Developmental neurotoxicity of reserpine exposure in zebrafish larvae (Danio rerio)

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Abstract
Reserpine is widely used for treatment of hypertension and schizophrenia. As a specific inhibitor of monoamine transporters, reserpine is known to deplete monoamine neurotransmitters and cause decreased movement symptoms. However, how zebrafish larvae respond to reserpine treatment is not well studied. Here we show that swimming distance and average velocity are significantly reduced after reserpine exposure under various stimulatory conditions. Using liquid chromatograph-mass spectrometer analysis, decreased levels of monoamines (e.g., dopamine, noradrenaline, and serotonin) were detected in reserpine-treated larvae. Moreover, reserpine treatment significantly reduced the number of dopaminergic neurons, which was identified with th (Tyrosine Hydroxylase) in situ hybridization in the preoptic area. Interestingly, dopaminergic neuron development-associated genes, such as opa, opb, wnt1, wnt3, wnt5 and manf, were downregulated in reserpine treated larvae. Our data indicates that 2 mg/L reserpine exposure induces dopaminergic neuron damage in the brain, demonstrating a chemical induced depression-like model in zebrafish larvae for future drug development.

1. Introduction
Reserpine, a specific inhibitor of the vesicular monoamine transporter (VMAT), used as an anti-hypertension drug, is also widely used for treatment of schizophrenia (Busanello et al., 2011; Dutra et al., 2002; Nur and Adams, 2016). However, several studies reported that reserpine has multiple side effects, such as decreased movement symptoms (Chaudhury et al., 2015; Leao et al., 2015). Numerous investigations on the effects of reserpine in the central nervous system (CNS) have been determined after injection or feeding with 40 mg/L reserpine. In mammals, rats treated with 2.5 mg/kg reserpine exhibit a profound loss of 5-hydroxytryptamine (5-HT) innervation in cerebral cortex, 1 mg/kg dosage found decreased in striatal dopamine (DA) in weaver mutant and heterozygous mice after treatments, and 3 mg/kg treatment showed greatly potentiated dopamine toxicity in dopamine 1 receptor D(1)R−/− mice (Ares-Santos et al., 2012; Berger et al., 1989; Richter et al., 1993). What's more, the influence of locomotor activity caused by 40 mg/L reserpine is usually correlated with decreased levels of monoamines, such as dopamine, noradrenaline (NE) and serotonin (5-HT) (de Freitas et al., 2016; Haeggendal and Lindqvist, 1964; Khnychenko et al., 2017; Puttonen et al., 2017). These results indicated that reserpine played a critical role in dopaminergic neuron development.

In the CNS, dopaminergic neurons distribute in ventral midbrain, ventral tegmentum and substantia nigra. As the rate-limiting enzyme that catalyzes catecholamine biosynthesis, TH (Tyrosine hydroxylase) has been widely used as a marker to detect the dopamine system. Prominent evidence showed that reserpine suppressed the development...
and survival of dopaminergic neurons, and decrease the number of TH+ cells in the substantia nigra pars compacta in rat (Fukui et al., 2007; Santos et al., 2013; Stefanovic et al., 2016).

Various genes are involved in the dopaminergic development in vertebrate. Importantly, Wnt1, Wnt3a and Wnt5a are required for neuronal axon extension and differentiation (Castelo-Branco et al., 2003). A previous study found that DA neurons number are reduced in Wnt1+/− mice and Wnt1−/−; Wnt3a+/−; Wnt5a−/− mice showed a nearly complete loss of TH+ cells (Andersson et al., 2013). A recent research showed the role of mesencephalic astrocyte-derived neurotrophic factor (manf) in the dopamine system protects neurons from neurototoxic damage. Knock-down manf morphant displayed reduced dopamine level and decreased expression levels of two th transcripts in the diencephalon region (Chen et al., 2012). Orthopedia homeodomain protein (otp) a and otpb are correlated with dopamine neuron development and otpa and otpb mutants of zebrafish had a reduced th expression (Fernandes et al., 2013; Ryu et al., 2007).

