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Chronic early life stress alters the neuroimmune profile and functioning of the developing zebrafish gut

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ABSTRACT

Chronic early life stress (ELS) potently impacts the developing central nervous and immune systems and is associated with the onset of gastrointestinal disease in humans. Though the gut-brain axis is appreciated to be a major target of the stress response, the underlying mechanisms linking ELS to gut dysfunction later in life is incompletely understood. Zebrafish are a powerful model validated for stress research and have emerged as an important tool in delineating neuroimmune mechanisms in the developing gut. Here, we developed a novel model of ELS and utilized a comparative transcriptomics approach to assess how chronic ELS modulated expression of neuroimmune genes in the developing gut and brain. Zebrafish exposed to ELS throughout larval development exhibited anxiety-like behavior and altered expression of neuroimmune genes in a time- and tissue-dependent manner. Further, the altered gut neuroimmune profile, which included increased expression of genes associated with neuronal modulation, correlated with a reduction in enteric neuronal density and delayed gut transit. Together, these findings provide insights into the mechanisms linking ELS with gastrointestinal dysfunction and highlight the zebrafish model organism as a valuable tool in uncovering how "the body keeps the score."

1. Introduction

Historically regarded as disparate, mounting experimental and clinical evidence have demonstrated that the gut nervous and immune systems are dynamically engaged and coordinate environmental responses to achieve host defense and protection (Deak et al., 2015; Verheijden and Boeckxstaens, 2018; Jakob et al., 2020; Muller et al., 2014; Matheis et al., 2020). Neuroimmune dysfunction arises when chronic stress overwhelms the body's capacity to maintain homeostasis in part through prolonged activation of the sympatho-adrenal and hypothalamic-pituitary-adrenal (HPA) axes, which modulate immune responses in tissues (Jakob et al., 2020; Schreier and Chen, 2017; Dhabhar, 2014; Bam et al., 2016; Rusiecki et al., 2013; Ayaydin et al., 2016; Cunliffe, 2016; Smith et al., 2011). Though acute stress can enhance immune function, chronic stress can become physically damaging, negatively impacting physiological systems, and resulting in nervous system dysregulation and immune impairment (von Kanel et al., 2010). Chronic early life stress (ELS) is associated with the most severe health outcomes, indicating that stress becomes 'hard-wired' during development (Dube et al., 2009). In addition to the relatively well-described effects on the developing brain and immune systems, ELS has emerged as a potent modulator of the gut-brain axis and appreciated as an important etiological factor linked to the onset of functional gastrointestinal disorders later in life (Dube et al., 2009; Ilchmann--Diounou and Menard, 2020; Song et al., 2020; Bennett et al., 1998; Foster et al., 2017; Moeser et al., 2007; Kronman et al., 2021; Danese and S, 2017; Traina, 2019; Song et al., 2018). Despite these advances, the specific mechanisms linking ELS with perturbed gut function remain incompletely understood.

In addition to their recognized strengths of high fecundity, larval

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transparency, and genetic tractability, zebrafish are increasingly appreciated for their translational potential in ELS models (Champagne et al., 2010; Golla et al., 2020; Egan et al., 2009) (recently reviewed by Eachus et al. (2021)). Further, we and others have demonstrated that zebrafish are a powerful model to interrogate gut biology and neuroimmune interactions in health and disease (Graves et al., 2021; Earley et al., 2018; Ye et al., 2021). In this study, we introduced a novel model of ELS by applying unpredictable mild environmental stressors throughout zebrafish larval development and assessed behavior and the transcription of neuroimmune genes in the brain and gut at different time points. By taking a comparative transcriptomics approach, we demonstrated that ELS altered the enteric expression of neuroimmune genes in a similar-yet-distinct pattern to that of the developing larval brain. Whole mount immunofluorescence revealed a concomitant reduction in enteric neuron density with functional impacts to the developing gut, suggesting that ELS may impair gut neurodevelopment analogous to that described in the central nervous system in mammals and zebrafish. Altogether, these data provide key insights into how ELS impacts developing organisms from a tissue-centric lens and lays the groundwork for more mechanistic and cell-type specific studies in this valuable model system.

2. Materials and methods

2.1. Zebrafish husbandry

All experimental 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). Animals were housed in an AAALAC-accredited facility in compliance with the *Guide for the Care and Use of Laboratory Animals* as detailed on protocols.io (dx.doi.org/10.17504/protocols.io.bg3jjykn). Wild-type AB fish (from a stock population) were used in this study and were reared and maintained in the aquaria of the UNC-CH Zebrafish Aquaculture Core Facility under a 14 h light/10 h dark cycle at 28 °C. Experiments were performed prior to sex determination. For each experimental data set, stage-matched control siblings were used. Experimental subjects were randomly assigned by tank.

2.2. Early life stress procedure

ELS-exposed fish (experimental group) were subjected to one of three mild, unpredictable stressors twice daily during the light cycle beginning at 6 days post fertilization (dpf) until 11 or 30 dpf as adapted from a previously described stress paradigm (Golla et al., 2020). Control groups were raised identically without stressor exposure. Stressors used in this study included water turbulence (3 tank water changes, 3 min), chasing with a small transfer pipette (5 min), or strobe light flash. Strobe light flash was performed using a software-controlled 165 lumen LED (Samsung 3432 1.8t [FH341A]) applied for 10 min at 5Hz directly above the tank lid within a secondary enclosure to limit ambient light or unintended disturbance of non-experimental subjects. Stressor selection and time of day were predetermined and prescheduled for the entire stress period by random number generation. Four different experimenters applied stressors also following a randomized schedule. Whole fish were rapidly euthanized and snap frozen after a 24 h washout for cortisol extraction. Behavioral analysis and euthanasia for tissue harvest was performed after a 24 h and 48 h washout period, respectively. A complete 7- and 8-day washout prior to behavioral analysis and euthanasia for tissue harvest, respectively, was also performed as indicated.

