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LIFE SCIENCE AND BIOMEDICINE
NOVEL-RESULT
SUPPLEMENTARY-RESULT

Knockdown of vitamin D receptor genes impairs touch-evoked escape behavior in zebrafish

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Abstract

Vitamin D is a steroid hormone well-known for its role in calcium homeostasis and bone health. Biological actions of vitamin D are mediated through the vitamin D receptor (VDR) present in various cells and tissues. Vitamin D has been implicated in multiple aspects of neuromuscular functions. This study aimed to investigate the role of VDR signaling during early stage of locomotor development utilizing a gene knockdown approach. Zebrafish larvae deficient in VDR showed severe motor impairment and no obvious response to touch. These results indicate that VDR signaling is indispensable for the correct neuromuscular development and touch-evoked escape swimming behavior in zebrafish.

Keywords: neuromuscular; touch-evoked response; vitamin D; vitamin D receptor (VDR)

1. Introduction

Vitamin D has been known to play an essential role in regulating bone metabolism and maintaining calcium and phosphate homeostasis. The active form of vitamin D exerts its biological effects mainly by binding to the vitamin D receptor (VDR). VDR is expressed in most of the cells (Bouillon et al., 2008), which explains why VDR signaling has been implicated in a variety of physiological processes, including neuromuscular function. Indeed, the VDR has been found in the sensory and motor areas of the nervous system and muscles in both humans and rodent models (Bischoff et al., 2001; Eyles et al., 2005; Girgis et al., 2014; Prüfer et al., 1999). In addition, many studies indicate that vitamin D is related to various neurological and neuromuscular disorders (Di Somma et al., 2017; Dodig et al., 2017). VDR null mice display motor deficits and muscular impairments (Burne et al., 2005; Kalueff et al., 2004). It has been suggested that Schwann cells and the neuromuscular junctions (NMJs) are a target of VDR signaling (Sakai et al., 2015).

Zebrafish have become an effective animal model for studying neuromuscular functions since muscle activity can be assessed easily in the early stages of development, the first few days after fertilization (Sztal et al., 2016). In zebrafish embryos, two paralogs for VDR genes (*vdra* and *vdrb*) have been identified (Kollitz et al., 2014; Lin et al., 2012). Although previous studies have demonstrated that VDR signaling in zebrafish regulates heart development (Han et al., 2019; Kwon, 2016), ocular angiogenesis (Merrigan & Kennedy, 2017), and hematopoiesis (Cortes et al., 2016), its function in neuromuscular development is largely unknown.

2. Objective

The objective of the present study was to investigate whether loss of VDR affects neuromuscular activity in zebrafish embryo and larvae using touch-evoked response behavior analysis.

3. Methods

3.1. Animal care and use

The author asserts that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals. Zebrafish (*Danio rerio*) were reared and maintained at 28.5°C in accordance with the Animal Care and Use Committee at Texas A&M University. Larvae were staged as hours postfertilization (hpf) or days postfertilization (dpf) according to the established guidelines (Kimmel et al., 1995).

3.2. The knockdown of VDR genes

The VDR gene knockdown experiment was carried out using antisense morpholino oligonucleotides (MOs) as described previously (Kwon, 2016; Lin et al., 2012). To knockdown *vdra*, a translation blocker (5′-AAC GGC ACT ATT TTC CGT AAG CAT C-3′) was used. To knockdown *vdrb*, a splice blocker (5′-TCC ATC ACT AGC AGA CGA GGG AAG A-3′) targeting the intron2-exon3 (I2E3) junction was used. Zebrafish embryos were co-injected with 5 ng of *vdra* MOs and 5 ng of *vdrb* MOs at the one-cell stage. All MOs used here were obtained from Gene Tools, LLC (Philomath, OR). The experiments were conducted at least three times.

3.3. Touch-evoked response behavior analysis

Touch-evoked zebrafish movements were recorded using a CCD camera mounted on a dissecting microscope at 40 hpf and 6 dpf. For touch responses at 40 hpf, tactile stimuli were generated by the manual dechorionation using fine forceps. For touch responses at 6 dpf, tactile stimuli were elicited by touching the tail region of the larvae with forceps. Images were captured, converted into a video file, and analyzed individual frames of time-lapse. To assess behavioral phenotypes, at least 10 embryos were examined in each group. The phenotypes described in this study were completely penetrant.

