



RESEARCH ARTICLE

DOI: <http://dx.doi.org/10.14259/mn.v1i1.116>

Brief Embryonic Exposure to Prozac® is Correlated With Alterations in Embryonic Synaptic Gene Expression and Larval Behavioral Changes in *Danio rerio*

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Article Info: Received: April 12th, 2014; Accepted: April 23rd, 2014

ABSTRACT

Prozac®, a widely prescribed drug of the selective serotonin reuptake inhibitor class, is used to treat a number of disorders, including depression, panic attacks, obsessive compulsive disorder, and some eating disorders. Its widespread use raises concerns regarding the potentially harmful effects of embryonic exposure to the drug. A zebrafish model was utilized to study the effects of Prozac® on the developing nervous system. Here we specifically focused on embryonic synaptic remodeling events and the later-staged adolescent behavioral changes resulting from a brief window of embryonic exposure to the drug. While no morphological differences between experimental and control embryos were observed, changes in the expression of genes involved in synaptic transmission, including those for serotonin and dopamine transporters were detected. Later-staged adolescent behavioral effects were analyzed using tracking software. Drug-treated fish showed significant changes in swimming behavior, including decreased total distance moved, time spent mobile, velocity, and time spent in an inner zone in the testing well. Prozac®-treated embryos also exhibited changes in learning responses in a simple non-threatening habituation assay compared with controls. Using the zebrafish model, we demonstrate that a brief period of embryonic exposure to Prozac® alters the expression of genes involved in embryonic synaptic transmission and is associated with changes in later-staged adolescent behavior.

Keywords: Zebrafish; Development; Prozac®; Synaptic; Behavior

Introduction

Development of the nervous system is a delicate process directed by a highly organized sequence of cellular events consisting of neurogenesis, proliferation, migration, differentiation, and synaptogenesis. Individual neurons are incorporated into more complex neural networks, which ultimately determine behavior. These stages of neural development are both sequential and interdependent. Disruption of the developmental process by environmental toxins, pharmaceutical drugs, or other chemical compounds to which we are repeatedly exposed may have

negative long-term consequences. It has been demonstrated previously that fetal exposure to heavy metals and pesticides is associated with a number of learning and behavioral deficits including, attention deficit, hyperactivity, memory impairment, decreased IQ and motor dysfunction [1]. Those most extensively studied are lead, methylmercury, pesticides, alcohol, and other recreational drugs [2].

In general, exposure to environmental chemical compounds does not result in obvious morphological changes, but rather subtle changes at the molecular level, making the effects of exposure difficult to identify and characterize. Previous studies have shown that electrical stimulation of neurons alters expression of neurotransmitter-associated genes [3]. Expanding upon this work, this study examines whether a pharmacological challenge can produce similar results.

Today women are increasingly exposing their unborn

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children to another environmental challenge—prescription drugs. A study published last year by Boston University's Slone Epidemiology Center found that over the past three decades, first-trimester use of prescription medication increased by more than 60%, with the most dramatic increase observed for antidepressants [4]. Depression among pregnant women is not uncommon. In fact, prevalence rates of depression during pregnancy can range from approximately 7-13% [5]. While these medications have been approved and are generally considered safe to use during pregnancy, mounting evidence suggests that they may negatively affect an unintended target—the developing fetus. With over 33 million prescriptions issued in 2002, Prozac® is one of the most widely prescribed antidepressants [6]. It is also prescribed to treat anxiety disorders, obsessive-compulsive disorders, and some eating disorders. Its widespread use raises concerns regarding the potentially harmful developmental effects of embryonic exposure to the drug.

Prozac® is a drug of the selective serotonin reuptake inhibitor (SSRI) class. Depression is often associated with a lack of serotonin in the brain. As an SSRI, Prozac® exerts its effects by inhibiting the serotonin transporter, thereby preventing the reuptake of serotonin at the synapse and increasing overall serotonin levels in the brain. One of the major neurotransmitters in the central nervous system, serotonin (5-HT) is most commonly known for its role in controlling mood, but it also modulates fear, aggression, circadian rhythms, sleep patterns, anxiety, and locomotion [7]. Moreover, 5-HT may also function as an important developmental signal, regulating the development of serotonergic neurons as well as the tissues they target [8-10]. Serotonin has been shown to function at all levels of neural development, including neuronal proliferation, migration, differentiation and synaptogenesis [7,9,11,12]. Genetic models that target the 5-HT system demonstrate that transient changes in 5-HT levels modify neural connections in the brain and lead to permanent changes in adult behavior [9]. Serotonin depletion studies have found that reduced serotonin levels during development negatively affect body length, notochordal morphology, locomotor behavior, and the expression of genes involved in serotonin transmission, including the 5-HT receptor and 5-HT transporter [10].