Recently, the zebrafish model has proved to be beneficial for research on human brain disorders (Nguyen et al., 2014). Reserpine-treated zebrafish larvae showed an almost total disappearance of histamine-containing nerve fibers in the dorsal telencephalon, while adult zebrafish remarkably reduced activity 7 days later after reserpine exposure (Kyzar et al., 2013; Puttinen et al., 2017). Here, we propose that a 5-day reserpine treatment induces neurotoxic symptoms, including loss of dopaminergic neurons, decreased monoamine content, and reduced swimming distance. The amount of mRNA for DA development related genes, such as manf, otpa, otpb, wnt1, wnt3a, and wnt5a, were decreased in the 2 mg/L reserpine treated group. Therefore, we provide the evidence that reserpine neurotoxic damage with ensuing impaired brain functions and depleted storage of brain monoamine in early zebrafish larvae, and might establish a chemical induced depression-like model in zebrafish larvae, which can be further used for large-scale drug screening.

2. Materials and methods

2.1. Animals

Wild-type zebrafish embryos were obtained by natural spawning and cultured at controlled temperature (28 ± 1 °C) in the dark incubator. All experiments conducted on animals were in accordance with the guidelines of the Institutional Animal Care and Use Committee of Wenzhou Medical University.

2.2. Drugs

Reserpine from Sigma-Aldrich (catalog#82580, purity ≥ 99.0%) was dissolved in DMSO (Solarbio, catalog#D8370) of 4 mg/mL and stored at −20 °C before use. 4 mg/L reserpine stock solution was diluted to the exposure concentrations (0.5, 1, 2 mg/L) in E3 solution (0.25 M NaCl, 0.0085 M KCl, 0.0165 M CaCl₂·H₂O, 0.0165 M MgSO₄·7H₂O). DMSO was normalized at 0.05% per 10cm plate.

2.3. Treatment

The embryos (40/plate for behavior and whole-mount in situ hybridization or 70/plate for neurotransmitter measurement) were kept in E3 solution containing various concentrations of reserpine (0.5, 1, 2 mg/L) or control (0.05% DMSO) at 6 hours post fertilization (hpf). All control and treated groups were maintained in the dark constant temperature incubator for subsequence experiments. The medium was changed every 2 days. Larvae fish of 5 days post fertilization (dpf) were collected and used for the experiment.

2.4. Behavioral experiment

The high-throughput monitor systems (ViewPoint Life Sciences Lissieu, Calvados, Lower Normandy Region, France) were used for all behavioral experiments. The larvae fish were transferred to a 96-well plate (length × width: 1270 mm × 860 mm, well diameter 75 mm), with a total of 300 μL of E3 solution per well. Before the experiment, larvae fish were conditioned to the environment for 20 min. For the day-night behavioral experiment, the light value was set at 100% (8000 Lux). The illumination was on at 8:00 a.m. and off at 22:00 p.m. For the light and sound stimulation, the programming was set as follows: for 10 cycles, light/sound was on for 1 min and off for 1 min. The light was set at 100% (8000 Lux) and the sound was set at 440 Hz. All behavioral experiments started at 8:00 a.m. The data analyzed every 10 minute bins for the day-night behavioral experiment, and every 30 sec bins for the stimulation.

2.5. Neurotransmitter measure

The protocol for measure has been described previously (Chen et al., 2017). Briefly, the samples were homogenized in 500 μL acetonitrile (0.1 M formic acid) by ultrasound sonicator (Sientz, Ningbo, CN). After centrifuged 12,000g for 20 min at 4 °C, 200 μL supernatant was diluted in 2600 μL diluent (0.0234% formic acid, 5.385% methanol) and stored at −80 °C. The monoamines, including DA (Sigma-Aldrich, catalog#D081), 5-HT (Sigma-Aldrich, catalog#14927), 5-HIAA (Sigma-Aldrich, catalog#H8876), NE (Sigma-Aldrich, catalog#A7257) were measured by LC-MS (liquid chromatograph-mass spectrometer). The standard curves were linear from 1 ppb to 200 ppb, in order to normalize the data. The data is reported as percent of the average of the control group.

