EARLY WEANING STRESS AND POSTNATAL AGE INFLUENCE THE TIME COURSE AND NATURE OF INTESTINAL MAST CELL ACTIVATION IN PIGLETS

By

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ABSTRACT

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Early life adversity (ELA), such as abuse, neglect and household dysfunction are known risk factors for increased disease risk and the development of immune disorders, such as Irritable Bowel Syndrome (IBS) and Inflammatory Bowel Disease (IBD) later in life in human. Early life stress also impacts agricultural species, like swine, and has been shown to lead to increased susceptibility to disease and to the development of acute and chronic immunologic and enteric disease later in life. In response to ELA, piglets exhibit similar pathophysiology's (leaky gut, enteric and immune system dysfunction, diarrhea) as humans. While there is a long-term impact of ELA in both human and animals, the mechanism is unknown. Mast cells (MCs) are critical innate immune cells which orchestrate the pathogenesis of many immunological disorders and are highly activated in response to psychological stress, supports their role as key modulators of stress-induced disease. Previous studies from our lab has shown that MCs can trigger intestinal permeability and inflammation. The goal of this thesis was to determine the time course of intestinal MC activation in response to stress and whether age of piglets at the time of weaning influences MC activity. Our work provides insight into understanding the early mechanism that could be initiating changes in gut development in response to weaning-induced stress. This works also suggest that preventative measures and strategies in swine production may in fact need to be administered early before the weaning process.

This is dedicated to my mom and dad. Thank you for always supporting me.

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KEY TO ABBREVIATIONS

ELA	Early Life Adversity
EWS	Early Weaning Stress
FGID	Functional Gastrointestinal Disorders
GI	gastrointestinal
CRF	corticotropin releasing factor
MC	mast cell
HR	Histamine receptors
H1R	Histamine 1 receptor
H2R	Histamine 2 receptor
H4R	Histamine 4 receptor
CMA1	chymase
MCT7	tryptase
DAO	diamine oxidase
HNMT	histamine-N-methyltransferase
MPO	myeloperoxidase

CHAPTER 1

Introduction

Early Life Adversity Increases Disease Risk in People and Animals

Early Life Adversity (ELA) or childhood trauma is defined as exposure to psychological, physical, and/or emotional abuse such as neglect, poverty or household dysfunction or infections during early life. Traumatic events during childhood has emerged as a significant risk factor for increase susceptibility to disease and development of debilitating immune disorders (allergy, asthma, obesity, diabetes, functional gastrointestinal disorders) later in life (1-7). Similar to people, there is increasing evidence that early life stress in animals, such as those associated with agricultural production practices, increases disease risk across the life span (8-10). Early weaning stress (EWS) is a common practice used in porcine production where piglets are separated from their mother at an early age during a crucial period in development(11). Research in our laboratory has shown that EWS in piglets can lead to increase disease susceptibility and the development of gastrointestinal (GI) disturbances, like intestinal permeability, relapsing diarrhea, and inflammation later in life(9, 11). Given the pathophysiologic similarities seen in human GI disorders, such as irritable bowel syndrome, our laboratory is interested in elucidating the mechanisms that are orchestrating disease susceptibly and development of GI disturbances later in life. The acute mechanisms driving the differences in stress-induced GI responses in early weaned piglets is unknown. My thesis research is focused on exploring the early mechanisms driving the differences in stress-induced GI responses to establish a new potential target for therapeutic and preventative strategies for both pigs and humans.

Factors impacting ELA-induced Disease Risk: Number and intensity stressors and biological sex

The number and intensity of adverse childhood experiences (ACE) is correlated with risky health behaviors and disease onset later in life (2). The frequency of childhood adversities and magnitude is correlated with increase disease severity and negative outcomes in adulthood. Higher prevalence of adverse childhood experiences are also known to be correlated with a diminished quality of life, decreased overall welfare, less resources, and most strikingly decreased life expectancy by up to twenty years (12, 13). In particular, the nature of childhood adversity (physical and/or sexual abuse) predisposed individuals to 1.5-2 times greater risk of developing GI disturbances and GI disorders (1). There is also increasing evidence that women experience increased prevalence of GI disturbances (bloating, constipation, abdominal pain) and a higher prevalence of ELA compared to male counterparts (3, 14-16).

Despite this well-established link between ELA and disease risk, the biological mechanisms by which ELA triggers long-lasting disease risk remains unknown. The rest of this review will focus on early life adversity, functional GI disorders and models of early life adversity.

ELA and Functional Gastrointestinal Disorders

Functional gastrointestinal disorders (FGID) currently affect approximately 25 million Americans. FGID's are characterized as chronic heterogenous GI disturbances with no atypical structural or biochemical defects (17-19) according to the Rome Foundation diagnosis criteria. There are four established categories of FGIDs: Functional dyspepsia, belching disorders, nausea and vomiting, and rumination syndrome(20). Individuals with FGIDs may experience singular or multiple GI symptoms such as constipation, diarrhea, bloating, belching, abdominal pain, and incontinence(18).

One of the most commonly studied GI disorders that often overlaps with the symptoms listed above, is irritable bowel syndrome (IBS), currently affecting 10-15% of individuals globally(21).

The hallmark of IBS is chronic abdominal pain combined with abnormal bowel habits (diarrhea (IBS-D), constipation (IBS-C) or both (IBS-M)). IBS is also commonly comorbid with other medical conditions (i.e. other FGIDs, psychiatric disorders, fibromyalgia, chronic fatigue and pelvic pain) and symptomology (visceral hypersensitivity) (22-25). Although the mechanism of IBS remains unknown due to incomplete understanding of disease pathophysiology there is growing evidence that characterizes IBS as a disruption in brain-gut axis communication. This disruption in brain-gut axis has several well documented pathophysiologic factors: irregular motility, increased intestinal permeability, visceral hypersensitivity and immune activation(26-30) that has also been shown in animal models of early life adversity(9, 11, 31, 32). Similarly, in agricultural animal species, early life stressful management practices, such as early weaning, negatively impacts GI development and increases disease susceptibility into adulthood.

FGIDs are a major public health concern in both humans and animals. Current therapeutic approaches are scarce and specifically only target GI symptoms. Diagnostic testing is also limited given that FGIDs do not present with structural or biochemical abnormalities (inflammation, swelling). Although there is growing evidence that early life adversity may be a contributing factor to disease susceptibility there is still a need for more targeted approaches for preventative and intervention strategies.

The Importance of the Gastrointestinal Developmental Critical Period

To understand how early changes in GI development result in long term alterations, it is first important to understand the developing gut during this time. The early postnatal period (first three month after birth) is a highly malleable time for the developing GI tract, in both humans and pigs(10). During this critical window of development, there is establishment of the microbiome, intestinal barrier integrity, immune system, and enteric nervous system. The intestinal barrier is relatively mature after birth but is still permeable and resolves (less permeable) with age. Neonates are relatively dependent on the passive transfer of antigens

from breast milk through the permeable intestinal lining. Breast milk provides exposure to antibodies and white blood cells that allow for the modulation of the neonate's intestinal microbes. Neonates also become tolerant to commensal microbes and nutrition. During this time, the enteric nervous system undergoes formation of neurocircuits, neurogenesis, gangliogenesis, and apoptosis that increases neurons expressing acetylcholine and neurite growth. If these events in gut development are no longer quiescent and homeostasis is lost, the brain and gut axis becomes hyperresponsive. This hyperresponsiveness is thought to lead to disease susceptibility and contribute to the development of GI diseases later in life. Therefore, understanding the underlying mechanism that leads to disease susceptibility in adulthood is important to elucidate therapeutics and preventative measures.

Early Weaning Stress in piglets as a Model of Early Life Adversity

Early weaning is a common practice in the porcine agricultural industry and is a significant stressor on piglets during a critical period of postnatal development. In nature piglets are slowly weaned at about 3-4 months of age (33-35) and are no longer dependent on the sows milk for passive immunity. In non-natural environments like commercial weaning, piglets are weaned at younger ages, about 14 days to 30 days of age, often determined by several factors: lactation space, disease status, and weaning schedule. During the weaning process piglets undergo numerous stressors such as changes in diet, maternal and littermate separation, temperature variations, compromised air quality, commingling with new littermates (crowding), fighting for social order, new environment, vaccinations and transportation stress (36). These abrupt stressors have both short term and long-term effects that increase disease susceptibility (transmissible gastroenteritis, *Haemophilus parasuis*, Porcine reproductive and respiratory syndrome virus, etc) (36-38), lead to a decrease in feed intake, performance, and higher mortality rates(36). This stressful experience has implications for disruption and dramatic

changes in the GI tract, brain and gut axis, immune system, and enteric nervous system development.

Many studies addressing weaning have focused on the early post-weaning period between 1 d and 14 d post-weaning. Many studies have reported similar findings in that weaning induces marked changes in intestinal morphology (villus blunting, increased crypt depth), and intestinal inflammation (39), which coincided with reductions in growth rate. Studies investigating functional changes to the intestine at weaning have shown that early weaning caused impaired barrier function, characterized by increased intestinal permeability. Previous research from our laboratory showed that early weaned pigs (weaned at 21 d of age) exhibited heightened intestinal permeability compared with pigs weaned at 28 d of age measured at 1 day post-weaning (40, 41). Further, these studies showed that activation of the corticotropin releasing factor (CRF) system was upregulated in the intestinal mucosa of early weaning piglets and that intestinal permeability was reduced by pretreating piglet with CRF receptor antagonist drugs (40).

More recently, studies have focused on the long-term effects of early weaning stress in pigs. Pohl et al (2017) showed that early weaning leads to long-term persistent intestinal permeability, increased numbers of intestinal mast cells and chronic diarrhea (9). Medland et al 2016 showed that early weaning induced long-lasting changes to the enteric nervous system characterized by heightened cholinergic activity and heightened nerve-induced secretory activity (42). Further, these studies showed that biological sex plays an important role in that early weaned female pigs exhibit greater intestinal permeability, enteric nervous system hypersensitivity and diarrhea, compared with male castrates. Together previous literature demonstrates that weaning induced stress leads to long-term pathophysiologic, immunologic and psychologic alterations in adulthood and that mast cells may be at the forefront of orchestrating these cascades of events.

Mast Cells

Mast cells (MC) are critical innate immune effector cells that play a major role in modulating disease severity, progression, and symptoms. Mast cells are derived from CD34+ hematopoietic progenitor cells in the bone marrow(43). Once circulating in the blood, MC progenitors travel to various mucosal or connective tissues, found in the digestive tract, central nervous system in the brain, cutaneous layers of the skin, respiratory tract, and cardiovascular system(44). Unlike other immune cells, mast cells undergo maturation in the periphery which is influenced by the microenvironment. Mast cells can be found positioned in close proximity to blood vessels, lymphatic tissues, terminal nerve endings, and at host-environment interfaces, such as the epithelium in the skin and intestinal mucosa(10, 45). The positions of MC allow them to play a key role in host defense against pathogens, wound healing, and inflammation(45-47). However extreme mast cell activation is known to be detrimental in inflammatory diseases like asthma, allergy, and functional GI disorders (irritable bowel syndrome)(48-51).

Mast cell populations are heterogenous and differ between species, anatomical location, function, and protease content(44). Mast cells contain granules with tightly packed compounds called mediators such as cytokines, histamine, serotonin, and mast cell specific serine proteases (chymase, tryptase and carboxypeptidases). Mast cells mediators are preformed or newly synthesized and can change composition if the mast cell is activated in response to a stimulus. In humans, MC phenotype is characterized corresponding to protease content in two subclasses (MCT, tryptase only, and MCTC, tryptase, chymase, carboxypeptidase). In mice, MC are characterized by location, mucosal (MMC) MC's containing mostly chymase or connective tissues (CTMC) containing both tryptase and chymase(47). This difference in phenotypic composition can impact and determine mast cells activity, response to stimuli, and mediator release during inflammatory response.

MC Activation

Mast cells can be activated by both endogenous and exogenous stimuli to induce degranulation of prestored mediators or production of newly synthesized mediators (52). Mast cells are also capable of recycling their granules in a process called regranulation(53). Typical mast cell degranulation is prompted by allergen binding IgE with cross linkage of the FCεRI receptor(54). This leads to the influx of intracellular calcium, cytokine, and arachidonic metabolite production within seconds to minutes of stimulation, releasing preformed mediators. Mast cell degranulation can also occur through non-IgE mediated mechanism including stimulation with anaphylatoxins, calcium ionophores, or drugs. Strategically colocalized to nerve fibers, neurons, lymph nodes, and blood vessels(9). Mast cells are also activated by neurotransmitters (acetylcholine, serotonin, epinephrine), neuropeptides (substance P, vasointestinal peptide, horseradish peroxidase) and growth hormones (corticotropin-releasing hormone, stem cell factor, urocortin)(55). De novo synthesis of mast cell mediators (cytokines, chemokines, etc) occurs after MC degranulation in a secondary stimulus response. Unlike degranulation, de novo synthesis response time varies; minutes to hours depending on the mediator(56). For example, mast cells activation by pathogenic lipopolysaccharides through the toll-like receptor 4 leads to cytokine production and not rapid degranulation(57, 58). It is through the activation and degranulation process that mediators are rapidly or transiently released prompting a response. Therefore, mast cell activity is important for bidirectional communication in the inflammatory response.

Mast Cell Mediators in disease

Mast cell mediators are immunomodulators that influence the innate and adaptive immune responses through various receptors and pathways as mentioned above. Mast cell mediators such as chymase, tryptase and histamine can impact and initiate the inflammatory response through dual proinflammatory and protective anti-inflammatory roles(47).

Tryptase and chymase are well known mast cell specific proteases that comprise approximately 25% of mast cell granule content(47, 59, 60). Given that tryptase is exclusively in MC's it is a well-known indicator of mast cell activity and a recognized clinical marker of anaphylaxis(61, 62). Some proinflammatory actions of tryptase and chymase are neutrophil and eosinophil recruitment, cytokine and chemokine activation, and degradation of cell-to-cell junctions and the extracellular matrix.(44) Protective actions of proteases are degradation of cytokines, chemokine and toxins, IgE cleavage, tissue homeostasis/remodeling, wound healing, and parasite clearance(61). Each of these biological actions can regulate the activity of other mediators to impact disease processes in anaphylaxis, autoantibody-mediated arthritis(63), parasite and bacterial infections, and asthma(45).

Histamine is another major prestored mast cell mediator and accounts for approximately 10% of the granule's total composition. Histamine is a biogenic amine that is synthesized from the amino acid L-histidine by the metabolic action of Histidine Decarboxylase (HDC). Histamine is released into circulation relatively quickly within seconds to minutes and is known to increase in plasma as quickly as 5-10 minutes(64). Degradation of histamine mediated by Diamine Oxidase (DAO) through deamination and by Histamine-N-MethylTransferase (HNMT) through methylation. DAO is stored in plasma membrane vesicles and expressed in the intestines, kidney and placenta. Reduced or compromised DAO levels has been linked to histamine intolerance(65) leading to various symptoms like congestion, diarrhea, vomiting headache and wheezing(66, 67). Reduced DAO levels have also been shown in GI disease like crohns and ulcerative colitis(68, 69) HNMT is stored in cytosolic compartments of cells and widely expressed in several tissues like the kidney, liver, spleen, colon, prostate, ovary, spinal cord cells, trachea and bronchi(65). Polymorphisms of HNMT has been shown to vary in histamine catabolism with a reduction in HNMT levels in bronchial epithelium of asthma patients(70, 71).

This indicates that the presence or absence of histamine degrading enzymes may be critical for the regulation of histamine in disease.

Once in circulation, histamine binds to its G protein coupled receptors subtypes, H1R-H4R, on multiple cell types. These receptors have differential expression across tissues and are found on a multitude of cell types such as neurons, bacteria, enterochromaffin like cells, epithelial cells and basophils(72). In disease states, histamine has been shown to play a deleterious role by the induction of allergic inflammation (mostly H1R mediated)(73, 74), colitis (partly H4R mediated in rodents)(75-77), anaphylaxis(78), diarrhea, and visceral hypersensitivity(65). Histamine has also been shown to play a beneficial role in pathogen clearance (mostly H2R mediated), allergen expulsion, gastric acid secretion, induction of tolerance (partly H2R mediated)(79), and wound healing(80).

