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Dalton J. Anderson
Georgia Southern University

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The Biobehavioral Effects of Embryonic Exposure to Neural Inflammation and Oxidative Stress in Zebrafish

An honors Thesis submitted in partial fulfillment of the requirements for Honors in Biology and Psychology

By

Dalton J. Anderson

Under the mentorship of Drs. Joshua Herrington and Robert Mans

ABSTRACT

The purpose of this research is to improve understanding of the neurodevelopmental effects of embryonic exposure to elevated inflammation and oxidative stress induced by the antipyretic drug acetaminophen (APAP). Our study was the first to examine the interactive effects of APAP and inflammation in zebrafish embryos and how the treatments affect brain development and larval behavior. Experimental groups of zebrafish larvae were exposed to lipopolysaccharide (LPS) to induce inflammation, APAP, or LPS + APAP and larval behavior was analyzed using Ethovision automated behavioral tracking software. We also measured changes in whole-brain Glycogen Synthase Kinase 3 Beta (GSK3B) and GSK3B phosphorylation, a common biomarker in ASD populations that has been implicated in abnormal brain development. Analysis of larval behavior revealed that the LPS and LPS + APAP groups displayed significantly less activity when exposed to a light/dark stimuli test. In addition, the LPS + APAP group showed significantly elevated ratios of pGSK3B/totalGSK3B compared to controls, which could be an indicator of atypical neuronal development.

ACKNOWLEDGEMENTS

I extend my deepest gratitude to my mentors, Dr. Herrington, and Dr. Mans, for their invaluable guidance and mentorship throughout my academic journey. You have both helped me to become a better researcher and taught me what it means to be a scientist. The combined expertise in the fields of biology and psychology you both share have allowed me to understand how all fields of science are important to one another and how to work in a team for a common goal. Thank you both for encouraging me through this research and believing in me, even when things didn't go as planned.

INTRODUCTION

APAP and Autism Spectrum Disorder

The embryonic stage of development is extremely sensitive for all organisms, especially during periods of prolific neurological growth. Insults to a developing organism via environmental exposures to teratogens could alter neuronal development and result in a developmental disorder, such as autism spectrum disorder (ASD). ASDs are typically diagnosed during early development and is characterized by many neurological and attention deficits, repetitive behavior, and impairment of language. Social interaction and communicative behavior are key identifiers for autism in adolescence according to the Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-5).

Since the approval of APAP for use during pregnancy, it has quickly become one of the most common over-the- counter (OTC) medications to treat pain, fever, and inflammation. APAP is also the only antipyretic medication recommended by physicians to alleviate pain and fevers during gestation. A recent meta-analysis and subsequent consensus of 91 physicians, scientists, and public health professionals has indicated that a growing body of literature supports a link between APAP exposure during pregnancy and infancy and neurodevelopmental disorders in humans, such as autism spectrum disorder (ASD) and attention-deficit hyperactivity disorder (ADHD) (Bauer et al., 2021).

Zebrafish as a model organism

A recent article touted zebrafish as a viable animal model to uncover the neurophysiological mechanisms that underlie the core deficits of autism spectrum

disorder, and highlights that zebrafish portray comparative behaviors found in children diagnosed with autism disorder after pharmacological treatments or alterations to their neurodevelopment (Meshalkina et al., 2018). Previous studies have found that exposing Zebrafish embryos to non-lethal doses of APAP results in hepatotoxicity and altered behavior, (Selderslaghs, Hooyberghs, et al. 2013) and altered physiological development (Glasco, Wang, Kang & Funkhouser, 2022). An extensive review of the literature has found that no studies have examined the interactive effects of APAP and inflammation in zebrafish embryos and their effects on brain development and behavior. In addition, previous studies attempting to link APAP exposure to ASD/ADHD fail to look at both the neurological effects and behavioral effects following embryonic exposure to APAP and/or LPS alone (Reuter et al., 2016).