2.6. In situ hybridization

Whole mount in situ hybridization was performed using single-stranded RNA probes labeled with anti-digoxigenin-AP (Roche Diagnostics, catalog#11093274910) according to established protocols (Li et al., 2009). Riboprobes of th cDNA were generated as described (Sheng et al., 2010). The amplified fragments with lengths of 358 bp were used as templates to synthesize the riboprobe for whole mount in situ hybridization experiments (Primer sequences are listed in Table 1). Briefly, fixed and staged of embryos were prehybridized for 3 h, followed by hybridization with the indicated probe overnight. After a series of stringent washes, embryos were blocked in 2% bovine serum albumin and incubated with alkaline phosphatase-conjugated antibody overnight. After washes with phosphate-buffered saline with Tween-20, the samples were labeled with BCIP/NBT stock solution (Roche, DAKO, Carpinteria, CA).

### Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>th(358 bp)</td>
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<tr>
<td></td>
<td>5′-CAGGGACGTGGATTAGA-3′</td>
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<td>manf(67 bp)</td>
<td>5′-CCAGGCTAGCTAGTCTG-3′</td>
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<td></td>
<td>5′-TAAAGCGGCTGAGGAAACCC-3′</td>
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<td>otpa(70 bp)</td>
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<td>ophp(86 bp)</td>
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<td>5′-GCGCCGCCAAAAGCTTGGCG-3′</td>
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<td>wnt1(97 bp)</td>
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<td>wnt5a(63 bp)</td>
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<td>5′-TACACCGACCTGAGCAGC-3′</td>
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<td>EF1α(87 bp)</td>
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<tr>
<td></td>
<td>5′-ATCAGAGAAGATAGTGACCCGATTAC-3′</td>
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2.7. RNA extraction and cDNA synthesis

After 5 days of treatment, 30 larvae fish per plate were homogenized. Total RNA was isolated using RNAiso Plus, according to the procedure for animal tissues (TaKaRa, catalog #9108). Total RNA concentration was determined by Nanodrop (Thermo scientific). RNA was reverse-transcribed into cDNA using All-in-One cDNA Synthesis SuperMix (Bimake, catalog #B22403).

2.8. Quantitative PCR

All qPCR analyses were performed using the 2× SYBR Green qPCR Master Mix (Bimake, catalog #B21203). 10 ng cDNA was used as reaction samples. The reaction mixes were prepared according to the manufacturer’s protocol. The genes related to the function of the dopamine system were analyzed: man, otpa, otpb, wnt1, wnt3a, wnt5a (Huang et al., 2015). The gene EF1α served as an internal control (Tang et al., 2007). Primer sequences are listed in Table 1. Data were run on the software provided by Applied Biosystems. The quantification was done according to the 2-ΔΔCt method.
Fig. 2. Locomotor activities of larvae with alternating light and sound stimulation. (A) Reserpine-treated larvae showed decreased total locomotor activity distance in light stimulation (****p < 0.0001). (B) Reserpine-treated larvae showed decreased average velocity in light stimulation (****p < 0.0001). (C) The reserpine group was less sensitive to the control at each time point in light stimulation. (D) The total locomotor activity distance of reserpine treated groups were significantly decreased in sound stimulation (**p < 0.01, ***p < 0.001). (E) The average velocity of reserpine treated groups were significantly decreased in sound stimulation (**p < 0.01, ***p < 0.001). (F) The reserpine group was less sensitive to the control at each time point in sound stimulation. N = 24 in each concentration group per well. The distance was evaluated using Prism 6 from 3 independent experiments. Data presented as mean ± SEM. Statistical analyses were done using one-way ANOVA.
2.9. Statistics analysis

All experiments were independently repeated at least three times. The statistical analysis was performed using GraphPad Prism 6 (Graph Pad Software). Multi-groups were analyzed by one-way ANOVA followed by Dunnett’s test. Student’s t-test was used for qRT-PCR data. Statistically significant differences were considered at p < 0.05.

3. Results

3.1. Decreased locomotion after reserpine treatment

The neurotoxicity phenotype showed in higher dosage of reserpine includes locomotion behavior (Puttonen et al., 2017). Here, we measured the swimming distance induced by exposure of a lower dosage of reserpine. Compared with the control group, after 5 days of reserpine treatment, swimming distance significantly decreased in all treatment groups, (31.0% reduction, 36.9% reduction and 34.2% reduction in 0.5, 1, 2 mg/L group, respectively) (Fig. 1A). During day transition, the swimming distance decreased in all reserpine treated groups (Fig. 1C, 31.2%, 36.3% and 32.9% decreased, respectively). The similar trend also showed in the night situation (Fig. 1E, 33.1%, 36.0% and 33.6% reduction in each group). In addition to swimming distance, we also analyzed the average velocity of the entire 24 h, day and night hours. The treated group had slower velocity in comparison to the control group (Fig. 1B, D and F).

