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Zebrafish show long-term behavioral impairments resulting from developmental vitamin D deficiency

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ABSTRACT

Vitamin D has been shown in a wide variety of species to play critical roles in neurodevelopment. Vitamin D deficiency disrupts development of the brain and can cause lasting behavioral dysfunction. Zebrafish have become an important model for the study of development in general and neurodevelopment in particular. Zebrafish were used in the current study to characterize the effects of developmental vitamin D deficiency on behavioral function. Adult zebrafish that had been chronically fed a vitamin D deficient or replete diets were bred and the offspring were continued on those diets. The offspring were behaviorally tested as adults. In the novel tank diving test the vitamin D deficient diet significantly lowered the vertical position of fish indicative of more anxiety-like behavior. In the novel tank diving test swimming activity was also significantly decreased by vitamin D deficiency. Startle response was increased by developmental vitamin D deficiency during the early part of the test. No significant effects of vitamin D deficiency were seen with social affiliation and predatory stimulus avoidance tests. These results indicate a phenotype of vitamin D deficiency characterized by more anxiety-like behavior. This result was relatively specific inasmuch as few or no behavioral effects were seen in other behavioral tests.

1. Introduction

Vitamin D plays a variety of important roles in physiology, most notably helping to control intestinal absorption of calcium, which aids bone mineralization. In addition to this prominent well-known effect, vitamin D also provides important signaling during development, including playing key roles during neurodevelopment [1]. Vitamin D deficiency during brain development causes persistent impairment in behavioral function. For example, vitamin D deficiency during early development in rats has been found to cause long-lasting abnormalities in synaptic function in the hippocampus in parallel to impaired cognitive function [2]. Vitamin D deficiency can disrupt a wide variety of physiological functions, particularly neural systems during development. The zebrafish model can be useful for linking cellular and molecular mechanisms of vitamin D deficiency that impair behavioral function. First the behavioral phenotype of developmental vitamin D deficiency in the zebrafish must be determined.

Zebrafish have become an important model species for the study of molecular and cellular processes of neurodevelopment in vertebrates [3]. Their clear chorion and the availability of reporter systems makes visualization of the processes of neurodevelopment particularly accessible. Key to understanding the functional impact of perturbations of neurodevelopment is the assessment of behavioral function. Fortunately, a variety of behavioral tests have been developed to determine a spectrum of behavioral functions in zebrafish, from simple sensorimotor response to more complex emotional, social and cognitive behavior [4]. With the characterization of the functional behavioral dysfunction caused by developmental Vitamin D deficiency in the zebrafish, we can use this model to determine the critical mechanisms by which vitamin D causes those impairments.

The current study was conducted to determine the short- and long-term behavioral effects of early developmental vitamin D deficiency in zebrafish. Zebrafish with vitamin D deprivation during larval and juvenile development were compared with zebrafish which were fed a diet replete with vitamin D. The effects of early developmental vitamin D deficiency on persisting behavioral function effects on locomotion, sensorimotor response and habituation, emotional responsivity, and social behavior during adulthood.

