Npas4a expression in the teleost forebrain is associated with stress coping style differences in fear learning

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Learning to anticipate potentially dangerous contexts is an adaptive behavioral response to coping with stressors. An animal's stress coping style (e.g. proactive–reactive axis) is known to influence how it encodes salient events. However, the neural and molecular mechanisms underlying these stress coping style differences in learning are unknown. Further, while a number of neuroplasticity-related genes have been associated with alternative stress coping styles, it is unclear if these genes may bias the development of conditioned behavioral responses to stressful stimuli, and if so, which brain regions are involved. Here, we trained adult zebrafish to associate a naturally aversive olfactory cue with a given context. Next, we investigated if expression of two neural plasticity and neurotransmission-related genes (npas4a and gabbr1a) were associated with the contextual fear conditioning differences between proactive and reactive stress coping styles. Reactive zebrafish developed a stronger conditioned fear response and showed significantly higher npas4a expression in the medial and lateral zones of the dorsal telencephalon (Dm, Dl), and the supracommissural nucleus of the ventral telencephalon (Vs). Our findings suggest that the expression of activity-dependent genes like npas4a may be differentially expressed across several interconnected forebrain regions in response to fearful stimuli and promote biases in fear learning among different stress coping styles.

Animals frequently must overcome stressors and the ability to encode and recall these salient experiences is essential to an individual's survival. Within individuals, behavioral and physiological responses to stressors often co-vary, belonging to correlated suites of traits that are consistent across contexts and time1–4 (i.e. animal personality, stress coping styles; bold-shy axis, proactive–reactive axis). In addition to boldness, aggression, and stress physiology, studies demonstrate that proactive and reactive individuals also differ in learning and memory processes5–9. The more risk-prone proactive individuals tend to show faster acquisition of memories that require higher levels of activity, or paradigms with positive and rewarding valence10–16. In contrast, the risk-averse reactive individuals tend to show faster acquisition of aversive paradigms that require avoidance or reduced levels of activity17–19. Despite these findings, the neuromolecular mechanisms and regional brain activity underlying these stress coping style differences in learning are not well understood.

Recent work has suggested that neural plasticity and neurogenesis may be key mechanisms underlying divergent proactive–reactive responses to stress, but whether these processes are associated with differences in learning and memory is not understood10,21. While previous studies have characterized the whole-brain transcriptome of proactive and reactive individuals at baseline, the contribution of specific neural plasticity- and synaptic transmission-related candidate genes and their spatial expression patterns have yet to be examined during a learning and memory task22,23. Two particularly interesting candidate genes, npas4 and gabbr1 (npas4a and gabbr1a in teleosts) are essential in regulating neuronal excitability and molecular processes related to learning and memory such as long-term potentiation24–26. npas4 is an immediate early gene transcription factor that is predominantly expressed in the brain and enriched in the limbic regions. It is expressed through calcium signaling and is thought to induce primarily GABAergic inhibitory synapses in response to excitation and play an important role in homeostatic plasticity25. gabbr1 codes for a metabotropic GABA B receptor, which has also been shown to play an important role reducing neuronal excitability through G-protein signaling-dependent slow, long lasting hyperpolarization of postsynaptic cells. Further, deletion or altered expression of both of these genes has been shown to cause abnormal synaptic plasticity, neurogenesis, and impaired learning and memory.

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genes primarily related to neurometabolism and neurotransmission22,38–40. Additionally, these divergent behaviors between the strains are consistent across contexts and over time and have high repeatability36,41,42. During testing, fish were individually housed in 3-L tanks on a recirculating water system (Pentair Aquatic Eco-Systems) using UV and solid filtration on a 14:10 L/D cycle at a temperature of 27 °C. Fish were fed twice a day with Tetramin Tropical Flakes (Tetra, USA). All procedures were approved by the Institutional Animal Care and Use Committee of University of Nebraska at Omaha/University of Nebraska Medical Center (17-070-00-FC, 17-064-08-FC). All experiments were performed in accordance with relevant guidelines and regulations.