3.2. Reduced sensitivity to light/sound stimulation by reserpine-exposed larvae

To further elucidate the locomotion under stimulus, we observed that all three reserpine-treated groups showed a significant decrease in swimming distance under light stimulation, which is 54.2%, 59.2% and 51.5% reduction, compared to the control group with the stimulation of light (Fig. 2A). Upon the stimulation of sound, the behavioral test measured slower locomotion in the reserpine-treated group by 30.6%, 37.7% and 30.5% reduction, respectively (Fig. 2D). The average swimming distance showed the weaker sensibility to light or sound stimulation in the reserpine treated groups (Fig. 2C and F). Furthermore, the analyzed average velocity was also reduced compared to control group. Our measurements revealed that the larvae fish were less sensitive to the light and sound after reserpine treatment (Fig. 2B and E).

3.3. Monoamine levels were decreased in reserpine treated groups

As a specific inhibitor of monoamine transporter, reserpine reduced monoamine concentrations. To further examine this phenomenon in zebrafish larvae, monoamine contents were measured. We found that 2 mg/L reserpine exposure group showed 40.1% reduction of DA, 56.0% reduced NE and 14.9% decrease of 5-HT content, in comparison with control group (Fig. 3A, B and C). However, the metabolites of 5-HT, such as 5-HIAA level were not changed after reserpine treatment (Fig. 3D).

Fig. 3. Monoamine levels measured by LC-MS after reserpine treatment. (A) Dopamine levels are significantly decreased compared to the control group, (*p < 0.05). (B) NE content is reduced with reserpine treatment (*p < 0.05). (C) 5-HT levels are reduced in the treated group (*p < 0.05, **p < 0.01). (D) The 5-HIAA levels showed no significant difference compared with the control group. N = 3 in each group. The distance was evaluated using Prism 6 from 3 independent experiments. Data presented as mean ± SEM. Statistical analyses were done using t-test.
3.4. Reserpine treated group lead to a reduction in prethalamic DA neurons

To investigate the effect of reserpine on DA neuronal development, we analyzed \( th \) expression using whole-mount in situ hybridization in control and 2 mg/L reserpine-treated group. The reserpine-treated larvae resulted in a significant decrease in the number of pre-thalamic DA neurons (Fig. 4A). The applied quantification assessed dosage-induced 71.1% reserpine treated zebrafish displayed reduced DA number, compared with control group (Fig. 4B).

3.5. DA correlated developmental genes were downregulated

Many transcriptional target genes and the \( wnt \) signaling pathway have been reported to regulate DA development (Castelo-Branco et al., 2003; Chen et al., 2012; Ryu et al., 2007). Using qRT-PCR, we analyzed their expression after 2 mg/L treatment of reserpine in zebrafish larvae. Candidate target gene mRNA levels decreased in reserpine-treated groups, which is 47.4% reduction of \( \text{manf} \) (NM_001076629), 27.4% reduction of \( \text{otpa} \) (ENSDARG00000014201), 47.1% reduction level of \( \text{otpb} \) (ENSDARG00000058379), 35.0% reduction of \( \text{wnt1} \) (ENSDARG00000055554), 53.5% reduction of \( \text{wnt3a} \) (ENSDARG00000058822) and 44.7% reduction of \( \text{wnt5a} \) (ENSDARG00000014495) (Fig. 5).

4. Discussion

In this study, we demonstrated that a 5-day of reserpine treatment induced several motor and non-motor symptoms in zebrafish. Significant changes include inadequate motor function and depression-like behavior was observed in the reserpine-treated larvae. This might be due to the reduction of neurotransmitters (DA, NE, 5-HT) and loss of DA in the zebrafish brain. Mechanistically, DA related genes were downregulated, which indicates the neurotoxic effect of reserpine in early DA development. These results suggest that reserpine induce a depression-like behavior in a zebrafish model.

