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Microbiota-Derived Metabolites and Their Effects on Anxiety-Related Behavior

Author
Quicho, Michael Noah Banaag

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Microbiota-Derived Metabolites and Their Effects on Anxiety-Related Behavior

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Physiological Science

by

Michael Noah Banaag Quicho

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ABSTRACT OF THE THESIS

Microbiota-Derived Metabolites and Their Effects on Anxiety-Related Behavior

by

Michael Noah Quicho

Master of Science in Physiological Science

University of California, Los Angeles, 2023.

Professor Elaine Yih-Nien Hsiao, Chair

The microbiota-gut-brain (MGB) axis is a complex communication network connecting the central nervous system (CNS) with the gut microbiota. Lifestyle factors, including diet, can have a profound impact on the composition and function of the gut microbiome and hence modulates the MGB axis. The high-fat diet (HFD) is a major contributor to the obesity pandemic and is associated with increased susceptibility to neurological disorders, including anxiety and depression. The HFD is shown to decrease the abundance of beneficial bacteria and increase systemic levels of proinflammatory cytokines and lipopolysaccharides. Animal and human studies have demonstrated that HFD can induce anxiety-like behavior, as measured by elevated plus maze and open field tests in mice and rats. The underlying mechanism of this effect is believed to involve alterations in the MGB axis, as well as changes in the gut microbiota composition and metabolites. Obesity-induced chronic inflammation induces anxiety-like behavior by activating the immune system and altering neurotransmitter signaling. There is increasing focus on the role of limbic system inflammation in the pathogenesis of several metabolic and neurological disorders, especially concerning HFD. However, the biochemical

mechanisms and pathways that link high-fat consumption and CNS homeostasis with the pathology of neurological disorders are not well understood.

This thesis investigates the relationship between diet, specifically high-fat diet (HFD), and anxiety-like behavior in mice, focusing on the role of the microbiota-gut-brain (MGB) axis. A 10-week experiment was conducted, wherein male C57BL/6 mice were divided into HFD and control diet (CD) groups. The HFD group exhibited weight gain and increased anxiety-like behavior compared to the CD group, as assessed through various behavioral paradigms, including the open field test (OFT), elevated plus maze (EPM) test, and light/dark box (LDB) test. Notably, anxiety-like behavior was observed as early as three weeks into the HFD treatment. Additionally, the study explored the potential involvement of imidazole propionate (ImP), a microbiota-derived metabolite, in anxiety-like behavior. ImP supplementation induced anxiety-like behavior in mice, despite no significant differences in ImP levels between HFD-fed and CD-fed mice. Histological analysis suggests that ImP decreases cFOS expression in the hypothalamus, a brain region important in the regulation of appetite and insulin signaling. There were no changes in microglial morphology in specific brain regions associated with anxiety regulation in response to ImP. However, further investigations are needed to understand the precise mechanisms underlying the effects of ImP on the MGB axis. Overall, this thesis contributes to the understanding of the complex interplay between diet, the gut microbiota, and the CNS, highlighting potential therapeutic strategies targeting the MGB axis for anxiety and neurological disorders.

The thesis of Michael Noah Quicho is approved.

Patricia E. Phelps

Xia Yang

Elaine Yih-Nien Hsiao, Committee Chair

University of California, Los Angeles

2023

DEDICATION

For my family, Jeffrey Quicho, Louisa Quicho, Isabella Quicho, and Mason Quicho

In Loving Memory of Vicente Bautista Quicho (1942-2022)

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Acknowledgments

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Introduction

The microbiota-gut-brain (MGB) axis is a complex bidirectional communication network that links the central nervous system (CNS) with the gut microbiota (Cryan *et al.*, 2019; Martin *et al.*, 2018). The gut microbiome, which is the collection of microorganisms residing in the gastrointestinal tract, has been shown to produce a variety of gut-derived metabolites that can communicate with the CNS through various pathways, including the circulatory system, the enteric nervous system (ENS), the vagus nerve, and the immune system (Agirman and Hsiao, 2021; Marcobal *et al*., 2013). The impact of the gut microbiome on the host's physiological state is highlighted in germ-free mice, mice depleted of a gut microbiome, which exhibit several behavioral, social, and cognitive deficits (Sampson and Mazmanian, 2015). Further studies reveal that neurological deficits in germ-free mice can be rescued or partially rescued by restoring the gut microbiota. For instance, when germ-free mice are colonized with a conventional gut microbiota through fecal microbiota transplantation or cohousing with conventionally raised mice, their anxiety-like behavior and cognitive impairments improve (Luczynski *et al. 2016;* Bercik *et al., 2011*). Overall, this indicates that the gut microbiota plays a crucial role in shaping CNS function and behavior.

There is an abundance of research and clinical evidence highlighting the importance of a diverse and stable gut microbiome to maintain optimal health. Dysbiosis of the gut microbiota and their metabolites have been implicated in the pathogenesis of neuropsychiatric disorders, such as anxiety and depression (Foster and McVey Neufeld, 2013; Rogers *et al.*, 2016). Some microbes and gut-derived metabolites have been shown to have harmful effects on host physiology. For example, the gut-derived metabolite, 4-ethylphenyl sulfate (4EPS) has been shown to impair

oligodendrocyte myelination and induce anxiety-like behavior in mice (Needham *et al*., 2021). However, the precise mechanisms by which this gut-derived metabolite and others interact with the CNS and contribute to the development of neurological disorders require further investigation.

Conversely, certain bacterial species have shown the potential to alleviate anxiety-like behavior in mice. For example, social deficits were rescued through the supplementation of certain commensal microbes, like *Bacteroides fragilis*, which has been shown to ameliorate anxiety-like behavior in autism spectrum disorder (ASD) mouse models (Hsiao *et al*. 2013). Additionally, supplementation with *Enterococcus faecalis* restores social deficits and reduces corticosterone levels in antibiotic-treated mice (Wu *et al.*, 2021). These findings underscore the complex interplay between the gut microbiome and neurological disorders, suggesting that targeted manipulation of the gut microbiota and their metabolites may hold promise for developing novel therapeutic treatments.

Diet is a significant modulator of the gut microbiota-gut-brain axis and can profoundly impact the composition and function of the gut microbiome (Cotillard *et al*., 2013; Nemoto, Kubota, and Ohno, 2023). The high-fat diet (HFD), which is characterized by a high intake of saturated and trans fats and a low intake of fiber and micronutrients, has been growing in popularity due to globalization, particularly in Western countries (Malesza *et al.*, 2021). The prevalence of HFD increases each year as highly-processed and affordable food becomes more accessible in the developing world (Swinburn *et al.*, 2011). The HFD is a major contributor to the obesity pandemic and has been shown to increase the risk of various comorbid chronic diseases,

including cardiovascular disease, diabetes, and certain types of cancer (Castillo, Orlando, and Garver, 2017). The consequences of HFD are not just limited to physical health, but it is also associated with increased susceptibility to neurological disorders highlighted by a positive correlation between body mass index (BMI) and rates of anxiety and depression in the United States (Zhao *et al.* 2009). The HFD has been shown to modulate the gut microbiota composition and function by reducing gut microbial diversity, leading to gut dysbiosis and inflammation, which may contribute to impaired synaptic plasticity and increased susceptibility to anxiety-like behavior in mice (Daniel *et al.*, 2014; Liu *et al.*, 2015; Malesza *et al.*, 2021).

However, studies examining the relationship between HFD and anxiety-like behavior have produced conflicting results. Some animal and human studies have demonstrated that a HFD can induce anxiety-like behavior, as measured by elevated plus maze (EPM) and open field tests (OFT), in mice and rats (Dutheil *et al.*, 2015; Li *et al*., 2022; Pang *et al*., 2022; Picolo *et al*., 2021). In contrast, other studies have shown that HFD consumption had no effect on anxiety-like behavior in mice (Toyama *et al*., 2015, Zemdegs *et al.*, 2015). The underlying mechanism of how HFD affects anxiety-like behavior remains uncovered, but it is believed to involve alterations in the MGB axis and changes in the gut microbiota composition and metabolites (Bruce-Keller, Keller, and Morrison, 2008). Specifically, HFD decreases the abundance of beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium*, which are known to produce gamma-aminobutyric acid (GABA) and increase the levels of proinflammatory cytokines and lipopolysaccharides (LPS) (Turnbaugh *et al*., 2008). Other notable implications of the HFD include a decreased *Bacteroidetes/Firmicutes* ratio, an overall decrease in microbial alpha diversity, and alterations in the abundance of microbial taxa (Ley *et al.*, 2006). Obesity-induced

chronic inflammation induces anxiety-like behavior by activating the immune system and altering neurotransmitter signaling. Specifically, obesity-induced inflammation is shown to modulate several brain regions in the limbic system such as the hypothalamus (Krishna *et al*., 2015), amygdala, and hippocampus, which all can regulate the pathogenesis of several neurological disorders like depression and anxiety (Chevalier *et al.*, 2020; Guillemot-Legris and Muccioli, 2017; Kokras *et al.*, 2012). However, the understanding of the biochemical mechanisms and pathways connecting the consumption of HFD to CNS homeostasis regarding the development of neurological disorders remains limited.