The serotonin transporter (SERT), which is the target of SSRIs like Prozac®, plays an important role in the regulation of serotonin levels. It has been found that SERT is expressed in serotonergic neurons and transiently expressed in non-serotonergic neurons throughout development [13]. This suggests that there is a window of vulnerability for drugs, like Prozac®, that target SERT. Previous studies have demonstrated that chronic, post-natal treatment with SSRIs produces anxiety-related behavior in mice, similar to that of 5-HT deficient mice [14]. Subsequent studies have demonstrated that prenatal and neonatal exposure can also cause anxiety- or depression- like symptoms in adulthood [15], as well as transient deficits in motor development [16]. However, research on the effects of a brief period of exposure to SSRIs early in development remains sparse.

The objective of this study was to characterize the effects of a brief period of embryonic exposure to Prozac® at the embryonic molecular and later-staged behavioral levels. A zebrafish model was utilized to study the effects of Prozac® on the developing nervous system [17]. Zebrafish are vertebrates, have high mating fecundity, are optically transparent during early development and their brain development has been fully studied. Here we specifically focused on embryonic synaptic remodeling events and the later-staged adolescent behavioral changes resulting from a brief window of embryonic exposure to the drug.

Materials and Methods

1. Zebrafish care and embryo handling

Zebrafish were maintained in a self-contained, re-circulating aquaculture ZMOD system (Marine Biotech®) at Sacred Heart University, Fairfield, CT. Zebrafish were kept on a strict 14:10 light-dark cycle, and fed on a diet of brine shrimp (Inve Aquaculture, Corp.) Embryos were generated by natural pairwise mating in standard zebrafish crossing tanks [18]. Embryos were collected in petri dishes in 30% Danieau Buffer (30 % Danieau prepared by adding 6 mL 50 X concentrated stock Danieau's Solution [169.475g NaCl, 2.61g KCl, 4.93g MgSO₄ 7H₂O, 7.085g Ca (NO₃)₂ 4H₂O, 0.5M HEPES at a pH of 7.6, autoclaved] to 994 mL of dH₂O) at 28 °C for 5 hrs before moving into drug treatment. Zebrafish were staged according to standard staging series [19].

2. Drug administration

A 2.9 mM stock solution of Prozac® (Sigma, St. Louis, MO, USA) was made by dissolving 0.01 g of Prozac® in 10 mL dH₂O. A 50 µM working solution was made by dilution in 30% Danieau Buffer. At approximately 5 hpf (hours post fertilization), or the sphere stage [19], embryos were moved from 30 % Danieau Buffer to working concentration of 50 µM Prozac®. Embryos were left in Prozac® solution for 48, 72, or 96 hours. Daily, debris was removed from petri dishes and fresh 30 % Danieau or 50 µM Prozac® was added to control and experimental embryos, respectively.

3. Live imaging of wild-type zebrafish

Live images of embryos were taken to examine changes in gross morphology as a result of embryonic exposure to Prozac®. After 48, 72, or 96 hours of drug or control treatment, embryos were manually dechorionated, anesthetized with Tricane, and visualized under a Leica Zoom 200 dissection microscope. Images of control and experimental embryos were taken at each of the three time points.

4. RNA Extraction

RNA was extracted from whole control and Prozac®-treated embryos at each of the three time points. For embryos at 48 hpf, 125 embryos were homogenized and used for RNA

extraction. At 72 hpf, 75 embryos were used and at 96 hpf 60 embryos were used.

