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Understanding the multidirectional axes of communication between the gut microbiome and the brain to augment human performance

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14. ABSTRACT

The overall goal of this project was to characterize host-microbe interactions following an acute stressor and examine whether this interaction mediates host stress recovery via mechanism(s) of the microbiota-gut-brain axis. Our hypothesis was based on the principle that acute stress will modulate host-microbe interactions at the level of the gastrointestinal tract as the host responds to and recovers from that stressor; and that identifiable patterns of host-microbe crosstalk would yield a molecular signature predictive of, and useful in modulating, host behavioural and cognitive outcomes via the microbiome-gut-brain axis.

We have characterized aspects of recovery from acute stress using common stress-related readouts in male and female conventionally-raised, germ-free (GF), as well as ex-GF (i.e. GF mice that have been colonized with a conventionally-raised murine microbiota) mice. Substantial differences in the reaction and recovery of the enteric L-tryptophan metabolic pathway were identified in the gastrointestinal tracts of conventional and GF mice of both sexes. Specifically, the conversion of L-tryptophan to neuroactive molecules, including the neurotransmitter serotonin, was significantly elevated post-stressor in the colon of the gastrointestinal tract of male but not female conventional mice. This stress-induced effect was absent in GF male mice but was restored in ex-GF mice, thereby highlighting a role for the microbiome in influencing stress-responsivity at the level of the gastrointestinal tract. Metabolomic profiling revealed that levels of tryptophan in the cecum decreased after stress in all groups. An elevation of microbially-produced caecal tryptamine was found in conventional and ex-GF animals in response to stress and there was an increase in indoleacetate and a reduction in indolepropionate levels only in conventional and ex-GF animals followi

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Understanding the Multidirectional Axes of Communication between the Gut Microbiome and the Brain to Augment Human Performance

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Summary

The overall goal of this project was to characterize host-microbe interactions following an acute stressor and examine whether this interaction mediates host stress recovery via mechanism(s) of the microbiota-gut-brain axis. Our hypothesis was based on the principle that acute stress will modulate host-microbe interactions at the level of the gastrointestinal tract as the host responds to and recovers from that stressor; and that identifiable patterns of host-microbe crosstalk would yield a molecular signature predictive of, and useful in modulating, host behavioural and cognitive outcomes via the microbiome-gut-brain axis.

We have characterized aspects of recovery from acute stress using common stress-related readouts in male and female conventionally-raised, germ-free (GF), as well as ex-GF (i.e. GF mice that have been colonized with a conventionally-raised murine microbiota) mice. Substantial differences in the reaction and recovery of the enteric L-tryptophan metabolic pathway were identified in the gastrointestinal tracts of conventional and GF mice of both sexes. Specifically, the conversion of L-tryptophan to neuroactive molecules, including the neurotransmitter serotonin, was significantly elevated post-stressor in the colon of the gastrointestinal tract of male but not female conventional mice. This stress-induced effect was absent in GF male mice but was restored in ex-GF mice, thereby highlighting a role for the microbiome in influencing stress-responsivity at the level of the gastrointestinal tract. Metabolomic profiling revealed that levels of tryptophan in the cecum decreased after stress in all groups. An elevation of microbially-produced caecal tryptamine was found in conventional and ex-GF animals in response to stress and there was an increase in indoleacetate and a reduction in indolepropionate levels only in conventional and ex-GF animals following acute stress. Both host and microbial tryptophan metabolic pathways were thus markedly altered by stress. To our knowledge, this is the first time that a direct effect of acute stress on microbial metabolism has been demonstrated, with changes in indoleacetate and indolepropionate, metabolites produced exclusively by gut microbes. The microbiome was also found to affect host brain response to acute stress in a region- and sex-dependent manner with key host enzymes involved in the metabolism of tryptophan and production of serotonin also found to be affected by the microbiome.

The global metabolic profile following stress exposure in GF animals was also markedly different and colonisation of GF animals restored the metabolic profile in general in the cecum to conventional levels but not in the mucosa. In particular, there were reductions in butyrate and acetate, short chain fatty acids (SCFAs) produced exclusively by gastrointestinal microorganisms which have an important role in host physiology. While this could reflect reduced microbial production in response to acute stress, changes in the tricarboxylic acid (TCA) cycle in the colonocytes of conventional and ex-GF groups after stress exposure were also observed. This stress-induced shift in colonocyte metabolism is subverted in the absence of gut microbiota but exaggerated in the ex-GF group suggesting that early-life colonisation is essential to regulate gastrointestinal energy demands following stress exposure.

