THE ROLE OF MAST CELLS IN GUT PHYSIOLOGY, BRAIN CHEMISTRY, AND BEHAVIOUR

By

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A Thesis Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements for the Degree

Master of Science

McMaster University

DESCRIPTIVE NOTE

McMaster University MASTER OF SCIENCE (2017) Hamilton, Ontario

TITLE: The Role of Mast cells in Gut Physiology, Brain Chemistry, and Behaviour

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NUMBER OF PAGES: IX, 48

LAY ABSTRACT

Stress affects the immune system, which influences the body. Mast cells of the immune system are involved in several stress-induced changes in the body. They can also influence the brain and behaviour. We investigated how mast cells influence the changes that occur in the gut, brain, and behaviour during stress. Using mouse models, we prevented mast cells from activating during stress and looked at this effect on gut movement, changes in brain chemistry, and behaviour. We also compared the behaviour of mast cell deficient mice and deficient mice that gained mast cells. We found that by preventing mast cells from activating during stress, we can prevent several stress-associated changes in gut movement, brain chemistry, and anxiety behaviour. We also found that mast cells affect anxiety and social behaviour. These results suggest that mast cells impact the body, brain chemistry, and behaviour in stress and non-stress conditions.

ABSTRACT

Background and Research Aim: Stress affects the immune system, which influences host physiology. Mast cells have been associated with several stress-induced changes in gut physiology. Mast cells also have the potential to influence the brain and behaviour. We investigated how mast cells influenced the body, brain, and behaviour during stress.

Methodology: We investigated the behaviour of mast cell deficient animals and deficient animals that received whole bone marrow (WBM) transplants. We also studied the effects of mast cell stabilization during stress on changes in gut motility, via *ex vivo.* recordings of intestinal segments, and brain, via behavioural measurements and flow cytometry analysis of proinflammatory monocyte trafficking to the brain.

Results: Mast cell deficiency leads to several behavioural changes related to activity level, exploration, and sociability. Furthermore, deficient animals that received WBM transplants demonstrated social and anxiety-like behaviour that differed from their deficient counterparts. Mast cell stabilization during stress prevented many of the stress-induced changes in gut motility commonly observed in the intestine. Mast cell stabilization during stress also prevented proinflammatory monocyte trafficking to the brain and was associated with reduced anxiety-like behaviour.

Conclusion: Our findings support the role of mast cells in baseline behaviour, suggesting the presence of mast cells is needed for normal social and anxiety-related functioning. We also found that mast cell activation contributes to stress-induced intestinal dysmotility, suggesting that mast cells should be a target for interventions of stress-related gut motility disorders, such as irritable bowel syndrome. Lastly, our findings on the role of mast cells in monocyte trafficking and anxiety adds to our knowledge of neuroimmune interactions during stress and supports a potential role for mast cells in anxiety-related mood disorders, where stabilization of mast cells during stressful events may be of benefit.

ACKNOWLEDGEMENTS

I would like to thank everyone who helped me obtain my MSc degree.

First, thank you Dr. Paul Forsythe for your supervision and continuous support throughout my graduate studies. Your guidance was invaluable and I have truly learned a lot from you. You have not only taught me to be a better researcher, but have given me the opportunity to experience the scientific process as I have never been able to before.

I would like to thank Dr. John Bienenstock and Dr. Wolfgang Kunze for agreeing to be my committee members and providing helpful feedback whenever I needed it. Dr. Bienenstock, thank you for always making yourself available and offering support for not just my research, but all my educational endeavors. Thank you Dr. Kunze for helping me grasp many of the concepts in my field of study and motivating me throughout my experiments.

Thank you to everyone in the Brain-Body Institute and St. Joseph's Healthcare research department that helped me during my MSc. journey. Many of you directly helped with experiments, contributing to data collection and analysis; while others trained me on lab skills and offered research advice. I would not have been able to accomplish this much in such a short time without your help.

Finally, I would like to thank my family for their support over the last two years. You have been there to encourage me during difficult times and help me celebrate those happy moments. I would especially like to acknowledge my mother for always supporting me. I am thankful for all you have done.

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Term	Abbreviation
Aggressor interaction score	AI score
Autism associated disorders	ASDs
Blood brain barrier	BBB
Chronic social defeat	CSD
Claudin-5	Cldn5
Complementary DNA	cDNA
Corticosterone	CORT
Corticotropin releasing hormone	CRH
Diameter maps	Dmaps
Defeated mice that drank Ketotifen	KD
Defeated mice that drank water	WD
Effect size	ES
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
Gastrointestinal	GI
Interleukin 4	IL-4
Interleukin 6	IL-6
Interleukin 10	IL-10
Intraperitoneal	IP
Irritable bowel syndrome	IBS
Light/dark test	LDT
Mast cell deficient mice	MCD
Mast cell deficient mice that received whole bone marrow transplant	MCR
Mouse mast cell protease-1	mMCP-1
Mice with normal mast cell populations	MCC
Occludin	Ocldn
Open field test	OFT
Phosphate-Buffered Saline	PBS
Posttraumatic stress disorder	PTSD
Propagating contractile cluster	PCC
Real-time polymerase chain reaction	RT-qPCR
Sociability score	SS
Standard error of the mean	SEM
Tight junction	tj
Tumour necrosis factor α	TNFα
Non-defeated mice that drank Ketotifen	KC
Non-defeated mice that drank water	WC
Whole bone marrow	WBM

CHAPTER 1: GENERAL INTRODUCTION & LITERATURE REVIEW

1.1 Stress & The Immune System

Stress affects our immune system. It is not a coincidence that we are more likely to catch a cold when we are stressed (Cohen, Tyrrell, & Smith, 1991). Stressors have long been associated with altered immune function (Calabrese, Kling, & Gold, 1987; Khansari, Murgo, & Faith, 1999; Kiecolt-Glaser & Glaser, 1991). For example, psychological stress is associated with increased production of proinflammatory cytokines, including tumour necrosis factor α (TNFα) and interleukin 6 (IL-6), and suppression of immunoregulatory cytokines, such as IL-4 and IL-10 (Maes et al., 1998). Psychological stress has also been linked to increased expression and reduced function of T and B lymphocytes (Dorian et al., 1982). Changes in the immune system do not occur in isolation, with altered immune function affecting other aspects of host physiology. In the body, psychological stress-induced immune dysregulation is associated with increased susceptibility to viral infections, impaired wound healing, and increased risk of cancer (Godbout & Glaser, 2006). Altered immune function can also affect the brain and behaviour. Changes in the immune system has been linked to depression, a disorder where stress is not only a common risk factor, but where most initial episodes of major depression begin after encountering a stressor (Raison, Capuron, & Miller, 2006). With its clear connection to stress and its versatility in both peripheral and central physiology, cells of the immune system are prime targets for stress intervention.

1.2 Mast Cells: Overview

Of the cells composing the immune system, mast cells are strong candidates for stress intervention. Mast cells have many qualities that make them facilitators of both upstream and downstream signalling between the nervous system and host physiology. Mast cell precursors travel in the blood before entering tissue and taking on tissue-specific matured phenotypes (Chen, Grimbaldeston, Tsai, Weissman, & Galli, 2005). They are found throughout the body including the brain and peripheral nervous system, making their widespread location an asset when mediating communication between the nervous system and the rest of the body (Dropp, 1976; Olsson, 1971). Mast cells also release a wide array of mediators, including neurotransmitters, cytokines, and chemokines, which enables communication with a variety of cell types (Marshall, 2004).

There has been extensive research on mast cells in stress. Human mast cells have functional receptors for the stress hormone corticotropin releasing hormone (CRH), initiating mediator release upon CRH binding (Cao et al., 2005). Furthermore, mast cells are key mediators in many of the changes observed in the stressed gastrointestinal (GI) tract, including increases in esophagus permeability, gut mucin release, intestinal permeability, and development of gastric

ulcers (Castagliuolo et al., 1996; Ogle & Hui, 1995; Santos, Benjamin, Yang, Prior, & Perdue, 2000; Zhong et al., 2015). In addition to their involvement in stress-induced changes in peripheral physiology, they have also been linked to stress-induced changes in the blood brain barrier (BBB) (Esposito et al., 2001). When mast cells are inhibited or absent in stressed animals, many of the observed effects in these areas are either reduced or do not occur.

Mast cells are linked to changes in the brain and behaviour. Studies investigating the baseline effects of mast cell deficiency and stabilization in mice have shown an influence of mast cells on anxiety-like behaviour. The absence or inhibition of brain mast cells increases anxiety-like behaviour. This effect is observed over multiple behavioural tests (Nautiyal, Ribeiro, Pfaff, & Silver, 2008). Research with mastocytosis patients have also alluded to a role of mast cells in emotional regulation (Kushnir-Sukhov, Brittain, Scott, & Metcalfe, 2008). The population of patients with mastocytosis have an abnormal bimodal distribution of serotonin expression, with either high or low levels observed (Kushnir-Sukhov, Brown, Wu, Kirshenbaum, & Metcalfe, 2007). Serotonin levels significantly correlate with psychiatric symptoms, such as depression and anxiety, where lower serotonin levels are associated with higher rates of symptoms (Kushnir-Sukhov et al., 2008). These examples suggest that mast cells are not only involved in altering brain chemistry, but their influence may manifest in mood disorders.

1.3 Models to Investigate Mast Cells *in Vivo*

There are several methods to investigate mast cell function *in vivo*. Researchers commonly use pharmacological stabilizers to prevent activation of mast cells and observe the resulting effects in their experimental model. These stabilizers include Doxantrazole, Cromolyn Sodium Salt, and Ketotifen. Each stabilizer has unique attributes that aid in investigating mast cell function. For example, Doxantrazole is a non-discriminatory stabilizer that crosses the BBB, Cromolyn is more selective for mucosal tissue mast cells and does not cross the BBB, and Ketotifen is thought to cross the BBB and be more specific for connective tissue mast cells (Caldwell, Mahmood, & Weatherall, 1978; Kanwar & Kubes, 1994; Lisle, Meldi, Roach, Flynn, & Sewell, 2009; Norris, 1996; Oka, Kalesnikoff, Starkl, Tsai, & Galli, 2012; Pearce, Befus, Gauldie, & Bienenstock, 1982; Tamai & Tsuji, 2000; Yamazaki et al., 1994). In addition to stabilizers, mast cell deficient models are available to test the necessity of mast cells *in vivo.* One of these models is the $Kit^{W/}Kit^{W-v}$ model. These mice have been used for decades as a model of mast cell deficiency (B. Y. Kitamura, Go, & Hatanaka, 1978; Y. Kitamura, 1989). Their mast cell deficiency arises from mutation in the W allele at the c-kit locus. This mutation leads to decreased c-kit tyrosine-dependent signalling and mast cell deficiency. Researchers can test the physiological and behavioural difference of these mice compared to controls to investigate the necessity of mast cells in an experimental model. Mast cell deficiency can also be partially repaired in these mice through the transfer of wild type bone marrow or cultured bone marrow derived mast cells (Tanzola, Robbie-Ryan, Gutekunst, & Brown, 2003). By reconstituting the animal, researchers can test the effects of adding mast cells in their model. It is important to note that $Kit^{W/}Kit^{W-v}$ are not only mast cell deficient, but express other physiological changes, such as altered coat color,

anemia, and sterility (Besmer et al., 1993; Russell, 1979). Nevertheless, these mice are great models for investigating physiology and behaviour in the absence of mast cells and in cases of reconstitution.

THESIS GOAL

I will investigate the role of mast cells in:

1. Stress-induced changes in gut motility.
2. Stress-induced changes in brain chemistry and behavior resulting from psychosocial stress.
3. Baseline exploratory, anxiety, and social behaviour.

HYPOTHESIS

I hypothesize that mast cells influence baseline behavior and contribute to stress-induced changes in gut physiology, brain chemistry, and behavior.

CHAPTER 2: MAST CELLS IN STRESSED-INDUCED CHANGES OF GUT MOTILITY

2.1 Introduction

Environmental stress has pronounced effects on the brain-gut axis and autonomic nervous system. Stress exposure has been linked to several GI symptoms, such as dyspepsia, diarrhoea, and abdominal pain. The connection between stress and the gut goes beyond symptomology, with experimental stressors evoking both functional and structural changes in the gut (Konturek PC, Brzozowski T, 2011). Amongst these changes, intestinal motility has been repeatedly shown to be altered in organisms exposed to stress (Thomas F Burks, Peterson, Williams, & Kramer, 1989; Konturek PC, Brzozowski T, 2011; C. West et al., 2016; Christine West, Stanisz, Wong, & Kunze, 2016). Intestinal dysmotility resulting from stress may not arise from direct communication between the brain-gut axis and gut physiology, but be mediated by other biological pathways. The immune system has the potential to be one of these pathways because it is not only influenced by stress, but also interacts with the gut (Dhabhar, 2014; Khan & Collins, 2004; Mayer, 2011; Padgett & Glaser, 2003).

Mast cells are candidates for mediating brain-gut communication leading to stress-induced dysmotility. They are found adjacent to neurons in the gut and contain a range of mediators capable of facilitating a wide array of physiological responses, including granule-associated factors like histamine and serotonin, lipid-derived factors, and cytokines (Marshall, 2004; Stead et al., 1987). Mast cells also have surface receptors for CRH and are linked to changes in the stressed gut (Cao et al., 2005). The alterations in gut mucin release, permeability, and development of gastric ulcers that occur with stress are inhibited or absent in mast cell-free models or organisms that have had their mast cells stabilized prior to stress exposure (Castagliuolo et al., 1998; Ogle & Hui, 1995; Santos et al., 2000; Wallon et al., 2008). In addition to their involvement in stress-induced gut physiology, mast cells have also been linked to other cases of gut dysmotility, such as motility changes due to parasite infections and postoperative ileus after abdominal surgery (De Jonge et al., 2004; Serna, Porras, & Vergara, 2006a). However, to date the contribution of mast cells to motility changes following stress exposure has not been investigated.