A Qiagen RNEasy RNA Extraction kit and protocol for purification of total RNA from animal cells using spin technology were used (catalogue #: 74104) with the following changes. Control and drug treated embryos were placed in a 15 mL snap-cap tube. Remaining water or Prozac® solution was removed with a filter-free pipet. RLT + BME was prepared by adding 14 µL BME to 1400 µL RLT and inverting to mix. 700 µL RLT-BME was added to each tube, and the solution was homogenized. The homogenizer was cleaned 4 times for 30 seconds each in DEPC water before, between, and after homogenization of samples. Post-homogenization foam was allowed to settle, and all liquid was transferred to a 1.5 mL eppendorf tube, which was centrifuged at 14,000 rpm for 3 minutes. The supernatant was transferred to a new 1.5mL eppendorf tube. 600 µL of 70 % ethanol diluted in RNase free water was added to each tube and mixed well. 700 µL of each sample was applied to a spin column and centrifuged for 15-30 seconds at 10,000 rpm, after which the flow-through liquid in the collection tube was discarded. This was repeated for the remaining 700 µL lysate. 700 uL luffer RW1 was added to each column and spun 10000 rpm for 15s, after which the flow-through and collection tube were discarded. 500 µL buffer RPE was added and each column was placed in a new collection tube. The column was spun at 10,000 rpm for 15 seconds and the flow-through was discarded. Another 500 µL of buffer RPE was added and the columns were centrifuged for 2 minutes, after which the flow-through was discarded. To air dry, the columns were spun for an additional minute at 14,000 rpm. The column was placed in a clean eppendorf tube. The RNA was eluted off the column by applying 20 µl of RNase free water directly to the membrane. After sitting for 2 minutes, the samples were spun for 1 minute at 10,000 rpm to elute the RNA, which was quantified and stored in the freezer.

5. cDNA Preparation

A Bio-Rad iScript cDNA synthesis kit (catalogue #: 170-8890) was used according to the manufacturers specifications. 1 µg/µl

of RNA sample was used for each reaction. All solutions used for cDNA synthesis were prepared in 0.5 µL PCR tubes on ice. Solution was a total of 20µL: 1 uL enzyme RT, 4 uL Buffer 5Xiscript, 1 µg/µL RNA, and sterile water.

6. PCR and Gel Electrophoresis

Platinum Blue PCR Supermix from Invitrogen (catalogue #: 12580-015) was used according to the manufacturers specifications. Briefly, 45µl Platinum Blue Supermix, 2 µl cDNA, 1.5 µl 20 µM forward primer and 1.5 µl 20 uM reverse primer were combined in a 0.5 µl standard PCR tube. PCR conditions were standard only changing the T_m, as reflected by the primers. The PCR program used was: Warm up: 94 °C for 2 minutes, Denature: 94 °C for 30 seconds, Anneal: dependent of T_m's set for primers for 30 seconds, Elongation: 72 °C for 1 minute, Amplification: Repeat step 2-5 35 times. Primers were all ordered from IDT Technologies (www.idtdna.com) and designed by IDT SciTools PrimerQuest from sequences imported from PubMed. Primer sequences are shown in Table 1. PCR products were electrophoresed in a standard 1 % agarose gel and visualized using a DNA trans illuminator.

7. Larval and adult behavioral testing

Embryos were generated by natural pairwise mating, collected, and treated as described above. After 48, 72, or 96 hours of embryonic exposure to 50 µM Prozac®, fish were released from drug treatment and raised in normal fish water until the juvenile and adult stages when behavior was analyzed.

Zebrafish behavior was analyzed using DanioVision® tracking software by Noldus. Six control fish and 6 Prozac®-treated fish were placed into individual test wells in a 12-well plate. The plate was placed into the machine. The following parameters of swimming behavior were measured: total distance moved, time spent mobile, time spent immobile, velocity, angular velocity, meander, and time spent in an inner zone in the testing well. Learning behavior was

Table 1: Forward and reverse primer sequences to genes involved in synaptic transmission

Gene	Accession #	Product Size (bp)	Forward Primer	Reverse Primer
<i>bactin1</i>	NM_131031.1	686	TTGGCATGGGACAVA AAGACTCCT	ACCGCAAGATTCCATA CCCAGGAA
<i>slc6a4b</i> (5-HT transporter)	NM_001177459	984	TTGGATTTGCGGTAG ACCTCGGAA	TCGACCCAACCATGTT TGCAATGG
<i>htr1aa</i> (5-HT receptor)	NM_001123321	698	ATGTGGCCAACATATC TCATCGGCT	GTTGTTTGGCAGGCTC AGGTGATT
<i>slc6a3</i> (Dopamine transporter)	NM_131755.1	735	TGCTCAACGACACGT ACAAGACCA	AGAGCTCTCGATGTTT GTGCAGGA
<i>dbh</i> (dopamine beta hydroxylase)	NM_001109694.2	703	AGTACAGCGGCTCCT GTGATTCAA	AGGTAATCTGTGTCCA CGTGGCTT

examined through a simple non-threatening habituation assay. Fish were allowed to adjust to the test well for five minutes. Then, a light stimulus was presented 3 times, with 1 minute between each stimulus. Total distance moved was measured during the 10 seconds before and after the stimulus.