**Methods**

**Subjects.** Zebrafish are utilized in a variety of laboratory studies to understand the neural, genetic, and pharmacological mechanisms of learning and memory43–46. Both wild and laboratory strains of zebrafish display the proactive and reactive stress coping styles, which have distinct genetic architectures and neuroendocrine responses27,28,46. Here we used the high-stationary behavior (HSB; reactive) and low-stationary behavior (LSB; proactive) zebrafish strains to study the association between npas4a and gabbr1a expression and fear learning differences between proactive and reactive stress coping styles. Starting from wild-caught zebrafish, the HSB and LSB strains were generated and are maintained by artificial selection for opposing amounts of stationary behavior in the open field test36. Specifically, we bred fish together that spent at least 66.7% exhibiting stationary behavior (200 s out of a 300 s trial, fish did not exceed speed of 0.1 cm/s) during the trial period to produce a High Stationary Behavior (HSB) line and bred those spending at most 16.7% time displaying stationary behavior to produce a Low Stationary Behavior (LSB) line. Consistent with proactive (LSB) and reactive (HSB) stress coping styles, HSB fish show elevated behavioral and cortisol stress responses compared to LSB fish36,46. LSB fish developed larger caudal regions and faster swimming performance37. Whole-brain transcriptomic differences included genes primarily related to neurometabolism and neurotransmission22,38–40. Additionally, these divergent behavioral profiles between the strains are consistent across contexts and over time and have high repeatability36,41,42.

We trained fish from the HSB and LSB strains to associate alarm substance exposure with a context in one training trial, followed by a second assessment trial in the absence of the alarm substance. We then quantified npas4a and gabbr1a forebrain expression to investigate their potential link with differences in conditioned fear responses between alternative stress coping styles. We predict that an increased conditioned fear response in reactive zebrafish will be associated with increased expression of neural plasticity-related genes in the dorsal and medial portions of the dorsal telencephalon (Dm, Dl) and the dorsal, ventral, and supracommissural portions of the ventral telencephalon (Vd, Vv, Vs), putative homologues of the mammalian basolateral amygdala, hippocampus, striatum, lateral septum, and bed nucleus of the stria terminalis, respectively36–39.

**Alarm substance.** We created a single batch of alarm substance as previously described47. In brief, 20 randomly selected donor fish (wild type) were euthanized by rapid chilling followed by light abrasion of lateral skin cells on one side of each donor fish, ensuring that no blood was drawn. Donor bodies were then individually soaked in 10 mL of DI water for 10 min. A total of 200 mL was filtered, diluted in half, and stored in aliquots at –20 °C until use.

**Contextual fear learning.** To test learning, we utilized a validated contextual fear conditioning paradigm30. Briefly, zebrafish were tested individually in a 16 × 16 × 10 cm arena filled with 1.4 L of system water. The arena was surrounded by opaque white plastic on the bottom and sides to serve as the contextual stimulus. Animals were removed from group housing and placed into individual housing 72 h prior to the training session. Each learning trial was 15 min long and was divided into three subsections. Fish acclimated to the chamber for the
first five minutes, followed by five minutes of recording pre-exposure behavior (conditioned fear response for second trial). After these 10 min, 1 mL of alarm substance (AS) or distilled water (DI) was administered into the water through plastic tubing that came from outside of the testing arena. Following alarm substance exposure, the unconditioned fear response was recorded for five minutes. Between trials, fish were placed back into their individual housing, the testing arenas were rinsed out, and were refilled with 1.4 L of fresh system water. Fish underwent two training trials with 30 min between trials. The second training trial was stopped after the second five minute block (conditioned response). Fish immediately had their forebrains removed or were decapitated and frozen on dry ice and stored at ~80 °C for qPCR and ISH, respectively. We selected the second trial for gene expression analyses because we previously showed that out of four training trials, the second trial was both the earliest trial and one that resulted in the most prominent proactive–reactive behavioral differences during fear conditioning before both lines achieved similar conditioned responses. These differences during training were also associated with stronger fear memory recall 96 h following training50.

