Dynamic Orexinergic Responses to Environmental Stressors in the Zebrafish Gut

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ABSTRACT

Objectives: The hypothalamic hypocretin (*hcrt*)/orexinergic system modulates sleep/wake cycles, arousal, and feeding. While orexinergic circuits have been well-described in the central nervous system, debate remains whether a *bona fide* circuit exists in the enteric nervous system. The primary objective of this study was to investigate the expression of *hcrt* and the *hcrt* receptor (*hcrtr2*) in the zebrafish gut using transcriptomic approaches; the secondary objective was to determine whether gut *hcrt/hcrtr2* expression is modulated by chronic stress or feeding state.

Results: qPCR analysis revealed robust transcription of *hcrt* and *hcrtr2* in the zebrafish gut, with significantly higher expression in the distal gut compared to the proximal gut. We also found that an overnight fast significantly increased expression of gut *hcrt* and *hcrtr2* and that the distal gut is more responsive to the feeding state than proximal gut. Moreover, both brain and gut *hcrt/hcrtr2* transcription was significantly impaired in fish exposed to chronic early life stress.

Conclusions: Our results describe for the first time the presence of an orexinergic system in the zebrafish gut, and that local expression is responsive to feeding state and is reduced following chronic stress.

INTRODUCTION

Figure 1. The function of orexinergic system.[5]

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 (**Figure 1**)[1] Hypocretin (*hcrt*)-producing neurons are found in lateral hypothalamus and have widespread anatomical projections which also plays a large role in organismal stress responses. In the brain. In zebrafish, the hcrt network comprises ~16-60 neurons with one hypothalamic nucleus for the hypocretin gene.[2]

The presence of hypocretin producing neurons in 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 qPCR for a transcriptional approach to investigate the presence of *hcrt*.

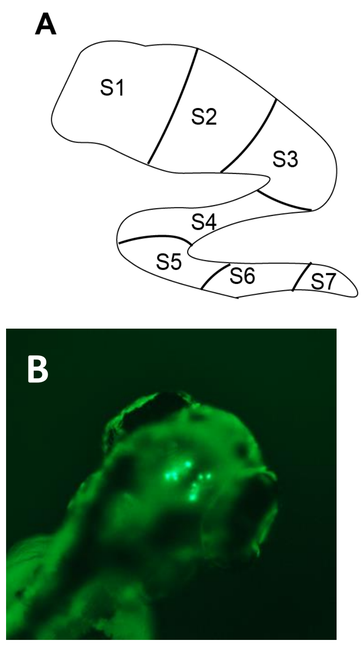

Figure 2. A) Anatomical Regions of the zebrafish gut.[6] B) *In vivo* characterization of hcrt expression in the brain in *hcrt:gfp-nbt:dsred* zebrafish at 3 dpf.

METHODS & MATERIALS

Zebrafish Husbandry: All procedures performed in this study were reviewed and approved by the University of North Carolina Chapel Hill (UNC-CH) Animal Care and Use Committee (protocol #20-241 and #23-178). Fish were reared and maintained in the AAALAC-accredited UNC-CH Zebrafish Aquaculture Core Facility under a 14h light / 10h dark cycle at 28°C in compliance with the Guide for the Care and Use of Laboratory Animals. N=31 Wild-type AB zebrafish were used. On the day before qPCR experiments, food was left accessible (control) or removed overnight (fasted).

RNA Extraction and qRT-PCR: Freshly resected and cleaned gut tissue was quickly minced and homogenized (QIAshredder, Qiagen) prior to RNA extraction (RNeasy® Mini Kit, Qiagen). cDNA synthesis was accomplished with iScript® Reverse Transcriptase (Bio-Rad Laboratories, Berkeley, CA, USA) and using 250 ng starting RNA per sample. Brain (n=6) and gut (n=10) cDNA samples derived from juvenile fish (stressed *vs*. unstressed tank mates) from our previously published study (Graves et al., 2023) were also used in this study. qPCR was conducted using SsoAdvanced™ SYBR Green Supermix and *hcrt*- and *hcrt2r*-specific primers. Data were collected and processed using CFX Connect™ and CFX Manager™ (Bio-Rad Laboratories, Berkeley, CA, USA). Gene expression was calculated using the ΔΔCT algorithm and compared to a reference gene (*ef1α*).