2.3. Behavioral assessment

Fish were individually placed in a holding tank of system water (novel tank) and video recorded (12 MP camera, 30fps, Samsung Electronics, Suwon-si, KR) for 5 min. Analysis of videographic data was performed by three independent experimenters and using EthoVision XT Software. The following behaviors were scored: latency (time until first dive [s]), total time spent in the top half of the tank (s), total time spent in the bottom half of the tank (s), total number of entries into the top (n), total distance travelled (cm), and average velocity (cm/s). All behavioral assessments were conducted at the same time of day (10 a.m.–12 p.m.) to minimize effects of circadian rhythm on behavior.

2.4. Whole body cortisol

Cortisol extraction was performed on homogenized whole body samples of $n = 4$ control and $n = 5$ ELS-exposed fish as previously described (Measuring Endocrine). Briefly, cortisol was reconstituted in 500 μL of 1X PBS after ether evaporation and incubated overnight at 4 °C and measured using a human salivary high sensitivity cortisol assay kit (Catalog No. 1–3002; Salimetrics, LLC, Pennsylvania, USA). Each sample was run in technical duplicates and average absorbance values were used to calculate concentrations based on a standard curve and four parameter logistic regression. Concentrations were then normalized by dividing total cortisol per fish by the fish weight.

2.5. Tissue harvest and RNA isolation

Following euthanasia, fish length (standard length, SL) was recorded using a standard ruler. Brain and/or gut were quickly harvested, intestinal contents removed and grossly minced in Buffer RLT (Qiagen, Hilden, DE) on wet ice. Gut was enriched for muscularis tissue by gentle reduction of the mucosae using an ultrafine soft-bristled artists' brush. Length measurements and organ harvest were conducted under low magnification on a Leica M125C fitted with 0.8X LWD PLAN objective (Leica Microsystems, Wetzlar, DE). Tissue was homogenized using QiaShredder columns and RNA was extracted using an RNeasy® Mini Kit and according to manufacturer protocols (Qiagen). RNA was analyzed for quantity and quality using a NanoDrop™ One Microvolume UV–Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Tissue was extracted from a total of $n = 11$ control and $n = 12$ ELS-exposed fish (3–5/group) and gene expression analysis was conducted on individual samples.

2.6. Gene expression and analysis

Gene expression was quantified using the NanoString™ nCounter® Gene Expression Profiling System according to manufacturer protocol (NanoString Technologies, Inc., Seattle, WA, USA). 100 ng of RNA was used as starting template for a 40-gene custom NanoString™ nCounter® CodeSet probe panel (Table S1). Template-probe hybridization was conducted at 65 °C for 18 h prior to quantification on the nCounter Prep Station and Digital Analyzer. Resulting data were analyzed by ROSALIND® (https://rosalind.bio/), with a HyperScale architecture developed by ROSALIND, Inc. (San Diego, CA, USA). Read Distribution percentages, violin plots, identity heatmaps, and sample MDS plots were generated as part of the QC step. Normalization, fold changes and p-values were calculated using criteria provided by Nanostring. ROSALIND® follows the nCounter® Advanced Analysis protocol of dividing counts within a lane by the geometric mean of the normalizer probes from the same lane. Housekeeping probes to be used for normalization are selected based on the geNorm algorithm as implemented in the NormqPCR R library (Perkins et al., 2012). ROSALIND performs a filtering of Cell Type Profiling results to include results that have scores with a p-value greater than or equal to 0.05. Fold changes and p-values are calculated using the fast method as described in the nCounter® Advanced Analysis 2.0 User Manual.

2.7. Whole mount immunostaining, imaging and enteric neuron quantification

Whole mount *ex vivo* processing of gut tissue was adapted from our previously published protocol (Graves et al., 2021). Following euthanasia, gut was quickly harvested, opened longitudinally, and cleaned under low magnification prior to immersion in 10% neutral buffered formalin (16 h, 4 °C). Following fixation, tissue was transferred to a permeabilization solution containing 0.5% Triton X-100 (Sigma-Aldrich) in 1X Dubelco's PBS (PBT) (O/N, 4 °C) and blocked in PBT containing 5% normal goat serum (O/N, 4 °C). Primary antibody (Mouse α-HuC/HuD neuronal protein, clone 16A11; Thermo Fisher) was applied in fresh blocking solution at a 1:250 dilution (72hr, 4 °C) and washed in PBT (6x, 10 min/wash) prior to secondary antibody (AlexaFluor 647 goat anti-mouse IgG (H + L), Thermo Fisher) application at a 1:500 dilution for 1–3 h at room temperature, protected from light. After final PBT washes (6x, 10 min/wash), samples were mounted in medium (VECTASHIELD® PLUS Antifade Mounting Media, Vector Laboratories, Newark, CA, USA) on glass slides and covered with a 1.5 thickness coverslip. Imaging of the proximal gut was performed on a Zeiss LSM 800 Upright confocal microscope fitted with a Plan-Apochromat 20x/0.8 M27 dry objective using Zeiss ZEN V2.3 (blue edition) software (Carl Zeiss AG, oberkochen, Baden-Wirttemberg, DE). At least 10 confocal micrographs were obtained per subject and averaged to obtain a composite density for each subject. Resulting confocal micrographs were analyzed using FIJI V2.9.0/1.53t software (Schindelin et al., 2012). Neurons were quantified as discrete HU + labelled cell bodies located in the muscularis region using the multi-point function and are reported as number of neurons per area quantified (neuronal density). Quantifications were performed in a blinded fashion by at least two experimenters.