As such, the current study will examine both the neurological and behavioral effects of allometrically scaled doses of LPS/APAP during embryonic development. In a similar study using zebrafish, it was found that inducing valproic acid or fluoxetine (a widely used pharmacological model of ASD) during early life stages caused inattentive and circling behavior mimicking ASD behaviors in humans (Dwivedi et al., 2019). In addition, that study found damage to neurological signaling pathways and decreased mGluR5 proteins (Dwivedi et al., 2019).

Another previous study on zebrafish found that dosing zebrafish larvae with APAP throughout embryonic development resulted in behavioral abnormalities, including lack of response to external stimuli, and altered movement patterns (David & Pancharatna, 2009). A similar study found when LPS is used with a non-opioid analgesic and antipyretic agent similar to APAP, the swimming patterns are very erratic, and the

zebrafish showed signs of bottom dwelling which is a characteristic of anxiety or social avoidance (Lee et al., 2022).

The current experimental study aims to mirror the procedures of the Dwivedi et al., (2019) study; however, we will expose zebrafish embryos to APAP and LPS and observe changes in whole-brain Glycogen Synthase Kinase 3 Beta (GSK3B) phosphorylation, and total GSK3B. Inflammation and subsequent use of APAP during pregnancy and infancy is more clinically relevant to humans, and abnormal GSK3B activity is a clinically relevant and thought to be involved in the etiology of ASD (Khanzada, Butler et al., 2017).

After review of previous studies mentioned above which examined the effects of inflammation and APAP on zebrafish larvae, we were able to create several a-priori hypotheses for the current experiments, which are as follows:

H1.) The LPS and LPS + APAP groups will display significantly decreased activity in the light-dark test compared to the control group.

H2.) The APAP group will display significantly more activity in the light-dark test compared to the control and LPS and LPS + APAP groups.

H3.) The LPS group will display significantly more pGSK3B than the control group.

H4.) LPS + APAP group will display a significantly higher ratio of pGSK3B/totalGSK3B.

METHODS

Subjects

For the experimental study, a total of 400 zebrafish embryos were used to test three different treatment groups, with 100 zebrafish in each experimental group. Our final sample was N=202, with nearly half of our sample expiring before behavioral tests took place at 96h post-fertilization (hpf). Zebrafish were raised at the Georgia Southern University Developmental Psychobiology Laboratory with full IACUC approval.

The first group served as our untreated controls and were reared in standard zebrafish egg water, the second were exposed to 10mcg/ml of LPS at 4hpf though egg water absorption causing an inflammatory response. The third treatment group were exposed to 3.31 mmol/ml of APAP at 24hpf through absorption, inducing oxidative stress. The fourth group was exposed to the same amount of LPS at 4hpf then the same amount of APAP at 24 hours to mimic the effects of taking APAP (a generator of oxidative stress) after inflammation occurs. For all groups, behavioral analysis occurred at ~96hpf and a western blot analysis was used to test if the treatments had effect on GSK3B pathways in the brain.

Light/ Dark Testing

Behavioral analysis serves as a pivotal methodological approach for evaluating deviations in behavior within zebrafish. Light-dark cycle testing involves subjecting zebrafish larvae to controlled conditions within specialized chambers equipped with an overhead camera system to observe larval activity. During the light-dark test, zebrafish larvae are positioned atop UV dark-activated lights within 96-well plates and undergo

alternating cycles of light and darkness, each lasting 10 minutes, repeated for a duration of one hour. The overhead camera meticulously tracks the behavioral patterns of individual zebrafish within the wells, enabling the identification and documentation of any aberrations to the activity of the fish during the transitions between light and darkness with Noldus Ethovision automated behavioral tracking software. Automated tracking of zebrafish larvae offers valuable insights into the behavioral responses of zebrafish under specific conditions, facilitating nuanced assessments of treatment efficacy and behavioral anomalies associated with our pharmacological treatments.

