EFFECTS OF EARLY LIFE STRESS AND MAST CELLS ON BRAIN FUNCTION AND BEHAVIOR IN THE MOUSE AND PIG

By

Kaitlin Durga

A DISSERTATION

Submitted to
Michigan State University
In partial fulfillment of the requirements
for the degree of

Physiology – Master of Science

2020

ABSTRACT

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Depression is a leading cause of disability worldwide, yet available treatments are ineffective for nearly half of treated patients. Depression has been linked to early life adversity and is exacerbated by stress, so uncovering how stress affects mood-related brain regions is critical to improve our understanding of depression etiology and potentially improving treatment. Depression patients often display reduced hippocampal volume, and many animal models of depression display a reduction in hippocampal neurogenesis that is reversed by chronic exposure to antidepressants. Using a pig and mouse model, we were able to examine the effects of early life adversity on adult neurogenesis, and mast cell FosB on behaviors respectively. Our examination of female, castrated male, and intact male pigs, who underwent either early weaning or late weaning allowed us to see not only the effects of early life adversity, but any possible sex-specific effects as well. We found that early weaned female pigs expressed a significantly reduced number of new neurons in their hippocampi compared to their late weaned counterparts. Using a transgenic mouse model which had FosB floxed out of all mast cells, we observed the behavioral outcomes in various assays, including social interaction, elevated plus maze, and sucrose preference. We found that male mice homozygous for the mutation had a significantly increased preference for sucrose compared to the wildtype mice.

For my family and friends who have always been there. Thank you.

ACKNOWLEDGEMENTS

This research would not have been possible without the funding from our R01 grant from the National Institute of Child Health and Human Development (NICHD R01-072968). This work was also supported in part by a 5 semester long TA-ship, in total, for faculty members Dr. Erica Wehrwein and Dr. John Zubek.

This work's completion was contributed to significantly by Dr. Natalia Duque-Wilckens, as well as other members of the lab of Dr. Adam Moeser.

I am so fortunate to have worked with such a talented group of individuals over the past two years. I have grown as a person and as a scientist to a level of magnitude that would not have been possible without my lab mates, and, notably, my advisor, Dr. A.J. Robison. His attentive mentorship style was defining of my graduate school experience, and for that, I am so grateful. He pushed me to work harder, smarter, and with diligence. Additionally, I would like to thank my committee members, Dr. Michelle Mazei-Robison, Dr. Adam Moeser, and Dr. Erica Wehrwein. They all offered a great deal of suggestions, critiques, and encouragement along the way, all of which made my thesis that much more impactful.

Not only have I been lucky enough to work alongside such an amazing group of people, but I have also formed some strong friendships as well. While I have enjoyed the company of all of my colleagues, first to mind is my dear friend, Haley Lynch. I would like to thank her, not only for making the lab space just a bit brighter with her presence, but also her unwavering love for sushi. It goes without saying that my time in graduate school would have been drastically different without her presence. I also have to thank the PSL 475

teaching team for their friendships as well. Dr. Erica Wehrwein, Yvonne Ogrodzinski, CJ McDonnel, Keeler Steele, Jake Canfield, and Hannah Zawisa have all become such great friends of mine. Their support through good and stressful times have provided me a safe and loving space where their best intentions are clear. Their commitment to providing a meaningful experience in PSL 475 for the students is unmatched, in my eyes, by any class I have seen in my 7 years at Michigan State University. I cannot put into words how special this team and class is. They are all so near and dear to my heart, and have taught me so much.

Last, but certainly not least, thank you to my family, particularly my mother, Tammy, my father, Joe, and my sister, Lauren. I cannot say thank you enough. Their support throughout not only my journey through graduate school, but through everything preceding, is unparalleled. Even in times of disagreement, their love is clear and strong. I would not have been able to achieve this accomplishment without them.

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CHAPTER 1: INTRODUCTION

Major Depressive Disorder (MDD) is a mood-related disease that becomes more prominent each year in the United States and worldwide. Characterized by a persistently low mood, feelings of guilt, hopelessness, and countless accompanying symptoms, it goes without saying that depression is a painfully draining diagnosis to live with. According to the National Survey on Drug Use and Health, approximately 17 million people in the US experienced at least one major depressive episode during the year of 2017 [1]. With this many instances, depression is now the leading cause of disability in the US and is a major contributing factor to suicidality, which is a leading cause of death in the US [2].

Due to treatment costs and factors such as loss of productivity in the workforce as a result of depressive episodes, the country is facing an extreme economic burden, estimated to be greater than 200 billion dollars per year [3]. To offset these societal challenges, it is important to treat depression effectively and efficiently to reduce the occurrences of disability and suicide attempts. Problematically, available treatments only work for some depression patients, while up to two-thirds of them remain symptomatic and virtually unresponsive to the first line of treatment [4]. Not only does this contribute to the aforementioned rates of disability, suicide, and lost workplace productivity, but it also leaves room for major disruption in an individual's daily activities. The molecular and cellular underpinnings of these inconsistencies in treatment efficacy are unknown, and this represents a substantial hurdle to overcome in physiology, neuroscience, and translational research.

Although there are studies that suggest a genetic link for depression, causing some to be predisposed, some cases seem to be attributed to environmental factors. A common risk factor for the onset of depression later in life is the occurrence of early life adversity.

Early life adversity is characterized as toxic stress that occurs during childhood, resulting from traumatizing experiences including instances of abuse, neglect, or sexual abuse [5]. The occurrence of this adversity increases the likelihood of the development of depressive mood disorders [6]. While the molecular mechanism for this relationship remains unknown, there are numerous correlates between those who have suffered adverse conditions at a young age and those who struggle with depression. One of the common findings in depressed patients is reduced hippocampal volume [7, 8] and this is found in those exposed to early life adversity as well [9].

The hippocampus is a brain region located in the medial temporal lobe and is an extension of the limbic system. A bilateral structure, the hippocampus curves into an ‘S’ shape and extends from the dorsal to the ventral side of the brain. Often associated with learning and memory, the hippocampus works in close association with other cortical structures such as the amygdala and the thalamus for the purpose of memory consolidation [10]. The hippocampus also has implied roles in psychiatric diseases, such as schizophrenia and depression [11,12].

The decrease in hippocampal volume in depressed patients has lead researchers to explore the effects of anti-depressant drugs on the hippocampus as well as adult neurogenesis in this region. Although it is a topic of controversy in the field, there are researchers who provide a compelling argument that neurogenesis continues through adulthood in the dentate gyrus of the hippocampus [13,14]. With this idea in mind,

reduced hippocampal volume in early life adversity and/or depression may be the result of reduced neurogenesis within the dentate gyrus: it is intuitive that without this accumulation of new neurons, the size of the hippocampus in a depressed patient would be considerably smaller compared to that of a healthy individual. Animal models of stress show a reduction in hippocampal volume, consistent with what is seen in human patients [15], and chronic administration of antidepressants, specifically fluoxetine, reverse this atrophy [16].

In addition to hippocampal atrophy, reduced neuronal proliferation is apparent after stress takes place. Bromodeoxyuridine (BrdU) is a marker of proliferation that is often used in immunohistochemistry (IHC) to identify new cells [17]. This type of staining is common in assessing levels of neurogenesis. After stress, mice and rats exhibit a reduced rate of neurogenesis in the dentate gyrus [18, 19]. The occurrence of adult neurogenesis may be contributing to the effects that antidepressants have in depressed patients. After chronic administration of fluoxetine, neurogenesis in the dentate gyrus increases in rats and mice [17, 20].

Early life adversity increases susceptibility to stress-related disorders later in life [21], and this introduces many other complications in coping with the symptoms of depressive episodes. Interestingly, not all people who undergo these early life stressors experience negative symptoms later in life, and this apparent compensation for the effects of stress is termed “resilience.” An important distinction that has yet to be made is that between the biochemical and cellular factors driving resilience and the factors driving susceptibility to the deleterious effects of early life stress. Critically, these different behavioral outcomes of stress can be modelled in mice following a variety of stress paradigms, one of the most

frequently used being Chronic Social Defeat Stress (CSDS). During this protocol, adult male C57 mice are placed into the home cage of a larger and territorial male retired breeder CD1 mouse, which reacts aggressively [22, 23]. Typical aggressive behaviors seen during the bouts of interaction are tail rattling, pinning, and biting on the back of the submissive mouse [24,25]. Depending on the experimental design and desired behavioral assessments, the social defeat stress duration can vary; however, C57 mice are typically subjected to a new aggressor daily for ten minutes each day for ten days. These aggressive encounters are quite stressful for the C57 intruder mice, and the effects of stress can be assessed in a variety of ways.

After C57 intruder mice have gone through CSDS, they will exhibit behaviors indicating that they are either susceptible or resilient to the effects of the stress [26]. Normally, mice find social interaction to be rewarding, especially with novel mice [27]. CSDS susceptible mice will demonstrate anhedonic behaviors including social avoidance as measured by a Social Interaction (SI) test [23]. The SI assay involves the mouse being placed in an open arena with prescribed corner and interaction zones, and its motion is tracked using a digital video camera and tracking software. The mouse is allowed to explore the arena for two bouts of two and a half minutes, one with and one without a target mouse enclosed in a mesh cylinder that allows for visual and olfactory stimulation from the target mouse [28]. Susceptible mice will spend less time interacting with a social target than with the empty enclosure, indicating social withdrawal, whereas resilient or unstressed mice spend more time with a social target.

Additionally, sucrose preference (SP) is measured for the purpose of detecting anhedonia, as susceptible mice will experience decreased sucrose preference [29]. In

this assay, each mouse is given access to two water bottles in their home cage, one containing normal drinking water, while the other contains a 1% sucrose water solution. Normal mice will consume >80% of their total liquid intake from the sucrose bottle, while mice susceptible to CSDS typically have a much lower sucrose preference, indicating lack of pursuit of pleasure, anhedonia. Critically, chronic administration of the antidepressant fluoxetine, a common selective serotonin reuptake inhibitor (SSRI), abolishes the effects of CSDS in susceptible mice [23].