There is increasing focus on the role of limbic system inflammation in the pathogenesis of several metabolic and neurological disorders, especially concerning HFD (Schachter *et al*., 2018; Saltiel and Olefsky, 2017). Fenestrated capillaries within the hypothalamus allow for direct access to microbiome-derived circulating factors, thus the hypothalamus is important for sensing molecules that typically would not cross the blood brain barrier (BBB) (Banks, 2016). The microbiome is essential in modulating the permeability of the BBB by regulating the expression of tight junctions, and chronic immune activation can disrupt the BBB's permeability (Martin *et al.*, 2018). The arcuate nucleus of the hypothalamus, which regulates feeding behavior and insulin responses, is primarily susceptible to gut-derived metabolites, including free fatty acids, that can induce inflammation by activating toll-like receptors on microglia (Thaler *et al.*, 2012). Microglia are also rapidly recruited to the paraventricular nucleus (PVN) of the hypothalamus in mice under chronic psychological stress (Ataka *et al.*, 2013). Consumption of the HFD can increase the production of gut-derived metabolites and cytokines that can directly affect the activity of microglia, the resident immune cells of the CNS (Erny *et al.*, 2015; Tapia-González *et*

al., 2011; Wen *et al.*, 2011). Growing evidence suggests that changes in microglia function and morphology contribute to the overall cognitive and social deficits induced by HFD (Bocarsly *et al.*, 2015; Cope *et al.*, 2018; Hao *et al.*, 2016; Spencer *et al.*, 2019), especially as it pertains to depression and anxiety (Seguella *et al*., 2021; Yirmiya, Rimmerman, and Reshef, 2015). Mice fed a long-term HFD exhibited anxiety-like behavior accompanied by microglia morphology and hippocampal structure alterations. However, it has been shown that treatment with butyrate, *L. reuteri* (Zhuang *et al.*, 2022), or obeticholic acid (Wu *et al.*, 2021) ameliorates anxiety-like behavior in mice fed HFD through the activation of microglia. Microglia isolated from HFD mice secrete more pro-inflammatory cytokines such as TNF-α than that secreted by standard diet (SD) mice (Puig *et al.*, 2012). Therefore, microglia are an important therapeutic target for modulating metabolic disorders (Wen *et al.*, 2011).

Recent studies have identified a novel gut microbiota-derived metabolite, imidazole propionate (ImP), as a potential mediator of HFD-gut-brain axis interaction. Increased levels of ImP were found in mice that consume the ketogenic diet (KD), which is a type of HFD (Olson *et al.*, 2018). Additionally, mini-pigs that consumed a choline-deficient HFD also exhibited increased ImP levels (Lützhøft *et al.*, 2022). Levels of ImP were also found to be elevated in obese humans (Osadchiy *et al.*, 2023). ImP is a product of histidine metabolism by the gut-bacteria-produced enzyme, urocanate reductase (urdA), and has been shown to have detrimental effects on metabolic and neurological health (Molinaro *et al.*, 2023; van Son *et al.*, 2021). ImP is largely associated with the pathogenesis of diabetes mellitus (Molinaro *et al.*, 2020; Peters *et al.*, 2023), as it has been shown to induce insulin resistance through the mTORC1 pathway and the inhibition of AMPK (Koh *et al.*, 2018; Koh *et al.*, 2020). ImP has also been shown to induce

intestinal inflammation by activating toll-like receptor 4 (TLR4) leading to intestinal barrier impairment (Chen *et al*., 2022; Könner and Brüning, 2011; Wu *et al*., 2022).

Despite the effect of ImP on metabolic function, whether the metabolite can influence the MGB and induce behavioral changes is unknown. Therefore, this study aims to investigate the microbiome-dependent effect on anxiety behavior, emphasizing gut-derived metabolites like ImP on the microbiota-gut-brain axis. In this study, we evaluated the effect of a 10-week HFD (60% kcal fat) and a 3-week HFD (60% kcal fat) consumption on anxiety-like behavior, limbic system activity, microbiota-derived metabolites, and inflammation. Additionally, we measured the same variables in mice administered ImP for 2 weeks via an osmotic pump. Microglia cultures were used to evaluate the effect of gut-derived metabolites on microglial density and morphology. The findings of this study could provide novel insights into the mechanisms by which the HFD affects the MGB axis and its role in the pathogenesis of anxiety-like behavior. Studying the potential therapeutic benefits of gut-derived metabolites on the CNS could provide new avenues for the prevention and treatment of anxiety and other neurological disorders. Further investigation into the MGB axis and its role in neurological function has the potential to revolutionize our understanding of the relationship between the gut and the brain and improve human health outcomes.

Materials and Methods

Mice and Diet

Male C57BL/6J mice, aged 8-10 weeks, were housed in the UCLA Health Sciences Barrier Facility under controlled conditions, including a constant temperature and light/dark cycle. The mice were co-housed with same-sex littermates. Two dietary groups were formed: a control diet (CD, D12450Ji, n=12, 900.00g carbohydrates, 10% kcal fat) and a high-fat diet (HFD, D12492, n=12, 197.00g carbohydrates, 60% kcal fat). Both groups had similar amounts of protein and fiber, with differences in fat content (45g for CD and 270g for HFD). The mice had *ad libitum* access to water and were maintained on their respective diets for either 3 weeks or 10 weeks.

Osmotic Pump Implant

Adult male C57BL/6J mice, aged 2-3 months, underwent intraperitoneal implantation of Alzet osmotic pumps which accurately delivered either 300ug/day of Imidazole Propionate (n=9) or vehicle (1% DMSO in water; n=9). Previous research has shown that diabetes patients have as much as a ten-fold increase in ImP compared to controls (Koh *et. al.*, 2018). Therefore, this concentration of ImP was chosen since it gives the best approximate fold change between the experimental and control groups. Prior to surgery, mice received a mixture of lidocaine and bupivacaine to minimize the pain. All surgeries were conducted aseptically and mice were anesthetized via isoflurane inhalation. Osmotic pumps delivered either ImP or vehicle over the course of 14 days before behavioral testing and euthanization.

Open Field Test (OFT)

The open field test (OFT) is used to evaluate exploratory and anxiety-like behavior in mice. Mice were acclimated to the experimental room 45 minutes before each experiment. Each mouse was carefully placed into the center of a white plastic box of 50cm x 50cm x 40cm to freely explore for 10 minutes. The arena is brightly lit to provoke the natural tendency of mice to avoid open, brightly lit spaces as an index of anxiety. For the analysis, the arena is delimited into two zones: the center zone and the peripheral zone. Anymaze tracking software is used to measure the time spent in the center, the time spent in the periphery, the total distance traveled, and the overall speed. The apparatus was thoroughly cleaned and deodorized between each trial.

Elevated Plus Maze (EPM) Test

The elevated plus maze (EPM) test is commonly used to assess anxiety-like behavior in mice. All mice were acclimated to the behavioral testing room 45 minutes before each experiment. The EPM apparatus is elevated 50 cm above the ground and consists of two-open arms and two-closed arms that form a plus shape. Each arm is 30 cm x 6 cm x 25 cm in size. The arms of the apparatus are brightly illuminated to induce anxiety-like behavior. The mouse was carefully placed in the center of the apparatus facing one of the open arms. The mouse was allowed to explore freely for 5 minutes. Anymaze video tracking software was used to measure the time spent in the open arms, the time spent in the closed arms, the number of entries into the open arms, and the speed of the mouse. The apparatus was thoroughly cleaned and deodorized between trials.

Light/Dark Box (LDB) Test

The light/dark box (LDB) test is a commonly used method to evaluate anxiety-like behavior in mice. All mice were acclimated to the behavioral testing room 45 minutes before each experiment. The LDB apparatus is 45 cm x 91 cm x 38 cm in size and is composed of two compartments: one dark and one illuminated. The two compartments are separated by a small opening of 10 cm x 10 cm. Each mouse was carefully placed in the center of the illuminated compartment and was allowed to freely explore for 10 minutes. Anymaze video tracking software was used to measure the time spent in the light compartment and the number of transitions between the compartments. The LDB apparatus was thoroughly cleaned and deodorized between each trial.