The purpose of this study was to employ restraint stress, a model that has reliably produced symptoms of dysmotility in past studies, to examine the effects of mast cell stabilization on stress induced dysmotility (Thomas F Burks et al., 1989; C. West et al., 2016; Christine West et al., 2016; Zhang et al., 2014). Restraint stress induces different changes in the small intestine and colon. We can quantify these changes by measuring changes in the propagating contractile clusters (PCCs) of the intestines. PCCs are composed of pressure waves

that migrate aborally along the intestines (Husebye, 1999). In the small intestine, restraint stress increases the amplitude of PCCs, but decreases their frequency and velocity. While, restraint stress increases all PCC characteristics in the colon (Thomas F Burks et al., 1989; C. West et al., 2016). We will investigate if mast cells are involved in this process.

2.2 Research Aim

To investigate the effect of mast cell stabilization using Doxantrazole and Cromolyn Sodium Salt on restraint stress-induced changes in gut motility.

2.3 Materials and Methods

Animals

Adult male Swiss Webster mice (6-8 weeks old, 20-30 g Charles River Laboratories, Wilmington, MA, USA) were housed 3-5/cage with food and water available *ad libitum* and a 12 hour light/dark cycle. Mice were given 1 week to acclimatize prior to the beginning of any experiments. All procedures were approved by the Animal Research Ethics Board of McMaster University (permit 08-08-35).

Drugs and Substances

Mast cell stabilizers Doxantrazole (1mg/ml) (Sigma-Aldrich, Oakville, ON) and Cromolyn Sodium Salt (100mg/kg) (Sigma-Aldrich) were dissolved in Phosphate-Buffered Saline (PBS) and delivered as an intraperitoneal (IP) injection prior to stress exposure.

Experimental Design

Mice undergoing stress exposure were given a 0.5 ml IP injection of either Doxantrazole, Cromolyn Sodium Salt, or PBS, housed in their cage for 30 minutes, and then placed in a wire mesh restraint device for 1 hour. Control groups received the same IP injections, but were housed in their cage for 1 hour after the injection. After the hour of restraint stress or housing, mice were euthanized and a 4 cm long segment of jejunum or colon was removed and placed into the well of an organ bath perfusion system and the segment's PCC frequency, velocity, and amplitude were assessed.

Gut Motility Recordings

The oral and anal ends of the 4 cm long gut segments were cannulated and attached to a manifold using silicone tubing. Oxygenated Krebs buffer, composed of (mmol/L): 118 NaCl, 4.8 KCl, 25 $NaHCO_3$, 1.0 NaH_2PO_4, 1.2 $MgSO_4$, 11.1 glucose, and 2.5 $CaCL_2$ bubbled with carbogen gas (95% O_2 and 5% CO2), flowed through the tubing, intestinal segment, and well of the perfusion system. Enteric nervous system-dependent PCCs were evoked by creating a pressure differential

between the inflow and outflow sections of the segment. The jejunal segment's pressure differential was 2 hPa (cm H_2O) and 2.2 hPa at the inflow and outflow, respectively. The colon's pressure differential was 2-3 hPa at the inflow and the outflow was raised 0.2 cm above the inflow.

The segment's motility was digitally recorded on a camcorder (JVC) located 10 cm above the segment. The video recordings were used to create spatiotemporal diameter maps (Dmaps) that were then analyzed for PCC frequency, velocity, and amplitude as previously described in (C. West et al., 2016; Wu et al., 2013). Briefly, PCCs on Dmaps were identified as broad bands that indicated coordinated contractile clusters moving from the oral to anal end of the segment (Wu et al., 2013). PCC frequencies were found by plotting an envelope around individual contractions, finding the peak of each contraction as the point where its derivative is zero, and using the intervals between peaks of the envelope to calculate the frequency. PCC velocity was found by measuring the slope of the contractions (distance/time) and PCC amplitude was found by measuring the difference between gut diameter before and after contractions.

Statistics

To avoid the limitations of null hypothesis testing and discern the biologically relevant effects of the treatments, a measure of effect size (ES) was used to compare the groups (Cumming, 2014). Although Cohen's d is a widely-used representation of ES, the calculation for d may produce a biased effect size with sample sizes less than 20. With some control groups having sample sizes of less than 20, Hedges's g was chosen to calculate ES instead of Cohen's d. Hedges's g provides a more unbiased measure when considering small sample sizes and was deemed more suitable in this context to measure ES (Cumming, 2013; Hedges, 1982)**.** Differences of $g < 0.2$ were deemed as trivial effect sizes, g of 0.2 – 0.5 was a small effect size, g of 0.5 – 0.8 was a moderate effect size, and $g > 0.8$ was a large effect. All data is presented as mean +/- Standard Error (SEM).

2.4 Results

Stress Modulates Intestinal Motility

We first compared control (PBS IP injection alone) (Control) with restraint stress after PBS IP injection (Stressed). PCCs were present in all segments in both stressed and control treatments. As described in West et al., 2016, jejunum Dmaps in the stressed condition appeared highly disorganized and irregular when compared to Control Dmaps. While, Stressed colon Dmaps generally consisted of more frequent PCCs and active motility changes than Control colon Dmaps.

Stress induced a decrease in PCC velocity for the jejunum (ES small, $g = 0.30$) and colon (ES trivial, $g = 0.17$, Figure 1). PCC frequency decreased in the jejunum (ES moderate, $g = 0.55$) and increased in the colon (ES moderate, $g = 0.69$) following restraint stress. PCC amplitude

showed the same stress-induced changes in both types of segments, increasing in the jejunum (ES large, g = 1.40) and colon (ES moderate, g = 0.69).

Mast Cell Stabilizers have minimal effects on normal Intestinal Motility

We found trivial or small differences when comparing the Control group with the non-stressed group that received Doxantrazole (Dox Control) (Figure 1), Mice in the Dox Control group had increased PCC velocity for jejunum (ES trivial, g = 0.01) and colon (ES small, g = 0.48). Their PCC frequency was also comparably decreased (ES trivial, g = 0.03) in the jejunum and increased (ES trivial, g = 0.18) in the colon. While, their amplitude was slightly decreased in the jejunum (ES trivial, g = 0.089) and colon (ES trivial, g = 0.16).

Like Doxantrazole, Cromolyn Sodium Salt injections produced small or trivial effects on intestinal motility (figure 2). When comparing the Control group with the non-stressed group that received Cromolyn Sodium Salt (Crom Control), PCC velocity was decreased in the jejunum (ES small, g = 0.25) and colon (ES trivial, g = 0.16) of Crom Control mice. PCC frequency was also decreased in the jejunum (ES small, g =0.32) and colon (ES small, g = 0.37). This trend continued in measurements of PCC amplitude, with Crom Control mice having lower values than Control mice for jejunum (ES small, g =0.35) and colon (ES trivial, g = 0.13).

Mast Cell Stabilizers Attenuate Stress-Induced Dysmotility

Doxantrazole altered the effects of restraint stress (Dox Stressed) on motility (Figure 1). When compared to the Stressed group, the Dox Stressed group had greater PCC velocity for jejunum (ES moderate, g = 0.72) and a decreased PCC velocity for colon (ES small, g = 0.32). The Dox Stressed group also had PCC frequencies that were greater for jejunum (ES large, g = 0.83) and less frequent for colon (ES large, g = 1.01). The PCC amplitude was also comparably decreased in the Dox Stressed group for both jejunum (ES large, g = 1.67) and colon (ES large, g = 1.00).

Motility differences between mice that received Cromolyn Sodium Salt (Crom Stressed) and the Stressed group were not as prominent as those with the Dox Stressed group, however, differences were still present (Figure 2). The PCC velocity for the Crom Stressed group was greater than the Stressed group in the jejunum (ES small, g = 0.30) and colon (ES small, g = 0.34). The Crom Stressed group also had an increased PCC frequency in the jejunum (ES trivial, g = 0.19) and decreased frequency in the colon (ES moderate, g = 0.62). The most pronounced differences were seen in PCC amplitude, where the Crom Stressed mice had a much smaller PCC amplitude in the jejunum (ES large, g = 1.44) and colon (ES large, g = 1.55) when compared to Stressed mice.

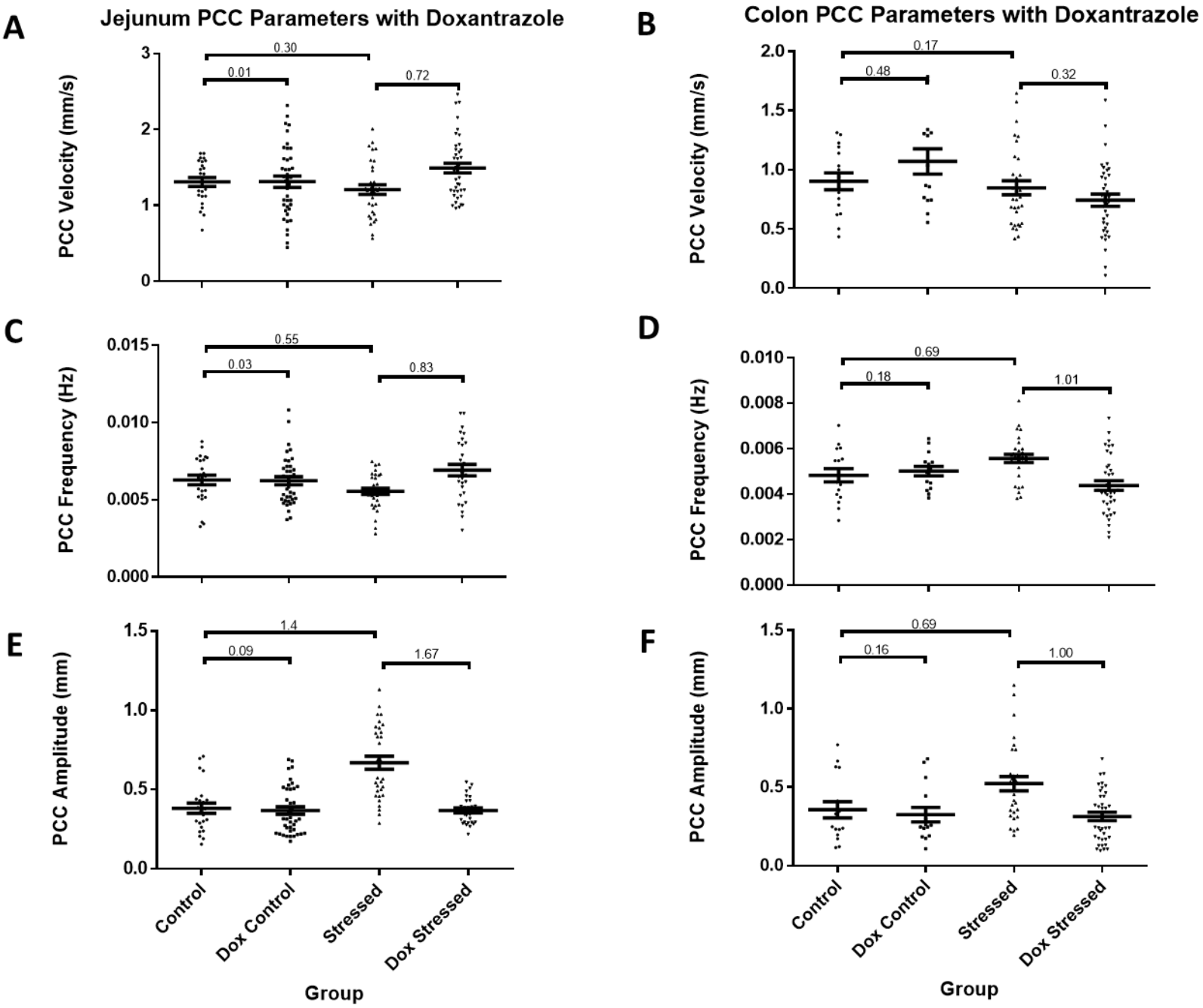

Fig. 1. Mean (± SEM) jejunum **A)** PCC velocity **B)** PCC frequency or **C)** PCC amplitude and colon **D)** PCC velocity **E)** PCC frequency or **F)** PCC amplitude of mice that were given Doxantrazole (Dox Control, n = 38-40 for jejunum and n = 14-15 for colon, Dox Stressed, n =29-40 for jejunum and n = 36-37 for colon) or PBS (Control, n = 24 for jejunum and n = 16 for colon, Stressed, n = 32 for jejunum and n = 30-32 for colon) prior to undergoing restraint stress.

