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The Neuroinflammatory Effects of Chronic Unpredictable Stress on Zebrafish, Danio rerio

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This thesis is dedicated to Wydene Stewart & Rosa Finney.

Contents

ABSTRACT

Stress is a state of threatened homeostasis counteracted by various physiologic and behavioral responses aimed to maintain or restore balance. As such, stress acts as a motivator to perform during the challenges of life to survive. Chronic perturbations to the stress response homeostasis without relief can lead to dysregulation, thus attenuating organ systems and structures and causing significant damage {1]. Individuals who undergo psychological trauma endure an acute and transient experience, which results in minimal functional impairment, but some suffer from a chronic condition called posttraumatic stress disorder (PTSD). Individuals who have PTSD are likely to experience intense stress, fear, anxiety, and helplessness, resulting in a permanent or temporary psychological wound characterized by physical, cognitive, emotional, or behavioral changes. In this study, we will be exploring the physiologic and behavioral effects of chronic stress on functionally distinct brain areas related to reward and aversion, the neuromodulator dopamine (DA), and DA's critical role in mediating behaviors used to meet survival needs.

In this study, we used the zebrafish to model PSTD by implementing a chronic unpredictable stress paradigm to simulate a traumatic experience. We measured behavior differences using an anxiety-like behavior assay, the Light-Dark Preference Test, and attempted to validate our findings using immunohistochemistry and microscopy to observe brain changes in regions of interest involving aversion. Though experimental zebrafish did respond to stressful stimuli, exhibiting typical anxiety-like behaviors, there was no significant difference amongst our groups. Multiple behaviors were present but unquantifiable due to experimental error. Additionally, we found there to be no significant difference in the effect of PTSD on the brain. However, post ad-hoc tests indicated individual differences amongst experimental groups for the average time in the light compartment statistic, the number of crosses into the light compartment statistic, and the single pairwise difference in tyrosine hydroxylase (TH) expression, suggesting that stress still may induce anxiety behaviors and affect the neurocircuits that modulate stress. These outliers prompt additional trials with larger sample sizes.

I: Introduction

Stress

All organisms encounter dangers to homeostasis, which must be met with adaptive responses to survive. This homeostatic equilibrium is regularly challenged by antagonistic stimuli that present intrinsically or extrinsically, real or perceived. These stimuli are known as stressors, the cause of an autonomic response that produces physical or mental tension as an effect of an environmental, biological, or psychological barrier. Thus, stress is a state of threatened homeostasis counteracted by various physiologic and behavioral responses aimed to maintain or restore balance. As such, stress acts as a motivator to perform during the challenges of life to survive [1].

Animals with a limbic system, a complex system of nerves and networks in the brain that controls basic emotions, express what is known as the classic "fight-or-flight" response [2]. This acute stress response is an interplay between connected neuroendocrine, cellular, and molecular infrastructures. It is the effect of a cascade of hormones that influence the secretion of norepinephrine, epinephrine, dopamine, cortisol, and other messengers. These chemical messengers' signals affect several organ systems; elevating heart rate, blood pressure, influencing digestive function, increasing muscle tension, suppressing the immune response, and many other effects. Chronic perturbations to the stress response homeostasis without relief can lead to dysregulation, thus attenuating organ systems and structures and causing significant damage [1]. Research suggests that chronic stress can bring about or worsen disease and disease symptoms, linking stress to cardiovascular disease, coronary heart disease, exacerbation of autoimmune diseases, and elevation of pro-inflammatory cytokines that may adversely affect the mental health of susceptible individuals [3,4]. This thesis aims to explore the physiologic and behavioral effects of chronic stress on multiple areas of the brain.

The Mesocorticolimbic Pathway

The brain is the most complex organ in the body. It is a network made of billions of neurons that acts as the center of activity. It regulates heart rate and respiration, controls the fine motor skills required to write or draw, and governs the compounding functions that summate one's ability to read. The brain is the integrator between perception and reality. From its evolution, stress has turned its reinforcement pathway for survival into a conduit for pleasure and reward as well as fear and aversion. The impact of chronic stress on the brain presents in various ways, leading to neurodegeneration, loss of control over fine motor skills, behavioral maladaptation, and the production of negative consequences such as general anxiety disorder and posttraumatic stress disorder [5].

This study of biological responses to stress will focus on two regionally and functionally distinct areas related to reward and aversion: the mesocortical pathway and the mesolimbic pathway, each of which plays a different role in affecting neural circuits; governing reward, memory, motivation, and higher-order cognitive control. Each structure within our combined pathway of interest, the mesocorticolimbic pathway, is critical in driving essential aspects of basic survival behaviors. Together, the ventral tegmental area (VTA), the nucleus accumbens (NAc), amygdala, and prefrontal cortex (PFC) function to monitor internal homeostasis, mediate memory, mediate learning, and experience emotion.

Several major efferent projections, located near the midline, extend from the VTA to create what is known as the reward circuit [6]. The mesolimbic and mesocortical pathways are two of the most prominent, extending to limbic and cortical areas. These projections to the NAc, PFC, and amygdala serve to relay whether environmental stimuli are rewarding or aversive. They are considered integral to reward behaviors and cognitive functions and are particularly active in circumstances of arousal, stress, and motivation. The functions of the nucleus accumbens (NAc)

have not been fully elucidated, but its role in the reward circuit is recognized due to its connections with the VTA. While the exact contribution of the nucleus accumbens in processing reward is not completely clear, it is thought that this basal forebrain structure likely plays a role in memory processing and learning about punishments, rewards, and the stimuli that are associated with them [3,4]. Experiments have shown that levels of dopamine in the NAc rise anytime a positive or negative event occurs, suggesting that dopamine signaling may be involved with storing information about environmental stimuli associated with different types of experiences and potentially prioritizing levels of aversion or reward [7]. In this regard, dopamine is considered to play a significant role in the stress response. The ability to form associations between predictive environmental stimuli and rewarding or aversive outcomes is an essential aspect of learned behavior. This suggests that the NAc acts as a vital part of the reward/aversion system from its contribution to motivationally relevant anticipation.

The amygdala is an almond-shaped collection of nuclei found within the medial temporal lobe [8]. This paired subcortical brain structure has been shown to play a prominent role in mediating many aspects of emotional recognition and behavior and is primarily associated with fear and other emotions related to aversive stimuli. However, recent studies have paired it with positive emotions promoted by rewarding stimuli [3]. In humans, the amygdala is the fundamental structure responsible for multimodal reflexive responses and performs significant roles in the formation of short-term memories and long-term storage of memories [9].

The prefrontal cortex (PFC), located at the front of the frontal brain, is one of the final structures to mature during nervous system development. There are many competing theories on the functions of the PFC, as it is implicated in numerous complex behaviors. Because it makes up over 10% of the volume of the brain and is highly interconnected with much of the brain, the PFC is categorized as a multimodal association area [10]. The prefrontal cortex is especially interconnected with brain regions involved in executive functions such as attention, working memory, decision-

making, and impulse control. These higher-level cognitive processes are displayed with outstanding proficiency in humans, significantly contributing to personality development, moderating social behavior, predicting future events, complex planning, and prioritizing competing and simultaneous information. It is this structure that helps critically define the socio-emotional and executive functions that make human cognition unique.

Dopamine

Dopamine (DA) is a neurotransmitter within the brain that is often referred to as the pleasure chemical [11]. While this is in part true, dopamine has a wide array of utility and plays a role in many other behaviors and functions. Regarding evolution, dopamine is an ancient chemical messenger that is conserved among vertebrates and invertebrates [12]. Its signaling is a critical element of (but not limited to) cognition, learning, memory, reward, aversion, motivation, and voluntary movement [11]. Because of this, dopamine is used to signal organisms to fulfill basic survival needs, and dopamine is released when organisms experience stress. In this study, we will be exploring how excessive activation of the stress response affects dopamine and dopamine synthesis within the mesocorticolimbic pathway.

DA generally functions as a slow-acting neuromodulator, controlled by regulatory mechanisms common to monoamine neurotransmitters, and is synthesized from the amino acid tyrosine [13]. Tyrosine is transported to DAergic neurons, where a series of reactions involving tyrosine hydroxylase (TH) and dopa decarboxylase convert it to L-dopa and then dopamine [13]. After synthesis, dopamine is transported from the cytosol into synaptic vesicles by the vesicular monoamine transporter (VMAT2). It is stored in these vesicles and released into the synaptic cleft following the depolarization of its host neuron.

Once in the synapse, dopamine stimulates neurons by binding to and activating cell surface receptors. These can be postsynaptic dopamine receptors located on dendrites or presynaptic autoreceptors located on the membrane of an axon terminal of the presynaptic neuron. The

resulting action potential triggers the release of second messengers in the postsynaptic neuron. Dopamine molecules are then unbound from their postsynaptic receptors and released back into the synaptic cleft. The dopamine molecules are reabsorbed into the presynaptic cytosol by dopamine transporters or plasma membrane monoamine transporters. Once back in the cytosol, dopamine can either be broken down by a monoamine oxidase or repackaged by VMAT2 [13].

DA acts in a complex interplay of genetic and environmental factors to facilitate homeostasis and neurodevelopment. It has been found present within the brain prior to synaptogenesis, and activation of DA receptors during development alters brain structure and connectivity with long-term anatomical and behavioral effects [14]. DA has been shown to be critical for many processes that drive learning and memory, including motivation, incentive salience, and avoidance behavior, and is key in fear learning.

Disruption of DA synthesis or interaction can affect neuronal structure, function, or connectivity and can alter developmental trajectory in mesolimbic and mesocortical pathways. Experiments have demonstrated that neurodevelopmental alterations in areas with prominent DA innervations can result in long-lasting and sometimes irreparable effects [15-20]. Growing evidence is beginning to implicate DA and DA receptor damage and malfunction in the cause of neuropsychiatric disorders like Parkinson's disease and schizophrenia [15-20]. In vivo neuroimaging research has revealed that the same DAergic circuitry involved in rodent contextual fear conditioning and extinction is dysfunctional in humans with posttraumatic stress disorder [21,22].

Cortisol and Inflammation

Stress is a whole-body reaction. A key element in this adaptive response is the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which sends chemical messengers known as glucocorticoids throughout the body. These hormones promote gluconeogenesis, increase immune activity, inhibit nonessential processes such as reproductive function, and act as a counter-measure to the primary response. Collectively, the increase in glucocorticoids creates a stimulating and

immunosuppressive response that facilitates fight or flight behaviors to remove an organism from immediate danger while later restoring bodily homeostasis.

Glucocorticoids are understood to participate in anti-inflammatory and immunosuppressive actions, but the over-activation of the stress response can lead to excessive glucocorticoid production and detrimental effects in a wide range of tissues [23]. Chronic activation of the stress response results in an increase of circulating glucocorticoids in the central nervous system (CNS). This dysregulation is thought to exert pro-inflammatory effects on DAergic innervations leading to impairment and atrophy in the PFC and amygdala in people with posttraumatic stress disorder [24-27].

Cortisol is the primary stress hormone in humans and a potent anti-inflammatory hormone that prevents tissue and nerve damage [14]. Its production is upregulated during the stress response, and its dysfunction is likely to result in widespread inflammation following the reactivation of an acute pro-inflammatory stress response [14]. While an adaptive coping response would permit a return to normal levels of epinephrine, norepinephrine, and cortisol, a maladaptive response causes excessive or prolonged cortisol secretion, creating a fear-based memory of the stressful stimulus that is sensitized and readily reactivated by future stressors [14]. The preceding cortisol dysfunction would then result in unregulated inflammation following reactivation of the stress response, which may contribute to a cycle of inflammation, depression, and pain. This implicates pain, a stressor, in the reactivation of the pro-inflammatory stress response, now unmodulated due to cortisol dysfunction.

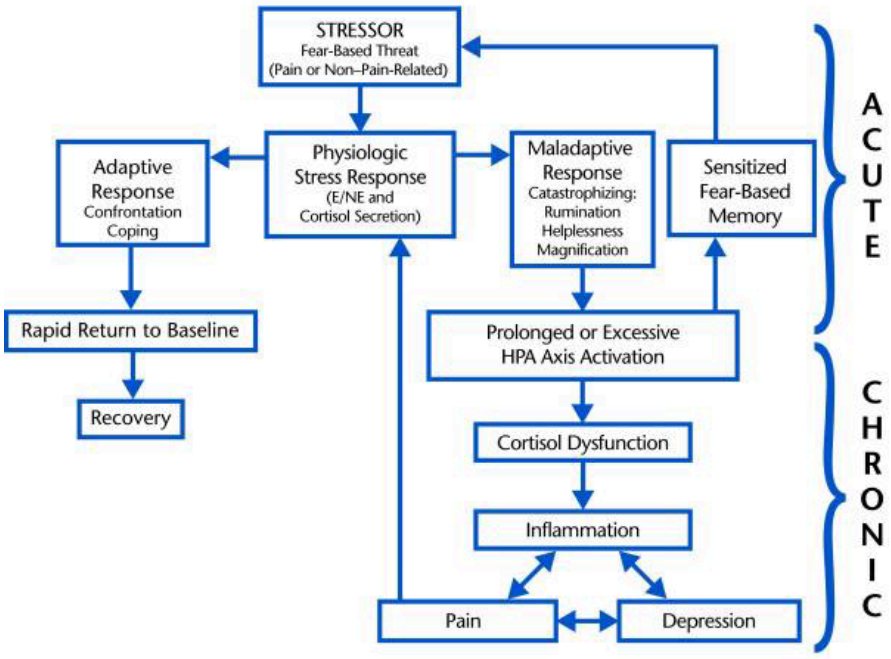


Figure 1. Proposed role of stress-related hypothalamic-pituitary-adrenal (HPA) axis activation. Acute stress response: pain or non–pain-related stressor activates a normal physiologic stress response. Chronic stress response: prolongs cortisol and epinephrine/norepinephrine (E/NE) secretions [due to maladaptive coping response to acute stress] results in cortisol dysfunction. [14].

Studies have shown that molecular messengers used to mediate immunity and inflammation, known as cytokines, are associated with stress-related chronic pain and hypocortisolism [15,28]. Following injury, localized secretion of inflammatory cytokines initiates the healing process, lowering the nociceptor thresholds to elicit a protective pain response [16]. However, an injury that coincides with chronic reactivation of sensitized stress responses may result in a persistent inflammatory response that halts cellular repair and sensitizes nociceptors, increasing pain sensitivity. Moreover, the subsequent impairment of cortisol's anti-inflammatory function may intensify, exhausting HPA response, leading to stress-induced hypocortisolism, and prolonging a formerly short-term inflammatory response. Furthermore, chronic psychological inputs, fear-based threats that are absent of direct physical harm share this compromised feedback loop. Ultimately, chronic reactivation of the stress response by unregulated inflammatory messengers and

heightened emotional responsiveness may compound the effects of inflammation, reinforce a conditioned stress response, and amplify the maladaptive cycle [15].

