Behavioural Brain Research 311 (2016) 219–227

Contents lists available at ScienceDirect

Behavioural Brain Research

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

Research report

Microbial modulation of behavior and stress responses in zebrafish larvae

Daniel J. Davis[a], Elizabeth C. Bryda[a], Catherine H. Gillespie[a], Aaron C. Ericsson[a,b,*]

[a] Department of Veterinary Pathobiology, University of Missouri, Columbia, MO 65201, USA
[b] University of Missouri Metagenomics Center (MUMC), University of Missouri, Columbia, MO65201, USA

HIGHLIGHTS

- Microbiota modulates anxiety-related behavior in zebrafish larvae.
- Larval stress responses are dramatically blunted in absence of microbiota.
- *L. plantarum* attenuates anxiety-related behavior in conventionally-raised larvae.
- Zebrafish larvae are a valuable tool for research on the gut-brain axis.

ARTICLE INFO

Article history:
Received 10 March 2016
Received in revised form 18 May 2016
Accepted 19 May 2016
Available online 20 May 2016

Keywords:
Zebrafish
Microbiota
Microbiome
Germ-free
Gnotobiotic
Anxiety
Stress
Behavior
Gut-brain axis

ABSTRACT

The influence of the microbiota on behavior and stress responses is poorly understood. Zebrafish larvae have unique characteristics that are advantageous for neuroimmune research, however, they are currently underutilized for such studies. Here, we used germ-free zebrafish to determine the effects of the microbiota on behavior and stress testing. The absence of a microbiota dramatically altered locomotor and anxiety-related behavior. Additionally, characteristic responses to an acute stressor were also obliterated in larvae lacking exposure to microbes. Lastly, treatment with the probiotic *Lactobacillus plantarum* was sufficient to attenuate anxiety-related behavior in conventionally-raised zebrafish larvae. These results underscore the importance of the microbiota in communicating to the CNS via the microbiome-gut-brain axis and set a foundation for using zebrafish larvae for neuroimmune research.

1. Introduction

Zebrafish (*Danio rerio*) larvae are an emerging high-throughput model system for neurobehavioral studies. After hatching from their chorion between 2 and 3 days post fertilization (dpf), the larvae inflate their swim bladder and exhibit numerous neurobehavioral phenotypes by day 4–5 dpf [1]. Many of these larval behaviors have been correlated with those seen in human neurological processes and disorders, such as anxiety [2], learning [3], fear [4], sociability[5], and psychosis[6]. One of the best characterized behaviors in zebrafish larvae is anxiety-related behavior, often measured by thigmotaxis or the tendency to remain close to vertical surfaces (i.e., "wall-hugging"). This behavior is evolutionarily conserved and exhibited by a wide range of species, including rodents [7], fish [8–11], and humans [12]. Thigmotactic behavior is a well-validated index of anxiety in zebrafish larvae since anxiolytic and anxiogenic drugs significantly attenuate and enhance this behavior, respectively [2,13–17]. Zebrafish larvae also exhibit physiological responses to stress similar to those seen in mammals, wherein responses to stress rely heavily on the hypothalamic-pituitary-adrenocortical (HPA) axis and its synthesis of glucocorticoids. Reports have demonstrated functional and anatomical parallels between the zebrafish hypothalamic-pituitary-interrenal (HPI) axis and the mammalian HPA axis [18–20]. Cortisol is the primary corticosteroid in both zebrafish and humans, unlike rodents wherein corticosterone is the main corticosteroid. During a stress response, the HPI axis is activated and there is an elevation of

* Corresponding author at: University of Missouri, Department of Veterinary Pathobiology, 4011 Discovery Drive, S123B Columbia 65201, USA.
E-mail address: ericssona@missouri.edu (A.C. Ericsson).

http://dx.doi.org/10.1016/j.bbr.2016.05.040

plasma cortisol which is mediated via activation of melanocortin type 2 receptor (*mc2r*) by ACTH binding [21]. Steroidogenic acute regulatory protein (StAR), and 11β-hydroxylase, both involved in the final steps of cortisol synthesis, are also increased in response to acute stressors [21,22].

Factors that play a role in the development of the corticoid system and stress responses during early life of vertebrates are not well understood. Recently, there have been many studies suggesting bidirectional influences between the microbiota and neurological development and stress-related behavior [23–26]. For example, social stress can alter the structure of the intestinal microbiota [27] while alterations in the microbiota can affect models of neurological disorders [28] and behavior [29]. Clarke et al. have also shown that the early life microbiota plays a critical role in affecting CNS signaling [30]. Similarly, long-lasting differences in the microbiota have been identified in rodents exposed to early life stress [31]. Furthermore, anxiety-related behavior and symptoms of stress have been shown to decrease in both mammals and zebrafish following the ingestion of particular probiotic *Lactobacillus* strains [24,32]. For example, *Lactobacillus plantarum* has been shown to reduce anxiety-related behavior and reduce stress-associated inflammatory cytokine levels in mice [33]. Interestingly, this particular strain has been shown to be highly adherent to gut epithelium during colonization of zebrafish [34,35].