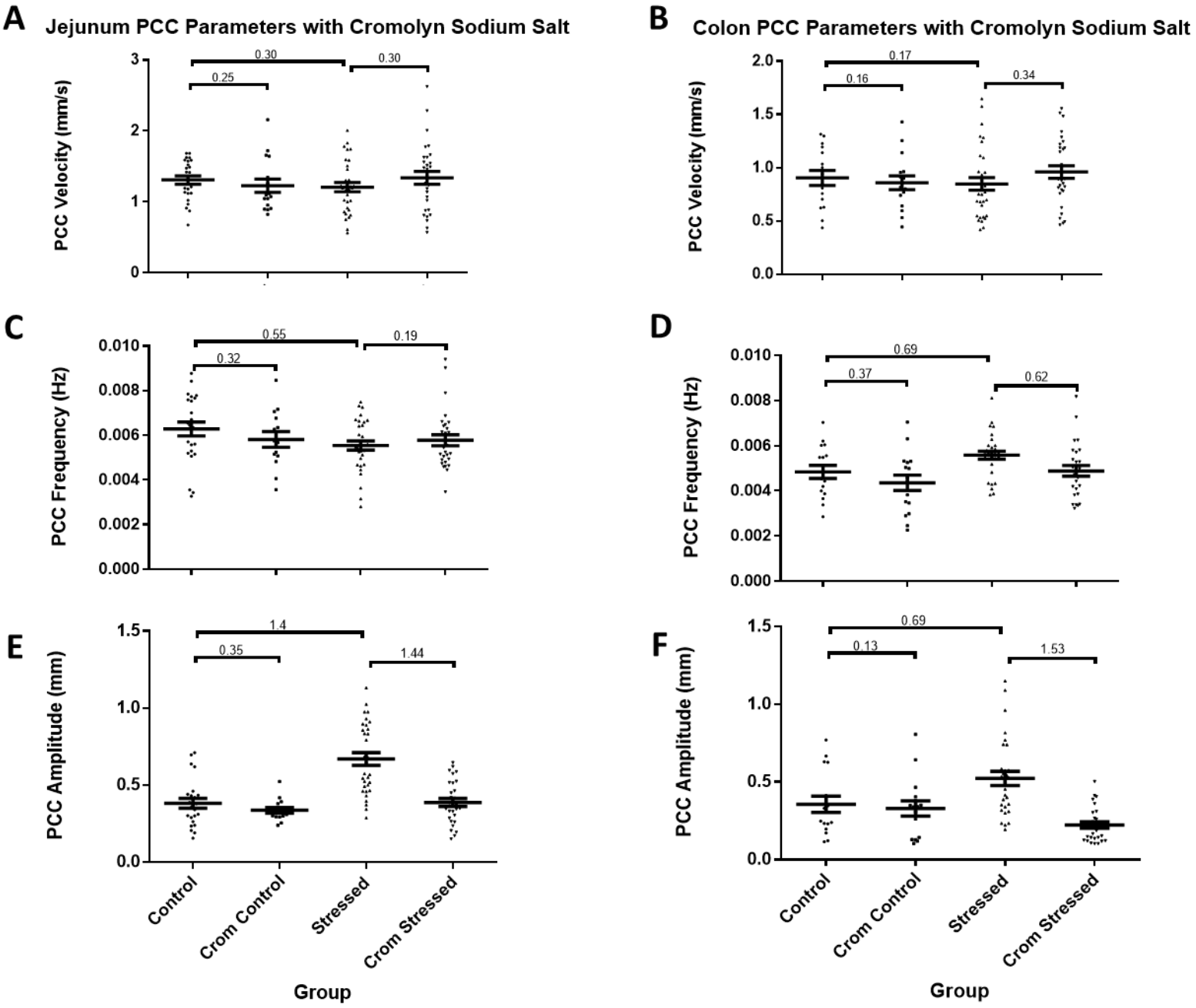

Fig. 2. Mean (± SEM) jejunum **A)** PCC velocity **B)** PCC frequency or **C)** PCC amplitude and colon **D)** PCC velocity **E)** PCC frequency or **F)** PCC amplitude of mice that were given Cromolyn Sodium Salt (Crom Control, n = 14-16 for jejunum and n = 16 for colon, Crom Stressed, n = 32 for jejunum and n = 30-32 for colon) or PBS (Control, n = 24 for jejunum and n = 16 for colon, Stressed, n = 32 for jejunum and n = 30-32 for colon) prior to undergoing restraint stress.

2.5 Discussion

We have shown that pre-treatment with mast cell stabilizing drugs prevents much of the restraint stress-induced PCC motility changes in the jejunum and colon. Given several stress-induced physiological changes in the gut involve mast cell activation (Castagliuolo et al., 1998; Ogle & Hui, 1995; Santos et al., 2000; Wallon et al., 2008), it is not surprising that motility would follow a similar trend. In addition to the general involvement of mast cells, the presence of preventative effects with Cromolyn Sodium Salt, a drug that does not cross the BBB, suggests that at least some of the dysmotility is mediated locally by the activation of peripheral, mucosal mast cells (Norris, 1996; Pearce et al., 1982).

Restraint stress induced dysmotility in the jejunum and colon, replicating previous findings and supporting the reliability of the restraint stress procedure (T. F. Burks, Peterson, Williams, & Kramer, 1989; C. West et al., 2016). The only discrepancy occurred with PCC velocity, where restraint stress produced a small comparable decrease in velocity between the stressed and control groups in the jejunum and trivial differences in the colon. These findings contrast other reported findings that show large decreases in PCC velocity in the jejunum and increases in the colon. The inability to replicate these effects may be attributed to differences in methodology, for we used restraint stress following IP injection, which is itself a stressor.

The stabilizers had minimal effects on baseline motility. When comparing control mice that received either stabilizer or PBS, there were minor differences observed between groups. These results suggest that the stabilizers do not have a large effect on motility on their own and mast cell activation may not be required for baseline motility in the jejunum and colon.

Although the stabilizers did not exert effects on non-stressed animals, they greatly altered motility in stressed mice. Mast cell stabilization prior to restraint stressed prevented many of the characteristic changes in PCC parameters observed after stress. Doxantrazole pre-treatment prevented most of the stress-induced changes in the jejunum and colon. When compared to the Stressed group, the Dox Stress group showed moderate to large differences in the jejunum and colon's PCC characteristics that were closer to control levels. Furthermore, pre-treatment with Cromolyn Sodium Salt reproduced many of the trends observed with Doxantrazole. These effects support the role of mast cell degranulation contributing to the stress-induced motility changes in the gut. The common trends seen amongst stabilizers were especially evident in the PCC amplitude of both gut segments and PCC frequency in the colon. However, the effects of Cromolyn Sodium Salt on the other parameters were not as pronounced as Doxantrazole. The differences between stabilizers should not be attributed to the dosage selected for Cromolyn Sodium salt because 100 mg/kg is equal or greater than previously reported doses of that drug in mouse IP injections (Costa et al., 2008; Forbes et al., 2008; Huang, Pang, Karalis, & Theoharides, 2003). Differences between stabilizers may be attributed to their mechanisms of action. Cromolyn Sodium Salt is a discriminative mast cell stabilizer that mainly targets mucosal mast cells, while Doxantrazole is more non-discriminatory, targeting both mucosal and connective tissue mast cells (Lisle et al., 2009; Oka et al., 2012). Doxantrazole affecting a larger range of PCC

parameters in both segments may imply that both types of mast cells have a role in mediating the effects of stress on motility.

This study's findings conflict with previous work in mast cell deficient mice that showed no involvement of mast cells on colon transit following restraint stress (Castagliuolo et al., 1998), however, the methodology is very different between studies (*ex vivo.* motility study versus *in vivo*. transit study). By analyzing PCC characteristics in an *ex vivo.* model, we directly monitored the effects of our manipulation on the gut in an isolated system, minimizing the potential for interference from other stress-induced mechanisms that would occur in an *in vivo.* model. Furthermore, they used a mast cell deficient model (Kit^{W}/Kit^{W-v}), while we used normal mice that had their mast cell pharmacologically stabilized. When compared to normal mice, mast cell deficient mice have both structural and functional differences in their intestine, such as deficits in the development of interstitial cells of Cajal and irregular peristaltic wave patterns (Der-Silaphet, Malysz, Hagel, Arsenault, & Huizinga, 1998; Ward, Burns, Torihashi, & Sanders, 1994). These differences make comparing the two mouse models much more difficult. Therefore, the differences in methodologies may account for the diverging results.

Our findings support the need to further investigate the role of mast cells in GI motility associated with stress, such as irritable bowel syndrome (IBS) (Konturek PC, Brzozowski T, 2011). There are several established links between mast cells and IBS. Patients with IBS have increased mast cell numbers in their small and large intestines (Giovanni Barbara et al., 2004; Guilarte et al., 2007) and IBS is associated with differential mast cell activation and mediator release (G. Barbara et al., 2004). Furthermore, the use of the mast cell stabilizer Ketotifen significantly increases the threshold for discomfort in hypersensitive IBS patients, while also reducing complaints of abdominal pain and diarrhoea (Klooker et al., 2010). Our findings that mast cell stabilization prevents changes in stress-induced gut motility, a prominent feature of IBS, is congruent with the other cases of mast cell involvement in the illness and supports the notion for mast cells to be a target for IBS therapy (Konturek PC, Brzozowski T, 2011). In addition to general motility, having both stabilizers prevent stress-induced increases in PCC amplitude, a characteristic associated with abdominal pain, supports mast cell involvement in motility-induced pain for IBS patients (Sarna, 2010). Although rodent studies are not directly transferable to humans, we have shown that Cromolyn Sodium Salt has potential preventative effects on stress-induced dysmotility. Cromolyn Sodium Salt has been used clinically for more than 40 years, making the transferability of this study to a clinical setting more plausible (Bernstein et al., 1972). Future studies should investigate mast cell-centered interventions to relieve symptoms of IBS and other stress-related motility disorders.

CHAPTER 3: THE EFFECTS OF MAST CELL STABILIZATION DURING PSYCHOSOCIAL STRESS

3.1 Introduction

Stress is linked to a variety of human psychopathologies, including depression, anxiety disorders, and posttraumatic stress disorder (PTSD) (McLaughlin, Conron, Konenen, & Gilman, 2010; Thornton & Prescott, 1999). For example, bullying is a prominent psychosocial stressor that contributes to mood disorders. Bullying is heavily psychological in nature, many times occurring through indirect aggression and social manipulation (Björkqvist, 2001; Lagerspetz, Björkqvist, & Peltonen, 1988; Whitney & Smith, 1993). Bullying in youth leaves the victims feeling submissive and more likely to express signs of depression, low self-esteem, social withdrawal, and increased anxiety (Björkqvist, Ekman, & Lagerspetz, 1982; Hawker & Boulton, 2000; Kaltiala-Heino, Fröjd, & Marttunen, 2010; Kowalski & Limber, 2013). Bullying is not only restricted to youth. Adults go through workplace bullying and experience similar outcomes. Victims of workplace bullying show signs of PTSD, depression, sociophobia, and are more prone to commit suicide (Björkqvist, 2001; Hauge, Skogstad, & Einarsen, 2010).

Researchers use animal models to investigate the underlying physiology of psychosocial stress. A prominent animal model of psychosocial stress is the chronic social defeat (CSD), a rodent resident-intruder paradigm that involves daily aggressive interactions and housing with an aggressive conspecific (Golden, Covington, Berton, & Russo, 2011). CSD results in the subject becoming submissive and developing altered behaviour, including depressed behaviour, social impairment, and increased anxiety-like behaviour (Bharwani et al., 2016; Golden et al., 2011; A. J. Keeney & Hogg, 1999; Rygula et al., 2005; Yang et al., 2017). Recent research with CSD has implicated a major role of the immune system in the development of anxiety-like behaviour. More specifically, recent findings on proinflammatory, glucocorticoid-insensitive, bone marrow-derived monocytes show that these cells are released during stress, travelling to various parts of the body, including the heart, lungs, and brain. (Bailey, Kinsey, Padgett, Sheridan, & Leblebicioglu, 2009; Stark et al., 2001; Wohleb, Mckim, Sheridan, & Godbout, 2014). Peripheral Ly6C^{hi} CD11b^{+} cells contribute to anxiogenic behaviour when they traffic to the brain. When proinflammatory monocyte trafficking to the brain is blocked in defeated mice, they exhibit decreased anxiety-like behaviour compared to defeated mice with regular trafficking (Wohleb et al., 2011; Wohleb, Powell, Godbout, & Sheridan, 2013). The pathway of this process has been eluded to in previous works (Wohleb et al., 2014). It is suggested to involve activating the stress response of the brain, followed by initiating the hypothalamic-pituitary-adrenal axis and sympathetic nervous system, then releasing proinflammatory monocytes, which migrate to the brain and further augment mood disorders.

Although a monocyte trafficking pathway to the brain has been identified, it is far from fully defined and additional immune cells may take part in this process. This study is designed to investigate if mast cells are part of peripheral Ly6C^{hi} CD11b^{+} cell trafficking to the brain during

stress. Mast cells have several characteristics that make them likely to be involved in this pathway. They play a role in downstream stress signaling that facilitates several changes in the gut of stressed animals (Castagliuolo et al., 1998; Ogle & Hui, 1995; Santos et al., 2000; Wallon et al., 2008). They are also associated with anxiety-like behaviour. Mast cell deficient mice or mice that have had their mast cells pharmacologically stabilized display significantly altered baseline anxiety-like behaviour when compared to controls (Nautiyal et al., 2008). Mast cells have also been linked to increased BBB permeability following stress, where inhibition of mast cell activation during stress attenuates permeability changes in stressed subjects (Esposito et al., 2001). By regulating the BBB during stress, mast cells may influence trafficking of peripheral cells to the brain, such as monocytes. This study will employ a mast cell stabilizer to test how stabilization of mast cells during psychosocial stress alters resulting physiology and behaviour.

