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Brief embryonic strychnine exposure in zebrafish causes long-term adult behavioral impairment with indications of embryonic synaptic changes ☆

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ABSTRACT

Zebrafish provide a powerful model of the impacts of embryonic toxicant exposure on neural development that may result in long-term behavioral dysfunction. In this study, zebrafish embryos were treated with 1.5 mM strychnine for short embryonic time windows to induce transient changes in inhibitory neural signaling, and were subsequently raised in untreated water until adulthood. PCR analysis showed indications that strychnine exposure altered expression of some genes related to glycinergic, GABAergic and glutamatergic neuronal synapses during embryonic development. In adulthood, treated fish showed significant changes in swimming speed and tank diving behavior compared to controls. Taken together, these data show that a short embryonic exposure to a neurotoxicant can alter development of neural synapses and lead to changes in adult behavior.

1. Introduction

Many environmental chemicals have been linked to childhood and adolescent behavioral deficits and studies have demonstrated the negative effects of environmental toxicants on developmental disabilities (Bellinger et al., 1992; Lanphear et al., 2000; Mendola et al., 2002; Stein et al., 2002; Needleman, 2004; Slotkin, 2004; Koger et al., 2005; Slotkin et al., 2006). One of the difficulties when investigating environmental toxicants is that many cause no overt morphological deformities, but demonstrate behavioral abnormalities. A study on the organophosphate pesticide chlorpyrifos (Dursban®) in zebrafish embryos demonstrated that adults exposed to low doses at embryonic stages where morphologically normal, but demonstrated learning deficiencies (Levin et al., 2004; Roy and Linney, 2007). While this study showed that toxicant exposure during early development can lead to adult behavioral deficits, it did not relate those deficits to specific perturbation of neuronal signaling in the developing embryonic nervous system. Furthermore, the behavioral tests were conducted infancy, and so do not address the issue of the persistence of the effect of treatment. Studies with ethanol and lead have also demonstrated early embryonic exposure affects neurobehavioral function in zebrafish, but again, no early neural molecular changes are demonstrated (Carvan et al., 2004) and the behavioral testing was rudimentary. These studies suggested the hypothesis that chemical perturbation of the developing nervous system alters the delicate balance of neurotransmitters in the developing fetal brain, which can be observed as long-lasting effects on behavior (Roy and Linney, 2007).

A neurotransmitter important to neural development is glycine, which inhibits firing of brainstem and spinal cord motor neuron (Legendre, 2001). This is a critical process to counterbalance the firing of glutamatergic excitatory neurons and is essential for regulating motor rhythm which underlies locomotive behavior (Legendre, 2001). The inhibitory actions of glycine on the brain and spinal cord result from an increase in chloride conductance. Additional glycine left in the cleft is reabsorbed into the presynaptic neuron by glycine transporters embedded in the membrane surface of the presynaptic neuron (glycine transporter 2) or in glial processes in close association with these neurons (glycine transporter 1). Studies in knockout mice, which lack the glycine transporter 1 and cannot reabsorb glycine suffer severe sensorimotor deficits. GLYT1$^{-/-}$ mice demonstrate hyporesponsiveness to tactile stimuli due to hyperactive glycine-mediated signaling (Gomeza et al., 2003; Aragon and Lopez-Corcuera, 2005). Additionally, knockout mutations of glycine transporter 2 lead to high levels of glycine in the synaptic cleft resulting in muscular spasticity, impaired motor coordination and tremor (Eulenburg et al., 2005). In other vertebrate species, like zebrafish, mutations in glycine transporter 1 lead to the *shocked* phenotype which results in motor deficits, spontaneous coiling of the trunk, an absence of the fish escape response and a lack of swimming behavior (Cui et al., 2005). Furthermore, mutations in the postsynaptic glycine receptor subunits have also demonstrated defects. Mutations in the glycine

☆ Sadly, Dr. Cerutti recently passed away.

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receptor beta-subunit results in a loss of glycinergic inhibitory neural signaling resulting in the *bandoneon* phenotype which causes trunk muscles to fire excitatory neurotransmitters concurrently (Hirata et al., 2005).

Strychnine antagonizes the inhibitory actions of glycine (Legendre, 2001; Hirata et al., 2005) by blocking the binding of glycine to its postsynaptic chloride channel receptor (Hirata et al., 2004; Hirata et al., 2005). Thus the channel does not open, preventing propagation of the inhibitory signal. In zebrafish, strychnine exposure demonstrates an "accordion" phenotype (Hirata et al., 2004), characterized by simultaneous contraction of the bilateral trunk muscles along the length of the body. Studies of behavior in 30 day old Japanese medaka (*Oryzias latipes*) exposed to strychnine demonstrate changes in equilibrium, general activity, and startle response (Rice et al., 1997).

The zebrafish model system provides a powerful tool to study early neurotoxic challenge and the impact on adult behavior. Zebrafish develop *ex utero*, allowing toxicant treatment at time windows beginning immediately post-fertilization. Once treated, embryos can be removed from the toxicant and raised in non-treated water to adulthood (3 months). Behavioral testing can be performed at any time during development. Most importantly, however, zebrafish are vertebrate organisms that develop in accordance with the vertebrate plan like humans. Thus, research investigating how environmental toxicants affect zebrafish neural development and influence juvenile and adult behavior is applicable to human development.