Dissecting Larvae

To accurately delineate the brain structures within the zebrafish larvae intended for dissection, we consulted the graphical representations detailed in the work of Hildebrand, D. et al. (2017), enabling comprehensive visualization of the entire brain anatomy. The dissection process involved the utilization of micro dissecting forceps, a light microscope, two petri dishes, and ice to ensure precision and adherence to established protocols. Sacrificing and dissection followed the methodology outlined by Ferreira, J., et al. (2018), employing rapid cooling for a duration of 3-5 minutes to achieve humane euthanasia. Subsequently, utilizing micro dissecting forceps, the larvae's eyes were excised, and the body was dissected at the Weberian apparatus, proximal to the "neck" region. Two intact brains were carefully extracted and deposited into a 1.5mL microcentrifuge tube, where they were homogenized for 1 minute with TPER buffer solution. Following homogenization, the samples were briefly subjected to centrifugation

(30 seconds - 1 minute) and promptly transferred to a freezer maintained at -20 degrees Celsius to preserve biochemical integrity.

RESULTS

Activity Level

Activity was quantified as total time spent active in seconds for the duration of the light/dark experiment. Our data was found to violate assumptions of normality in a homogeneity of variance test ($p < .001$). As such, an Independent-Samples Kruskal-Wallis analysis of variance (ANOVA) test was employed to examine the differences in activity between the experimental groups (control, LPS, APAP, & LPS + APAP). The Kruskal-Wallis ANOVA confirmed a main effect of our treatment on activity levels of the zebrafish larvae, (3, $N = 202$) = 41.248, $p < .001$. Subsequent pairwise comparisons employing the Mann-Whitney *U* test (with the Bonferroni correction) indicated that the control group displayed significantly higher levels of activity compared to both the LPS group ($U = 55.02$, $p < .001$) and the LPS + APAP group ($U = 44.98$, $p < .001$) while no significant difference in activity was found compared to the APAP group ($U = -9.273$, $p > .05$). The APAP group activity was found to be significantly higher than both the LPS ($U = -64.29$, $p < .001$) and the LPS + APAP group ($U = 54.25$, $p < .001$).