In FGID's, like irritable bowel syndrome, in humans and early weaning stress in pigs, mast cells numbers are elevated in the intestinal mucosa. Although, there is an increase in MC numbers, MC mediators release during the stress response is not fully understood. Understanding the dual roles of MC mediators is important for the insight into the mechanistic aspect of disease pathophysiology's and development of therapeutics and disease targets.

Major role of MC's in disease and the stress response

Early life stress has been shown to induce mast cell activation and degranulation. Mast cells numbers and activity are increased in stress disorders such as functional GI disorders and allergy. Similarly, in agricultural animal species, early life stressful management practices, such as early weaning, contributes to ELA and negatively impacts GI development and increases disease susceptibility into adulthood(31). Our laboratory has previously demonstrated that weaning in pigs from their mother at less than 23 d of age (early weaning stress; EWS), a common practice in pig production, induces significant stress and leads to long-term increased

risk to functional diarrhea, increased intestinal permeability, and altered mast cell activity into adulthood(9-11).

Given the lifelong consequence GI disease risk, ELA represents a significant public health concern with major economic and welfare implications. While the long-term negative impacts of ELA on GI pathophysiology and increased disease risk has been established(8, 10), very little is known regarding the early GI responses to ELA that trigger long-term developmental alterations in GI development and increased GI disease risk throughout life. However, recent studies from our laboratory showed that EWS in piglets causes rapid loss of intestinal barrier function (increased intestinal permeability)(31) and intestinal inflammation(81) that is more severe in EWS pigs compared with pigs weaned later at 28 d of age. Further, we found that mast cells are also highly activated in response to weaning stress. Therefore, MCs are strategically positioned as potential initiators of ELA-induced GI barrier dysfunction, inflammation and long-term GI development. However, the significance of MC activation as an early immune mechanism driving GI dysfunction has not been explored.

Conclusion

In summary, multiple studies have revealed that early life adversity is a risk factor for GI and immune diseases across the lifespan in both humans and animals. The biological mechanism by which this occurs is unknown. Given that mast cells are a critical innate effector cells that is rapidly activated, triggers intestinal permeability, and inflammation in the stress response makes it an ideal target to study. Therefore, understanding the mechanism in which mast cells contribute to intestinal permeability and inflammation is important to target for disease prevention and therapeutics. The major goal of my master's thesis research is to demonstrate how ELA impacts the time course and magnitude on GI mast cell responses in an early life adversity model, EWS piglets.

REFERENCES

REFERENCES

1. Leserman J, Drossman DA. Relationship of abuse history to functional gastrointestinal disorders and symptoms: some possible mediating mechanisms. Trauma Violence Abuse. 2007;8(3):331-43.

2. Felitti VJ, Anda RF, Nordenberg D, Williamson DF, Spitz AM, Edwards V, et al. Relationship of childhood abuse and household dysfunction to many of the leading causes of death in adults. The Adverse Childhood Experiences (ACE) Study. Am J Prev Med. 1998;14(4):245-58.

3. Bradford K, Shih W, Videlock EJ, Presson AP, Naliboff BD, Mayer EA, et al. Association between early adverse life events and irritable bowel syndrome. Clin Gastroenterol Hepatol. 2012;10(4):385-90.e1-3.

4. Drossman DA, Li Z, Andruzzi E, Temple RD, Talley NJ, Thompson WG, et al. U.S. householder survey of functional gastrointestinal disorders. Prevalence, sociodemography, and health impact. Dig Dis Sci. 1993;38(9):1569-80.

5. Gern JE, Visness CM, Gergen PJ, Wood RA, Bloomberg GR, O'Connor GT, et al. The Urban Environment and Childhood Asthma (URECA) birth cohort study: design, methods, and study population. BMC Pulmonary Medicine. 2009;9(1):17.

6. Tomasdottir MO, Sigurdsson JA, Petursson H, Kirkengen AL, Krokstad S, McEwen B, et al. Self Reported Childhood Difficulties, Adult Multimorbidity and Allostatic Load. A Cross-Sectional Analysis of the Norwegian HUNT Study. PLOS ONE. 2015;10(6):e0130591.

7. Liu S, Hagiwara SI, Bhargava A. Early-life adversity, epigenetics, and visceral hypersensitivity. Neurogastroenterol Motil. 2017;29(9).

8. McLamb BL, Gibson AJ, Overman EL, Stahl C, Moeser AJ. Early weaning stress in pigs impairs innate mucosal immune responses to enterotoxigenic E. coli challenge and exacerbates intestinal injury and clinical disease. PLoS One. 2013;8(4):e59838.

9. Pohl CS, Medland JE, Mackey E, Edwards LL, Bagley KD, DeWilde MP, et al. Early weaning stress induces chronic functional diarrhea, intestinal barrier defects, and increased mast cell activity in a porcine model of early life adversity. Neurogastroenterol Motil. 2017;29(11).

10. Pohl CS, Medland JE, Moeser AJ. Early-life stress origins of gastrointestinal disease: animal models, intestinal pathophysiology, and translational implications. Am J Physiol Gastrointest Liver Physiol. 2015;309(12):G927-41.

11. Moeser AJ, Pohl CS, Rajput M. Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs. Anim Nutr. 2017;3(4):313-21.

12. Anda RF, Dong M, Brown DW, Felitti VJ, Giles WH, Perry GS, et al. The relationship of adverse childhood experiences to a history of premature death of family members. BMC Public Health. 2009;9:106.

13. Brown DW, Anda RF, Tiemeier H, Felitti VJ, Edwards VJ, Croft JB, et al. Adverse childhood experiences and the risk of premature mortality. Am J Prev Med. 2009;37(5):389-96.

14. Chang L, Toner BB, Fukudo S, Guthrie E, Locke GR, Norton NJ, et al. Gender, age, society, culture, and the patient's perspective in the functional gastrointestinal disorders. Gastroenterology. 2006;130(5):1435-46.

15. Meleine M, Matricon J. Gender-related differences in irritable bowel syndrome: potential mechanisms of sex hormones. World J Gastroenterol. 2014;20(22):6725-43.

16. Drossman DA, Leserman J, Nachman G, Li ZM, Gluck H, Toomey TC, et al. Sexual and physical abuse in women with functional or organic gastrointestinal disorders. Ann Intern Med. 1990;113(11):828-33.

17. Mukhtar K, Nawaz H, Abid S. Functional gastrointestinal disorders and gut-brain axis: What does the future hold? World J Gastroenterol. 2019;25(5):552-66.

18. Talley NJ. Functional gastrointestinal disorders as a public health problem. Neurogastroenterol Motil. 2008;20 Suppl 1:121-9.

19. Drossman DA. Functional Gastrointestinal Disorders: History, Pathophysiology, Clinical Features and Rome IV. Gastroenterology. 2016.

20. Tack J, Talley NJ, Camilleri M, Holtmann G, Hu P, Malagelada JR, et al. Functional gastroduodenal disorders. Gastroenterology. 2006;130(5):1466-79.

21. Disorders IFfG. Facts about IBS aboutibs.org: International Foundation for Gastrointestinal Disorders, Inc.; [updated 11-24-16. Available from: https://www.aboutibs.org/facts-about-ibs.html.

22. Choung RS, Herrick LM, Locke GR, 3rd, Zinsmeister AR, Talley NJ. Irritable bowel syndrome and chronic pelvic pain: a population-based study. J Clin Gastroenterol. 2010;44(10):696-701.

23. Morriss RK, Ahmed M, Wearden AJ, Mullis R, Strickland P, Appleby L, et al. The role of depression in pain, psychophysiological syndromes and medically unexplained symptoms associated with chronic fatigue syndrome. J Affect Disord. 1999;55(2-3):143-8.

24. Whitehead WE, Palsson O, Jones KR. Systematic review of the comorbidity of irritable bowel syndrome with other disorders: what are the causes and implications? Gastroenterology. 2002;122(4):1140-56.

25. Sperber AD, Atzmon Y, Neumann L, Weisberg I, Shalit Y, Abu-Shakrah M, et al. Fibromyalgia in the irritable bowel syndrome: studies of prevalence and clinical implications. Am J Gastroenterol. 1999;94(12):3541-6.

26. Tap J, Derrien M, Tornblom H, Brazeilles R, Cools-Portier S, Dore J, et al. Identification of an Intestinal Microbiota Signature Associated With Severity of Irritable Bowel Syndrome. Gastroenterology. 2017;152(1):111-23 e8.

27. Ohman L, Tornblom H, Simren M. Crosstalk at the mucosal border: importance of the gut microenvironment in IBS. Nat Rev Gastroenterol Hepatol. 2015;12(1):36-49.

28. Simren M, Tornblom H, Palsson OS, van Tilburg MAL, Van Oudenhove L, Tack J, et al. Visceral hypersensitivity is associated with GI symptom severity in functional GI disorders: consistent findings from five different patient cohorts. Gut. 2018;67(2):255-62.

29. Tornblom H, Van Oudenhove L, Sadik R, Abrahamsson H, Tack J, Simren M. Colonic transit time and IBS symptoms: what's the link? Am J Gastroenterol. 2012;107(5):754-60.

30. Manabe N, Wong BS, Camilleri M, Burton D, McKinzie S, Zinsmeister AR. Lower functional gastrointestinal disorders: evidence of abnormal colonic transit in a 287 patient cohort. Neurogastroenterol Motil. 2010;22(3):293-e82.

31. Smith F, Clark JE, Overman BL, Tozel CC, Huang JH, Rivier JE, et al. Early weaning stress impairs development of mucosal barrier function in the porcine intestine. Am J Physiol Gastrointest Liver Physiol. 2010;298(3):G352-63.

32. Fuentes IM, Walker NK, Pierce AN, Holt BR, Di Silvestro ER, Christianson JA. Neonatal maternal separation increases susceptibility to experimental colitis and acute stress exposure in male mice. IBRO Rep. 2016;1:10-8.

33. English P, Fowler, VR, Baxter, S, Smith, B. . The growing and finishing pig: Improving efficiency. . Farming Press Ltd Ipswich, Suffolk. 1988.

34. Newberry R, Wood-Gush, DGM. The suckling behaviour of domestic pigs in a semi-natural environment. Behav Res Ther. 1985;95:11-25.

35. Counsilman JJ L, LM. The definition of weaning. Anim Behav. 1985;33:1023-4.

36. States THSotU. An HSUS Report: The Welfare of Piglets in the Pig industry. 2010.

37. Fangman TT, Roderick. (1997). Segregated early weaning. Journal of Swine Health and Production.5. .

38. C. P. Disease of high-health pigs: Some ideas on pathogenesis. . 1995:16–7.

39. Brown DC, Maxwell CV, Erf GF, Davis ME, Singh S, Johnson ZB. The influence of different management systems and age on intestinal morphology, immune cell numbers and mucin production from goblet cells in post-weaning pigs. Vet Immunol Immunopathol. 2006;111(3-4):187-98.

40. Moeser AJ, Ryan KA, Nighot PK, Blikslager AT. Gastrointestinal dysfunction induced by early weaning is attenuated by delayed weaning and mast cell blockade in pigs. Am J Physiol-Gastr L. 2007;293(2):G413-G21.

41. Moeser AJ, Blikslager AT. Mechanisms of porcine diarrheal disease. J Am Vet Med Assoc. 2007;231(1):56-67.

42. Medland JE, Pohl CS, Edwards LL, Frandsen S, Bagley K, Li Y, et al. Early life adversity in piglets induces long-term upregulation of the enteric cholinergic nervous system and

heightened, sex-specific secretomotor neuron responses. Neurogastroenterol Motil. 2016;28(9):1317-29.

43. Jamur MC, Oliver C. Origin, maturation and recruitment of mast cell precursors. Front Biosci (Schol Ed). 2011;3:1390-406.

44. Welle M. Development, significance, and heterogeneity of mast cells with particular regard to the mast cell-specific proteases chymase and tryptase. J Leukoc Biol. 1997;61(3):233-45.

45. Abraham SN, St John AL. Mast cell-orchestrated immunity to pathogens. Nat Rev Immunol. 2010;10(6):440-52.

46. Maltby S, Khazaie K, McNagny K. Mast Cells in Tumor Growth: Angiogenesis, Tissue Remodeling and Immune-modulation. Biochim Biophys Acta. 2009;1796:19-26.

47. Pejler G, Rönnberg E, Waern I, Wernersson S. Mast cell proteases: Multifaceted regulators of inflammatory disease. Blood. 2010;115:4981-90.

48. Wouters MM, Vicario M, Santos J. The role of mast cells in functional GI disorders. Gut. 2016;65(1):155-68.

49. Boeckxstaens G. Mast cells and inflammatory bowel disease. Current Opinion in Pharmacology. 2015;25:45-9.

50. Galli SJ, Tsai M. IgE and mast cells in allergic disease. Nature Medicine. 2012;18(5):693-704.

51. Hamilton MJ, Frei SM, Stevens RL. The Multifaceted Mast Cell in Inflammatory Bowel Disease. Inflammatory Bowel Diseases. 2014;20(12):2364-78.

52. Galli SJ, Grimbaldeston M, Tsai M. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. Nat Rev Immunol. 2008;8(6):478-86.

53. Balseiro-Gomez S, Flores JA, Acosta J, Ramirez-Ponce MP, Ales E. Transient fusion ensures granule replenishment to enable repeated release after IgE-mediated mast cell degranulation. J Cell Sci. 2016;129(21):3989-4000.

54. Alber G, Kent UM, Metzger H. Functional comparison of Fc epsilon RI, Fc gamma RII, and Fc gamma RIII in mast cells. J Immunol. 1992;149(7):2428-36.

55. Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. Nat Immunol. 2005;6(2):135-42.

56. Burgoyne RD, Morgan A. Secretory granule exocytosis. Physiol Rev. 2003;83(2):581-632.

57. Varadaradjalou S, Feger F, Thieblemont N, Hamouda NB, Pleau JM, Dy M, et al. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells. Eur J Immunol. 2003;33(4):899-906.

58. Okumura S, Kashiwakura J, Tomita H, Matsumoto K, Nakajima T, Saito H, et al. Identification of specific gene expression profiles in human mast cells mediated by Toll-like receptor 4 and FcepsilonRI. Blood. 2003;102(7):2547-54.

59. Schwartz LB, Irani AM, Roller K, Castells MC, Schechter NM. Quantitation of histamine, tryptase, and chymase in dispersed human T and TC mast cells. J Immunol. 1987;138(8):2611-5.

60. Schwartz LB, Lewis RA, Austen KF. Tryptase from human pulmonary mast cells. Purification and characterization. J Biol Chem. 1981;256(22):11939-43.

61. Hallgren J, Pejler G. Biology of mast cell tryptase - An inflammatory mediator. Febs J. 2006;273(9):1871-95.

62. Nadel JA. Biology of Mast-Cell Tryptase and Chymase. Ann Ny Acad Sci. 1991;629:319-31.

63. Nigrovic PA, Binstadt BA, Monach PA, Johnsen A, Gurish M, Iwakura Y, et al. Mast cells contribute to initiation of autoantibody-mediated arthritis via IL-1. Proc Natl Acad Sci U S A. 2007;104(7):2325-30.

64. Stephen T. Holgate DHB, Martin K. Church, Fernando D. Martinez. Allergy 4th Edition. Allergy. 4 ed: ELSEVIER SAUNDERS; 2012. p. i-iii.

65. Maintz L, Novak N. Histamine and histamine intolerance. Am J Clin Nutr. 2007;85(5):1185-96.

66. Sattler J, Hafner D, Klotter HJ, Lorenz W, Wagner PK. Food-induced histaminosis as an epidemiological problem: plasma histamine elevation and haemodynamic alterations after oral histamine administration and blockade of diamine oxidase (DAO). Agents Actions. 1988;23(3-4):361-5.

67. Jarisch R, Wantke F. Wine and headache. Int Arch Allergy Immunol. 1996;110(1):7-12.

68. Mennigen R, Kusche J, Streffer C, Krakamp B. Diamine oxidase activities in the large bowel mucosa of ulcerative colitis patients. Agents Actions. 1990;30(1-2):264-6.