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2. Material and methods

2.1. Zebrafish housing and diets

All zebrafish (*Danio rerio*, AB* strain) in this study were housed and cared for according to standard protocols approved by the North Carolina State University (NC State) and Duke Institutional Animal Care and Use Committees. Adult zebrafish broodstock were maintained at appropriate densities in 9 L tanks as part of a recirculating aquatics system under a 14:10 h light:dark cycle. Water temperature was maintained at 28.5 ± 0.5 °C with a pH between 6.8 and 7.5. The parental F0 fish were co-housed and raised on a standard larval laboratory diet (11.5 iu/g Vitamin D) until 2 months of age were random sample of 61–83 juvenile zebrafish were assessed for standard length (SL) to screen for growth. Based on the average SL, any fish that was ± 2 standard deviations (SD) away from the mean was removed from the population. The remaining fish were equally divided and placed into nine, 9 L, mixed-gender tanks. The tanks were grouped in sets of three so that there were three tanks per diet. Upon transfer into their new housing, the 2-month old zebrafish began their new diet consisting of either a vitamin D null diet (0 iu/g VDD) or a vitamin D enriched diet (400,000 iu/g) (VD3) [5]. All zebrafish were kept on their designated engineered diet throughout the rest of their lifespan. To ensure that VDD zebrafish did not exchange water with VD3 sufficient fish, they were kept in their own quarantine system set to the same parameters as the main system. Demonstration of vitamin D deficiency in this model was established using LC/MS/MS as previously described [5]. Briefly, VDD zebrafish contained attenuated concentrations of both 1,25(OH)2D3 (0.0047 ± 0.0018 VDD versus 217.95 ± 33.4748 VD3 Sufficient) and the primary vitamin D metabolite 25(OH)D3 (below limit of detection VDD versus 0.2276 ± 0.0405 VD3 Sufficient). Confirmation of the vitamin D deficiency is fully described [5]. Briefly, vitamin D levels, *cyp* gene expression, microCT analyses, and calcium levels are described, confirming a state of vitamin D deficiency in the vitamin D deficient dietary group. We agree that this assessment is critical to demonstrating that our dietary protocol results in vitamin D deficiency in this species. Using a series of studies including analytical assessments of whole-body vitamin D and vitamin D metabolites, *cyp* gene expression, microCT analyses of bone, and whole-body calcium levels we have shown that zebrafish fed a vitamin D null diet are severally vitamin D deficient by six months. These data are included in a recent manuscript that is currently under revision/review with Scientific Reports. We have cited this reference including our analytical data demonstrating attenuated levels of 1,25(OH)2D3 and 25(OH)D3 in the vitamin D null fed cohort within the methods section of this manuscript.

At 6mpf, after 4 months on either the VDD or VD3 diet, zebrafish from each dietary group were spawned and offspring were collected for larval behavior testing. The offspring were tested at 5–7 months of age on the behavioral test battery as described below. The offspring continued the vitamin D diets as described above. All tests were conducted between 11:00 AM (2-h post lights-on) and 6:00 PM, with testing time counterbalanced across experimental groups (Fig. 1).

2.2. Behavioral test battery

Adult offspring of the vitamin D deprived and control zebrafish (N = 19–20 per dietary group) were tested at the age of 5–7 months in a series of 4 behavioral assays; novel tank dive test, tap test, shoaling test, and predator avoidance test, to evaluate several emotional, social and cognitive functions. Each assay was conducted on a separate day. All testing was conducted between 10 AM and 5 PM during the light phase of their diurnal cycle. Testing times were counterbalanced across the vitamin D groups. Fish tanks designated for testing were transferred to the behavior testing room, and let acclimate for 30–60 min. Freshly made system water was used in all testing apparatuses. A high definition camcorder (VIXIA HFR700; Canon Inc., Tokyo, Japan) was used for video recording in all assays, and the videos were processed by the EthoVision XT® software (Noldus) for fish tracking and activity analysis.

2.2.1. Novel tank diving test

Adult zebrafish were tested for novel environment response and recovery based on the method employed previously in our laboratory [6,7] with modifications (Fig. 2). The experimental set-up consisted of two adjacent 1.5 L plastic tanks (Aquatic Habitats) filled with system water to a depth of 10 cm. Each tank was a trapezoid: 22.9 cm along the bottom, 27.9 cm at the top, 15.2 cm high and 15.9 along the diagonal side. It was 6.4 cm wide at the top, and tapered to 5.1 cm at the bottom. The tanks were video recorded from the side. At the beginning of each trial two fish were individually placed in the testing tanks (one in each tank) and recorded for 5 min. Measurements extracted were the total distance traveled in cm/min and the mean distance from the tank floor in cm for each min. A dive recovery value was calculated for each treatment group by subtracting the distance from the tank floor in the first minute of testing from the mean distance in the last 4 min of testing.