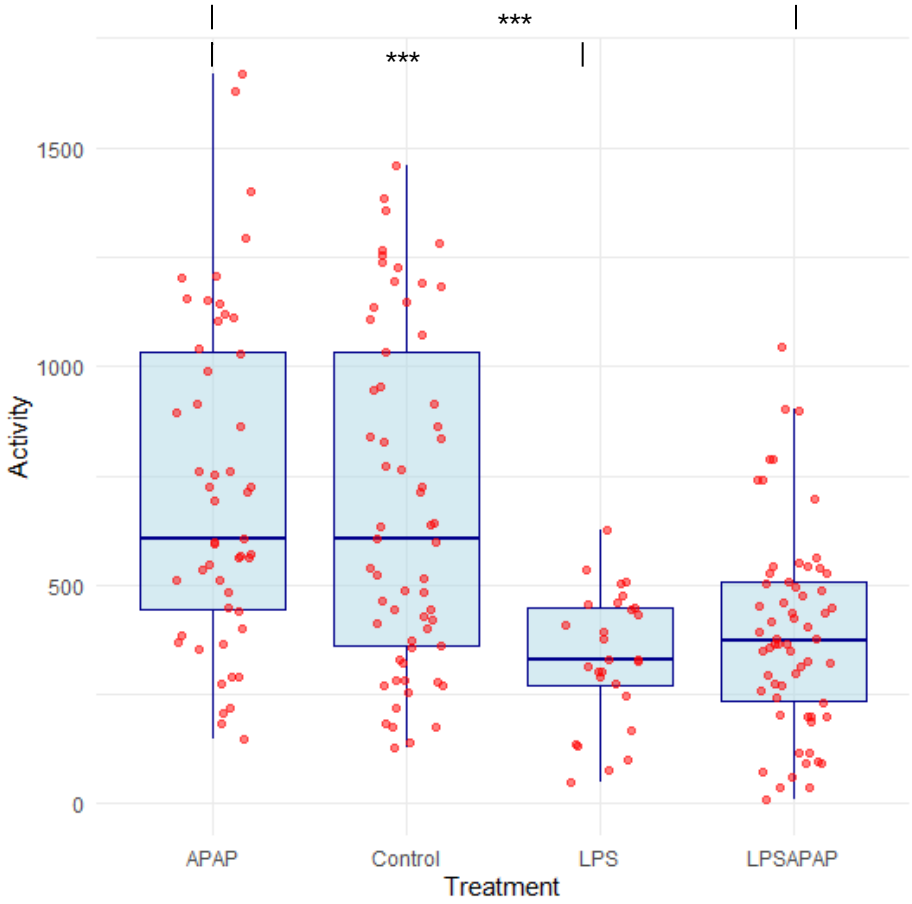

Fig 1. Activity levels in the APAP and Control groups were not found to be significantly different, while the LPS and LPS + APAP groups were found to be significantly less active than both the APAP and Control groups. No differences were found between the APAP/Control or LPS/LPS + APAP groups.

Western Blot Analysis

Phosphorylated GSK3B levels (pGSK3B) and total GSK3B were analyzed using Western Blot analysis. Image J software quantified the differences between the intensity of the bands in each column. GAPDH was used as a loading control for pGSK3B and total GSK3B analysis. A one-way analysis of variance (ANOVA) found that there was no significant effect of group on pGSK3B ($p > .05$). However, a significant effect of group

was found for total GSK3B, $F(3, 1.41) = 3.42$, $p = .043$ and for the pGSK3B/totalGSK3B ratio, $F(3, 4.376) = 3.42$, $p = .043$. Bonferroni-corrected post-hoc t-tests revealed significant differences in total GSK3B between the LPS and APAP groups, $p = .046$, and in the pGSK3B/totalGSK3B ratio between the control and LPS + APAP groups, $p = .049$.

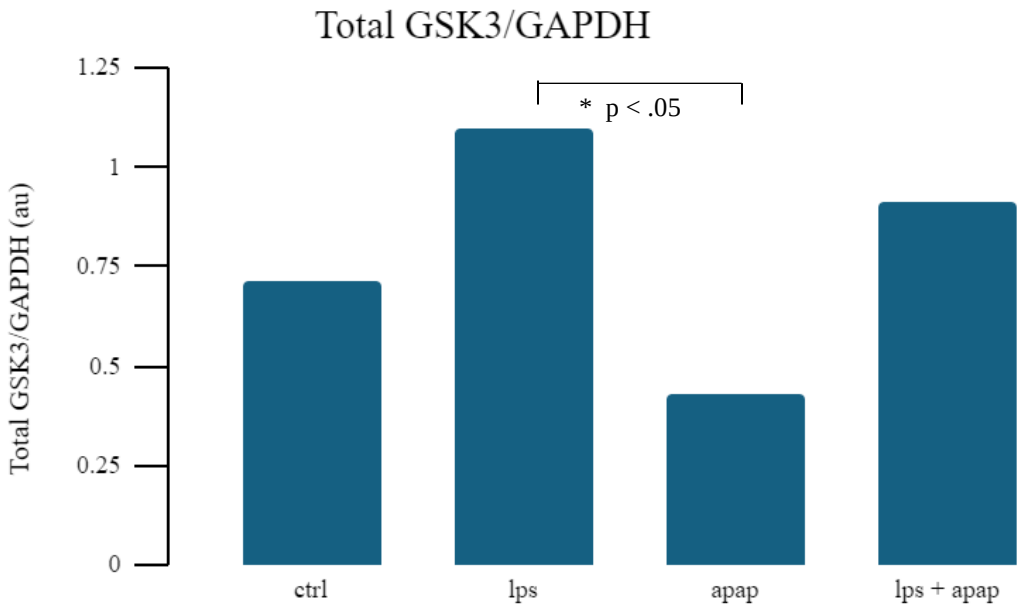

Fig 2. Analysis of our Western Blot data found that total GSK3B levels were elevated in the LPS treated group compared to the APAP treated group.