Post-Traumatic Stress Disorder

Psychological trauma involves the witnessing of a traumatic or life-threatening event. Individuals who witness these traumas are likely to experience intense stress, fear, anxiety, and helplessness, which can result in a permanent or temporary psychological wound characterized by physical, cognitive, emotional, or behavioral changes. Many who undergo psychological trauma endure an acute and transient experience, which results in minimal functional impairment, but some suffer from a persistent condition that is responsible for significant life changes called posttraumatic stress disorder (PTSD) [29].

PTSD is a common mental health condition that occurs in individuals who have experienced or witnessed a frightening or stress-inducing event and have difficulty psychologically recovering. The probability of developing PTSD depending on one's social background, home country, and kind of traumatic event experienced [30,31]. It affects more than 3 million US citizens each year in myriad ways, which include but are not limited to personal violence, sexual assault, war, serious accidents, and natural disasters. Per the American Psychiatric Association, 3.5% of US adults and nearly 1 in 11 people will be diagnosed with PTSD in their lifetime [29]. The condition can persist throughout the lifetime of afflicted individuals, with symptoms that are categorized into three groups:

- Avoidance – Numbing, withdrawal, confusion, dissociation, and depression
- Hyper-arousal – Insomnia, agitation, irritability, impulsivity, and anger
- Re-experience – Flashbacks, nightmares, and intrusive thoughts

These symptoms are brought on by triggers, resulting in the reliving of memories of the traumatic event along with intense emotional and potentially physical responses. Symptoms may start within one month of the traumatic event but can be latent for several years beyond the event and vary from person to person.

PTSD symptoms were once considered a normal response to extreme circumstances. However, the presence of symptoms for an extended period beyond one month is indicative of an abnormal neurological adaptation. As stated previously, stress is a normal biological and psychological response experienced when encountering a stressor. The normal stress response is a series of physical, psychological, and behavioral reactions that enable an organism to overcome a challenge then return to homeostasis. For people who have PTSD, the stress response is heightened and can lead to physical and psychological stress beyond that of a typical timeline. People with PTSD tend to struggle with symptoms in situations where a person without the disorder have no stress response. In fact, individuals who have PTSD maintain many of the psychological symptoms of stress chronically, even when there is no stressor around.

The Diagnostic and Statistical Manual of Mental Disorders 5th Edition (DSM-5) recognizes several criteria for a PTSD diagnosis [32]. The PTSD criteria are as follows:

A. **Exposure to a stressor** – The individual was either directly or indirectly (witnessing, learning, or exposure to aversive details) exposed to trauma.
B. **Intrusion symptoms (one required)** – The trauma is persistently re-experienced via recurrent memories, nightmares, flashbacks, psychological distress, or physiological reactivity to traumatic reminders.
C. **Persistent avoidance (one required)** – Avoidance of trauma-related stressors: recurrent trauma-related thoughts or environmental reminders such as people, activities, and places that act as visual reminders.
D. **Negative alterations in cognition and mood (two required)** – Inability to recall key features, persistent (and often distorted) negative beliefs and expectations about oneself or the world, persistent distorted blame of self or others, persistent negative trauma-related emotions, markedly diminished interest in pre-traumatic activities,

feeling alienated from others and constricted affect (persistent inability to experience positive emotions).

E. **Alterations in arousal and reactivity** – Disturbances to arousal and reactivity that began or worsened after the trauma are characterized by aggression, self-destructive or reckless behavior, hyper-vigilance, exaggerated startle response, and difficulty concentrating or sleeping.

F. **Duration** – Criteria B-D must be present for at least one month.

G. **Functional significance** – Trauma-related symptoms must cause psychological, social, or functional impairment.

Increasing the understanding of the neurobiology of PTSD is fundamental for the development and improvement of safe and effective treatments [33,34]. Currently, the information about the pathophysiological mechanisms underlying PTSD remains poor [34]. Growing evidence suggests that multiple neural systems may be involved in the development and persistence of PTSD, but most of the research in the field is focused on noradrenergic pathways and the effects of norepinephrine [34,35]. Interestingly, further evidence of the effects of the norepinephrine precursor, DA, shows that the DAergic system also controls behavioral responses to stressful situations [36].

The mesocorticolimbic pathway is one of the principal DA sources in the brain, and its structures have been shown to play important roles in fear conditioning and the acute and chronic stress response [37-41]. Various coping strategies to stressful events are sustained by fluctuations of DA levels in the nucleus accumbens (NAc), the amygdala increases dopamine transmission, which consolidates traumatic memory during application of stress, and manipulation of midbrain DAergic transmission alters resilience to stress [42-44]. Due to the interconnectivity of the mesocorticolimbic pathway and posttraumatic stress disorder, this DAergic pathway has the potential to reveal answers about the over-activation of the stress response and is pertinent for scientific investigation.

Zebrafish: A model for Post-Traumatic Stress Disorder

In 1897, Ivan Pavlov established that dogs could pair a neutral cue to a biologically relevant stimulus. After repeated predictive pairings, Pavlov's dogs were trained to recognize the sound of a bell as an indicator for the presence of food [45]. Like food, stress is also a biological motivator, and neutral cues can take on great salience when their association with stress predicts threats to homeostasis. Mounting an appropriate and adaptive response to threats to homeostasis is necessary to acquire rewarding stimuli and evade danger. Animals rapidly learn behavioral responses to identify the environmental cues that aid in maintaining homeostatic equilibrium. Conversely, the efficacy of adaptive behavioral responses may suffer due to chronic stress, subsequently producing unsuccessful interpretations of benign and dangerous scenarios, inducing anxiety disorders as well as ill-effective survival mechanisms [14,46]. In anxiety disorders, such as posttraumatic stress disorder, the adaptive stress response fails to extinguish, and reminders of traumatic events can cause pathological conditioned fear reactions long after and far removed from the inciting stimulus. The resulting outcome can severely impact the quality of life, and the compounding effects serve as detrimental and deleterious to recovery.

Since the 1970s, the exploration of the zebrafish model has taken place in several disciplines of science, such as genetics, developmental biology, cognitive neuroscience [47,48]. Where the rodent model's anatomical, biological and genomic homology to humans has made it the traditional model organism for many decades, its use has been burdened by challenging husbandry, difficulties with *in utero* manipulation, and costly high throughput screening [49]. The zebrafish (*Danio rerio*) has provided an alternative model to mitigate these shortcomings [50]. As a vertebrate, the zebrafish model provides more information than can be obtained from cell lines and invertebrate studies while remaining at a low-cost and high-throughput compared with mammalian models. Since being introduced for biomedical research purposes by Streisinger et al.

in 1981, the zebrafish model has taken the place of more complex vertebrates in several disciplines such as genetics, developmental biology, and pharmacology [47].

Zebrafish are small and prefer to be housed in large groups, requiring less space and fewer resources to maintain in comparison to rodents. The zebrafish is an oviparous organism with high fecundity, breeding every ~8 days, producing tens to hundreds of eggs each breeding session. The production of many offspring eases efforts to repeat experiments concurrently, giving confidence to result accuracy. Sequencing of the zebrafish genome began in 2001, and the reference genome was published in 2013 [51]. This has revealed that ~70% of human genes have at least one zebrafish ortholog, and ~84% of genes known to be associated with human disease have a zebrafish counterpart [51]. The genetics tools that have developed since then now provide a stage for the creation of informative transgenic and knockdown/knockout lines. As screening for germline transmission generally bottlenecks in the generation of transgenic lines, the high fecundity of zebrafish allows for more rapid screening and development [51]. This provides an important platform to study genes linked to human disorders, thus allowing effective modeling of human diseases and neurobehavioral disorders [48,51].

The use of zebrafish in the field of neuroscience continues to increase, and several recent reviews have highlighted both the strengths and weaknesses of using zebrafish to study neuroactive compounds and brain disorders [51-54]. Although there are neuroanatomical differences between zebrafish and humans, comparable features of the CNS allow for results to be generalized. The zebrafish brain has many analogous regions to those of mammals, and the complexity of both juvenile and adult zebrafish brains has been well documented [51,52]. For example, both mammal (rodent) and zebrafish thalamic DAergic nuclei are in the diencephalon with ascending projections to the telencephalon. Furthermore, homologs of the mammalian midbrain region such as the zebrafish posterior tuberal, ventral telencephalic, and dorsal telencephalic nuclei have been determined as functionally equivalent to the mammalian VTA and NAc, demonstrating evolutionary

conservation [55,56]. In addition to brain morphology, the neurochemistry and endocrine responses linked to zebrafish neuroactivity are highly homologous to other vertebrates [51]. The zebrafish CNS uses many of the same neurotransmitters that are responsible for higher-order cognitive function also found in mammals, including DA, norepinephrine, serotonin, acetylcholine, GABA, and glutamate [57,58]. Although the zebrafish CNS is more simplistic than the rodent model, experimentation has shown that it can mediate the same complex behaviors involving the same classical conditioning suggested to be linked to posttraumatic stress disorder that are sought to be elucidated in humans [59].

Chronic Unpredictable Stress Paradigm

Stress is a feeling of psychological or physiologic tension. It is a common state during which many feel a biological pressure to act. Stress is how our body responds to physiological demands, environmental challenges, and emotional conflict. Activation of the stress response leads to physiologic and behavioral changes that seek to reestablish homeostasis and improve coping with stressful situations. In the modern age, everyday stressors pang us in the form of deadlines, competitions, gridlock traffic, physical or verbal disputes. Within limits, stress acts as a positive nudge to cover our basic needs for survival. However, prolonged and constant stress exposure can have a deleterious effect on health. A lack of adaptation to excessive stress exposure poses a risk for the development of many psychopathological conditions, affecting physiology, mood, productivity, and overall quality of life.

Researchers using animal models have found that chronic stress elicits mood disorders, and thus, the chronic unpredictable stress (CUS) paradigm has been popularized as a standard protocol used to understand the neurobiological mechanisms underlying the consequences of chronic stress exposure [58]. The protocol follows methodical and repeated exposure to varied and unpredictable stress events used to induce behavioral characteristics observed in patients with anxiety, depression, and related mood disorders [62,63]. In rodents, researchers using the CUS paradigm have

observed downregulated plasma cortisol levels consistent with that of cortisol levels present in mood disorders such as PTSD and chronic fatigue syndrome [64]. Pairing the zebrafish model and CUS paradigm provides a cost-efficient and high throughput alternative capable of robust and well-documented stress-induced behaviors mimicking affective disorders observed in rodents and humans [62,65]. In this experiment, we aimed to further expound on the characterization of a specific stress disorder in PTSD using the CUS paradigm.

Anxiety-Like Behavior Assay

The modeling of anxiety and other mood-related disorders in zebrafish has been reliant upon the research and recognition of behavioral phenotypes [62]. The observation and analysis of these behaviors play an important role in providing insights into neural pathways, physiological biomarkers, and (epi)genetic underpinnings of normal and pathological brain function [63]. Several anxiety models have been developed to elicit the robust number of fear-related behaviors observed in the zebrafish. For this experiment, we sought to observe fish scototaxis, or innate preference of dark vs. bright areas, using the light-dark test (LDT) [60]. The light-dark preference model is an established anxiety model based on the marked preference for dark environments presented by many teleost fish [66]. This test seeks to provide a conflict situation in which the subject must choose between the anti-anxiety behavior we seek to quantify, an innate motivation to explore novel and potentially hostile environments, and crypsis, the subject's natural preference for protected areas and avoidance of detection. The LDT measures locomotor activity in both environments as an index of anxiety. We chose this test in tandem with the CUS paradigm to observe the anxiogenic effects of chronic stress events on the zebrafish.

Research Question

This work sought to elucidate the relationship between chronic stress induction, anxiety behavior, and neuroinflammation by addressing the following question: What are the neurological and behavioral effects of unpredictable environmental stress on the zebrafish mesocorticolimbic pathway?

In this study, we identify the activity of c-fos, an inflammatory mediator, and tyrosine hydroxylase, a catecholamine precursor enzyme, within the mesocorticolimbic reward pathway. We hypothesized that stress activates key areas in the brain related to reward/aversion and memory, but chronic stress results in a decline in expression of c-fos and TH receptors in various brain regions, more specifically in dopaminergic projections in the fore and midbrain. These essential brain areas receive and integrate sensory stimuli to drive behaviors related to survival, including feeding, mating, migration, and avoidance, and thus deleterious effects were expected to cause a negative change from the normal behavioral phenotype.

The purpose of this study was to explore the inflammatory effects of chronic unpredictable stress on specific DAergic brain regions, elucidate the subsequent influence on survival behaviors those regions are responsible for, as well as further develop connections to human disease by presenting zebrafish as a viable model for posttraumatic stress disorder. We hoped to contribute to the existing data on this subject by providing additional data on the effects of traumatic experiences on the brain using a vetted animal model.

II: Materials & Methods

2.1. Animals & Maintenance

Adult zebrafish of randomly bred genetically heterogeneous 'wild-type' strains were obtained from a local distributor (Optimum Aquarium, Kennesaw, GA 30144). All fish were acclimated to the laboratory environment for a minimum of 10 days, housed in 10 liter (L) aquaria at a density of ~ four fish per 1L, and then individually and adjacently housed within 3 L tanks at least 48 hours prior to behavioral testing. Zebrafish used in these studies were ~6 months old and maintained in a circulating system equipped with biological, chemical and mechanical filtration, aeration, and sterilization by UV light (Pentair Aquatic Habitats). Mounted LED lights provided illumination during a 14 h/10 h light/dark cycle. Tank water consisted of reverse osmosis deionized H_2O with supplemented dissolved sea salts (Instant Ocean) and was maintained at ~26-28 C^o. Water parameters (pH, ammonia, temperature, hardness, nitrate/nitrite, and chlorine) were maintained at the recommended amounts [67]. Fish were fed twice daily Zeigler zebrafish diet (Pentair Aquatic Ecosystems). All animals were experimentally naïve prior to testing. Behavior was recorded by USB GoPro Camera (saved as MP4 files for subsequent analysis) mounted to an overhead shelter. All protocols for animal use, housing, and care were approved and carried out per the Institutional Animal Care and Use Committee of Kennesaw State University.