69. Schmidt WU, Sattler J, Hesterberg R, Roher HD, Zoedler T, Sitter H, et al. Human intestinal diamine oxidase (DAO) activity in Crohn's disease: a new marker for disease assessment? Agents Actions. 1990;30(1-2):267-70.

70. Yamauchi K, Sekizawa K, Suzuki H, Nakazawa H, Ohkawara Y, Katayose D, et al. Structure and function of human histamine N-methyltransferase: critical enzyme in histamine metabolism in airway. Am J Physiol. 1994;267(3 Pt 1):L342-9.

71. Yan L, Galinsky R, Bernstein J, Liggett S, Weinshilboum R. Histamine N-methyltransferase pharmacogenetics: Association of a common functional polymorphism with asthma. Pharmacogenetics. 2000;10:261-6.

72. Smolinska S, Jutel M, Crameri R, O'Mahony L. Histamine and gut mucosal immune regulation. Allergy. 2014;69(3):273-81.

73. Lichtenstein LM, Gillespie E. The effects of the H1 and H2 antihistamines on "allergic" histamine release and its inhibition by histamine. J Pharmacol Exp Ther. 1975;192(2):441-50.

74. Thurmond RL, Gelfand EW, Dunford PJ. The role of histamine H1 and H4 receptors in allergic inflammation: the search for new antihistamines. Nat Rev Drug Discov. 2008;7(1):41-53.

75. Wechsler JB, Szabo A, Hsu CL, Krier-Burris RA, Schroeder HA, Wang MY, et al. Histamine drives severity of innate inflammation via histamine 4 receptor in murine experimental colitis. Mucosal Immunol. 2018;11(3):861-70.

76. O'Reilly M, Alpert R, Jenkinson S, Gladue RP, Foo S, Trim S, et al. Identification of a histamine H4 receptor on human eosinophils--role in eosinophil chemotaxis. J Recept Signal Transduct Res. 2002;22(1-4):431-48.

77. Hofstra CL, Desai PJ, Thurmond RL, Fung-Leung WP. Histamine H4 receptor mediates chemotaxis and calcium mobilization of mast cells. J Pharmacol Exp Ther. 2003;305(3):1212-21.

78. Akdis CA, Blaser K. Histamine in the immune regulation of allergic inflammation. J Allergy Clin Immunol. 2003;112(1):15-22.

79. Gao CX, Major A, Rendon D, Lugo M, Jackson V, Shi ZC, et al. Histamine H2 Receptor-Mediated Suppression of Intestinal Inflammation by Probiotic Lactobacillus reuteri. Mbio. 2015;6(6).

80. Inan A, Sen M, Surgit O, Ergin M, Bozer M. Effects of the histamine H2 receptor antagonist famotidine on the healing of colonic anastomosis in rats. Clinics (Sao Paulo). 2009;64(6):567-70.

81. Overman EL, Rivier JE, Moeser AJ. CRF induces intestinal epithelial barrier injury via the release of mast cell proteases and TNF-alpha. PLoS One. 2012;7(6):e39935.

CHAPTER 2

Influence of Piglet Postnatal Age on Intestinal Mast Cell Activation Induced by Weaning Stress

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Keywords

Early weaning stress, early life adversity, histamine, histamine receptors, chymase, tryptase

Abstract

Early life adversity (ELA) is a risk factor for later life emergence of functional and inflammatory GI disorders in people and animals. In this study we compared the early GI immune responses in piglets exposed to early weaning (EW) at 15-16d of age, a form of ELA we've previously shown results in altered GI developmental and health trajectories similar to ELA in humans, with that of late weaned piglets (LW: 26 d of wean age). Mast cells (MC), a critical innate immune cell activated by stress and a modulator of GI immune regulation, were activated early and differentially expressed in early weaned and late weaned pigs. Compared with late weaned piglets, early weaned piglets exhibited higher levels of plasma histamine during the first 1h post-weaning which coincided with a significant upregulation in histamine degrading enzymes diamine oxidase expression in jejunum mucosa. Early weaned pigs exhibited a reduction of mast cell tryptase by 24 hours. However, LW piglets exhibited a greater induction of chymase expression, compared to EW piglets. These data show weaning stress in piglets induces early GI MC activation which precedes immune cell recruitment and intestinal inflammation and age has a significant impact on the level and nature of this response. Understanding how EW and LW differentially regulate early GI immune responses may lead to a more mechanistic understanding of the GI and immune disorders linked with ELA.

Introduction

Early life adversity (ELA), such as abuse, neglect and household dysfunction are significant risk factors (3, 4, 82, 83) for the development of debilitating chronic immune disorders, such as functional gastrointestinal disorders (FGID) including irritable bowel syndrome and inflammatory bowel disease later in life (3, 4, 84). Despite this well-established link between ELA and disease risk in humans and animal models of early life adversity, the biological mechanisms by which ELA triggers long-lasting disease risk remains unknown.

The early postnatal period of gastrointestinal (GI) development, during the first three months after birth, is highly malleable and quiescent(11). During this critical window of development there is continuous programming in the enteric nervous system, establishment of the microbiome, intestinal barrier integrity, and the immune system. If quiescence (continuous uninterrupted programming) during GI development is disrupted, negative long-lasting disturbances like diarrhea, intestinal permeability, and inflammation occurs in adulthood (9, 84, 85). This has been shown for human GI disease and animal models of ELA, including weaning stress (EWS) in pigs and neonatal maternal stress (NMS) in rodents. In neonatal maternal stress models, pups are separated from their dam for approximately 3 hours a day during days 2-14 or days 4-20 after birth; this time period varies depending on rodent species (10). NMS has been shown to increase intestinal permeability, susceptibility to disease (colitis), visceral hypersensitivity and mast cell activation(32, 86-88). NMS is the most well studied and characterized model of ELA adversity that mimics some of the major pathophysiology related to human GI disease but is not without limitations. In contrast, pigs are more biologically comparable to humans, in terms of central nervous and enteric nervous system complexity, size, diet, anatomy and development (10). Thus EWS porcine model is well suited to study ELA. Also studying EWS in pigs has a dual purpose as it explains the effects of ELA in agricultural animal species which are an important resource. During the EWS piglets are early weaned at 14-18 days of age and compared to later weaned, control (24 or greater days of age) (10).

Compared to piglets that are gradually weaned at 3 months of age in nature, EWS piglets experience multiple abrupt changes such as change in diet, maternal and littermate separation, commingling with new littermates (crowding), fighting for social order, new environment, vaccinations, and transportation stressors(89). These abrupt stressors have both short term and long-term effects that impact lead to a decrease in feed intake, performance and disease susceptibility. In previous studies, EWS was shown to also increase intestinal permeability, diarrhea, susceptibility to infection, and mast cell activation (9, 31, 42, 90) like GI disturbances in humans. While previous studies on early weaning stress focused on the long-term effects of EWS on intestinal permeability, mast cell numbers and activation, the short-term effects of EWS and mechanism have not been elucidated. The objective of the present experiments was to determine the influence of age on weaning stress-induced MC activity in relation to intestinal barrier function and inflammatory responses. By defining the time course and profile of MC mediator activation induced by EWS, we hope these studies will provide the foundation for future research focused on targeting specific mast cell pathways for the prevention of ELA-induced GI injury and long-term GI development and disease throughout the lifespan.

Methods

Animals and experimental design

The University Institutional Animal Care and Use Committee (IACUC) at Michigan State University approved all studies. Yorkshire cross-bred female and male-castrated nursing piglets were obtained from the Michigan State University Swine and Teaching Unit and used in these experiments. At 10-12 d of age, litters from parity 3-4 sows were weighed randomly assigned to experimental groups. Experimental variable which constituted the experimental groups included wean age: 15 days of age; early weaning (EW) or 26 days of age; late weaning (LW), and post-weaning time point: 0h (unweaned), 30 min, 1h, 3h, 8h, and 24h post-weaning. At respective weaning ages, piglets were weaned from the sow, transported to a nearby swine nursery and housed in nursery pens. Weaned pigs were offered ad libitum access to water. To recapitulate production conditions and prevent the potential confounding effects of feed was withheld from all pigs for up to 24h, At each collection time point, selected piglets n=5-8 pigs/weaning age group/time point were sedated with a combination of xylazine (1.5 mg im) and ketamine (11 mg/kg im) followed by euthanasia with an overdose of intravenous pentobarbital via a catheterized ear vein, followed immediately by tissue sample collection and preparation for histological and gene analyses.

Mast Cell Staining and Counting

Jejunum tissues were collected from EWS and LWC at 15d and 26d of age during a time course (0h, 0.5h,1h,3h,8h,24h) postweaning immediately after euthanasia and fixed in Carnoy's fixative (60% ethanol-30% chloroform-10% glacial acetic acid) for 48h and then transferred to a 70% ethanol/1%sodium azide solution until further processing for routine histological evaluation. Samples were removed and placed in 50% ethanol before processing. For quantification of mucosal mast cells, longitudinal cross sections of jejunum (n=4-6 animals/treatment groups) were sectioned (10 um) then paraffin-embedded and stained with toluidine blue by the Michigan State University Investigative Histopathology Laboratory. Mucosal mast cells were counted in 10

fields per subject and corrected for lamina propria area in the villus tip and villus crypt, using Image J (U.S. NIH, Bethesda, MD, USA). Submucosal mast cells were counted in 10 fields per subject and corrected for lamina propria area using Image J (U.S. NIH, Bethesda, MD, USA). Counts were performed on n=24 EWS and LWC pigs with 4-6 Male-C and females' pigs per group.

Immunohistochemical analyses

Jejunum tissues were taken immediately after euthanasia and stored in Carnoy's fixative (60% ethanol-30% chloroform-10% glacial acetic acid) until processing for routine histological evaluation. Paraffin blocks were sectioned (10 um thick). Sections were prepped and immunohistologically labeled for Histamine Receptors1, 2 and 4 with either anti – goat HR1 (orb331289, Biorbyt) at 1:100 dilution; or polyclonal anti- rabbit HR2 (NLS1175, Novus Biologicals) at 1:100 dilutions; or polyclonal anti- rabbit HR4 (LS-C146254, Lifespan Biosciences) at 1:200 dilution. Detection of HR1 in sections was performed by using secondary anti-mouse-on-HRP Polymer for 10 minutes and treatment with Romulin AEC ; Detection of HR2 and H4R was performed by using secondary anti-rabbit-on- Farma HRP Polymer for 30 minutes and treatment with Betazoid DAB. Slides were counter stained with CATHE Hematoxylin at 1:10 and 1:5 dilution. All sample preparation and labeling was performed by Michigan State University's Investigative Histopathology Laboratory. Total mucosal area of histamine receptor positive and integrated density of Histamine receptors were determined to generally asses the number of positive cells to Histamine receptors and the intensity with which histamine receptors was expressed, respectively.

Gene expression analysis

Immediately after euthanasia, segments of ileum, jejunum, and colon were removed from the pig, cut open longitudinally and rinsed generously with 0.87% saline to remove any digesta prior to sample collection. The scrapings of the exposed mucosa were collected, immediately flash

frozen in liquid N2 and stored at -80°C until gene analysis. Total RNA samples were isolated from frozen intestinal mucosal scrapings using the Qiagen RNeasy Mini kit. First-strand cDNA was synthesized from 1µg RNA using Thermo Scientific Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. Semi-quantitative, real-time PCR was used to determine the relative quantities of transcripts of the genes of interest. The genes encoding the 60S ribosomal subunit (RPL4) were selected and validated as suitable internal reference genes. The relative gene expressions of histamine family genes histamine receptor 1 (H1R), histamine receptor 2 (H2R), histamine receptor 4 (H4R), diamine oxidase (DAO) and histamine-N-methyltransferase (HNMT); mast cell tryptase (MCT7) and mast cell chymase (CMA1) were determined. Primer sequences for all genes are provided as a Supporting Information file (S1 Table). All PCR reactions were subjected to a melt curve analysis to validate the absence of nonspecific products. The data are presented as 2-ΔΔCT in gene expression relative to control group, normalized to the RPL4 reference gene before statistical analysis.

Histamine and diamine oxidase ELISA

Blood samples were obtained from pigs via jugular venipuncture at 0h, 30min, 3h, 8h, and 24 h postweaning prior to euthanasia. Pigs were sedated prior to blood collection to minimize the stress of sampling procedures. All samples were taken at the same time of day to minimize the effects of diurnal rhythms. Plasma was separated by centrifugation (30 min, 10,000 g) aliquoted and then was stored at -80°C until analysis. Plasma levels of histamine and diamine oxidase were determined by using commercial ELISA kits (Histamine, Oxford Biomedical Research, Oxford, MI; porcine diamine oxidase, MyBiosource, San Diego, CA).

Statistical analyses

Data were reported as means ± SE. All data were analyzed using a 2-Way ANOVA using GraphPad Prism version 8 for Windows, (GraphPad Software, San Diego, CA, USA). A post

hoc Tukey's test was used to determine the effects of weaning treatment, weaning age, or interaction and any specific difference between groups. Differences were considered significant at $P<0.05$ to 0.001.

Results

Effect of wean age on plasma histamine responses to weaning

In the present study, we measured plasma histamine levels as an index of mast cell activation in early weaned and late weaned pigs. At baseline (unweaned) 15 d old piglets exhibited higher ($P<0.05$) plasma histamine levels compared with 26 d old piglets (Figure 1). In early weaned piglets, plasma histamine levels increased (by 65%) at 1-hour postweaning and returned to baseline (unweaned) levels by 3 h post-weaning. In contrast, late weaned pigs did not exhibit elevated plasma histamine in response to weaning. Taken together, this data indicates that compared with late weaned piglets, early weaned piglets have higher basal and weaning-induced plasma histamine levels, indicative of greater mast cell activation.

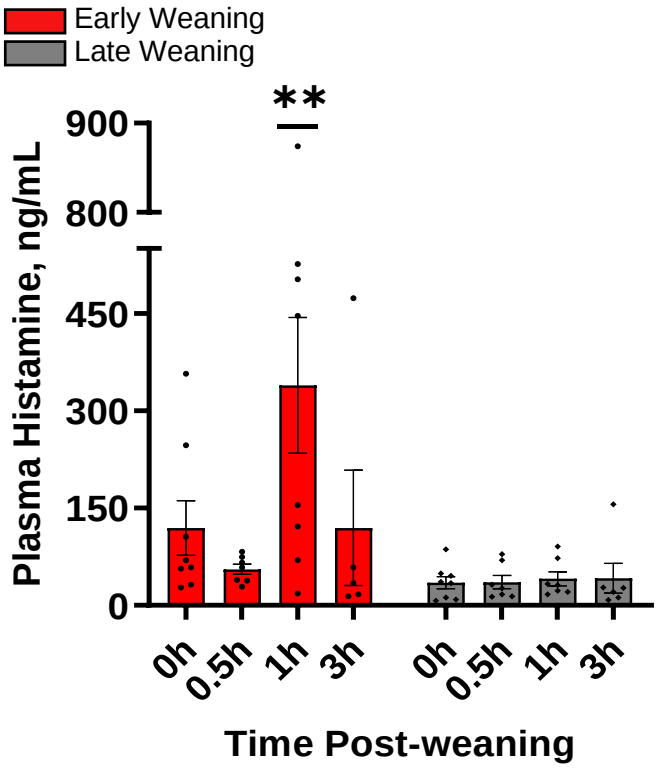

Figure 1. Influence of weaning age on plasma histamine concentrations at 0h, 0.5h, 1h, 3h postweaning. Histamine levels were determined by ELISA in plasma. All values are relative to 0h control. LWC, Late Weaned. EWS, Early Weaned. Data were analyzed with a 2-Way ANOVA Tukey's post hoc test. *P<0.05, **P<0.01, *** P<0.001 vs. Time 0 within each wean age group.