Total sample sizes consisted of 46 LSB (N = 28 males, 18 females) and 46 HSB (N = 28 males, 18 females) individuals. Of this total, we used 10 HSB individuals (N = 5 AS, 5 DI, all males) and 10 LSB individuals (N = 5 AS, 5 DI, all males) for qRT-PCR analysis. We used the remaining fish for ISH analysis. A total of 12 LSB (N = 6 males, 6 females) and 12 HSB (N = 6 males, 6 females) individuals received alarm substance CS-US reinforcements as the experimental group. For the DI water control group, we used 12 HSB (N = 6 males, 6 females) and 12 LSB (N = 6 males, 6 females) fish. To control for possible effects of the paradigm and handling, independent of treatment group, 12 HSB (N = 6 males, 6 females) and 12 LSB (N = 6 males, 6 females) were habituated to the same single housing as other groups, but did not undergo behavioral testing.

**Behavioral analysis.** All trials were video-recorded from above and later analyzed with Noldus Ethovision XT (Noldus XT, Wageningen, Netherlands). For each trial, we quantified freezing time as an indicator of the conditioned response. We examined freezing because it is one of the most consistent and conserved behaviors used to assess stress-related behaviors and fear learning and memory47. Additionally, freezing was the most reliable indicator of proactive–reactive differences in contextual fear conditioning in our prior study30. The subject was considered frozen if it moved less than 0.5 cm/s.

**qRT-PCR.** Preparation, execution, and analysis of the qRT-PCR of forebrain *npas4a* and *gabbr1a* expression followed previously established methods38,39. Gene expression was normalized to an endogenous housekeeping gene, *ef1a*, which has shown to be stable across sex, age, and chemical treatment in zebrafish48. See the supplemental methods for detailed parameters.

**ISH.** Brain samples were sectioned on a cryostat at 16 µm onto four serial series. Tissue fixation parameters, probe synthesis, and ISH conditions were based on established protocols49,50. We used digoxigenin (DIG)-labeled probes for *Npas4a* and *Gabbr1a* genes. All individuals were processed simultaneously (one gene at a time) to avoid any potential colorimetric development differences across individuals due to batch effects. Riboprobes showed specific binding with high expression using the antisense probe, proportionally reduced expression in the 1:25 cold-competition condition, and no expression in the sense and no probe conditions (Fig. S1). See supplemental methods for detailed parameters.

**Brain region analysis.** Brain section images were captured at 4X using a Nikon Eclipse monochrome camera (Q12). For each brain region, we used Nikon NIS Elements Version 4.6 software to measure a standardized rectangular box within the borders of each brain region and measured the mean intensity of *npas4a* and *gabbr1a* expression within the box. Brain regions were defined using a published teleost brain atlas and are displayed in Fig. S251. The researcher (M.R.B.) was blinded to the treatment and strain conditions when collecting and analyzing images. We quantified gene expression by measuring optical density (OD) of the digoxigenin labeled probes, an established semi-quantitative measure of gene expression in other systems49. For each slide, we normalized the mean intensity of all measures to the background (mean intensity of slide area not containing tissue), which produced a fractional transmittance value for each brain region in each section. Fractional transmittance was mathematically converted to optical density by the equation $\text{OD} = 2 - \log(\text{fractional transmittance})$. See supplemental methods for additional details.

**Statistics.** All statistics were performed using SPSS software (Version 24). To analyze freezing behavior we used a repeated measures two-way ANOVA with strain and treatment group as between-subjects factors. For analyzing qRT-PCR gene expression we used a multivariate general linear model (GLM) with normalized *npas4a* and *gabbr1a* expression as dependent variables, and strain and treatment as between-subject factors. For analysis of ISH OD measurements we used a multivariate GLM with the OD of the five brain regions as dependent variables and strain and treatment group as between-subjects factors. There were not any effects of sex on learning and memory in a previous nor the current study (3-way repeated measures ANOVA: $F_{\text{sex x trial}} = 0.40 \ p = 0.531; \ F_{\text{sex}} = 0.57 \ p = 0.456$), so we removed sex as a variable to simplify the model30. Individual groups were compared with simple effects testing. To account for multiple comparisons we applied the Benjamini–Hochberg correction to determine significance52. For all significant differences ($p < 0.05$) we also report the effect sizes (Cohen’s d (d)) for t-tests and partial eta-squared ($\eta_p^2$) for ANOVAs53. All effect sizes were medium or large effects53–55.
Ethical approval. The manuscript has been reviewed and approved by all listed authors for publication. All procedures were approved by the Institutional Animal Care and Use Committee of University of Nebraska at Omaha/University of Nebraska Medical Center (17-070-00-FC, 17-064-08-FC).