SSRIs work by blocking the serotonin transporter, in turn allowing for the accumulation of serotonin in the synapse [30]. Despite the effects it has in mice, SSRIs only improve outcome in about half of depressed patients [31]. Further, in those it does help, several weeks of treatment in patients are required before an improvement is noticed in mood [30]. CSDS studies reveal that mice also require repeated exposure to fluoxetine to induce the antidepressant behavioral effects [32], similar to the course of treatment and response in humans. In contrast, many other behavioral models (i.e., forced swim test or tail suspension) show immediate effects of antidepressants, indicating a lack of face validity, and reaffirming the necessity for chronic stress models like CSDS. Indeed, while chronic exposure to SSRIs reverse anhedonic behaviors, acute treatment can actually be anxiogenic [33].

In addition to modelling depressive-like behavioral changes in response to adult stress, early life adversity can be modeled in animals using neonatal maternal separation (NMS) or by early weaning, which is amenable to many animal models, from mice to pigs [34, 35]. In NMS stress, newborn pups are repeatedly removed from the dam's cage for a predetermined amount of time daily over a time course of multiple days [36]. Pups

undergoing this stress are separated from the dam every day from postnatal day 2 to 14 [37]. In contrast, early weaning is a premature permanent separation of pups from the dam [35], typically at 14 days after birth, whereas normal weaning occurs within the range of 21 to 30 days [38, 39]. These methods are important in observing the behavioral outcomes that result from this type of stress while the animal is still in the developmental stages but can also be used as early life stressors to study subsequent effects on behavior and stress susceptibility in adulthood.

The response that animals have to CSDS depends on earlier experiences. Mice that undergo NMS stress are more likely to be susceptible to developing depressive phenotypes following CSDS during adulthood [40]. This includes behaviors like reduced SI ratio, reduced sucrose preference, and increased immobility during the forced swim test (FST). FST is an assay used to measure an animal's motivation to escape. The mouse is placed in a cylinder filled with water that is inescapable. The mouse is then timed until it gives up and becomes immobile, exhibiting helplessness [41]. This test is often used to test the efficacy of antidepressants, as acute administration often reverses the learned helplessness phenotype that is displayed as floating [42].

The effects of stress, both in early life and adulthood, are not limited to the brain, and the physiology of many body systems can be affected by stress. The gut-brain axis refers to the bidirectional communication between the Enteric Nervous System (ENS) and digestive tract and the Central Nervous System (CNS) [43]. The ENS works with the CNS to coordinate proper digestive reflexes and gut motility [44]. The proper communication between these two systems is essential for life [45], however with such extensive and varied connections between them, there is room for error.

Irritable Bowel Disease (IBD), and the subtypes Crohn's Disease and Ulcerative Colitis, is a chronic condition hallmarked by abdominal pain, irregular bowel movements, food avoidance, and other issues [46]. There is no cure for IBD, only treatments for symptoms, including serious surgical procedures. Physiologically, this condition is characterized by flare-ups that can come and go without warning, often having a significantly negative impact on quality of life [47, 48]. The onset of IBD has a high comorbidity with depression and anxiety [49]. In fact, within the IBD patient population, the rates of anxiety and depression diagnoses are higher than within the general population [50]. One treatment that is common in IBD patients, even those who do not suffer from depression as well, are antidepressants, namely SSRIs [51]. Without having any obvious negative effects on IBD, antidepressants improve inflammatory markers in animal models of the disease [52]. Mast cells, a type of immune cell, are suggested to play a role in this inflammation seen in IBD. Activation of the gut-brain axis by stressful events can lead to the release of proinflammatory molecules by mast cells, including histamine [53]. The release of these molecules leads to increased permeability of the intestinal epithelium [54], which is a major factor in the mucosal inflammation that is seen in IBD [53]. Early life stress, in addition to increasing the risk for depression, is also a risk factor for IBD [55]. In NMS models of early life stress in rodents, colonic hypersensitivity is induced along with an increase in the number of mast cells in the colon [56]. However, the potential role of mast cells in the gut-brain axis and in behavioral responses to stress remains unknown.

In the following chapters, I present experiments designed to: 1) establish the effects of early life adversity (early weaning) on neurogenesis in the pig hippocampus, providing a novel and significantly more translational model for studies of ELA effects on the gut-brain

axis; and 2) establish the role of mast cells in stress-induced changes in mouse behavior, including uncovering novel mechanisms of transcriptional regulation in mast cells underlying their roles in these models. Thus, my thesis lays the groundwork for ongoing studies that may potentially illuminate novel cellular and molecular mechanisms of stress-induced susceptibility to mood disorders and potentially open up new avenues for their diagnosis and treatment.

CHAPTER 2: MATERIALS AND METHODS

Pigs

For the experiments discussed in chapter 3, pigs were split into early weaned and late weaned groups, being separated from their sow at 15 days and 28 days postpartum, respectively. Juvenile pigs were then sacrificed at 20 weeks of age using sodium pentathol injection into the heart. Brains were removed using a Stryker Autopsy Saw, and whole hippocampi were dissected and fixed in 10% formalin for IHC (see below). Portions of hippocampus, prefrontal cortex, and nucleus accumbens were microdissected using custom tissue punches and frozen on dry ice for Western blotting (see below).

All experiments were approved by the Institutional Animal Care and Use Committee at Michigan State University and performed in accordance with AAALAC and NIH guidelines.

Mice and Genotyping

The animals used in chapter 4 were adult male and female mice (>8 weeks). Adult mice were group housed 4-5 per cage in a 12 h light/dark cycle and provided ad libitum food and water. We used a *Mast Cell Protease 5-cre* mouse line, which expresses cre recombinase in the mast cells, and crossed them to a cre-dependent *Floxed FosB* mouse line [91], to produce progeny that lack *FosB* expression specifically in mast cells, as well as wildtype littermates. Mice were genotyped using standard PCR using the following primers:

- Floxed FosB:

  FB loxPu sequence: 5' – GCT GAA GGA GAT GGG TAA CAG – 3'

  LIPz sequence: 5' – AAG CCT GGT GTG ATG GTG A – 3'

LNEo1 sequence: 5' – AGA GCG AGG GAA GCG TCT ACC TA – 3'

- MCPT5-Cre:

  MCPT5-Cre Forward: 5’ – ACA GTG GTA TTC CCGGGG AGT GT – 3’

  MCPT5-Cre Reverse: 5’ – GTC AGT GCG TTC AAA GGC CA – 3’

  MCPT5-Ex1-DO3: 5’ – TGA GAA GGG CTA TGA GTC CCA – 3’

  All experiments were approved by the Institutional Animal Care and Use Committee at Michigan State University and performed in accordance with AAALAC and NIH guidelines.

Immunohistochemistry (IHC)

Whole sections of hippocampi were collected from each pig and were post-fixed for 24 hours in 10% formalin, cryopreserved in 30% sucrose, and frozen and sliced into 35 μm sections. The hippocampal slices were stored in PBS with 0.01% sodium azide until IHC was performed. The following primary anitbodies were used: Anti-FosB (1:1000, mouse, ab11959, Abcam), and anti-doublecortin (1:200, goat, sc-8066, Santa Cruz). The following corresponding secondary antibodies were then used: Donkey anti-mouse Ig biotin (1:500, 715-065-150, Jackson Immunoresearch) and Donkey anti-goat Ig biotin (1:600, 705-065-147, Jackson Immunoresearch). 3,3’-diaminobenzidine staining was then performed per manufacturer’s instructions (Vector Laboratories), and digital images were taken using traditional light microscopy. Prior to quantification, image files were blinded by a fellow experimenter.

Western Blots

Following euthanasia, punches of prefrontal cortex (PFC), nucleus accumbens (NAc), and hippocampus (HPC) were collected and flash frozen. HPC punches were thawed in

500 µl of RIPA buffer (pH 7.4), and PFC and NAc punches were thawed in 200 µl of RIPA buffer with phosphatase and protease inhibitor cocktails (Sigma P8340-1ML 1:100, Sigma P0044 1:100, Sigma P5726 1:100). The samples were then subjected to a DC protein assay (Biorad 5000112EDU). Lamelli buffer at a 5X concentration was added to each sample, and 20 µg of protein were loaded into each lane of a 4-15% precast Biorad gel. Protein was then transferred to PVDF membranes at 60 mV for 35 minutes at room temperature. Membranes were blocked in 5% nonfat dry milk in 1X PBS-Tween20 for 1 hour prior to an overnight primary incubation at 4C with Anti-FosB (FosB 5G4, 1:500, rb, #2251S, Cell Signaling Technologies) in 5% Bovine Serum Albumin in 1X PBS-Tween20. The following day, membranes were rinsed and incubated with Donkey anti-rb HRP (PI-1000; 1:40,000; Vector) followed by development with Western Substrate Dura and imaged with a film developer. Films were then quantified using ImageJSoftware and normalized to a total protein stain.

Mouse Behavioral Tasks

All behavioral tests were performed under red light conditions after an hour habituation, except sucrose preference which was tested in the home cage. Social Interaction and Elevated Plus Maze tasks were performed at approximately 17:00 hours. At the time of behavior analysis, mice were singly housed across the span of 4 days.

Elevated Plus Maze

Elevated plus maze (EPM) behavior was measured using a custom-built apparatus (aluminum base with polyvinylchloride arms) based on plans from ANY-maze (www.anymaze.com; Stoelting). The maze was laid out in the shape of a plus sign as viewed from above. It consisted of four gray-colored, interconnected runways (5 × 35 cm)

with two open and two closed arms, elevated 45 cm from the ground. Closed arms were enclosed on the long sides of the runway by two 15 cm high gray walls. Animals were placed into the center of the maze facing an open arm, and behavior was video recorded for 5 min. The percentage of time spent in the open arms and the percentage of entries into the open arms were quantified as assessments of anxiety-like behavior [87].

Social Interaction

For the social interaction test, we measured the time spent in the interaction zone during the first (target absent) and second (target present) trials; the *interaction ratio* was calculated as 100 × (interaction time, target present)/(interaction time, target absent). Target mice were other C57 mice of the same sex as the C57 mice being tested for interaction [88].