Immunohistochemical Imaging

All mice were anesthetized via isoflurane and cardially perfused with cold phosphate-buffered saline (PBS). To allow for c-Fos expression, mice brains were dissected from mice 90 minutes after undergoing behavioral testing. Brains were fixed in 4% paraformaldehyde (PFA) for 24 hours, then immersed in 30% sucrose for 24-48 hours before being frozen in optimal cutting temperature compound (OCT) at -80℃. Brains were cryosectioned using a Leica cryostat using standard cryostat techniques. 30um coronal sections were collected and immersed in PBS with 1% sodium azide in 24-well plates. All sections are stored at 4°C until processing. Sections were then washed and blocked (0.5%PBS-T, 10% donkey serum) for one hour at room temperature. Following blocking, sections were stained with primary antibodies for c-FOS and NeuN overnight on 200g rotation at 4°C. Microglia were stained with the primary antibody ionized calcium-binding molecule (IBA1, Fujifilm Wako Chemicals 019-19741) with a 1:1000 ratio. The primary antibody solution is removed and brain sections are washed with PBS three times on

200g rotation at room temperature for 10 minutes. Brain sections are then stained with a secondary antibody solution (cFOS, 1:100, anti-rabbit; NeuN, 1:1000, anti-guinea pig) for two hours on 200g rotation at room temperature. The secondary antibody solution is removed and brain sections are washed with PBS three times on 200g rotation at room temperature for 10 minutes. Sections are then stained with DAPI (1:1000) for 10 minutes on rotation at room temperature for 10 minutes. Sections are then washed with PBS and manually mounted onto glass slides using Prolong™ Diamond antifade mounting media.

Confocal Imaging and Analysis

Confocal imaging of the hypothalamus, amygdala, and hippocampus was performed using a Zeiss LSM 780 with Zen Black software at a magnification of 20x. Neuronal activity was measured using ImageJ by manually counting co-localized c-Fos expression within defined neuronal cell bodies labeled by NeuN and DAPI. Each brain region of interest was outlined using ImageJ, and all counting was normalized to the area. Every brain region was completely counted and was not split into brain subregions. The average amount of c-Fos expression amongst 6 sections per brain sample was measured. Images acquired were within one plane since images did not incorporate any z-stacks. Microglia imaging was performed at 20X magnification. The microglial density of *in vitro* cell cultures was measured using ImageJ.

Microglia Cultures

Brains from P3 C57BL6 mice were collected and disassociated using a Neural Tissue Dissociation Kit (P) (Miltenyi, Cat #130-092-628). Cells were then suspended on a 70um cell strainer and washed with HBSS with Ca/Mg. Cells were then centrifuged at 300g for 10 minutes.

The centrifuged cells were resuspended in magnetic-activated cell sorting (MACS) buffer (PBS-0.5% fetal bovine serum). 10uL of CD11b Microbeads (Cat#130-093-636) were then added to the cell solution. Cells were then suspended onto LS columns (Cat#130-042-401) and rinsed with 3mL of MACS buffer until microglia were collected and eluted from the column. $1x10^5$ isolated microglia cells were then placed on coverslips (in 24 well plate) coated in 0.01% Poly-L-Lysine. The microglia TIC medium was prepared according to Bohlen et al. (*Bohlen et al.*, 2017). The TIC medium consists of DMEM (GlutaMAX Life Technologies), N-Acetyl Cysteine (Sigma-Aldrich), Insulin (Sigma-Aldrich), Apo-Transferrin (Sigma-Aldrich), Sodium Selenite (Sigma Aldrich), Cholesterol (Sigma-Aldrich), Heparan Sulfate (Ams Bio), Mouse M-CSF (Peprotech), Mouse TGFb1(Peprotech), Mouse IL-34 (BioLegend), and Mouse CX3CL1 (Biolegend). Half of the TIC medium was changed after 3 days. 10mM of ImP or the vehicle solution (1% DMSO in water), were administered to the cultures shortly after the medium change. 3 days after metabolite administration, cell cultures were stopped by fixating the cells in PFA, and the medium supernatant was collected for further analysis. Untreated culture wells and treated culture wells were paired since they are derived from the same brain samples. Thus, untreated culture wells were normalized to allow for accurate cross-comparison to treated wells.

ELISA Assay

Levels of inflammatory cytokines of microglia culture media samples were detected using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's directions. Levels of IL-6 were measured using ELISA kits (Thermo Fisher).

Data Analysis

All data statistical analyses and comparisons were performed using a two-tailed unpaired Student's T-test ($P<0.05$ considered statistically significant) using GraphPad Prism. All data are represented as mean with standard deviation (SD). $*P < 0.05$ is considered statistically significant with $**P < 0.01$ and $***P < 0.001$. Non-significant differences are represented by "ns."

Results

10-Week High-Fat Diet (HFD)Treatment Induces Weight Gain and Anxiety-Like Behavior in Mice

To evaluate the impact of a high-fat diet (HFD) on anxiety-like behavior in mice, we conducted a 10-week experiment using male C57BL/6 mice aged 8-10 weeks. The mice were randomly assigned to two groups: the HFD group, which received a diet consisting of 60% kcal fat, and the control group (CD), which received a diet containing 10% kcal fat. The diets were provided *ad libitum* throughout the study period. After the 10-week dietary treatment, we assessed anxiety-like behavior in the mice using three behavioral paradigms: the open field test (OFT) (Kraeuter, 2019), the elevated plus maze test (EPM) test (Carola *et al.*, 2002), and the light/dark box (LDB) test (Arrant, Schramm-Sapyta, and Kuhn, 2013) (Figure 1A). Additionally, we monitored the changes in body weight between the HFD-fed and CD-fed mice over the course of the 10 weeks. As early as two weeks into the dietary treatment, the HFD-fed mice showed a significant increase in body weight compared to the CD-fed mice (Figure 1B).

Before the dietary treatment, we conducted baseline measurements of anxiety-like behavior in the OFT to ensure there were no initial differences between the two groups, thus suggesting that all effects observed are diet-induced. (Figure 1C). During the OFT, HFD-fed mice exhibited a higher frequency of fecal boli (Calvo–Torrent, Brain, and Martinez, 1999; Hall, 1934), suggesting increased stress compared to CD-fed mice (Figure 1D). Furthermore, HFD-fed mice spent less time in the center and more time in the periphery of the OFT, indicative of anxiety-like behavior (Figure 1E). In the EPM, HFD-fed mice spent more time in the closed arms of the apparatus than the exposed arms, further indicating anxiety-like behavior (Figure 1F). In the

LDB, HFD-fed mice spent more time in the dark compartment compared to the light compartment, supporting the presence of anxiety-like behavior (Figure 1G). Throughout all three behavioral paradigms, HFD-fed mice exhibited reduced locomotor activity, as evidenced by decreased distance traveled and increased immobility time. Overall, HFD-fed mice displayed greater anxiety-like behavior than CD-fed mice, as measured by the OFT, EPM, and LDB.

Mice were sacrificed 90 minutes after behavioral testing to allow for the expression of cFOS. To analyze possible differences in neuronal activity between HFD-fed mice and CD-fed mice, we performed immunohistochemistry (IHC) staining for cFOS and NeuN. cFOS+ cells were manually counted in limbic system brain regions. There was no statistically significant difference in the number of cFOS+ cells in HFD-mice and CD-mice in the hypothalamus and hippocampus (Figures 2A, 2B).

3-Week Short-Term High-Fat Diet Induces Anxiety-Like Behavior in Mice

We investigated the onset of anxiety-like behaviors in response to a high-fat diet (HFD) and sought to determine the timeframe during which these behaviors manifest. Based on our earlier observation of significantly increased weight gain induced by the high-fat diet after 2 weeks, we opted for a dietary treatment duration of 3 weeks in 8-10 week old male C57BL/6 mice. This choice was informed by the estimated timeframe of approximately 2 weeks for the microbiome to undergo a shift in response to dietary changes (Olson *et. al.*, 2018). Following the 3-week dietary treatment, the mice underwent three behavioral tests: the Open Field Test (OFT), Elevated Plus Maze (EPM), and Light-Dark Box (LDB) experiments (Figure 3A).

Before administering the dietary treatment, we conducted baseline assessments of anxiety-like behaviors using the OFT, ensuring no discernible differences in behaviors between the two experimental groups (Figure 3B). During the OFT, the number of fecal boli was measured as an indicator of a stress-related response, and no statistically significant difference was observed between the mice fed the control diet and those fed the HFD (Figure 3C). Notably, in the OFT, both the HFD-fed and CD-fed mice exhibited comparable levels of anxiety-like behavior and locomotion (Figure 3D).