Our data further revealed novel cell-type-specific changes in the innate immune system in response to acute stress, which in turn are impacted by the microbiota. This indicates that the microbiota influences the priming and recovery of the innate immune system to an acute stressor and may inform future microbiota-targeted therapeutics aimed at modulating stress-induced immune activation in stress-related disorders. Microbiota-depleted animals also displayed region-specific alterations in gastrointestinal permeability as a consequence of acute stress exposure. Cumulatively, our results suggest that acute stress induces rapid changes at the host-microbe interface along the microbiome-gut-brain axis and that the microbiota is responsible for directing host and microbial metabolism of tryptophan and other metabolites along specific and physiologically relevant homeostatic pathways.

Introduction

The warfighter is required to immediately respond to the rapidly changing landscape of the battlefield thereby requiring superior resiliency to high physical and emotional stress. The bacteria found in the human gut (i.e. the microbiota) are increasingly recognized to affect cognition and emotional responses[1] (e.g. anxiety, depression). Although host-microbiota interaction is known to be altered under conditions of chronic stress[2], and such perturbations are associated with decreased mental and emotional welfare[3], little is known regarding the impact of a novel acute stressor on host-microbiota communication or the implications for host cognition and mental resilience. Targeting the gut microbiome to promote stress resilience and optimize human performance in stressful environments is an important objective but one which requires further research to ensure the delivery of tangible benefits.

The acute phase of stress represents a critical temporal window long-recognized to strongly influence warfighter mental and physical capability[4]. In civilian populations, the acute stress reaction is known to modulate pain tolerance[5] and elicit neuroendocrine responses that modulate behaviour[6]. Yet, many of these host-derived neuroactive molecules (i.e. monoamines such as serotonin) are also important signalling molecules in host-microbiota interaction at the intestinal interface, with roles in affecting host cognition and emotion via the microbiota-gut-brain axis[7]. Indeed, probiotic bacteria synthesize and secrete these same host-derived molecules[8]. Studies performed in animals that lack a microbiota (i.e. germ-free, GF) revealed distinct stress responses compared to conventionally-raised animals, highlighting a role for the microbiota in modulating host stress response[9]. Interestingly, ~95% of the body's supply of serotonin is found in the digestive tract, and evidence from our laboratory has demonstrated that chronic host stress alters colonic levels of this neurochemical[10]. As colonic serotonin has been demonstrated to alter gut motility and affect fecal output, understanding relevant host-microbe crosstalk influential of the gut serotonergic system will prove useful towards addressing stress-associated gastrointestinal disorders. Host biosynthetic pathways surrounding the metabolism of L-tryptophan are widely-recognized to strongly influence behaviour and cognition[3] (**Figure 1**). Additionally, the gut microbiota has been demonstrated to modulate host gastrointestinal serotonin synthesis[11]. Thus, stress-induced changes of the host-microbe interaction in the gastrointestinal serotonergic system represents a tractable target for the improvement of resilience to stress. However, there is a paucity of data in understanding host-microbe interaction immediately following an acute stressor and how the microbiota may be manipulated during this time period to augment performance in stressful situations.

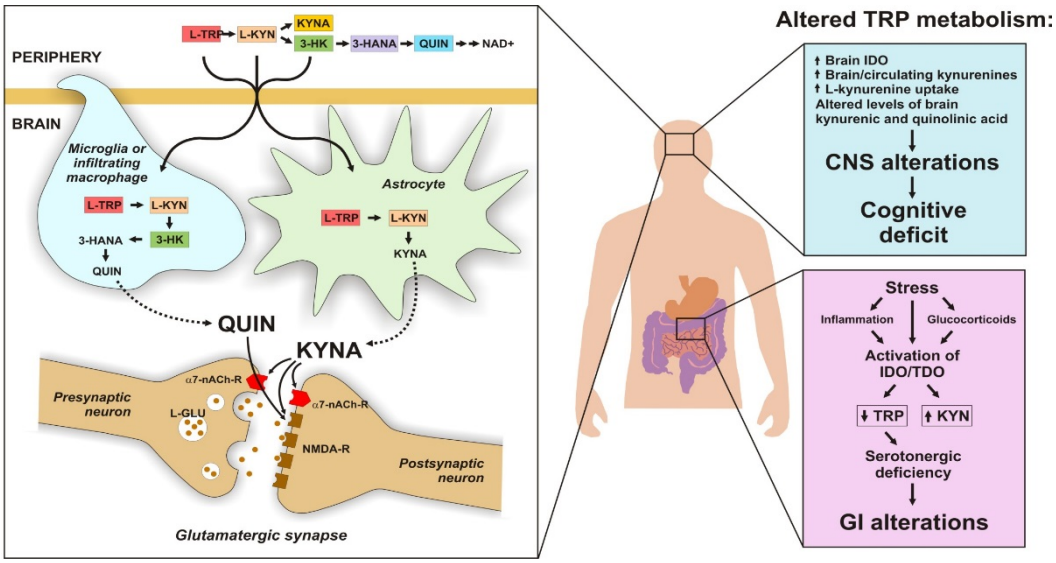

Figure 1. Tryptophan metabolism in host health Stress-induced alterations in tryptophan metabolism within the central nervous system (CNS) and the gastrointestinal tract are important to host health. Stress can activate kynurenine (KYN) production pathway enzymes indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO), shunting tryptophan away from serotonin production and instead generating kynurenine. Quinolinic acid (QUIN) and kynurenic acid (KYNA), metabolites of kynurenine, can exert excitotoxic and protective effects, respectively, in the CNS.