3.2 Research Aim

To investigate the effect of administering a mast cell stabilizer, during CSD on:

1. $Ly6C^{hi}$ $CD11b^{+}$ cell trafficking to the brain.
2. Exploratory, anxiety, and social behaviour between treatment groups.
3. Baseline Corticosterone levels between treatment groups.
4. Genetic markers of BBB permeability between groups.

3.3 Materials and Methods

Animals

Male C57BL/6 (6-8 weeks old) and CD-1 mice (retired breeders) were acquired from Charles River Laboratories. All animals were housed in standard conditions following a 12 hour light-dark cycle with *ad libitum* access to food and water. Mice acclimated to the housing facility for at least seven days prior to beginning any experiments. All experiments followed the Canadian Council on Animal Care's guidelines and were approved by McMaster Animal Research Ethics Board.

Experiment Outline

C57BL/6 mice received either water or the mast cell stabilizer, Ketotifen, dissolved in water (Sigma-Aldrich) (20 mg/kg/day), while undergoing CSD according to standard defeat procedure (Figure 3) (Golden et al., 2011; Pothoulakis et al., 1993; Serna, Porras, & Vergara, 2006b). Ketotifen was added to the drinking water two days prior to starting defeat and continued until endpoint. Ketotifen dosing was regularly monitored and adjusted until the end of the experiment. Once the social defeat procedure was over, mice were singly housed and underwent a battery of daily behavioural testing, including the open field test (OFT), light/dark test (LDT), sociability, and aggressor interaction test. Following behavioural testing, blood and

brain samples were collected for analysis of serum corticosterone (CORT) levels, brain Ly6C^{hi} CD11b^{+} monocyte levels, and brain tight junction (tj) gene expression using real-time polymerase chain reaction (RT-qPCR).

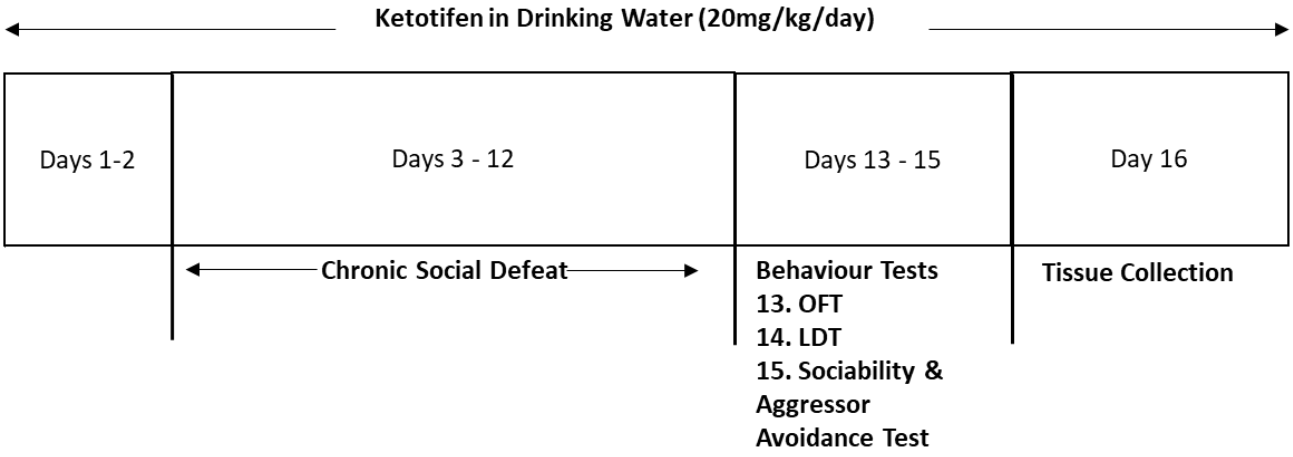

Fig. 3. Outline for "The Effects of Mast Cell Stabilization During Psychosocial Stress" study.

Chronic Social Defeat

CSD is a model of chronic psychosocial stress that induces anxiety-like behavior, social avoidance, and submissive behavior (Golden et al., 2011; Kudryavtseva, Bakshtanovskaya, & Koryakina, 1991). Social defeat occurred over 10 days. Each day, the C57BL/6 mouse was placed in the home cage of a different CD1 mouse (aggressor) that has been previously screened to be aggressive against male C57BL/6 mice. The animals interacted for 2-3 minutes, with multiple aggressive bouts occurring during this time. After 2-3 minutes, the C57BL/6 was housed with the aggressor for 24 hours, separated by a Plexiglas barrier to allow sensory stimuli between animals, but prevent physical contact. After 24 hours, the process was repeated with a different aggressor. Control mice were housed in pairs, separated by a Plexiglas barrier, and underwent daily cage rotations to mimic the handling and changing of housing conditions that occurred with defeated mice.

Behavioural Tests

Open Field Test

The OFT is used to determine general activity level, exploratory behavior, and anxiety-like behavior between groups (Hall, 1934). Testing occurred during the dark cycle. After one hour of habituation in the testing room, mice were singly housed inside an empty Plexiglas chamber (18 x 38 cm) and allowed to explore the chamber for 30 minutes. Using photo beam sensors around the chamber (Motor Monitor; Kinderscientific), basic movement/activity (measured by the number of times a mouse crossed a beam sensor), total distance travelled in the center of the

chamber (in), time spent in the center (sec), number of entries into the center, and rearing counts were collected. Chambers were cleaned between animals.

Light/dark Test

The LDT is used to determine differences in rearing and anxiety-like behavior between groups (Crawley & Goodwin, 1980). Testing occurred in the dark cycle. After one hour of habituation in the testing room, mice were singly housed in a clear Plexiglas chamber (18 x 35 cm) with two compartments: an illuminated compartment (light zone) and a dark compartment (dark zone). Each testing session lasted 10 minutes. Using Kinderscientific motor monitor software, entries into the light zone, time spent in the light zone, distance travelled in the light zone, and rearing counts were collected. Chambers and compartments were cleaned between animals.

Sociability Test

The sociability test is used to determine the presence of social impairment between treatment groups (Moy et al., 2004). All tests took place during the light cycle and begun after a minimum of 30 minutes habituation in the testing room. The test occurred in a three-chamber Plexiglas apparatus (each chamber being 24.5 cm L x 44 cm W x 30 cm H) with dividing walls that included small openings for the mouse to access each chamber. The chambers were monitored by a video camera placed above the apparatus and each chamber was virtually divided into two halves: an interaction zone and a non-interaction zone (EthnoVision XT; Noldus). The test involved two trials: a habituation trial and a sociability trial. Once a trial was over, the subject was returned to its home cage and the next trial was prepared. In the habituation trial, the mouse was placed in the center chamber with the openings to the other chambers blocked. The center chamber was empty, except for a wire cage located in the interaction zone. The mouse explored the chamber for 2.5 minutes. In the sociability trial, a stranger mouse of the same age, gender, and strain of the subject was placed in a wire cage and added to the interaction zone of one of the outside chambers. An identical wire cage with a novel object was placed in the interaction zone of the other outside chamber. The barriers that separated the center chamber from the outside chambers were removed and the subject was placed in the center chamber. The mouse explored the chambers for 10 minutes, while video tracking software recorded the time spent in each chamber and in within-chamber zones. Sociability scores (SS) were calculated by dividing the time spent in the mouse-chamber's interaction zone by the time spent in the object-chamber's interaction zone. All equipment was cleaned between subjects.

Aggressor Interaction Test

The aggressor interaction test is used to assess resiliency to the social defeat (Golden et al., 2011). Following the sociability trial, all mice and cages were removed and a novel aggressor in a wire cage was placed in the center chamber's interaction zone. The openings to the outside chambers were blocked and the subject explored the center chamber for 2.5 minutes. Tracking software (EthnoVision XT; Noldus) measured the amount of time the subject spent in the interaction zone during the trial. An aggressor interaction (AI) score was calculated by dividing the time spent in the interaction zone when the aggressor was present by the time spent in the

zone during the sociability test's habituation trial. AI scores that were significantly lower than those of control animals indicated mice that were susceptible to the CSD. All equipment was cleaned between subjects.

Serum Extraction and Corticosterone Analysis

All mice were euthanized before noon. Following decapitation, trunk blood was taken and kept on ice. Blood samples were centrifuged (3000 rpm for 10 minutes at 4 °C) and serum was removed to measure CORT levels using immunoassay (Enzo Life Sciences, NY, USA). All samples were measured in duplicates and the average value was taken to represent the CORT levels for each sample.

RNA Extraction and RT-qPCR Analysis

After decapitation, the brain was extracted from the skull and the left half of the brain was stored in RNAlater® solution for RT-qPCR (Ambion, Life Technologies,CA, USA). The tissue incubated overnight at 4 °C and was then transferred to –20 °C storage to await additional processing. The tissue was thawed and macrodissected to remove the hippocampus and rostral cortex (this included the left frontal and prefrontal cortex). RNA was extracted using the a mirVana miRNA Isolation kit (Invitrogen, ThermoFisher, ON, CA) and assessed with a NanoDrop® Spectrophotometer ND-1000. Using SuperscriptIII™ First-Strand Synthesis Supermix (Invitrogen, CA, USA), one µg of RNA was converted into complementary DNA (cDNA) and diluted by a factor of five to be used in RT-qPCR. The RT-qPCR reaction used the PowerUp™ SYBR®Green Master Mix (Applied Biosystems, Life Technologies, Austin, TX, USA) and ROX™ Passive Reference Dye. All reactions were performed in the fast mode (uracil-DNA glycosylase [UDG] activation 50 °C, 2 min; Dual-Lock™ DNA polymerase 95 °C, 2 min; denaturation 95 °C, 1 s; annealing/ extension 60 °C, 30 s; number of cycles: 40) using QuanStudio3™ (Applied Biosystems). All primers were taken from previous work on brain tj gene expression (Leclercq et al., 2017). Primer sequences in the 5' to 3' orientation are as follows: Claudin-5 (Cldn5) forward, TCAGCTTCCCGGTCAAGTACTC, reverse, CCGCCCTTAGACATAGTTCTTCTT, Occludin (Ocldn) forward, TGAACAGCCCCCCAATGT, and reverse, TCAACTCTTTCCGCATAGTCAGAT. Primers were used at a final concentration of 300 nM. Transcripts were normalized to endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and analyzed using the ΔΔCt method.

Flow Cytometry

Flow cytometry was conducted as described by (Wohleb et al., 2013). Briefly, $Ly6C^{hi}$ $CD11b^{+}$ cell populations were analyzed using flow cytometry. Cells were isolated from whole-brain homogenates passed through a 70 µm cell strainer and monocytes were separated using standard protocol of particle gradient centrifugation (2000 RPM, 22°C, 20 mins). Cells were collected from the interphase between 70% and 30% and washed two times with cold PBS. The cells were plated 1 million cells/well. FC receptors were blocked with anti-CD16/CD32 (eBioscience, ThermoFisher, ON, CA). Cells were then washed and incubated for 30 mins with the CD11b, Ly6C, and CD45 antibodies (PerCP-Cy5.5, PE-Cy7, and APC-Cy7, respectively) (eBioscience, Thermofisher, ON, CA). After incubation, cells were washed and resuspended in

FACS buffer. Analysis was performed with FACSCanto (Becton Dickinson, Oakville, ON, Canada) and FlowJo (TreeStar, Ash-land, OR, USA).

Statistics

All experiments were analyzed using two-way factorial ANOVA followed by Bonferroni corrected post-hoc tests. Measures in figures are reported as mean +/- SEM and statistical significance is denoted as # ($P < 0.10$), * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$). In addition to null hypothesis testing, ES was calculated using Hedges's g for all corrected comparisons where $P < 0.1$ (Hedges, 1982). Differences of $g < 0.2$ were deemed as trivial effect sizes, g of 0.2 – 0.5 was a small effect size, g of 0.5 – 0.8 was a moderate effect size, and $g > 0.8$ was a large effect.

3.4 Results

Ketotifen Prevented Ly6C^{hi} CD11b^{+} Monocyte Trafficking to the Brain Following CSD

Whole brains were taken at the end of the experiment and used to measure Ly6C^{hi} CD11b^{+} monocyte levels in the brain. There was a significant increase in monocyte levels of control defeated mice (WD) when compared to control non-defeated mice ($F(1,28) = 8.885$, $p = 0.0059$, Bonferroni-corrected t-test, WD vs. WC, $p = 0.0276$, ES large, $g = 1.45$) (figure 4). This increase in monocyte levels was not seen in defeated mice treated with Ketotifen (KD), indicating that stress-induced trafficking was prevented by drug administration (Bonferroni-corrected t-test, WD vs. KD, $p = 0.0177$, ES large, $g = 1.54$). There were no significant differences between non-defeated mice and non-defeated mice treated with Ketotifen (KC).

CSD Induced Deficits in Exploratory and Social Behaviour

Following CSD, mice were subjected to a battery of behavioural tests. The first differences between groups were observed in the OFT, where defeated mice showed less movement than control mice (main effect of defeat, $F(1,53) = 9.579$, $p = 0.0031$, Bonferroni-corrected t-test, WD vs. WC, $p = 0.0528$, ES large, $g = 0.95$) (Figure 5A). In addition to reduced activity, the rearing behaviour of defeated mice was significantly reduced compared to controls (main effect of defeat, $F(1,53) = 24.79$, $p < 0.0001$, Bonferroni-corrected t-test, WD vs. WC, $p < 0.0001$, ES large, $g = 1.75$) (Figure 5B). This difference was also seen in the LDT (main effect of defeat, $F(1,52) = 8.980$, $p < 0.0042$, Bonferroni-corrected t-test, WD vs. WC, $p = 0.06$, ES large, $g = 1.04$) (Figure 5C). These results suggest an overall reduction in exploratory behaviour in defeated animals.