Reserpine has been extensively used to generate movement inability
models, particular in orofacial dyskinesia (OD) models in rodents, in which it causes motor and non-motor symptoms (Burger et al., 2004; de Freitas et al., 2016; Haeggendal and Lindqvist, 1964; Leao et al., 2015). Interestingly, there are some studies showed that the involuntary tremulous jaw movements induced by reserpine similar to tremor-related symptom found in patients with PD (Dawson et al., 2000; Menzaghi et al., 1997). Independent of reserpine as model of different diseases, motor ability is the main symptom observed in the experiment. Previous researches showed that the swimming distance is significantly decreased after exposure to 40 mg/L reserpine (Puttonen et al., 2017). Here, our findings support decreased locomotor activity in larvae also appeared in consecutive 5 days of 2 mg/L reserpine treatment immediately, including the average distance and velocity, which indicated the neurotoxicity of reserpine in zebrafish might occurred earlier and lower concentration. Additionally, we specifically analyzed locomotion at each time point and observed that larvae were less sensitive to light and sound stimulation suggesting that reserpine affected the reaction activity between the larvae and environment stimulation. Therefore, our findings demonstrated that under the lower dosage, reserpine decreased motor activity and sensitive in treated zebrafish larvae.

Movement inability was commonly divided into hypokinetic and hyperkinetic. Most of hypokinesia are associated with the

Fig. 5. Screening of related-genes expression levels were analyzed by qPCR. (A) The expression of manf was significantly reduced in 2 mg/L reserpine (**p < 0.01), (B) The expression of otpa decreased in 2 mg/L reserpine (*p < 0.05), (C) The expression of otpb was downregulated in 2 mg/L reserpine (**p < 0.01), (D) The downregulation of wnt1 in 2 mg/L reserpine (*p < 0.05), (E) The decreased expression of wnt3a in 2 mg/L reserpine (*p < 0.05), (F) The expression of wnt5a was significantly decreased in 2 mg/L reserpine (**p < 0.001), The distance was evaluated using Prism 6 from 3 independent experiments. Data presented as mean ± SEM. Statistical analyses were done using t-test.
neurodevelopment. Recent research build the related model in zebra-
fish by rotenone and paragaut has revealed that loss of dopaminergic
neurons is one of the major pathological manifestations in movement
inability, including adult and larvae (Nellore and P, 2015; Wang et al.,
2017). Here, we found the neurotransmitters, like DA, NE and 5-HT, are
decreased after 5 days persistent reserpin treatment, which is consist-
sent with previous findings that reserpin treatment would inhibited
whole monoamine levels (Puttenen et al., 2017). Particularly, all three
neurotransmitters displayed significant change in 2 mg/L reserpin
group, suggesting that this concentration might be the limited activity
dosage in dopaminergic system. Furthermore, based on our in situ hy-
bridization data, DA neurons are depleted in reserpin-treated larvae
brain, indicating that dopaminergic neurons vulnerable to reserpin-
treated, similar to that observed in 6-OHDA-treated larvae, another
neurotoxin to DA neurons (Feng et al., 2014). These data indicating that
brain, indicating that dopaminergic neurons vulnerable to reserpine-
dosage in dopaminergic system. Furthermore, based on our

5. Conclusion
Taken together, our results provide strong evidence that dopami-
nergic neuron development is inhibited by treatment with reserpin, as
exhibited by a near complete depletion of TH immunoreactivity fol-
lowing reserpin treatment. The reduction in dopamine-dependent
locomotion suggests that the loss is functionally relevant. Low-dosage
reserpin treatment can potentially induce hypocoactivity in larvae.
The depression-like phenotype, caused by dopaminergic loss, may be useful
for studying the reserpin treatment neurotoxicity, and finding solu-
tions to develop novel drugs and prevent the side effect.

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Declaration of Competing Interest
None.

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factor otpa and otpb paralogous genes function during dopaminergic neuron
development (Huang et al., 2015), suggesting that reserpin
inhibiting expression of the manf, oop, and wnt genes, which might lead to
the loss of DA neurons in the zebrafish larvae. In summary, exposure to
reserpin could induce impairment of the dopaminergic system and
contribute to hypokinetic behaviour.

None.