2.2 Chronic Unpredictable Stress Paradigm

All experiments were conducted between the feeding hours of 11:00h and 16:00h. To measure the locomotive activity of the subjects, individual zebrafish were placed into a 10 L observation tank partitioned into two sections, one solid black and the other solid white, and recorded for 12mins. Subjects were divided into three experimental groups and a control based on the number of stress events the groups were subjected to. A modified version of the Chakravarty et al. chronic unpredictable stress paradigm was implemented [62]. Our preliminary study concluded in a 100% death rate before the end of the trial timeframe. We scaled back the two stress events per

day found in the literature to a single stress event to maintain a sample size capable of being analyzed. Thus, our experimental groups were within a 14-day timeframe and as; 2, 4, and 8 stress events. The experimental groups were subjected to a variety of chronic stressors, such as restraint stress (RS), social isolation (SI), over-crowding (OC), tank change (TC), cold stress (CS), chasing (C), heat stress (HS), and dorsal body exposure (DBE). Each stressor was administered within fresh system water to avoid cross-contamination. Stressors were administered unpredictability using randomly determined administration time, changing the time and sequence of stressors daily during the 14 days of the stress paradigm. Stressors were administered such that no group experienced the same stressor more than once to avoid habituation. The stressors were administered as follows: each animal was restrained (RS) for an hour in a 2-ml microcentrifuge tube with perforations at both ends for free water flow, exposed to heat stress (HS) and cold stress (CS) by transfer to new tanks maintained at 33 °C and 20 °C, respectively, for 30 min; socially isolated (SI) in separate beakers for 60 min; over-crowded (OC) with ten animals in a 250-ml beaker containing only 150 ml water for 60 min; kept in housing tanks with low water levels to expose the animal's dorsal body (DBE) for 2 min; transferred from one tank to another (TC) six consecutive times; and chased (C) by a net for 8 min. Aeration and temperature were controlled during the presentation of each stressor, except during heating and cooling stresses. The non-stressed control group was maintained in the same room during the 14-day stress period.

2.3 Anxiety-Like Behavior Assay

A modified scototaxis (light/dark preference test) protocol was performed following the final stressor event of the eight-stressor events group to analyze the behaviors of the control and stressed groups [62]. Fish were transported from the housing tank and individually placed into a 10L observation tank distinctly partitioned in half with solid black tape on one side and white tape on the opposite side. All overhead lights were off, and only ambient light was present during the recording. As the fish was placed in the tank, they could choose to enter the black (dark) or white

(light) side of the tank. Scototaxis behavior was captured using the GoPro - HERO7 Black camera. The preference of each fish for the dark and light compartments was recorded over a 12-min test period with front and top-down views.

2.4 Immunohistochemistry & Microscopy

After animals received behavioral testing, they were removed carefully from the observation tank and transferred into a water-filled beaker placed into refrigeration at 4 ^{o}C. Following ~15mins at 4 ^{o}C, subjects received tactile tests for responsiveness and were observed for operculum movement. When fish were no longer responsive to physical stimulation and the operculum movement ceased, their heads were removed using a scalpel placed at a right angle to the tip of the pectoral fin. Zebrafish brains were harvested and stored at 4 ^{o}C in 4% formaldehyde in PBS overnight before sectioning. Fixed brains were then washed three times for 10 mins and then transferred into a solution of 30% sucrose in PBS (w/v) until the tissue sank to the bottom of the container. The following day, the brains were transferred into Tissue-Tek O.C.T., frozen using liquid nitrogen, and maintained at -20 ^{o}C until processing. Transverse sections through the mesencephalon region of the brain were taken at 20 μm and collected on charged slides.

Tissues were stained using an immunohistochemistry procedure previously optimized to show expression of TH throughout the mesencephalon (Ganser et al., 2013). For TH and C-Fos expression, mouse anti-TH1 monoclonal antibody (Immunostar) and mouse anti-C-Fos monoclonal antibody (Immunostar) were applied at a concentration of 1:1000, then counterstained with super clonal Alexa Fluor 568 goat anti-mouse secondary antibody (Invitrogen, green) at 1:1000. Completed slides were observed using a Zeiss LSM 700 laser scanning confocal microscope. 1-3 repeated trials were used for each treatment with 9-25 tissue-sections per slide. Pictures were taken with a z-stack of each tissue section. Average expression was in the form of fluorescence arbitrary units (FAU) for the TH, C-Fos (red, 555 channel), and DAPI (blue, 405 channel) stains. Total FAU was scored manually by providing a ratio of two circled regions of interests, the

periventricular nucleus of posterior tuberculum (TPp) and posterior commissure (Cpost) on each cross-section using the FIJI software imaging analysis tool. This ratio was done to provide a comparison of DAergic to non-DAergic signaling within a cross-section to compensate for differences in laser intensity and gain used due to stain fade over time.

2.5 Statistical Analysis

Behavioral Analysis

Following the chronic unpredictable stress experiment, video recordings were taken of the light-dark preference test and scored manually using an impartial third party. The parameters measured were as follows: average time spent in light compartment and numbers of crosses into the light compartment. We expected to see a decreasing trend of exploratory behavior as the number of stress events increased. A one-way ANOVA ($p < 0.05$) was run using Graph Pad Statistical Software (Prism 9) to establish the existence of any statistical differences between control and experimental groups. To determine where were significant differences in variables occurred, if any, a liberal and conservative pairwise comparison was performed using the least significant difference test and Tukey's HSD posthoc comparison. Table 1 below lists the treatments and the number of fish per treatment in each behavioral experiment.

Mortality

Death rates were determined by manually scoring losses in the group population over the 14-day timeframe of the CUS paradigm. We expected to see a downward trend of death as stress increased over time. A one-way ANOVA ($p < 0.05$) was run using Graph Pad Statistical Software (Prism 9) to establish the existence of any statistical differences between control and experimental groups. To determine where were significant differences in variables occurred, if any, pairwise comparisons using the Bonferroni test and Tukey's HSD posthoc comparison were performed.

Table 1. Behavioral Assay Treatment Groups

Chronic Unpredictable Stress	n	Light-Dark Preference Test	n
Control	42	Control	8
2 Stress Events	42	2 Stress Events	7
6 Stress Events	42	6 Stress Events	7
8 Stress Events	42	8 Stress Events	8

Tissue Samples

Samples were rendered using a virtual stack in FIJI ImageJ. A z-projection of each image was enabled to sum slices. The freehand selection tool was used to identify and outline two regions of interest, the periventricular nucleus of posterior tuberculum (TPp) and posterior commissure (Cpost), in each image, and a measurement of the mean gray value of each region of interest was taken. A ratio of TPp mean gray value to Cpost mean gray value was taken for each image from each experimental group for TH and c-Fos stained tissues. This was done to provide standardization to each image's data and compensate for changes in laser gain and intensity.

Figure 1. Control Brain TH Stain:

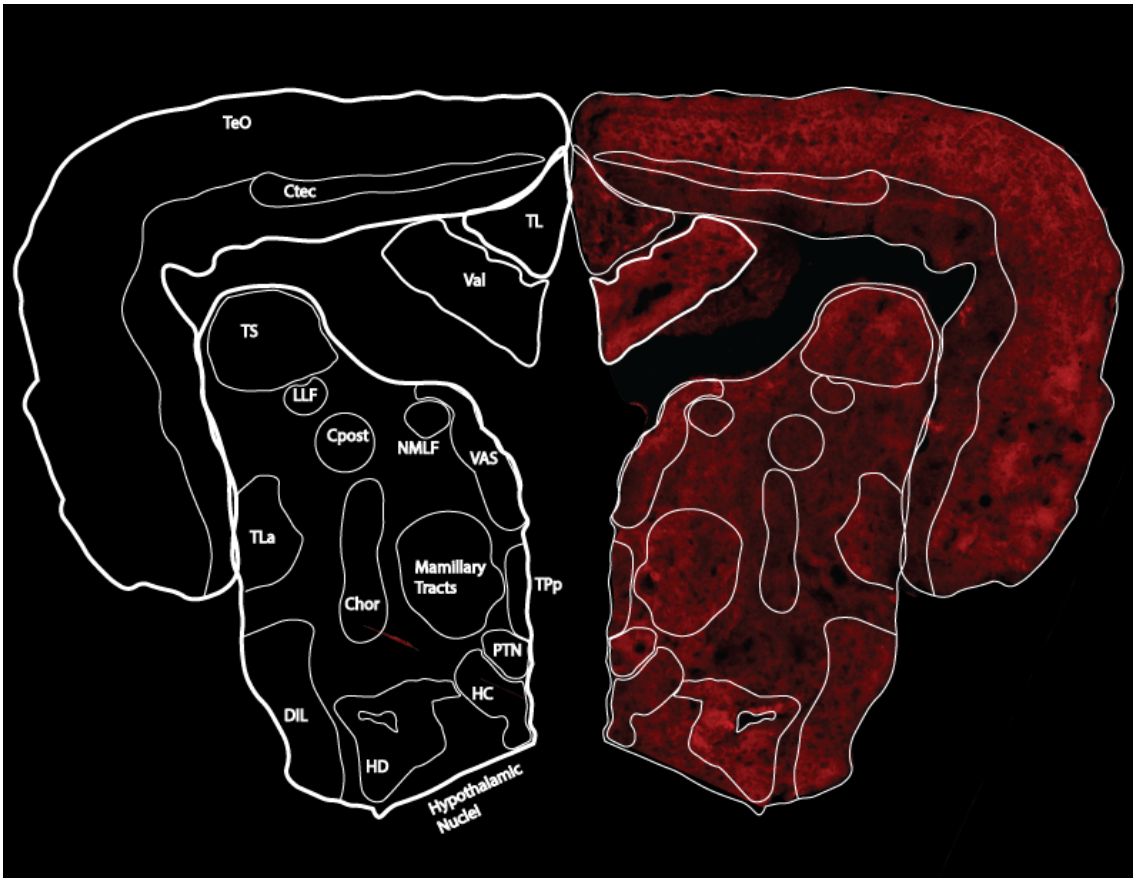


Figure 1. Representative midbrain nuclei map to serve as reference to identify areas of interest concerning tyrosine hydroxylase and c-Fos staining. We specifically focused on the posterior tuberculum (TPp) to analyze dopamine synthesis and regional inflammation because of overstimulation. We used the vascular lacunae of the area postrema (VAS), mammillary tracts, a periventricular nuclei (Hc) to orient our search for the TPp.

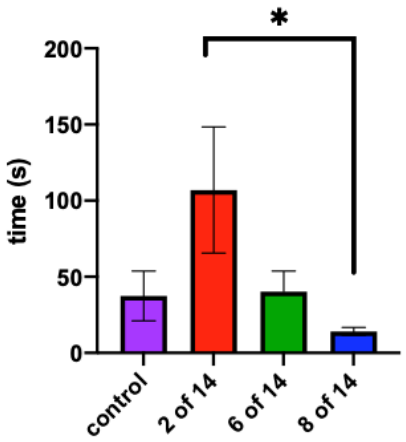
III: Results

Anxiety-like Behavior

For the behavior study, we examined if the time a fish spent in the light compartment was related to the number of stress events the fish received over the course of the study. When stressed, most zebrafish seek darker areas of their environment, possibly where they are hidden from the sight of predators. We did a one-way ANOVA to assess whether there were differences in the average time spent in the light compartment per cross/visit among the treatment groups (n=22). The ANOVA indicated a significant difference in the average time spent in the light compartment (p

= 0.0375), and Bonferroni-corrected pairwise comparisons indicate that fish that were stressed eight times during the fourteen-day experimental period spent significantly less time in the light compartment compared to the fish undergoing two days of stress over the experimental period (p = 0.0360). **Figure 2** shows the average time spent in the light compartment among experimental groups. We only detected a statistically significant difference between the 2-stress event vs 8-stress event treatment groups. The 2-stress group spent significantly more time in the light per visit into the light compartment than the 8-stress event group. While these data reflect the trend we expected to see, the comparison against the control makes this inconclusive without additional data points

Figure 2. Average Time in Light:



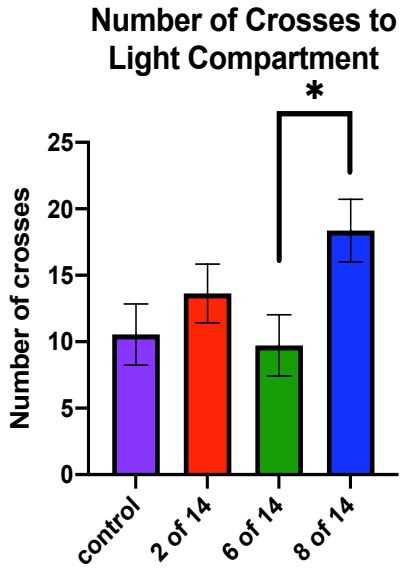
Bonferroni's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
control vs. 2 of 14	-69.59	-157.6 to 18.37	No	ns	0.2127
control vs. 6 of 14	-2.929	-90.89 to 85.03	No	ns	>0.9999
control vs. 8 of 14	23.19	-64.77 to 111.1	No	ns	>0.9999
2 of 14 vs. 6 of 14	66.66	-22.27 to 155.6	No	ns	0.2760
2 of 14 vs. 8 of 14	92.78	3.848 to 181.7	Yes	*	0.0360
6 of 14 vs. 8 of 14	26.12	-62.82 to 115.0	No	ns	>0.9999

Figure 2. The average time spent in the light compartment of the tank (n=22). A one-way ANOVA indicates a significant difference in the average time spent in the light compartment (p = 0.0375). While a Bonferroni pairwise comparison shows that fish stressed twice within the fourteen-day experimental period spent significantly more time in the light compartment when compared to fish undergoing eight days of stress over the experimental period (p = 0.0360). All other pairwise comparisons were not significant.

Stressed zebrafish in a light-dark test are expected to generally avoid bright or exposed areas. Our conjecture was that as stress events increased there would be an increase in avoidant behavior and therefore a decrease in exploration. We observed subjects within a bi-compartmental

tank for how many times they would venture beyond the environment most suited for their natural inclination to be hidden in the dark. We performed a one-way ANOVA to assess whether there were differences in the number of crosses from the dark compartment into light compartment among the treatment groups (n=22). The ANOVA indicated a significant difference in the number of crosses to light compartment ($p = 0.0402$), and Tukey multiple comparisons test indicated that fish that were stressed eight times during the fourteen-day experimental period had a significantly greater number of crosses into the light compartment compared to the fish undergoing six days of stress over the experimental period ($p = 0.0479$). **Figure 3** shows the number of crosses to the light compartment among experimental groups. The data indicated a statistically significant difference between the 6-stress event vs 8-stress event treatment groups. The 6-stress group crossed the light threshold significantly fewer times than the 8-stress event group.