In addition to exploratory behaviour, defeated mice also showed social deficits. In the sociability test, defeated mice received a much lower SS than controls (main effect of defeat, $F(1,49) = 6.555$, $P = 0.0136$, Bonferroni-corrected t-test, WD vs. WC, $p = 0.0243$, ES large, $g = 1.15$) (Figure 6A). A lower SS suggests social impairment. Unlike the control mice, defeated mice did not have a preference for interacting with a conspecific over an object (Moy et al., 2004). Defeated mice also had an overall reduced AI score when compared to controls (main effect of defeat, $F(1,51) = 34.46$, $p < 0.0001$, Bonferroni-corrected t-test, WD vs. WC, $p < 0.0001$, ES large,

g = 1.81) (Figure 6B). A lower AI score especially when it is below 1, indicates that most of the defeated mice were susceptible to CSD (Golden et al., 2011).

Although exploratory and social deficits were observed in defeated mice, there were no apparent differences in anxiety-like behaviour between WD and WC mice in either the OFT or LDT (Figure 7). For example, there were no significant differences in time spent in the center of the chamber in the OFT (no main effect of defeat, $F(1,51) = 1.784$, $p = 0.1876$, Bonferroni-corrected t-test, WD vs. WC, $p > 0.8$) (Figure 7A) or distance travelled in the light zone in the LDT (no main effect of defeat, $F(1,52) = 0.6643$, $p = 0.4188$, Bonferroni-corrected t-test, WD vs. WC, $p > 0.7$) (Figure 7B).

Deficits in exploratory and social behaviour have been previously observed in CSD studies and may be a common result of defeat (Bharwani et al., 2016; Bharwani, Mian, Surette, Bienenstock, & Forsythe, 2017; Golden et al., 2011).

Ketotifen treatment Affects Anxiety-like Behaviour

Although we did not demonstrate an effect of social defeat on anxiety-like behaviour, Ketotifen treatment influenced this behaviour in non-defeated and defeated mice. When compared to WC mice, the KC group spent significantly less time in the center of the chamber during the OFT (no main effect of treatment or interaction, Bonferroni-corrected t-test, WC vs. KC, $p = 0.0387$, ES large, $g = 1.04$) (Figure 7A), suggesting that Ketotifen induces anxiogenic behaviour at baseline. Contrary to the KC group, KD mice displayed anxiolytic behaviour when compared to WD mice. In the LDT, KD mice had greater entries into the light zone (no main effect of interaction, $F(1,51) = 2.953$, $p = 0.0918$, Bonferroni-corrected t-test, KD vs. WD, $p = 0.0876$, ES large, $g = 0.83$) (Figure 7C), and travelled a significantly greater distance in the light zone (no main effect of interaction, $F(1,52) = 2.394$, $p = 0.1279$, Bonferroni-corrected t-test, KD vs. WD, $p = 0.024$, ES large, $g = 1.03$) (Figure 7B). There was also an apparent difference in time spent in the light zone between groups, but post hoc analysis did not reveal significant differences (main effect of interaction, $F(1,49) = 5.517$, $p = 0.0229$, Bonferonni-corrected t-test, KD vs. WD, $p = 0.1368$) (Figure 7D).

Apart from anxiety-like behaviour, there were no clear behavioural differences between treatment groups.

Ketotifen Affects Corticosterone Levels

Baseline CORT levels were measured from trunk blood taken from mice four days after the last day of defeat. The CORT levels from the WD group were similar to WC mice, suggesting that baseline CORT is similar to non-defeated levels four days after defeat (no main effect of defeat, $F(1,26) = 0.0105$, $p = 0.919$, Bonferroni-corrected t-test, WD vs. WC, $p > 0.8$) (Figure 8). There was a main effect of Ketotifen treatment, however, post hoc tests did not reveal statistically significant differences (main effect of treatment $F(1,26)$, $p = 0.0296$, Bonferroni-corrected t-test, KC vs. WC, $p > 0.5$, Bonferroni-corrected t-test, KD vs. WD, $p = 0.1071$).

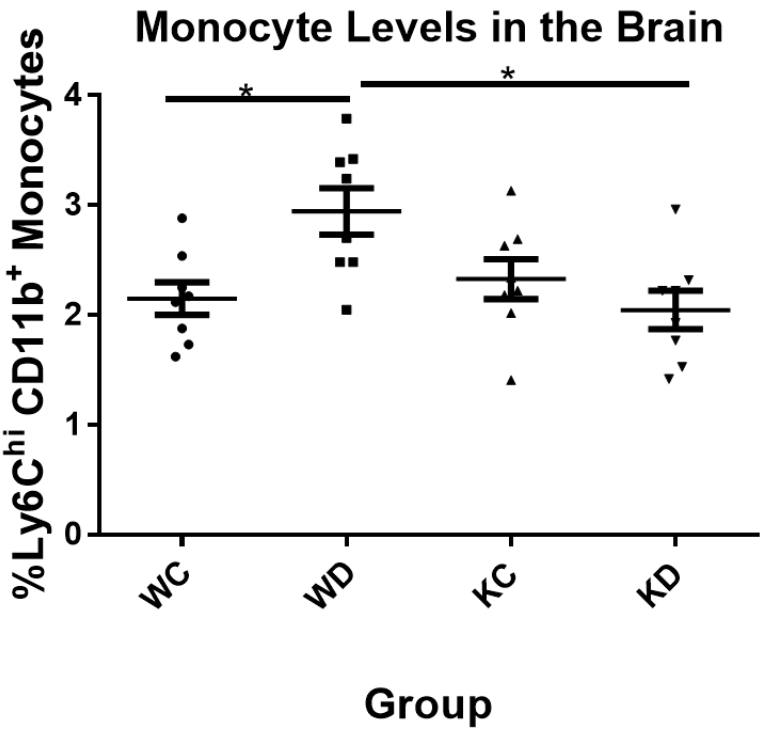

Fig. 4. Mean (± SEM) Percentage of $Ly6C^{hi}$ $CD11b^{+}$ in the brain (n = 8).

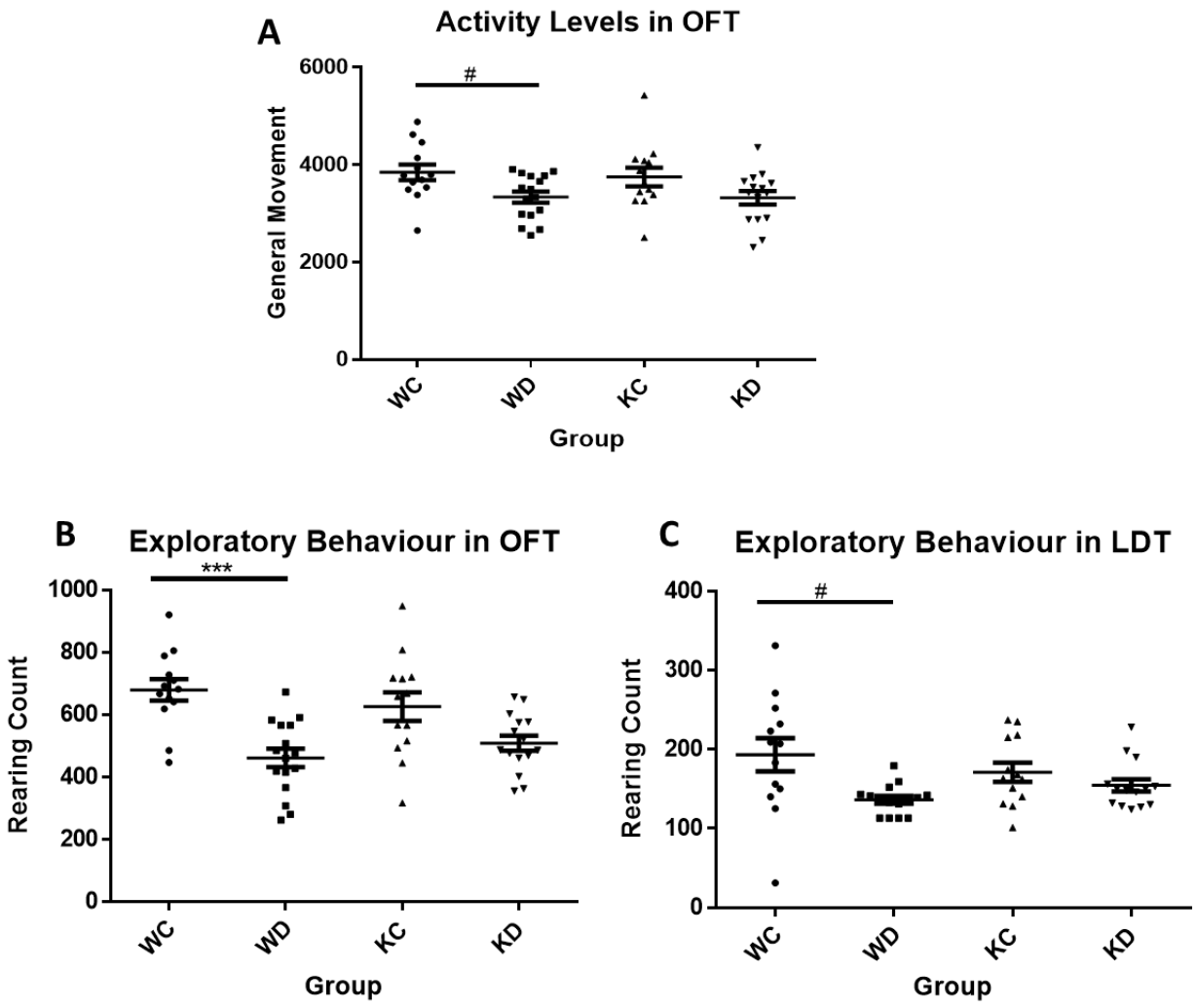

Fig. 5. Mean (± SEM) **A)** General movement in OFT (WC, n = 13, WD, n = 16, KC, n = 13, KD, n= 15). **B)** Rearing behaviour in OFT (WC, n = 13, WD, n = 16, KC, n = 13, KD, n= 15). **C)** Rearing behaviour in LDT (WC, n = 13, WD, n = 15, KC, n = 13, KD, n= 15).

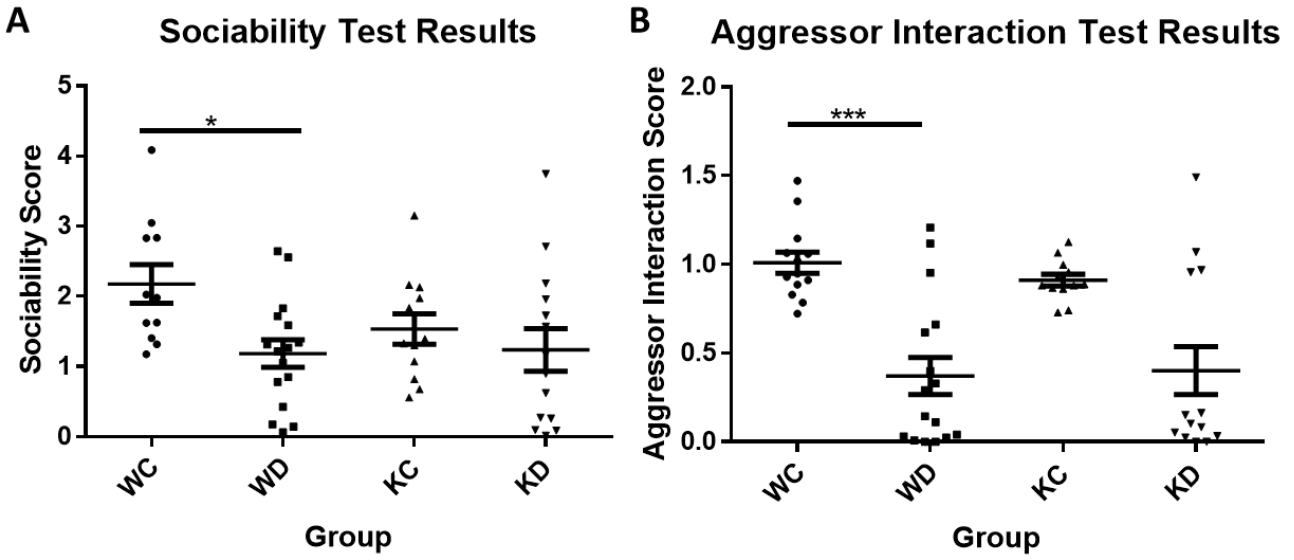

Fig. 6. Mean (± SEM) **A)** Sociability scores in Sociability test. (WC, n = 11, WD, n = 16, KC, n = 12, KD, n = 14). **B)** Aggressor Interaction Score in Aggressor Interaction Test (WC, n = 13, WD, n = 16, KC, n = 12, KD, n = 14).