2.2.2. Sensorimotor startle tap response and habituation test

Sensorimotor startle response and habituation were tested using a custom-built apparatus and based on a protocol developed in our laboratory [8,9](Fig. 3). We have found this test to be sensitive to the long-lasting effects of early life pertubations caused by low-level exposure to pesticides and flame-retardant chemicals [4,9–12]. The testing apparatus consisted of a flat white 23 × 39 cm surface with white 23 × 27 cm visual blocking barriers between each of the eight test arenas. On the flat surface were attached eight clear cylindrical arenas, 5.7 cm in diameter, which were made of Plexiglas and arranged in a 2 × 4 setup. Each arena was clear with horizontal bottoms and slightly angled sides to enable complete visibility to the camera fixed overhead. Opaque screens separated the arenas, isolating subjects from each other to eliminate shoaling behavior. Each arena contained 40 ml of system water (17 mm depth) that was replaced after each trial. The apparatus was video recorded from above. Below each arena was a centrally located 24-volt DC push solenoid that provided a sudden tap when activated by the EthoVision XT® software. At the beginning of each trial eight fish were individually placed in the testing arenas and the testing sequence was initiated. The testing sequence consisted of a 30-s acclimation period followed by 10 consecutive taps at 1 min intervals. Measurements extracted were total distance traveled (mm) per the 5-s period after (post) each tap. The log transform was used to normalize the distribution of the data. The choice of post-tap 5-s time segments was based on pilot tests conducted in our lab during method development [9] and were found to provide consistent and sensitive measures of startle activity.

2.2.3. Social shoaling test

Social attraction was assessed in a test adapted from the one originally developed by Gerlai and co-workers [13]. This specific test procedure was adapted in our laboratory [4,11,12], with modifications. We have found this test to be sensitive to the long-term effects of early developmental exposure to pesticides and flame retardants [4,14,15]. The tank is a 512 × 327 mm (L x W, outer measurements) rectangle. The sides and bottom are made of 12.7 mm thick transparent acrylic sheets. The bottom sheet is sandblasted to reduce glare, and divided into a 5 × 3 grid by a network of slots that are 6.35 mm wide and 10 mm deep. The grid slots continue up the walls of the tank, and along the walls on the inner bottom perimeter. Three 16 mm x 31.4 mm black partitions were inserted to create two adjacent lanes across the tank width. Two 49.5 cm LCD monitors flanked the narrow ends of the two lanes. A digital video camcorder was placed above the tank.

Adult fish were singly isolated in 1.5-L tanks surrounded by opaque

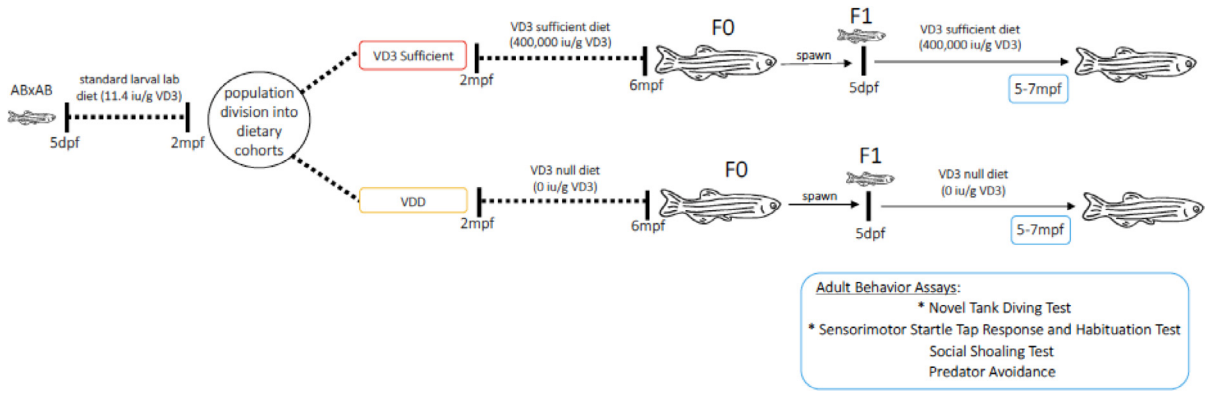

Fig. 1. Timeline of the study.

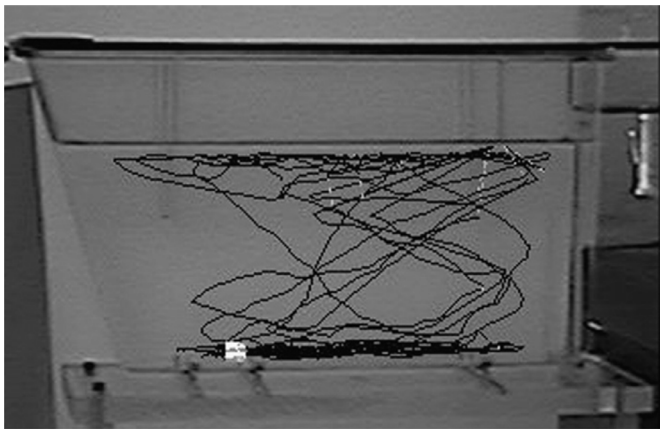

Fig. 2. Novel tank diving test [6,7].