Results

Contextual fear learning. In the conditioned fear response period during acquisition testing, there was a significant trial × treatment group interaction effect for freezing ($F_{1, 64} = 54.86, p = 3.59 \times 10^{-10}, \eta^2_p = 0.46$). The alarm substance group showed increased freezing between trials at a faster rate than the DI control group (Fig. 1). Additionally, there was a significant trial × strain × treatment group interaction ($F_{1, 64} = 5.88, p = 0.018, \eta^2_p = 0.08$) where treated HSB fish increased freezing behavior at a faster rate than LSB fish. HSB fish exposed to alarm substance froze significantly more than LSB fish at trial two ($t(32) = 4.23, p = 1.81 \times 10^{-4}, d = 1.45$), but did not significantly differ at trial one ($t(32) = 1.05, p = 0.303$). Full model results are presented in Table 1.

qRT-PCR. There was a significant effect of strain on both $npas4a$ ($F_{1, 16} = 11.72, p = 0.003, \eta^2_p = 0.42$) and $gabbr1a$ ($F_{1, 16} = 7.29, p = 0.016, \eta^2_p = 0.31$) forebrain expression. There was a significant effect of treatment for $npas4a$ ($F_{1, 64} = 5.88, p = 0.018, \eta^2_p = 0.08$) where treated HSB fish increased freezing behavior at a faster rate than LSB fish. HSB fish exposed to alarm substance froze significantly more than LSB fish at trial two ($t(32) = 4.23, p = 1.81 \times 10^{-4}, d = 1.45$), but did not significantly differ at trial one ($t(32) = 1.05, p = 0.303$). Full model results are presented in Table 2. In HSB fish, $npas4a$ gene expression was significantly higher in the AS group compared to the DI group ($p = 0.003, d = 2.34$; Fig. S3). There were no effects of treatment on $npas4a$ expression in LSB fish ($p = 0.918$).

In situ hybridization. Treatment effects on $npas4a$ OD. There was a significant effect of treatment condition on $npas4a$ OD in the Dm ($F_{2, 66} = 6.20, p = 0.003, \eta^2_p = 0.16$), DI ($F_{2, 66} = 7.13, p = 0.002, \eta^2_p = 0.18$), Vv ($F_{2, 66} = 3.38, p = 0.040, \eta^2_p = 0.09$), and Vs ($F_{2, 66} = 3.93, p = 0.024, \eta^2_p = 0.11$). In the Dm, $npas4a$ OD was signifi-
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F group interaction effects in the Dm (impairs the formation of new fear and contextual memories33,58–60. Our findings suggest that plays a similar role in learning and memory as its mammalian homolog, npas4a found that and integral to fear learning and memory across species32–35. Similar to rodents, lesioning the teleost Dm and Dl

| Strain | npas4a F (p, ηp²) | gabbr1a F (p, ηp²) |
|--------|------------------|-------------------|
| Intercept | 393.93 (1.08 × 10⁻¹², 0.96) | 364.98 (1.94 × 10⁻¹², 0.96) |
| Strain | 11.72 (0.003, 0.42) | 7.29 (0.016, 0.31) |
| Treatment | 11.72 (0.003, 0.42) | 4.30 (0.055) |
| Strain × Treatment | 2.32 (0.147) | 3.88 (0.066) |

Table 2. Results of multivariate GLM for forebrain expression of npas4a and gabbr1a from qPCR. Bold text indicates p < 0.05.