However, when assessing anxiety-like behaviors using the EPM, we noted a higher prevalence of such behaviors in the HFD-fed mice compared to the CD-fed mice. This was evident from the reduced time spent by the HFD-fed mice in the center of the maze (Figure 3E). Furthermore, in the LDB experiment, the HFD-fed mice exhibited a lower frequency of entries into the light zone and spent less time in the light zone compared to the CD-fed mice (Figure 3F).

These behavioral tests collectively demonstrated that anxiety-like behaviors start to manifest around the 3-week mark of consuming the high-fat diet. It is worth noting that the 3-week short-term (STHFD) mice exhibited anxiety-like behavior, albeit to a lesser extent than the 10-week long-term (LTHFD) mice, indicating a progressive increase in anxiety-related responses with prolonged exposure to the HFD. Interestingly, no difference in c-Fos expression was observed in the hypothalamus of STHFD-fed mice compared to CD-fed mice (Figure 4). This was indicated by IHC staining of brain samples 90 minutes following a 90-minute LDB test.

Imidazole Propionate Supplementation Induces Anxiety-Like Behavior in Mice After 2 Weeks

Imidazole Propionate (ImP) is an interesting candidate microbiome-derived metabolite that has been implicated in diabetes, cardiovascular disease, and obesity. We measured ImP levels in the serum of the LTHFD-fed mice and the CD-fed mice and found no difference (Figure 5A). Despite this, we decided to investigate any potential links between ImP, HFD, and anxiety-like behavior. To evaluate imidazole propionate's role in inducing anxiety-like behavior, we implanted mice with an Alzet osmotic minipump that delivered 300μg of ImP per day over 14 days. This osmotic pump ensures the accurate release of ImP over the course of the experiment. Body weight was also measured over the course of ImP supplementation, but there was no significant difference between the two groups. OFT and EPM were performed on day 14 of the ImP supplementation (Figure 5B). During the OFT, ImP-supplemented mice had increased fecal boli than control mice, thus suggesting increased stress in ImP-supplemented mice (Figure 5C). ImP-supplemented mice also exhibited more anxiety-like behavior with decreased speed and distance traveled in the center of the OFT (Figure 5D). Even though there was no difference in time spent between the open and closed arms of the EPM, ImP-supplemented mice exhibited an increase in time immobile with a decrease in distance traveled in the closed arms of the EPM (Figure 5E)

Following behavioral testing, mice were sacrificed and tissues were collected for histological analysis. Brains were cut and stained for cFOS and NeuN to analyze differences in neuronal activation. cFOS+ and NeuN+ positive cells were imaged using confocal microscopy and manually counted. Interestingly, ImP-supplemented mice exhibited fewer cFOS+ cells in the hypothalamus than that of control mice (Figure 6A). The hypothalamus is responsible for regulating a plethora of functions relating to metabolic disorders including hunger, glucose, and

insulin levels (Kokras *et al.*, 2012). However, there was no difference in cFOS+ cells in the amygdala, which is responsible for processing fear and emotions (Figure 6B). Since it has been reported in the literature that microbiota-derived metabolites can affect microglia, the brain's resident immune cells, we also stained brains for IBA1 to evaluate changes in microglia morphology and abundance (Duan *et al.*, 2021; Spencer *et al.*, 2019; Tapia-González *et al.*, 2011). We observed no differences in microglia morphology visually using LEICA imaging. Additionally, manual counting of IBA1+ positive cells using confocal microscopy confirmed no differences in microglia density in the hypothalamus or the amygdala between the two groups (Figure 6C).

Imidazole Propionate Supplementation Does Not Alter Microglial Morphology or Activity in In Vitro Primary Microglia Culture

To evaluate the direct effects of ImP on microglia, we generated primary microglia cultures using magnetic-activated cell sorting (MACS) (Figure 7A). We then supplemented the *in vitro* microglia cultures with 10mM of Imidazole propionate or the vehicle solution (1% DMSO in water). The cultures were fixated and stained with DAPI and IBA1 3 days following the supplementation with the metabolite. Using confocal microscopy, we observed no differences in microglial morphology or abundance between the two cultures. To analyze potential differences in cytokine release from the two cultures, we performed an ELISA for IL-6 using the supernatants of the microglia cultures and found no difference between the treated and control cultures (Figures 7B, 7C). The *in vitro* microglia culture experiments were conducted twice, each with a technical replicate of 2. The obtained data align consistently with our *in vivo* results, indicating no significant alteration in microglia density overall.

Discussion

The microbiota-gut-brain (MGB) axis is a complex bidirectional communication network that plays a crucial role in regulating brain function and behavior (Cryan *et al.* 2019; Martin *et al.*, 2018). It involves intricate interactions between the gut microbiota, the gut epithelium, the enteric nervous system, and the central nervous system (CNS) (Collins *et al.*, 2012). The MGB axis is involved in various physiological processes, including nutrient metabolism, immune regulation, and neurobehavioral modulation (Morais *et al.*, 2021). Dysregulation of the MGB axis has been implicated in the pathogenesis of several neurological disorders, including anxiety, depression, and neurodevelopmental disorders (Foster *et al.*, 2013; Rogers *et al.*, 2016; Wu *et al.*, 2021).

In this study, we aimed to investigate the impact of a high-fat diet (HFD) on the MGB axis and its potential role in the development of anxiety-like behavior. The prevalence of high-fat diets in modern society has raised concerns about their detrimental effects on health, including their impact on brain function and behavior (Gesù *et al.*, 2022; Zhuang *et al.*, 2022). Emerging evidence suggests that diet composition, particularly the consumption of high-fat diets, can influence the composition and function of the gut microbiota, leading to alterations in MGB axis signaling and subsequent behavioral changes (Wu *et al.*, 2023).

Consistent with previous studies (Li *et al.*, 2022; Zhuang *et al.*, 2022), our results demonstrated that HFD consumption induced anxiety-like behavior in mice, as assessed by the elevated plus

maze (EPM), open field test (OFT), and light/dark box (LDB) test. Although previous studies have demonstrated that a 10-week high-fat diet (HFD) can induce anxiety-like behavior, the specific timeframe for the onset of this phenotype and the effects of a 3-week HFD treatment have not been previously investigated. In our study, we discovered that mice fed a high-fat diet exhibited anxiety-like behavior as early as 3 weeks into the HFD treatment. This finding highlights the rapid development of the anxiety phenotype in response to the HFD regimen. The emergence of anxiety-like behavior within 3 weeks of HFD consumption supports the notion that diet can modulate brain function and behavior. These behavioral changes are consistent with the growing body of literature linking dietary factors, particularly high-fat diets, to the development of anxiety disorders in both preclinical and clinical studies (Dutheil *et al.*, 2016).

Interestingly, while the HFD-induced anxiety-like behavior was evident, we did not observe concurrent changes in limbic system activity, as evidenced by no alteration in c-Fos expression within the hypothalamus and hippocampus. These brain regions are known to play a crucial role in the regulation of anxiety and stress responses. The hypothalamus is responsible for numerous homeostatic functions, including regulating appetite and insulin levels, and previous research has shown hypothalamic neuroinflammation in obese animal models and humans (Ataka *et al.*, 2013; Guillemot-Legris and Muccioli, 2017; Thaler *et al.*, 2012; Wu *et al.*, 2021). Neuroinflammation and decreased synaptic plasticity have also been reported in the hippocampus in response to HFD (Hao *et al.*, 2016; Krishna *et al.*, 2015; Puig *et al.*, 2012; These findings suggest that the anxiety-like behavior observed in our study may not solely rely on changes in traditional limbic system activity but could involve more complex mechanisms. The anxiety-like phenotype observed in HFD-fed mice may potentially involve the activation of subcellular brain regions

that were not covered in our large-scale analysis. To gain further insight into the mechanisms underlying anxiety-like behavior, it may be necessary to employ high-resolution techniques that can analyze specific subregions of the central nervous system (CNS).

Furthermore, our study examined the role of the gut microbiota-derived metabolite, imidazole propionate (ImP), in the MGB axis and anxiety behavior. ImP is a product of histidine metabolism by certain gut bacteria and has been implicated in the pathogenesis of metabolic and neurological disorders (Chen *et al.*, 2022; Koh *et al.*, 2018; Molinaro *et al.*, 2023). Interestingly, we did not observe increased levels of ImP in mice fed HFD, and this may be due to the composition of the diet we administered. It is plausible that the diet we employed might have been deficient in crucial precursors necessary for the biosynthesis of ImP, as evidenced by higher ImP levels observed in alternative high-fat diet models (Lützhøft *et al.*, 2022). However, we decided to evaluate the effects of ImP supplementation anyways as it is used as a model for diabetes, and elevated ImP levels have been reported in humans with metabolic disorders.