Sucrose Preference

A standard two-bottle choice procedure was assessed across 4 days. Singly housed mice were first given access to two bottles of drinking water on the top of their home cage for 1 day to assess baseline drinking and to acclimate the mice to the bottles. Then, mice were given two-bottle choice, in which one of the bottles was replaced with a 1% sucrose solution for the remaining 3 days. Each bottle was weighed daily and alternated to the opposite side of the cage topper to verify there was no side bias. Sucrose solution consumed per day as a percentage of total liquid consumption was measured as an index of anhedonia [87].

CHAPTER 3: EARLY WEANING LEADS TO ALTERATIONS IN ADULT NEUROGENESIS WITH POSSIBLE SEX INFLUENCES

Introduction

Women are more susceptible than men to developing mood disorders[69], and figure 1 demonstrates consistently increased disease prevalence among adult women across ages [70]. However, the potential molecular and cellular mechanisms underlying this disparity are unknown. As discussed in Chapter 1, mouse models have been used to demonstrate sex-differences in both behaviors associated with depressive disorders and sex differences in the brain regions underlying those behaviors. However, the mouse model is limited by the extreme differences in physiology between mice and humans, and thus there is need for study of sex differences in physiology using large animal models whose brains are much more similar to those of humans. Therefore, we investigated potential sex differences in markers of neurogenesis and neuronal activity in the hippocampus of pigs.

There is a noticeable reduction in hippocampal volume in patients who have endured early life adversity, as well as those who suffer from depression, which may be a result of reduced neurogenesis [7, 8, 9]. Early life stress affects neurogenesis in mice and rats alike. Following prolonged neonatal maternal separation stress, rats exhibit a prolonged suppression of adult neurogenesis [75]. Following early life stress, mice are often found to be more susceptible to developing depressive phenotypes after a chronic social defeat stress paradigm [40]. However, these effects can be reversed with the use of antidepressants such as fluoxetine [32]. Fluoxetine also results in an increase in

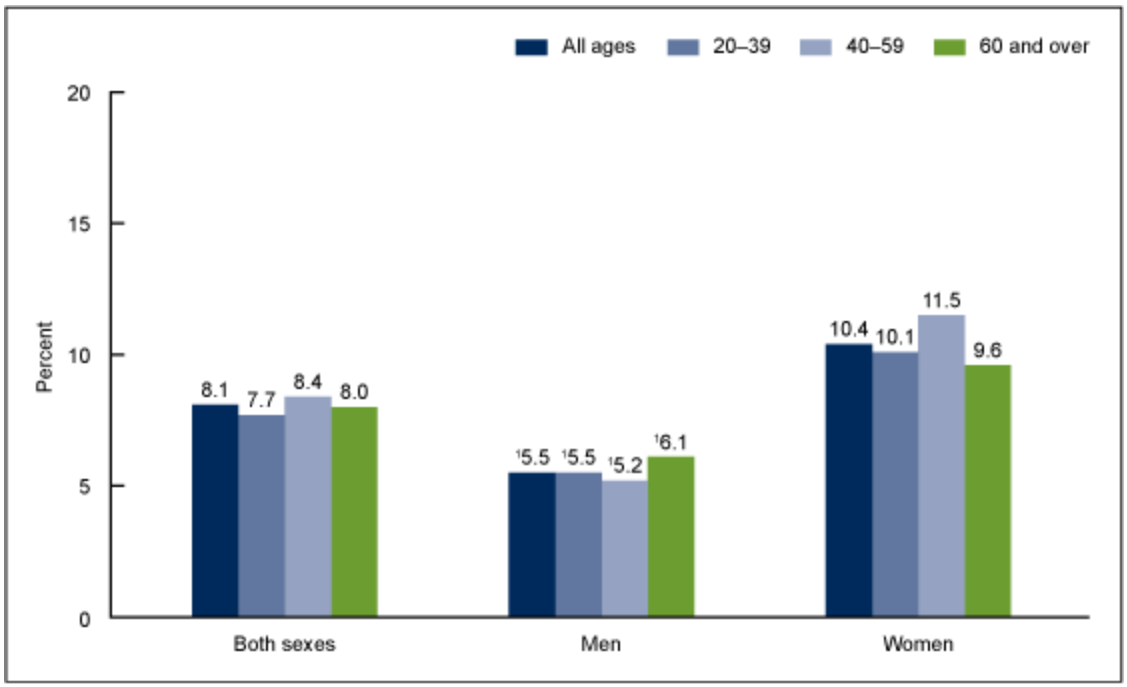

Figure 1 | Depression in the United States Incidences of depression in the United States in men and women ages 20 and older, years 2013-2016. Figure courtesy of CDC [70].

neurogenesis in these mice, reversing the hippocampal atrophy that is observed along with the behavioral deficits [16, 17, 20]. The use of rodents and other lower animal models to study neurogenesis has become a subject of great interest in the past few years, as a number of studies indicate the adult neurogenesis clearly observed in rodents is either not present or present at greatly reduced levels in humans [73]. These discrepancies in the field are what drove our experiments in the direction of using a higher order organism, such as the pig, to obtain more information regarding adult neurogenesis.

The human brain is very complex, and different stimuli may have drastically different effects on neuronal proliferation, migration, and survival [74]. Because adult neurogenesis is very easily observed in rodent models and remains very controversial in humans, rodents may not the most translationally relevant model. Therefore, considering that the pig is much more closely related to humans, both evolutionarily and in brain anatomy and

physiology, we sought to determine whether pigs have indicators of adult neurogenesis and whether early life stress alters markers of neurogenesis in adult pigs.

We performed IHC for FosB in the dentate gyrus of pigs in order to quantify cells positive for the transcription factor. In figure 2, our lab previously demonstrated that the presence of FosB in the hippocampus, specifically the dentate gyrus, is necessary for neurogenesis to occur in mice during adulthood [17]. On the left side of the figure, the effects of floxing FosB are seen by the decreased number of BrdU positive cells. BrdU is a marker of newly proliferated cells. However, BrdU is not specific to neuronal prolifieration. Doublecortin, a protein specific to new neurons, is shown on the right side of the figure with DAB staining. Again, there is a significant decrease in the number of Doublecortin positive cells in the

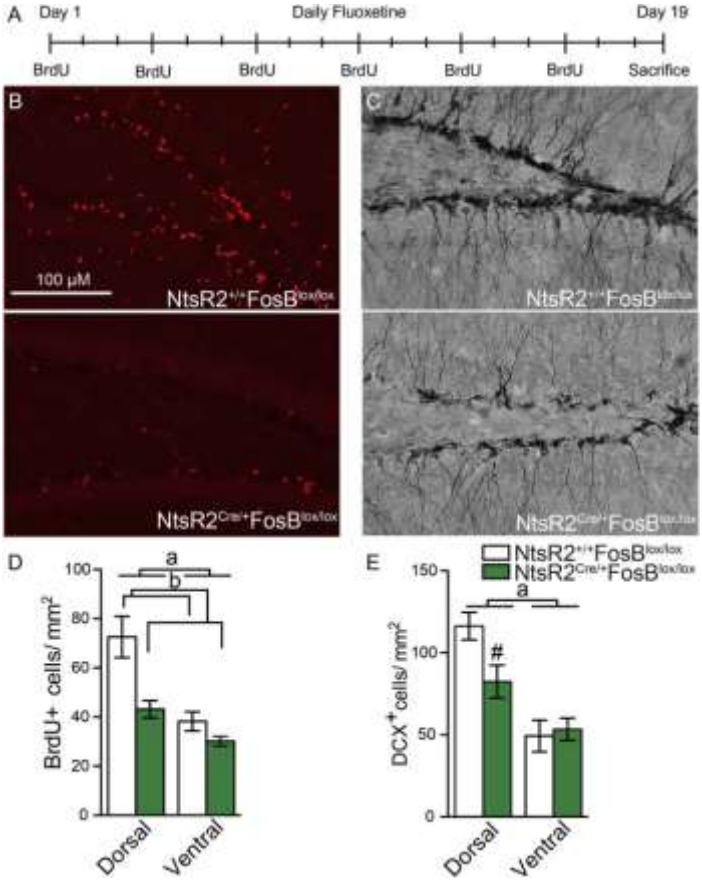

Figure 2 | Role of FosB in neurogenesis Quantification of Doublecortin positive cells in the dentate gyrus of the mouse hippocampus with and without floxing of FosB. Floxed FosB mice showed decreased neurogenesis (published in [17]).

dentate gyrus as a result of floxing FosB. Therefore, as early life stress is associated with reduced hippocampal neurogenesis in rodents and humans, we hypothesized that there would be a reduced presence of FosB in early weaned pigs compared to late weaned pigs. As seen in a CSDS model of stress, FosB mediates resilience to the stress test and results in a lesser depressive phenotype [32]. If it is found that early life stress results in a reduction in FosB, this could allow for a stronger understanding of stress susceptibility on a molecular level. In addition to individual cell counts, we used western blots to quantify both FosB, and its truncated form, ΔFosB, in the nucleus accumbens and prefrontal cortex.

The nucleus accumbens is an important brain region within the ventral striatum, and is critical in reward, most associated with drugs of abuse [58]. The mesolimbic pathway, also known as the reward pathway, is a dopaminergic pathway important in motivation for many essential behaviors, including eating, drinking, and reproduction [59]. Dysfunction in this pathway, however, may cause anhedonia in cases of depression. Both acute and chronic restraint stress cause an increased induction of FosB and ΔFosB within the nucleus accumbens [60]. Coupled with a quantifiable increase in FosB gene products, acute and chronic restraint stress result in elevated corticosterone levels, and depressive behaviors such as a disinterest in eating resulting in weight loss, and decreased sucrose preference [61, 62].

The prefrontal cortex is a brain region implicated in executive functioning, including goal-directed behaviors [63]. In cases of depression, changes in synaptic plasticity occur in this region [64]. Following chronic restraint stress, dendritic shortening occurs within the medial prefrontal cortex [65]. Dendritic shortening corresponds with reduced synaptic

plasticity via the induction of long-term depression [66]. This impacts the type and quantity of synapses that occur within the affected brain region. Synaptic plasticity is the brain's ability to modify responses to stimuli, either increasing or decreasing [67]. This being affected in the prefrontal cortex following chronic stress may lead to significant behavioral changes. Additionally, there is evidence that shows ΔFosB in this region promotes susceptibility to stress, resulting in exaggerated depressive phenotypes [68].