| Strain effects on npas4a OD. There was a significant main effect of strain on the OD of npas4a in the Dm (F₁,₆₆ = 7.66, p = 0.007, ηp² = 0.10), DI (F₁,₆₆ = 8.82, p = 0.004, ηp² = 0.12), and Vv (F₁,₆₆ = 5.16, p = 0.026, ηp² = 0.07). HSB fish overall had higher OD of npas4a in each of the three brain regions. Additionally, HSB fish exposed to AS had significantly higher npas4a OD compared to LSB fish exposed to AS in the Dm (p = 0.001, d = 1.25), DI (p = 0.001, d = 1.65), and Vv (p = 0.039, d = 0.65; Fig. 2A–C). Full model results are presented in Table 3. Representative raw images for npas4a expression in the Dm, DI, and Vs are shown in Figs. 3, 4, and 5. Npas4a expression in the Vv and Vd are shown in Figs. S5–S6.

| Strain specific treatment effects on gabbr1a OD. For gabbr1a OD, there were significant strain × treatment group interaction effects in the Dm (F₁,₆₆ = 3.31, p = 0.043, ηp² = 0.09), Vv (F₁,₆₆ = 7.70, p = 0.001, ηp² = 0.19), Vd (F₁,₆₆ = 6.95, p = 0.002, ηp² = 0.17), and Vs (F₁,₆₆ = 3.89, p = 0.025, ηp² = 0.11). For each of those regions, there were no significant differences between any treatment groups for HSB fish. However, for LSB fish the DI group had significantly lower gabbr1a OD compared to the BL (p = 0.003, d = 1.37) and AS (p = 0.024, d = 1.00) groups in the Dm, BL (p = 0.023, d = 1.02) and AS (p = 0.003, d = 1.60) groups in the Vs, and the BL (p = 0.015, d = 1.06) and AS (p = 0.003, d = 1.37) groups in the Vv (Fig. S7). The BL group had a significantly higher gabbr1a OD compared to the DI (p = 0.003, d = 1.71) and AS (p = 0.030, d = 0.99) groups in the Vs. Full model results are presented in Table 4. Representative raw images for gabbr1a expression across all of the brain regions and treatment groups are shown in Figs. S8–S12.

Discussion
Expression of neural plasticity-related genes (e.g. npas4, gabbr1a) has been broadly implicated as a key process underling activity-dependent expression of npas4a, gabbr1a in reactive fish relative to proactive (LSB) individuals (Fig. 1)30. Further, we found that npas4a expression was significantly higher in several key forebrain regions of reactive zebrafish. Altogether, our findings suggest that npas4a plays a similar role in learning and memory as its mammalian homolog, and may be an important regulator of proactive–reactive differences in learning and memory.

ISH analysis showed that npas4a expression was significantly higher in reactive fish in the Dm, DI, and Vs (Fig. 2A–C). The Dm (BLA), DI (hippocampus), and Vs (BNST) are key sites of experience-dependent plasticity and may be an important regulator of proactive–reactive differences in learning and memory across species32–35. Similar to rodents, lesioning the teleost Dm and DI impairs the formation of new fear and contextual memories35,38–40. Our findings suggest that npas4a-dependent plasticity within these brain regions may be a key underlying mechanism regulating differences in fear learning and memory capabilities between stress coping styles. In a prior study using the same conditioning paradigm, we showed that reactive zebrafish acquired a conditioned fear response faster than proactive zebrafish28. The higher activity-dependent expression of npas4a in reactive individuals observed in this study may promote higher levels of neural plasticity, resulting in salient and fearful experiences to be encoded into memory more quickly28,61. We predict that npas4a knockout experiments would produce similar learning and memory deficits as in rodents, and are needed to establish a direct causal role in zebrafish. More recently, specific glutamatergic populations of Dm cells have been shown to be required for fear conditioning33. Our study is not able to distinguish between cell types expressing npas4a, which would be an important future direction to better characterize the specific circuits regulating proactive–reactive differences in learning. In selectively bred proactive and reactive trout, the Dm and DI regions have also been shown to display differing monoaminergic and cortisol responses to acute stress62,63. This suggests that higher expression of npas4a in these brain regions may play important roles in constraining variation across a number of behavioral contexts.