dividers for 30 min before being netted into the tank lanes described above and recorded for 7 min. During the first 2 min of the test, each monitor screen displayed a background of static ovals approximately the size of an adult zebrafish colored with the pattern and of a typical zebrafish. At the end of the first two min one of the two monitors began to display a color video recording of a real zebrafish shoal for the remaining 5 min. The other monitor was plain white. Measurements extracted were total distance traveled in cm per min, and the mean distance in cm per min to the side of the tank on which the video of real zebrafish was displayed. A pre-post video difference value was calculated for each treatment group by subtracting the average distance from the tank side in the 2 min after the video started playing from the average distance in the 2 min before the video began, thus providing a measure of social shoaling behavior.

2.2.4. Predator avoidance test

Threat recognition and evasion behavior were tested using a testing apparatus and set-up as described in the previous section (Shoaling test). The test procedure was based on a protocol developed in our laboratory [10–12,15] with modifications. Individual fish were placed in the tank and recorded for 9 min consisting of 1 min acclimation followed by 8 min of alternating minute-long stimulus/no stimulus (NS) events. The stimulus was a PowerPoint™ presentation showing either a blue slow-growing dot (4 s) or a red fast-growing dot (1 s) appearing repeatedly on one of the screens. The blue dot appeared in the first two stimulus events and the red dot appeared in the last two stimulus events. Measurements extracted were total distance traveled in cm/min, and the mean distance in cm/min to the side of the tank on which the stimulus was displayed. A flee response value was calculated separately for the blue and red stimuli, and for each treatment group, as the difference in average distance from the tank side between trial minutes in which the dot stimulus was presented and minutes in which there was no stimulus.

2.3. Statistical analysis

All statistical analyses were performed in Superanova/Statview (SAS Institute, Cary, NC, USA). *Post hoc* comparisons were done using Dunnett's test, unless stated otherwise. Significance was set at $p < 0.05$ for all analysis of variance (ANOVA) and post hoc comparisons. Larval locomotor activity at 6 dpf was analyzed with two-way ANOVA, with treatment as the between-subjects factor, and either illumination phase as the within-subjects factor, or minute as the within-group repeated measure. Distance moved (cm per 1-min increment or 10-min illumination phase) was the dependent variable. Two-way ANOVA was used to analyze total distance traveled and mean distance from the tank floor in the novel tank diving test; total distance traveled pre- and post-tap in

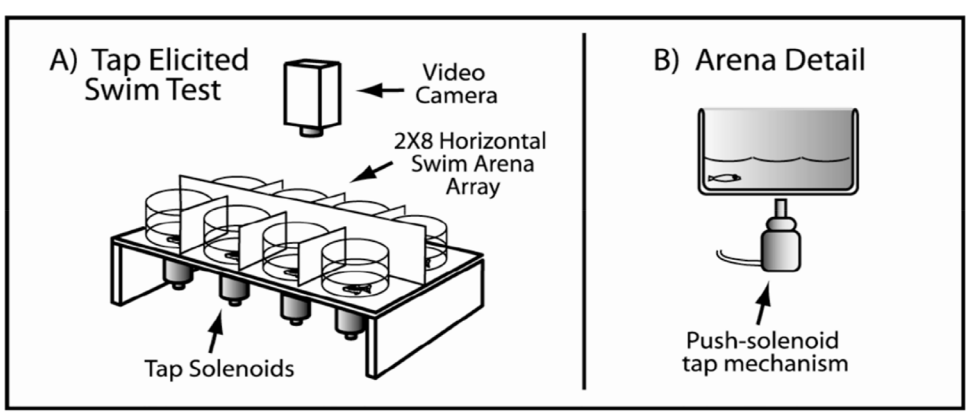

Fig. 3. Tap startle test [8,9].