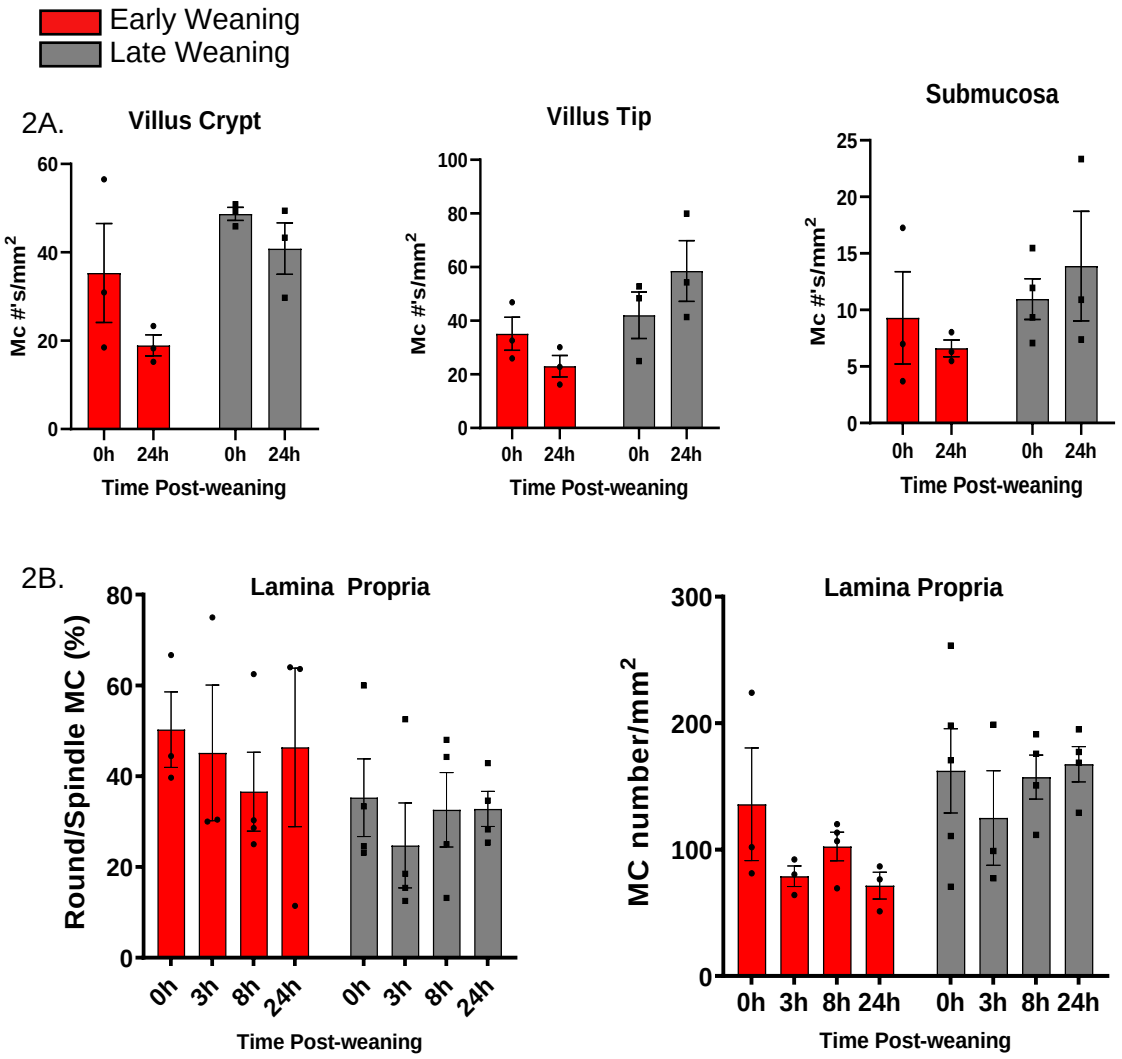

Figure 2. Influence of weaning age on jejunal mast cell numbers, localization and morphology at 0h, 3h, 8h, 24h postweaning. Jejunum sections from pigs were stained with Toluidine blue and toluidine blue cells were quantified to determine mast cell numbers. (A) Jejunum mast cell localization in villus crypt, tip, and submucosa of 15d early weaned and 26d late weaned pigs weaned at 0h and 24h post weaning. (B) Jejunal mast cell morphology and mast cell numbers in lamina propria of 15d early weaned and 26d late weaned pigs weaned at 0h, 3h, 8h, 24h post weaning. Data were analyzed with a 2-Way ANOVA Tukey's post hoc test. *P<0.05, **P<0.01, *** P<0.001 vs. Time 0 within each wean age group.

Effects of piglet age and weaning on intestinal mast cell numbers and morphology

To determine whether heightened plasma histamine levels in early weaned piglets coincided with increased intestinal mast cell activity, we analyzed mast cell numbers and morphology in early weaned and late weaned pigs. Mast cell morphology is a potential indicator of mast cell activity in terms of activation status and motility(91). Jejunal mucosal sections were stained with the Toluidine blue to identify mast cells and mast cells numbers and localization in the villus tip lamina propria, crypt lamina propria, submucosa and muscle layer was assessed. Mast cells were localized to the villus tip lamina propria, crypt lamina propria, submucosa, muscle layers, but the highest concentration of mast cells were found in the lamina propria of the crypt region in both 15 d and 26 d old piglets (Figure 2A). Piglet age had a significant effect on mast cell number with younger piglets (15 d old piglets) having lower numbers of toluidine blue positive mast cells compared with 26 d old pigs (Fig 2A). In response to weaning, a reduction in jejunal mast cell numbers was observed in early weaned piglets, indicative of mast cell activation and loss of granule staining properties. The morphology of mast cells determined by rounded vs fusiform shape, also differed by age with 15 d old piglets exhibiting a higher percentage of rounded mast cells compared with 26 d old pigs (Fig 2B).

Intestinal mucosal histamine degrading enzyme expression in response to early weaning stress in piglets

Extracellular histamine levels is regulated predominantly by two major histamine degrading enzymes, which are diamine oxidase (*DAO*) and histamine-n-methyltransferase (*HNMT*). To determine if elevated histamine responses in early weaned pigs was due to reduced histamine degrading enzymes, we measured DAO and HNMT gene expression in jejunum, ileum and colon mucosa. In early weaned pigs, DAO was upregulated at 3h (4.1- Fold increase; $p<0.001$) and 8h (3.38-Fold increase; $p<0.05$) and then declined by 24h in the jejunum (Figure 3A). In late weaned pigs, DAO was upregulated at 8h (Figure 3) and increased by ~2.76-fold ($p<0.05$) at 8h in late weaned controls. There was no significant difference in DAO expression in the ileum

(Figure 4) or colon (Figure 5) or in the plasma. There was no significant difference in HNMT expression in the jejunum, ileum, and colon of early weaned stress or late weaned control piglets.

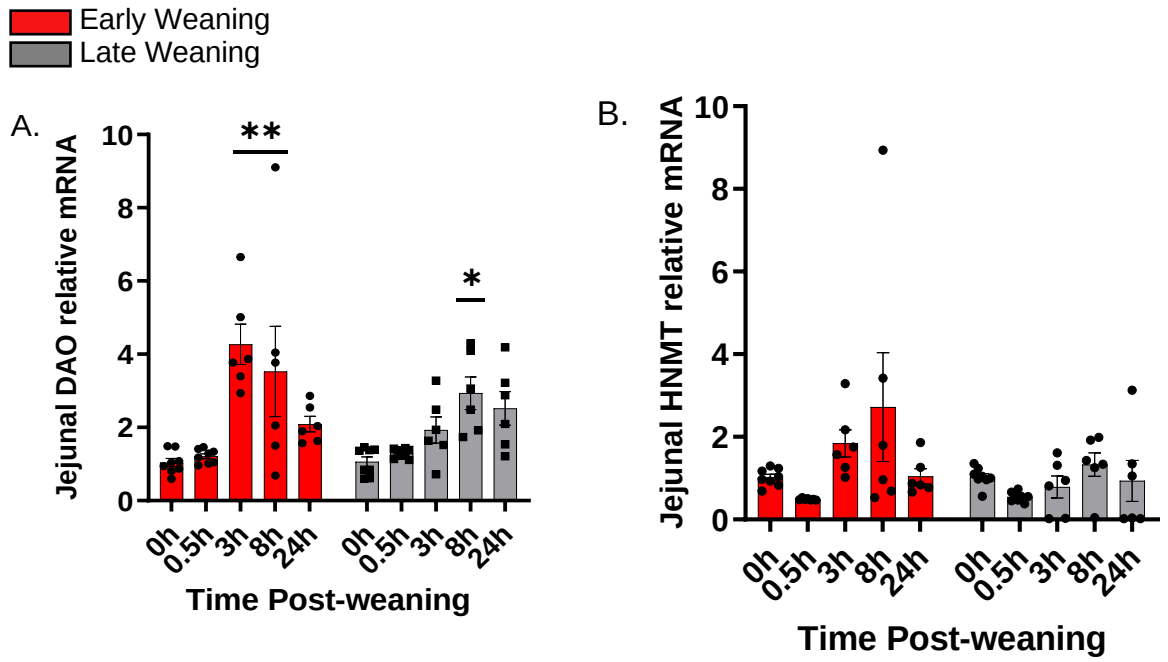

Figure 3. Effect of wean age on histamine degrading enzyme expression in jejunal mucosa from piglets. Piglets were weaned either at 15 d of age (early weaning stress; EWS) or 26 d of age (Late weaning control; LWC). Data were expressed as the mean ± SE fold increase in mRNA expression for (A) DAO, (B) HNMT at each post-weaning time point, relative to the Time 0 (unweaned controls) within each wean age group. Data were analyzed with a 2-Way ANOVA Tukey's post hoc test. *P<0.05, **P<0.01, *** P<0.001 vs. Time 0 within each wean age group.

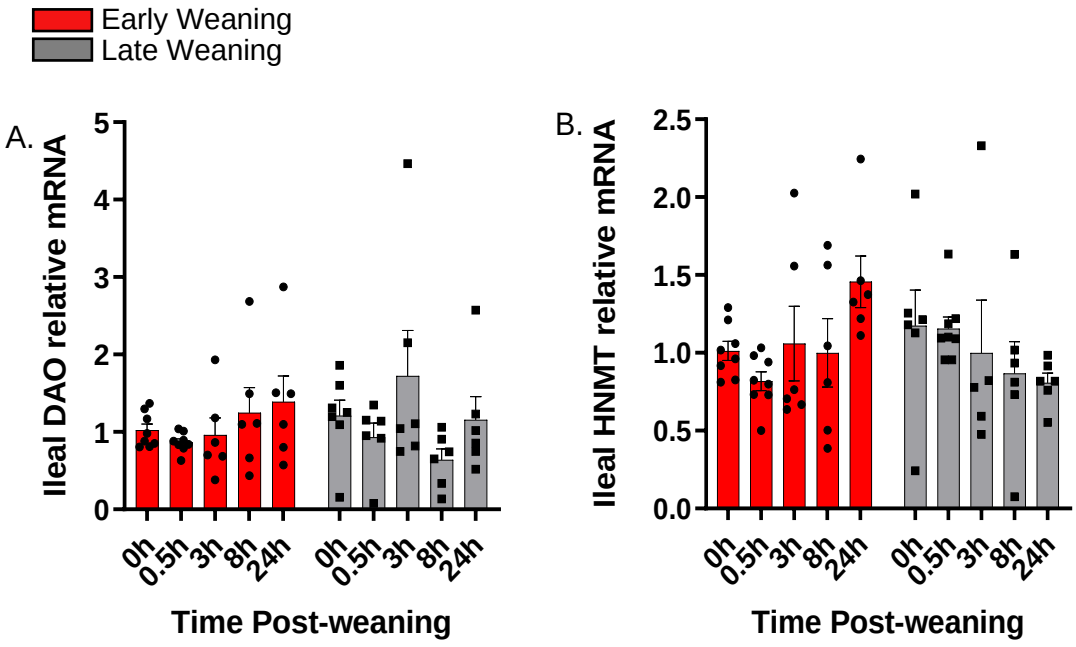

Figure 4. Effect of wean age on histamine degrading enzyme expression in ileal mucosa from piglets. Piglets were weaned either at 15 d of age (early weaning stress; EWS) or 26 d of age (Late weaning control; LWC). Data were expressed as the mean ± SE fold increase in mRNA expression for (A) DAO, (B) HNMT at each post-weaning time point, relative to the Time 0 (unweaned controls) within each wean age group. Data were analyzed with a 2-Way ANOVA Tukey's post hoc test. *P<0.05, **P<0.01, *** P<0.001 vs. Time 0 within each wean age group.

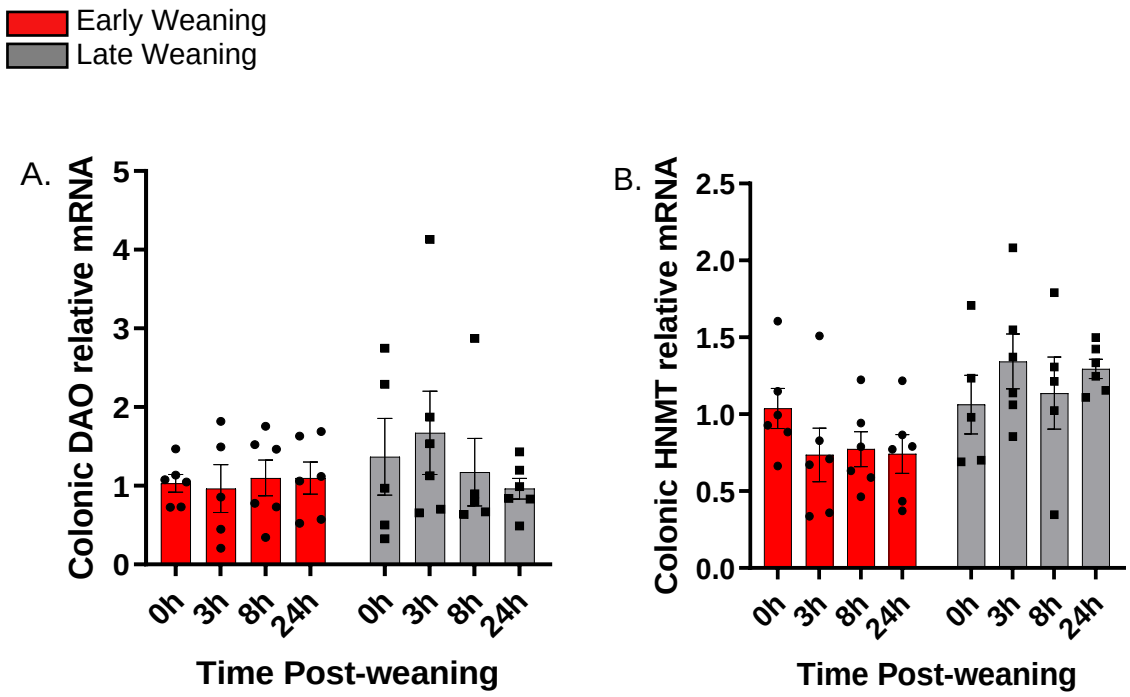

Figure 5. Effect of wean age on histamine degrading enzyme expression in colonic mucosa from piglets. Piglets were weaned either at 15 d of age (early weaning stress; EWS) or 26 d of age (Late weaning control; LWC). Data were expressed as the mean ± SE fold increase in mRNA expression for (A) DAO, (B) HNMT at each post-weaning time point, relative to the Time 0 (unweaned controls) within each wean age group. Data were analyzed with a 2-Way ANOVA Tukey's post hoc test. *P<0.05, **P<0.01, *** P<0.001 vs. Time 0 within each wean age group.

Effects of piglet age and weaning on intestinal mucosal histamine receptor subtype gene expression

Given the heightened histamine levels and mast cell activation observed in EW vs LW piglets, we next investigated the effects of piglet and weaning on downstream histamine receptor expression. We measured histamine receptor subtypes H1R, H2R and H4R gene expression at 0h, 3h, 8h, and 24h post-weaning in ileum, jejunum, and colon via qPCR. In the jejunum H1R receptor gene expression was not significantly altered in early weaned pigs or late weaned piglets(Figure 6A). In jejunum H2R gene expression, exhibited a 2.6 fold ($p<0.05$), increase within 3h post-weaning and remained elevated over the 24h ($p<0.0001$) time period post-weaning (Figure 6B) in early weaned stress piglets compared to the 0h post-weaning time point. In contrast, no significant changes in H2R expression were observed in LW piglet jejunal mucosa. Jejunal mucosal H4R gene expression also increased significantly by 3 h post-weaning reaching a peak expression at 8h (~4.4 fold increase) postweaning (Figure 6C) in early weaned piglets compared to late weaned 0h post-weaning time point with no change in H4R expression in late weaned controls.