Surprisingly, the administration of ImP via osmotic pumps induced anxiety-like behavior, suggesting a potential role for ImP in anxiety regulation. This finding indicates that ImP, despite its non-elevated levels in the HFD condition, can independently induce anxiety-like behavior. It is unknown if ImP influences the MGB axis and anxiety behavior through other pathways. However, previous studies have implicated ImP in the modulation of insulin signaling and intestinal inflammation (Koh *et al.*, 2018).

To explore potential mechanisms underlying the ImP-induced anxiety-like behavior, our study further investigated the role of microglia, the resident immune cells of the CNS, in mediating the effects of ImP on anxiety-like behavior. Microglia have emerged as important regulators of brain function and have been implicated in the pathogenesis of neurological disorders (Cope *et al.*, 2018; Tapia-González *et al.*, 2011; Yirmiya, Rimmerman, and Reshef, 2015). They play a crucial role in maintaining the homeostasis of the CNS by surveilling the environment and responding to various stimuli, including changes in the gut microbiota composition (Wen *et al.*, 2011). In our study, we found that ImP supplementation did not lead to changes in microglial density within specific brain regions involved in anxiety regulation. While the abundance and density of microglia were unaffected by ImP supplementation, the activity of these microglia was not extensively measured. We did measure the levels of the anti-inflammatory cytokine, interleukin-6 (IL-6), produced by the microglia cultures in response to ImP supplementation via ELISA, but found no difference (Rose-John, Winthrop, and Calabrese, 2017). Microglial activation could be a response to changes in gut microbiota composition and the subsequent release of microbial-derived molecules like ImP that can activate the innate immune system. Future experiments should extend the scope of cytokine analysis and incorporate morphological analyses. These investigations would allow for a comprehensive evaluation of microglial activation in response to ImP. By doing so, we may potentially unveil an immune-mediated pathway that connects the gut microbiota with anxiety-like behavior. This expanded approach would provide valuable insights into the mechanisms underlying the relationship between the gut microbiota, immune responses, and anxiety-related phenotypes.

ImP has been shown to induce insulin resistance and impair intestinal barrier function, both of which have been associated with metabolic and neurological disorders (Koh *et al.*, 2018; Molinaro *et al.*, 2020). Therefore, it is plausible that ImP-induced alterations in insulin signaling and intestinal barrier integrity contribute to the dysregulation of the MGB axis and the development of anxiety-like behavior. Future studies should aim to elucidate the specific molecular pathways and cellular mechanisms through which ImP exerts its effects on the MGB axis.

In conclusion, our study provides novel insights into the impact of HFD on the MGB axis and its role in the development of anxiety-like behavior. We demonstrate that HFD consumption leads to the emergence of anxiety-like behavior, highlighting the intricate interplay between diet, the gut microbiome, and brain function. These findings suggest that modulation of the MGB axis through diet modification or targeting specific gut-derived metabolites may have therapeutic potential for the prevention and treatment of anxiety and other neurological disorders.

Figures

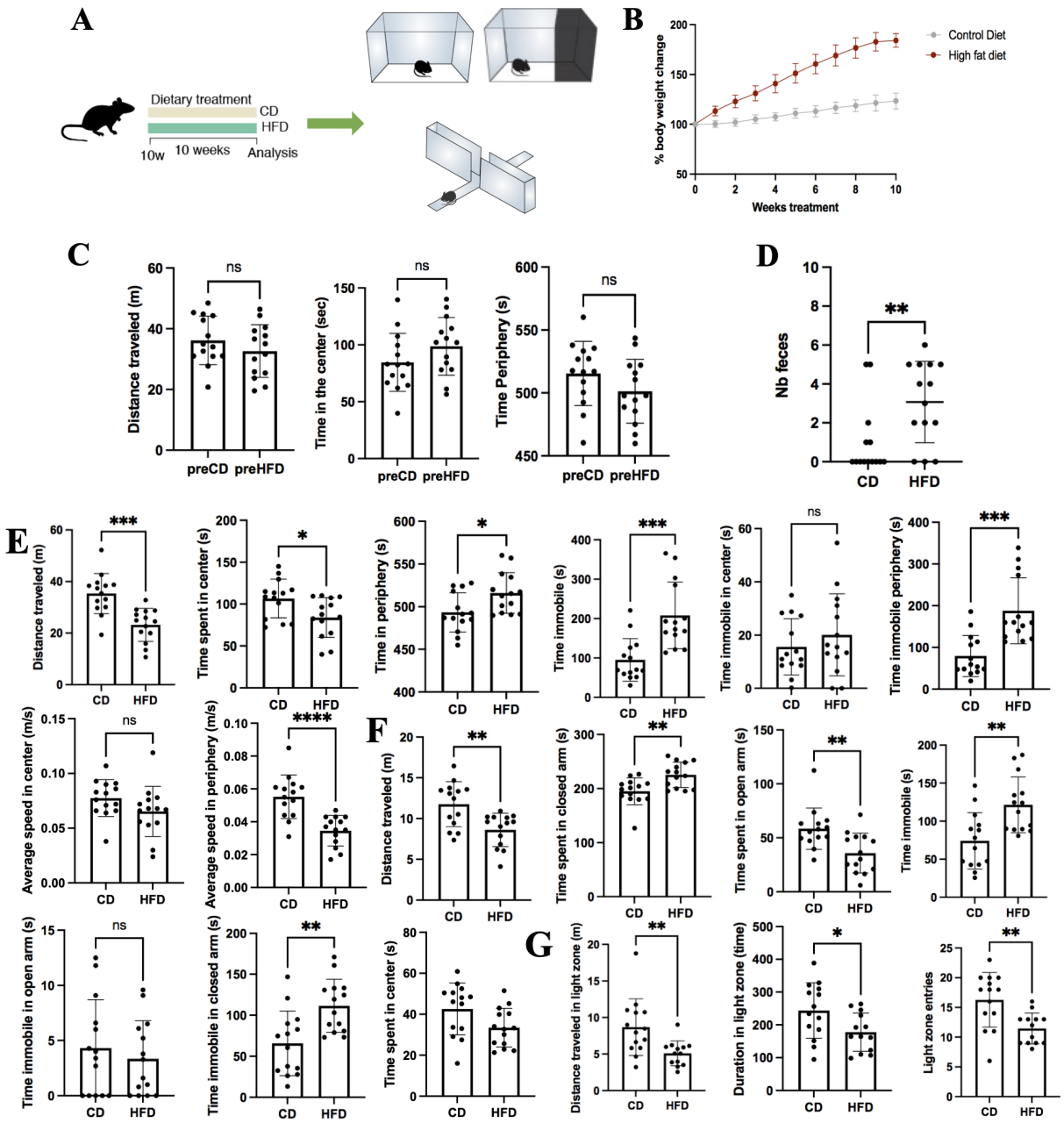


Figure 1: 10-week HFD Induces Anxiety-like Behavior in Mice | **A.** 8-10 week-old male C57BL/6 mice were treated with either HFD (n=12) or CD (n=12) for 10 weeks, during which body measurements were taken. **B.** Body weight measurements of HFD and CD mice over the 10-week dietary treatment. **C.** Baseline OFT behavior. **D.** Number of fecal boli dropped by HFD-fed and CD-fed mice during the OFT. **E.** Measures of locomotion and anxiety-like behavior of HFD-fed and CD-fed mice in OFT. **F.** Measures of locomotion and anxiety-like behavior of HFD-fed and CD-fed mice in EPM test. **G.** Measures of locomotion and amount of time spent in the bright and dark compartments of HFD-fed and CD-fed mice in the LDB test.