The microbiota signals to the central nervous system (CNS) via several potential pathways. Likely mechanisms of communication include production of various metabolites that pass through the intestinal barrier into the circulatory system, and/or metabolites produced by microbes that can signal through the immune system [36]. Moreover, afferent pathways of the vagus nerve from the enteric nervous system (ENS) to the CNS have been implicated as a major route of communication between the microbiota and CNS [24]. Within the ENS, enteric glial cells outnumber enteric neurons by 4:1, and they are thought to play crucial roles in maintaining the intestinal epithelial barrier and regulating immune responses in the mucosa. Kabouridis et al. showed that the postnatal arrangement and ongoing supply of glial cells in the intestinal mucosa are regulated by the microbiota in mice, further indicating that this is a key pathway in the microbiota-gut-brain axis [37]. The development of intestinal innervation follows characteristic steps in zebrafish as in other vertebrates. Neural crest cells give rise to the bilateral vagal pathway through various signals, including hormonal cues, neurotrophins, and direct interactions with other cells. Development of the vagal extrinsic innervation of the gut occurs well before the onset of feeding which is comparable to other vertebrate species [38]. Despite architectural differences between the ENS of zebrafish and mammals, most of the molecular mechanisms underlying ENS development and function are conserved among species [39]. These functional similarities have allowed human ENS disorders (such as Hirschsprung's disease, Goldberg-Shprintzen Syndrome, and Bardet-Biedl syndrome) to be modeled in zebrafish as they have been modeled in rodents [40,41].

Zebrafish larvae have unique characteristics that could help elucidate mechanisms involved in the microbiota-gut-brain axis. The optical transparency of zebrafish allows for in vivo visualization of labeled bacteria interacting with host cells [42]. Furthermore, the *ex utero* development of zebrafish allows for easy manipulation of microbial contact and investigation of microbial involvement throughout development. Lastly, the neurobehavioral similarities with mammals and the ability to readily produce and control gnotobiotic zebrafish larvae make them a valuable model for neuroimmune studies.

Here, to investigate the ability of the microbiota to influence anxiety-related behavior and stress responses in zebrafish larvae, thigmotaxis (a measure of anxiety-related behavior in zebrafish larvae) was assessed in larvae raised in germ-free or conventional environments, and stress responses were evaluated via cortisol production in reaction to an osmotic stress challenge. Zebrafish larvae were examined at 6 dpf, an age at which high-throughput behavioral screening can be accomplished using multi-well plates. It has been shown that enteric innervation is well-developed before the onset of feeding (5–6 dpf) [38], allowing for gnotobiotic studies to be easily controlled without the complications of maintaining sterility during feeding. Additionally, effects of probiotics were examined in zebrafish larvae in conjunction with anxiety-related behavior and stress responses. The current data demonstrate that the microbiota modulates locomotor behavior and thigmotactic behavior in larvae, and that the stress response of zebrafish larvae relies heavily on the presence of the microbiota. Moreover, supplementation with the probiotic *Lactobacillus plantarum* was sufficient to mitigate thigmotactic behavior. These results underscore the importance of microbes in gut-brain signaling to modulate behavior and appropriate responses to stress, and provide a foundation for the use of zebrafish larvae in neuroimmune studies.

2. Methods

2.1. Animals

Wild-type zebrafish purchased from Aquatica BioTech (Sun City Center, FL) were used for this study. Multiple breeders were placed into a breeding tank overnight to spawn. Eggs were collected immediately after fertilization and evenly divided into separate groups for subsequent treatment. Due to the high number of zebrafish larvae that can be collected at one time, sufficient numbers of embryos were collected for all treatment groups for any given experiment on the same day. This is ideal for eliminating variation between clutches and for eliminating variation in the microbial content of the fish water used for non-sterile larvae groups. Germ-free (GF) embryos were generated by following a previously published method [43]. Briefly, embryos were collected in sterile fish water containing 250 mg/mL amphotericin B, 5 μg/mL kanamycin, and 100 μg/mL ampicillin (AB-fish water). After sorting to remove unfertilized embryos, viable embryos were transferred to a tissue culture hood and gently washed 3 times in AB-fish water. Embryos were immersed in 0.1% PVP-Iodine solution for 2 min, and then immediately washed 3 times with sterile fish water. After washing, the embryos were immersed in 0.003% bleach solution for 1 h before being washed an additional 3 times with sterile fish water. Finally, the embryos were transferred into sterile tissue culture flasks and maintained in a 28.5 °C incubator. Conventionalized (CV) embryos followed the same sterilization process as GF embryos with the exception of being housed in conventional fish water rather than sterile fish water. Conventionally-raised (CR) embryos were collected and maintained in conventional fish water without undergoing the sterilization process. Zebrafish larvae were raised in their respected environment at a density of ~1 larvae/mL until 6 days post fertilization (dpf), when all tests were performed. Sterility monitoring was conducted according to previously reported procedures for generating gnotobiotic zebrafish larvae [43]. *Lactobacillus* administration was done following a previously published protocol [44]. Briefly, probiotic bacteria were grown for 24 h in MRS media at 37 °C, centrifuged at 4000*g* for 5 min, and then washed with sterile fish water. At 4 dpf, zebrafish larvae were exposed to 2×10^7 CFU/mL of *Lactobacillus plantarum* (USDA-ARS, Washington DC) by injecting the bacteria directly into the water of the larvae housing flask. Zebrafish larvae were allowed to be exposed to *L. plantarum* for 2 days (until testing at 6 dpf).

2.2. Microbial DNA extraction and quantification

Microbial DNA was extracted according to an adapted previously published protocol [45]. Immediately following euthanasia,