8. Live imaging of transgenic zebrafish

fli1-gfp transgenic zebrafish were obtained from the Lawson Lab (UMASS Medical School) and used to examine the effects of embryonic exposure to Prozac® on the developing vascular architecture. Transgenic embryos were generated by natural pairwise crossing of transgenic adults. Embryos were collected in 30 % Danieau, and experimental embryos were treated with 50 μ M Prozac® at approximately 5 hpf. To inhibit pigmentation, control embryos were transferred from 30 % Danieau to 30 % Danieau + 1XPTU at approximately 8 hpf. 30 % Danieau + PTU was prepared by adding 6 mL 50XDanieau and 20 mL 50XPTU to 974 mL diH₂O. Experimental embryos were transferred to 50 μ M Prozac® 1XPTU, prepared by adding 3.5 mL 2.9 mM stock Prozac® solution to 196.5 mL 30 % Danieau + 1XPTU.

After 48, 72, or 96 hours of drug or control treatment, embryos were manually dechorionated, anesthetized with Tricane, and visualized under a fluorescent microscope with a digital camera. Images of control and experimental embryos were taken at each of the three time points.

Results

A. Embryonic exposure to 50 μ M Prozac® was not associated with gross morphological changes in wild-type or transgenic embryos:

Zebrafish embryos were generated by natural pairwise mating

and subjected to treatment with 50 μ M Prozac® solution or control water at the sphere stage of development, or approximately 5 hpf. Embryos were left in treatment for 48, 72, or 96 hours. Changes in gross morphology were examined by taking images of live embryos at each of the time points. Prozac® treatment did not appear to cause any overt morphological defects or delays in development (Figure 1).

Transgenic embryos were utilized to examine morphology more closely. *fli1-gfp* transgenic fish enabled visualization of the vascular architecture. Embryos were generated by natural pairwise mating of transgenic adults and subjected to treatment with control water or 50 μ M Prozac®. After 48 hours, images of live embryos were taken under a fluorescent microscope. There were no observable differences in vascular architecture or delays in development between control and Prozac®-treated fish (Figure 2).

B. Embryonic exposure to 50 μ M Prozac® was associated with changes in synaptic gene expression:

RT-PCR analysis was performed to examine the expression of neurotransmitter-related genes in control and Prozac®-treated embryos. Experimental embryos were treated with 50 μ M Prozac® for 48, 72, or 96 hours until they were subjected to whole embryo total RNA extraction. cDNA was prepared from RNA isolated from whole embryos and utilized in RT-PCR. Primers for genes involved in neurotransmitter transmission were chosen (Table 1).

There were no observed differences in expression of *5-HT receptor* or *dopamine beta hydroxylase* (Figure 3). However, obvious downregulation of *dopamine transporter* was observed