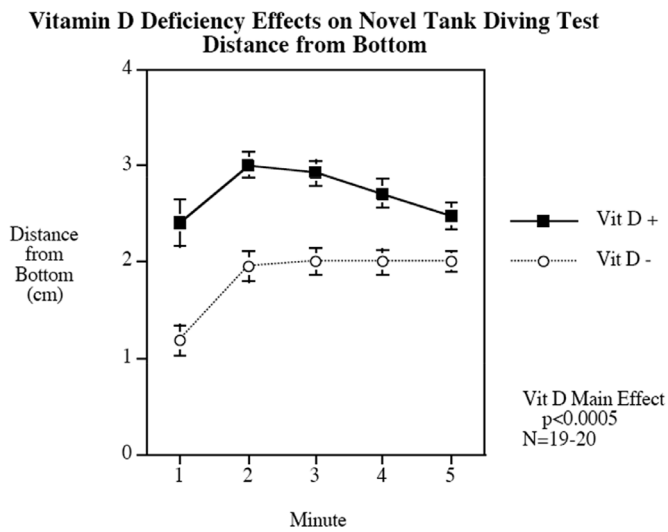

Fig. 4. Effects of early-life vitamin D deficiency on novel tank diving test in adults: mean distance (cm) from the bottom (mean ± sem). Zebrafish with vitamin D deficiency during development dwelled significantly ($p < 0.0005$) closer to the bottom of the tank than controls, indicative of a more anxious type of behavior.

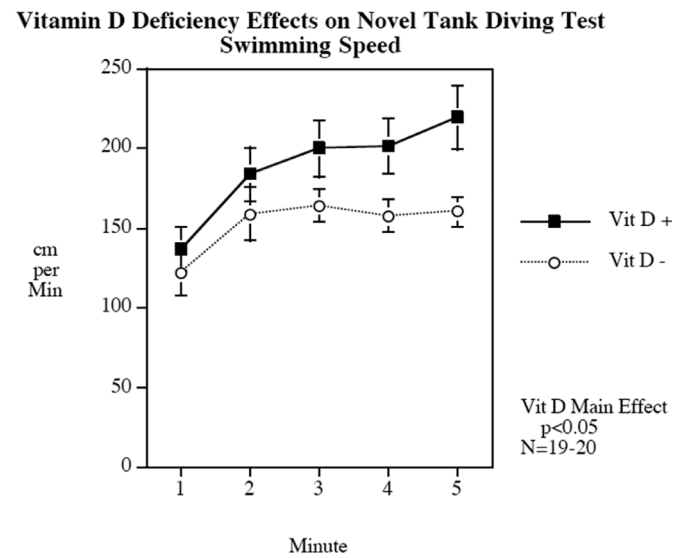

Fig. 5. Effects of early-life vitamin D deficiency on novel tank diving test in adults: swimming speed (mean ± sem). Zebrafish with vitamin D deficiency during development swam significantly ($p < 0.05$) more slowly than controls.

the tap test; and distance traveled and mean distance from tank side for the shoaling and predator avoidance test. Vitamin D deficiency treatment was the between subjects factor. Time (minute) or tap number was the repeated measure, and distance (cm per minute) as the dependent variable. Two-way ANOVA was also used to analyze shoaling test before and after video 2-min intervals. Again, vitamin D deficiency treatment was the between subjects factor, the 2-min intervals as the repeated measure, and average distance from tank side as the dependent variable.

3. Results

3.1. Novel tank diving test

The novel rank diving test demonstrated significant dietary effects between VDD and VD3 at 6 months in age (Fig. 4). Vitamin D deficient fish kept to a significantly ($F(1,37) = 37.29$), $p < 0.0005$) lower position in the tank. There was also a significant interaction of vitamin D status x minute ($F(4148) = 3.43$, $p < 0.025$). Vitamin D deficiency had a significant effect at each minute of the test with $p < 0.0005$ for each of the first three minutes, $p < 0.001$ for minute 4 and $p < 0.025$ for minute 5. Vitamin D-deficient fish dwelled closer to the bottom of the tank during the earlier part of the test compared to control fish. The vertical position of the fish in the tank showed a robust effect of vitamin D diet. As shown in Fig. 5, swimming activity (cm/min) was significantly decreased by vitamin D deficiency ($F(1,37) = 4.88$, $p < 0.05$) and there was also a significant main effect of minute within the session ($F(4148) = 12.38$, $p < 0.0005$) with activity increasing from the first minute to the later minutes of the session.