Thus, the purpose of this chapter was to quantify both hippocampal neurogenesis and FosB expression in the brains of male and female pigs with or without early life stress.

Results

In our first experiment, we used coronal slices of the hippocampi collected from female, intact male, and castrated male pigs, all of which had been subjected to either early weaning, at 15 days postpartum, or late weaning, at 28 days postpartum. To quantify neurogenesis, we stained each sample for doublecortin (DCX), a protein that is only present in neuronal precursor cells [57]. Cells were determined to be DCX positive if they were within the region of interest, the subgranular zone of the dentate gyrus, and were stained completely black, as displayed in figure 3. This experiment was designed to test the hypothesis that animals that undergo early life adversity experience a reduction in adult neurogenesis.

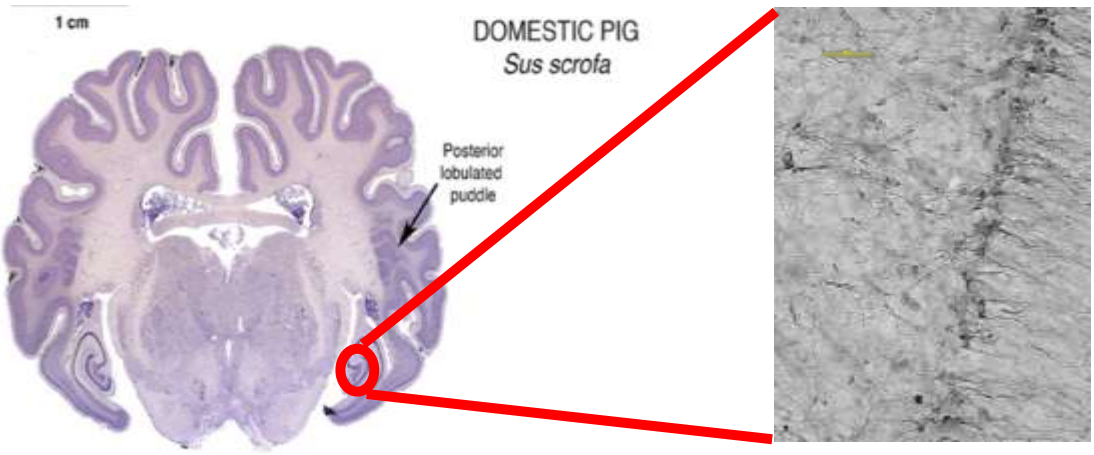

Figure 3 | Location of hippocampus Example image of Doublecortin staining of hippocampal slice from an early weaned castrated pig. Images were taken at 20x magnification. Yellow scale bar represents 100 µm.

Adult neurogenesis is reduced in early weaned females

We found a significant decrease in the average number of DCX positive cells in the hippocampus of early weaned female pigs compared to their late weaned counter parts; however, no difference was apparent in either the intact or castrated males ($F_{(2,29)}$=1.541; p=0.0252, p=0.7918, and p=0.8873, respectively). Additionally, there were no significant differences between sexes ($F_{(2,59)}$=0.2864, p=0.7520). Each group had 6 individuals, aside from the late weaned females which had 5. These data were analyzed with a Two-Way ANOVA.

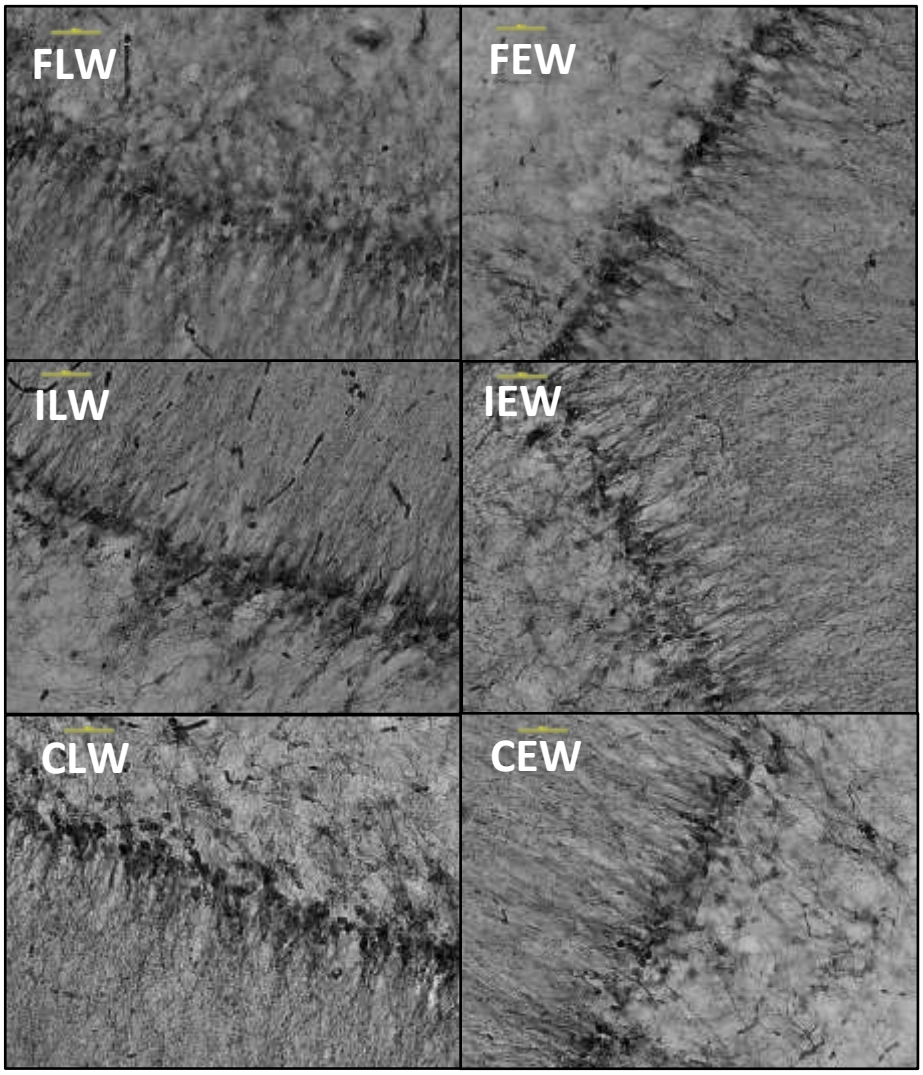

Figure 4 | Doublecortin staining Example images of doublecortin staining of the dentate gyrus from late weaned and early weaned female pigs (FLW and FEW), intact males (ILW and IEW), and castrated males (CLW and CEW). Pictures taken at 20x magnification. Yellow scale bar represents 100 µm.

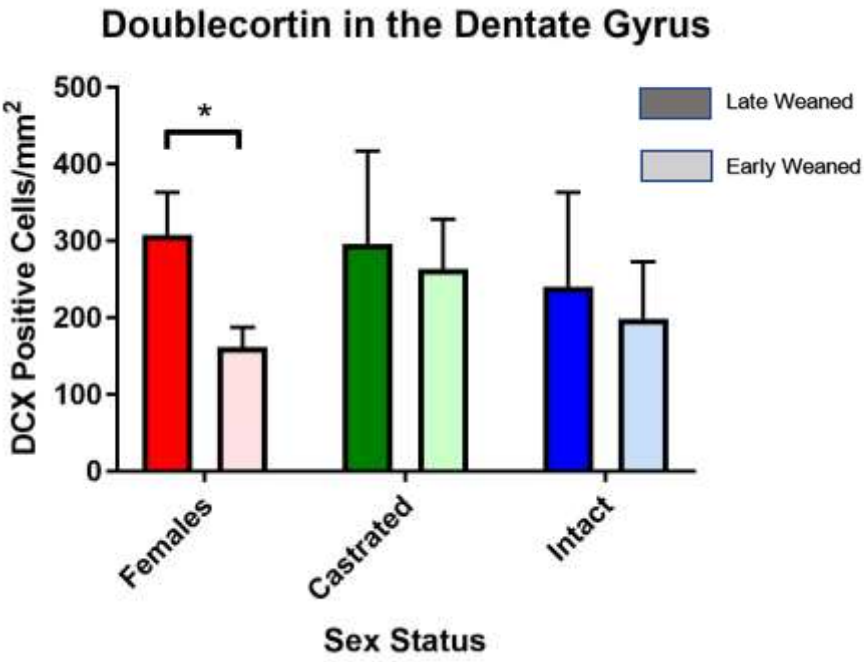

Figure 5 | Early weaned females show reduced neurogenesis
Doublecortin (DCX) positive cells in the dentate gyrus of early and late weaned female, castrated male, and intact male pigs. A significant decrease was observed in DCX+ cells in early weaned females. * denotes $p<0.05$.

FosB in the CA1 and dentate gyrus of pig hippocampus

Using DAB staining, we performed an IHC experiment to show FosB positive cells in the CA1 and dentrate gyrus of early and late weaned pigs. No significant differences were found in FosB expression in the CA1 and dentate gyrus of the hippocampus between early and late weaned pigs ($F_{(1,19)}=1.062$, $p=0.3158$ and $F(1,20)=0.2595$, $p=0.6160$). However there does appear to be an interaction of sex, showing a trend in the CA1 of increased FosB in castrated males ($F_{(2,19)}=3.474$, $p=0.0518$). No such trend exists within the CA1 ($F_{(2,20)}=0.03963$, $p=0.9612$). Due to the difference in FosB positive cells in the CA1 between females and castrated males, we can begin to think about what specifically in females causes the reduced amount of FosB. Interestingly, these results do not mirror what we saw from the data in figure 4, with respect to preliminary data. Early weaned

females showed a reduced amount of neurogenesis compared to late weaned females. This led us to expect a similar decrease in FosB positive cells in early weaned females as well. Instead, the number of FosB positive cells remained constant between late and early weaned females across both regions analyzed.