Figure 3. Number of Crosses to Light Compartment:



Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
control vs. 2 of 14	-3.095	-11.50 to 5.306	No	ns	0.7695
control vs. 6 of 14	0.8144	-7.586 to 9.215	No	ns	0.9942
control vs. 8 of 14	-7.822	-16.22 to 0.5787	No	ns	0.0773
2 of 14 vs. 6 of 14	3.909	-4.672 to 12.49	No	ns	0.6326
2 of 14 vs. 8 of 14	-4.727	-13.31 to 3.854	No	ns	0.4762
6 of 14 vs. 8 of 14	-8.636	-17.22 to -0.05505	Yes	*	0.0479

Figure 3. The number of crosses over the light compartment threshold (n=22). A one-way ANOVA indicates a significant difference in the number of crosses to light compartment ($p = 0.0402$), A Tukey multiple comparison showed that fish stressed eight times within the

fourteen-day experimental period crossed significantly more often into the light compartment when compared to fish that experienced six days of stress over the experimental period (p = 0.0479). All other pairwise comparisons were not significant.

When it became clear that our initial analyses were not significant, we analyzed our data for difference in total time spent within the light compartment. We expected the scototaxis behavior to strongly influence the zebrafish positioning within the bi-compartmental tank, and reveal a paired relationship between stress events and time spent in the dark. There was no such trend suggested by the data. A one-way ANOVA showed no differences within the percent of total time in light compartment among the treatment groups (n=22, p = 0.2062). **Figure 4** shows the percent of total time in light compartment among experimental groups and indicates no significant p values. We expected a downward trend in total time spent within the light compartment as stress events increased. We observed qualitative differences that suggest this trend to be true but only in comparison to the stress receiving groups. In comparison to the control group, each variable group qualitatively spent more time within the light compartment, contrary to our hypothesis. More data may strengthen the trend amongst the stress receiving groups, but further examination may be necessary to determine the cause of the control group being less exploratory than stressed groups.

Figure 4. Percent of Total Time in Light Compartment:

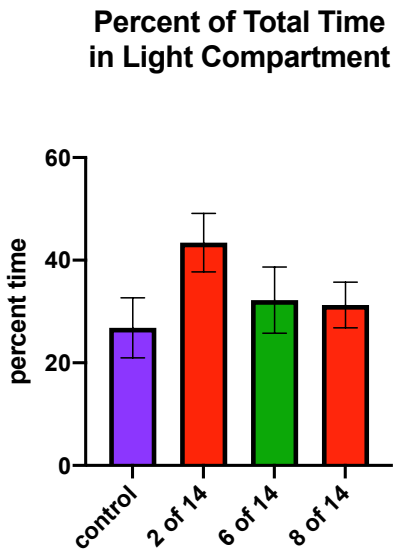


Figure 4. Percent of total time in light compartment of the tank (n=22). A one-way ANOVA indicated no significant difference in percent of total time in light compartment (p = 0.2062). A qualitative downward trend in exploring the light compartment amongst the stress groups suggests a larger sample size may show significance.

Survivorship

During multiple iterations of our experiment, our zebrafish were prone to large die-offs within our experimental colonies. We observed 100% mortality within our preliminary trial, during which we modified the CUS paradigm to promote endurance within our 14-day timeframe. The original CUS paradigm saw subjects stressed twice a day for 14 days. Our adjustment created 3 stress event variables; 2 stressors within 14 days, 6 stressors within 14 days, and 8 stressors within 14 days. Each stress event was evenly distributed within a 7-day timeframe such that each group had 6, 3, and 2 rest day intervals between stressors. We hypothesized that the decrease in stress in comparison to the original paradigm would lead to an increase in survivorship and there would be a trend demonstrating an increase in mortality as a consequence of increased stress events. At the end of our experiment we pooled the numbers from our experiment and duplicates into one data set and plotted a linear regression. This analysis showed a significant difference in the slopes of each variable. Within our mortality, we observed the trend we hypothesized in that an increase from no stress to 8-stress events was related to increased mortality. An ANOVA and Tukey's multiple comparisons tests ($p<0.0001$) demonstrated that all mortality rate pairwise comparisons were significantly different.

Figure 5. Mortality Results:

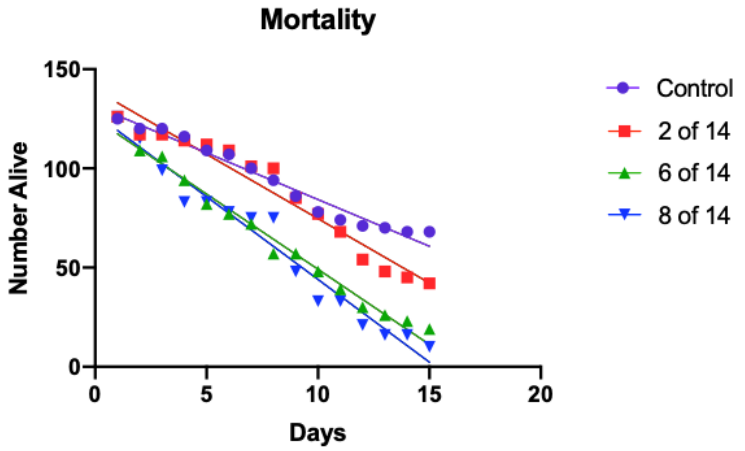


Figure 5. The effects of increasing stress on mortality (n=123). An ANOVA and Tukey multiple comparisons test indicated a significant difference in mortality ($p < 0.0001$). The data we obtained shows that mortality increases as stress increased over time. This is consistent with our hypothesis that an increase in stress will lead to an increase in death.

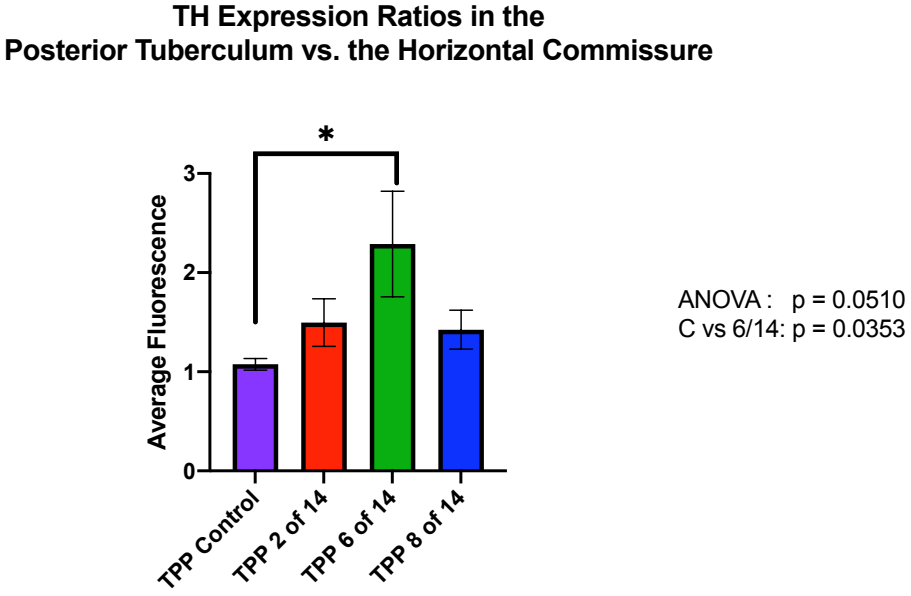
Immunofluorescent Mapping

We sought to further quantify the observed behavioral changes using immunofluorescence to stain for differences within regions of the brain involved in stress mediation. We observed three areas; the posterior tuberculum (TPp), posterior commissure (Cpost), and the horizontal commissure (Chor). Our original experimental design was set up to compare DA-ergic areas only but as the analysis of our subjects took more time, our staining began to fade, and we adjusted our laser setting to compensate. To standardize our imaging, we chose to observe DA-ergic and non-DA-ergic areas to generate a ratio of fluorescence to compare amongst our experimental groups. To mitigate the effects of artifact staining in our non-DA-ergic areas, we chose two areas, the Cpost and the Chor to further compare. We hypothesized that as stress events increase, we expected to see an increase in our fluorescence ratio, demonstrating an increase in enzymatic activity of tyrosine hydroxylase and c-Fos, until the overstimulation and inflammation results in cell death and therefore no activity.

The first ANOVA measuring TH expression, **Figure 6**, indicated there was no significant difference between ratios of control or experimental groups (0.0510). However, multiple post-hoc pairwise comparisons found significance between the control and 6-stress groups (p=0.0353).

There appears to be a qualitative trend, as the graphic demonstrates an increase in the ratio as stressors increase until the 8-stress group, suggesting that a larger sample size may yield different statistical results. This potential trend is consistent with our hypothesis that stress increases enzymatic activity of tyrosine hydroxylase in the brain.

Figure 6. TH Expression Ratio in the Posterior Tuberculum vs the Horizontal Commissure



Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
TPP Control vs. TPP 2 of 14	-0.4206	-1.473 to 0.6317	No	ns	0.7013
TPP Control vs. TPP 6 of 14	-1.213	-2.361 to -0.06475	Yes	*	0.0353
TPP Control vs. TPP 8 of 14	-0.3493	-1.402 to 0.7030	No	ns	0.8044
TPP 2 of 14 vs. TPP 6 of 14	-0.7923	-1.886 to 0.3009	No	ns	0.2222
TPP 2 of 14 vs. TPP 8 of 14	0.07130	-0.9208 to 1.063	No	ns	0.9973
TPP 6 of 14 vs. TPP 8 of 14	0.8636	-0.2296 to 1.957	No	ns	0.1618

Figure 6. The TH expression ratio in the posterior tuberculum (TPp) and the horizontal commissure (Chor). A one-way ANOVA indicated no significant difference in TH activity amongst all experimental groups. (p = 0.0510). Contrariwise, a Tukey multiple pairwise comparison shows that tissue from fish stressed six time within the fourteen-day experimental period had significantly more TH activity when compared to controls (p = 0.0360).

There were no statistically significant differences detected when measuring the ratios of TH expression within the posterior commissure (TPp) and the posterior commissure (Cpost) amongst experimental groups (**Figure 7**). However, unlike the TPp vs Chor ratios, there appears to an inverted qualitative trend between staining expression and stress. Barring the control, ratios using

the Cpost show a downward trend. Because this data was taken using the same TPp values for TH expression, this suggest that Cpost, while expected to be non-DA-ergic, is different from the Chor. This was not hypothesized prior to experimentation and suggests that further testing may help elucidate the observed pattern.

Figure 7. TH Expression Ratio in the Posterior Tuberculum vs the Posterior Commissure:

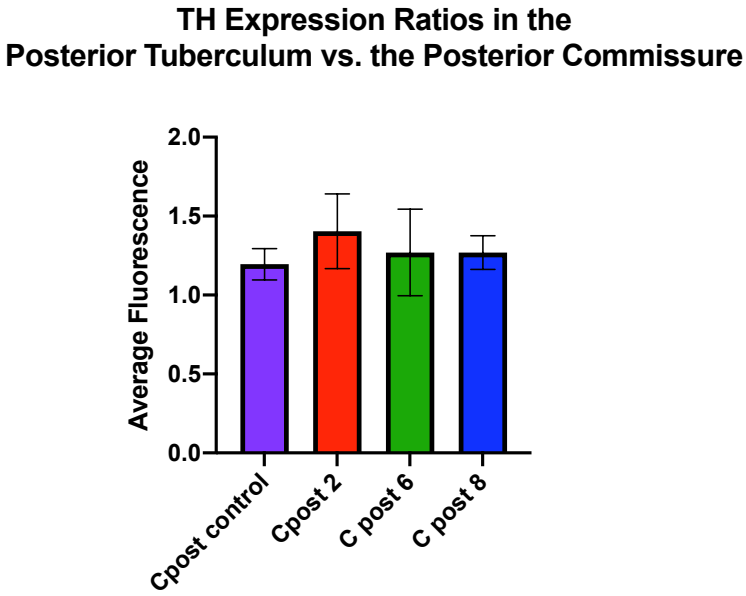


Figure 7. The expression ratio in the posterior tuberculum vs the posterior commissure. A one-way ANOVA indicated no significant difference amongst experimental groups ($p = 0.8813$). All other pairwise comparisons were not significant. Nevertheless, the graphic suggests an unexpected qualitative downward trend, showing an inverse relationship between stress and Cpost TH expression, that requires further analysis to provide insight.

To measure inflammatory activity brought on by stress, we stained brain tissues with the inflammatory marker c-fos and analyzed the same DA-ergic and non-DA-ergic areas we believed to be active during the stress response and stained to search for TH expression, the TPp, Chor, and Cpost. Once again original experimental design was set up to compare DA-ergic areas only but as the analysis of our subjects took more time, our staining began to fade, and we adjusted our laser setting to compensate. We standardized our fluorescence findings using a ratio of DA-ergic to Non-DA-ergic areas.

The first ANOVA measuring c-Fos expression, **Figure 8**, indicated no significant difference between ratios of control or experimental groups (p=0.9615) and no post -hoc tests offered any contradicting suggestions. Like the TH activity ratio for TPp vs Cpost, there appears to an inverted qualitative trend between staining expression and stress. The graphic demonstrates a decrease in the fluorescence ratio as stressors increase from the 2-stress group to the 8-stress group. We believe this may suggest potential trend but not necessarily a relationship with TH activity. It does not align with our hypothesis that an increase in stress would result in an increase in inflammation markers. Using a double staining method and different staining wavelengths, where we were limited by stains using the same wavelength (red, 555 channel), may further provide information regarding any relationship between TH and c-Fos activity.

Figure 8. c-Fos Expression Ratio in the Posterior Tuberculum vs the Horizontal Commissure:

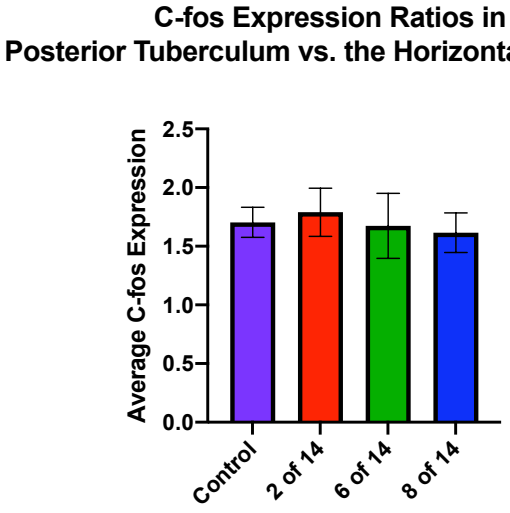


Figure 8. The c-Fos expression ratio in the posterior tuberculum (TPp) vs the horizontal commissure (Chor). A one-way ANOVA indicated no significant difference amongst these groups (p = 0.9615). All pairwise comparisons were also not significant. However, an inverted qualitative trend suggests that a larger sample size may be effective in providing a significant different between experimental groups.