3.2. Sensory-Motor tap response and habituation test

Mean activity before the startling stimulus was not significantly affected by diet condition (Fig. 6). All fish did show a startle response after the taps. There was a significant main effect of trial ($F(9324) = 3.36$, $p < 0.001$) with a pronounced habituation in startle response over the series of ten taps. The main effect of developmental vitamin D deficiency was not significant but there was a significant interaction of vitamin D diet x trial ($F(9324) = 2.24$, $p < 0.025$). The vitamin D deficient group showed slower but greater habituation of startle (i.e. a wider range in startle responses), with an exaggerated

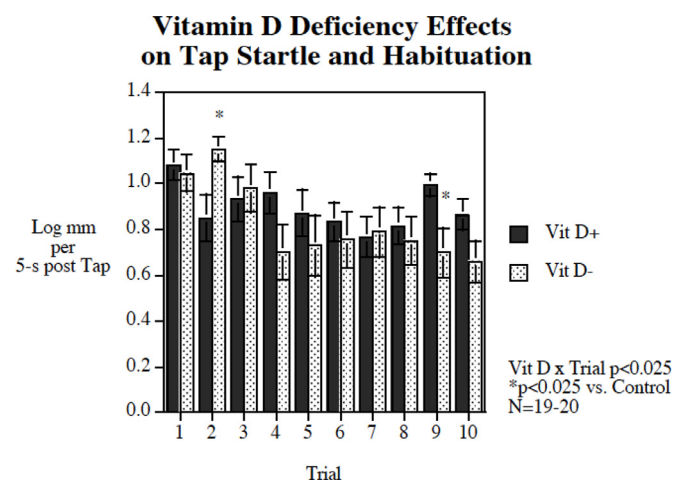

Fig. 6. Effects of early-life vitamin D deficiency on tap sensorimotor response and habituation. Activity five seconds after each tap for each of the ten taps in the session log mm/5-s post-tap (mean ± sem). There was a vitamin D deficiency x minute interaction with significant ($p < 0.025$) hyper-responsivity during an early trial (second) and hypo-responsivity on a later trial (ninth).

startle response following early taps and a muted startle response following later taps.

3.3. Shoaling test

There were no significant effects of developmental vitamin D diet on behavior of 6 month adult zebrafish in the shoaling test in terms of either the distance from the video of shoaling fish or the speed of swimming (cm/min) regardless of the average position in the tank. The shoaling test was validated by the internal positive control showing that it was performing as intended as indicated by the significant attraction (3.33 ± 1.51 cm) toward the shoal video relative to the baseline distance from the screen before the shoal video began ($F(1,36) = 5.01$, $p < 0.05$) with no significant interaction with vitamin D deficiency.

3.4. Predator avoidance test

There were no significant effects of developmental Vitamin D status

on behavior of adult zebrafish in the predator avoidance test stimulus distance from predator screen. There was an interaction of vitamin D diet x stimulus presence ($F(1,36) = 3.32$, $p < 0.08$) that prompted follow-up tests of the simple main effects of vitamin D diet on trials with the predator stimulus vs. those without the predator stimulus. When the predator stimulus was on, vitamin D-deficient fish remained slightly further away from the screen (25.3 ± 0.7 cm) compared to the control fish (23.7 ± 1.0 cm), but this was not significant. No significant Vitamin D effect was seen with swimming speed in this test, but the vitamin D deficient fish (231.0 ± 7.8 cm/min) were nearly significantly ($F(1,34) = 3.79$, $p = 0.06$) slower than those not deficient (300.5 ± 13.0 cm/min). The shoaling test was validated by the internal positive control showing that it was performing as intended as indicated by the significant main effect of the fish retreating away from the stimulus when present vs. not present ($F(1,34) = 273.82$, $p < 0.0005$) with the fish being 16.9 ± 0.6 cm from the screen when there was no predator stimulus and 24.4 ± 0.5 cm from the screen when the predator stimulus was shown.

4. Discussion

This study shows that vitamin D deficiency during early development causes specific behavioral impairment in zebrafish. This impairment manifested across multiple tests of behavioral function in adult zebrafish. This study shows that developmental vitamin D deficiency produced persisting neurobehavioral dysfunction in zebrafish.