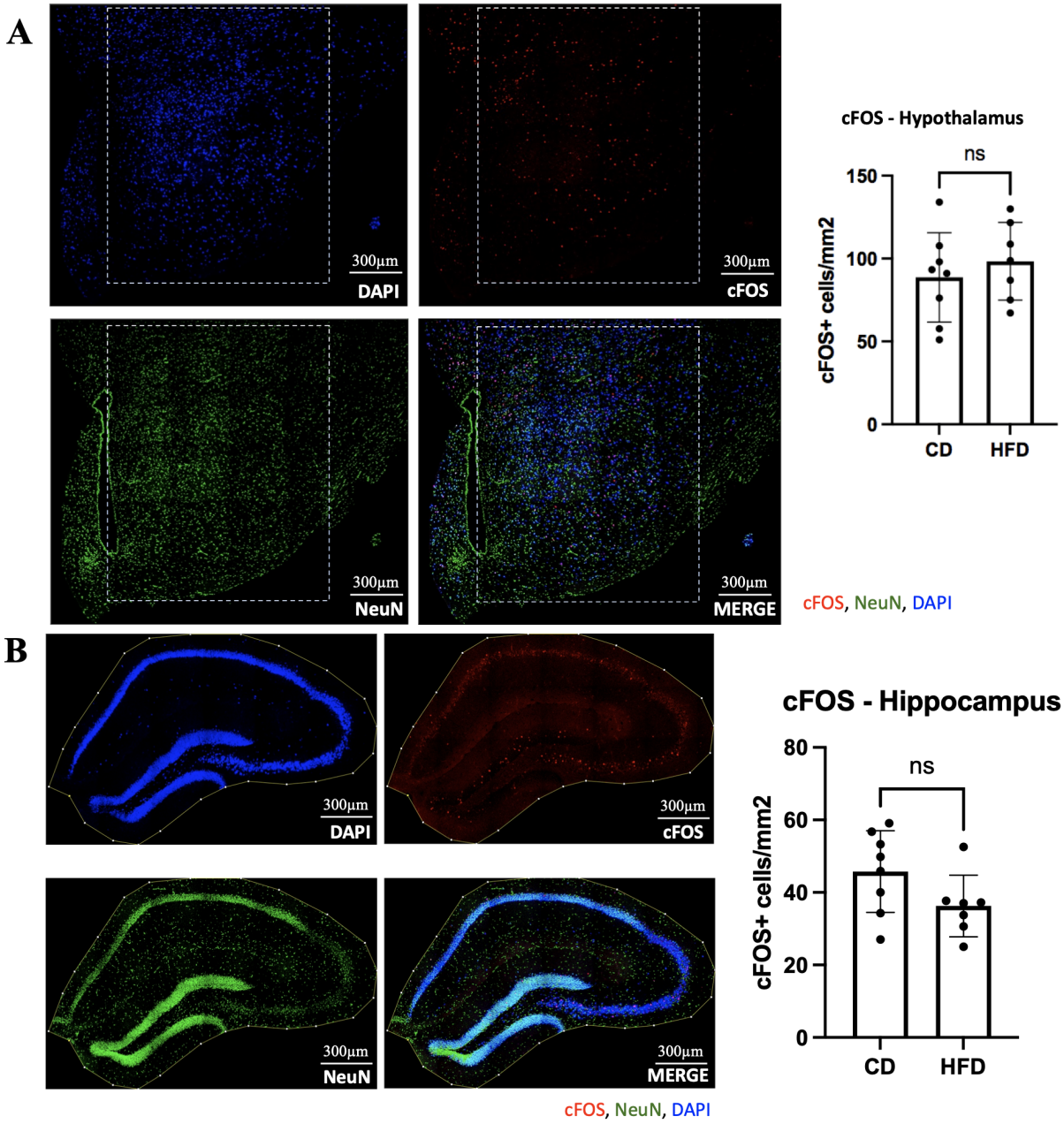


Figure 2: 10-week HFD Has No Significant Effect on Neuronal Activation in the Hypothalamus and Hippocampus in Response to Testing for Anxiety-like Behaviors. | A. Representative images of the hypothalamus. Brain samples were collected 90 minutes following LDB testing to allow for cFOS expression. Blue represents DAPI-stained nuclei, green represents NeuN-stained neurons, and red represents cFOS activation**.** Manual quantification of cFOS-positive neurons in the CD-fed and HFD-fed mice per hypothalamic area. Quantification represents NeuN+ cells as there was very little discrepancy between cFOS+ cells and cFOS+NeuN+ cells. **B.** Representative images of the hippocampus. Blue represents DAPI-stained nuclei, green represents NeuN-stained neurons, and red represents cFOS activation. Manual quantification of cFOS-positive neurons in the CD-fed and HFD-fed mice per hippocampal area.

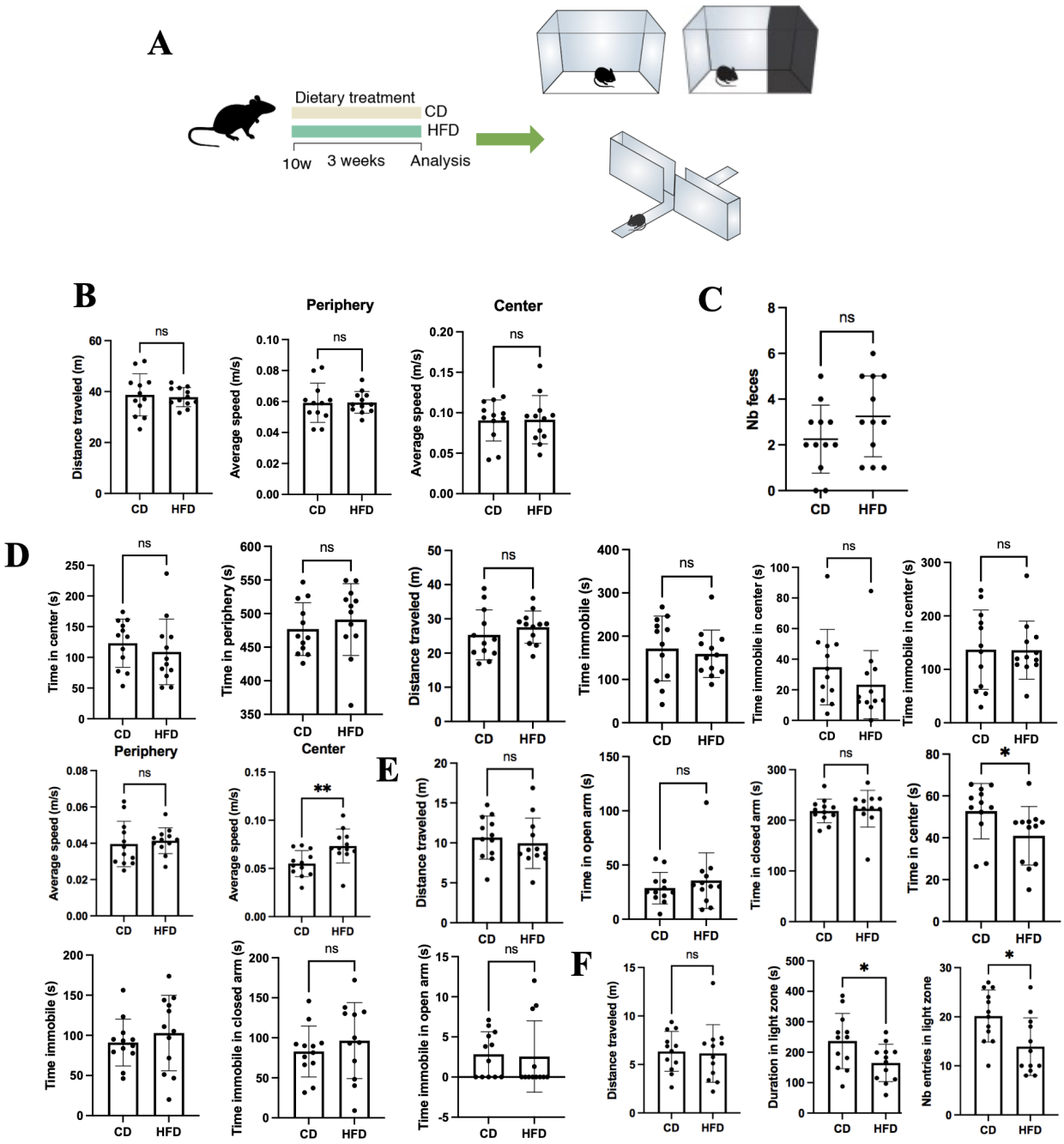


Figure 3: 3-week HFD Diet Treatment Induces Anxiety-Like Behavior. | **A.** 8-10 week-old male C57BL/6 mice were treated with either HFD (n=12) or CD (n=12) for 3 weeks. **B.** Baseline OFT behavior. **C.** Number of fecal boli dropped during the OFT by HFD-fed and CD-fed mice. **D.** Measures of locomotion and anxiety-like behavior of HFD-fed and CD-fed mice in OFT. **E.** Measures of locomotion and anxiety-like behavior of HFD-fed and CD-fed mice in EPM. **F.** Measure of locomotion and amount of time spent in bright and dark compartments in the HFD-fed and CD-fed mice during the LDB test.