Statistical Analysis: Statistical analysis and graphical representation was carried out using GraphPad Prism® V9.4.1 (GraphPad, La Jolla, CA, USA). Tests for normality and lognormality were performed on all datasets. Student's T-test or one-way ANOVA was employed for 2-group or >2-group comparisons as appropriate, unless otherwise specified. Values are reported as the mean±standard error of the mean (S.E.M.) ; $p \leq 0.05$ was considered statistically significant.

RESULTS

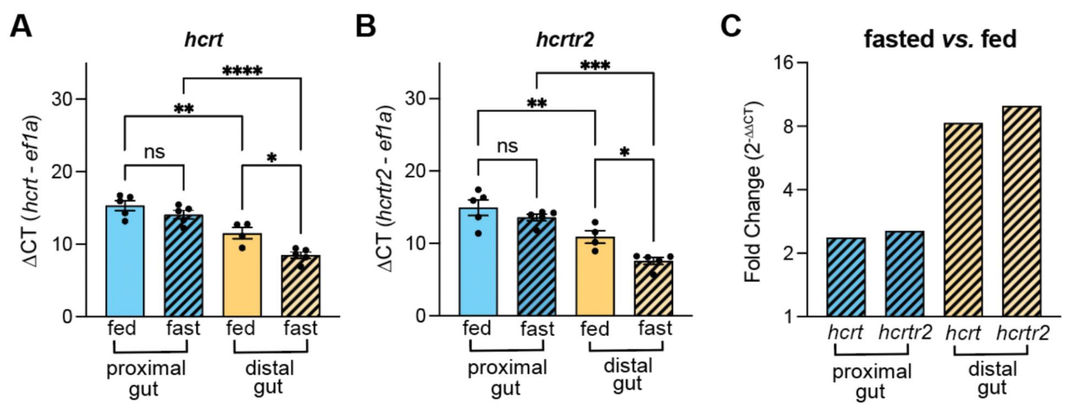

Figure 3. Transcriptional levels of *hcrt* and *hcrtr2* in the zebrafish gut are modulated by feeding state and exposure to chronic early life stress. A) *hcrt* and **B**) *hcrtr2* expression relative to a housekeeping gene (*ef1α*) in the proximal (blue) and distal (orange) gut of fasted (striped) and fed (solid) adult zebrafish. **C**) Fold change expression of *hcrt* and *hcrt2* comparing fasted (n = 5) *vs*. fed (n = 5) zebrafish in the proximal and distal gut. Each dot represents one animal. *p<0.05; **p<0.01; ***p<0.001 (by A and B: Kruskal-Wallis (Nonparametric one-way ANOVA) with Dunn's multiple comparisons test); ****p<0.0001

Acute fasting increases gut *hcrt* and *hcrtr2* expression

To determine whether the proximal and distal gut expresses similar levels of *hcrt* and *hcrtr2* as well as to determine whether fasting modulated gut expression of *hcrt* and *hcrtr2*, qRT-PCR was performed on gut tissue following an overnight fast (<24h) and compared to tankmates receiving a morning feeding. Robust expression of *hcrt* was observed with significantly lower abundance in the proximal half of the gut (S1-S3) compared to the distal half (S4-S7); *hcrt* expression was increased in the fasted group with the most pronounced upregulation of *hcrt* observed in the distal gut of fasted fish (**Figure 3A**). A similar pattern of expression was observed for *hcrtr2* (**Figure 3B**). Summatively, fasting induced a greater than 2-fold change in the expression of *hcrt* (FC: 2.4) and *hcrtr2* (FC: 2.5) in the proximal gut; in the distal gut, *hcrt* and *hcrtr2* expression increased by 8.3- and 10.0-fold, respectively (**Figure 3C**).