Here, we treated zebrafish embryos for short embryonic time windows with strychnine. We examined changes in neurons by analyzing the expression of synaptic transporter, receptor and processing enzymes for various neurotransmitters at embryonic stages via PCR analysis and demonstrate changes in expression of glycinergic, GABAergic and glutamatergic related genes between 24 and 96 hrs after treatment. Treated siblings were raised in normal fish water until adult stages where they were tested for behavioral deficits using test methods developed in our labs. The adult fish showed significant changes in swimming speed and tank diving behavior in a novel tank diving test. Taken together, our data suggest that a short embryonic exposure to a toxic chemical is significant enough to cause changes in embryonic neural synapses and lead to deficits in adult behavior.

2. Methods

2.1. Embryo handling

Embryos were generated by natural pair-wise mating in zebrafish mating boxes (Westerfield, 1994). Embryos were placed in Petri dishes in 30% Danieau Buffer (50× Danieau's Solution [169.475 g NaCl, 2.61 g KCl, 4.93 g $MgSO_4$ 7 H20, 7.085 g $Ca(NO_3)2$ 4 H_20, 0.5 M Hepes at a pH of 7.6, autoclaved]. 30% Danieau's solution was prepared by mixing 6 ml of the 50× concentrated solution into 1 l of dH_2O) at 28 °C for 5 hrs before moving into drug treatment. Zebrafish were staged in accordance with standard staging series (Kimmel et al., 1995).

2.2. Drug administration

Strychnine (Sigma, St. Louis, MO, USA) was dissolved in distilled water to a stock concentration of 10 mM. A 1.5 mM working stock was made by dilution in 30% Danieau Buffer. Strychnine dose was chosen from previous studies (Granato et al., 1996; Hirata et al., 2005). Although our dose was higher, in verbal communications with Granato, we used a different form of strychnine salt. However, we increased the dose until our embryos mimicked their published "accordion" phenotype without any morphological abnormalities. At 5 hpf (hours post fertilization) embryos were moved from 30% Danieau Buffer to working concentration of 1.5 mM strychnine. At 24 hpf, embryos were manually dechorionated with forceps. To verify strychnine was effective, the "accordion" phenotype was checked by manually touching embryos delicately with forceps (Hirata et al., 2004). Embryos were left in strychnine solution for 24, 48, 72 and 96 hrs time periods until they were subjected to RNA extraction. Increased time exposures were used to determine if longer treatments resulted in stronger phenotypes. Treatment in strychnine for longer time periods resulted in death. At the 96 hr time point, the longest treatment window, the embryos appeared healthy with no morphological defects, yet displayed the "accordion" phenotype. Embryos to be raised to adulthood for behavioral testing were treated for 18 and 29 hrs, removed from strychnine treatment, washed with 30% Danieau Buffer and placed in Danieau Buffer until they were 5 days old after which they were raised in standard fish water (Westerfield, 1994). Control embryos were collected and raised in 30% Danieau Buffer only. At 24, 48, 72 and 96 hrs, they were subjected to RNA extraction. Siblings raised for behavioral testing were kept in 30% Danieau buffer until they were 5 days old after which they were raised in standard fish water as described above for the strychnine treatments.

2.3. Molecular biology methods

RNA Extraction was performed with a Qiagen RNEasy RNA Extraction kit, protocol for purification of total RNA from animal cells using spin technology was used (catalogue #: 74104) with the following modifications. Control and drug treated embryos were placed in a 15 ml snap-cap tube and residual water was removed. 700 µl RTL + BME was added to each tube and homogenized for 30 seconds. After standard processing the RNA was eluted off the column by adding 20 µl of RNAse free water directly to the membrane. After sitting for 2 min, the samples were spun for 1 min at 10,000 rpm to elute the RNA which was quantified and stored at −80°. For embryos at 24 hpf, 75 embryos were homogenized, at 48 hpf, 60 embryos and at 72 and 96 hpf, 40 embryos were homogenized for RNA extraction.

cDNA preparation was conducted with a Bio-Rad iScript cDNA synthesis kit (catalogue #: 170-8890) according to the manufacturer's specifications. 1 µg/µl of RNA sample was used for each reaction.

PCR and Gel Electrophoresis was conducted with a Platinum Blue PCR Supermix from Invitrogen (catalogue #: 12580-015) and used according to the manufacturer's specifications. Primers were all ordered from IDT Technologies (www.idtdna.com) and designed by IDT SciTools PrimerQuest from sequences imported from PubMed. Primer sequences and Tm's are shown in Table 1. A standard 1% agarose gel was performed.

2.4. Novel tank dive test

Adult zebrafish, 3 months of age, were placed individually in either of two unfamiliar ("novel") 1.5-liter plastic tanks filled with 1350 ml of tank water. The tanks were trapezoidal in shape and extended 22.9 cm along the bottom and 27.9 cm in length cross the top. The tanks were trapezoidal in shape with one vertical side and one slanted side. The slanted side of the tank was approximately 15.9 cm in length and the opposing side was 15.2 cm long (Fig. 1). The novel tank dive test has been described previously (Levin et al., 2007). The two tanks had the short side next to each other with a barrier between them and a solid white 60 cm × 122 cm plastic board that was positioned behind the tanks. The two tanks were separated from each other in terms of water contact and visual access as shown in Fig. 1. Thus, each fish was tested independently. The video image was divided into lower, middle, and top swimming areas using the EthoVision™ program (Noldus Information and Technology, Wageningen, Netherlands). The trial series was started with a 5-min trial duration for testing. The video signal was transmitted through an 8 mm Samsung Camcorder. The video was then transmitted to the computer for analysis. There was one 5-min. trial per fish.