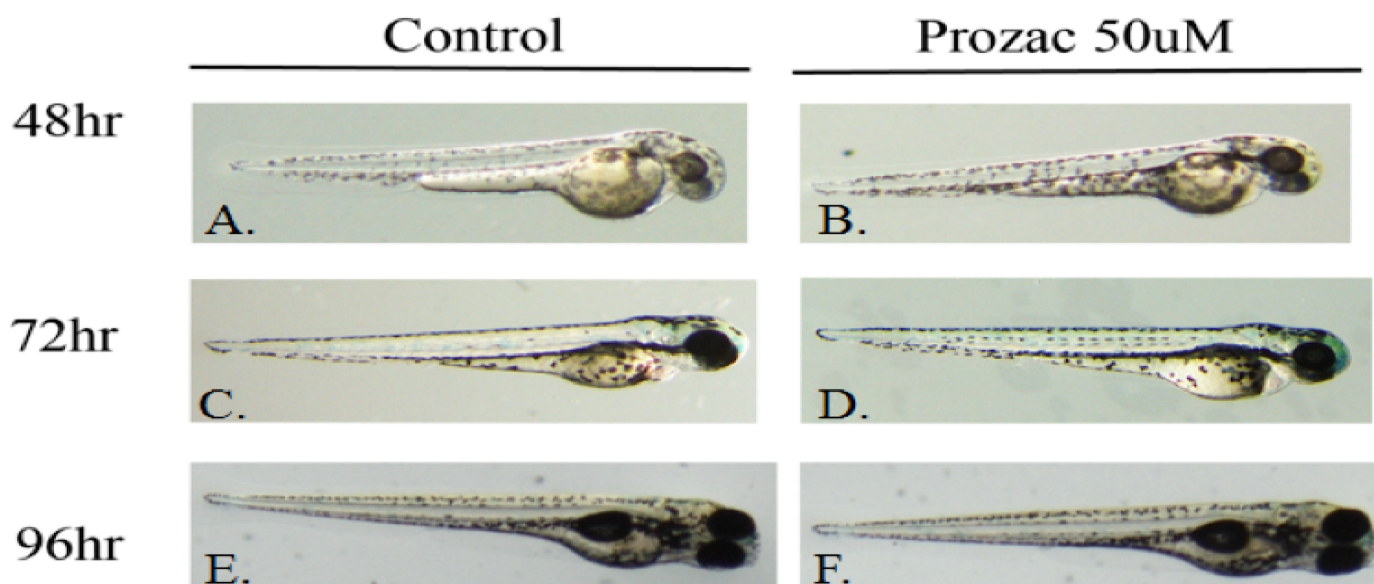


Figure 1: Images of live control (A,C,E) and Prozac-treated embryos (B,D,F) at 48, 72, and 96 hours. No morphological changes or delays in development were observed in drug-exposed embryos compared to controls.

after 72 and 96 hours of embryonic exposure to Prozac®. 5-HT transporter was also downregulated at 72 hr, but upregulated at 96 hr in Prozac®-treated embryos, however these results were not as strong as seen with the dopamine transporter (Figure 3).

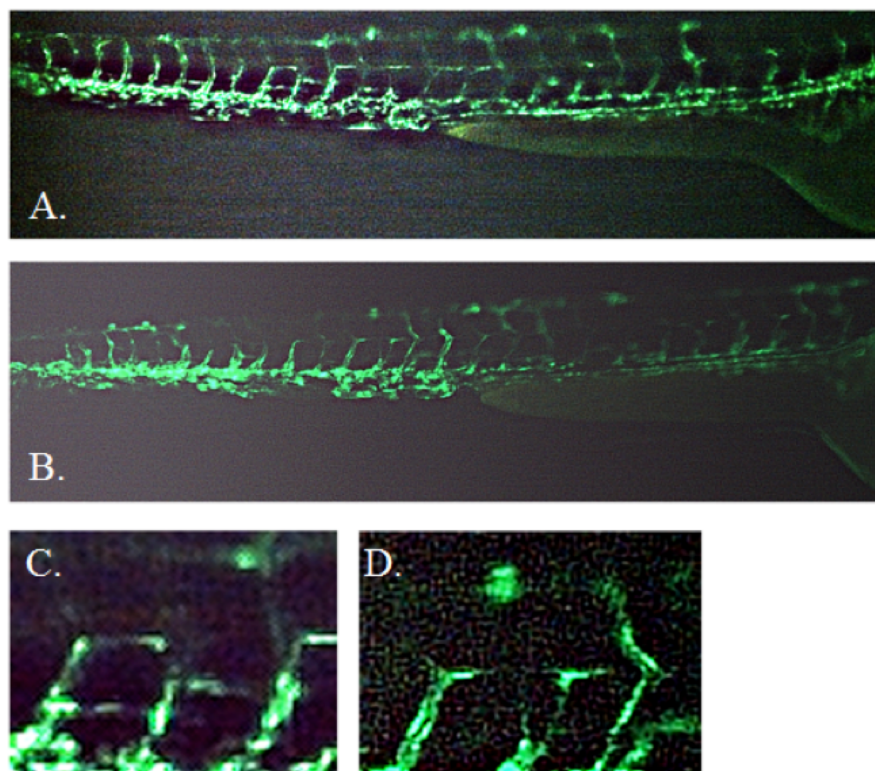


Figure 2: Control (A,C) and Prozac-treated (B,D) *fli1-gfp* transgenic embryos at 48 hours. No morphological changes in vascular architecture or delays in development were observed.

