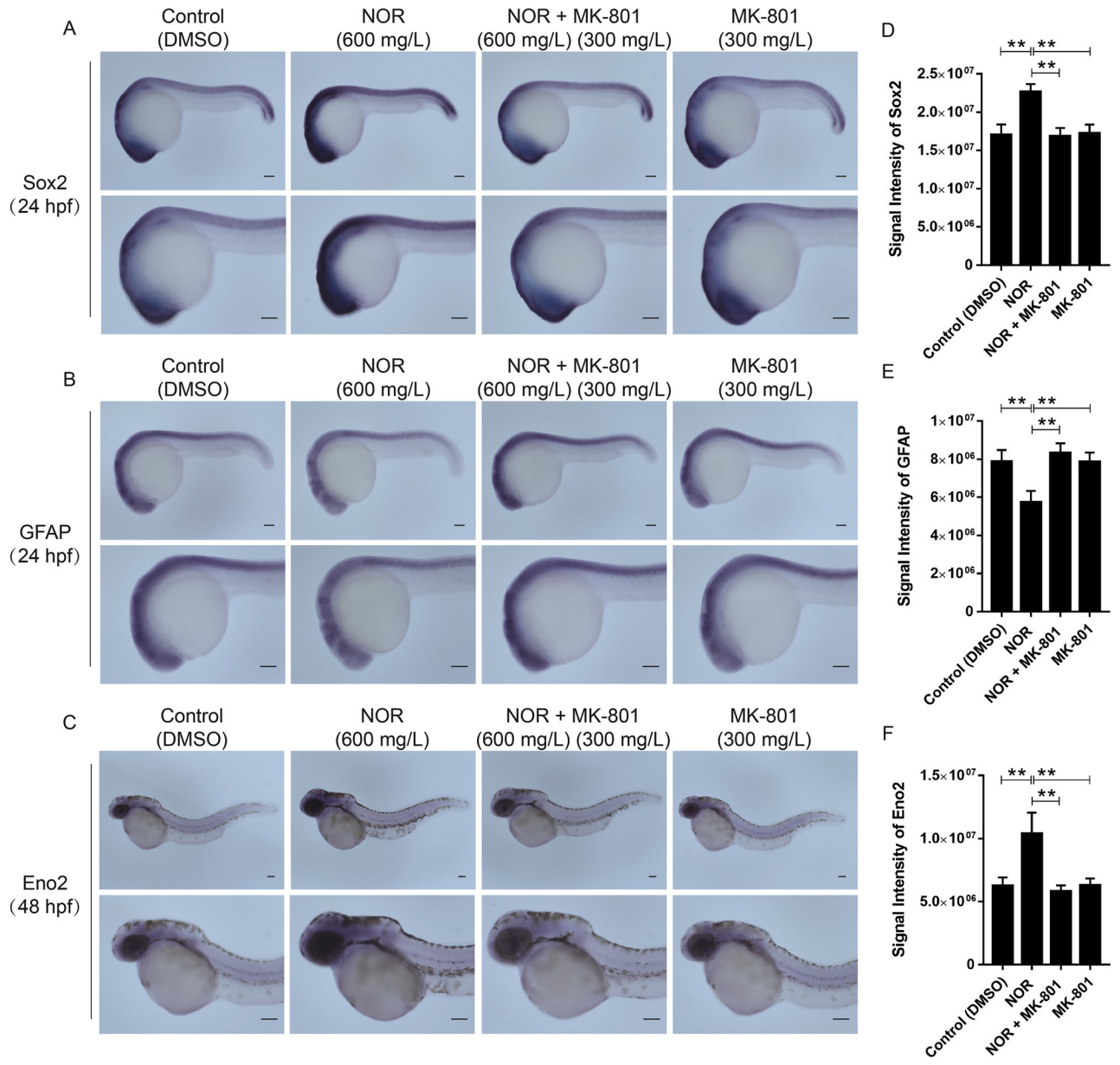


Fig. 5. MK-801 rescued NOR-induced abnormal neurodifferentiation in the brain of zebrafish embryos.
(A and D) The expression of Sox2 (SC marker) was remarkably induced by NOR at 24 hpf ($p < 0.01$). Upon co-exposure to NOR and MK-801, Sox2 transcription showed no evident difference from that of the control ($P > 0.05$). (B and E) NOR inhibited the expression of GFAP (glial cell marker) ($p < 0.01$), whereas this inhibitory effect was entirely blocked by MK-801($p < 0.01$). (C and F) The upregulation of the expression of Eno2 (mature neuron marker) induced by NOR was reversed by co-exposing the embryos to NOR and MK-801. Scale bar = 100 μm.

developing brain can result in brain malformations (Yamaguchi and Miura, 2015). As shown in our study, NOR can induce obvious cell apoptosis in the developing brain of zebrafish embryos. Given the critical role of apoptosis in the developing CNS, the apoptosis inducing effects of NOR could play an important role in its developmental neurotoxicity. However, the relationship between the apoptosis inducing effects of NOR and other pathological processes induced by NOR (e.g., the disrupted balance of neuro-glial differentiation) is unclear, and needs further investigation to reveal.

In the CNS, the process of apoptosis is complex, and relies on neurotransmitter activity. Abnormal patterns of apoptosis can be triggered by the altered neurotransmitter activity (Creeley, 2016). As a neurotransmitter receptor of glutamate, NMDA receptors are thought to be responsible for excitotoxicity and subsequent downstream events like neuroinflammation and apoptosis, and have been implicated in many important human pathologies, ranging from amyotrophic lateral sclerosis, Alzheimer's and Parkinson's disease, epilepsy, trauma and stroke to schizophrenia (Carvajal et al., 2016). The neurotoxicity in adult animals induced by FQs was thought to be related to the activation of NMDA receptors (Schmuck et al., 1998). Extracellular magnesium ions can bind tightly to NMDA receptor to block its activation by preventing ion permeation. This blocking effect is removed by FQ by its chelation with magnesium (Sousa et al., 2014). In our study, we discovered that NMDA receptor antagonists, MK-801, could antagonize the upregulation of apoptosis and abnormal neurodifferentiation in the brain of zebrafish embryos exposed to NOR. These results for the first time suggest that the developmental neurotoxicity of NOR is also medicated by the activation of NMDA receptors.