4. Results

To investigate the effects of the loss of VDRs on motor development, antisense MOs against *vdra* and *vdrb* (*vdra/b* MO) were co-injected into the one-cell embryo. During the hatching period, over 94% of the wild-type control embryos hatched by 3 dpf and 100% of them hatched on the next day ($n = 17$). It was noticeable that *vdra/b* MO-injection resulted in a reduction of hatching rate, 70% at 3 dpf. At 5 dpf, the hatching rate of *vdra/b* MO-injected larvae was still less than 81% ($n = 31$). At 40 hpf, 100% of control embryos showed rapid escape swimming behaviors in response to tactile stimuli generated in the manual dechorionation (Figure 1a–e; Supplementary Video S1). In contrast, 100% *vdra/b* morphants exhibited either a very weak or no muscle contraction and all of them failed to escape in response to touch during or after the dechorionation (Figure 1f–j; Supplementary Video S2).

To determine whether the impairment of touch-evoked escape in *vdra/b* morphants was due to the delayed onset of touch-responsiveness, the tactile response of zebrafish larvae at 6 dpf was examined. Upon touch at the tail, 100% of *vdra/b*-depleted larvae at 6 dpf displayed no obvious muscle contraction and none of them showed the escape response observed in 100% of control larvae (Figure 2; Supplementary Videos S3 and S4). *vdra/b* knockdown caused significantly reduced locomotor activity and perturbed development of the swim bladder. All of the *vdra/b* MO-injected larvae at 6 dpf had severe defects in free-swimming.

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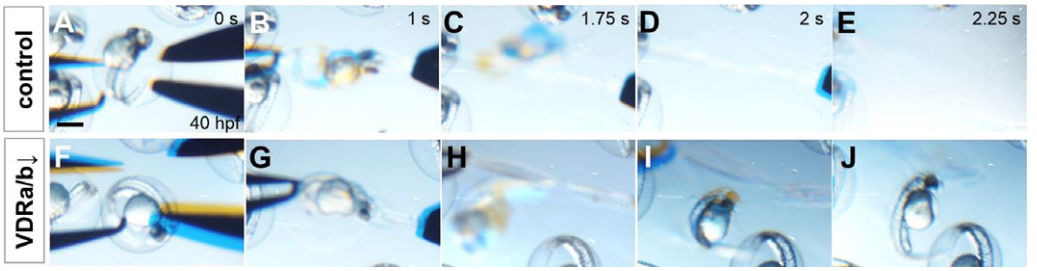

Figure 1. Knockdown of VDRs impairs touch-evoked behaviors. Video frames showing touch-evoked escape response at 40 hpf of wild-type control (a–e) but not of *vdra/b* MO-injected (f–j) embryos. The time of the frame is shown in the top right portion. The mechanosensory stimulation during the manual dechorionation causes control embryos to escape rapidly and exit the field of view (d and e). In contrast, *vdra/b* MO-injected embryos do not exhibit any escape response and fail to exit the field of view at the same time frames (i and j). Scale bar = 500 μm.

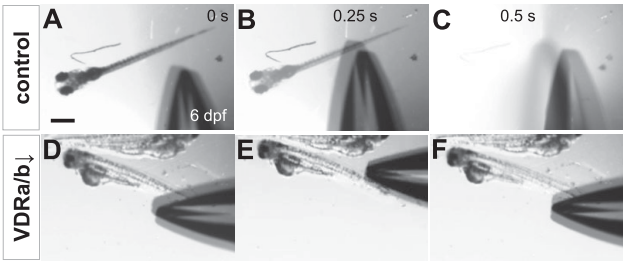

Figure 2. Loss-of-VDRs results in loss of touch-evoked escape swimming behaviors. Video frames showing touch-evoked response at 6 dpf of wild-type control (a–c) but not of *vdra/b* MO-injected (d–f) larvae. While the stimulation by forceps causes the wild-type larva to swim rapidly away and fully exit the field of view (c), *vdra/b* MO-injected larvae exhibit no touch-evoked response and remain in the same field of view (f). Scale bar = 500 μm.

5. Discussion

In the current study, VDR signaling was found to be involved in neuromuscular development and touch-evoked escape swimming behavior. In zebrafish embryos, spontaneous muscle contractions start to be noted around 17 hpf (Saint-Amant & Drapeau, 1998). Even before hatching, the zebrafish embryos acquire the ability to respond to touch by 24–27 hpf (Carmean & Ribera, 2010). The twitch contraction reaches the peak frequency, which is a driving force of releasing embryos from the chorion during the hatching period around 3 dpf. Delay in hatching found in *vdra/b* morphants could be due to the impaired muscle activity (Skobo et al., 2014).