The major objectives of this project are: **to characterize the host-microbe metabolic crosstalk immediately following an acute stressor; and examine whether this interaction mediates host stress response via mechanism(s) of the microbiota-gut-brain axis, including the gastrointestinal and central (e.g. brain) serotonergic systems, alterations in gastrointestinal permeability and the innate immune system**. The significance of these changes, and whether they represent a potential microbiome-based therapeutic target, lies in the implications for modulation of host cognitive performance and behaviour following an acute stressor. **Specifically, we have tested the hypothesis that host sex and the gut microbiota determine susceptibility, at multiple levels of the microbiome-gut-brain axis, to an acute stressor**.

Methods, Assumptions and Procedures

Adult male and female (10-11wks/age; n=7-8 mice/time-point) C57/BL6 conventional, germ-free (GF), and ex-GF mice (*equivalent to CGF and ColGF in some published figures*) were randomly allocated to either control or stress groups. The stressor consisted of a perforated 50mL tube into which a single mouse was inserted and restrained for a period of 15min. Immediately following the 15min stress, the mouse was removed from restraint, returned to its home cage and allowed to recover undisturbed for 0 or 45min before being culled (**Figure 2**). Tissues were harvested immediately following cull and stored at -80^{o}C until analysis. Plasma corticosterone was assayed via ELISA; 5-HT and its main metabolite, 5-HIAA were measured in both distal ileum and proximal colon of the

gastrointestinal tract, as well as the frontal cortex, hypothalamus, and hippocampus regions of the brain using HPLC. Cecum contents (representative of bacterial metabolism) and mucosal scrapings (representative of host metabolism) were sent for **metabolomic analysis**. Principal component analysis (PCA) and random forest analysis (RFA) were applied to the data in order to transform the large number of variables into a smaller number of components, thereby providing a high-level overview of the dataset and providing possible biomarkers accordingly to the stratification of the groups.

Flow cytometry was performed to assess stress induced alterations in the innate immune system using the BD FACSCalibur. Data were analysed using FlowJo (Version 10). Cell populations were selected as following: blood/splenic LY6Chi Monocytes: CD11b+, LY6Chi; blood/spleen LY6Cmid Monocytes: CD11b+, LY6Cmid, SSClow, CCR2+; blood/splenic LY6Clow Monocytes: CD11b+, LY6C−, SSClow, CX3CR1+; blood/splenic Granulocytes: CD11b+, SSChi; splenic Macrophages: CD11b+, LY6G-, MHC-II+, F4/80+; MLN LY6Chi Monocytes: CD11b+, LY6C+, LY6G-; MLN Neutrophils: CD11b+, LY6C+, LY6G+; MLN Macrophages: CD11b+, LY6C−, F4/80+; MLN Dendritic cells: CD11c+, MHC-II+. The investigated cell populations were normalised to overall cell levels.

Intestinal permeability was assessed ex vivo in adult male (10-11 wks/age; n=8 mice) C57/BL6 conventional mice exposed to an antibiotic cocktail in the drinking water (ampicillin 1mg/ml + gentamicin 1 mg/ml + vancomycin 0.5mg/ml + imipenem 0.25mg/ml) for 5 days (time determined previously). After 5 days, mice were randomly allocated to either control or stress groups. The stressor consisted of a perforated 50mL tube into which a single mouse was inserted and restrained for a period of 15min. Immediately following the 15min stress, the mouse was removed from restraint, returned to its home cage and allowed to recover undisturbed for 0 or 45min before being culled (Figure 2). Representative samples of distal ileum and proximal colon were harvested in Kreb's buffer 1X and used to measure the intestinal permeability to macromolecules ex vivo with the technique of the using chambers. Other tissues were harvested immediately following cull and stored at -80^{o}C. Results were also analysed by student's t-test or ANOVA, where applicable, and statistical significance was set at $p<0.05$. Future potential analyses are included in **Table 1**.