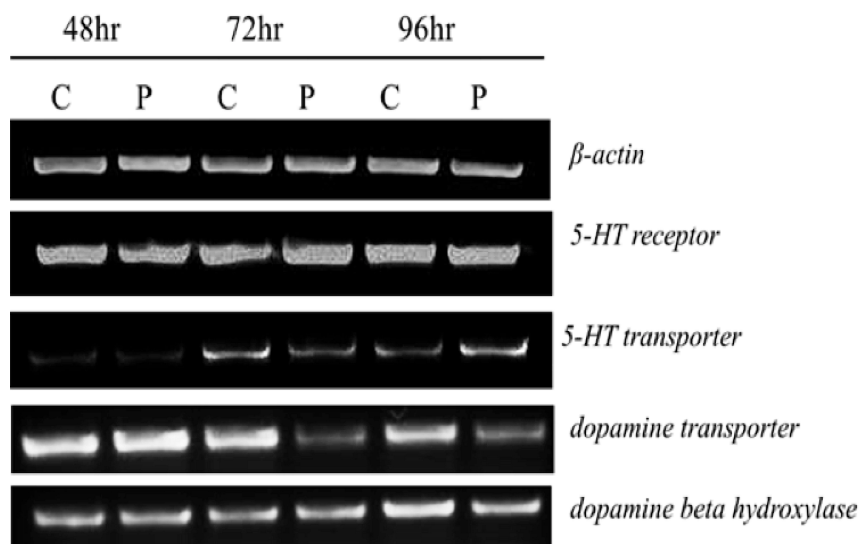


Figure 3: Relative expression of serotonergic and dopaminergic genes in control and Prozac-treated embryos at 48, 72 and 96 hours. Downregulation of dopamine transporter was observed at 72 and 96 hours. Downregulation was also observed in 5-HT transporter at 72 hours, while the gene was upregulated at 96 hours. *β-actin* is a loading control.

C. Embryonic exposure to 50 μ M Prozac® was associated with changes in juvenile behavior

After 48, 72, or 96 hours of treatment with 50 μ M Prozac®, fish were released from drug treatment and raised until they were subjected to behavioral testing as two-week old juveniles. DanioVision® tracking software by Noldus was used to measure zebrafish larval behavior. 13-day juveniles exposed to Prozac® for 72 hours during embryonic development exhibited significant changes in swimming behavior. Specifically, Prozac®-treated fish exhibited significantly decreased total distance moved (Figure 4A), time spent mobile (Figure 4B), velocity (Figure 4C), and duration in an inner zone in the testing well (Figure 4D) ($p < 0.05$). Moreover, drug-treated fish showed significantly increased time spent immobile (Figure 4E). There were no significant differences between control and drug-treated embryos for angular velocity or meander (Figures 4F, 4G).

Prozac®-treated fish also exhibited altered response in a simple non-threatening habituation assay. Control embryos tended to decrease distance moved during the 10 seconds after the stimulus for each time the stimulus was introduced. Interestingly, Prozac®-treated fish responded oppositely, tending to increase distance moved during the 10 seconds after the stimulus each time the stimulus was introduced (Figure 5).

Discussion

Drug-treated wild-type and transgenic embryos did not exhibit any morphological differences or delays in embryonic development when compared with controls of the same age. This is consistent with the hypothesis that neurotoxic agents do not necessarily lead to overt, phenotypic changes. However, drug-induced changes were observed at the molecular level. PCR analysis indicated that embryonic exposure to Prozac® affects the expression of serotonergic and dopaminergic genes during development. Specifically, downregulation of dopamine transporter was observed at 72 and 96 hpf, while downregulation of 5-HT transporter was observed at 72 hpf, with upregulation observed at 96 hpf. Interestingly, previous studies have reported long-term reduction