Together with the muscular and motor impairments observed in VDR knockout mice (Burne et al., 2005; Kalueff et al., 2004), these findings indicate that VDRs play an essential role in neuromuscular activity and locomotor behavior. The specific mechanism remains to be elucidated, but it may be explained by the effects of vitamin D on calcium homeostasis or maintenance of peripheral nerve axons and acetylcholine receptor clusters in NMJs (Lin et al., 2012; Sakai et al., 2015).

6. Conclusion

This study demonstrates that knockdown of VDRs causes impairment of muscle performance and elimination of touch-evoked escape swimming response in zebrafish embryos and larvae. Thus, the VDR signaling is required for the correct neuromuscular activity during early development. These results suggest the role of VDR signaling in locomotor behavior appears to be well-conserved between mammals and fish.

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Conflict of Interest. The author declares no conflicts of interest.

Data Availability Statement. The data that support the findings of this study are available as Supplementary Material.

Supplementary Materials. To view supplementary material for this article, please visit http://dx.doi.org/10.1017/exp.2021.22.

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Peer Reviews

Reviewing editor: Dr. Mariana Bexiga
University of Coimbra Center for Neuroscience and Cell Biology, Coimbra, Portugal, 3004-504

This article has been accepted because it is deemed to be scientifically sound, has the correct controls, has appropriate methodology and is statistically valid, and has been sent for additional statistical evaluation and met required revisions.

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Review 1: Knockdown of vitamin D receptor (VDR) genes impairs touch-evoked escape behavior in zebrafish

Reviewer: Dr. Ayca Ergul
Hacettepe Universitesi, Ankara, Turkey, 06532

Date of review: 14 September 2021

Conflict of interest statement. Reviewer declares none

Comments to the Author: This is a concise and interesting work. I watched the videos and they clearly shows the difference in the tactile response. The difference is dramatic. I think this would be a beneficial finding for zebrafish researchers.

Score Card

Presentation

4.0 /5

Is the article written in clear and proper English? (30%)	4/5
Is the data presented in the most useful manner? (40%)	4/5
Does the paper cite relevant and related articles appropriately? (30%)	4/5

Context

4.0 /5

Does the title suitably represent the article? (25%)	4/5
Does the abstract correctly embody the content of the article? (25%)	4/5
Does the introduction give appropriate context? (25%)	4/5
Is the objective of the experiment clearly defined? (25%)	4/5

Analysis

4.0 /5

Does the discussion adequately interpret the results presented? (40%)	4/5
Is the conclusion consistent with the results and discussion? (40%)	4/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)	4/5

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Review 2: Knockdown of vitamin D receptor (VDR) genes impairs touch-evoked escape behavior in zebrafish

Reviewer: Dr. Ana Valentim PhD

Universidade do Porto Instituto de Investigação e Inovação em Saúde, Porto, Portugal, 4099-002

Date of review: 15 September 2021

Conflict of interest statement. Reviewer declares none.

Comments to the Author: The following study investigates what the absence of vitamin D can induce to the reaction to a touch stimulus in zebrafish embryos and larvae, using morphants. It is a simple well-written study that relates the lack of vitamin D and locomotor impairment, which have been described in mammals. Thus, in the conclusion, the author should highlight the contribution of the experiment, that this mechanism involving vitamin D is conserved. In addition, the authors need to clarify some methodological parts: how many embryos and larvae were used in each group (add to methodology); make it clear that the touch response of 40hpf is the manually dechorionation of embryos, as the way it is, it seems that the touch is going to be performed afterward; clarify what was the part of the larvae body that received the touch, as this needs to be standardized. At the results, the authors need to provide the percentage of animals that respond to the stimulus in each group, and the ones which responded weakly in both embryos and larvae experiments. The videos are a good addition to the paper, and I wonder if the 6 dpf morphants swim at all, so they can eat and survive, do you have that information?

Some sentences on the results should have been put in the discussion, such as page 3 line 1-3, and line 8-10 (starting in "which" until Carmean et al 2010), so the authors can consider re-writing the discussion to include these literature ideas.

Score Card

Presentation

4.6 /5

Is the article written in clear and proper English? (30%)	5/5
Is the data presented in the most useful manner? (40%)	4/5
Does the paper cite relevant and related articles appropriately? (30%)	5/5

Context

4.8 /5

Does the title suitably represent the article? (25%)	5/5
Does the abstract correctly embody the content of the article? (25%)	4/5
Does the introduction give appropriate context? (25%)	5/5
Is the objective of the experiment clearly defined? (25%)	5/5

Analysis

Does the discussion adequately interpret the results presented? (40%)	4/5
Is the conclusion consistent with the results and discussion? (40%)	5/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)	3/5

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