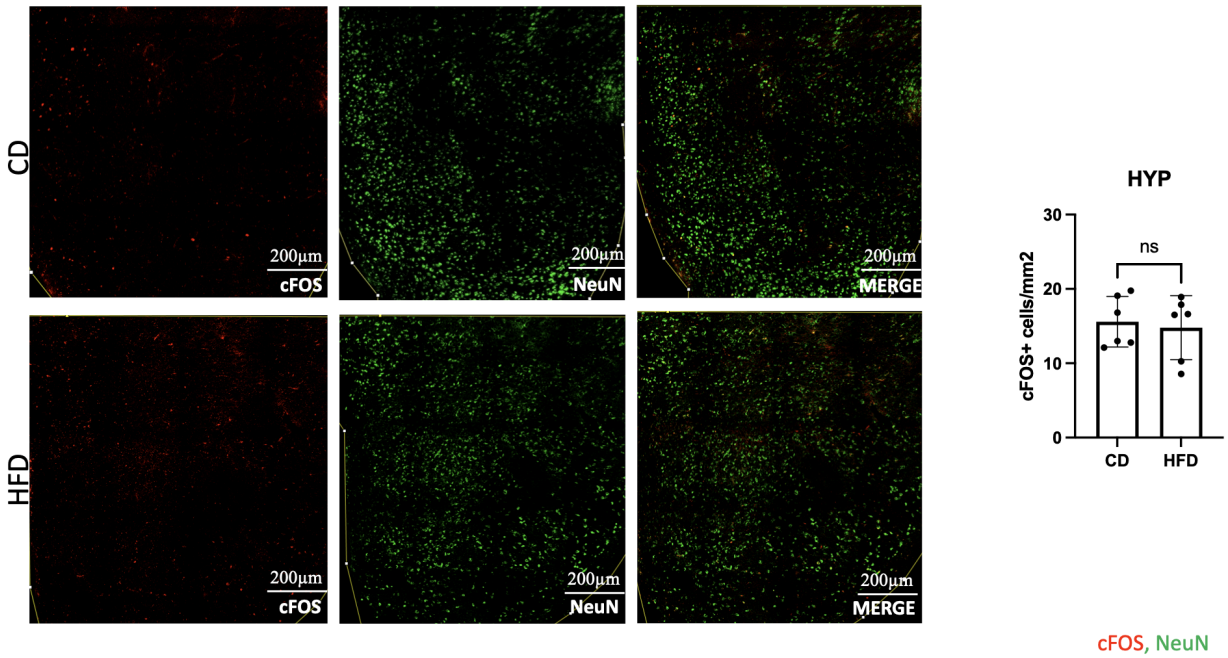


Figure 4: 3-Week HFD Has No Significant Effect on Hypothalamic Neuronal Activation in Response to Testing for Anxiety-like Behaviors. | Representative images of the hypothalamus of HFD-fed and CD-fed mice. Red represents cFOS activation and green represents NeuN-stained neurons. Manual quantification of cFOS-positive neurons in the CD-fed and HFD-fed mice per hypothalamic area. Quantification also represents NeuN+ cells as there was very little discrepancy between cFOS+ cells and cFOS+NeuN+ cells.

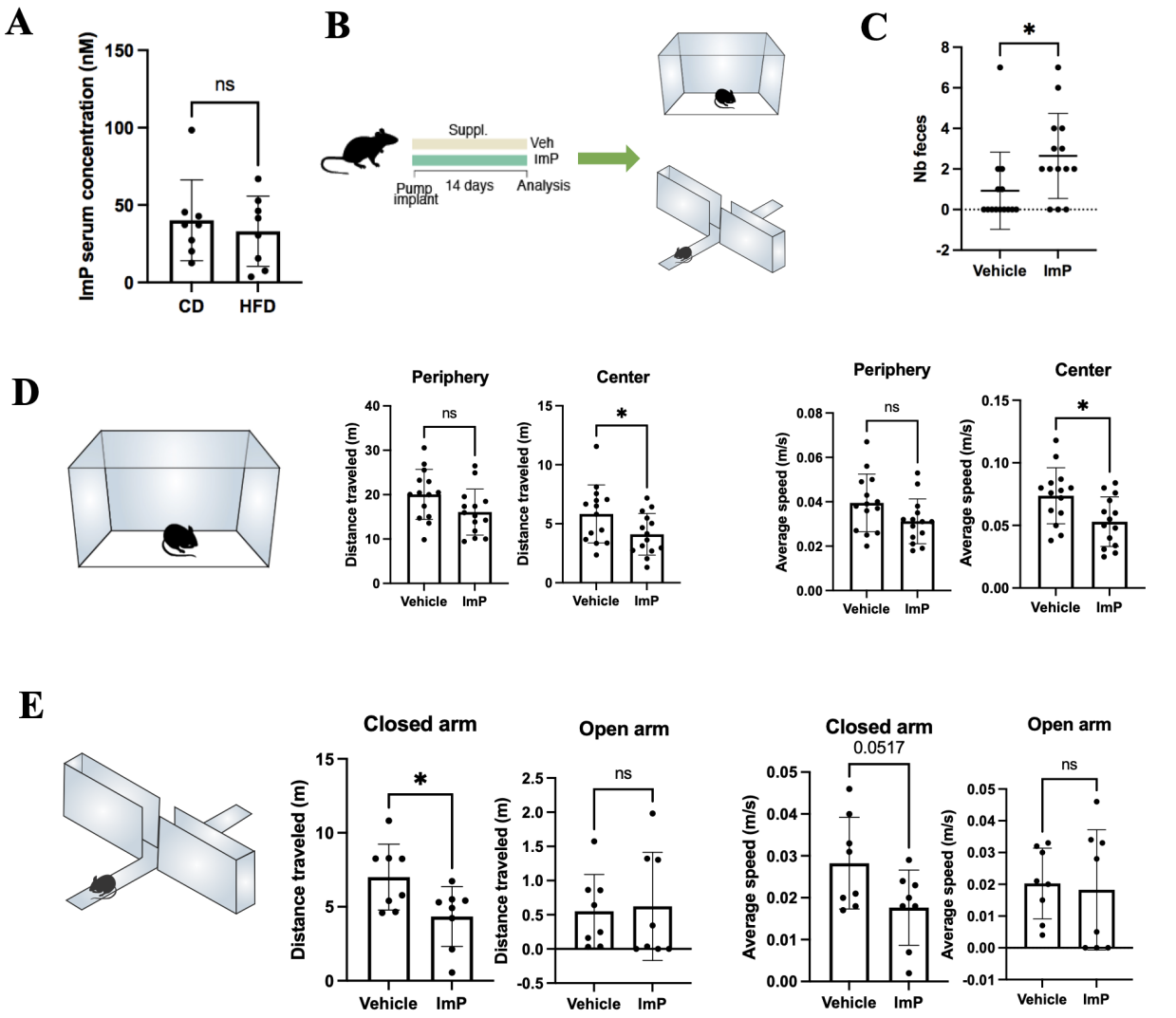
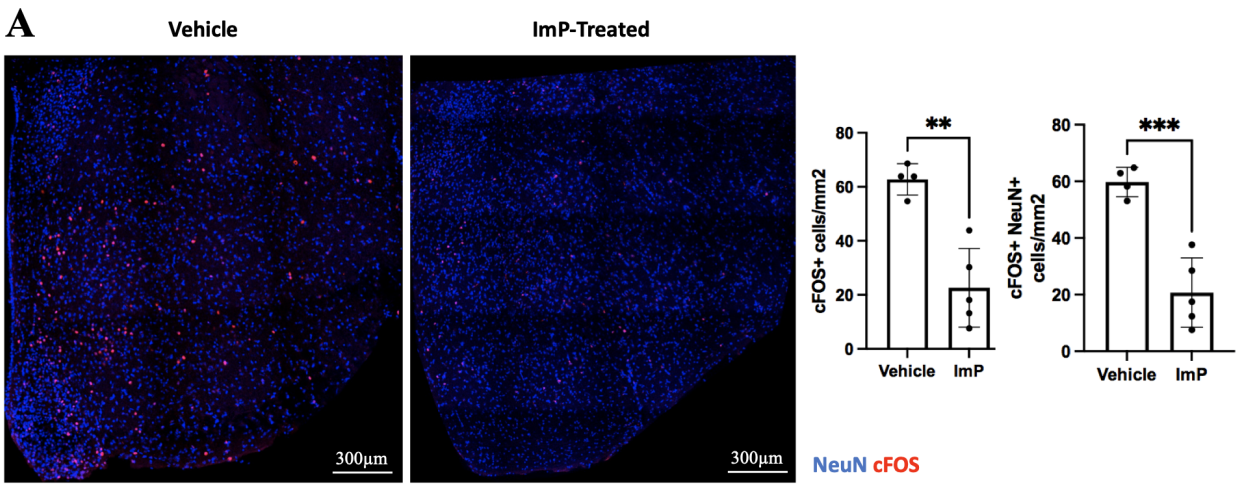