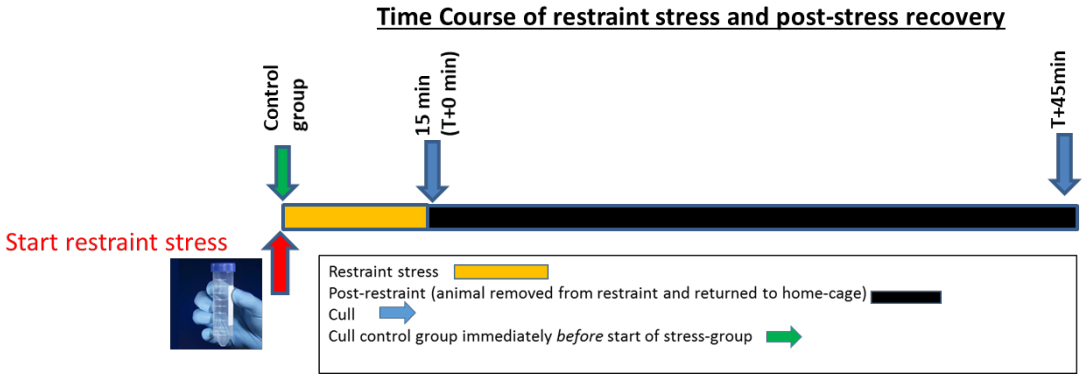

Figure 2: **Experimental design for induction and recovery from an acute stressor**

Results and Discussion

The Host Stress Response System: HPA Axis Alterations

Under germ-free (GF) conditions, the murine HPA-axis response to acute stress was shown to be exaggerated compared to that of conventional mice, suggesting the importance of host-microbe interactions in governing this core stress response system[9]. However, little is known about the role of the microbiome in the gastrointestinal and central serotonergic responses to acute stress. Corticosterone, a hallmark murine hormone produced following activation of the HPA-axis, was assessed immediately and at 45min following cessation of restraint stress. It was demonstrated that the magnitude of the corticosterone stress response is distinct between male and female conventional mice (**Figure 3**). Moreover, in the absence of the microbiome, a sex-dependent effect was observed in that only male GF mice had comparatively higher post-stressor corticosterone compared to conventional mice immediately post stressor. Colonization of male GF mice normalized the corticosterone response to conventional-like levels.

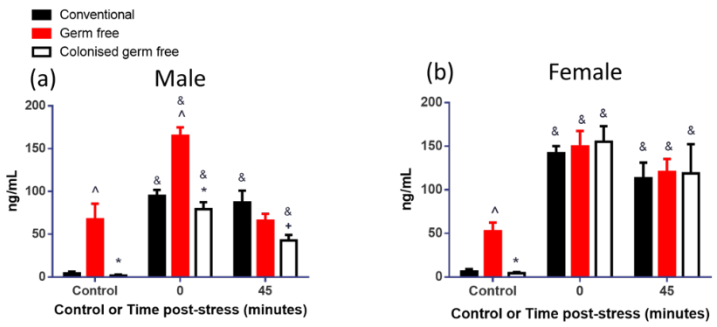

Figure 3: **Germ-free status affects acute stressor CORT response in a sex-dependent manner** Germ-free status alters corticosterone (ng/mL) response to acute stress in male (a) and female (b) mice in a sex-dependent manner. Corticosterone was determined in murine plasma using ELISA as described in Methods. Bar graphs depict mean ± SEM of each group of mice and represent an n=7-9/mice/sex/group. Symbols indicating significant differences at $p<0.05$ level: &=difference between timepoint X and respective control; ^ = between same-sex GF and conventional; * = between same-sex GF and colonised GF; + = between same-sex colonised GF and conventional.

Host Serotonin and Tryptophan Metabolism

A major finding of this project is that the acute stressor elicited a sex- and microbiome-dependent gastrointestinal serotonergic response (**Figure 4**). Acute stress caused a significant increase in colonic serotonin of male conventional mice, an effect that was absent in male GF mice but restored in ex-GF mice. We did not observe a stress-induced change in colonic serotonin of female mice. Similarly, we found the acute stressor to cause a change in 5-HIAA in male conventional and ex-GF mice, but not male GF mice. This stress effect was also absent in the colon of female mice. This highlights a previously unknown role for the microbiome in determining serotonergic responsivity of the colon to acute stress in a sex-dependent manner. These results are consistent with previous results from our laboratory where chronic-stress induced production of colonic serotonin in C57/BL6 conventional male mice[10].