While the BNST has been shown to be important for aversive learning in rodents64,65, the function of the Vs and specifically of npas4a expression in the Vs is not well understood in regards to learning and memory.
We found that similar to the Dm and Dl, npas4a expression within the Vs is likely important for fear learning, and is associated with differences between proactive and reactive stress coping styles. Supporting this, a previous study found that increased activity and npas4 expression in a population of corticotropin-releasing factor neurons in the BNST was associated with increased stress resiliency and prevention of a post-traumatic stress disorder-like phenotype in rodents[66]. This suggests that npas4a expression in the Vs may play an important role in how individuals experience and cope with stress differently. Interestingly, the Vs has been shown to have connections with both the Dm and Dl, and to the hypothalamus and other brainstem areas that are essential

**Figure 2.** Expression of npas4a in the Dm (A), Dl (B), and Vs (C). We measured expression of high stationary behavior (HSB) and low stationary behavior (LSB) fish at baseline (BL) or exposed to either alarm substance (AS) or distilled water (DI) during training. Bars represent mean ± 1 SE. Bars labeled with different letters indicate p < 0.05. × Indicates a significant strain main effect. ^Indicates a significant within-treatment group strain difference.
for eliciting behavioral and endocrine stress responses. While this study only assessed gene expression across select forebrain structures, future studies should investigate other downstream structures and consider the role of glucocorticoids and the hypothalamus–pituitary–adrenal axis (hypothalamus-pituitary-interrenal in teleosts). This is particularly promising as glucocorticoid differences have been well-characterized between proactive and reactive stress coping styles, though to a lesser extent related to learning and memory.

The DI treatment groups showed significantly lower npas4 expression compared to the AS treatment group in the Dm, Dl, and Vs (Fig. 2a,c). This suggests npas4 is expressed in a treatment-specific manner associated with the learned conditioned fear response in the AS group. Unexpectedly, npas4 expression in the DI group

| Region | F(p, ηp²) | F(p, ηp²) | F(p, ηp²) | F(p, ηp²) | F(p, ηp²) |
|--------|-----------|-----------|-----------|-----------|-----------|
| Dm     | 266.15 (4.36 × 10⁻⁴, 0.80) | 236.22 (4.36 × 10⁻⁴, 0.78) | 295.70 (4.36 × 10⁻⁴, 0.82) | 282.1 (4.36 × 10⁻⁴, 0.81) | 286.57 (4.36 × 10⁻⁴, 0.81) |
| Dl     | 7.66 (0.007, 0.10) | 8.82 (0.004, 0.12) | 5.16 (0.026, 0.07) | 0.77 (0.383) | 2.64 (0.109) |
| Vv     | 6.20 (0.003, 0.16) | 7.13 (0.002, 0.18) | 3.38 (0.040, 0.09) | 1.61 (0.208) | 3.93 (0.024, 0.11) |
| Vd     | 1.78 (0.177) | 3.02 (0.055) | 0.91 (0.406) | 4.51 (0.015, 0.12) | 1.59 (0.212) |
| Vs     | 1.78 (0.177) | 3.02 (0.055) | 0.91 (0.406) | 4.51 (0.015, 0.12) | 1.59 (0.212) |

Table 3. Results of multivariate GLM of npas4 optical density across the five forebrain regions. Bold text indicates p < 0.05.

Figure 3. Representative images of Npas4a expression in the Dm for low stationary behavior (LSB) and high stationary behavior (HSB) fish at baseline (BL) or exposed to either alarm substance (AS) or distilled water (DI) during training.
was significantly lower than the BL group in the Dm and Vs. Other studies have found that acute injection of corticosterone or chronic restraint and social isolation stressors can decrease npas4 expression in the rodent prefrontal cortex and hippocampus and lead to a variety of behavioral deficits including learning and memory71–73. It is unclear whether this decrease in expression is maladaptive, or whether it is an adaptive homeostatic response to stress74. It is unlikely that our results can be explained by physical isolation, as the baseline group was also socially isolated for the same duration. However, it is possible that handling stress could explain the reduction in npas4 expression for the DI group.