The final ANOVA for c-Fos expression also showed that there were no significant differences when measuring the ratios of expression within the posterior commissure (TPp) and the posterior commissure (Cpost) amongst experimental groups (**Figure 9**). However, unlike the c-fos TPp vs Chor ratios, there appeared to be an upward trend in c-fos expression as stress increase, stopping

at the final stress group. Because these data were taken using the same TPp values for c-Fos expression, this suggests that Cpost and Chor, while expected to be non-DA-ergic, may have different activity levels as a consequence of stress. While the upward trend in c-Fos expression within the TPp and Cpost aligns with our hypothesis, the comparisons within the sample, amongst other non-DA-ergic regions, and against our TH expression tissues makes this inconclusive without means to measure TH and c-Fos expression in the TPp, Chor, and Cpost within the same sample.

Figure 9. c-Fos Expression Ratio in the Posterior Tuberculum vs the Posterior Commissure

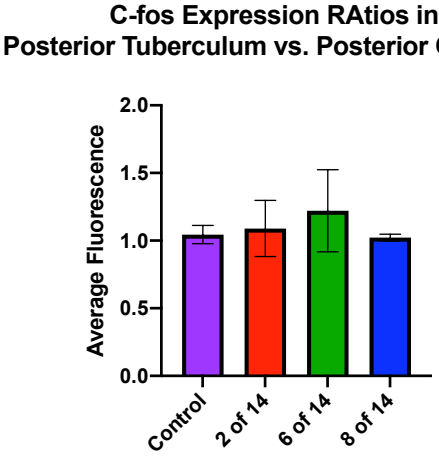


Figure 9. The average time spent in the light compartment of the tank. A one-way ANOVA indicated a significant difference in the average time spent in the light compartment ($p = 0.0375$). While a Bonferroni pairwise comparison shows that fish stressed twice within the fourteen-day experimental period spent significantly more time in the light compartment when compared to fish undergoing eight days of stress over the experimental period ($p = 0.0360$). All other pairwise comparisons were not significant.

Collectively, immunofluorescent data did not support our hypothesis that increasing stress events would cause a change in behavior as a result of changes stress-related regions within the brain. We were unable to demonstrate significant differences during our analysis of TH expression beyond specific pairwise comparisons and we were unable to demonstrate any significant difference amongst any groups expressing c-fos. Of the two analyses, the relationship shared between c-Fos expression and TH expression remains unidentified. Furthermore, the difference between the expression of c-fos and TH in the horizontal commissure compared to the posterior commissure suggest a potential inverse trend in expressions. Because the immunofluorescent data

did not result in a significant difference amongst any of our analyses, we believe the observed trends could be an effect of the stress or staining error. At this time, little is known to justify that observation and additional experimentation is required to be certain of any trends' existence.

IV: Discussion

An organism's survival often depends upon specific behavioral responses used to navigate dangerous conditions or acquire rewarding stimuli. Organisms need appropriate behavioral responses to specific stimuli. Inappropriate responses could mean missed opportunities or harm. A malfunction of the neural circuit responsible for response output can result in perpetuated misinterpretation of environmental cues and, thus, inappropriate behavioral response. Constant stress can lead to perturbations of the circuit the lead down this destructive path of inappropriate stress response. What we expected to see after a prolonged period of stress exposure was evidence of brain changes in the regions responsible for the stress response. We sought to validate our hypothesis that the brain regions responsible for the stress response would be negatively impacted by chronic stress by showing the inappropriate behavioral outcome and poor physiologic outcomes. Stress tests were run for 14 days to induce anxiety-like behavior in zebrafish, where we also predicted a physical change in the brain. Following the chronic stress tests, we observed the fish for quantifiable stress behaviors.

We measured anxiety as an effect of chronic stress by comparing lengths of time stressed subjects were willing to place themselves in an unfamiliar situation as opposed to a familiar and protected one. Fish that are in unfamiliar environments or that detect stress are known to seek refuge in dark areas where they cannot be seen by potential predators. Thus, we expected that the more incidents of stress experienced by the fish, the less they would venture to the light compartment of the tank. Because of overstimulation, crossing into the light area could indicate: 1) An absence of stress response, 2) A misinterpretation of the environment due to physiological miscues or dysfunction of the stress response circuits, or 3) Risky behaviors as a complete

dysfunction of the stress response system. These expectations are drawn from classic studies in which zebrafish responses to stressors was increased vigilance and the seeking of refuge [61]. From the 504 subjects at the beginning of the paradigm, we record several examples of typical anxiety behaviors, including erratic swimming, home base behavior, hyperactivity, freezing, and possible risk-taking behaviors. These behaviors were observable but not always quantifiable due to limitations of our experimental setup, indicating that the stress paradigms do, indeed, elicit typical stress responses, though they are not predictable within individual fish.

All fish behaviors were measured in the experiments following the end of a 14-day stress paradigm. This practice was done to simulate the effects of posttraumatic stress by allowing for a period following the traumatic event to take place before analyzing to determine any long-lasting anxiogenic effect. Fish were tested using a bi-compartmental test arena for which a tank was divided into a dark side and a light side, so the amount of time spent in the light side (risky/open side) was quantified. Aside from measuring behaviors specifically related to environment, subjects from all experimental groups exhibited behaviors consistent with anxiety-like behaviors found within the literature [65]. However, there was no statistical difference amongst the groups in how much of the behavior they displayed. This could possibly be due to a lack of intensity in the modified chronic unpredictable stress paradigm. The unaltered stress paradigm saw experimental groups stressed twice a day rather than once a day. During our preliminary trials using the unaltered stress paradigm, the stress protocol to which we initially modeled our experiment resulted in overwhelming mortality [62]. This occurred twice. Because we were unable to replicate these outlined stress paradigms, our altered protocol was not as intense as that reported by Chakravarty et al. (2013). Even so, the stressors presented to the fish in the final stress paradigm showed significant increases in mortality among fish subject to more stressors versus control fish.

We believe the significance and pattern demonstrated by the mortality analysis is clear evidence that there is a physiological component, that is not the brain, suffering from dysfunctions

leading to death. Metabolic elements of the stress response are controlled by HPA axis and effect the liver. The functions of the liver are critical and death occurs as an effect of its disruption. The increased glucocorticoid production by the adrenal cortices has been experimentally confirmed to induce lipid deposition within the liver, leading to death [69]. Chronic stress has been shown to disrupt hepatic function in mammals but there is currently little available research published regarding other vertebrates, like fish [70]. Exploration into the effects of chronic unpredictable stress on zebrafish metabolism may elucidate our death rate findings.

The final responses of the experimental fish to the behavior tests did not follow our predictions. Though we do see significant differences in time spent in the "unsafe" compartment versus the dark and safe side, the observation that fish subject to two stressors spent the most average time in the light compartment is puzzling. We expected that all groups that were subject to any amount of stress would practice heightened vigilance and seek refuge in the dark tank compartment, and once sufficient time passed for habituation, the fish may begin to explore the tank. In each of the light versus dark experiments, control fish spent the least amount of time in the light compartment compared to all other experimental groups. Because stress behavior is complex, we hypothesize there to be an increase in stress behaviors being exhibited by the 6 and 8-stress event groups but those behaviors could present in a way that is not indicative of the aversive behavior we anticipated. This result begs the question of whether the stress response system, being attuned to previous stressors, was not able to respond to situations calling for increased vigilance reliably.

We would expect to see changes in the brain indicative of these altered stress systems. One possible chemical response to the system would be the activation of dopaminergic pathways necessary to both quickly flee from stressful situations or to put a stressor into memory to react appropriately next time. Not only are these pathways modulated by dopamine (DA) secretion, they are also activated by other catecholamines like norepinephrine (NEPI) and epinephrine (EPI), both

of which are derived from tyrosine hydroxylase (TH) like dopamine. We expected the experience of stress to activate pathways governed by catecholamines, thus resulting in an increased presence of the precursor enzyme, TH in the brain, especially in the diencephalic – midbrain – hindbrain circuits. These diencephalic – midbrain - hindbrain dopaminergic neurons in the human reward, learning, and memory pathways mirror zebrafish DA circuits that govern survival responses in the hypothalamus, tuberculum, and descending connections to the cerebellum. The tuberculum structure in the zebrafish brain is not a brain structure present in amniotes. However, the tuberculum is homologous to the midbrain dopaminergic pathways originating from the mammalian periaqueductal gray and other nigrostriatal paths. [68]

The brains of our experimental fish, however, did not indicate significantly higher expression of TH compared to controls in areas typical for measuring DA expression. An ANOVA measuring differences among experimental groups in the ratio of TH expression between the posterior tuberculum (TPp, a DAergic nucleus) and the horizontal commissure (Chor, an area absent of DA) indicated that DA expression was not significantly different among experimental groups ($p = 0.0510$). Tukey's pairwise comparisons, however, revealed significantly higher DA expression ratios in the TPp of fish receiving six stressors compared to controls. These data suggest that an increase in sample size may have yielded more statistically significant results, but more importantly, all stressed fish averaged higher TH expression compared to controls, even though only one group differed significantly. These data suggest a positive correlation between stress and TH expression.

To further explore brain changes due to stress, we measured the expression of C-fos, a pro-inflammatory marker that is present in the brains of mammalian stress models [23,25]. Though our assays indicate no significant difference in C-fos expression, there seem to be qualitative differences in brain nuclei that modulate acute stress responses like the vascular lacunae of the area postrema (VAS). The VAS acts much like the vomit center in humans where toxin-sensing chemoreceptors

induce an immediate, often lifesaving evacuation response. Because fish do not vomit, turning on the VAS triggers an immediate stress response. In most of our experimental fish brains, the VAS showed distinct TH and C-fos expression. This could indicate the triggering of the VAS-escape system but nothing more. In the brains of mammalian models, C-fos is expressed in the pathways known for immediate and chronic stress responses, so areas of the midbrain – hypothalamic – limbic or locomotor pathways indicate overworked neurons undergoing inflammatory responses [25]. In the short term, inflammation can skew communication among stress response circuits. In the long term, inflammation reduces neuronal plasticity and may ultimately result in apoptosis [25].

Future Directions

Though our results were not wholly significant, our data compels more questions that only further experiments may be able to answer. A more in-depth exploration and quantification of specific anxiety behaviors would likely give a better indication of responses to stressful situations after a stress paradigm. Within that future experiment, the stress paradigm itself should likely be extended if subjects can survive. The questions we experienced with the Chakravarty et al. (2013) paradigm centered around the home tanks the fish were maintained in following their stressors. The experimental N in this protocol were impossible for us to sustain over the 14-day timeline. We wondered if the fish were placed back into a circulating aquarium system after being stressed, would their stress hormones and pheromones also circulate to the other fish, nullifying our brain immunohistochemistry assays. Perhaps measures of cortisol in the individual fish taken directly following each stress incident would be a more accurate measure of the stress response. Per the measures of TH expression in the TPp, we recognize that a larger sample size would tell a more accurate story, as well as quantification of expression in more than one brain area.

Integrative Significance

This study also attempts to untangle modern neurological problems from ancient and evolutionarily conserved brain circuits by providing research methods designed to confront the

complexity of modeling behavioral disorders in vertebrates. Thus, problems like PTSD, OCD, addiction, and dissociative means of handling stress responses can be viewed from the lens of a group of neurons meant only to maintain a fish's physiology and fitness. Due to the synergistic nature of biology, we took an integrative approach to define the characteristics of the subjects involved in our research questions. The question "Can zebrafish suffer from posttraumatic stress disorder?" was posed from multiple perspectives and encouraged subsequent questions that we sought to address to contribute to the never-ending scientific compendium. What molecular factors activate the sympathetic nervous system to influence the stress response? Does constant stimulation of the stress pathway trigger an immune response? What are the neurophysiological consequences of malfunction in stress response signaling? Are there any behavioral changes brought on by a damaged stress response pathway? Techniques and methods from endocrinology, immunology, neurophysiology, behavioral biology, and other disciplines were applied to our research to gain a better understanding of how trauma influences behavior.

In our experiment, we sought to use a multidimensional study to address our hypothesis that chronic stress exposure has deleterious effects on the zebrafish brain. Observing animal behavior is a standard research method used across multiple disciplines of science to gather insight on the effects of a subject's environment or the introduction of an agent or action onto the subject. We believed that examining anxiety-like behavior in our subjects would illuminate the locations of perturbations that could potentially occur within neural pathways of the treated fish, as well as indicate how these perturbations manifested.

In addition to our observational study, we believed that specific changes in stress behaviors were due to physiological changes in the neural pathway responsible for the stress response. To understand if and how chronic unpredictable stressors were influencing the stress response, we chose to analyze the dopamine precursor tyrosine hydroxylase and the inflammatory marker c-fos using immunohistochemical techniques and microscopy to antibody stain cross-sections of

zebrafish brains and visualize the potential effects of trauma on pathway responsible for "fight-or-flight" and anxiety-like behaviors.

The compilation of skills and techniques featured provides an intersectional approach to our research project. These studies on zebrafish allow us to look at the effects of stress on sensory, integrating, and efferent behavioral pathways to look to new indicators for measuring stress, altering consequential behaviors of stress, like risk-taking and anxiety behaviors. Using methods across scientific disciplines allowed us to look at our research questions from different perspectives, generating an integrative and multifaceted project. For us to understand the cellular and molecular consequences of stress lie in inflammation in the brain circuits necessary for our survival, may lead to therapies or interventions to mitigate the brain's harmful responses to stress.