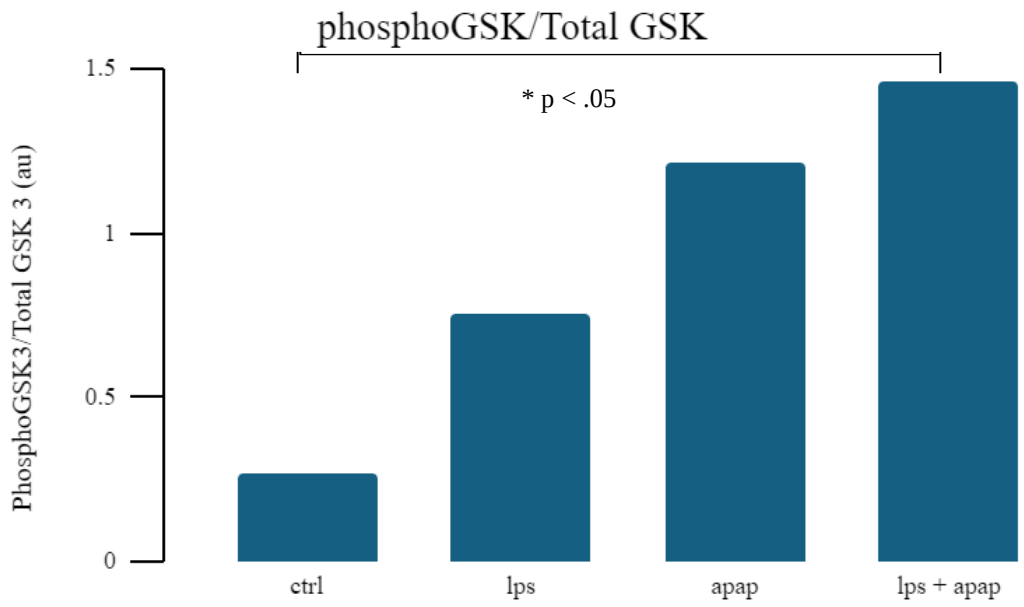

Fig 3. Analysis of our Western Blot data found that pGSK3B/total GSK3B level ratios were elevated in the LPS + APAP group compared to the control group.

DISCUSSION

The findings from this thesis study underscore the nuanced impact of lipopolysaccharide (LPS) and acetaminophen (APAP) on embryonic zebrafish behavior and neural development, offering implications for understanding human neurodevelopmental disorders such as Autism Spectrum Disorder (ASD). Contrary to the hypothesized protective role of APAP against inflammation-induced abnormalities, the data reveals that APAP alone does not mitigate the inflammatory effects of LPS, potentially due to suboptimal dosing or overwhelming inflammatory signals. Importantly, our behavioral results support the authors' *a-prior* prediction that the LPS group and LPS + APAP group would display significantly lower levels of activity compared to the control and APAP

group; however, the results suggest that APAP treatment did not significantly elevate activity levels compared to the control group.

Of note is our result that the LPS + APAP treatment was both significant in altering embryonic behavior compared to controls and was correlated with increased ratios of phosphorylated Glycogen Synthase Kinase 3 Beta (pGSK3B) to total GSK3β. An elevated pGSK3B to total GSK3B ratio is associated with atypical neural development, such as the over proliferation of neuronal growth, the elongation of oligodendrocytes which could alter neuronal growth trajectories, and disruption of apoptosis, or targeted neuronal cell death (Hur, & Zhou, 2010; Wang, et al., 2012). Our findings align with broader literature indicating a possible connection between prenatal exposure to APAP and increased ASD and ADHD risks, suggesting that such exposure could influence critical neurological pathways during embryonic development. The study's innovative use of zebrafish as a model organism provides a controlled environment to examine these effects, bridging gaps in existing research and highlighting the intricate interactions between pharmaceutical interventions and developmental biology in the context of neurodevelopmental health.

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