2.8. Gut transit

After a morning feeding, fish were transferred to a 10 cm × 20 cm x 12 cm holding tank containing 1.5L of fresh system water and a biologically inert, sterilized stainless-steel mesh insert (1250 μm pore diameter) designed to prevent coprophagia placed ~2.5 cm from the bottom of the tank. Fish (3–6 fish/time point; $n = 38$ total) were sacrificed at 0 (immediately upon transfer to new tank after feeding), 2, 4, 6 and 8 h after food withdrawal, immersed in 10% neutral buffered formalin (16hr, 4 °C) and then transferred to 70% EtOH. The gut was carefully resected and imaged in such a manner as to not disturb intestinal contents under light microscopy (ZEISS Axio Zoom V16, Carl Zeiss AG, Oberkochen, DE). Gut transit was calculated as length of gut containing intestinal contents as a percentage of total gut length using the line measurement tool in FIJI (Schindelin et al., 2012).

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism® V9.4.1 (GraphPad, La Jolla, CA, USA). Descriptive statistics and tests for normality and lognormality were conducted on all data sets. Student's T-test or one-way ANOVA were used as appropriate to compare differences between groups unless otherwise indicated. For lognormal data, the log of the data was taken to normalize the data prior to statistical tests. Data shown are representative from at least 2 independent experiments with at least 3–5 replicates per group, number of experimental subjects are noted in each figure and figure legend. All values are expressed as mean ±S.E.M. unless otherwise indicated. Differences were considered statistically different at $p < 0.05$. For cortisol assays, normalized cortisol concentrations (ng/g) were transformed lognormally and differences between groups were considered statistically significant at $p < 0.05$ using a Kolmogorov-Smirnov test. Data are reported as the natural log (Ln) of ng/g cortisol.

3. Results

3.1. Chronic stress throughout larval development results in lasting anxiety-like behavior

Though zebrafish are a validated model to study the physiological effects of ELS, most studies have been limited to the embryonic or early larval period, or prolonged stressors are only applied for a relatively short duration of time (2–7 days in duration) (Golla et al., 2020; Chin et al., 2022; Marcon et al., 2018). To determine the impact of chronic ELS throughout larval development we introduced environmental stressors to wild-type AB zebrafish beginning at 6 dpf and terminating after either 1 week, at 30 dpf, or at 30dpf and followed by a 7d washout (Fig. 1A). One of three mild stressors were randomly selected and introduced at random times during the light cycle (Fig. 1B).

We first assessed whether chronic ELS impacted fish behavior in a novel tank test. Twenty-four hours after the final stress exposure, an anxiety-like phenotype was observed in stress-exposed, but not control, subjects as indicated by a significant increase in bottom dwell time, significant reduction in top dwell time and significantly reduced frequency of surface entries over a 5-min recording period that was not due to a lack of locomotion (Fig. 2A–D, Fig. S1A). Notably, bottom-tank preference was observable immediately post stress stimulus exposure that waned within a few minutes after stress withdrawal (Fig. 2E). Further, stress-exposed fish exhibited persistent, though blunted, anxiety-like responses after a 7d washout, indicating lingering behavioral changes in response to ELS (Fig. 2F, Fig. S1B). In addition to changes in fish behavior, we also observed a modest-yet-significant (~10%) growth deficit following chronic (30 day), but not shorter term (1 week), ELS that subsided following washout (Fig. 2G) that correlated with a significant increase in basal cortisol levels (Fig. 2H). Together, these data demonstrate that zebrafish are susceptible to mild chronic early life stress beginning at 6 dpf and extending throughout larval development.

3.2. Chronic ELS alters neuroimmune gene expression in a time- and tissue-dependent manner

Although ELS is associated with gastrointestinal dysfunction, the underlying mechanisms of how ELS might transcriptionally program neuroimmune genes in the developing gut are unknown. To test the hypothesis that ELS alters neuroimmune gene expression in a tissue-specific and time-dependent manner, we transcriptionally profiled mRNA from the gut of zebrafish exposed to ELS for 1 week and from the gut and brain of zebrafish exposed to ELS until 30 dpf and control siblings using a custom 40-gene mRNA Expression Panel (Table S1). Genes were selected based on literature review of known or suspected transcriptional targets of the stress response irrespective of species and on genes associated with gut inflammation.

We found that ELS resulted in differential expression of key neuroimmune genes that differed depending on developmental stage and tissue type (Fig. 3A–D). After 1 week of ELS, gut expression of *s100b* (S100 calcium-binding protein B), *mthfr* (methylfolate reductase), *slc1a4* (solute carrier family 1 member 4), and *csf1ra* (colony stimulating factor 1 receptor, a) were significantly increased, whereas there was a significant downregulation of *irf1b* (interferon regulatory factor 1b), *nr3c1* (glucocorticoid receptor), and *nfkb2* (nuclear factor kappa- B2) when compared to control stage-matched siblings (Fig. 3A, D). Following chronic ELS (until 30 dpf), gut expression of *casp3a* (caspase 3a), *dnmt1* (DNA methyltransferase), *nfkb2*, *c1qb* and *c1qc* (complement component 1, q subcomponent, B and C chain), *ptenb* (phosphatase and tensin homolog B), and *nr3c1* were significantly upregulated compared to controls (Fig. 3B, D). A similar but distinct expression profile was observed in the brain of chronic ELS-exposed subjects including significant upregulation of *casp3a, dnmt1*, *nfkb2*, *ptenb*, and *nr3c1* as well as upregulation of *slc1a4* (solute carrier family 1 member 4), *sirt1* (sirtuin 1),