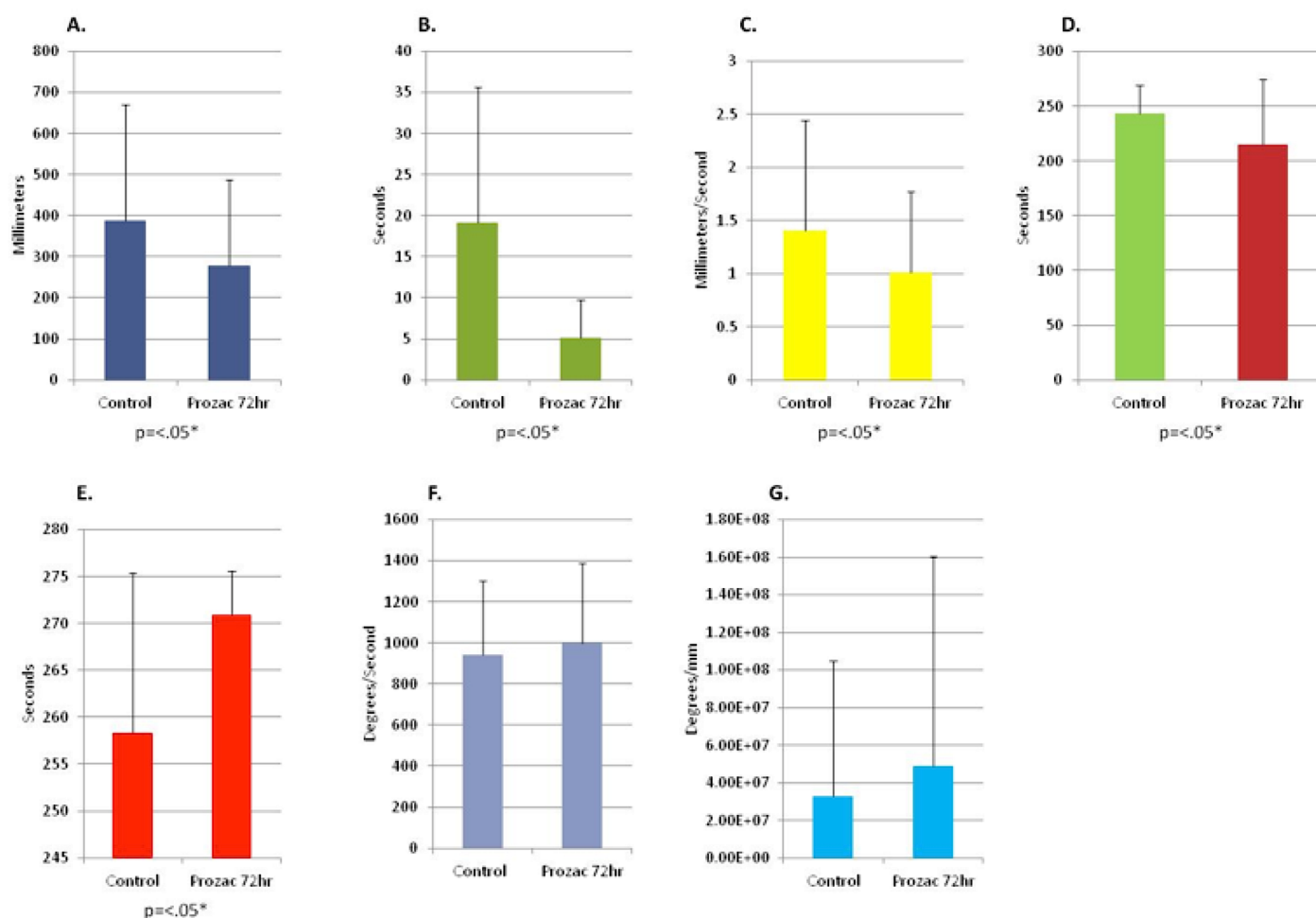


Figure 4: Swimming behavior of 72hr Prozac[®]-treated and control 13-day juveniles. Prozac[®]-treated embryos exhibited a significant decrease in total distance moved (A), time spent mobile (B), velocity (C), and duration in inner zone (D). Drug-treated fish showed a significant increase in time spent immobile (E). There were no significant differences between control and drug-treated fish for angular velocity (F) or meander (G).