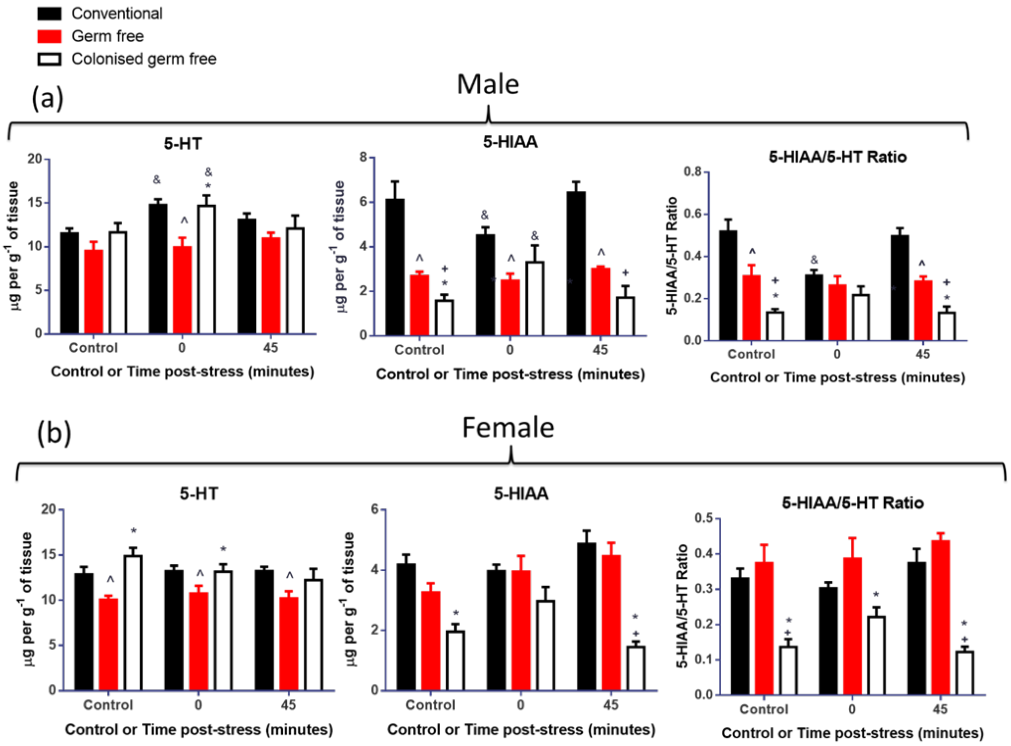

Figure 4: Germ-free status mediates sex-dependent change in gut 5-HT, and its main metabolite 5-HIAA Germ-free status is strongly influential of male (a) and female (b) colonic 5-HT, and its main metabolite 5-HIAA. 5-HT and 5-HIAA concentrations were determined using high performance liquid chromatography (HPLC) as described in Methods. Bar graphs depict mean ± SEM of each group of mice. Symbols indicating significant differences at $p<0.05$ level: &=difference between timepoint X and respective control; ^ = between same-sex GF and conventional; * = between same-sex GF and colonised GF; + = between same-sex colonised GF and conventional.

As the microbiome-gut-brain axis also concerns the effects of the microbiome on the central nervous system, we investigated whether the brain serotonergic response to acute stress is dependent on the microbiome (**Figure 5)**. Stress-microbiome interactions were identified in multiple regions of the brain, including the frontal cortex. We observed a sex-dependent change in conventional male but not female frontal cortical 5-HT. The stress-induced effect was absent in GF mice of both sexes. In

comparison to conventional mice, GF mice displayed elevated and lowered 5-HT and 5-HIAA, respectively. As reduced frontal cortical 5-HT turnover has been strongly associated with psychosocial stress, we identified a stress-induced increase in the 5-HIAA/5-HT ratio, a measure of 5-HT turnover, an effect that was absent in GF mice. This highlights a previously unknown role for the microbiome in affecting 5-HT turnover in the frontal cortex.