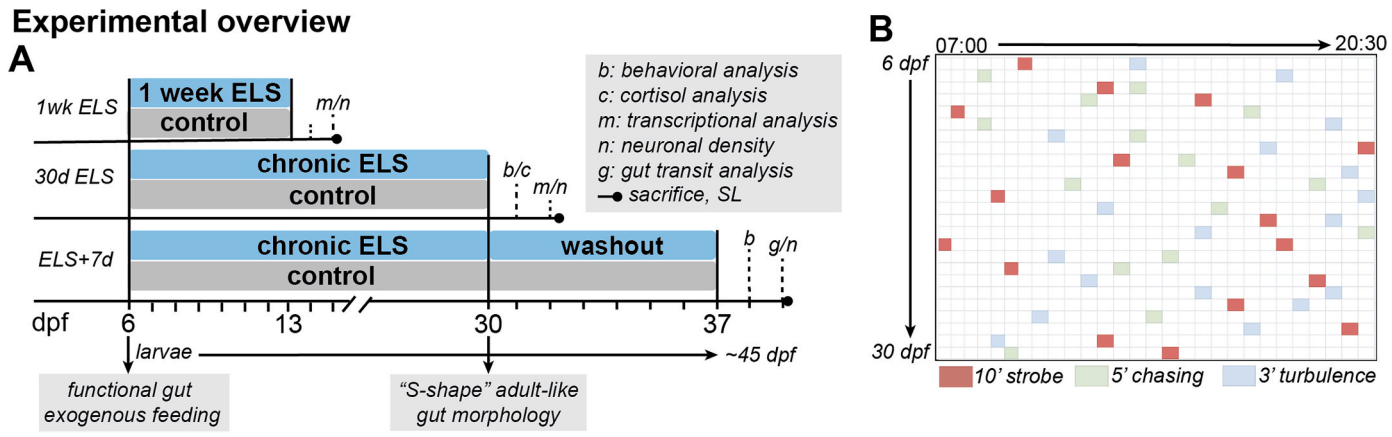

Fig. 1. (**A**) Schematic overview of the experimental design and experimental endpoints in relationship to fish and gut development. (**B**) Example randomized stress schedule showing time of day (x-axis) in 30-min blocks and individual stressors (red box: 10-min strobe light; green box: 5-min chasing; blue box: 3-min tank turbulence). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

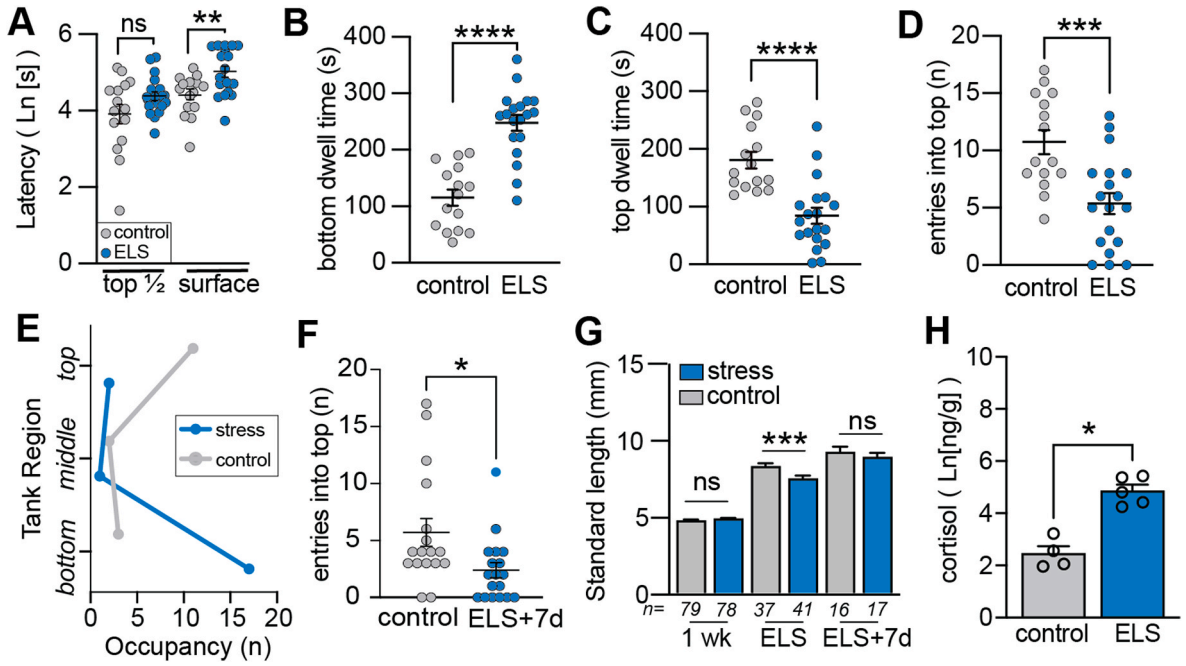

Fig. 2. Chronic ELS throughout larval development transiently hinders growth and results in anxiety-like behavior. (**A-D**) Behavioral analysis of zebrafish exposed to chronic ELS until 30 dpf quantifying (**A**) latency (Ln [s]) to the top ½ or surface (**B**) total time spent in the bottom of the tank (s), (**C**) total time spent in the top of the tank, and (**D**) number of entries into the top of the tank over a 5-min recording period. (**E**) Acute behavioral response to strobe stress recorded in the first minute following stress withdrawal showing greater bottom tank occupancy in the stressed group (blue line) as compared to control fish (grey line). (**F**) Behavioral analysis of zebrafish exposed to ELS until 30 dpf and following a 7-day washout (ELS+7d) quantifying number of entries into the top of the tank. (**G**) Standard length (SL) measurements taken at sacrifice of zebrafish exposed to ELS for 1 week (1wk), until 30 dpf (ELS) or until 30 dpf and following a 7-day washout (ELS+7d). (**H**) Whole body cortisol measurement of fish exposed to chronic ELS until 30 dpf. Data shown are (**B**) cumulative or (**C-G**) representative of 2–3 independent experiments. For **E** and **G**, number of experimental subjects (*n*) are noted. For **A-D** and **F** (n=15–20/group), as well as **H** (n = 4–5/group), each dot represents one subject. Data are presented as mean±SEM * $p < 0.05$, *** $p < 0.001$, and **** $p < 0.0001$ by Welch's T-Test (**A**), Mann Whitney *U* Test (**B-G**) or as described in Methods (**H**). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

adrb2a (adrenoreceptor beta 2) and downregulation of *lyz* (lysozyme) (Fig. 3C and D). We observed both unique and common genes differentially regulated by tissue type and stress duration (Fig. 3E). We applied multidimensional scaling to visualize transcriptional similarity among all samples and found that while chronic ELS results in the differential expression of key neuroimmune genes irrespective of tissue type (*e.g.* increased expression of *casp3a* in both the brain and gut, Fig. 3D), tissue type is the main driver of similarities in gene expression profile independent of developmental stage (dpf) (Fig. 3F).