In the novel tank diving test, the vertical position of the fish in the tank showed robust effect of vitamin D deficiency with fish raised on a vitamin D-deficient diet keeping to a significantly lower position in the tank, indicative of more anxiety-like behavior. Swimming activity was also significantly decreased by developmental vitamin D deficiency in this test. These two effects of vitamin D deficiency in the novel tank diving test seemed to be separate since there was a more pronounced bottom-dwelling behavior of vitamin D-deficient fish than vitamin D-replete fish early in the session compared to later with the reverse pattern over time being seen with vitamin D deficiency effects on swimming speed.

In the sensory-motor tap response and habituation test there was a significant interaction of vitamin D diet by trial. The vitamin D-deficient group showed an initially slower habituation to the startle, but showed a greater habituation by the end of the test session. With the predator avoidance test, there was a nearly significant effect of vitamin D diet with vitamin D-deficient animals showing a greater flee response.

These behavioral effects were selective. Developmental vitamin D deficiency caused specific behavioral effects with pronounced effects in the novel tank diving task and more subtle effects in the tap startle and habituation test, but no apparent effects in the other behavioral tests. This suggests that the behavioral effects seen were not just the results of non-specific structural or sensorimotor impairments that impeded overall responding. Rather, vitamin D deficiency during development resulted in a behavioral phenotype that differed most markedly from that of the controls when responding to a novel environment. The finding that no significant effects related to developmental vitamin D deficiency were detected in the larval light/dark motility test indicated that this early test lacked sensitivity to predict the deficits seen later in life.

Impaired muscle function does not seem to be the source of the effects seen. Each of the tests has a concurrent measure of overall locomotor activity in addition to the behavioral response to changing environmental conditions. Differential treatment effects on the locomotor activity and response to environment measures is evidence for the specificity of behavioral effect. In the Novel Tank Diving Test, vitamin D treatment had significant effects on both vertical position and speed of swimming but the timing of these effects is quite different. At the beginning of the test there is a large group difference in vertical position but none in swimming speed while at the end of the test there is a large group difference in swimming speed but a smaller difference in vertical position. In the tap startle test, the vitamin D deficiency effect is expressed in a differentially altered startle response across the session with increased response early in the session and decreased later. Vitamin D deficiency induced lowered swimming activity was not seen in the other tests of the battery.

Other investigators have also found that developmental vitamin D deficiency can lead to long-term neurobehavioral effects. Vitamin D plays a key role in normal neurophysiological development, and early developmental deficiencies have been linked to multiple neuropsychiatric disorders [1,16–18]. Epidemiological studies have found associations between vitamin D deficiency during development and autism [19] as well as neural tube defects [20,21].

Experimental studies in rodent models has shown that early life dietary vitamin D deficiency was increased anxiety-like behaviors in mice [22]. This agrees with the results of the present study, in which the adult zebrafish with a history of developmental vitamin D deficiency showed greater anxiety-like behavior in novel environments. This is similar to the finding in the current study of increased bottom-dwelling in the novel tank diving test. Developmental vitamin D deficiency in rats disrupted pre-pulse inhibition of the acoustic startle response [23]. This may be related to the finding in the current study that developmental vitamin D deficiency disrupts tap startle response and its habituation on zebrafish.

This study provides further support for the use of the zebrafish model to help determine the molecular mechanisms underlying neurobehavioral development, specifically with regards to vitamin D and vitamin D receptor (VDR)-dependent signaling. In addition, because of the low cost of the zebrafish model, it could be key in providing a functional screen for determining which environmental chemicals and drugs run the risk of disrupting neurobehavioral function with developmental exposure. This is advantageous particularly coupled with high-throughput *in vitro* screens such as those already utilized to identify compounds with the potential for interfering with VDR signaling [24].

The results of the current study demonstrate the behavioral impairments in adults that result from dietary vitamin D deficiency throughout development. While this deficiency was not severe enough to increase lethality or produce overt dysmorphogenesis, it did cause significant behavioral effects that emerged in adulthood. Pronounced effects were observed on the novel tank diving task alongside more subtle effects with startle habituation, together indicative of increased anxiety-like behavior. No significant effects were seen in tests of other behavioral domains. This indicates that developmental vitamin D deficiency results in relatively selective later-life neurobehavioral effects.

Declaration of Competing Interests

None.

Acknowledgments

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