Because, we were also interested in measuring site specific changes in gene expression across multiple intestinal regions. Therefore, we measured histamine receptor expression in ileum and colon. In the ileum, the mRNA transcripts for H2R were upregulated at 3h and 8h in both early weaned stressed piglets and late weaned controls when compared to 0h post weaning time point (Figure 7). H4R gene expression increased 8h postweaning in early weaned stress piglets with no significant difference in late weaned control piglets. Specifically, H2R gene expression was increased at 3h (~2.06-fold increase; $p<0.05$) and 8h (~2.09-fold increase; $p<0.05$) in early weaned stress piglets and ~2.98-fold at 3h and ~2.5-fold at 8h in late weaned controls. H4R mRNA transcripts were upregulated (2.98-fold) at 3h and (~3.08-fold) at 8h postweaning in early weaned piglets, but not late weaned piglets when compared to their respective 0h post-weaning timepoint (Figure 7C). In the colon, there was no significant difference in H1R or H2R

expression between early weaned stress or late weaned control piglets when compared to 0h time point. H4R gene expression in the colon was upregulated (~1.9-fold) at 8h in early weaned piglets when compared to 0h time point postweaning (Figure 8); however, no significant differences were observed in late weaned piglets.

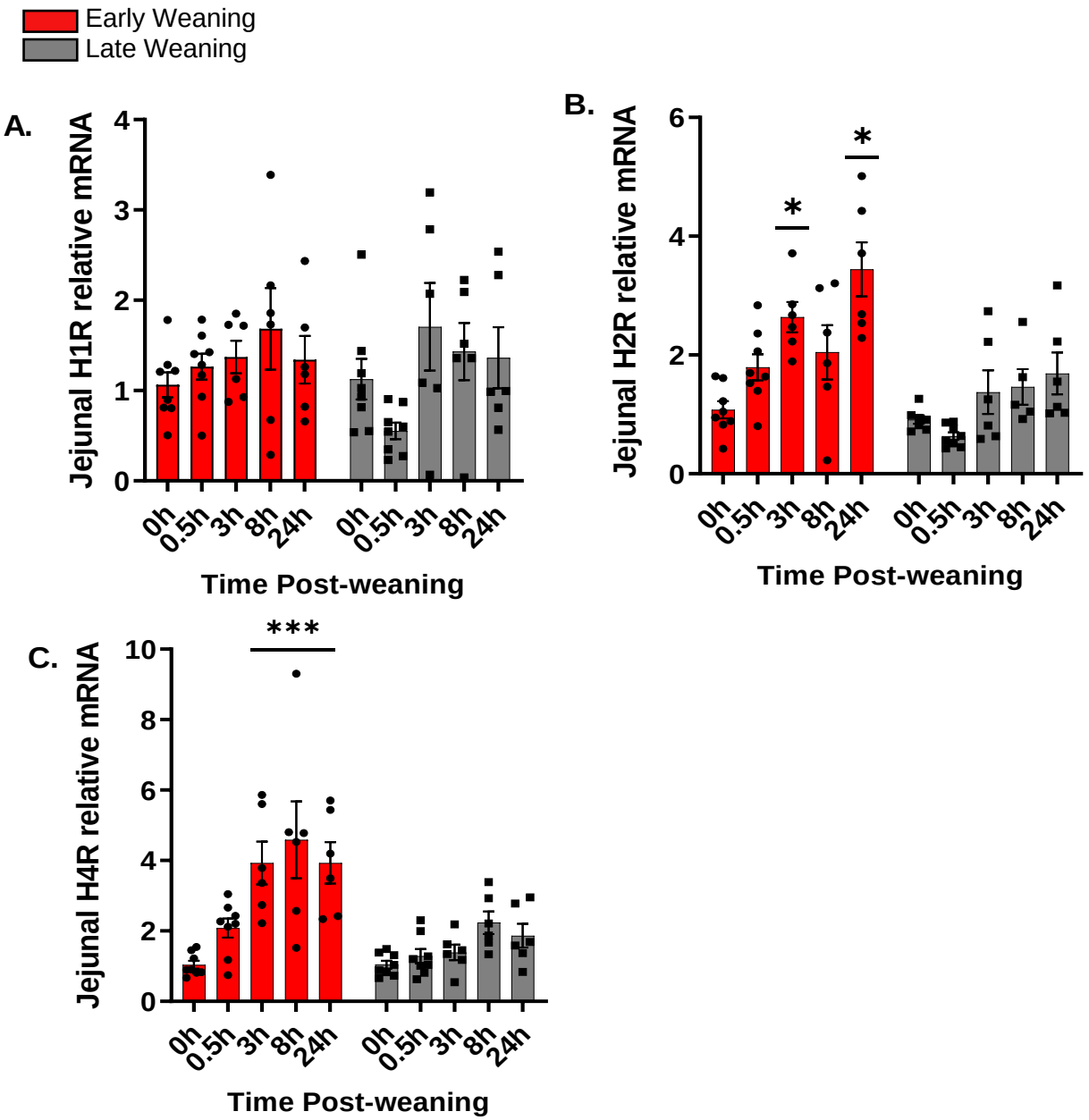

Figure 6. Effect of wean age on histamine receptor subtype expression in jejunal mucosa from piglets. Piglets were weaned either at 15 d of age (early weaning stress; EWS) or 26 d of age (Late weaning control; LWC). Data were expressed as the mean ± SE fold increase in mRNA expression for (A) *H1R*, (B) *H2R*, and (C) *H4R* at each post-weaning time point, relative to the Time 0 (unweaned controls) within each wean age group. Data were analyzed with a 2-Way ANOVA Tukey's post hoc test. *P<0.05, **P<0.01, *** P<0.001 vs. Time 0 within each wean age group.

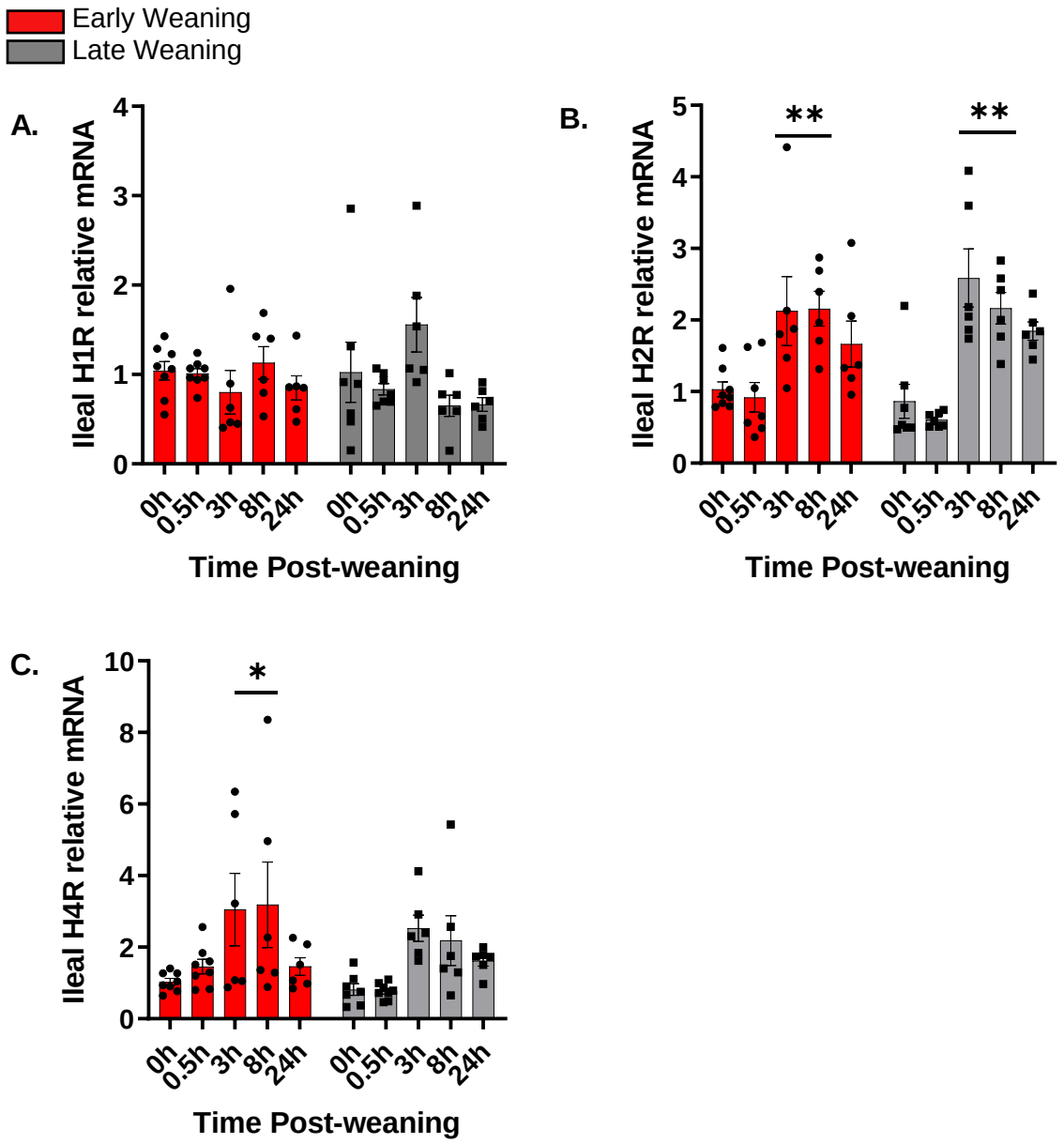

Figure 7. Effect of wean age on histamine receptor subtype expression in ileal mucosa from piglets. Piglets were weaned either at 15 d of age (early weaning stress; EWS) or 26 d of age (Late weaning control; LWC). Data were expressed as the mean ± SE fold increase in mRNA expression for (A) H1R, (B) H2R, and (C) H4R at each post-weaning time point, relative to the Time 0 (unweaned controls) within each wean age group. Data were analyzed with a 2-Way ANOVA Tukey's post hoc test. *P<0.05, **P<0.01, *** P<0.001 vs. Time 0 within each wean age group.

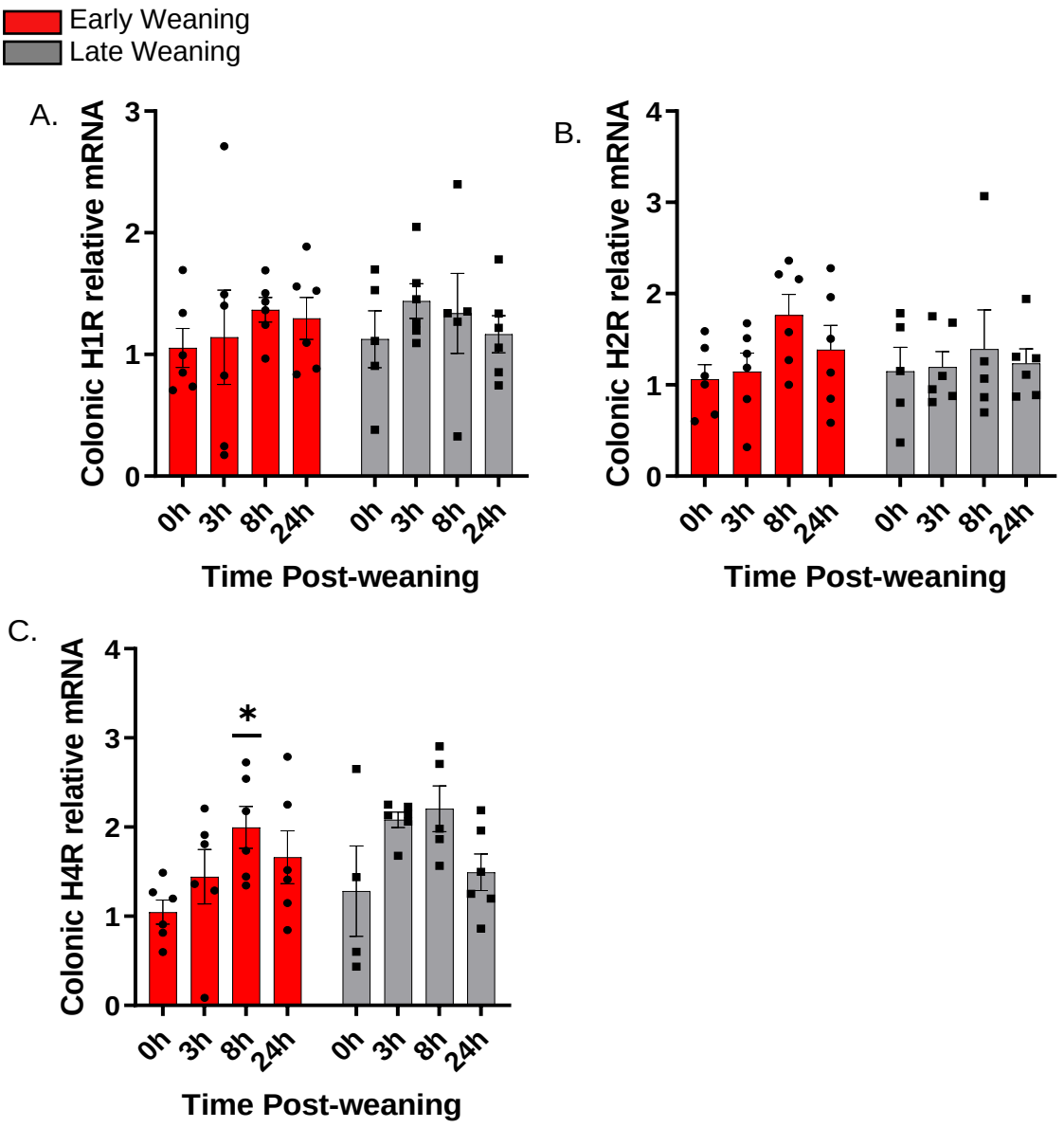

Figure 8. Effect of wean age on histamine receptor subtype expression in colonic mucosa from piglets. Piglets were weaned either at 15 d of age (early weaning stress; EWS) or 26 d of age (Late weaning control; LWC). Data were expressed as the mean ± SE fold increase in mRNA expression for (A) H1R, (B) H2R, and (C) H4R at each post-weaning time point, relative to the Time 0 (unweaned controls) within each wean age group. Data were analyzed with a 2-Way ANOVA Tukey's post hoc test. *P<0.05, **P<0.01, *** P<0.001 vs. Time 0 within each wean age group.

Intestinal mucosal mast cell tryptase expression in response to early weaning stress in piglets

Mast cells are critical innate immune effector cells that are known to be activated in response to weaning stress and release mast cell specific proteases that are involved in the inflammatory response. We measured the jejunum, ileum and colon mucosa of both late weaned control and early weaned stress piglets at 15 days and 26 days of age for tryptase (*MCT7*) gene expression levels. In ileal mucosa from EW piglets, there was a 66% downregulation of tryptase gene expression at 24hr compared with 0h post weaning time point with no significant difference in late weaned control when compared to the 0h post weaning time point (Figure 9). There was no significant difference in tryptase expression in the jejunum or colon of early weaned stress or late weaned control piglets when compared to 0h post weaning time point.