Figure 5: 14-Day Imidazole Propionate (ImP) Supplementation Induces Anxiety-Like Behavior. | **A.** ImP serum concentration (nM) of CD-fed and HFD-fed mice. **B.** Adult male C57BL/6 mice were intraperitoneally implanted with an Alzet osmotic pump that released 300ug of ImP per day over the course of 14 days. **C.** Number of fecal boli dropped during OFT of vehicle-treated mice and ImP-treated mice. **D.** Distance traveled and average speed in OFT of vehicle-treated mice and ImP-treated mice. **E.** Distance traveled and average speed in EPM of vehicle-treated mice and ImP-treated mice.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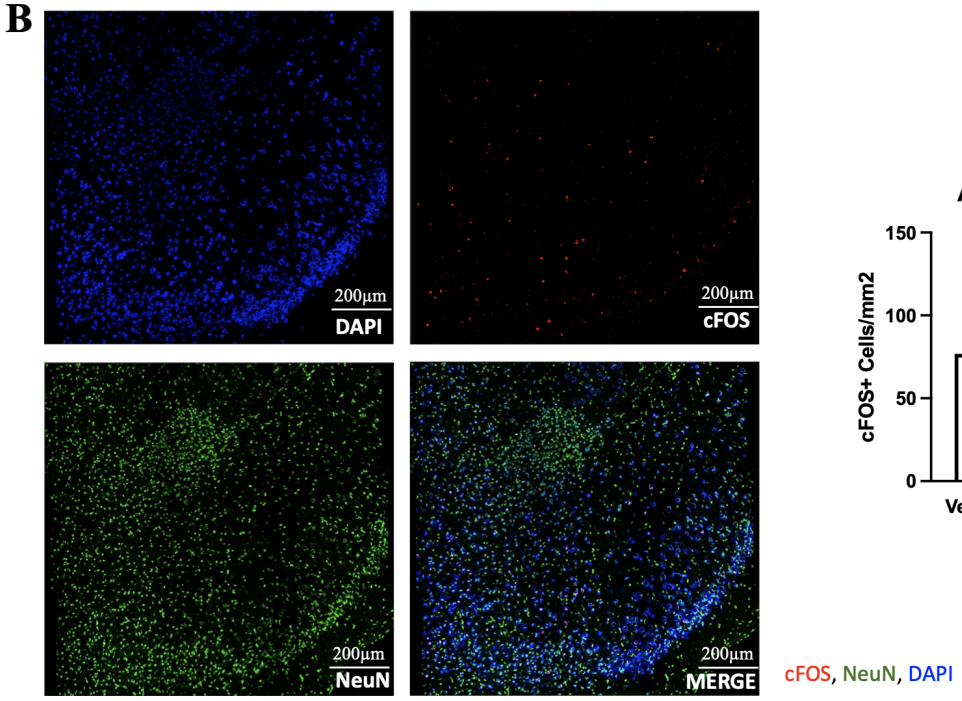
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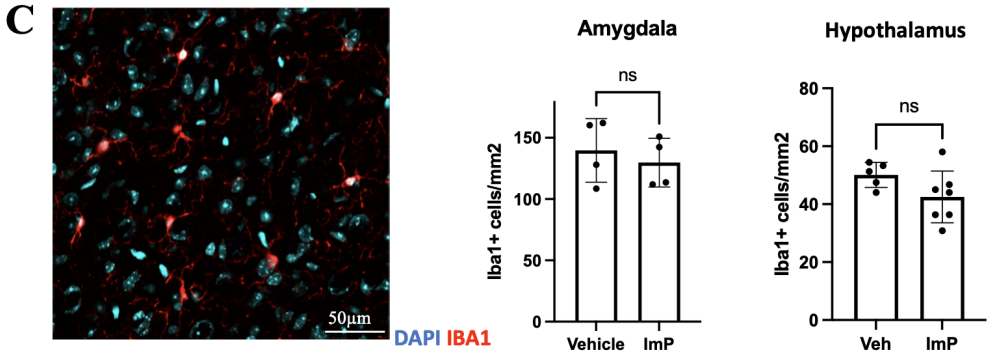
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Figure 6: 14-Day ImP Decreases Hypothalamic, but not Amygdalar, Neuronal Activation in Response to Testing for Anxiety-Like Behaviors. | A. Representative images of the hypothalami of vehicle-treated mice and ImP-treated mice. Blue signifies NeuN- stained neuronal cell bodies and red signifies c-FOS-positive cells. cFOS-positive cells were manually counted per hypothalamic area. **B.** Representative images of the amygdala of ImP-treated mice. Blue signifies DAPI-stained cell bodies, green represents NeuN-stained neuronal cell bodies, and red signifies c-FOS expression. CFOS-positive cells were manually counted per amygdala area. C. Representative image of the amygdala of ImP-treated mice. Microglia are stained with IBA1 and are represented in red. Cell bodies are stained with DAPI and are represented in blue. Microglia were manually counted per amygdala and hypothalamic area.

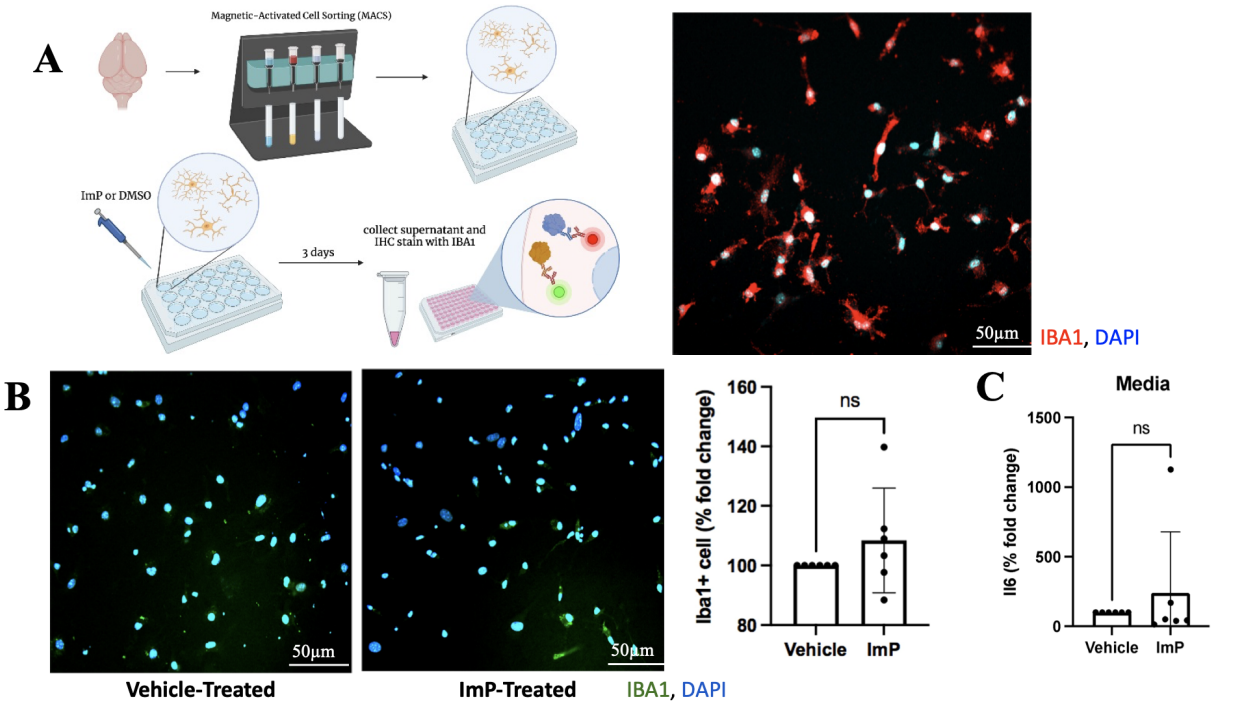

Figure 7: ImP has No Significant Effect on the Density and Activation of Cultured Microglia. | **A.** P3 brains from C57BL/6 mice were harvested and microglia were cultured using magnetic-activated cell sorting (MACS) and sustained using media detailed in Bohlen *et al.*. Microglia cultures were treated with 10mM of ImP or vehicle solution (1% DMSO in water). Representative image of microglia culture with IBA1-stained microglia in red and DAPI-stained cell bodies in blue. **B.** Representative images of vehicle-treated and ImP-treated microglia cultures with IBA-1 stained microglia in green and DAPI-stained cell bodies in blue. About 500,000 microglia cells were plated each well. Treated and untreated wells were paired together so that they are both derived from the same brain samples. Treated wells were directly compared to normalized untreated control wells with a technical N of 2 **C.** IL-6 ELISA of vehicle-treated microglia culture supernatant and ImP-treated microglia culture supernatant.

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