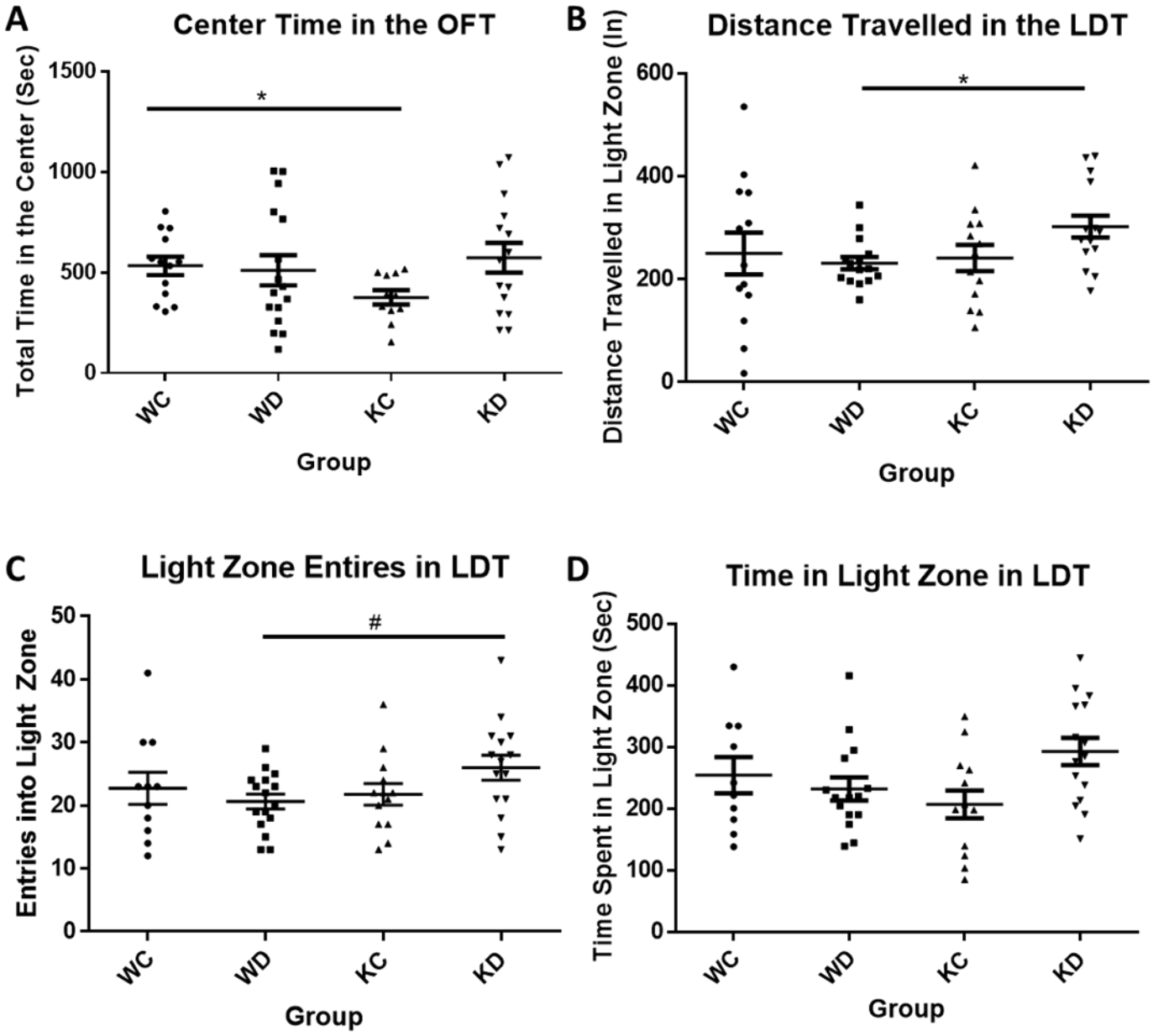

Fig. 7. Mean (± SEM) **A)** Time spent in the center (sec) of the OFT (WC, n = 13, WD, n = 16, KC, n = 11, KD, n = 15). **B)** Distance travelled in the light zone (In) of the LDT (WC, n = 13, WD, n = 15, KC, n = 13, KD, n = 15). **C)** Number of entries into the light zone in the LDT (WC, n = 11, WD, n = 16, KC, n = 13, KD, n = 15). **D)** Time spent in the light zone during the LDT (WC, n = 10, WD, n = 15, KC, n = 13, KD, n = 15).

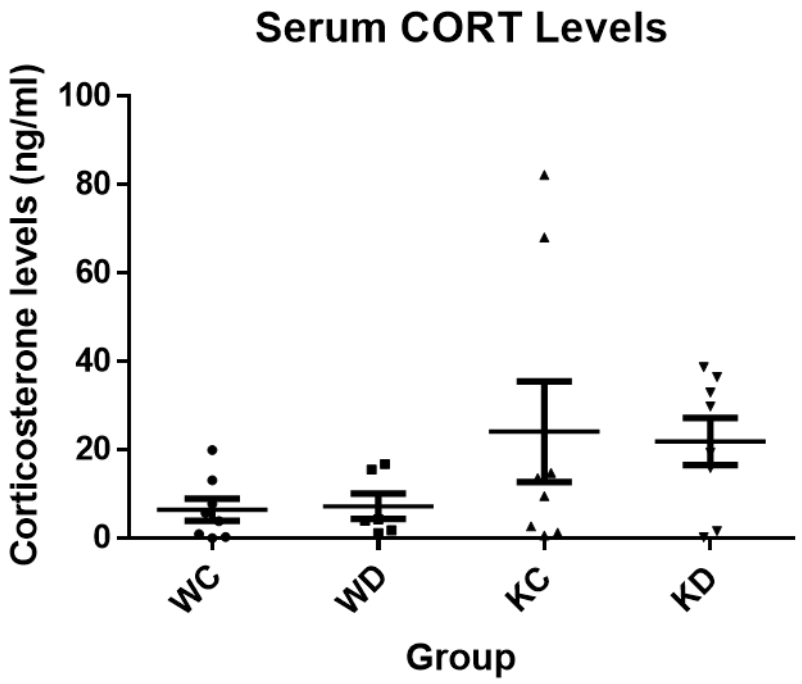

Fig. 8. Mean (± SEM) Corticosterone levels (ng/ml) measured from trunk blood samples (WC, n = 8, WD, n = 6, KC, n = 8, KD, n = 8).

3.5 Discussion

Psychosocial stress is linked to elevated anxiety in humans. Through the CSD model, trafficking of $Ly6C^{hi}$ $CD11b^{+}$ cells from the periphery to the brain is shown to influence anxiety-like behaviour (Björkqvist, 2001; Wohleb et al., 2011, 2013). We report that this pathway may also be associated with mast cells, with treatment using the mast cell stabilizer Ketotifen preventing significant increases in the brain population of these cells in KD mice compared to WD mice.

With the physiological differences apparent between the defeated groups, behavioural testing was conducted to investigate overt changes in phenotype. When compared to WD mice, the KD group reduced anxiety-like behaviour. This finding is consistent with previous demonstration of decreased proinflammatory peripheral monocyte trafficking to the brain (Wohleb et al., 2011, 2013). Although these effects were observed between the anxiety-like behaviour of KD and WD mice, the results must be interpreted with caution as the WD mice did not show significantly increased anxiety-like behaviour when compared to WC mice. Nevertheless, the results of the LDT, a longstanding test for anxiety-like behaviour, showed clear anxiolytic effects of Ketotifen in defeated mice (Crawley & Goodwin, 1980). KD mice had more light zone entries and travelled a greater distance in the light zone than the WD group. With these results in mind, Ketotifen treatment is most likely having anxiolytic effects that are comparably different from defeated mice that only drank water.

In contrast to the anxiolytic effects of Ketotifen in defeated mice, treatment seemed to have anxiogenic effects at baseline. KC mice spent less time in the center of the OFT's chamber than WC mice. These findings are consistent with a previous study that investigated baseline behaviour in mast cell deficient mice and mice that have had their mast cells pharmacologically stabilized (Nautiyal et al., 2008). They found increased baseline anxiety-like behaviour in mast cell deficient and stabilized mice when compared to controls. The contrasting role of mast cells on anxiety in stress versus non-stress environments suggests that mast cells may act in a context-dependent manner. Their activity maybe necessary for normal anxiety levels in a non-stress environment, while their inhibition is helpful in stressful situations. More research is needed to fully understand these effects.

Although ketotifen treatment affected anxiety-like behaviour, it did not affect other stress-induced behavioural changes, including exploratory and social behaviours. These findings may indicate that proinflammatory monocyte trafficking to the brain is not a major contributor towards these behaviours or that they may only contribute to anxiety behaviours, as has been demonstrated by Wohleb et al., 2013.

In addition to behavior, baseline CORT levels were potentially affected by Ketotifen treatment. Baseline CORT levels of WD mice were comparable to WC mice, suggesting that if CORT levels were elevated post defeat, they returned to control levels four days after the end of CSD. This is a likely result, with previous work on social defeat showing a rise in CORT levels that return to normal a week after defeat (A. Keeney et al., 2006). Although baseline CORT was at control levels in WD mice, Ketotifen-treated mice still showed somewhat elevated CORT levels.

However, these elevations were not statistically significant, despite ANOVA results showing a main effect of treatment. If there is a potential effect of Ketotifen treatment on CORT, then it could be the baseline effect of ketotifen or an effect that is specific to stress, where Ketotifen hinders the reestablishment of CORT levels following stress. Additional research with a larger sample size is needed to fully discern what effect Ketotifen may be having on CORT.

The effects of Ketotifen in this study lend support to the involvement of mast cells in anxiety disorders. Mast cells may have a role in both inducing and preventing anxiety disorders and a careful control of their activity may be important for mental health. However, this study is not fully conclusive, for Ketotifen has also been implicated as a histamine H1 antagonist (Yokoyama et al., 1993), suggesting these effects could be the work of histamine and not fully mast cell-dependent. To investigate if mast cells are being targeted, we conducted a separate experiment using passive systemic anaphylaxis. Mice that drank Ketotifen did not exhibit symptoms of anaphylaxis upon antigen challenge (data not shown). Anaphylaxis has long been associated with mast cell activation and preventing this result with Ketotifen suggests the drug is targeting mast cells (Martin, Galli, Katona, & Drazen, 1989). However, the results of this test does not remove the possibility that Ketotifen maybe targeting multiple targets, such as mast cells and separate H1 receptors. Future research with additional stabilizers and mast cell deficient models are needed to fully identify this relationship. If mast cell stabilization is preventing peripheral monocyte trafficking to the brain, then this maybe through BBB permeability. By stabilizing mast cells during stress, we may have prevented increases in BBB permeability that could be needed for monocyte trafficking (Esposito et al., 2001). Half-brains were collected for RT-qPCR analysis for BBB-associated genes. Analysis of these brains have not been completed at this time. The relative expression of these genes between groups may shed light on a possible mechanism for this study's findings.

This study has expanded on our knowledge of the contribution of the immune system to the brain and psychopathologies related to anxiety. The effects of Ketotifen have suggested a possible role of mast cells in the physiology of the central nervous system and behaviour during psychosocial stress. Our findings have potential implications for future investigations in psychosocial stress to not only look at the influence of the brain and its associated nervous system, but the immune system in anxiety disorders. Cells of the immune system are not just useful in host defense against infection, but are potential targets for defense against stress-induced psychopathologies.

CHAPTER 4: THE INFLUENCE OF MAST CELLS ON BASELINE BEHAVIOUR

4.1 Introduction

Mast cells are important components of the immune system. They have been heavily studied in host defence and disease, where they play a prominent role in allergic reactions and infection (Bischoff, 2007; Galli & Tsai, 2010; Matsuda, Nakano, Kiso, & Kitamura, 1987). In allergic reactions, mast cells are responsible for the early phase of type I hypersensitivity and recruitment of other immune cells to the site of inflammation (Bischoff, 2007; Galli & Tsai, 2010). They have also been linked to features of asthma and asthmatic symptoms in chronic asthma models (Boyce, 2003; Yu et al., 2006). In host defense, mast cells are key contributors of parasitic resistance, including ticks and nematodes (Galli & Tsai, 2010; Matsuda et al., 1987). They are also involved in host defense of bacterial infections, promoting clearance and overall protection of the host (Galli & Tsai, 2010).

Unlike the role of mast cells in allergic reactions and host defense, their impact on the central nervous system has not been extensively investigated. They have been linked to anxiety-like behaviour, where mast cell deficient and pharmacologically stabilized mice exhibit increased anxiety-like behaviour compared to controls. This elevated anxiety-like behaviour is thought to be due to mast cells acting on the brain (Nautiyal et al., 2008). Links have also been made between mast cells and autism associated disorders (ASDs). Patients with mastocytosis express a 10-fold increase in the prevalence of ASD compared to the general population. Many patients with ASD also suffer from food allergy and have other allergic symptoms, suggesting mast cell activation (Theoharides et al., 2012; Theoharides, Stewart, Panagiotidou, & Melamed, 2016). While these observations suggest that mast cells can modulate the central nervous system, more work is needed to investigate the role of mast cells in brain physiology and behaviour *in vivo*.

The goal of this study is to further explore the involvement of mast cells in modulating brain function by comparing the behaviour of mast cell deficient mice with healthy controls and deficient mice that have undergone mast cell reconstitution.

4.2 Research Aim

To investigate the role of mast cell deficiency and reconstitution on baseline exploratory, anxiety, and social behaviour in mice.

4.3 Materials and Methods

Animals

Male WBB6F1/J-KitW/KitW-v/J mice (4 – 5 weeks old) and +/+ controls were acquired from Jackson Laboratories (Bar Harbor, ME, USA). Animals were housed in standard conditions under a 12 hour light-dark cycle with food and water available ad libitum. They remained in the housing facility for a minimum of 7 days prior to beginning any experiments. Experiments followed the Canadian Council on Animal Care's guidelines and were approved by McMaster Animal Research Ethics Board.