It is important to note that the relationship between npas4 expression and behavior could also represent stress coping style differences to an acute stress response, given that the assessment trial for conditioned fear response is roughly 30 min following the first exposure. Some studies have shown differences in freezing post-alarm substance exposure36, though we did not find any unconditioned fear response differences with the current paradigm. While this could partially explain our findings, our previous study using similar repeated alarm substance exposure showed that proactive and reactive zebrafish developed a conditioned fear memory that was context-specific and persisted for at least 4 days30.

Figure 4. Representative images of Npas4a expression in the Dl for low stationary behavior (LSB) and high stationary behavior (HSB) fish at baseline (BL) or exposed to either alarm substance (AS) or distilled water (DI) during training.

While qRT-PCR findings showed strain effects in gabbr1a expression, there were no strain differences in any of the analyzed brain regions for the ISH analysis. This suggests that the strain differences in forebrain gabbr1a expression are driven by other brain regions not investigated in this study. Therefore, gabbr1a expression within the Dm, Dl, Vv, Vs, and Vd does not appear to be associated with development of a conditioned fear response. Other studies have suggested that GABAergic signaling may be more important for consolidation, reconsolidation, or extinction of fear memories35. Future studies should assess how GABA B receptor expression may influence other phases of fear conditioning, or other paradigms using positive reinforcement.
Learning to predict and cope with potentially dangerous environments is essential to an individual's survival. Proactive and reactive stress coping styles represent alternative strategies for coping with stress and differ in a number of behavioral contexts, including learning and memory. Our study suggests that brain-region specific expression patterns of *npas4a* may underlie differences in fear learning between proactive and reactive stress coping styles. These findings advance our understanding of the neuromolecular mechanisms underlying stress-coping style differences in cognition and highlight neuroplasticity's key role in regulating alternative adaptive behavioral responses to stress. Additionally, as proactive and reactive individuals share potentially conserved mechanisms underlying other stress coping behaviors, this suggests that these brain regions may also constrain behavioral variation in a number of disparate contexts.

**Figure 5.** Representative images of *Npas4a* expression in the Vs for low stationary behavior (LSB) and high stationary behavior (HSB) fish at baseline (BL) or exposed to either alarm substance (AS) or distilled water (DI) during training.

|       | Dm    | Di    | Vv    | Vd    | Vs    |
|-------|-------|-------|-------|-------|-------|
|       | F(p, np²) | F(p, np²) | F(p, np²) | F(p, np²) | F(p, np²) |
| Intercept | 121.69 (1.27 × 10⁻¹⁶, 0.65) | 107.89 (1.61 × 10⁻¹⁵, 0.62) | 147.75 (1.68 × 10⁻¹⁸, 0.69) | 153.60 (6.87 × 10⁻¹⁹, 0.70) | 134.16 (1.49 × 10⁻¹⁷, 0.67) |
| Strain  | 0.44 (0.509) | 2.91 (0.093) | 0.166 (0.685) | 0.59 (0.444) | 0.12 (0.736) |
| Treatment | 3.28 (0.044, 0.09) | 2.69 (0.076) | 1.52 (0.227) | 0.91 (0.410) | 5.88 (0.004, 0.15) |
| Strain x treatment | 3.31 (0.043, 0.09) | 1.51 (0.229) | 7.70 (.001, .19) | 6.95 (0.002, 0.17) | 3.89 (0.025, 0.11) |

**Table 4.** Results of multivariate GLM of gabbr1a optical density across the five forebrain regions. Bold text indicates $p < 0.05$. 
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Author contributions
M.R.B. and R.Y.W. conceived and designed the experiments, and wrote the manuscript. M.R.B. collected and analyzed the data.
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Competing interests
The authors declare no competing interests.

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