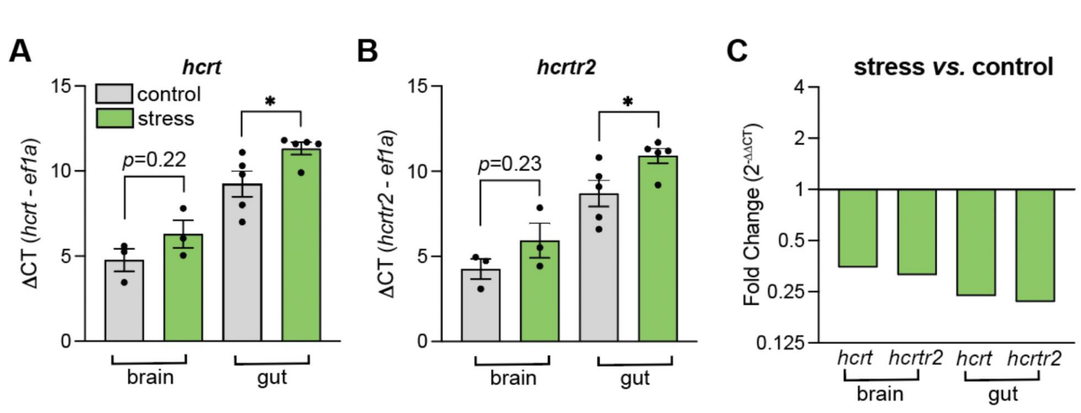

Figure 4. Stress exposure reduces *hcrt* and *hcrtr2* expression in the zebrafish brain and gut. A) *hcrt* and **B)** *hcrtr2* expression in the brain and gut of juvenile zebrafish in the absence (grey bars) or presence (green bars) of chronic early life stress. **C)** Fold change expression of *hcrt* and *hcrt2* in brain and gut tissues comparing stress *vs.* control. Each dot represents one animal. *p<0.05; Mann-Whitney *U* test.

ELS Reduces brain and gut *hcrt* and *hrctr2* expression

To determine whether the brain and gut express similar levels of *hcrt* and *hcrtr2* as well as to determine whether ELS modulated either expression of *hcrt* and *hcrtr2*, qRT-PCR was performed on brain and gut tissue on stress and control(unstressed) group. Robust expression of *hcrt* was observed, with significantly lower abundance in the gut compared to the brain. *hcrt* expression was increased in the fasted group with the most pronounced downregulation of *hcrt* observed in the gut of stressed fish (**Figure 4A**). A similar pattern of expression was observed for *hcrtr2* (**Figure 4B**). Transcription of *hcrt* and *hcrtr2* were significantly lower in the stressed group than in the control group for both brain and gut tissues (**Fig 4A-C**). Specifically, stress induced a less than 0.5-fold change in the expression of *hcrt* (FC: 0.35) and *hcrtr2* (FC: 0.31) in brain; in gut, *hcrt* and *hcrtr2* expression decreased by 0.24- and 0.22-fold, respectively (**Figure 4C**).

CONCLUSIONS

- Here, we demonstrate for the first time that orexin and its receptor is expressed in the zebrafish gut and this expression is more profound in the distal intestine.
- We show that fasting increases gut *hcrt* and *hcrtr2* expression, and provide new evidence that though both the proximal and distal gut increase *hcrt* and *hcrtr2* expression in the fasted state, the distal gut is more responsive to fasting than the proximal gut.
- Finally, using our recently published novel model of chronic early life stress[6], we show for the first time that chronic early life stress suppresses *hcrt/hcrtr2* expression in peripheral tissues including the gut.
- *Hcrtr2:hcrt* expression appear to be at fixed ratios (~1.2-1.4) suggesting the orexinergic system is tightly regulated
- People with PTSD frequently report insomnia and recurrent nightmares, which suggests that stress may induce REM fragmentation during sleep.[7]
- Future studies will explore the link between narcolepsy (loss of orexin neurons) and stress

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