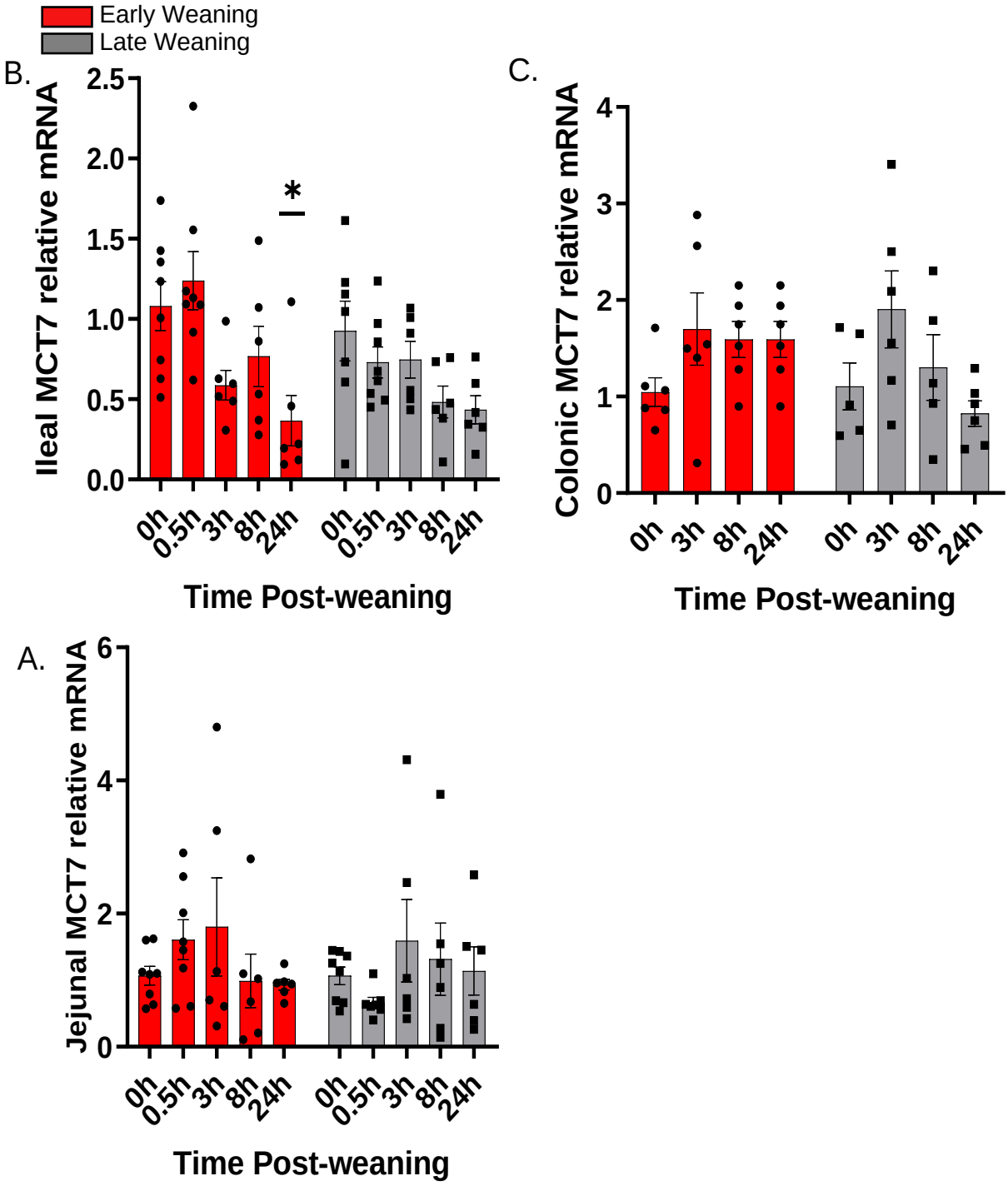

Figure 9. Effect of wean age on mast cell tryptase in jejunal, ileal, and colonic mucosa from piglets. Piglets were weaned either at 15 d of age (early weaning stress; EWS) or 26 d of age (Late weaning control; LWC). Data were expressed as the mean ± SE fold increase in mRNA expression in (A) Jejunum, (B) Ileal, (C) colon at each post-weaning time point, relative to the Time 0 (unweaned controls) within each wean age group. Data were analyzed with a 2-Way ANOVA Tukey's post hoc test. *P<0.05, **P<0.01, *** P<0.001 vs. Time 0 within each wean age group.

Intestinal mucosal mast cell chymase expression in response to weaning stress in piglets

Mast cells are critical innate immune effector cells that are known to be activated in response to weaning stress and release mast cell specific proteases that are involved in the inflammatory response. We measured the ileum and colon mucosa of both late weaned control and early weaned stress piglets at 15 days and 26 days of age for chymase (*CMA1*) gene expression levels. In ileal mucosa from LW piglets, there was an upregulation of chymase gene expression at 3hr compared with 0h post weaning time point with no significant difference in early weaned piglets when compared to 0h post weaning time point (Figure 10). Specifically, chymase was increased by 69% in late weaned piglets (Figure 10). There was no significant difference in chymase expression in the colon of early weaned stress or late weaned control piglets when compared to the 0h post weaning time point.

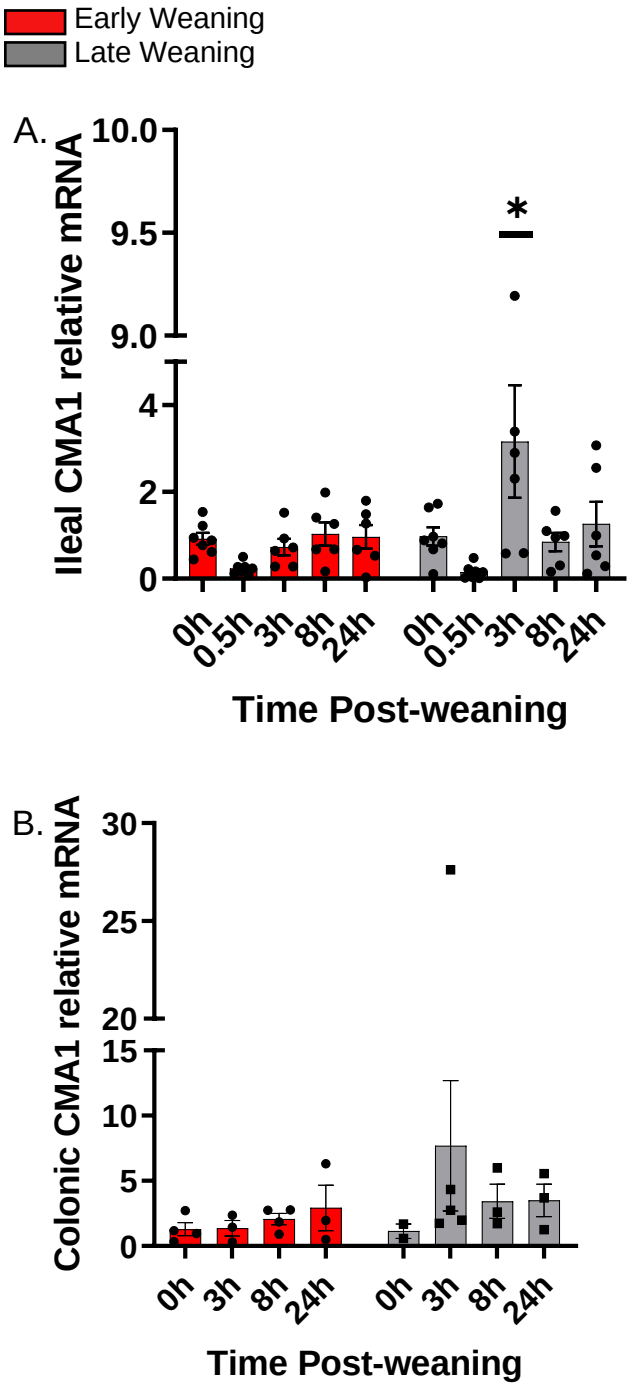

Figure 10. Effect of wean age on mast cell chymase in ileal and colonic mucosa from piglets. Piglets were weaned either at 15 d of age (early weaning stress; EWS) or 26 d of age (Late weaning control; LWC). Data were expressed as the mean ± SE fold increase in mRNA expression in (A) Ileal, (B) colon at each post-weaning time point, relative to the Time 0 (unweaned controls) within each wean age group. Data were analyzed with a 2-Way ANOVA Tukey's post hoc test. *P<0.05, **P<0.01, *** P<0.001 vs. Time 0 within each wean age group.

Histamine receptor subtype immunohistochemistry expression in jejunum mucosal

Given the differential gene expression in histamine receptors, with an upregulation of histamine receptors in early weaned pigs, we also wanted to know if weaning-induced stress impacts histamine receptor expression differentially expressed at the gut level in early and late weaned piglets. Therefore, we stained the jejunum mucosa for histamine 1 receptor, histamine 2 receptor, and histamine 4 receptor to determine the localization pattern. The staining pattern of Histamine 1 receptor (Figure 11 A, B) was localized to lamina propria cells, myofibroblast and muscle tissue. Histamine 4 receptor (Figure 13 A, B) was also localized to cells in the lamina propria. While Histamine 2 receptor (Figure 12 A, B) was localized to the epithelium and epithelial cells.

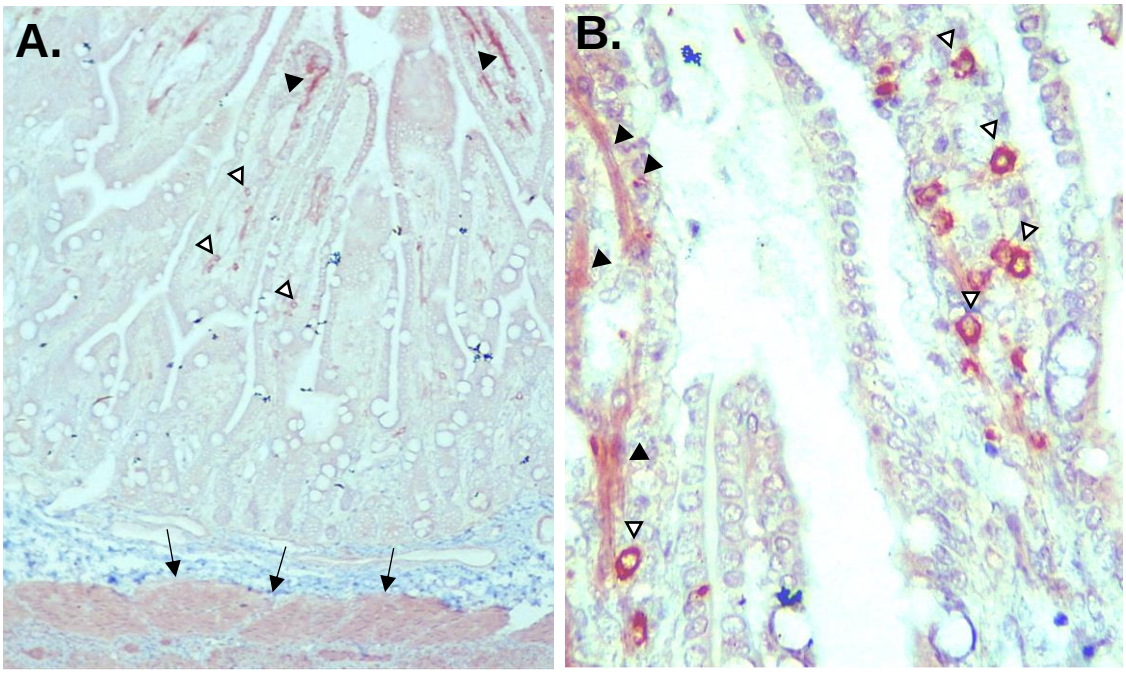

Figure 11. Histamine receptor immunohistochemical localization of the piglet jejunum mucosa in weaning induced stress piglets. (A) Longitudinal section of piglet mucosa with Histamine 1 receptor (H1R) expression in the muscle (black arrows). (B) magnified image demonstrating intensely stained myofibroblast (black arrowheads) and lamina propria cells (white arrowheads).

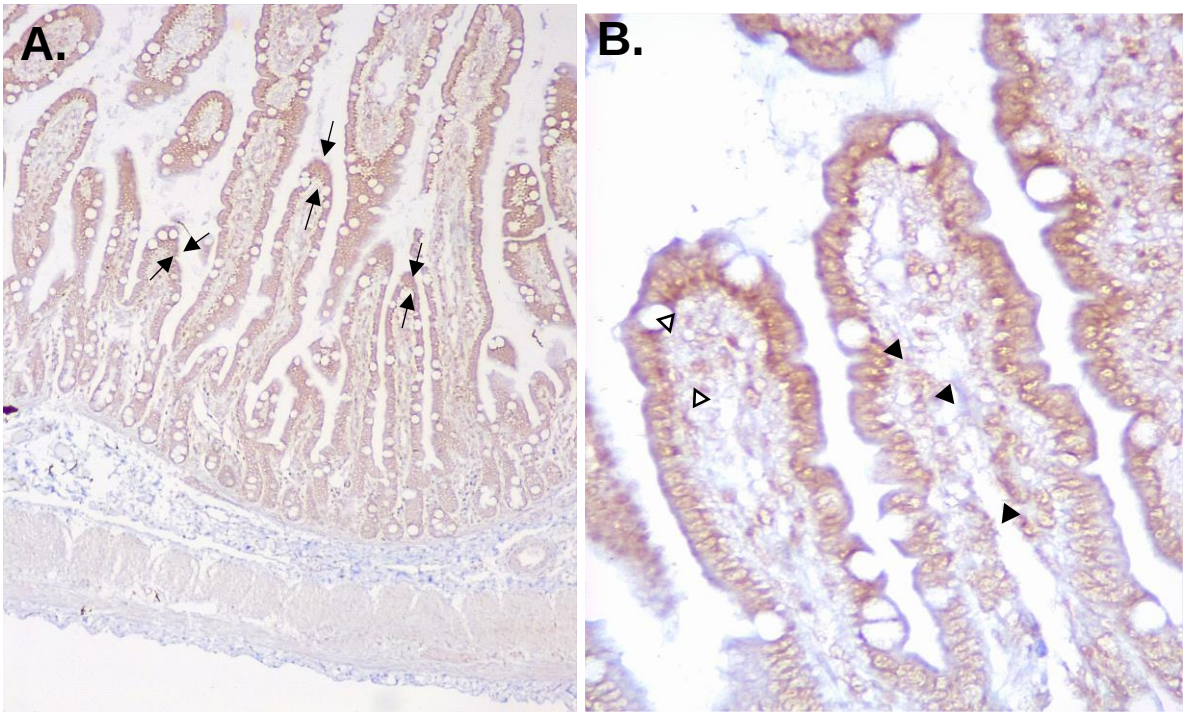

Figure 12. Histamine receptor immunohistochemical localization of the piglet jejunum mucosa in weaning induced stress piglets. (A) Longitudinal section of piglet mucosa with Histamine 2 receptor (H2R) expression in the epithelium and lamina propria cells (black arrows). (B) magnified image demonstrating intensely stained epithelium (white arrowheads) and lamina propria cells (black arrowheads).

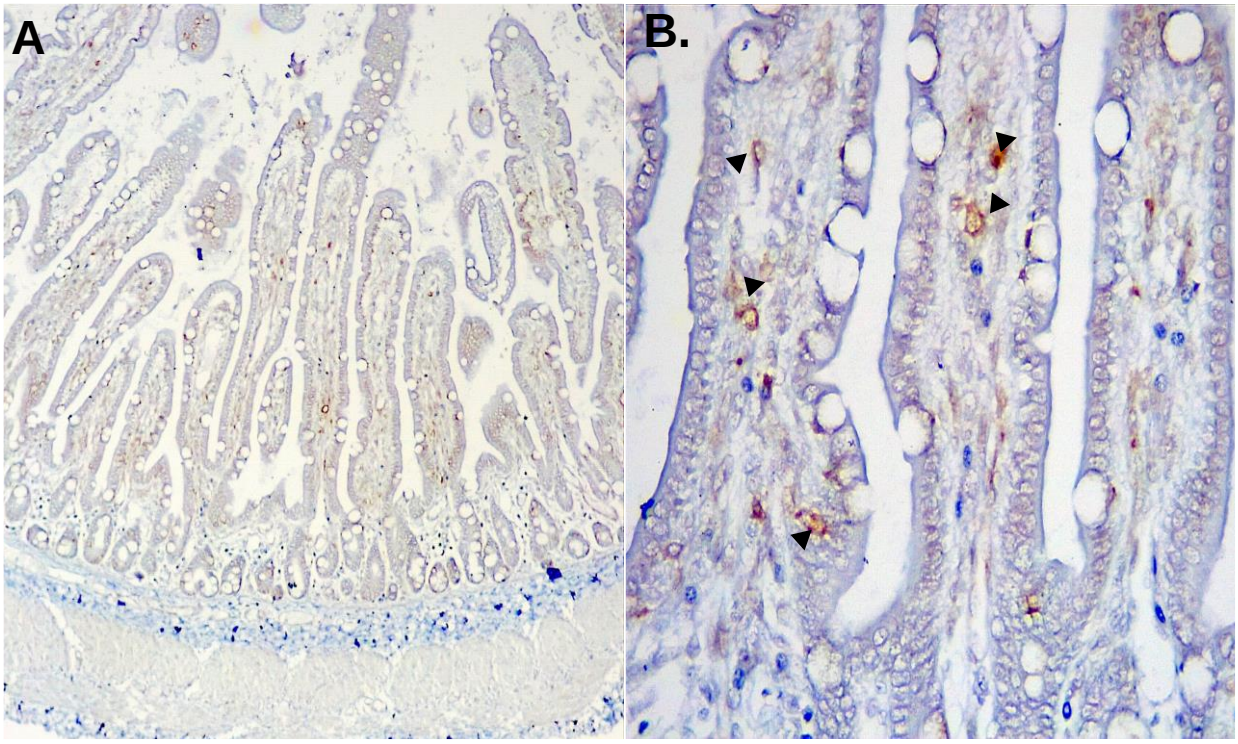

Figure 13 Histamine receptor immunohistochemical localization of the piglet jejunum mucosa in weaning induced stress piglets. (A) Longitudinal section of piglet mucosa with Histamine 4 receptor (H4R) expression in the jejunum mucosa. (B) magnified image demonstrating intensely stained lamina propria cells (black arrowheads).

