Characterization of Hypocretin Expression in Zebrafish

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Abstract

Objectives: Hypocretin modulates the circadian sleep/wake cycle, feeding behavior, and how organisms respond to stress. While hypocretin (hcrt) -containing neurons are found in the lateral hypothalamic (LH) area of the central nervous system, debate remains as to whether bona fide hypocretin producing neurons can be found in the enteric nervous system. In this study our goal was to characterize peripheral hypocretin expression focusing on the zebrafish gastrointestinal tract. **Methods**: We used fluorescent microscopy to image transgenic *hcrt:egfp+* zebrafish at different time points and also performed wholemount immunostaining (anti-*gfp* retrieval of endogenous gfp expression and co-labelled with a pan-neuronal antibody) and high resolution confocal imaging of *ex vivo* adult gut. **Results**: Image analysis revealed abundant *gfp+* hcrt expressing cells in the gut of adult zebrafish in the myenteric plexus layer, consistent with enteric nervous system expression of hcrt. The *gfp+* cells also exhibit maximal-neighbor patterning and more densely populate the proximal gut. **Conclusions**: To date, research on the existence of hypocretin-producing neurons in the enteric nervous system has been limited. Decades-old findings suggest a gut *hcrt+* population may be present, but these studies have largely relied on antibody retrieval at a time when rigorous antibody validation was less commonplace than today. Our data, by relying on endogenous expression of *gfp* under the *hcrt* promoter, provides evidence for a bona fide *hcrt+* population in the gut. In future studies, we plan to interrogate *hcrt+* innervation in the pharyngeal jaw and teeth of zebrafish.

Introduction

The hypocretin/orexin system is highly conserved from fish to man and plays a central role in the control of sleep and arousal as well as feeding and energy homeostasis.[1] Important to our lab, the hypocretin system also plays a large role in organismal stress responses. In the brain, hypocretin(hcrt1/hcrt2)-producing neurons are found in the lateral hypothalamus and have widespread anatomical projections. In zebrafish, the hcrt network comprises ~16-60 neurons with one hypothalamic nucleus for the hypocretin gene.[2] The presence of *bona fide* hypocretin producing neurons in the peripheral nervous system, including the enteric nervous system, has been debated. To date, the presence of *hcrt+* cells in the gut has been largely supported by indirect immunodetection using antibodies specific to hcrt.[3] In this study, we utilized a transgenic line that expresses *gfp* under the hypocretin promoter in order to determine the expression of hypocretin in peripheral tissues including the gut. The ENS of zebrafish is organized into two main plexuses, the myenteric plexus and the submucosal plexus. The myenteric plexus is located between the circular and longitudinal layers of the muscularis externa, while the submucosal plexus is located in the submucosa layer of the GI tract. These two plexuses contain neurons and glial cells.

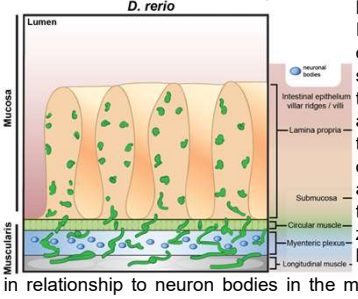


Fig1: Illustration showing the architecture and organization of the zebrafish gut in relationship to neuron bodies in the myenteric plexus and supporting cell types. Adapted from Graves *et al.*[4]

Materials and Methods

GFP sorting: Anesthetized 3 dpf fish from *hcrt:eGFP* and *hcrt:eGFP;nbt:DsRed* lines were sorted for *gfp+* or *gfp+Dsred+* fish using a Zeiss AxioZoom V16 Fluorescence Microscope.

Gut dissection: Fish were sacrificed and standard length (cm), phenotypes and sex were recorded. Hcrt *gfp* expression was confirmed by imaging brain tissue ex vivo. Guts were dissected and fixed in 10% neutral buffered formalin tubes for 72 h at 4 ° C, then stored in 70% ethanol at 4° C.

Whole mount immunostaining: Resected fixed whole guts from *hcrt:gfp* zebrafish were permeabilized with 0.5% TritonX-100 in DPBS (PBST), blocked with goat serum and 5% NGS in PBST for 24 h and primary antibodies (pAb Rabbit anti-GFP AbcAm Ab290; Mouse anti Ac Tubulin clone 6-116-1) were applied at a 1:200 dilution. After washing, secondary antibodies (Goat anti rabbit 488 life technologies A11034; goat anti mouse 647 life technologies 21236) were applied at a 1:500 dilution and protected from light (72h at 4° C followed by 1h RT). Guts were spread on slides, vacuum grease and 1.5 drops of mounting medium (Vectashield Plus H-1900), and a 1.5 coverslip were added prior to imaging.

Confocal Microscopy: Z stack confocal images were obtained on a Zeiss 800 Upright Microscope fitted with 20x dry objective and resulting images were analyzed with IMARIS 9.9.1.