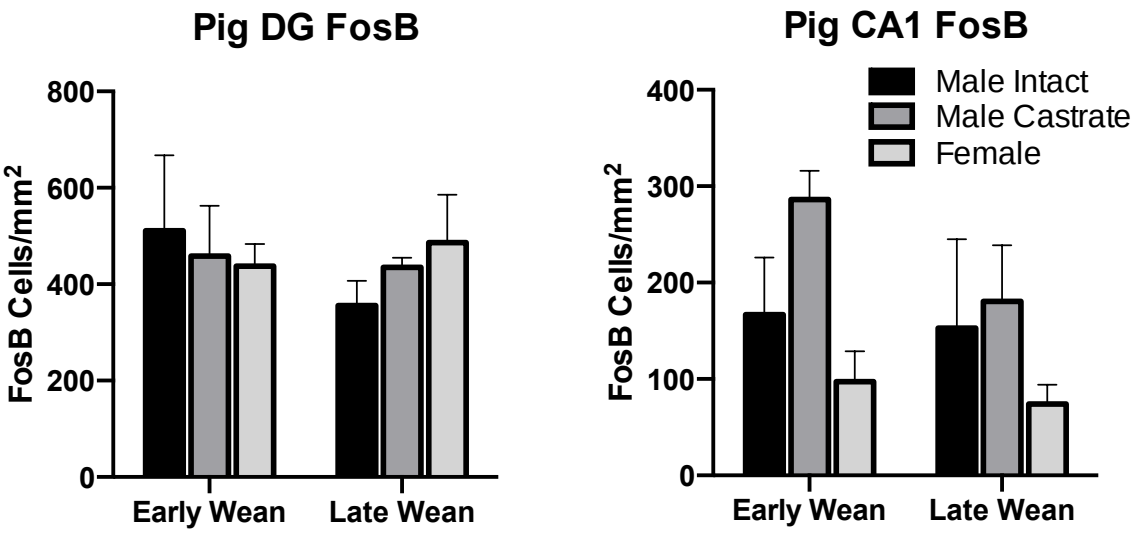

Figure 6 | FosB in the dentate gyrus and CA1 of the pig hippocampus Quantitation of FosB staining of dentate gyrus and CA1 revealed no significant differences between late weaned and early weaned pigs, regardless of sex or castration.

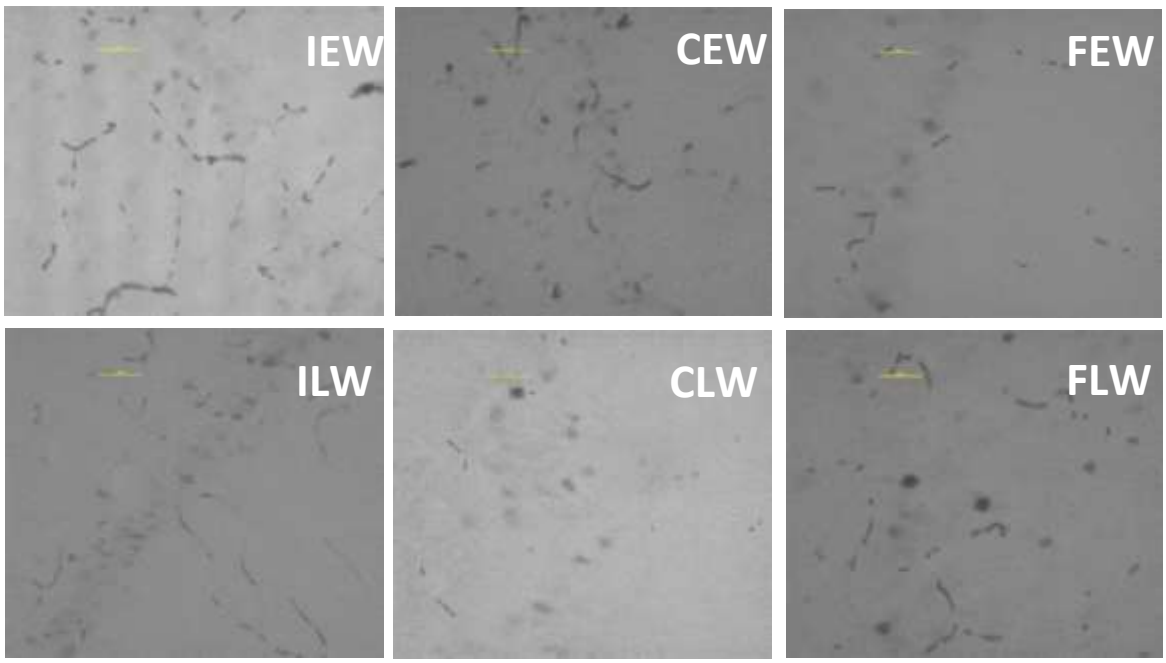

Figure 7 | Example images of FosB staining in CA1 FosB staining of CA1 from late weaned and early weaned female pigs (FLW and FEW), intact males (ILW and IEW), and castrated males (CLW and CEW). Images were taken at 20X magnification and yellow scale bar represents 100 µm.

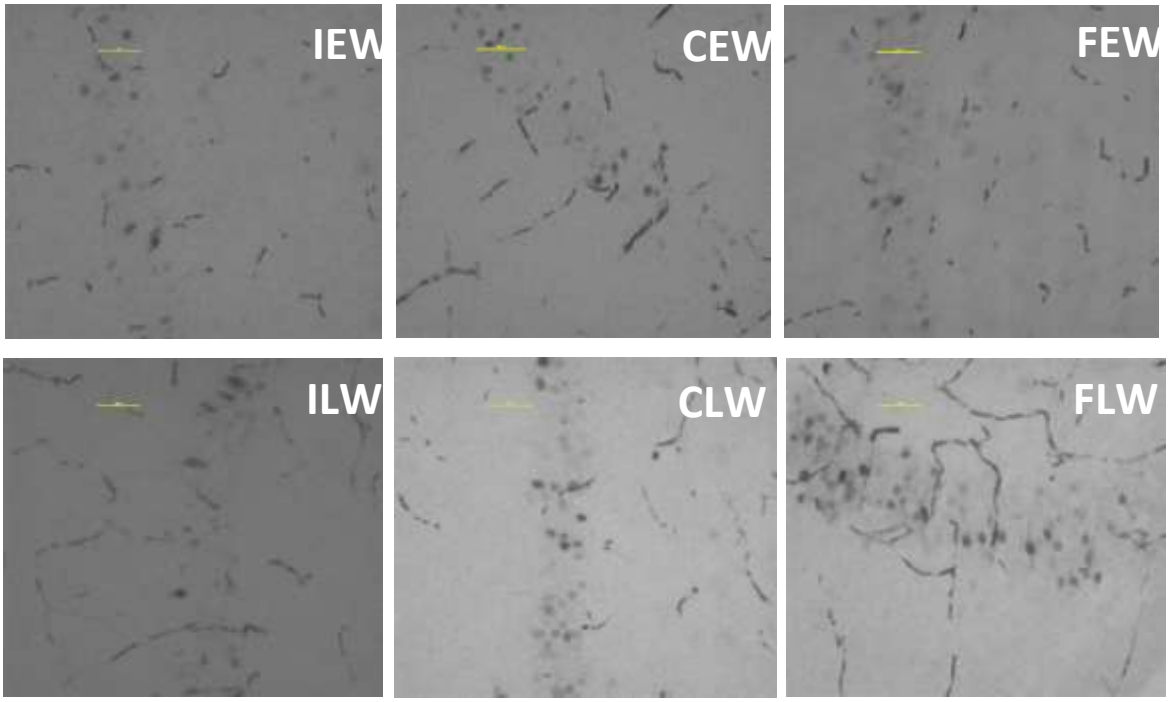

Figure 8 | Example images of FosB staining in dentate gyrus FosB staining of dentate gyrus from late weaned and early weaned female pigs (FLW and FEW), intact males (ILW and IEW), and castrated males (CLW and CEW).). Images were taken at 20X magnification and yellow scale bar represents 100 µm.

Possible sex differences in FosB gene product expression in hippocampus

As previous work in both rodents and humans has shown altered FosB gene expression in the accumbens, hippocampus, and prefrontal cortex following stress, we used Western blots to examine whether early life adversity changed FosB or ΔFosB in male or female pigs. Western blot revealed no differences in full-length FosB or ΔFosB in any of the hippocampus ($F_{(5,38)}$=0.763, p=0.5821 and $F_{(5,38)}$=1.276, p=0.2945). However, from this data set we do see a trend in decreasing FosB and ΔFosB in the early weaned castrated males compared to late weaned castrated males. All western blot data were analyzed using a Two-Way ANOVA, with an n of 8 early and late weaned females, and