Acknowledgments

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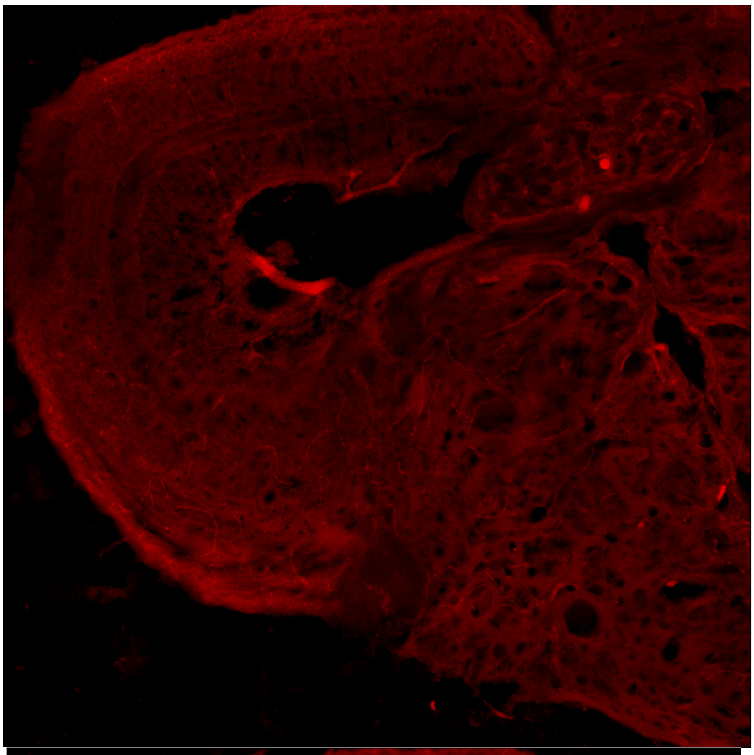
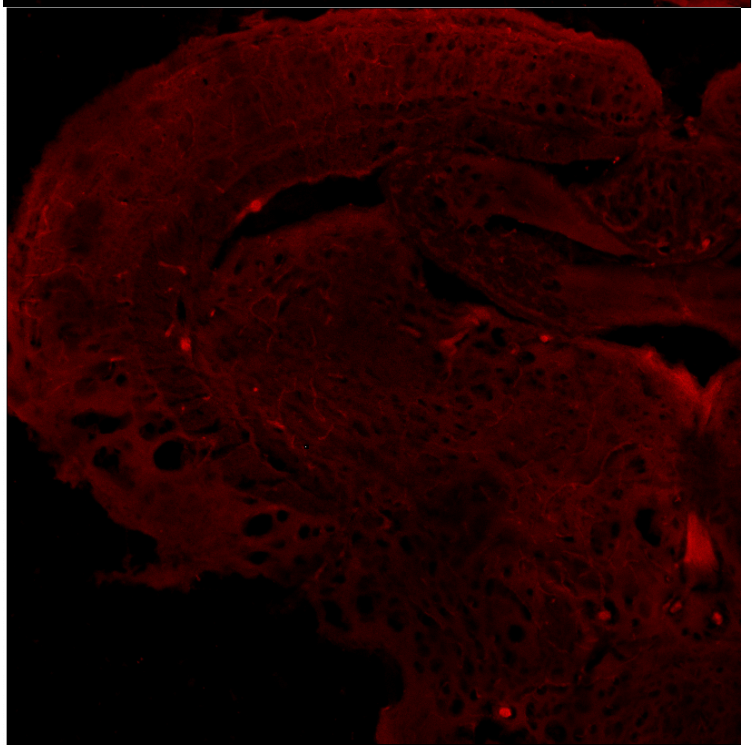
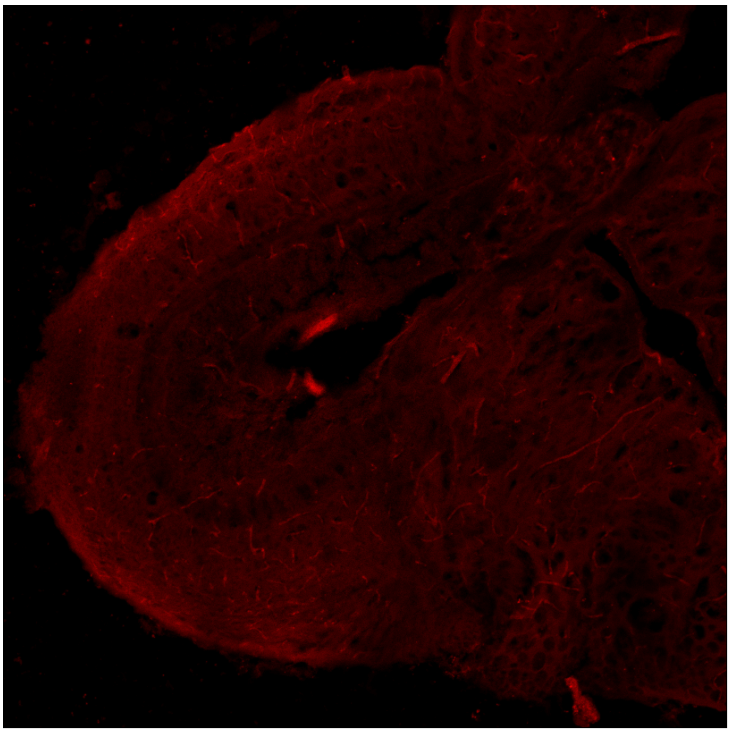
V: Appendix

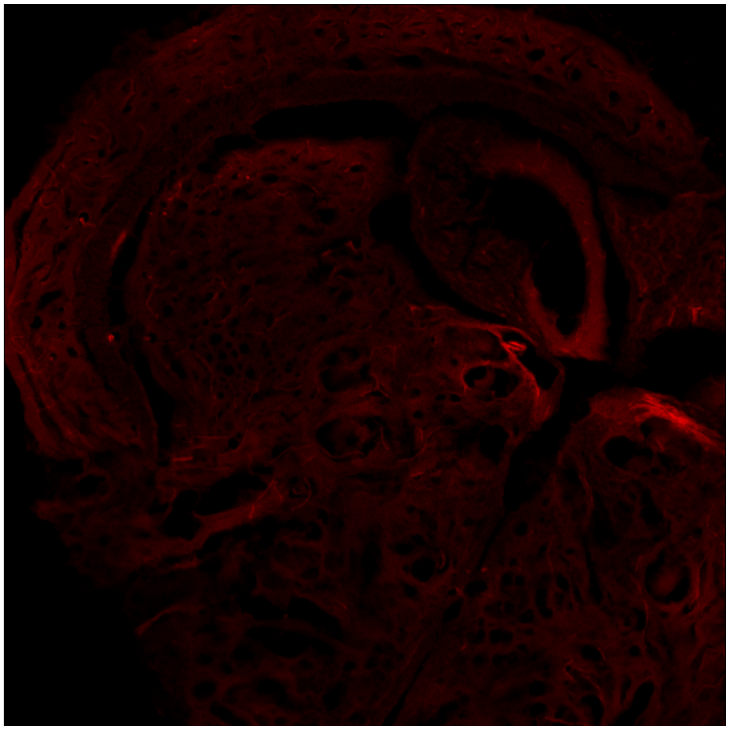
TH Control Brain Images

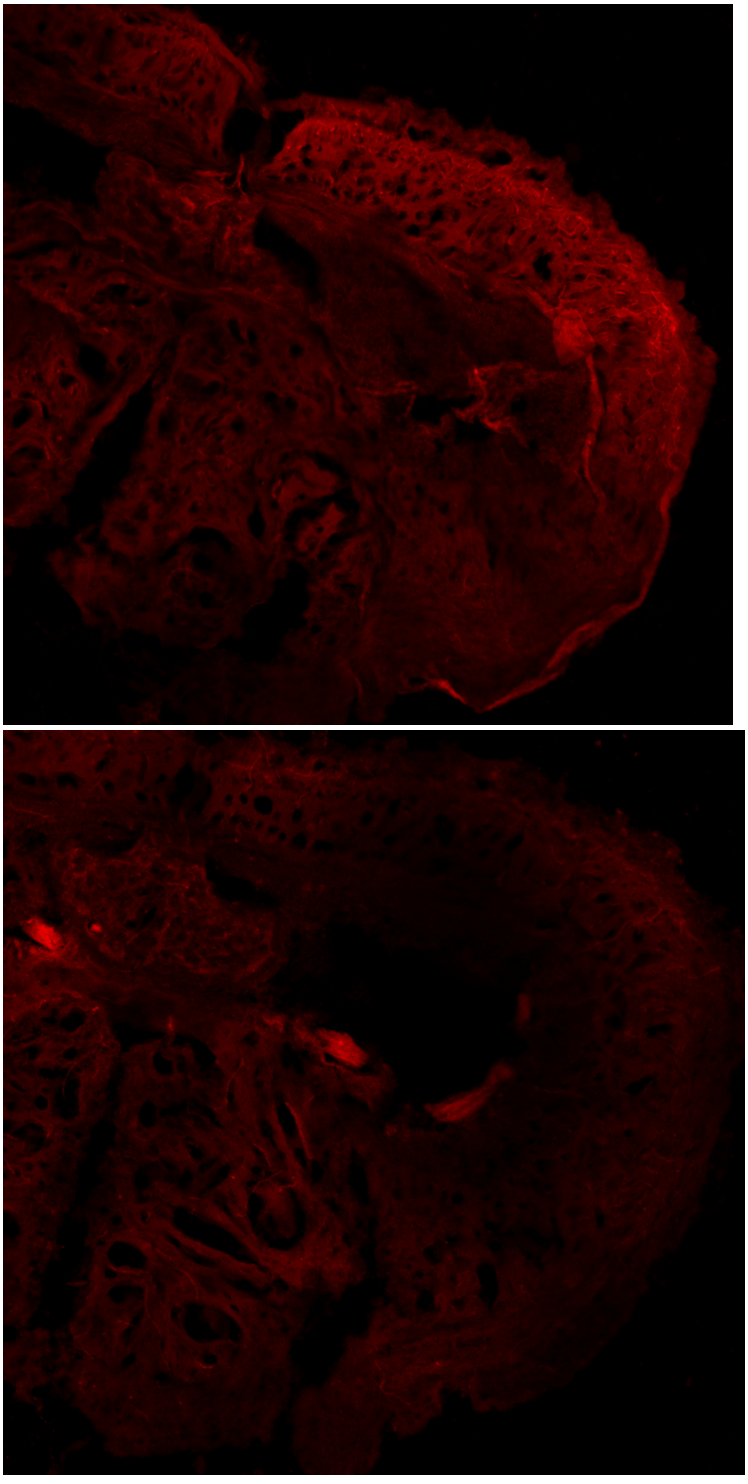






TH 2-day Stress Brain Images





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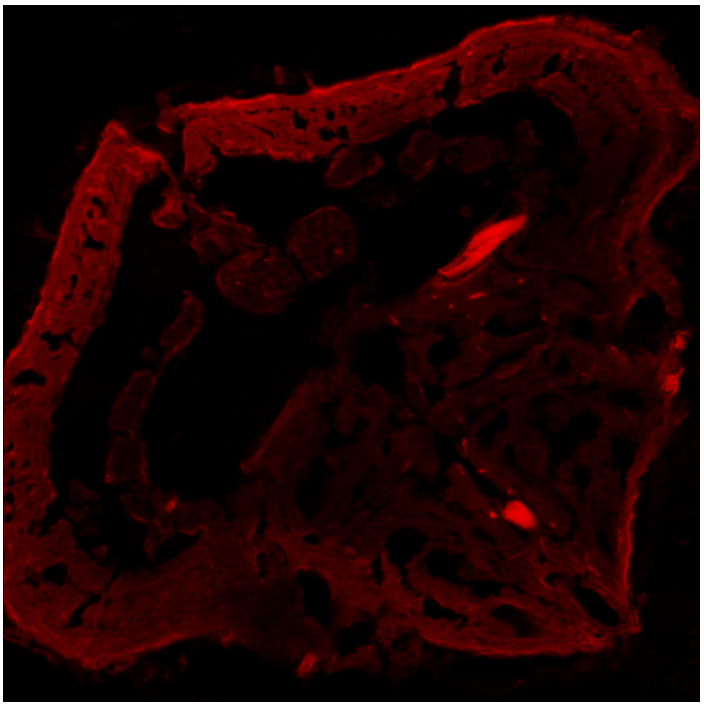

