General and Comparative Endocrinology xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Research paper

Germ cell depletion in zebrafish leads to incomplete masculinization of the brain

Ajay Pradhan, Per-Erik Olsson *

Biology, The Life Science Center, School of Science and Technology, Örebro University, SE-701 82 Örebro, Sweden

ARTICLE INFO

Article history:
Received 30 September 2017
Revised 2 February 2018
Accepted 2 February 2018
Available online xxxx

Keywords:
Reproduction
Sex differentiation
Steroid hormone
Dimorphism
Gonads

ABSTRACT

Zebrafish sex differentiation is under the control of multiple genes, but also relies on germ cell number for gonadal development. Morpholino and chemical mediated germ cell depletion leads to sterile male development in zebrafish. In this study we produced sterile males, using a *dead end* gene morpholino, to determine gonadal-brain interactions. Germ cell depletion following *dnd* inhibition downregulated the germ cell markers, *vasa* and *ziwi,* and later the larvae developed as sterile males. Despite lacking proper testis, the gonadal 11-ketotestosterone (11-KT) and estradiol (E2) levels of sterile males were similar to wild type males. Qualitative analysis of sexual behavior of sterile males demonstrated that they behaved like wild type males. Furthermore, we observed that brain 11-KT and E2 levels in sterile males remained the same as in the wild type males. In female brain, 11-KT was lower in comparison to wild type males and sterile males, while E2 was higher when compared to wild type males. qRT-PCR analysis revealed that the liver transcript profile of sterile adult males was similar to wild type males while the brain transcript profile was similar to wild type females. The results demonstrate that proper testis development may not be a prerequisite for male brain development in zebrafish but that it may be needed to fully masculinize the brain.

1. Introduction

In most organisms, sex is determined by sex chromosomes. In mammals, the molecular cascade initiated by the SRY gene, located on the Y chromosome, is the sex determining factor initiating testis differentiation (Koopman, 2005). Primordial germ cells (PGCs) also plays a crucial role in proper testis and ovary development (Kocer et al., 2009). Germ cells are the progenitors of spermatozoa and oocytes, and despite the sex chromosomal constitution (XX or XY), the gonadal environment in which they develop determine their sexual fate (Bowles and Koopman, 2010; Kocer et al., 2009). In mammals signals from somatic cells control the PGCs fate (Ross and Capel, 2005), but it is not clear if signals from the germ cells affect the soma (Rios-Rojas et al., 2015). In other organisms including medaka (Kurokawa et al., 2007), three-spined stickleback (Petersen et al., 2016) and zebrafish (Siegfried and Nusslein-Volhard, 2008; Slanchev et al., 2005; Tzung et al., 2015; Wong and Zohar, 2015), the germ cell number influence sex differentiation. In other species, such as the red-eared slider turtle (Dinapoli and Capel, 2007), goldfish (Goto et al., 2012) and loach (Fujimoto et al., 2010) germ cells do not appear to influence sex differentiation. Thus, the role of germ cells in sexual differentiation varies among species.

Zebrafish gonadal sex differentiation is under the control of multiple genes (Liew et al., 2012; Pradhan et al., 2012; Pradhan and Olsson, 2014, 2016; Rodriguez-Mari et al., 2010), but also relies on germ cell numbers. Knockdown of *dead end* (*dnd*), which is necessary for germ cell migration and survival, leads to the development of sterile male zebrafish (Siegfried and Nusslein-Volhard, 2008; Slanchev et al., 2005; Tzung et al., 2015; Wong and Zohar, 2015). Germ cells are also important in maintaining the sexual phenotype in adult zebrafish, as ablation of germ cells leads to sex reversal of adult females (Dranow et al., 2013). Adding to the complexity a study by Tzung et al. (2015) suggests that *dnd* injected fish show delayed gonad development compared to wild type males and that testis development in sterile males is disrupted, leading to underdeveloped testis with no signs of spermatozoa. However, developmental expression of the male specific genes *SRY-box 9a* (*sox9a*) and *anti-mullerian hormone* (*amh*) is not affected by germ cell depletion (Siegfried and Nusslein-Volhard, 2008). As the expression of early sex differentiation markers are not inhibited, this indicates that somatic cell and germ cell interactions are not a prerequisite for the onset of testis differentiation

* Corresponding author.
E-mail address: per-erik.olsson@oru.se (P.-E. Olsson).

https://doi.org/10.1016/j.ygcen.2018.02.001
0016-6480/

but is crucial for initiating the female developmental pathway. Furthermore, a study by Slanchev et al. (2005) argues that the germ cells in zebrafish are not important for gonad formation but, rather, essential for differentiation and survival of the testis.

In mammals, gonadal steroid hormones are involved in activation and organization of brain sexual differentiation (Phoenix et al., 1959), but the molecular mechanisms are not fully understood. Zebrafish has emerged as a valuable model system that could be used to understand brain sexual differentiation. However, in zebrafish, long term exposure to exogenous steroid hormones induce sex change (Orban et al., 2009; Schulz et al., 2007), while short term exposure induce behavioral changes in adult fish (Pradhan and Olsson, 2015). This suggests that the use of exogenous steroid hormones is not an ideal choice to unravel the interactions between the gonads and brain in regulating zebrafish brain sexual differentiation. Here we have used a *dnd* morpholino to generate sterile males and studied the role of gonads in shaping zebrafish brain sexual differentiation. The results suggest that zebrafish brain may be female by default and that proper testis development is necessary to fully masculinize the brain gene expression.