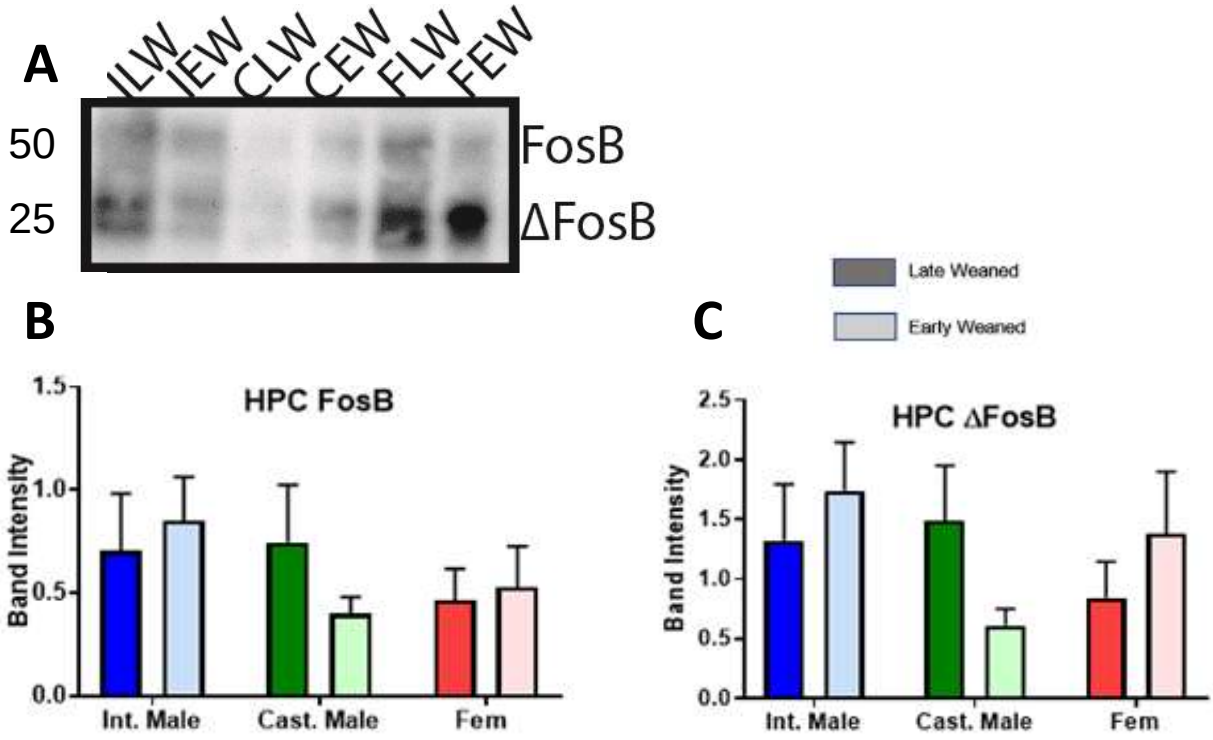

Figure 9 | FosB gene products in pig hippocampus A. Western blot of FosB and ΔFosB in hippocampi from female pigs (FLW and FEW), intact males (ILW and IEW), and castrated males (CLW and CEW). Quantification reveals no differences between sexes or weaning times in full length FosB (B) or ΔFosB (C).

an n of 7 intact and castrated males in both the early and late weaned groups.

The same analysis was performed using tissue from the nucleus accumbens. As shown, there were no significant differences between early and late weaned animals, suggesting that early life adversity had no effect on the prominence of FosB and ΔFosB in this brain region ($F_{(1,40)}$=1.264, p=0.2676 and $F_{(1,40)}$=0.05295, p=0.8192). FosB and ΔFosB were quantified in the prefontal cortex as well using western blots. Our results

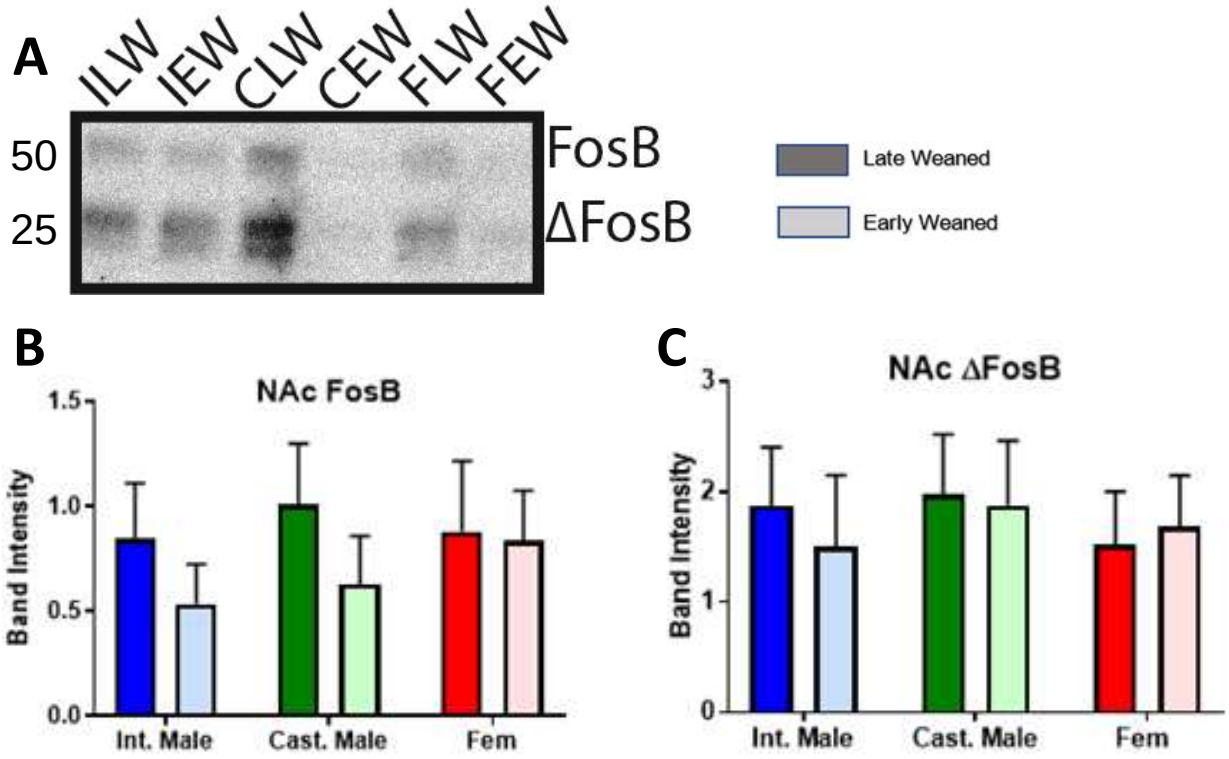

Figure 10 | FosB gene products in pig nucleus accumbens A. Western blot of FosB and ΔFosB in nucleus accumbens from female pigs (FLW and FEW), intact males (ILW and IEW), and castrated males (CLW and CEW). Quantification reveals no differences between sexes or weaning times in full length FosB (B) or ΔFosB (C).

from this analysis were similar to those from the NAc, as there were no significant differences in the presence of FosB or ΔFosB between the late and early weaning

groups ($F_{(1,40)}$=0.06858, p=0.7948). Again, this is telling that there is no effect of early life adversity on the presence of FosB gene products in this brain region.

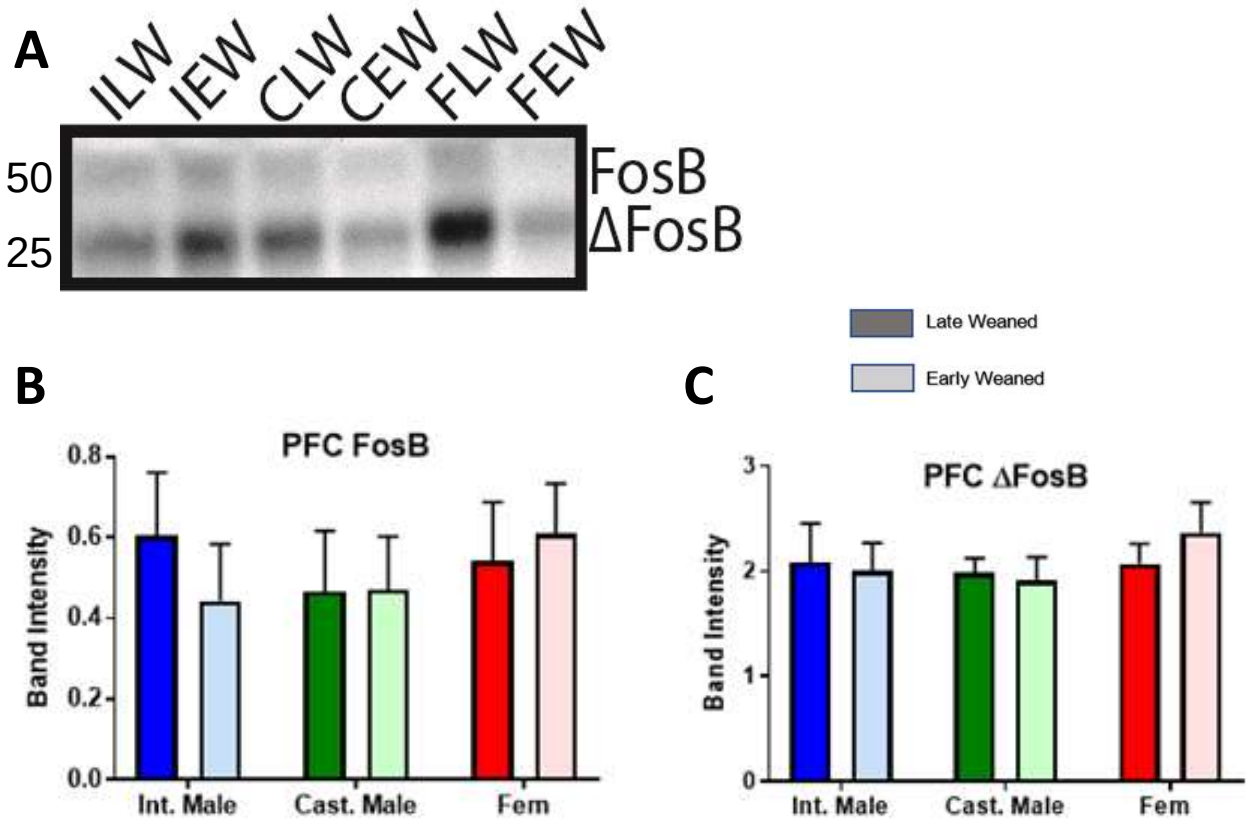

Figure 11 | FosB gene products in pig prefrontal cortex A. Western blot of FosB and ΔFosB in prefrontal cortex from female pigs (FLW and FEW), intact males (ILW and IEW), and castrated males (CLW and CEW). Quantification reveals no differences between sexes or weaning times in full length FosB (B) or ΔFosB (C).

Discussion

The reduced number of DCX positive cells in the early weaned females (Figure 5) may indicate reduced neurogenesis. These data suggest that females may be specifically vulnerable to the effects of early life adversity on hippocampal neurogenesis. This would then be a potential mechanism underlying the increased susceptibility of women to depression. Behavioral models for testing this in the pig are lacking, though it is possible

that future studies could observe whether early-weaned female pigs are more likely than males to exhibit failure to thrive, have reduced social interaction, or display a reduced social rank.