3.3. Chronic ELS reduces enteric neuronal density and delays gastrointestinal transit

ELS is appreciated to potently impact the developing central nervous system (Danese and S, 2017; Bielefeld et al., 2021; Catale et al., 2020) and reduced enteric neuronal density has been observed in a variety of inflammation-related contexts across species (Matheis et al., 2020; Becker et al., 2018; Saffrey, 2013; Stenkamp-Strahm et al., 2015; Furlan et al., 1999; Boyer et al., 2005). Together with our observations that chronic ELS was associated with a significant upregulation in genes

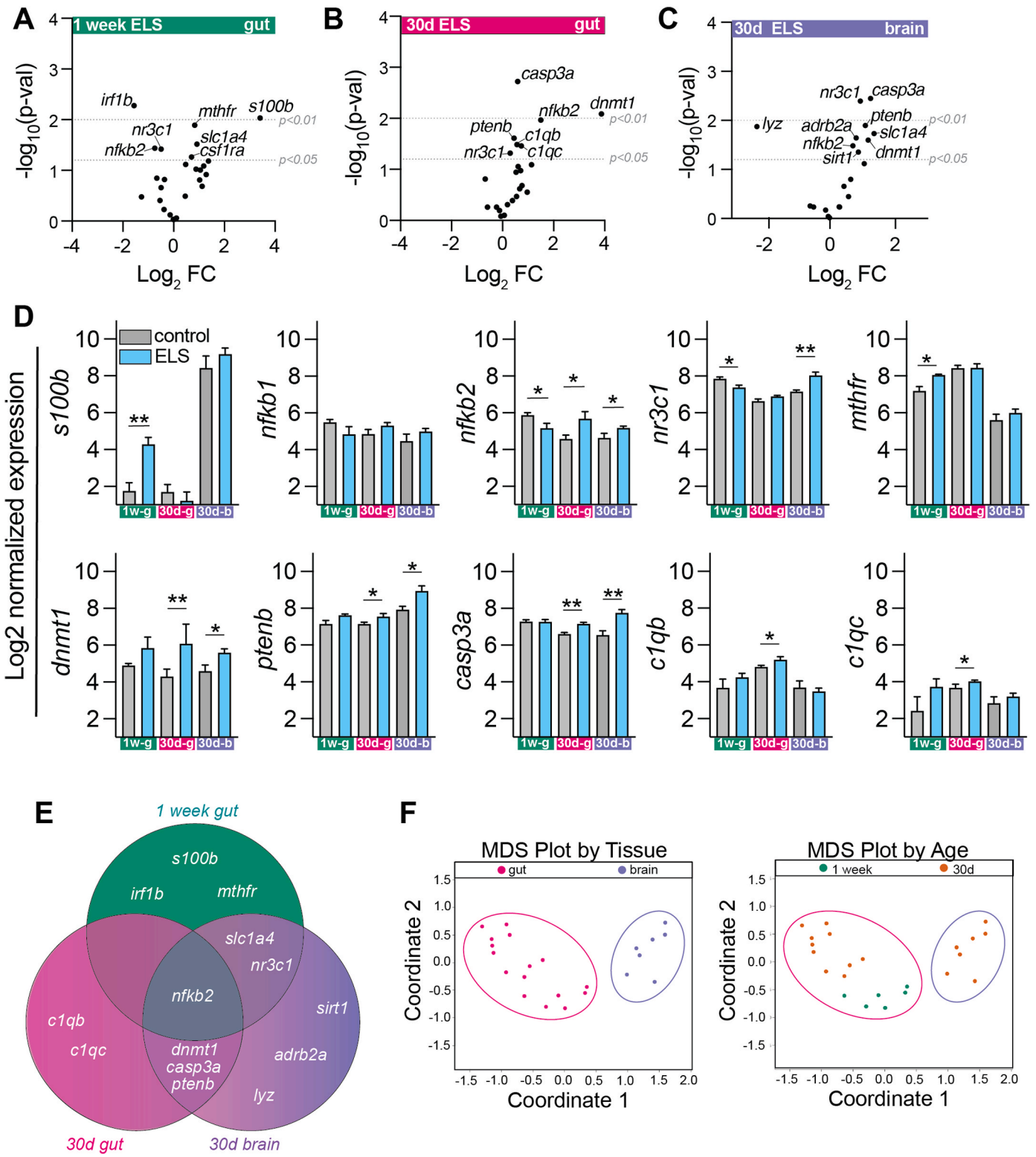

Fig. 3. Early life stress alters neuroimmune gene expression profiles in a time- and tissue-dependent manner. mRNA was isolated from whole *ex vivo* gut (n=3/group; total n=6) of zebrafish exposed to ELS for 1 week (1w-g [green]) and from whole *ex vivo* gut (n=5/group; total n=10) and brain (n = 3 control; n = 4 ELS; total n = 7) of zebrafish exposed to ELS until 30 dpf (30d-g [magenta] and 30d-b [purple], respectively) and stage-matched sibling controls. Gene expression was evaluated using NCounter® Analysis as described in *Methods*. (**A-C**) Data are shown as Volcano plots of the $\log_2$ transformed fold change (FC) in neuroimmune gene expression with associated p-value significance after normalization to housekeeping and control genes for (**A**) 1 week ELS gut, (**B**) ELS until 30 dpf gut, and (**C**) ELS until 30 dpf brain. (**D**) Data are shown as the normalized expression of key genes of interest. *p<0.05; **p<0.01 Mann-Whitney U Test. (**E**) Venn diagram summarizing significantly ($p < 0.05$) differentially expressed genes in a unique or common fashion to each tissue type and developmental stage. (**F**) Multidimensional scaling (MDS) plots by tissue type (left) and by developmental stage (right). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

associated with neuronal damage and remodeling including *dnmt1*, *ptenb* and *casp3a* in both the gut and brain, we wondered whether these changes were associated with a change in enteric neuron density. Using antibody-based immunolabelling (anti-HU C/D) and quantification of enteric neurons in whole mount *ex vivo* tissue, we found that chronic ELS throughout larval development was associated with a significant reduction in enteric neuron density when compared to control siblings that persisted following a complete 7d washout (ELS+7d) (Fig. 4A and B). To determine whether this reduced neuronal density was associated with functional changes in the tissue, we performed a transit assay and showed that ELS was associated with a persistent and significant delay in clearance of gut content (Fig. 4C and D).