Discussion

Early life adversity is a major risk factor for the development of functional GI disorders in both humans and livestock. The biological mechanisms by which ELA triggers long-lasting disease risk remains to be elucidated. Here we show age-related acute short-term changes in histamine release and differential expression of histamine receptors are induced by early weaning stress in piglets. This study also reveals that pigs exposed to early weaning stress exhibited no change in mast cell numbers during the time course but an increase in histamine receptor localization compared to late weaned piglets.

Age influences plasma histamine response to weaning

Compared with late weaned piglets, early weaned piglets exhibited an acute and marked increase in plasma histamine concentration at 1-hour post-weaning indicating a greater activation of mast cells. This is in line with our hypothesis that early weaned piglets are more susceptible to weaning stressors and exhibit more severe intestinal barrier dysfunction (increased intestinal permeability) which was shown to be mast cell dependent (11). These results are also in line with Ahrens et al. who conducted an experiment measuring plasma histamine concentrations after early weaning and immune challenge (immunization)(92). The time course of the plasma histamine response in early weaned pigs also revealed the transient nature of circulating histamine levels in early weaned pigs with higher baseline plasma histamine that decreased at 30 min prior to the rise at 1 h and subsequent returns to baseline levels by 3 h post weaning. Given that mast cell histamine can be released in seconds to minutes then become elevated into circulation as quick as 5-10 minutes (64) and that this response is transient, it is possible we may have missed a primary histamine response between 0 and 30 min and that the 1 h elevation may represent a secondary phase in plasma histamine levels. This may also explain why late-weaned pigs exhibited no rise in plasma histamine, which was unexpected as mast cell activation likely occurred in last weaned animals as well, based on intestinal measurements of histamine receptor expression. An earlier time course of plasma

histamine will be required to confirm this. However, in support of this, our histological analysis showed that mast cells from early weaned pigs were more activated in appearance at baseline (e.g. rounded mast cell phenotype) and that mast cell granule staining with toluidine blue was rapidly lost after weaning, indicative of mast cell degranulation. In other words, toluidine blue stains mast cell granules and does not always capture mast cells that have degranulated and maybe in the process of regranulation. Therefore, we have concluded that a decrease in mast cell numbers may be due to loss in granularity and can also represent an increase in mast cell activity in early weaned pigs. This suggest that early weaned piglets indeed have hyperactive mast cells which is contributing to the increased magnitude in GI disturbances in early weaned piglets compared to late weaned piglets.

Differential Histamine receptor expression and localization to the lamina propria in early and late weaned pigs

In the present study we showed that histamine receptor subtype expression is influenced by weaning and wean age. A robust induction of H2R and H24 gene expression was observed in early and late weaned pigs in the jejunum and ileum. Moreover, the induction of H2R and H4R appeared to be greater in early weaned pigs compared with late weaned pigs. It was surprising that H1R, the most highly expressed histamine receptor subtype that is most well-studied in inflammatory and allergic diseases (78) was not increased post-weaning. These data may indicate an important role of H2R and H4R in intestinal responses to weaning. H2R is most well-known for its regulation of histamine-mediated gastric acid secretion. We showed that H2R is expressed in the intestinal mucosa and IHC showed that H2R is localized to the epithelium, lamina propria cells and enteric ganglia. H4R had a slightly different localization pattern as it was mainly expressed in lamina propria cells. This expression pattern is in line with the literature(93, 94). The weaning induced recruitment of H2R-expressing and H4R-expressing immune cells to the intestinal mucosa that might contribute to the increased expression. The

precise role of histamine receptor subtype in weaning-induced intestinal injury in the pig remains to be elucidated.

The staining pattern of Histamine 1 receptor (Figure 11 A, B) was localized to lamina propria cells, myofibroblast and muscle tissue. Histamine 4 receptor (Figure 13 A, B) was also localized to cells in the lamina propria. While Histamine 2 receptor (Figure 12 A, B) was localized to the epithelium and epithelial cells.

Potential mechanisms of increased mast cell activation in the early weaned pig

In this study, we showed that early weaned piglets exhibited greater plasma histamine levels and tissue mast cell activation and downstream histamine receptor signaling. However, an important question that remains to be elucidated is why do younger pigs (early weaned) exhibit greater mast cells activation? Lenz et al 2018 showed in that mast cell numbers in the brain change during development when influenced by sexual differentiation(95). Liu et al 2005 also showed that mast cell numbers gradually changed during corneal development from birth to 21days in rodents (96). This indicates that mast cell numbers do in fact change during development in various tissue regions. Analysis of intestinal mast cell numbers between early and late weaned pigs showed that early weaned pigs have lower numbers of intestinal mast cells which is likely related to the less mature immune system in the early weaned piglet. However, these findings indicate that the higher level of mast cell activation in early weaned pigs is not due to increased mast cell numbers. We also hypothesized that higher histamine levels and subsequent receptor expression might be a result of decreased histamine degradative capacity in early weaned pigs so we measured levels of major histamine degrading enzymes DAO and HNMT. However, plasma DAO levels were similar between early and late weaned pigs and weaning induced gene expression for these enzymes that were greater in early weaned piglet intestinal mucosa compared with late weaned piglets. Some other potential mechanisms could be that early weaned pigs exhibited a heightened neuro-endocrine response

leading to enhanced release of mast cell stimuli such as neuropeptides including CRF and substance P. In support of this, Overman and Moeser et al showed that early weaning resulted in greater CRF levels and neuronal-mediated intestinal secretion (40, 81). Future studies investigating the precise mechanisms for elevated mast cell activation in the early weaned piglet will be critical for the design of targeted therapeutic strategies to limit excessive mast cell activation and intestinal injury in the weaned piglet.

Conclusion

Overall these data demonstrate that early weaning stress in pigs has a profound impact early during the weaning process. As previously shown by Moeser et al, physiologic changes at the gut level occur 24 hours post weaning(40). Here in this study we show that mast cell activation in early weaned piglets, shown by the heightened histamine release 1-hour post weaning, occurs relatively early in response to weaning stress. This adds to the growing evidence from our lab that mast cells are at the center of orchestrating acute and long-term changes in gut function. Further characterization of the histamine receptor histochemistry, regarding specific immune cells stained and their localization pattern, could also shed light on the downstream effects of mast degranulation. Modulation of mast cell activity and histamine regulation through its receptors may be a potentially new mechanism that leads to rapid and very early GI disturbances. This study also suggest that therapeutics and intervention strategies may need to be administered early before the process of weaning in piglets begins, particularly when piglets are still nursing. Therefore, early modulation of histamine receptors in stress-induced GI responses may provide a new target for therapeutics and preventative strategies in agricultural early weaning stress response and stress induced GI disease in humans.

APPENDIX

Table 1. Primer sequences and real-time PCR conditions used for gene expression analysis by qRT-PCR

Gene Name	Forward (5'-3')	Reverse (5'-3')
CMA1	TTCACCCGGATCTCCCAT-GA	GAGACACACACTCGGTCT-GG
DAO	Unique Assay ID (BIO RAD Primer)	qSscCID0005156
H1R	ATGGTCATCGCCTTCTGC-AA	GATGAGGGGGTTCAGTGT-GG
H2R	AGGAAAATAGCACCGGGT-CG	AGGCCCCTCGATGATTCT-CT
H4R	TTCTTCGAATTCCTGGCC-CC	TGGCTTTGGCACCTACTG-AG
HNMT	Unique Assay ID (BIO RAD Primer)	qSscCID0003833
MCT7	ATCGTGGGCGGAAAGGAAG	ATTGGTCGAGGCATCTCA-GG
RPL4	GGCGTAAAGCTGCTACCC-TC	GGATCTCTGGGCTTTTCA-AGATT

Table 2. Immunohistochemistry Protocol: Dilution Protocol

Primary Antibody:	Catalog #:	Dilution:	Vendor:	Time in primary
Goat anti – HR1	*orb331289*	*1:100*	*Biorbyt*	*Overnight at 4°C*
Rabbit Polyclonal anti – HR2	*NLS1175*	*1:100*	*Novus Biologicals*	*60 minutes*
Rabbit Polyclonal anti – HR4	*LS-C146254*	*1:200*	*Lifespan Biosciences*	*60 minutes*

Table 3. Immunohistochemistry Protocol: Retrieval Protocol

Primary Antibody:	Pretreatment Reagent:	Pretreatment Time:
Goat anti – HR1	Scytek Tris-EDTA pH 9.0	*30 seconds at 125C then 10 seconds at 90C*
Rabbit anti – HR2	Scytek Tris-EDTA pH 9.0	*30 seconds at 125C then 10 seconds at 90C*
Rabbit anti – HR4	Scytek Tris-EDTA pH 9.0	*30 seconds at 125C then 10 seconds at 90C*

Jejunum tissues were taken immediately after euthanasia and stored in Carnoy's fixative (60% ethanol-30% chloroform-10% glacial acetic acid) until processing for routine histological evaluation. Paraffin blocks were sectioned (10 um thick). Sections were placed on charged slides and dried at 56°C overnight were subsequently de-paraffinized and hydrated to distilled water; followed by Tris Buffered Saline pH 7.4 (Scytek Labs – Logan, UT) for 5 minutes for pH adjustment. Heat Induced Epitope Retrieval performed using the Dako Pascal Micro Processor controlled pressure cooker. (See table) Endogenous peroxidase was blocked in a 3% Hydrogen Peroxide / Methanol bath followed by running tap water distilled water rinses; followed by Tris buffered saline + Tween 20 (Scytek) for 5 minutes. Following pretreatment standard Micro-polymer staining performed at room temperature on the Biocare intelliPATH automated stainer. All staining steps followed by rinses in TBS Autowash Buffer (Biocare – Concord, CA). Non-specific protein blocked using Background Punisher (Biocare) for 5 minutes; primary antibodies were diluted in normal antibody diluent (Scytek) and incubated as listed in table above; followed by :Goat anti – HR1 ProMark™ Goat on Rodent HRP Probe and Polymer detection (Biocare) – 5 minutes. Reaction developed with Romulin AEC (Biocare) – 10 minutes followed by enhancement with DAB Sparkle (Biocare) – 1 minute. Counterstained with CATHE Hematoxylin diluted 1:10 (Biocare) – 5 minutes, dehydrated, cleared and mounted with synthetic mounting media. Rabbit anti-HR2 diluted in normal antibody diluent (Scytek) and incubated as in table above; followed by ProMark™ Rabbit on Farma HRP-Polymer detection (Biocare) – 30 minutes. Reaction developed with Betazoid DAB (Biocare) – 5 minutes. Counterstained with CATHE Hematoxylin diluted 1:5 (Biocare) – 5 minutes, dehydrated, cleared and mounted with synthetic mounting media.Rabbit anti-HR4 diluted in normal antibody diluent (Scytek) and incubated as in table above; followed by ProMark™ Rabbit on Farma HRP-Polymer detection (Biocare) – 30 minutes. Reaction developed with Betazoid DAB (Biocare) – 5 minutes. Counterstained with CATHE Hematoxylin diluted 1:5 (Biocare) – 5 minutes, dehydrated, cleared and mounted with synthetic mounting media.

REFERENCES

REFERENCES

1. Leserman J, Drossman DA. Relationship of abuse history to functional gastrointestinal disorders and symptoms: some possible mediating mechanisms. Trauma Violence Abuse. 2007;8(3):331-43.

2. Felitti VJ, Anda RF, Nordenberg D, Williamson DF, Spitz AM, Edwards V, et al. Relationship of childhood abuse and household dysfunction to many of the leading causes of death in adults. The Adverse Childhood Experiences (ACE) Study. Am J Prev Med. 1998;14(4):245-58.

3. Bradford K, Shih W, Videlock EJ, Presson AP, Naliboff BD, Mayer EA, et al. Association between early adverse life events and irritable bowel syndrome. Clin Gastroenterol Hepatol. 2012;10(4):385-90.e1-3.

4. Drossman DA, Li Z, Andruzzi E, Temple RD, Talley NJ, Thompson WG, et al. U.S. householder survey of functional gastrointestinal disorders. Prevalence, sociodemography, and health impact. Dig Dis Sci. 1993;38(9):1569-80.

5. Gern JE, Visness CM, Gergen PJ, Wood RA, Bloomberg GR, O'Connor GT, et al. The Urban Environment and Childhood Asthma (URECA) birth cohort study: design, methods, and study population. BMC Pulmonary Medicine. 2009;9(1):17.

6. Tomasdottir MO, Sigurdsson JA, Petursson H, Kirkengen AL, Krokstad S, McEwen B, et al. Self Reported Childhood Difficulties, Adult Multimorbidity and Allostatic Load. A Cross-Sectional Analysis of the Norwegian HUNT Study. PLOS ONE. 2015;10(6):e0130591.

7. Liu S, Hagiwara SI, Bhargava A. Early-life adversity, epigenetics, and visceral hypersensitivity. Neurogastroenterol Motil. 2017;29(9).

8. McLamb BL, Gibson AJ, Overman EL, Stahl C, Moeser AJ. Early weaning stress in pigs impairs innate mucosal immune responses to enterotoxigenic E. coli challenge and exacerbates intestinal injury and clinical disease. PLoS One. 2013;8(4):e59838.

9. Pohl CS, Medland JE, Mackey E, Edwards LL, Bagley KD, DeWilde MP, et al. Early weaning stress induces chronic functional diarrhea, intestinal barrier defects, and increased mast cell activity in a porcine model of early life adversity. Neurogastroenterol Motil. 2017;29(11).

10. Pohl CS, Medland JE, Moeser AJ. Early-life stress origins of gastrointestinal disease: animal models, intestinal pathophysiology, and translational implications. Am J Physiol Gastrointest Liver Physiol. 2015;309(12):G927-41.

11. Moeser AJ, Pohl CS, Rajput M. Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs. Anim Nutr. 2017;3(4):313-21.

12. Anda RF, Dong M, Brown DW, Felitti VJ, Giles WH, Perry GS, et al. The relationship of adverse childhood experiences to a history of premature death of family members. BMC Public Health. 2009;9:106.

13. Brown DW, Anda RF, Tiemeier H, Felitti VJ, Edwards VJ, Croft JB, et al. Adverse childhood experiences and the risk of premature mortality. Am J Prev Med. 2009;37(5):389-96.

14. Chang L, Toner BB, Fukudo S, Guthrie E, Locke GR, Norton NJ, et al. Gender, age, society, culture, and the patient's perspective in the functional gastrointestinal disorders. Gastroenterology. 2006;130(5):1435-46.

15. Meleine M, Matricon J. Gender-related differences in irritable bowel syndrome: potential mechanisms of sex hormones. World J Gastroenterol. 2014;20(22):6725-43.

16. Drossman DA, Leserman J, Nachman G, Li ZM, Gluck H, Toomey TC, et al. Sexual and physical abuse in women with functional or organic gastrointestinal disorders. Ann Intern Med. 1990;113(11):828-33.