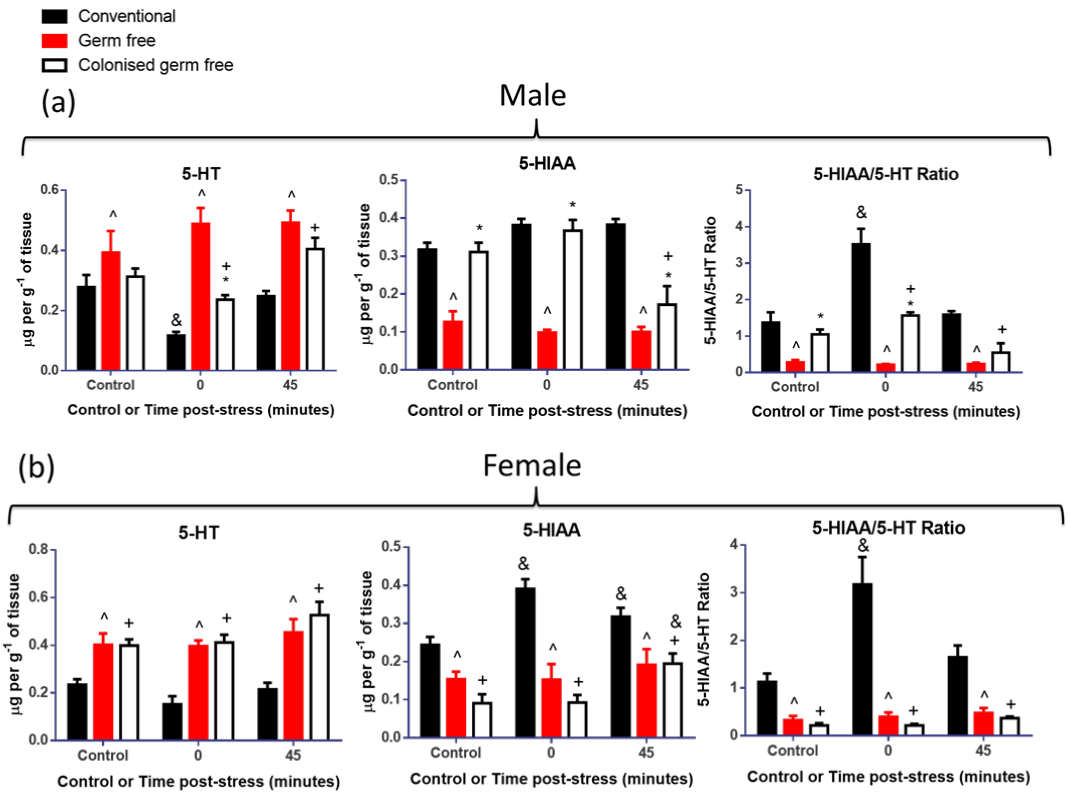

Figure 5: Germ-free status mediates sex- and region-dependent brain response to acute stress Germ-free status alters male (a) and female (b) CNS frontal cortex serotonergic system and its response to acute stress in a sex-dependent manner. 5-HT and 5-HIAA concentrations were determined using high performance liquid chromatography (HPLC) as described in Methods. Bar graphs depict mean ± SEM of each group of mice. Symbols indicating significant differences at $p<0.05$ level: &=difference between timepoint X and respective control; ^ = between same-sex GF and conventional; * = between same-sex GF and colonised GF; + = between same-sex colonised GF and conventional.

Metabolomics

Under germ-free (GF) conditions, the metabolic profile in both caecal contents and mucosal scrapings are markedly different in response to acute stress compared to that of conventional (Conv) or recolonized (ex-GF) mice, suggesting the importance of host-microbe interactions in governing this stress response (Figure 6). However, little is known about the specific role of acute stress on microbial metabolic response. Short chain fatty acids (SCFAs), metabolites exclusively produced by gut microbiota, were significantly affected by acute stress with a decrease in the cecal levels of acetate and butyrate in Conv and ColGF animals (Figure 6). Propionate was also decreased in ColGF. As expected, SCFAs were not detected in GF animals, confirming the absence of gut microbiota in these animals.