Experiment Outline

There were three treatment groups: mast cell deficient WBB6F1/J-KitW/KitW-v/J mice that received PBS injections (MCD), WBB6F1/J-KitW/KitW-v/J that received whole bone marrow (WBM) injections (MCR), and +/+ controls that received PBS injections (MCC). After acclimation, mice received tail vein injections with either PBS or WBM extracted from +/+ controls (Figure 9). All mice were housed for 11 weeks for reconstitution of mast cells to occur in the mice that received WBM injections (Tanzola, Robbie-ryan, Anne, Brown, & Brown, 2003). All mice went through a battery of behavioural tests, one occurring each day, organized as follows: OFT, LDT, Elevated plus Maze (EPM), and the sociability test. Following testing, mice were euthanized and sections of jejunum, ileum, and colon were taken to measure mast cell reconstitution in the intestines. The hypothalamus and pituitary were also extracted to measure mast cell reconstitution in the brain.

Day 1	Days 2 - 77	Days 78 - 81	Day 82
PBS/WBM Injection	**Group Housing**	**Behaviour Tests** **78. OFT** **79. LDT** **80. EPM** **81. Sociability Test**	**Tissue Collection**

Fig. 9. Outline for "The Influence of Mast Cells on Baseline Behaviour" study.

Whole Bone Marrow Transplant

To prepare WBM injections, +/+ control mice were euthanized and their femurs were flushed with ice cold PBS. Red blood cells were lysed using 1ml of ACK lysis buffer for 5 minutes. The extracted WBM cells were then centrifuged (1200 rpm for 5 mins at 20 °C) and resuspended in 1x PBS. MCR mice were injected in the tail vein with a minimum of 1.8×10^7 WBM cells in the 0.2 ml tail vein injection.

Behavioural Tests

Elevated Plus Maze

The EPM is a longstanding measure of anxiety-like behaviour in rodents (Komada, Takao, & Miyakawa, 2008; Pellow & File, 1986; Rodgers & Johnson, 1995). This t-shaped testing area is located high above the floor and includes two open arms and two closed arms. The test focuses on the mouse's natural fear for open and elevated areas along with their tendency to explore novel environments. The test lasts 5 minutes. Anxious mice spend a greater time in the closed arms than the open arms. Factors related to open arm activity, such as entries, time spent (sec), and distance travelled (in) in the open arms were measured to gauge the mouse's anxiety. The testing area was cleaned with water between mice.

Please refer to Chapter Three's Materials and Methods section for descriptions of the other behavioural tests.

Quantification of Intestinal Mast Cells

Following dissection, 2 cm pieces of jejunum, ileum, and colon were flushed and stored in 4% paraformaldehyde at 4 °C for 18-24 hours. Intestine segments were then transferred to a 30% sucrose solution and stored at 4 °C for 5-7 days. Segments were then snap-frozen using 2 methyl-butane and stored at -80 °C until further processing. Tissue analysis was conducted as previously described in (Castillo-Courtade et al., 2015). Briefly, the tissue was embedded in OCT compound (Tissue-Tek,Torrance, CA, USA) and frozen sections (20 µm) were cut using a crystat (-20 °C). Sections were then incubated with normal donkey serum (1:10; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 30 mins, followed by antiserum against mouse mast cell protease-1 (mMCP-1) for 12-18 hrs (1:5000; Moredun Scientific, Penicuik, UK), and FITC-conjugated sheep antidonkey IgG (1:200). Sections were washed in 0.01 M PBS after each incubation. The sections were mounted in sodium carbonate-buffered glycerol (pH 8.6) and examined using fluorescence microscopy (Zeiss Axio Observer.Z1, Zeiss Canada, Toronto, Ontario, Canada). Five non-consecutive sections of each segment were assessed for each mouse. The number of mMCP-1 positive cells per unit area was used to analyze differences between groups (Fiji image analysis software).

Quantification of Brain Mast Cells

Following decapitation, the hypothalamus and pituitary were dissected and placed in 10% buffered formalin. The brain regions were then embedded in wax and 5 µm sections were cut and stained with toluidine blue for quantification of mast cell numbers.

Statistics

All tests were analyzed using Welch-corrected two-tailed student's t test with post-hoc Bonferroni corrections for multiple comparisons. Measures in figures are reported as mean +/- SEM and statistical significance is denoted as # ($P < 0.10$), * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$). In addition to null hypothesis testing, ES was calculated using Hedges's g for all corrected

comparisons where $P < 0.1$ (Hedges, 1982). Differences of $g < 0.2$ were deemed as trivial effect sizes, g of 0.2 – 0.5 was a small effect size, g of 0.5 – 0.8 was a moderate effect size, and $g > 0.8$ was a large effect.

4.4 Results

Mast Cell Quantification in the Brain and Intestines

Brain areas and intestinal segments were processed; however, they have not been stained and analyzed. Without this data, we cannot confirm that MCD mice were mast cell deficient or that MCR mice had their mast cells reconstituted. However, the Mast cell deficient mouse strain and method of WBM transplantation selected is an established model of mast cell deficiency and reconstitution (Grimbaldeston et al., 2005a; B. Y. Kitamura et al., 1978; Tanzola, Robbie-ryan, et al., 2003). The longstanding use of this methodology in mast cell research lends support that this study's findings should be considered as preliminary results in the context of mast cell deficiency and reconstitution.

Mast Cell Deficiency Decreased Exploratory Behaviour

There were several behavioural differences between MCD and MCC mice. The first major difference was observed in the OFT. MCD mice showed significantly less activity than MCC mice (Bonferroni-corrected t-test, MCC vs. MCD, $p = 0.0122$, ES large, $g = 1.40$) (Figure 10A). They also showed a significant decrease in exploratory behaviour, as evident by decreased rearing count (Bonferroni-corrected t-test, MCD vs. MCC, $p = 0.009$, ES large, $g = 1.42$) (Figure 10B). This comparative decrease in rearing count was also reproduced in the LDT (Bonferroni-corrected t-test, MCD vs. MCC, $p = 0.0004$, ES large, $g = 2.70$) (Figure 10C).

Mast cell Deficiency Did Not Affect Anxiety-like Behaviour, but Induced Sociability Deficits

Although the OFT showed differences in mobility and exploration, there were no apparent differences in anxiety-like behaviour, with the distance travelled in the center of the field (Bonferroni-corrected t-test, MCD vs. MCC, $p > 1$, Figure 11A) and time spent in the center (Bonferroni-corrected t-test, MCD vs. MCC, $p > 0.5$, Figure 11B) being similar between MCD and MCC mice. There were also no differences in anxiety-like behaviour in the LDT or EPM, with the time spent in the light zone (Bonferroni-corrected t-test, MCD vs. MCC, $p > 1$, Figure 11C) and open arms (Bonferroni-corrected t-test, MCD vs. MCC, $p > 1$, Figure 11D) being similar between MCD and MCC mice.

Unlike in anxiety-like behaviour, there were social differences between deficient mice and controls. MCD mice had a significantly lower SS than MCC mice, suggesting deficits in social affiliation that accompanies mast cell deficiency (Bonferroni-corrected t-test, MCD vs. MCC, $p = 0.0208$, ES large, $g = 1.48$) (Figure 12).

Mast cell Reconstitution Induced Anxiety Deficits and Attenuated Social Impairments

Although mast cell deficiency did not induce anxiety-like behavioural changes, MCR mice showed increased anxiety-like behaviour when compared to their deficient counterparts. MCR mice had significantly fewer entries into the light zone in the LDT (Bonferroni-corrected t-test, MCD vs. MCR, $p = 0.0128$, ES large, $g = 1.54$) (Figure 13A) and travelled a smaller distance in the light zone (Bonferroni-corrected t-test, MCD vs. MCR, $p = 0.0802$, ES large, $g = 1.07$) than MCD mice (Figure 13B).

In addition to the effects of reconstitution on anxiety, MCR mice had greater sociability than MCD mice (Bonferroni-corrected t-test, MCD vs. MCR, $p = 0.0916$, ES large, $g = 0.95$). These results indicate that WBM transplants affected behaviour, inducing changes in MCR mice that were different from their mast cell deficient counterparts.

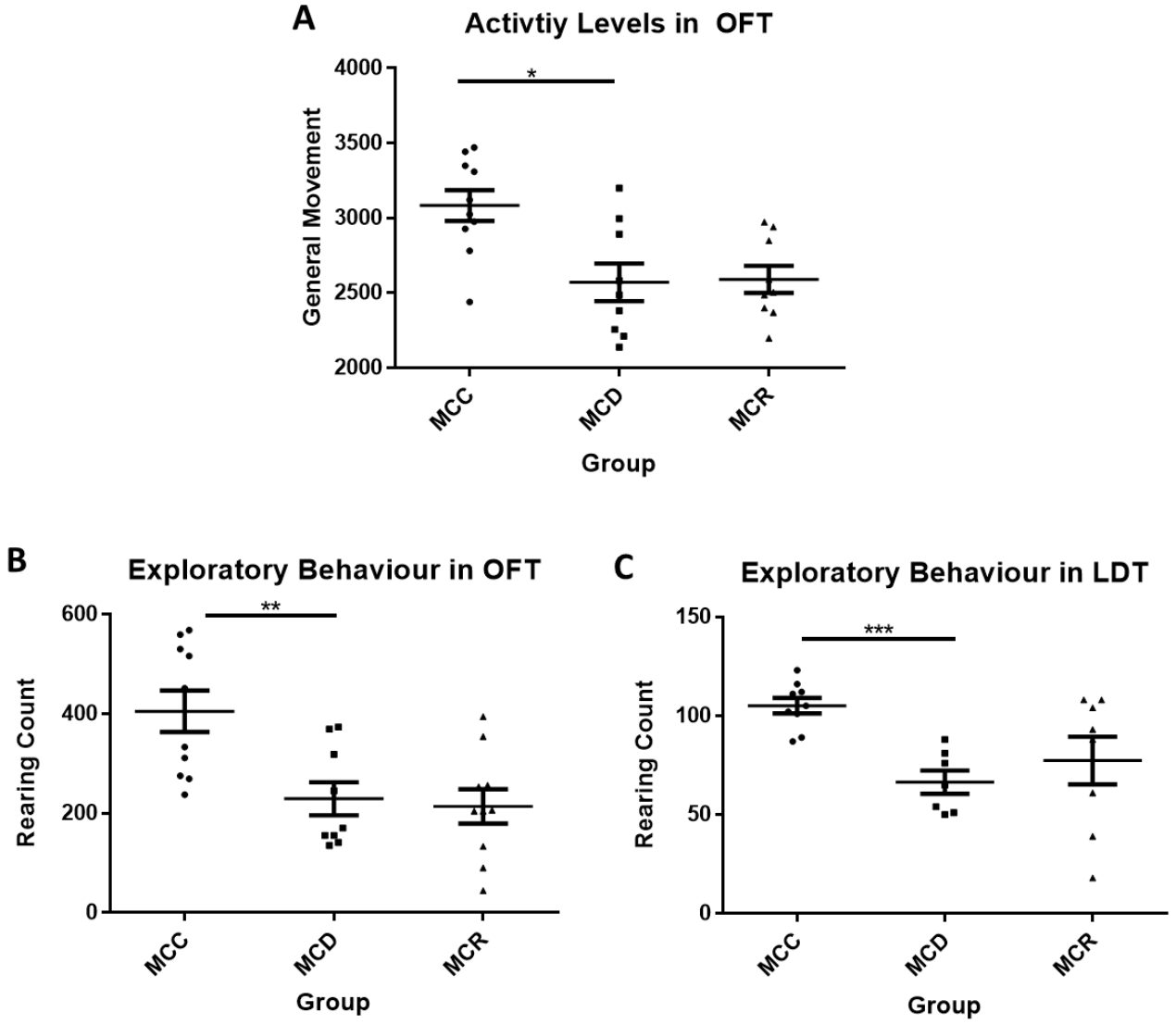

Fig. 10. Mean (± SEM) **A)** General movement in OFT (MCC, n = 10, MCD, n = 9, MCR, n = 9). **B)** Rearing behaviour in OFT (MCC, n = 10, MCD, n = 9, MCR, n = 10). **C)** Rearing behaviour in LDT (MCC, n = 9, MCD, n = 7, MCR, n = 8).