17. Mukhtar K, Nawaz H, Abid S. Functional gastrointestinal disorders and gut-brain axis: What does the future hold? World J Gastroenterol. 2019;25(5):552-66.

18. Talley NJ. Functional gastrointestinal disorders as a public health problem. Neurogastroenterol Motil. 2008;20 Suppl 1:121-9.

19. Drossman DA. Functional Gastrointestinal Disorders: History, Pathophysiology, Clinical Features and Rome IV. Gastroenterology. 2016.

20. Tack J, Talley NJ, Camilleri M, Holtmann G, Hu P, Malagelada JR, et al. Functional gastroduodenal disorders. Gastroenterology. 2006;130(5):1466-79.

21. Disorders IFfG. Facts about IBS aboutibs.org: International Foundation for Gastrointestinal Disorders, Inc.; [updated 11-24-16. Available from: https://www.aboutibs.org/facts-about-ibs.html.

22. Choung RS, Herrick LM, Locke GR, 3rd, Zinsmeister AR, Talley NJ. Irritable bowel syndrome and chronic pelvic pain: a population-based study. J Clin Gastroenterol. 2010;44(10):696-701.

23. Morriss RK, Ahmed M, Wearden AJ, Mullis R, Strickland P, Appleby L, et al. The role of depression in pain, psychophysiological syndromes and medically unexplained symptoms associated with chronic fatigue syndrome. J Affect Disord. 1999;55(2-3):143-8.

24. Whitehead WE, Palsson O, Jones KR. Systematic review of the comorbidity of irritable bowel syndrome with other disorders: what are the causes and implications? Gastroenterology. 2002;122(4):1140-56.

25. Sperber AD, Atzmon Y, Neumann L, Weisberg I, Shalit Y, Abu-Shakrah M, et al. Fibromyalgia in the irritable bowel syndrome: studies of prevalence and clinical implications. Am J Gastroenterol. 1999;94(12):3541-6.

26. Tap J, Derrien M, Tornblom H, Brazeilles R, Cools-Portier S, Dore J, et al. Identification of an Intestinal Microbiota Signature Associated With Severity of Irritable Bowel Syndrome. Gastroenterology. 2017;152(1):111-23 e8.

27. Ohman L, Tornblom H, Simren M. Crosstalk at the mucosal border: importance of the gut microenvironment in IBS. Nat Rev Gastroenterol Hepatol. 2015;12(1):36-49.

28. Simren M, Tornblom H, Palsson OS, van Tilburg MAL, Van Oudenhove L, Tack J, et al. Visceral hypersensitivity is associated with GI symptom severity in functional GI disorders: consistent findings from five different patient cohorts. Gut. 2018;67(2):255-62.

29. Tornblom H, Van Oudenhove L, Sadik R, Abrahamsson H, Tack J, Simren M. Colonic transit time and IBS symptoms: what's the link? Am J Gastroenterol. 2012;107(5):754-60.

30. Manabe N, Wong BS, Camilleri M, Burton D, McKinzie S, Zinsmeister AR. Lower functional gastrointestinal disorders: evidence of abnormal colonic transit in a 287 patient cohort. Neurogastroenterol Motil. 2010;22(3):293-e82.

31. Smith F, Clark JE, Overman BL, Tozel CC, Huang JH, Rivier JE, et al. Early weaning stress impairs development of mucosal barrier function in the porcine intestine. Am J Physiol Gastrointest Liver Physiol. 2010;298(3):G352-63.

32. Fuentes IM, Walker NK, Pierce AN, Holt BR, Di Silvestro ER, Christianson JA. Neonatal maternal separation increases susceptibility to experimental colitis and acute stress exposure in male mice. IBRO Rep. 2016;1:10-8.

33. English P, Fowler, VR, Baxter, S, Smith, B. . The growing and finishing pig: Improving efficiency. . Farming Press Ltd Ipswich, Suffolk. 1988.

34. Newberry R, Wood-Gush, DGM. The suckling behaviour of domestic pigs in a semi-natural environment. Behav Res Ther. 1985;95:11-25.

35. Counsilman JJ L, LM. The definition of weaning. Anim Behav. 1985;33:1023-4.

36. States THSotU. An HSUS Report: The Welfare of Piglets in the Pig industry. 2010.

37. Fangman TT, Roderick. (1997). Segregated early weaning. Journal of Swine Health and Production.5. .

38. C. P. Disease of high-health pigs: Some ideas on pathogenesis. . 1995:16–7.

39. Brown DC, Maxwell CV, Erf GF, Davis ME, Singh S, Johnson ZB. The influence of different management systems and age on intestinal morphology, immune cell numbers and mucin production from goblet cells in post-weaning pigs. Vet Immunol Immunopathol. 2006;111(3-4):187-98.

40. Moeser AJ, Ryan KA, Nighot PK, Blikslager AT. Gastrointestinal dysfunction induced by early weaning is attenuated by delayed weaning and mast cell blockade in pigs. Am J Physiol-Gastr L. 2007;293(2):G413-G21.

41. Moeser AJ, Blikslager AT. Mechanisms of porcine diarrheal disease. J Am Vet Med Assoc. 2007;231(1):56-67.

42. Medland JE, Pohl CS, Edwards LL, Frandsen S, Bagley K, Li Y, et al. Early life adversity in piglets induces long-term upregulation of the enteric cholinergic nervous system and

heightened, sex-specific secretomotor neuron responses. Neurogastroenterol Motil. 2016;28(9):1317-29.

43. Jamur MC, Oliver C. Origin, maturation and recruitment of mast cell precursors. Front Biosci (Schol Ed). 2011;3:1390-406.

44. Welle M. Development, significance, and heterogeneity of mast cells with particular regard to the mast cell-specific proteases chymase and tryptase. J Leukoc Biol. 1997;61(3):233-45.

45. Abraham SN, St John AL. Mast cell-orchestrated immunity to pathogens. Nat Rev Immunol. 2010;10(6):440-52.

46. Maltby S, Khazaie K, McNagny K. Mast Cells in Tumor Growth: Angiogenesis, Tissue Remodeling and Immune-modulation. Biochim Biophys Acta. 2009;1796:19-26.

47. Pejler G, Rönnberg E, Waern I, Wernersson S. Mast cell proteases: Multifaceted regulators of inflammatory disease. Blood. 2010;115:4981-90.

48. Wouters MM, Vicario M, Santos J. The role of mast cells in functional GI disorders. Gut. 2016;65(1):155-68.

49. Boeckxstaens G. Mast cells and inflammatory bowel disease. Current Opinion in Pharmacology. 2015;25:45-9.

50. Galli SJ, Tsai M. IgE and mast cells in allergic disease. Nature Medicine. 2012;18(5):693-704.

51. Hamilton MJ, Frei SM, Stevens RL. The Multifaceted Mast Cell in Inflammatory Bowel Disease. Inflammatory Bowel Diseases. 2014;20(12):2364-78.

52. Galli SJ, Grimbaldeston M, Tsai M. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. Nat Rev Immunol. 2008;8(6):478-86.

53. Balseiro-Gomez S, Flores JA, Acosta J, Ramirez-Ponce MP, Ales E. Transient fusion ensures granule replenishment to enable repeated release after IgE-mediated mast cell degranulation. J Cell Sci. 2016;129(21):3989-4000.

54. Alber G, Kent UM, Metzger H. Functional comparison of Fc epsilon RI, Fc gamma RII, and Fc gamma RIII in mast cells. J Immunol. 1992;149(7):2428-36.

55. Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. Nat Immunol. 2005;6(2):135-42.

56. Burgoyne RD, Morgan A. Secretory granule exocytosis. Physiol Rev. 2003;83(2):581-632.

57. Varadaradjalou S, Feger F, Thieblemont N, Hamouda NB, Pleau JM, Dy M, et al. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells. Eur J Immunol. 2003;33(4):899-906.

58. Okumura S, Kashiwakura J, Tomita H, Matsumoto K, Nakajima T, Saito H, et al. Identification of specific gene expression profiles in human mast cells mediated by Toll-like receptor 4 and FcepsilonRI. Blood. 2003;102(7):2547-54.

59. Schwartz LB, Irani AM, Roller K, Castells MC, Schechter NM. Quantitation of histamine, tryptase, and chymase in dispersed human T and TC mast cells. J Immunol. 1987;138(8):2611-5.

60. Schwartz LB, Lewis RA, Austen KF. Tryptase from human pulmonary mast cells. Purification and characterization. J Biol Chem. 1981;256(22):11939-43.

61. Hallgren J, Pejler G. Biology of mast cell tryptase - An inflammatory mediator. Febs J. 2006;273(9):1871-95.

62. Nadel JA. Biology of Mast-Cell Tryptase and Chymase. Ann Ny Acad Sci. 1991;629:319-31.

63. Nigrovic PA, Binstadt BA, Monach PA, Johnsen A, Gurish M, Iwakura Y, et al. Mast cells contribute to initiation of autoantibody-mediated arthritis via IL-1. Proc Natl Acad Sci U S A. 2007;104(7):2325-30.

64. Stephen T. Holgate DHB, Martin K. Church, Fernando D. Martinez. Allergy 4th Edition. Allergy. 4 ed: ELSEVIER SAUNDERS; 2012. p. i-iii.

65. Maintz L, Novak N. Histamine and histamine intolerance. Am J Clin Nutr. 2007;85(5):1185-96.

66. Sattler J, Hafner D, Klotter HJ, Lorenz W, Wagner PK. Food-induced histaminosis as an epidemiological problem: plasma histamine elevation and haemodynamic alterations after oral histamine administration and blockade of diamine oxidase (DAO). Agents Actions. 1988;23(3-4):361-5.

67. Jarisch R, Wantke F. Wine and headache. Int Arch Allergy Immunol. 1996;110(1):7-12.

68. Mennigen R, Kusche J, Streffer C, Krakamp B. Diamine oxidase activities in the large bowel mucosa of ulcerative colitis patients. Agents Actions. 1990;30(1-2):264-6.

69. Schmidt WU, Sattler J, Hesterberg R, Roher HD, Zoedler T, Sitter H, et al. Human intestinal diamine oxidase (DAO) activity in Crohn's disease: a new marker for disease assessment? Agents Actions. 1990;30(1-2):267-70.

70. Yamauchi K, Sekizawa K, Suzuki H, Nakazawa H, Ohkawara Y, Katayose D, et al. Structure and function of human histamine N-methyltransferase: critical enzyme in histamine metabolism in airway. Am J Physiol. 1994;267(3 Pt 1):L342-9.

71. Yan L, Galinsky R, Bernstein J, Liggett S, Weinshilboum R. Histamine N-methyltransferase pharmacogenetics: Association of a common functional polymorphism with asthma. Pharmacogenetics. 2000;10:261-6.

72. Smolinska S, Jutel M, Crameri R, O'Mahony L. Histamine and gut mucosal immune regulation. Allergy. 2014;69(3):273-81.

73. Lichtenstein LM, Gillespie E. The effects of the H1 and H2 antihistamines on "allergic" histamine release and its inhibition by histamine. J Pharmacol Exp Ther. 1975;192(2):441-50.

74. Thurmond RL, Gelfand EW, Dunford PJ. The role of histamine H1 and H4 receptors in allergic inflammation: the search for new antihistamines. Nat Rev Drug Discov. 2008;7(1):41-53.

75. Wechsler JB, Szabo A, Hsu CL, Krier-Burris RA, Schroeder HA, Wang MY, et al. Histamine drives severity of innate inflammation via histamine 4 receptor in murine experimental colitis. Mucosal Immunol. 2018;11(3):861-70.

76. O'Reilly M, Alpert R, Jenkinson S, Gladue RP, Foo S, Trim S, et al. Identification of a histamine H4 receptor on human eosinophils--role in eosinophil chemotaxis. J Recept Signal Transduct Res. 2002;22(1-4):431-48.

77. Hofstra CL, Desai PJ, Thurmond RL, Fung-Leung WP. Histamine H4 receptor mediates chemotaxis and calcium mobilization of mast cells. J Pharmacol Exp Ther. 2003;305(3):1212-21.

78. Akdis CA, Blaser K. Histamine in the immune regulation of allergic inflammation. J Allergy Clin Immunol. 2003;112(1):15-22.

79. Gao CX, Major A, Rendon D, Lugo M, Jackson V, Shi ZC, et al. Histamine H2 Receptor-Mediated Suppression of Intestinal Inflammation by Probiotic Lactobacillus reuteri. Mbio. 2015;6(6).

80. Inan A, Sen M, Surgit O, Ergin M, Bozer M. Effects of the histamine H2 receptor antagonist famotidine on the healing of colonic anastomosis in rats. Clinics (Sao Paulo). 2009;64(6):567-70.

81. Overman EL, Rivier JE, Moeser AJ. CRF induces intestinal epithelial barrier injury via the release of mast cell proteases and TNF-alpha. PLoS One. 2012;7(6):e39935.

82. Bright MA, Thompson LA. Association of Adverse Childhood Experiences with Co-occurring Health Conditions in Early Childhood. J Dev Behav Pediatr. 2018;39(1):37-45.

83. Chang XN, Jiang XY, Mkandarwire T, Shen M. Associations between adverse childhood experiences and health outcomes in adults aged 18-59 years. Plos One. 2019;14(2).

84. Chitkara DK, van Tilburg MA, Blois-Martin N, Whitehead WE. Early life risk factors that contribute to irritable bowel syndrome in adults: a systematic review. Am J Gastroenterol. 2008;103(3):765-74; quiz 75.

85. Danese A, Pariante CM, Caspi A, Taylor A, Poulton R. Childhood maltreatment predicts adult inflammation in a life-course study. Proc Natl Acad Sci U S A. 2007;104(4):1319-24.

86. Soderholm JD, Yates DA, Gareau MG, Yang PC, MacQueen G, Perdue MH. Neonatal maternal separation predisposes adult rats to colonic barrier dysfunction in response to mild stress. Am J Physiol Gastrointest Liver Physiol. 2002;283(6):G1257-63.

87. Barreau F, Ferrier L, Fioramonti J, Bueno L. New insights in the etiology and pathophysiology of irritable bowel syndrome: contribution of neonatal stress models. Pediatr Res. 2007;62(3):240-5.

88. Gareau MG, Jury J, Perdue MH. Neonatal maternal separation of rat pups results in abnormal cholinergic regulation of epithelial permeability. Am J Physiol Gastrointest Liver Physiol. 2007;293(1):G198-203.

89. Li Y, Song Z, Kerr KA, Moeser AJ. Chronic social stress in pigs impairs intestinal barrier and nutrient transporter function, and alters neuro-immune mediator and receptor expression. PLoS One. 2017;12(2):e0171617.

90. Pohl CS, Lennon EM, Li Y, DeWilde MP, Moeser AJ. S. Typhimurium challenge in juvenile pigs modulates the expression and localization of enteric cholinergic proteins and correlates with mucosal injury and inflammation. Auton Neurosci. 2018;213:51-9.

91. Lee J, Veatch SL, Baird B, Holowka D. Molecular mechanisms of spontaneous and directed mast cell motility. J Leukocyte Biol. 2012;92(5):1029-41.

92. Ahrens F, Sunkel Y, Pollmuller T, Bussemas R, Weissmann F, Erhard MH. Influence of two different stressors, weaning and immunization, on the plasma histamine level of organic farming piglets. Inflamm Res. 2009;58 Suppl 1:53-4.

93. Fabisiak A, Wlodarczyk J, Fabisiak N, Storr M, Fichna J. Targeting Histamine Receptors in Irritable Bowel Syndrome: A Critical Appraisal. J Neurogastroenterol. 2017;23(3):341-8.

94. Sander LE, Lorentz A, Sellge G, Coeffier M, Neipp M, Veres T, et al. Selective expression of histamine receptors H1R, H2R, and H4R, but not H3R, in the human intestinal tract. Gut. 2006;55(4):498-504.

95. Lenz KM, Pickett LA, Wright CL, Davis KT, Joshi A, McCarthy MM. Mast Cells in the Developing Brain Determine Adult Sexual Behavior. J Neurosci. 2018;38(37):8044-59.

96. Liu J, Fu T, Song F, Xue Y, Xia C, Liu P, et al. Mast Cells Participate in Corneal Development in Mice. Sci Rep-Uk. 2015;5(1):17569.