4. Discussion

In humans, chronic stress – defined as stress lasting weeks to months – is associated with profound impacts on long-term health across physiological systems (Song et al., 2018, 2020) and is particularly detrimental when it occurs early in life and throughout childhood (Dube et al., 2009). At present, studies investigating the impacts of ELS, including chronic stress, in zebrafish rarely extend beyond 1 week in duration and are largely limited to embryonic development (Golla et al., 2020). In this study, stress exposure began during the early larval stage at the onset of functional gut development and exogenous feeding (6 dpf) and extended through 30 dpf when the gut morphologically resembles that of adults, enteric neuroimmune networks are established (Graves et al., 2021) and juvenile transition has not yet occurred (Singleman and Holtzman, 2014) (Fig. 1). In addition to impacts on fish behavior, we observed a reduction in body size of stressed fish in agreement with previously published studies (Golla et al., 2020). Further, we demonstrate these changes are associated with significant increases in basal cortisol levels(Fig. 2). This novel ELS paradigm thus models the toxic childhood stress condition in which adverse experiences are prolonged and dramatically increase risk of long-term adverse health effects (Franke, 2014).

Using this paradigm, we explored the physiological impact of chronic ELS from a tissue-centric lens and employed a comparative transcriptomic approach that uncovered differential expression of several stress-related neuroimmune genes. Intriguingly, we observed a significant downregulation of gut *nfkb2* after 1 week of stress followed by a significant increase that was consistently observed in both the gut and brain after chronic ELS until 30 dpf (Fig. 3). Similar expression trends were seen for *nfkb1*, although these differences were not significant at any stage in gut or brain. NF-κβ, a master regulator of the immune system, has been repeatedly implicated in stress-related disease states such as post-traumatic stress disorder (PTSD) (Gupta and Guleria, 2022). Women with childhood abuse-related PTSD are reported to have increased peripheral NF-κβ pathway activity (Pace et al., 2012) and some studies suggest that NF-κβ may be a determinant of PTSD more globally (Hong et al., 2017). Studies in rodents have also shown that Nf-κβ signaling mediates stress-associated suppression of neurogenesis in the hippocampus by blocking the proliferation of neural stem cells (Koo et al., 2010). In the gut, NF-κβ regulates intestinal homeostasis in part through modulation of the microbiome (Zhang et al., 2022). Along these lines, recent work shows that ELS alters the gut microbiota and represents one – likely of many – mechanisms by which the ELS disrupts the gut-brain axis (Park et al., 2021; Kemp et al., 2021; Hantsoo and Zemel, 2021). Taken together, our data adds credence to previous work highlighting the central involvement of NF-κβ regulation in chronic stress and importantly suggests these effects are not limited to the central nervous or peripheral immune systems.

We also observed an upregulation of *nr3c1* expression in the brain following ELS until 30dpf (Fig. 3). In contrast, *nr3c1* expression was significantly downregulated in the gut following 1 week of ELS that was only moderately increased after ELS until 30dpf. Numerous studies indicate that aberrant modulation of glucocorticoid receptor gene *nr3c1* is involved in the pathophysiological processes associated with ELS (Palma-Gudiel et al., 2015; Holmes et al., 2019; McGowan et al., 2009) and recent work in a different zebrafish ELS model highlights the conservation of this important stress-response pathway (Chin et al., 2022). In addition to its role in regulating the hypothalamic-pituitary-adrenal axis, glucocorticoid also plays an important role in gut development (Majumdar and Nielsen, 1985). Together, the data presented in this study is concordant with existing literature highlighting the importance of ELS-associated glucocorticoid signaling in the CNS (Chin et al., 2022) and suggests that *nr3c1* modulation may also play an important role in the developmental programming of the 'stress-informed' gut.

ELS is well appreciated to modulate CNS neurogenesis and affect brain developmental programs with long-lasting functional impacts and profound effects on organismal behavior (Korosi et al., 2012; Salmina et al., 2021). Neurogenic regions involved in stress regulation, emotions, memory, and executive functioning including the hippocampus, amygdala, prefrontal cortex, and hypothalamus have been widely studied. In the hippocampus, ELS is associated with reduced volume, delayed development, reduced stem cell proliferation and impaired neurogenesis though reduced stem cell proliferation and autophagic death (Youssef et al., 2019; Jung et al., 2020). In the prefrontal cortex, total volume (Tyborowska et al., 2018) as well as spine and dendrite loss (Woo et al., 2021) has been reported. Changes in enteric neuronal density have also been described in a variety of pathophysiological and experimental contexts including enteropathogenic infection (Matheis et al., 2020), inflammatory bowel disease (Margolis et al., 2011), diabetes (De Freitas et al., 2008), chemical injury, and aging (Becker et al., 2018; Saffrey, 2013). While infection and inflammation have been reported to elicit overt neuronal death in some experimental settings (Matheis et al., 2020; Ye et al., 2020), the mechanisms underlying reduced enteric neuronal density is not well characterized in most studies. This, coupled with the more recent appreciation of post-natal enteric neurogenesis across species (Goto et al., 2013; Grundmann et al., 2019; Kulkarni et al., 2017) suggests that changes in enteric neuronal density could arise not only because of cell death, but also as a delay or failure in their development. Extending this work, here we found that chronic ELS is associated with a persistent reduction in enteric neuronal density and delayed gastrointestinal transit (Fig. 4). In unstressed siblings, we observed a lognormal increase in the enteric neuronal density that recapitulates our previous observations of normal gut growth and development at this stage (Graves et al., 2021). Enteric neuronal density was not significantly different between ELS-exposed or control siblings after just one week of stress exposure, whereas a significant reduction was observed in animals exposed to ELS until 30 dpf (~25%) that persisted even after a full 7d washout (~20% reduction compared to control siblings). While it is possible that the modest growth delay observed at 30 dpf (Fig. 2) could be a contributing factor, the magnitudes of differences observed do not directly compare (~25% neuronal density reduction versus ~10% difference in SL) and do not explain the persistent reduction observed after 1 week of washout when no significant difference in SL was observed. Future studies will address whether the reduced enteric neuronal density observed in ELS-exposed animals is a consequence of *bona fide* enteric neuron death or a delay in maturation and development from precursor populations.