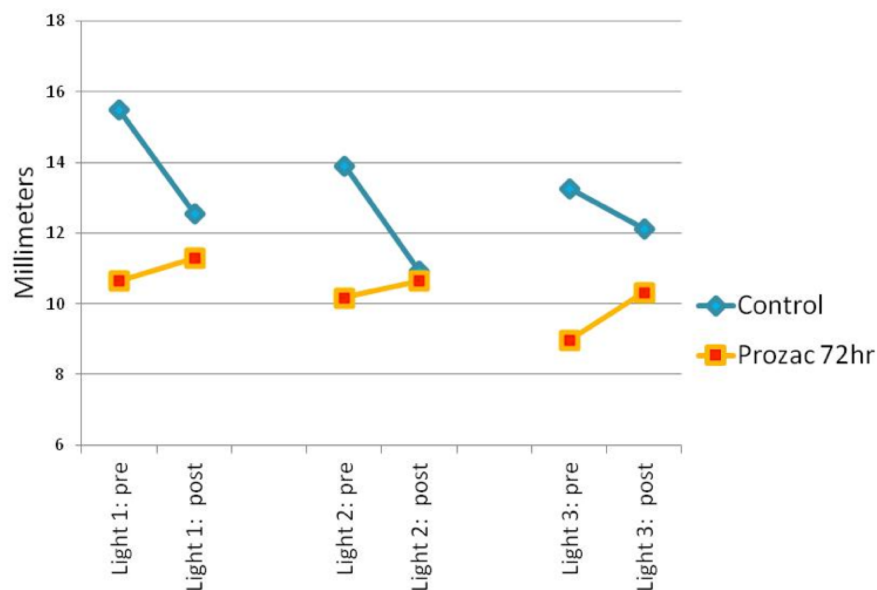


Figure 5: Learning response in a simple non-threatening habituation assay. Distance moved in millimeters was measured 10s pre/post-stimulus. Control fish were active during the 10s before the light stimulus, and less active after the stimulus. Prozac[®]-treated fish were hyperactive after the light stimulus. 13-day juveniles, 72hr Prozac[®], n=42

in *serotonin transporter* mRNA levels as a result of chronic, neonatal exposure to SSRIs [9]. The results of the present study suggest that *serotonin transporter* mRNA expression is transiently decreased in embryos exposed to Prozac® for a brief period of time and increased later in development. It is possible that early exposure to Prozac® gives the developing nervous system time to adjust to fluctuating serotonin levels. To further examine this issue, RT-PCR analysis of *serotonin transporter* should be carried out on RNA extracted from juvenile and adult fish.

The molecular alterations observed as a result of embryonic exposure to Prozac® were associated with behavioral changes later in life. Thirteen-day juveniles exposed to Prozac® for 72 hours after fertilization and then raised in normal fish water showed significant changes in swimming behavior and learning response in a simple non-threatening habituation assay. Interestingly, experimental embryos were hyperactive in response to the non-threatening light stimulus compared to controls. An important next step would be to assess adaptations of treated fish to chronic stimulation rather than just a brief light pulse. Of importance, drug-treated embryos spent significantly less time in the inner zone of the testing well. This assay is in some ways analogous to an open field test used to measure anxiety in rodents. Studies have shown that zebrafish prefer the edge of the testing well, a behavior known as thigmotaxis, and edge preference can be used as a measure of anxiety in zebrafish [20,21]. A previous study that examined the effects of Prozac®-exposure on thigmotaxis found that drug exposure did not influence edge preference [22]. In this study, however, fish were exposed to Prozac® at 7dpf, while our study examines the effects of exposure to Prozac® before and during nervous system development. Furthermore, the results of the present study are consistent with previous studies that have shown that prenatal exposure to SSRIs produced anxiety-like behaviors in adult mice [15]. To further address movement abnormalities, transgenic fish tracking neural synaptogenesis in real time could be used to determine impaired synaptogenesis.

Using the zebrafish model, we demonstrate that embryonic exposure to Prozac® may alter gene expression, which results in synaptic, but not morphological changes as well as behavioral changes later in life. Specifically, changes in expression of *dopamine transporter* and *serotonin transporter* and anxiety-like juvenile behavior were observed. Here, we do not suggest a specific mechanism, but rather suggest that Prozac® challenge may inappropriately influence synaptic modeling and thus, further neural differentiation. This may occur by influencing a suite of genes, not having a direct effect on any single gene or pathway to influence behavior. In the current study, protein levels were not addressed. Although we see synaptic gene changes, alterations at the protein levels could be occurring. To link alterations in the transcriptional level, synaptic protein markers should be addressed, but were out of the scope of this study.

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