Results

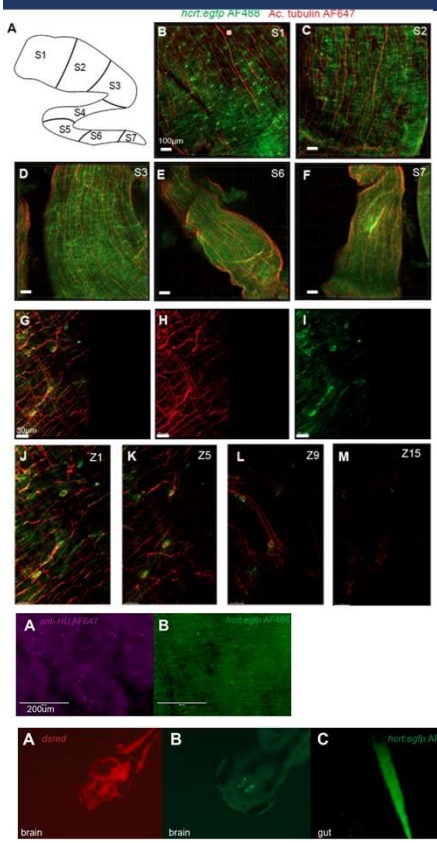


Fig 2. Regional distribution of Hcrt expressing cells in the enteric nervous system. A) Schematic of zebrafish gut showing major anatomical regions as described by Wang et al 2010.[5] For B-M green = all gfp labelled cells; red = all neural projections labelled with Acetylated tubulin. B) proximal intestine. For all B-F scale bar = 100 um. C) Hcrt expressing cells appear localized to the myenteric plexus of adult zebrafish and intimately associated with neural processes. Merged Z-stack (19 slices at 1.1 um per slice) image showing *hcrt:gfp* expression (green) together with acetylated tubulin (red). Individual channels are shown in B (Ac. tub) and C (hcrt). J-M shows individual slices (indicated on each panel) of a zoomed in area of Figure 2G.

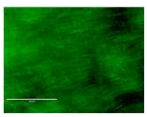
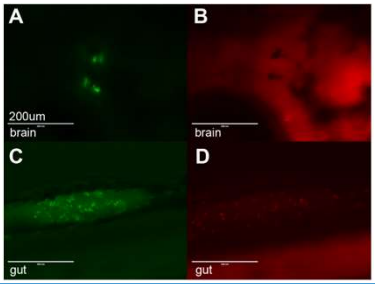


Fig 3. （middle) **Hcrt expression and neuronal cells in the S1 region of an aged zebrafish (SL = 2.8 cm), which shows few overlap.** A) Neuronal cells in the S1 region shown by anti-hu. B) hdcrt:gfp expression.

Fig 4. (lower right) **Hcrt expression in the S3 region of aged zebrafish.** *gfp* appears to have horizontal and vertical layers, which indicates supporting cells.

Fig 5. (lower left) **In vivo characterization of *hcrt:gfp-nbt:dsred* zebrafish at 3 dpf.** A) nbt expression in zebrafish, which labels the brain and spinal cord. B) hcrt expression in the brain. C) At the stage the gut isn't fully formed.

Fig 6. (bottom right) **In vivo characterization of *hcrt:gfp-nbt:dsred* zebrafish at 11 dpf.** A, B shows hcrt and nbt in the brain. C, D shows hcrt and nbt in the distal S5 region of the gut.



Discussions

In order to characterize the expression of hypocretin in the enteric nervous system of zebrafish, we used fixed adult tissue and stained for gfp and Acetylated tubulin (labels all neural processes) as described in Methods. After the whole amount immunostaining, confocal imaging was done. The images obtained show that Hcrt is expressed in the gut (Fig. 2).

First, we showed that there are *gfp+* hcrt producing cells in the gut of adult zebrafish (Fig 2). Specifically, we observed *hcrt:gfp* expressing cells with higher density in the proximal intestine. These cells appeared closely associated with the neural processes and regularly spaced. Given that signal was observed in the myenteric plexus (no signal observed toward the epithelium (Z15) we hypothesized that the hcrt cells could be of neuronal origin. To test this, we stained hcrt:gfp tissue with anti-gfp and an antibody that labels neuron bodies (anti-HU) and found minimal evidence of co-expression in aged animals by widefield fluorescent imaging (EVOS FL), suggesting a non-neuronal source of the hcrt:gfp expression observed in Fig 2 (Fig 3). Ongoing confocal imaging will provide further clarity. Another possibility would be that the gfp is labeling glial cells which may explain the expression pattern we observed (Fig 4).

Since Hcrt expression is present as early as 3 dpf in the LH (Fig 5), we conducted an in vivo analysis on embryonic zebrafish to determine at which stage we can see hcrt expression in the gut. We did not observe gut hcrt at this stage, which is expected because the gut isn't fully developed yet (HU+ gut neurons are first seen 5dpf at the onset of feeding). At 11dpf, there are hcrt and nbt expression in the brain, and the gut appears to have hcrt expression (Fig 6).

References

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