At the molecular level, significant expression differences in genes associated with the nervous system and neuro-regulation either preceded (*s100b, mthfr*) or co-occurred with (*dmnt1*, *casp3*, and *ptenb*) the changes we observed in enteric neuronal density (Figs. 3 and 4). First, we observed an early upregulation of enteric *s100b* expression after 1 week of stress that was not observed after chronic stress in the gut or brain (Fig. 3). Although the totality of its functions and mechanisms of action are incompletely understood, S100B is a well-known biomarker of brain injury (Goyal et al., 2013) with concentration- and time-dependent neurotoxic, neuroprotective, or neurotrophic properties (Langeh and Singh, 2021; Willoughby et al., 2004; Rodrigues et al., 2022). Clinically, increased serum S100B has been reported in

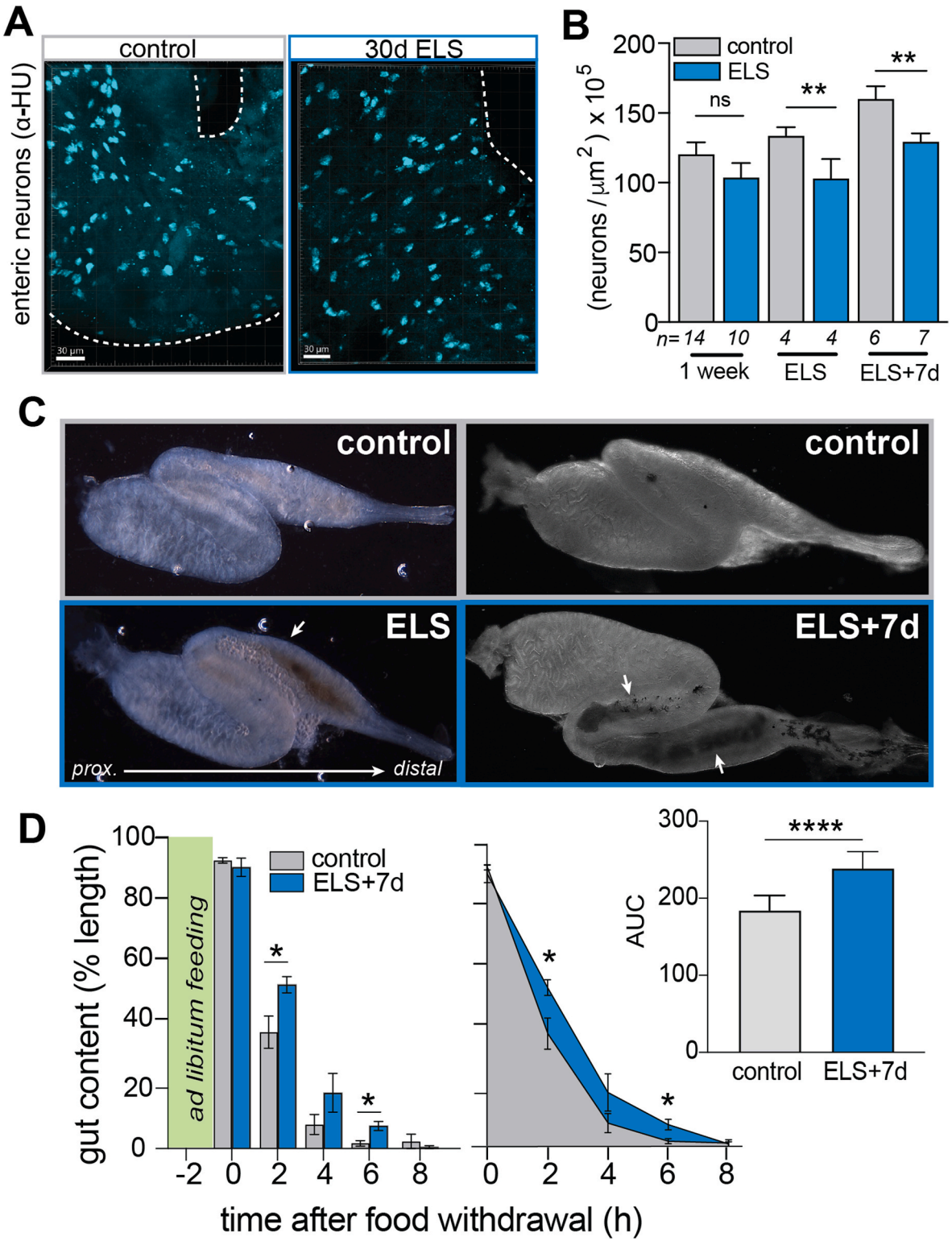

Fig. 4. Chronic ELS reduces enteric neuronal density and impedes gut transit. (**A**) Representative confocal images of whole mount gut tissue labeling enteric neuron bodies following chronic ELS (until 30 dpf). Scale bar = 30 µm. Dotted lines show boundary of gut tissue. (**B**) Quantification of enteric neuron density after 1 week (left), until 30 dpf, and until 30 dpf and following a 7-day washout (right) ELS compared to stage-matched control siblings (grey bar). $^{**}p < 0.01$ by Mann-Whitney *U* Test. Number of subjects (*n*=) is denoted on graph. (**C**) Representative wide field images of *ex vivo* gut tissue following chronic ELS (until 30 dpf, left) and after a 7d washout (ELS +7d, right) and compared to stage-matched control siblings. Short arrows regions of gut content. (**D**) Gut content (expressed as % of total gut length) following ad libitum feeding at the indicated times following food withdrawal. Data are expressed as comparisons between groups (left; control = grey bar; ELS +7d = blue bar) and area under the curve (right and inset, AUC). $^{*}p < 0.05$; $^{****}p < 0.0001$ by two-tailed *z*-test, $n = 19$ subjects per group (total of 38 individuals); $n = 3$–7 subjects per group per time point. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

adolescent patients with a history of childhood trauma (Falcone et al., 2015). Analogous to astrocytic production in the brain, S100B is largely produced by enteric glial cells (EGC) in the gut (Cirillo et al., 2011) and has been associated with gut inflammation (Di Liddo et al., 2020). Along these lines, 'activated' EGC, which are partially characterized by upregulation of S100B (Chow and Gulbransen, 2017), appear to trigger neurodegenerative processes (Seguella et al., 2019), but are also critical for neuronal maintenance, survival, and function (De Giorgio et al., 2012). Further, mounting evidence suggests that EGC (or their precursors) can serve as a source of neural progenitors (Laranjeira et al., 2011; McCallum et al., 2020; Kuil, NWindster, Bindels, Zink, van der Zee, Hofstra, Shepherd, Melotte, Alves, Administrator) and recent work has revealed a previously uncharacterized ENS progenitor population in zebrafish that gives rise to *s100b*-expressing EGC homologous to mammals (Kuil, NWindster, Bindels, Zink, van der Zee, Hofstra, Shepherd, Melotte, Alves, Administrator). Although we did not determine the cellular source of *s100b* in this study, our data suggest that *s100b* is involved in the early stress response prior to overt neuronal density changes in the gut. Follow up studies are needed to determine whether this increase in *s100b* is a result of progenitor skewing thereby limiting neuronal differentiation and maturation, EGC activation causing neurotoxicity, or a compensatory mechanism to prevent neuronal death that is eventually overwhelmed. In addition, we also observed a significant increase in the expression of two pleiotropic genes, *casp-3* and *ptenb,* in both the brain and gut after