Interestingly, we also observed that MK-801 alone could induce apoptosis in the brain of zebrafish embryos. These results are consistent with previous studies in which the administration of NMDA receptor antagonists, MK-801 and phencyclidine (PCP), in late fetal and early

postnatal period of life in rats increased neuronal death by widespread apoptosis in multiple brain regions (Hetman and Kharebava, 2006). These results together with the above observations imply that proper activation level of NMDA receptors is pivotal for the maintenance of the appropriate rate of apoptosis in the developing brain.

Based on this work, we conclude that NOR showed developmental neurotoxic effects on zebrafish embryos by inducing apoptosis and disrupting the balance between the neurodifferentiation and glial cell differentiation. We propose that these effects are mediated by the activation of NMDA receptors. Our new findings together with the knowledge on the excitatory actions of NOR on the adult brain would improve our understanding of the effects of NOR and other FQs on the CNS at both developmental and adulthood stages.

5. Conclusion

To conclude: (a) NOR significantly inhibited the hatching rate of zebrafish embryos, and increased the mortality rate and malformation rate of the embryos. (b) NOR induced obvious cell apoptosis in the brain of zebrafish embryos, and disrupted the balance of the neuro-glial differentiation during the early embryonic development of zebrafish brain. (c) Blocking NMDA receptors could rescue NOR-induced cell apoptosis and aberrant neurodifferentiation in the brain of zebrafish embryos. (d) Our results suggest that the activation of NMDA receptors mediates the developmental neurotoxicity of NOR.

Conflict of interest

The authors declare no conflict of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

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