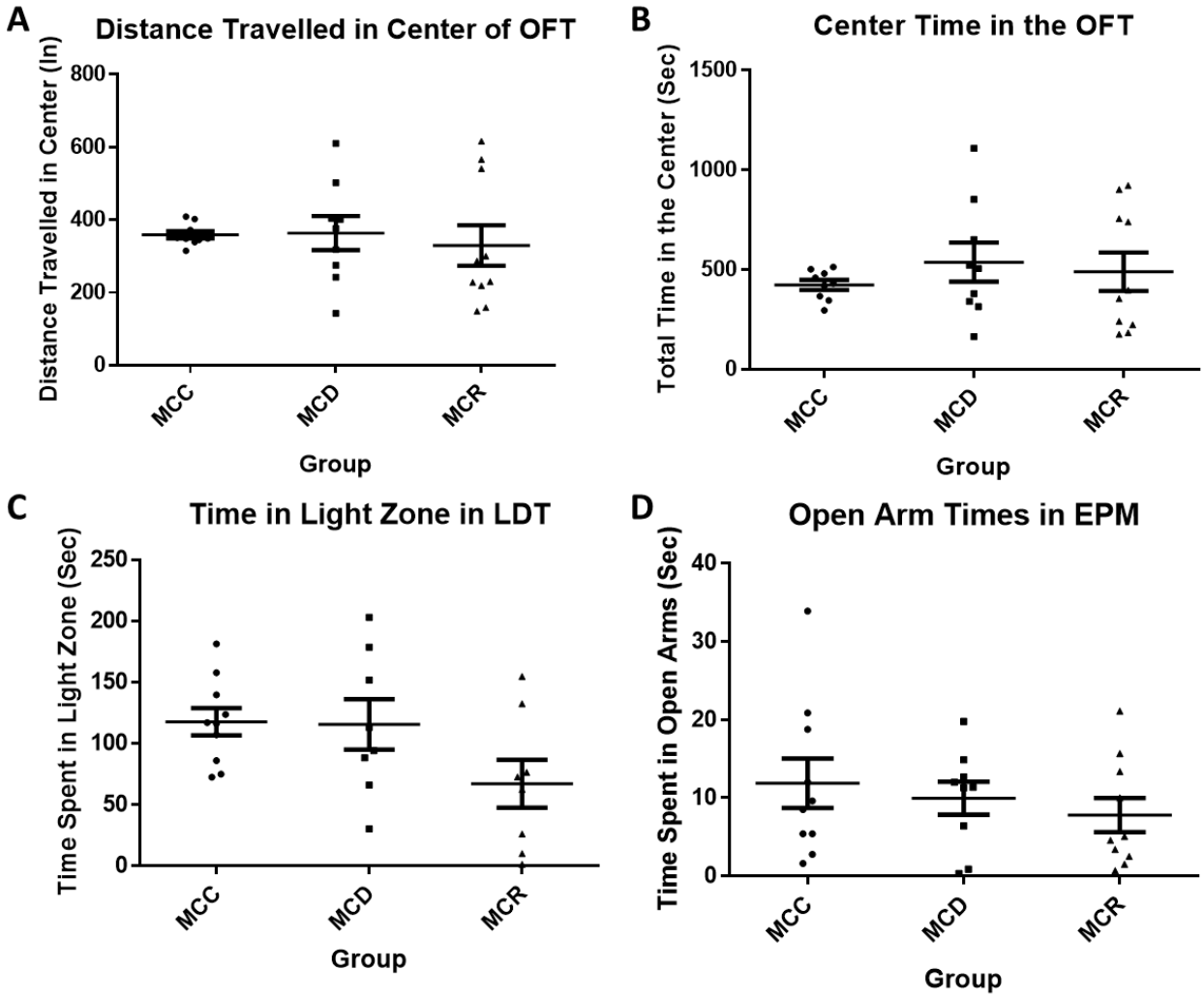

Fig. 11. Mean (± SEM) **A)** Distance travelled in the center area (In) of the OFT (MCC, n = 9, MCD, n = 9, MCR, n = 10). **B)** Time spent in the center area (sec) of the OFT (MCC, n = 9, MCD, n = 9, MCR, n = 10). **C)** Time spent in the light zone during the LDT (MCC, n = 10, MCD, n = 8, MCR, n = 8). **D)** Time spent in the open arm of the EPM (sec) (MCC, n = 10, MCD, n = 9, MCR, n = 10).

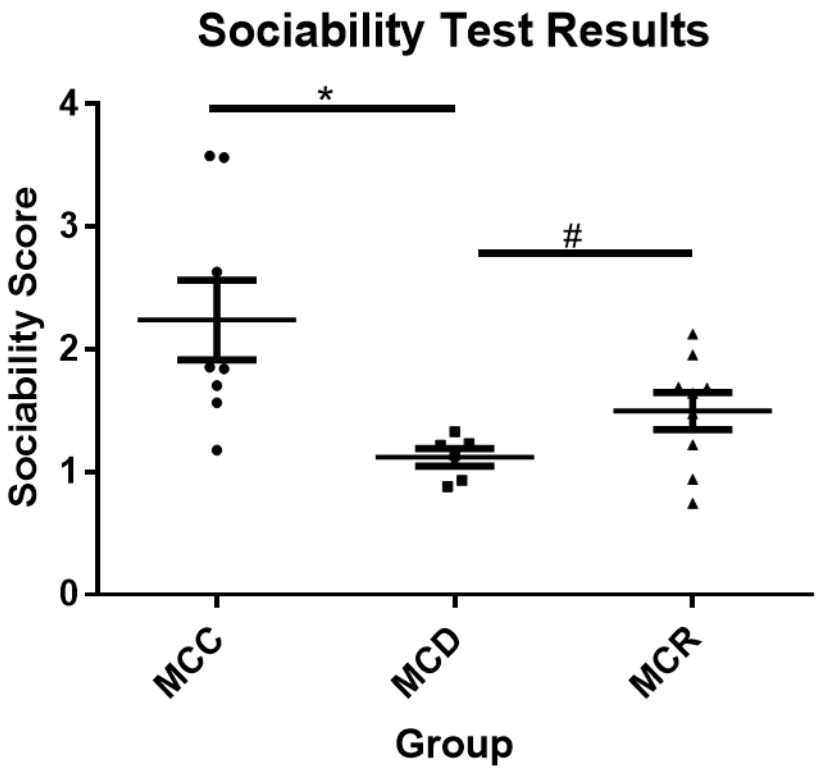

Fig. 12. Mean (± SEM) Sociability scores in the Sociability test (MCC, n = 8, MCD, n = 6, MCR, n = 9).

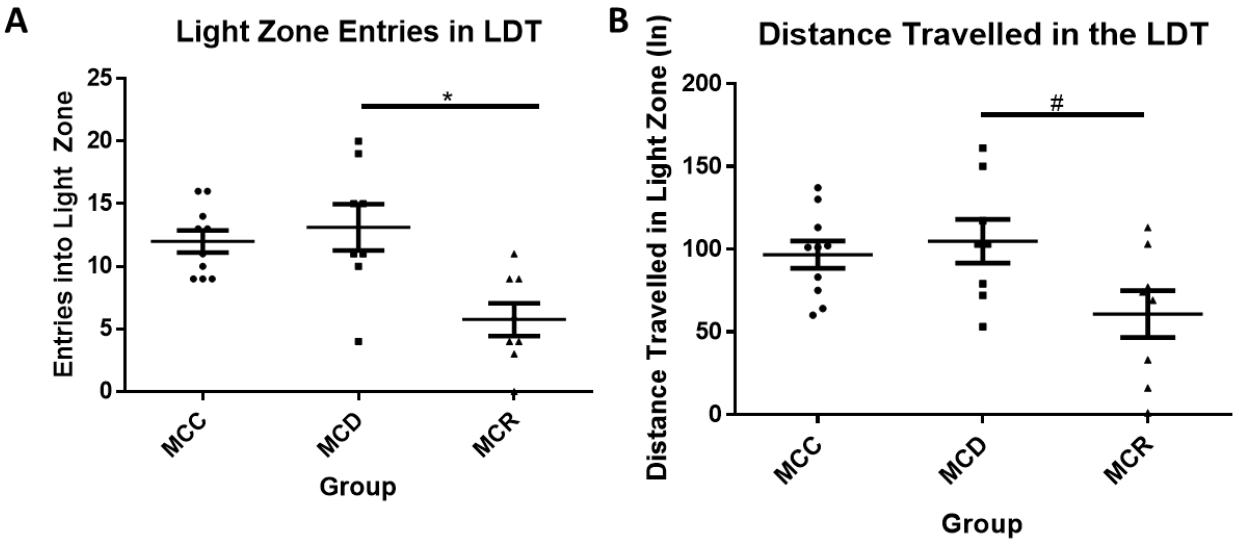

Fig. 13. Mean (± SEM) **A)** Entries into the light zone during the LDT (MCC, n = 10, MCD, n = 8, MCR, n = 8). **B)** Distance travelled in the light zone of the LDT (MCC, n = 10, MCD, n = 8, MCR, n = 8).

4.5 Discussion

This study provides preliminary findings that suggest mast cell deficiency and reconstitution induces several behavioural changes in the mouse. MCD mice had decreased activity levels and exploratory behaviour, as evident by their general movement and rearing behaviour. The source of these behavioural deficits is unknown, with WBM transplant failing to induce changes in these behaviours.

Although deficits were seen in activity and exploratory behaviour, MCD mice did not express anxiety-like behaviour that differed from controls. This result contrasts a previous report of mast cell deficiency inducing increased anxiety-like behaviour (Nautiyal et al., 2008). It is possible that with a greater sample size, we would be more likely to find differences in anxiety-like behaviour. However, Nautiyal et al., 2008 found comparable differences in their mast cell deficient study with a similar sample size, so this explanation is unlikely. A more plausible explanation is that difference in results stemmed from strain differences in the models used in each study. Our study used $Kit^{W/}Kit^{W-v}$ mice, while Nautiyal et al., 2008 used $Kit^{W-sh/W-sh}$ mice. Although both strains are mast cell deficient, there are physiological differences between them. For example, $Kit^{W/}Kit^{W-v}$ mice are anemic and sterile, while $Kit^{W-sh/W-sh}$ do not have these impairments (Besmer et al., 1993; Grimbaldeston et al., 2005b; B. Y. Kitamura et al., 1978; Lyon & Glenister, 1982; Nakano, Waki, Asai, & Kitamura, 1989; Russell, 1979; Tono et al., 1992). The unique attributes of each strain may have influenced their behaviour and resulted in the different findings. Interestingly, MCR mice did show anxiogenic behaviour when compared to MCD mice, suggesting that WBM reconstitution may lead to an increase in anxiety. This finding is supported

by past work on mast cell stabilization and reconstitution. WBM injection does not reconstitute mast cells in the brain, which means that MCR mice may have had functional peripheral mast cells, but no brain mast cells (Tanzola, Robbie-Ryan, et al., 2003). This model is comparable to mice that have their brain mast cells pharmacologically stabilized. Mice with stabilized brain mast cells exhibit increased anxiety-like behaviour compared to controls (Nautiyal et al., 2008). With this effect in mind, our results with MCR mice supports the notion that functioning peripheral mast cells in the absence of brain mast cells results in increased anxiety. These findings may suggest diverging functions of mast cells on behaviour that depend on location or the need for a balance of both central and peripheral mast cells for normal anxiety behaviour.

In addition to finding differences in anxiety-like behaviour, we observed changes in sociability between treatment groups. MCD mice exhibited a lower SS than controls, suggesting social impairment in these mice. This deficit was somewhat attenuated in mice that received WBM transplant. Mast cells have been implicated in disorders like ASD, which includes social deficits (Theoharides et al., 2012, 2016). These findings support the potential involvement of mast cells in social disorders, with the absence of mast cells influencing the social behaviour of mice. It is also noteworthy that the SS attenuation observed in the MCR group was probably due to peripheral mast cells, given brain mast cells are usually not reconstituted following WBM transplant (Tanzola, Robbie-ryan, et al., 2003). With the reconstitution in mind, this finding may suggest a novel role for peripheral mast cells on sociability. Future research is needed to investigate mast cells in the context of social behaviour, including conducting additional social tests and employing selective mast cell stabilizers to uncover the extent of this observed phenomenon.

This study provides insight into the role of mast cells on baseline behaviour. They have been shown to influence activity levels, exploratory, anxiety-like, and social behaviour. Furthermore, differences may exist between peripheral and central mast cells, with peripheral mast cells in the absence of central mast cells inducing anxiogenic behaviour and attenuating social deficits. Mast cells clearly have an impact on behaviour and future research should investigate how they affect brain physiology and their role in mood disorders related to ASD and anxiety.

CHAPTER 5: CONCLUSION

The studies conducted in this thesis were intended to increase our awareness of the neuro-immune interactions that occur in the body. Through investigating mast cells in both non-stress and stress conditions as well as in downstream and upstream signalling, we have achieved our goal.

Our gut motility experiments showed that mast cell stabilization prevents several characteristics of stress-induced dysmotility. By using stabilizers that target different mast cell populations, we found that many of the changes in motility were mediated by peripheral mast

cells, suggesting a more direct action of mast cells on gut physiology (Pearce et al., 1982). These findings are beneficial for disorders where stress causes gut dysmotility, such as IBS (Konturek PC, Brzozowski T, 2011). Our work indicates mast cells as a probable target in the treatment of IBS-associated dysmotility and pain. Future work should look at the stress-induced motility changes of other mast cell models, such as mast cell deficient animals, to further investigate the degree of which mast cells affect stressed gut physiology.

Our experiment using Ketotifen and CSD highlighted the role of mast cells in changes in brain chemistry and behaviour. Mast cell stabilization prevented proinflammatory monocyte trafficking to the brain and produced anxiolytic behaviour. These findings suggest a role for mast cells in anxiety-related mood disorders and present these cells as potential targets for intervention. This study also supported previous research on mast cell stabilization producing anxiogenic behaviour in non-stress conditions (Nautiyal et al., 2008). The opposing effects of mast cell stabilization in stress versus non-stress environments demonstrate the importance of these cells in anxiety and allude to a possible dual nature of mast cells that should be further investigated.

The study with mast cell deficient and potentially reconstituted mice gave us a better understanding of the effects of mast cells on baseline behaviour. We found that the presence of mast cells affected exploratory, anxiety, and social behaviour in mice. These findings support the role of mast cells in immune signaling to the brain and reinforce their importance for normal behaviour. Our findings may also indicate a potential balance between brain and peripheral mast cells, where an imbalance alters anxiety and social functioning. Future work should test these behaviours with mast cell stabilizers and further expand on the physiological changes that are seen in the behavioural phenotypes.

These studies have expanded our knowledge on mast cells in both downstream and upstream signalling between the nervous system and host physiology. I hope this work will be applied towards further investigation in immune interventions for stress-based ailments of both the brain and body.

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