Coupled with the previous findings provided from our lab using mouse models, it would be intuitive to predict that there would be reduced FosB gene products in the hippocampus of adult pigs who experienced early life stress if they have reduced neurogenesis. While there were no significant differences indicating any change in FosB expression across the varying sex and stress groups, there were some intriguing trends that could lay a groundwork for future studies. Both early and late weaned females appear to have reduced FosB in the CA1 of the hippocampus. This may tell us more about the molecular basis of the increased prominence of depression in women, as FosB expression is thought to drive resilience. In the hippocampus among the castrated males, there appears to be a reduction in both FosB and ΔFosB. This trend is not apparent in the intact males. This suggests that testosterone may protect against stress effects on FosB gene expression in males. In human studies, there is evidence of a protective role of testosterone in resilience to depressive episodes [71, 72]. This may be a substantial contributing factor to the differences in the rates of depression diagnosis between men and women.

CHAPTER 4: EFFECTS OF FOSB ON MAST CELL FUNTION AND BEHAVIOR IN MICE

Introduction

Mast cells serve many functions throughout the body. Largely responsible for immune responses, such as inflammation, they release histamine, cytokines, and other proinflammatory mediators from their many granules that are pre-packaged for secretion [53, 76, 77]. This mechanism is commonly associated with allergic reactions; however, it appears that it may be implicated in depression and anxiety as well [76, 78].

Mast cells are localized predominantly in areas of the body with high vasculature [79]. Notably, they are located along the blood vessels within the meninges of the brain [80]. The meninges are the collective three membranous layers that serve as a protective covering between the brain and the skull cap [81]. Being in such proximity with the blood supply surrounding the brain gives ample opportunity for mast cells to have a significant impact on the brain and its functions.

In human patients suffering from depression, there is a prevalence of neuroinflammation [82]. Additionally, mast cell activation appears to be increased in depressed patients, a phenomenon thought to be caused by the formation of inflammatory cytokines [83]. In a study of patients who suffer from functional dyspepsia, or indigestion, there is a positive correlation between the presence of anxiety and/or depression in these patients and the number of duodenal mast cells and degranulation [84]. In animals, however, models of external stressors such as learned helplessness and chronic mild stress show an increase in inflammation that precedes many depressive-like behaviors and changes in brain morphology, such as weight loss and hippocampal neurodegeneration[85].

Mast cells respond quickly to environmental stimuli, and their activation following a stressful event is almost instantaneous [79]. This leads to a rapid degranulation, and therefore secretion of various cytokines to facilitate the inflammatory response [79]. However, mast cell deficient mice, or sash mice, not only lack these signaling cascades, but also exhibit behavioral changes. Homozygous sash mice displayed more anxiety-like behaviors in both the open-field test and the elevated plus maze compared to their wild type and heterozygous control counterparts [86]. This same study also performed a blockade of only brain mast cells, leaving peripheral mast cells active. They found that this increased anxiogenic behaviors as well. These data suggest that mast cells act in mediating stress responses, and without them, these responses are exacerbated. Preliminary data from our lab (Figure 12) show that mast cells express high levels of the transcription factor FosB when activated, and that early life stress exacerbates this response. Moreover, we show that removal of FosB gene expression from mast cells increases their active secretion of histamine, suggesting that FosB acts as a break on mast cell activity. As mice lacking mast cells are susceptible to anxiety[86], it is possible that mice lacking FosB expression in their mast cells may have increased mast cell activity, making them less anxious. For the purpose of our study, we used an MCPT-5 (mast cell protease 5) Cre mouse line crossed with a *floxed FosB* mouse line in order to delete FosB expression specifically in mast cells systemically. Sucrose preference, social interaction scores and elevated plus maze data were collected for each mouse and compared across genotype and sex. We expected to find that removing FosB from mast cells would increase mast cell activation, and in turn would reduce the occurrence

of anxiogenic behaviors while increasing sucrose preference as well. All behavioral data were analyzed using a Two-way ANOVA.

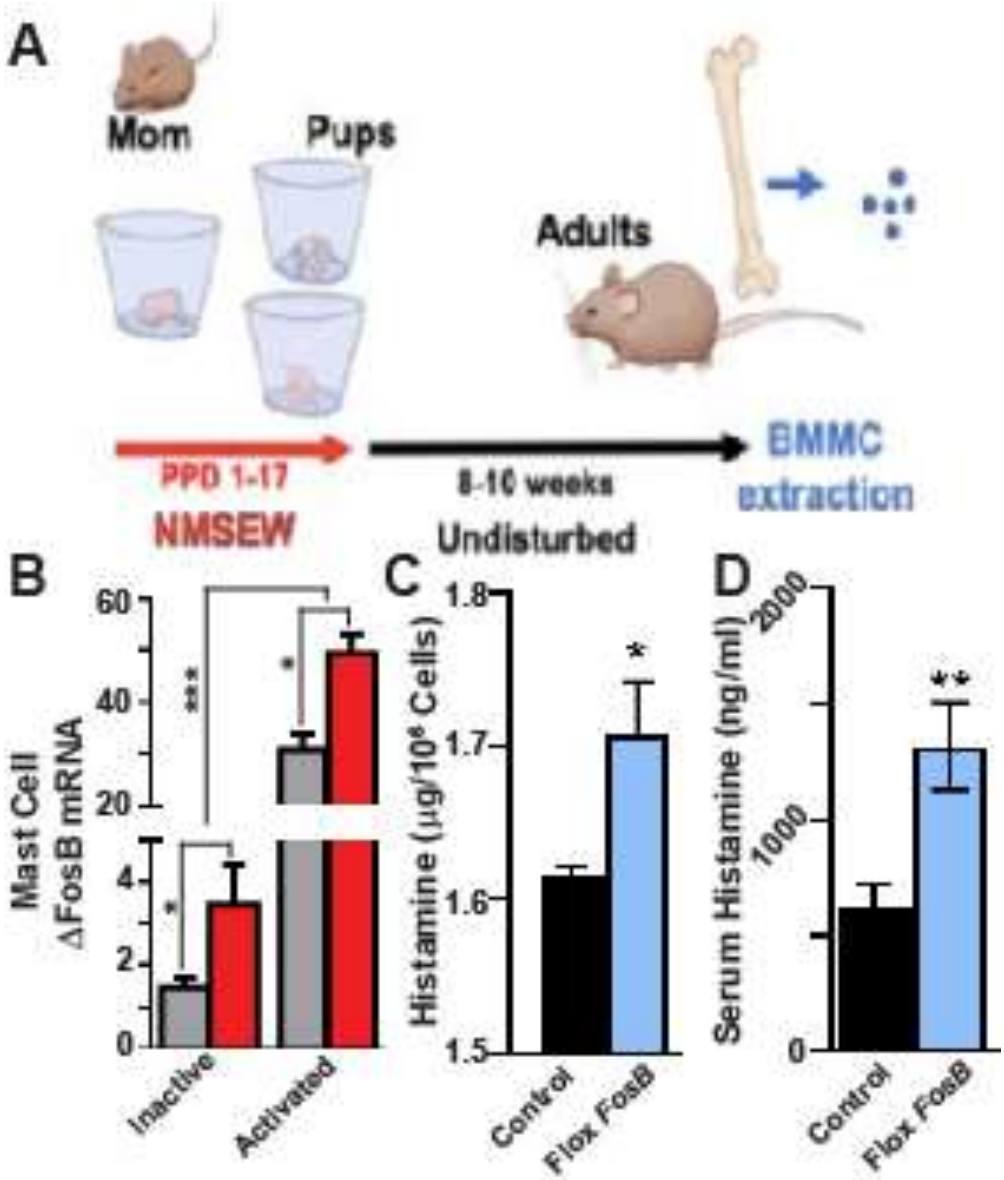

Figure 12 | Role of FosB in mast cells A. Schematic depicting early life stress and adult extraction and differentiation of bone marrow derived mast cells. B. qPCR reveals ΔFosB mRNA is increased upon mast cell activation and is higher in mast cells derived from stressed animals (red) than in control animals (gray). Mice lacking FosB gene expression specifically in mast cells have MCs that produce more histamine in culture (C) and have more histamine in their blood (D).

Results

Social Interaction Test

Mice that had FosB floxed from their mast cells exhibited no differences in their social interaction scores compared to the wild type mice, nor did we see an effect of sex ($F_{(1,41)}$=1.649, p=0.2063 and $F_{(1,41)}$=0.5329, p=0.4695). For this experiment, we had n-values of 12 wildtype and 14 homozygous females, along with 8 wildtype and 11 homozygous males. Contrary to our expected results, there were no effects on anxiety-like phenotypes in this behavior test.

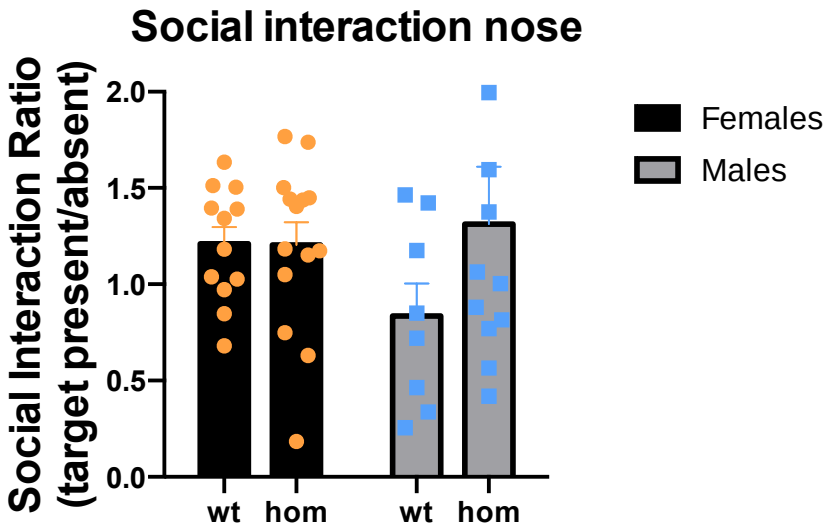

Figure 13 | Social interaction scores Social interaction scores of male and female mice from MCPT5-Cre lines, both wildtype and homozygous for the mutation.

Elevated Plus Maze

Our data from the elevated plus maze show no significant effect between mice homozygous for the mutation and wild type mice. However, the homozygous females do show an upward trend in their open/closed arm ratios on the maze, suggesting that floxing FosB from mast cells may reduce the occurrence of anxiogenic phenotypes in females

($F_{(1,42)}$=0.2952, p=0.0931). Our n values for this experiment were 12 wildtype and 14 homozygous females, and 9 wildtype and 11 homozygous males. Additionally, no differences were observed across sexes other than the homozygous female mice showed a trend for less anxiogenic behavior, while the male mice did not (F(1,42)=0.01189, p=0.9137).