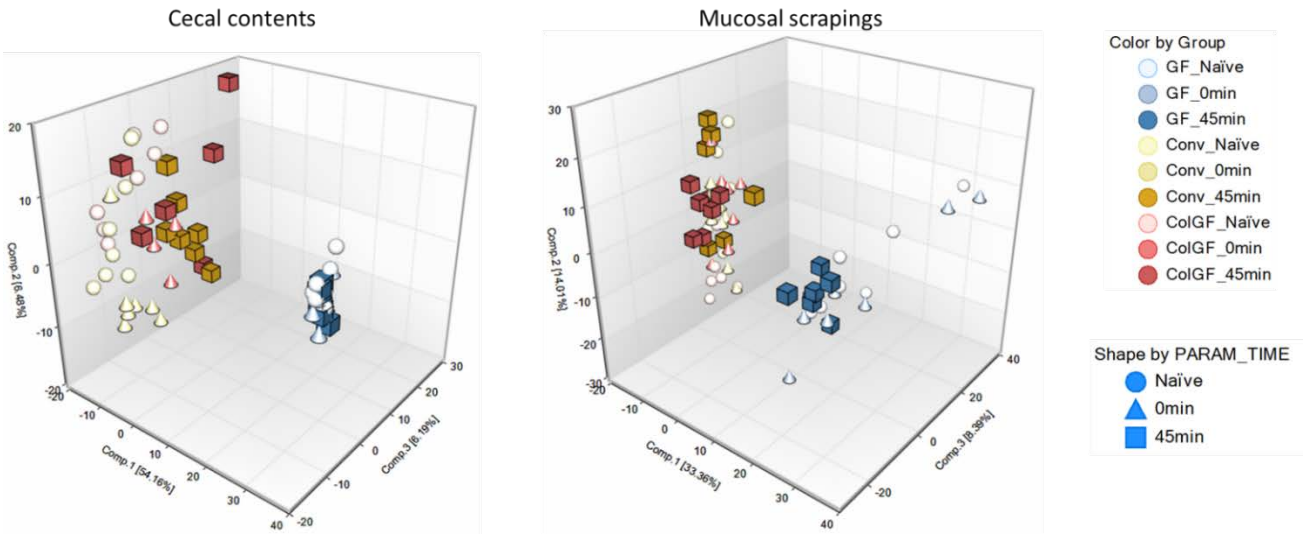

Figure 6: Metabolomic profile following acute stress PCA results from cecal contents and mucosal scrapings obtained from conventional (Conv, yellow), germ free (GF, blue) and ex GF (ColGF, red) show the markedly difference in the metabolic profile of GF animals before (Naïve) and after stress (0min, 45min), especially in the mucosa. Circles depict naïve results, triangles metabolites found immediately after stress, and squares the results from animals culled 45 minutes following stress exposure. and represent an n=7 mice/group.

It was demonstrated for the first time that there is a change in SCFA levels in response to acute stress in a short period of time. Moreover, colonization of male GF mice mostly normalized SCFA production in the cecum to conventional-like levels, except for propionate, which was higher even at baseline levels in ex-GF animals, suggesting small but potentially significant differences for host physiology depending on the timing of microbiota colonization. Considering the plethora of ways in which SCFAs can affect the host physiology, including depressive-, anxiety and stress-related behaviours, it is not surprising that these metabolites may be involved in the GI stress response. The observed change in SCFA levels in the cecum may be explained by a reduction in bacterial synthesis of these metabolites or by changes in host gastrointestinal metabolism. Colonocytes derive 60–70% of their energy supply from SCFA oxidation to ketone bodies and CO2, which are later used in TCA cycle to produce ATP10. It is well known that stress activates the “fight or fly” response in the organism, which leads to an increase in metabolism rate, glucose consumption and catabolism to generate energy. In the gut this probably might be translated into an increase in SCFAs uptake and oxidation to produce ATP through the TCA cycle in response to acute stress. This theory was confirmed following the observation of a decrease in the first intermediates of the TCA cycle (citrate and alpha ketoglutarate) and an increase in the last intermediates of the TCA (succinate and malate) in Conv and ColGF animals in response to stress (Figure 7). These changes are not observed in GF

animals, which in turn seemed to be obtaining their energy from other metabolic pathways such as glucolisis and glucogenolisis. However, the direct influence of stress over bacterial production of SCFAs can not be ruled out and further analysis must be carried out to shed light in this particular observation.

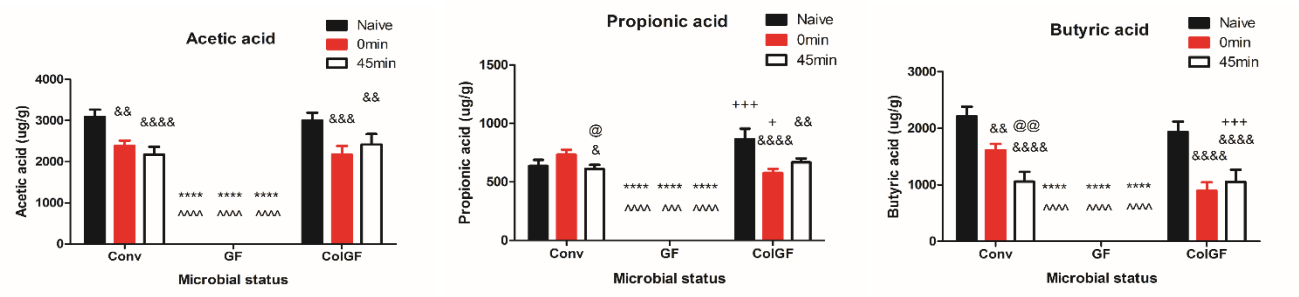

Figure 7: Impact of acute stress on short chain fatty acids (SCFAs). No short chain fatty acids (SCFAs) were detected in germ-free (GF) animals. Acute stress alters SCFAs (ug/g) levels in the cecum of conventional (Conv) and ex-GF (ColGF) mice in a time-dependent manner. SCFAs were determined in cecal contents as described in Methods. Bar graphs depict mean ± SEM of each group of mice and represent an n=7mice/group. Symbols indicating significant differences at $p<0.05$ level: &=difference between timepoint X and respective control; ^ = between GF and Conv; * = between GF and ColGF; + = between ex-GF and Conv.