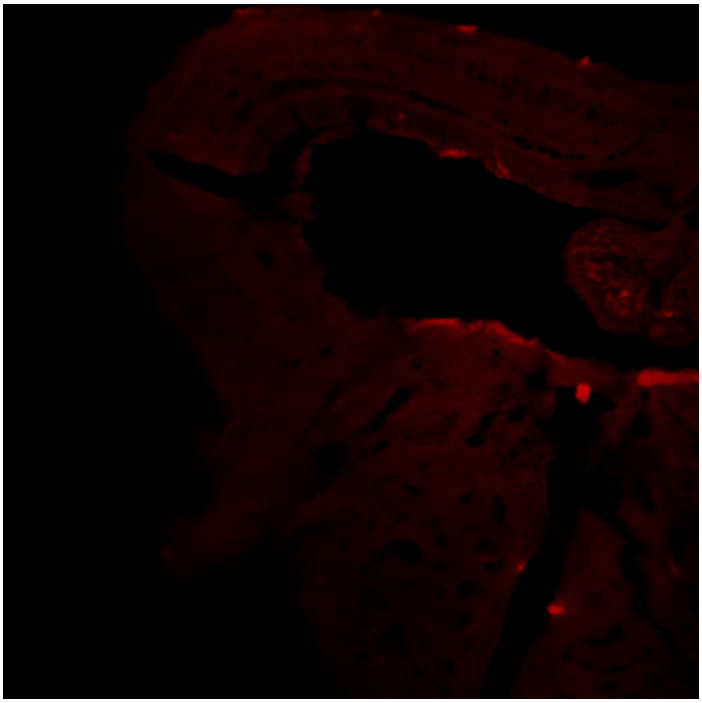

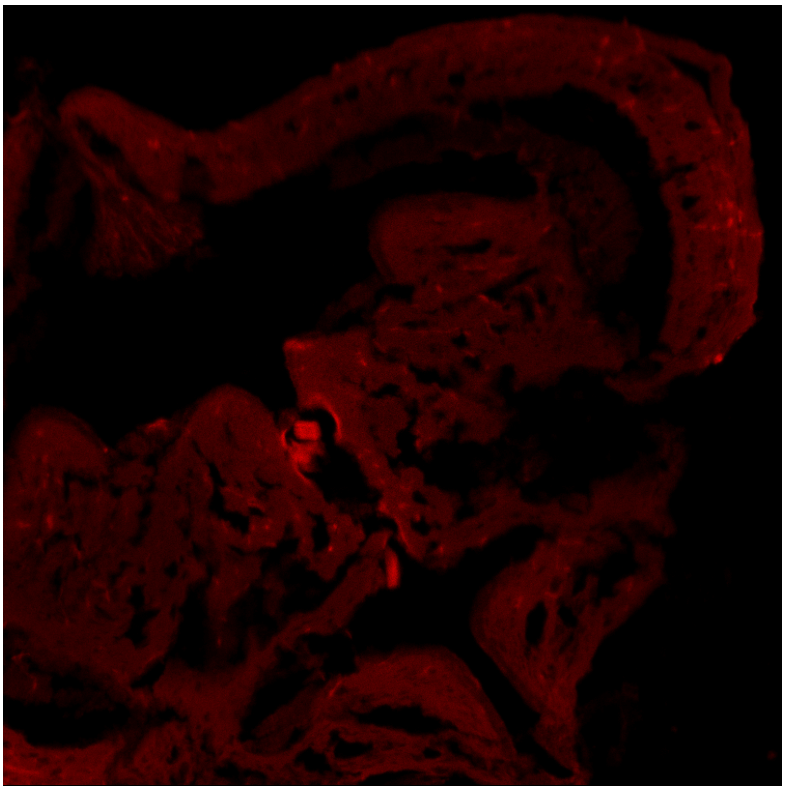

TH 8-day Stress
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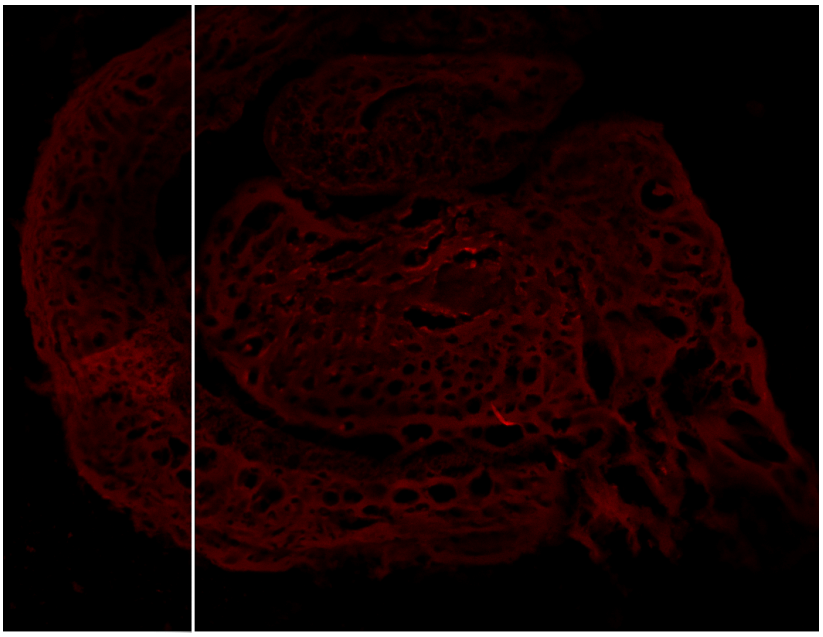
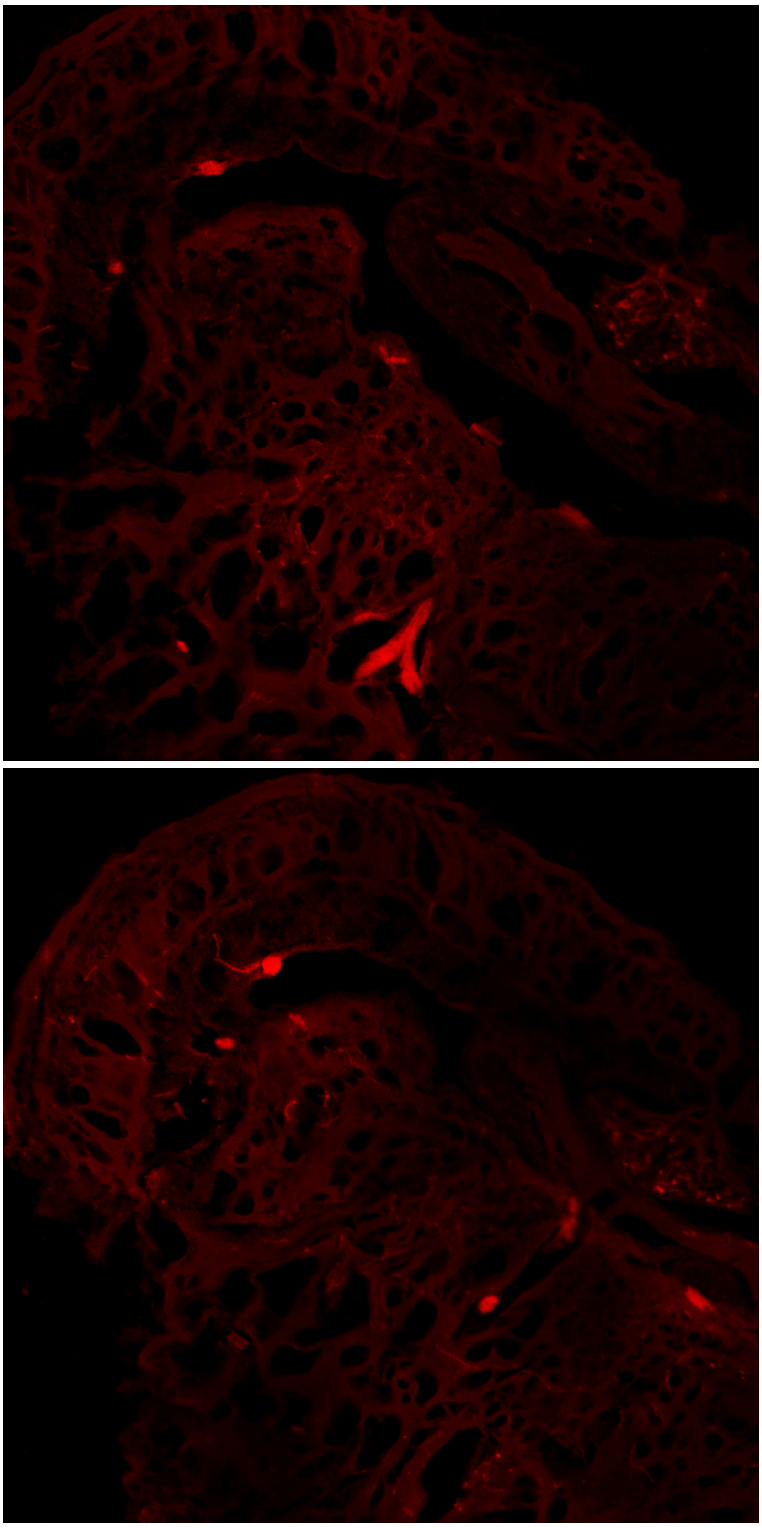
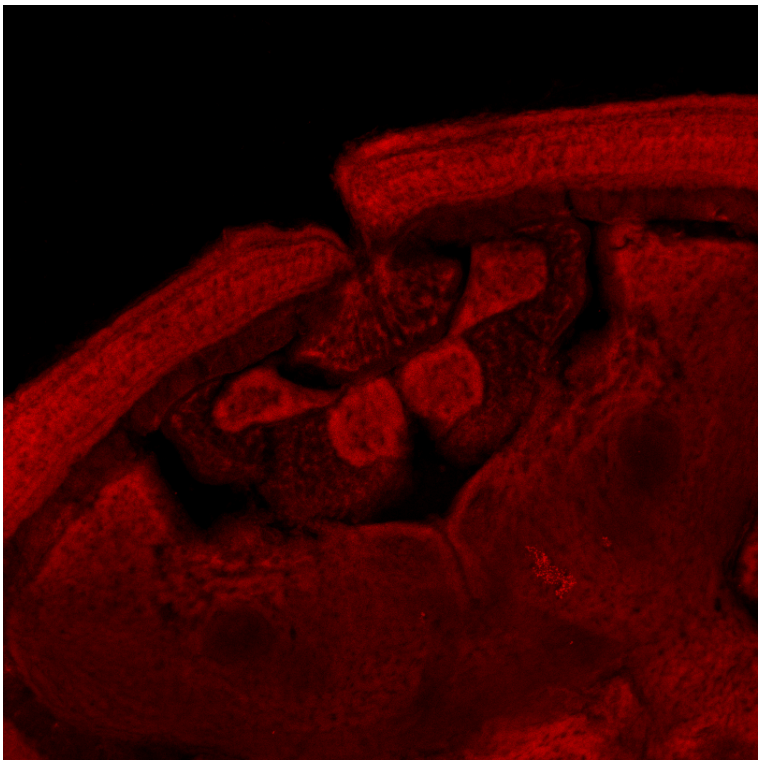
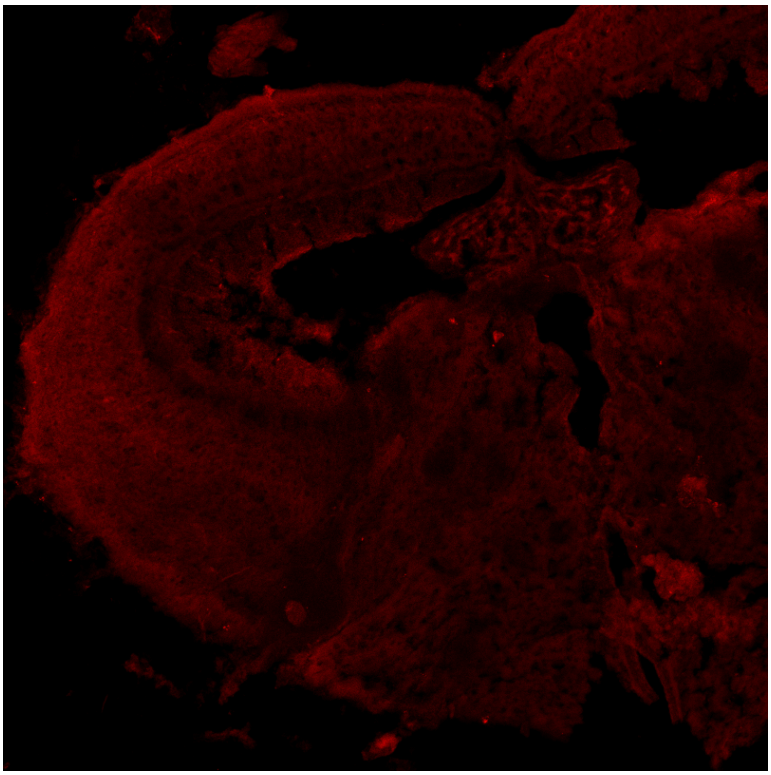
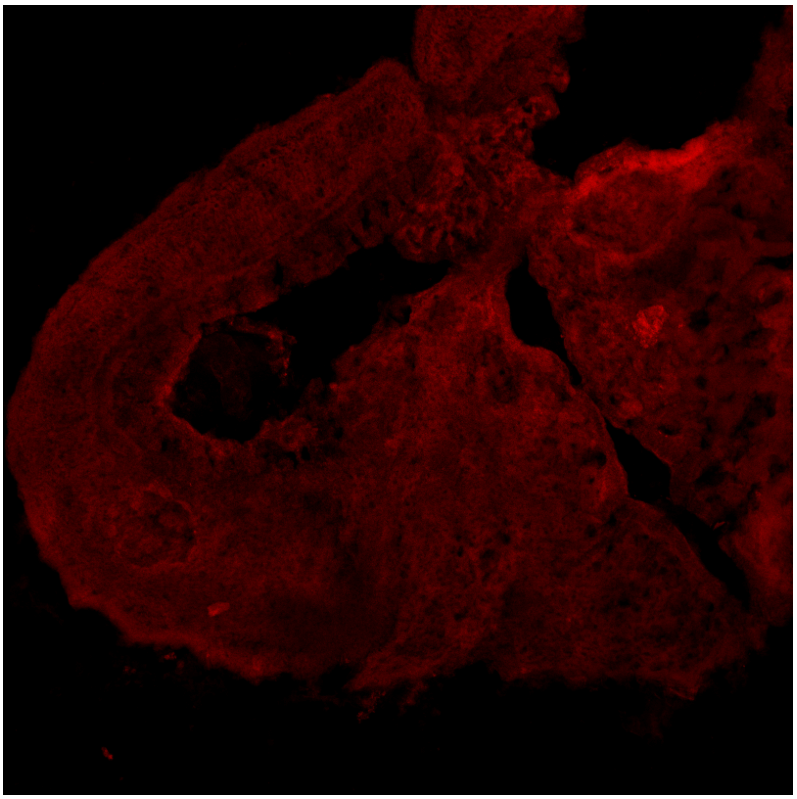