Figure 14 | Ratio of time spent on open arms to closed arms in elevated plus maze Open/closed arm ratios from elevated plus maze with MCPT5-Cre mouse line

Sucrose Preference

In addition to the social interaction test and elevated plus maze, we measured sucrose preference over the duration of the four days of behavioral data collection. We found that homozygous males exhibited a significant increase in sucrose preference compared to the wild type males, while females showed no difference ($F_{(1,49)}$=4.727, p=0.0346). Interestingly, although homozygous males displayed an increase in this behavior when compared to their wildtype counterparts, there was no difference between the homozygous males and either of the female genotypes ($F_{(1,49)}$=0.05574, p=0.8143). This

experiment had n values of 13 wildtype and 18 homozygous females, as well as 11 wildtype and homozygous males. A low sucrose preference is indicative of anhedonia, a common symptom of depression. Thus, a higher sucrose preference in male mice lacking FosB in their mast cells may indicate resilience to anhedonia.

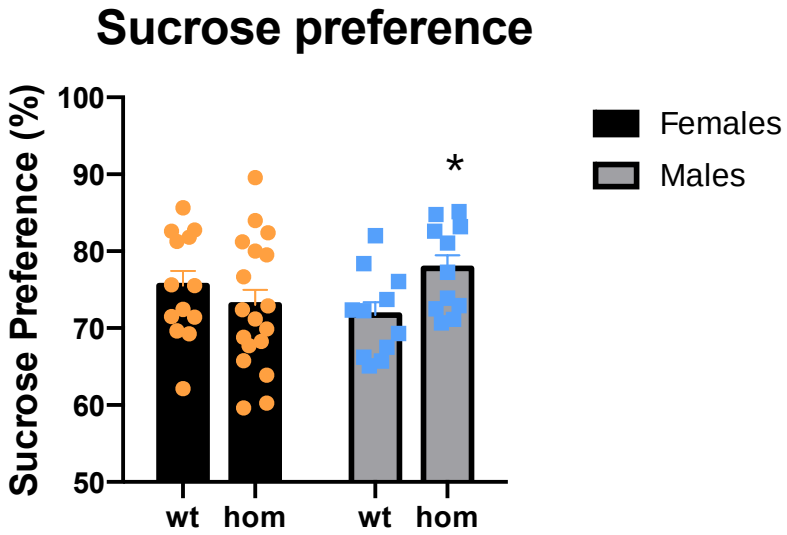

Figure 15 | Percentage of sucrose solution consumed out of total water consumption Sucrose preferences of wildtype and floxed FosB mice. *denotes p<0.05.

Discussion

No significant differences from this cohort of animals were found upon analysis of the data from the social interaction test. However, while the same can be said for the data collected from the elevated plus maze, there does appear to be an interesting trend between the male wildtype animals and the males homozygous for the mutation. Although not significant, homozygous males showed a greater open to closed arm ratio, suggesting that removing the FosB from the mast cells did attenuate their baseline anxiogenic behaviors to an extent. Sucrose preference significantly increased in homozygous males compared to wildtype males, but was consistant across all females.

From this we can gather that, in the males, removing FosB increased the desire for reward, or reduced anhedonia, a common symptom of depression. Collectively, from these behavioral assays, we have gathered intriguing data regarding how FosB is implicated in mast cell activation, as well as resulting behaviors. Our preliminary data already showed explicitly that when FosB is absent, mast cell activation is significantly increased, while other data exhibits that the lack of mast cells, and thereby their activity, increases anxiogenic behaviors. We observed a significant increase in sucrose solution consumption in homozygous male animals, as well as no increases in any anxiogenic behaviors, significant or otherwise. This data set provides a solid foundation to continue with larger cohorts for the purpose of increasing sample size and having a more conclusive picture of the molecular mechanisms of these behavioral changes.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Summary

The work presented here makes contributions to the field's understanding of mood disorders in terms of sex differences, as well as the effects of early life adversity on the likelihood of developing mood disorders. While it is well documented that depression is more commonly diagnosed in women than men, it is not understood why [69]. The same can be said for individuals who undergo early life adversity [6]. Using two animal models that demonstrate depressive phenotypes and cellular morphologies in both males and females, we were able to contribute several data sets that can provide more insight into these phenomena.

Our model of early life adversity using early weaned and late weaned pigs uncovered a possible sex-specific effect. While early weaned females showed a significant decrease in neurogenesis in the dentate gyrus, as measured by DCX staining, no difference was found in either of the male groups. We followed this up with a stain for FosB in the same region to see if our results mirrored the previous finding of FosB's necessity for neurogenesis to occur [17]. While no differences were observed between the early and late weaned groups in either the CA1 or the dentate gyrus, there did appear to be a drop in FosB in the CA1 in females compared to castrated males. Previous studies not only point to the role of FosB in mediating programmed cell death, or apoptosis, but also its implication in decreased cellular excitability in the dorsal hippocampal CA1 [89, 90]. Thus, it is possible that FosB activity was high enough in the dentate gyrus of the females that excessive apoptosis occurrs, thereby showing a decrease in neurogenesis in the dentate

gyrus as well. However, if this is the case, the reason that this did not occur in intact or castrated males is unclear.

When quantifying FosB gene products via western blot, we found that only early weaned castrated males had trends toward reduced amounts of both FosB and ΔFosB in the hippocampus. Interestingly, these results are not mirrored by either IHC experiment. Since we saw decreased neurogenesis in the early weaned females, we expected there to be a similar decrease in hippocampal FosB, but no differences were observed. It is difficult to say why there was no difference in FosB expression after early weaning, however something to take note of is the size of the hippocampal tissue punch that was homogenized for analysis. These tissue punches were quite large, and due to their size, I think that it is quite likely that our FosB quantification was flooded by other FosB that is present in other parahippocampal areas that we were not interested in. In the future, it would be best to take a more precise tissue punch from a more specific location, such as dentate gyrus or CA1, so we are only getting a quantification of FosB in our region of interest. Doing this may also make way for a significant decrease in FosB expression in early weaned castrated males. As for the nucleus accumbens and the prefrontal cortex, all groups were similar to each other, indicating that early life adversity had no effect on *FosB* expression in either region. In reflection upon existing literature, these results are not confounding. While it has been shown that changes occur in these regions in response to acute and chronic forms of stress, no changes have been observed in unstressed adult mice that experienced early maternal separation [61, 62, 67].

With our transgenic mouse model, we were able to observe the role of mast cell *FosB* expression in various behaviors. With respect to our preliminary data and what we know

about the behavioral outcomes of increased mast cell activation, we were expecting to see a decrease in anxiogenic behavior in mice homozygous for the mutation. This hypothesis was, in some cases, supported by our findings from the behavior experiments using the MCPT5-Cre x Floxed FosB mouse line. In the elevated plus maze, although not significant, the female mice homozygous for the mutation did show a reduced anxiogenic phenotype through increased exploration of the open arms on the maze. What we can gather from these data is that females may be more sensitive to mast cell activation than males. These data are encouraging for future studies with more cohorts using this transgenic model. With a larger sample size, a sex specific effect on this behavior may become clearer, as well as whether there is an effect of genotype on this behavioral outcome. This provides preliminary information for further studies in our lab regarding mast cell activation and behavioral outcomes.

Homozygous males showed a significant increase in sucrose preference compared to the wildtype mice, while there was no effect between the females. Sucrose preference measures desire for reward, and it is reduced in male animals that are susceptible to social defeat stress (CSDS), indicating a depression-like anhedonia in these animals. Thus, the increased sucrose preference in males lacking *FosB* in their mast cells may indicate resilience to stress or may be a result of altered metabolism or taste sensitivity. Future experiments will be needed to resolve these possible interpretations.

Finally, we assessed social interaction and found no differences across genotypes or sexes. This may indicate that mast cells play little role in regulating social behavior. As we have not yet generated conclusive data on whether social interaction is altered in mice lacking mast cells completely, further experiments will be needed to resolve this issue.

Future Directions

Along with behavior analysis, we performed perfusions followed by brain, meninges, and mesentery tissue collection. To add to our current findings, I would like to analyze the tissue for mast cell activation systemically, to see if the floxing of FosB did in fact increase mast cell activation in the mesentery from the gut as well as meningeal tissue. We could thus identify how systemic the effects of mast cell activation are in attenuating anxiety and depression-like phenotypes. Additionally, blood cortisol levels could be telling about the animal's intermittent stress levels throughout a behavioral experiment.

I would like to use this same mouse line in a chronic social defeat stress paradigm as well to further identify the effects of mast cell activation on susceptibility and/or resilience to stress. Following the CSDS paradigm, I would subject each experimental mouse to assessments of behavior, such as social interaction, elevated plus maze, and sucrose preference which would be measured for the duration of the study. I would expect to see more resilient behaviors from the mice homozygous for the mutation, such as an increased social interaction score, open/closed arm ratio, and increased sucrose preference, respectively.

Optogenetics may be useful here as well to observe the direct impact of mast cell activation on behavior while the animal is behaving. I would like to inject a virus that would specifically target mast cells in the brain and make them express Channelrhodopsin-2. I could optogenetically excite mast cells in the hippocampus specifically, or perhaps activate mast cells throughout the meninges by shing light through the shaved skull. This would allow me to cause mast cell activation and observe the immediate effects on behavior. I would perform this procedure on the wildtype mice after collecting baseline

data from them and use the light activation during the behavioral assays mentioned above. I would compare these findings to their baseline behaviors and those of the homozygous mice as well. I would expect to see an increase in social interaction ratios, open/closed arm ratios, and sucrose preference in both the homozygous mice, and the optogenetically altered mice compared to the wildtype baseline.

My work has helped to lay the foundation for the study of mast cell effects on brain and behavior in both mouse and pig models. There are many potential future experiments, and I will be excited to see how mast cells contribute to baseline and stress induced behaviors in the group's future studies.

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LITERATURE CITED

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