chronic ELS (Fig. 3). While both genes have been implicated in neuronal apoptosis and neurodegenerative disease (Ismail et al., 2012; Rideout and Stefanis, 2001), cell-death independent roles have also been described (D'Amelio et al., 2012). Here, caspase activation has also been shown to maintain progenitor quiescence in the gut (Arthurton et al., 2020) and are engaged during normal developmental pruning and neuronal turnover in the brain (Sokolowski et al., 2014). Taken together, our data suggest that – like the developing brain – ELS may also modulate neurogenesis in the enteric nervous system with effects on tissue (gut) functioning.

4.1. Limitations

Though this study presents intriguing and innovative findings, several limitations exist. First, we used a novel tank paradigm to assess anxiety-like behavior of larval zebrafish. While the data support our conclusion that the chronic ELS protocols adapted for use in this study did indeed illicit anxiety-like behaviors, we did not perform a complete characterization of behaviors nor did we assess behavior in other behavioral tests, such as the light-dark preference test (Magno et al., 2015). Relatedly, while we did assess some measures including behavior, standard length, and gut transit using following a complete 7-day washout, we did not conduct transcriptional assessments on these samples, nor did we interrogate behavioral or biological changes beyond a 7-day washout period. Recent studies have shown that zebrafish subjected to stressors between 4 and 6 dpf develop long-lasting anxiety dependent on glucocorticoid signaling (Chin et al., 2022). Given that our stress paradigm was initiated within this window of development, we anticipate that some behavioral changes will be long-lasting, and the sustained durability of behavioral responses to our chronic ELS paradigm is the subject of ongoing investigation.

In this study, we tested the hypothesis that neuroimmune genes with known or suspected central nervous system involvement in stress responses would also be altered in the gut, which contains the enteric nervous system. While we did observe significant transcriptional differences in key neuroimmune genes of interest, the mechanistic relevance of these changes is unclear; further, whether changes in some, or multiple, of these genes are directly responsible for the differences in motility observed is yet to be determined. Finally, the selection of only a subset of neuroimmune genes of interest represents a limitation in this study and ongoing work to conduct unbiased transcriptional analyses is forthcoming. Lastly, standardized protocols for the assessment of gut transit in zebrafish at the late larval stage (30 dpf) do not exist. To overcome this challenge, we developed a novel assay to assess transit of gut content which included the fabrication of a custom in-tank steel mesh insert. Given that this work is the first reported data using this assay, validation of this protocol will require follow-up study and implementation by other investigators.

5. Conclusions

Here, we introduce a new model of chronic ELS that commences at the onset of exogenous feeding and free swimming and extends throughout larval zebrafish development. Our findings demonstrate that chronic ELS impacts the transcription of neuroimmune genes in a tissue-dependent manner and has lasting consequences on gut function. This work advances our understanding of how "the body keeps the score."

Author contributions

Conceptualization, Experimental Design, Manuscript - original draft - C.L.G.; Experimentation, Data Analysis - C.L.G., E.N., D.T., O.K., Y.S., A.C., and A.S.Z.; Manuscript - editing and review – C.L.G. and A.S.Z.; Project Administration, Funding, Oversight, - C.L.G. and S.M.W.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2023.100655.

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