2. Materials and methods

2.1. Zebrafish husbandry

In the present study vas::EGFP transgenic zebrafish, expressing green fluorescence protein (GFP) under the control of the *vasa* promoter (Krovel and Olsen, 2002) and wild type zebrafish originally obtained from a local pet store in Sweden was used. Zebrafish were maintained as described previously (Pradhan et al., 2012). In brief, the zebrafish were kept in a recirculating system (Aquaneering) with a 14 h light/10 h dark cycle. The temperature was maintained at 25 ± 1 °C and the fish were fed twice with *Artemia salina* (Ocean Nutrition) and commercial flake food (Tetra). Other water quality parameters (nitrite, <0.3 mg/L; nitrate, 4 ± 1 mg/L; pH, 7.2 ± 0.2 and salinity, 0.04 ± 0.02) were also recorded. Five month old adult zebrafish with an average length of 3.86 ± 0.2 cm for males and 3.84 ± 0.2 cm for females and an average body weight of 0.48 ± 0.03 g for males and 0.55 ± 0.05 g for females were used for the experiments. Analysis was performed in parallel, hence the same animal was not used for behavior, gene expression or other end points. The use of experimental animals was approved by the Swedish Ethical Committee in Linköping (Permit 32-10).

2.2. Morpholino injection

The *dnd* (5′-GCTGGGCATCCATGTCTCCGACCAT-3′) and standard control (5′-CCTCTTACCTCAGTTACAATTTATA-3′) morpholinos (MO) were purchased (Gene Tools) and dissolved in water. Eggs at the one cell stage were collected from 3 to 4 couples and microinjected with morpholinos mixed with phenol red (Sigma) using a microinjector system (Narishige). To analyze the morpholino efficacy, Vas::EGFP was injected with the *dnd* morpholino and the GFP signal was visualized under Fluoview FV1000 scanning confocal laser microscope (Olympus). The efficacy was also analyzed using qRT-PCR in wild type zebrafish.

2.3. qRT-PCR analysis

Isolated samples were lysed in Trizol reagent (Sigma) and RNA extraction was performed using Direct-zol RNA miniprep kit (Zymogen). The DNase treatment was performed according to the kit instructions. The RNA quantity and quality was measured in a DS-11 spectrophotometer (DeNovix). RNA quality was further assessed using gel electrophoresis. cDNA was prepared from 1 μg RNA using the qScript cDNA synthesis kit (Quanta Biosciences) and qRT-PCR was performed using SYBR Green (KapaBiosystems). qRT-PCR was performed using CFX96 (BioRad) thermocycler and the thermocycling conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 2 s and 60 °C for 30 s. No reverse transcriptase control was also used along with the samples in qPCR analysis. Melting curves were analyzed to determine primer specificity and DNA contamination. For reference gene selection, 18S, *elongation factor* (*eef1a1*), *beta actin, tubulin* and *gapdh* was run for all the samples and analyzed using geNorm software to select the most stable reference gene. *eef1a1* and 18S were the most stable genes for brain samples and used as the normalizing control while *eef1a1* was the most stable gene for liver samples. Data analysis was performed using the ΔΔCt method (Schmittgen and Livak, 2008). Primer sequences were obtained from previous studies (Levi et al., 2012; Pradhan et al., 2013; Pradhan and Olsson, 2014). For liver qRT PCR analysis the sample size was 10 and for brain analysis 12 samples were used.

2.4. Histology

Adult zebrafish were euthanized using MS222 (Sigma) and testis samples from 4 wild type males and 4 sterile males were isolated. The testis samples were embedded in cryomold (Tissue Tek) and frozen in tissue freezing medium (Tissue Tek). Sectioning (10 μm) was performed using CM3050 cryostat (Leica Biosystems). The sections were transferred to superfrost ultra plus microscope slides (Thermo Scientific) and fixed in 4% paraformaldehyde. The sections were washed and stained using Hematoxylin and Eosin (Sigma). The sections were washed and dehydrated in an oven at 60 °C for 10 min and mounted using mounting media. The slides were observed under a BX51 microscope (Olympus) and images were captured using Color View camera (Olympus).

2.5. Hormone level

Adult zebrafish were euthanized and brain and gonadal tissue samples were isolated and snap frozen in liquid nitrogen. The samples were homogenized in ELISA dilution buffer and kept on ice for 15 min with intermittent vortexing for proper lysis. The samples were centrifuged for 15 min at 13000 rpm and at 4 °C. The supernatants were transferred to fresh tubes and the level of 11-ketotestosterone (11-KT) and estradiol (E2) were measured using an ELISA kit (Cayman) according to the manufacturer's instructions. The absorbance was measured using spectrophotometry using the Infinite F50 (Tecan). For 11-KT the assay detection range was 0.78–100 pg/ml while for E2 it was 6.6–4000 pg/ml. Four and seven independent samples were used for the detection of steroid hormone levels in the gonads and brain respectively.

2.6. Behavior analysis

Qualitative analysis of sexual behavior was performed as previously described (Pradhan and Olsson, 2015). In brief, 8 animals each from the wild type male, wild type female and sterile male groups were selected for sexual behavior analysis. One wild type male and one wild type female were placed in a spawning chamber during the evening. The following morning the behavior was analyzed and the released eggs were counted and checked for fertilization. Sexual behavior analysis (male chasing the female, female being chased, nipping, luring to a spawning site and egg release by the females) was observed visually for 20–30 min. For analysis of sterile male behavior, the same wild type females were grouped with the sterile males. Behavior analysis was performed 4 times for

Please cite this article in press as: Pradhan, A., Olsson, P.-E. Germ cell depletion in zebrafish leads to incomplete masculinization of the brain. Gen. Comp. Endocrinol. (2018), https://doi.org/10.1016/j.ygcen.2018.02.001