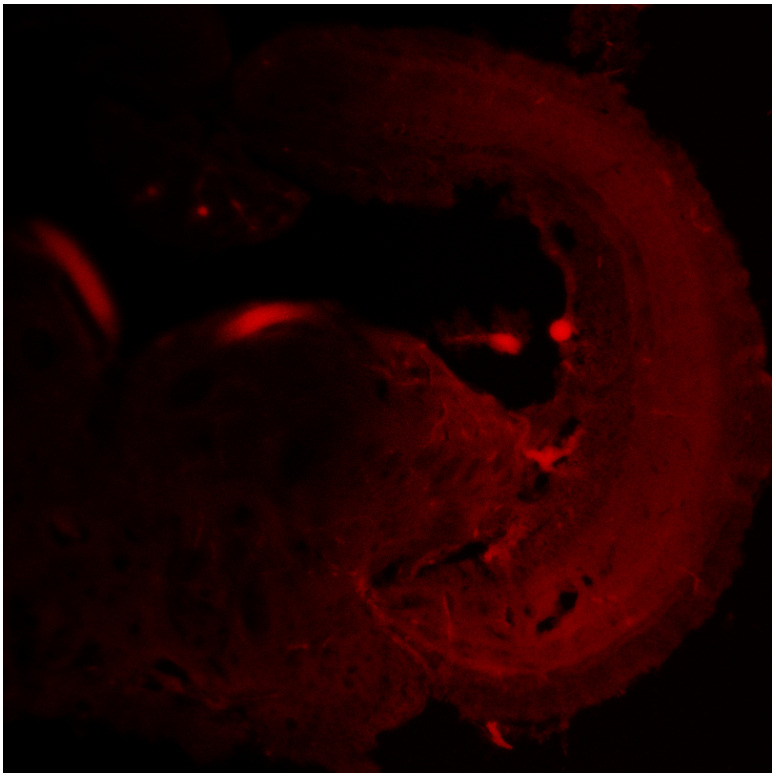
C-Fos Control Brain Images

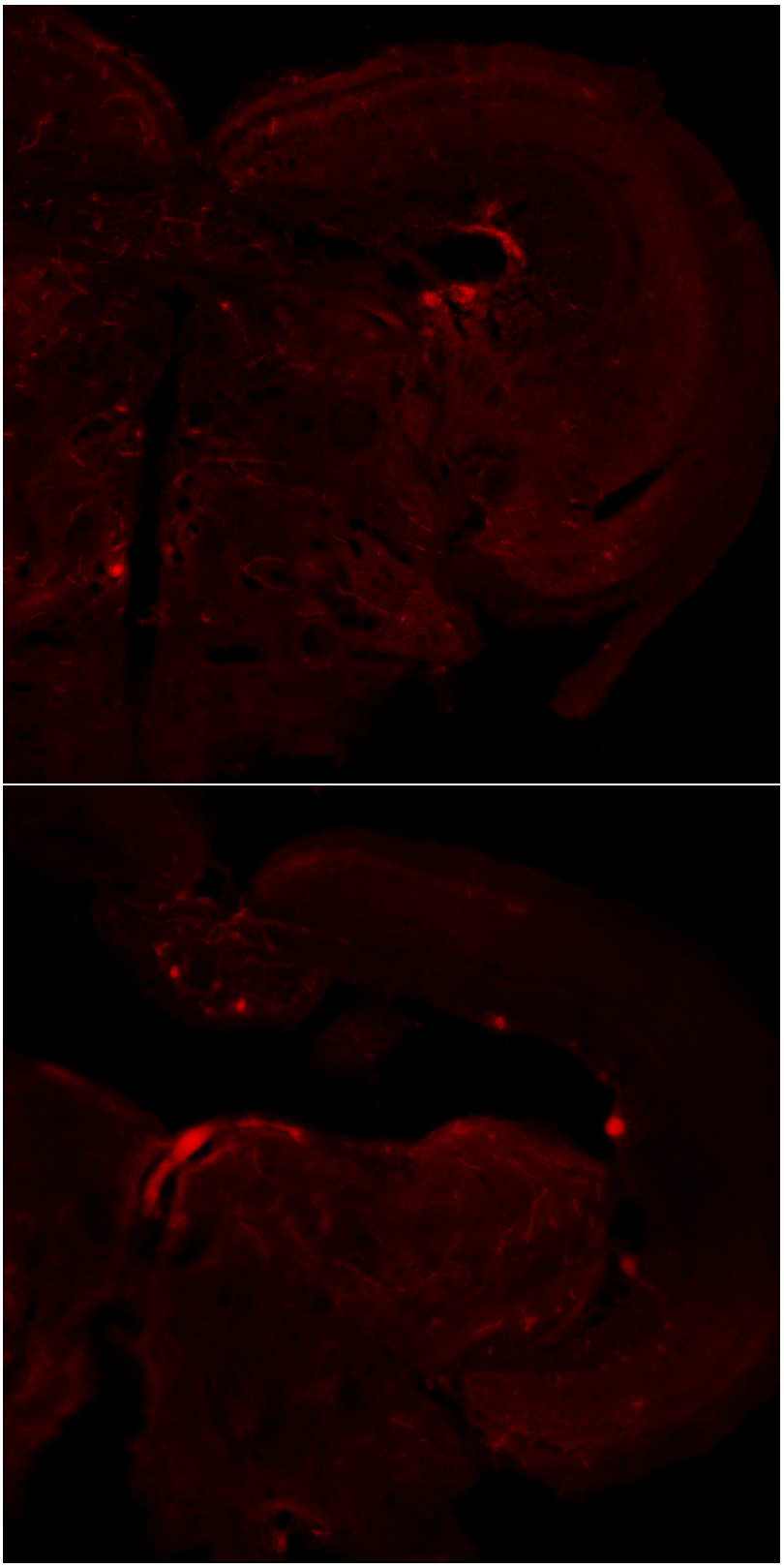




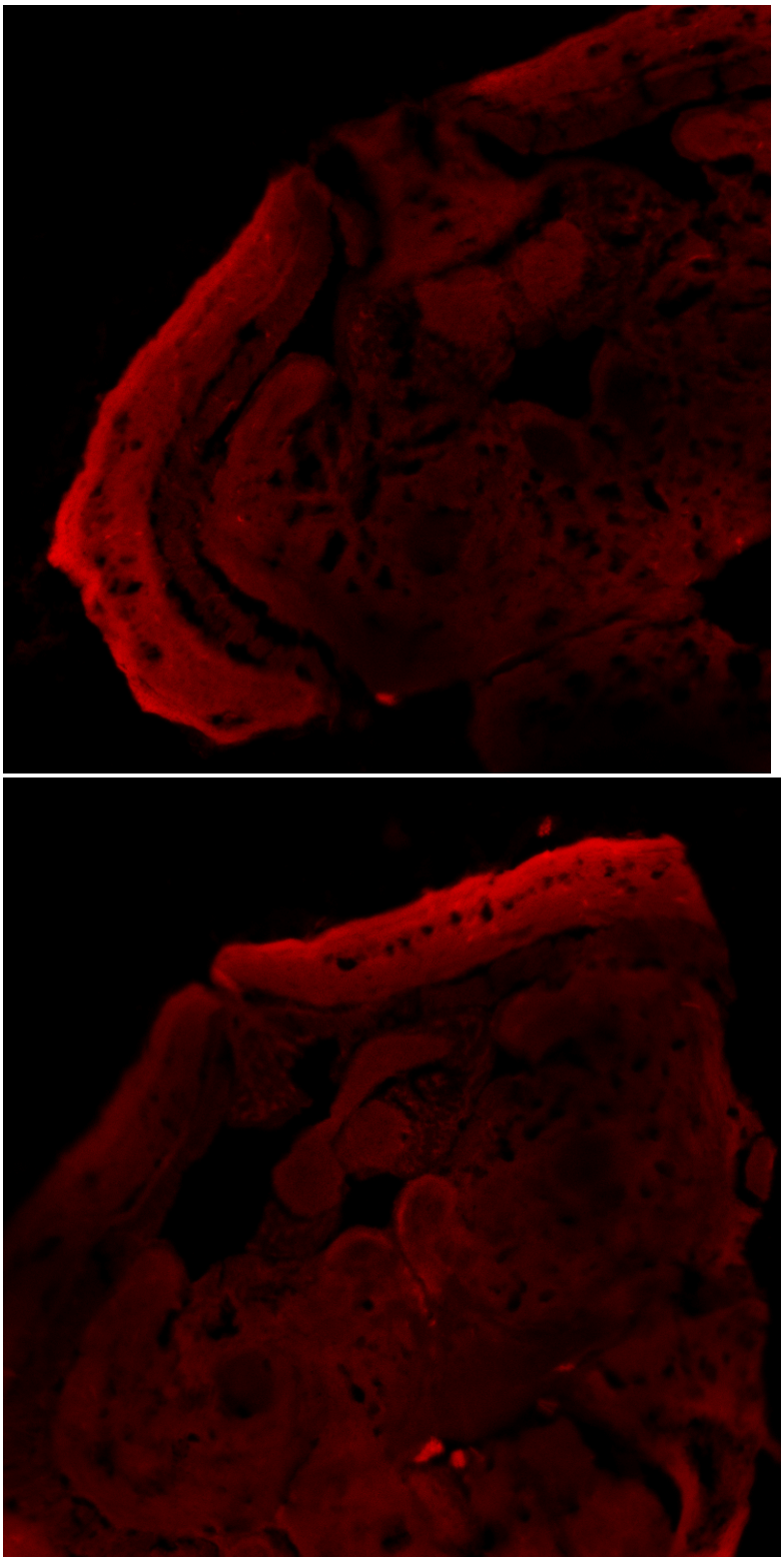


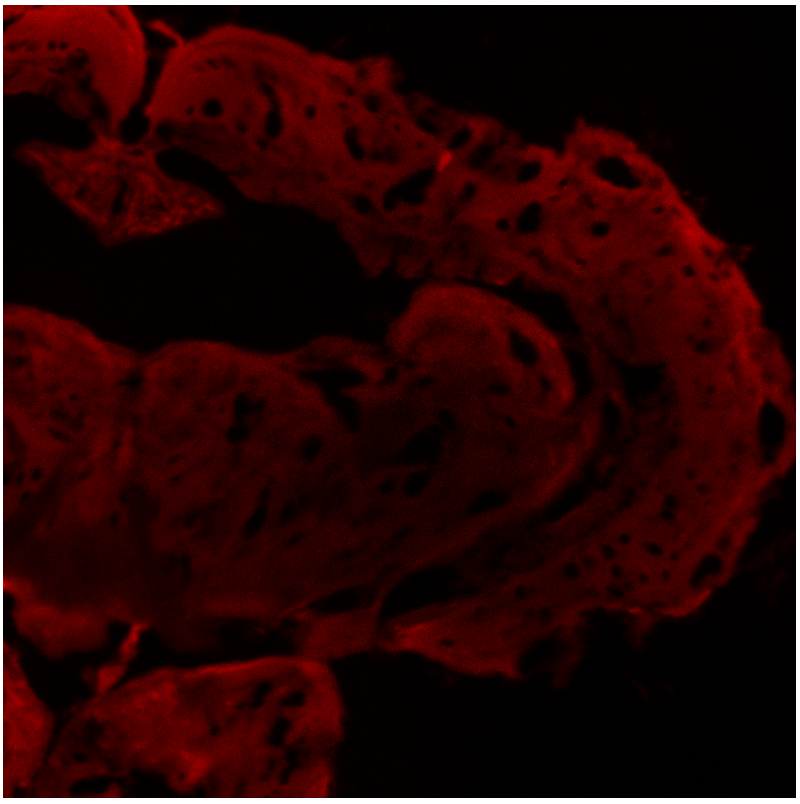
C-Fos 2-Day Stress Brain Images



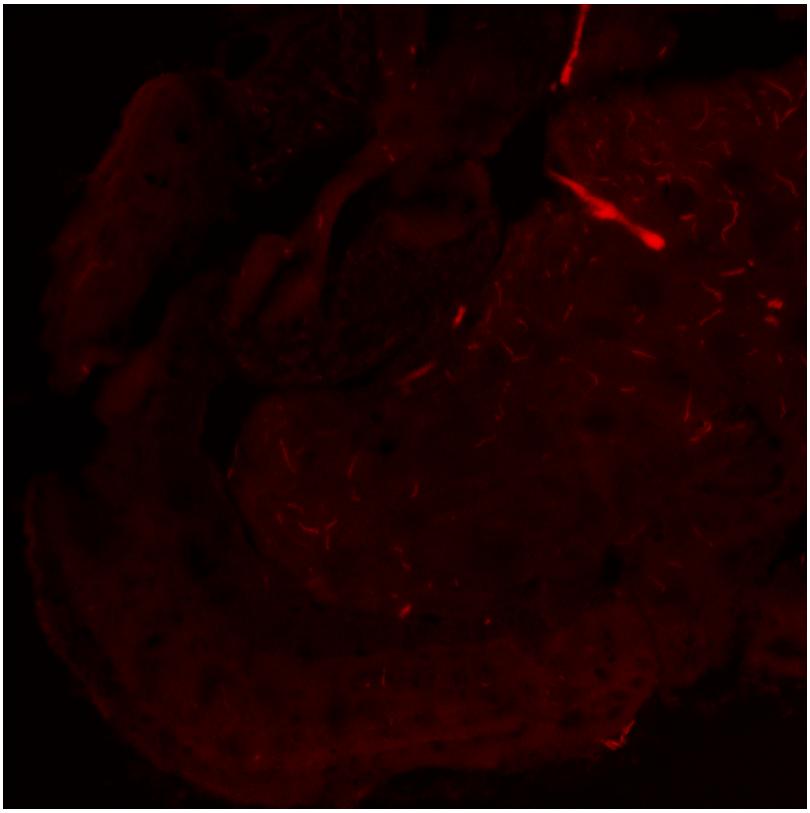


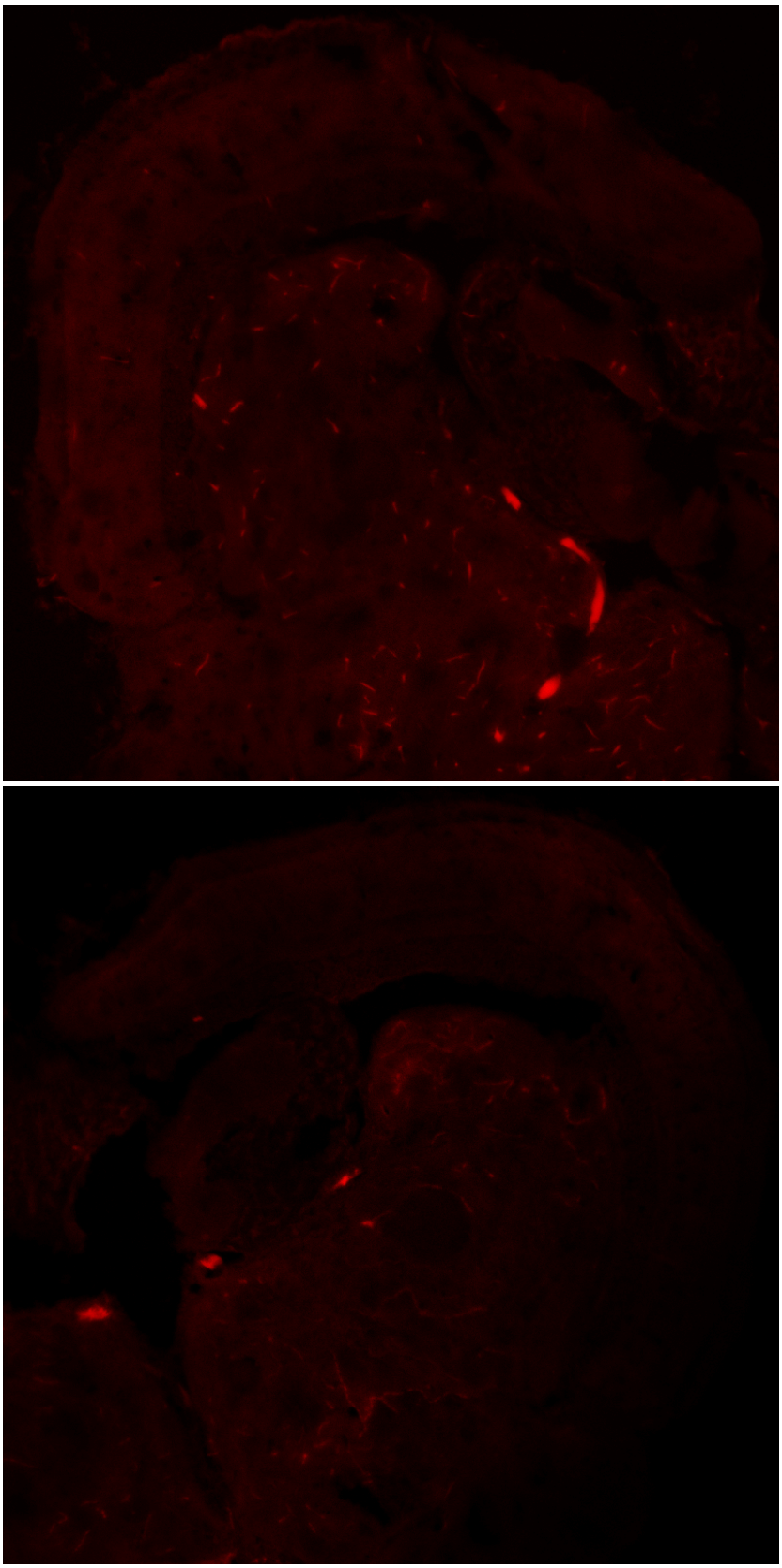
C-Fos 6-Day Stress Brain Images





C-Fos 8-Day Stress Brain Images





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