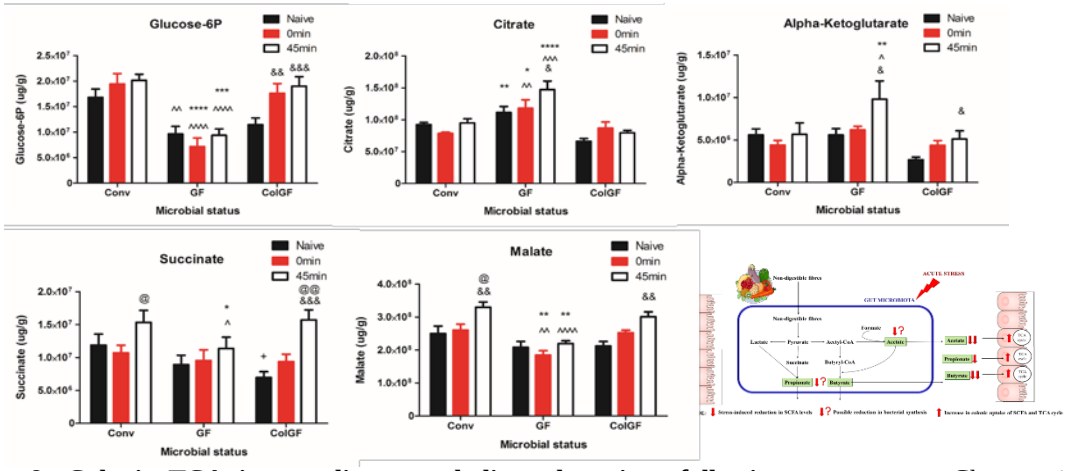

Figure 8: Colonic TCA intermediate metabolites alterations following acute stress Changes in colonic TCA intermediate metabolites were induced by acute stress, but the presence of microbiota seems to modulate those changes. The changes observed in Conv and ex-GF animals, with an increase in late intermediates such as succinate and malate, indicates a shunt of metabolism to produce energy in response to stress. The response to stress is exaggerated in the ex-GF group. This stress-induced shift in colonocyte's metabolism is subverted or delayed in the absence of gut microbiota, with increases in early intermediates of the TCA cycle but not in late intermediates observed in GF animals. TCA metabolites were determined in cecal contents as described in Methods. Bar graphs depict mean ± SEM of each group of mice and represent an n=7mice/group. Symbols indicating significant differences at $p<0.05$ level: &=difference between timepoint X and respective control; ^ = between GF and Conv; * = between GF and ex-GF; + = between ex-GF and Conv.

The acute stressor elicited a microbiome-dependent tryptophan metabolic response as well as changes in microbiota tryptophan metabolism (Figure 9). Acute stress caused a significant decrease in caecal tryptophan levels in all groups, suggesting that tryptophan is being absorbed by colonocytes and bacteria to produce different metabolites to deal with the stressor. In particular, tryptophan is being used in the mucosa of male Conv mice to produce serotonin and tryptamine, an effect that was absent in GF mice but restored in ex-GF animals. These results are consistent with previous results from this project and from our laboratory where acute and chronic stress induced production of colonic serotonin in C57/BL6 conventional male mice. At the same time, we observed a stress-induced decrease in colonic kynurenate and xanthurenate of Conv mice, meanwhile these metabolites are increased in response to stress in GF and ex-GF animals. This highlights the fact that recolonization of GF animals during adulthood could not completely restored the host metabolism to conventional normal levels, suggesting that absence of microbiota during early life can induce changes in the physiology of the gut that could not completely be rescued by later microbial colonization.

Acute stress is also capable of directly modulating bacterial metabolism. To our knowledge, this is the first time that a direct effect of acute stress on microbial metabolism is has been reported, with observed increases in indoleacetate and decreases in indolepropionate, tryptophan metabolites produced exclusively by gut microbes from tryptophan. According to caecal tryptophan reductions shown after stress, gut bacteria may be taking up tryptophan from the lumen to produce more indoleacetate, which is further absorbed by the mucosa, in detriment of indolepropionate synthesis. This is important because indoleacetate has is known to be a cytotoxic agent, meanwhile indolepropionate exhibits neuroprotective effects. However, the percentage of absorption of these compounds by the host has not been determined yet, and further studies need to be done to determine if they are suitable biomarkers for acute stress or can be used as targets to improve stress resilience.

Overall our results suggest that acute stress induces rapid changes in both microbial and host metabolism and that microbiota is responsible for directing host metabolism of tryptophan along specific pathways to maintain an adequate gastrointestinal response to stress. Furthermore, this project has demonstrated the potential of indoleacetate, indolepropionate and SCFAs, metabolites exclusively produced by gut microbiota, to be used as targets to improve stress resilience through microbiota modulation.

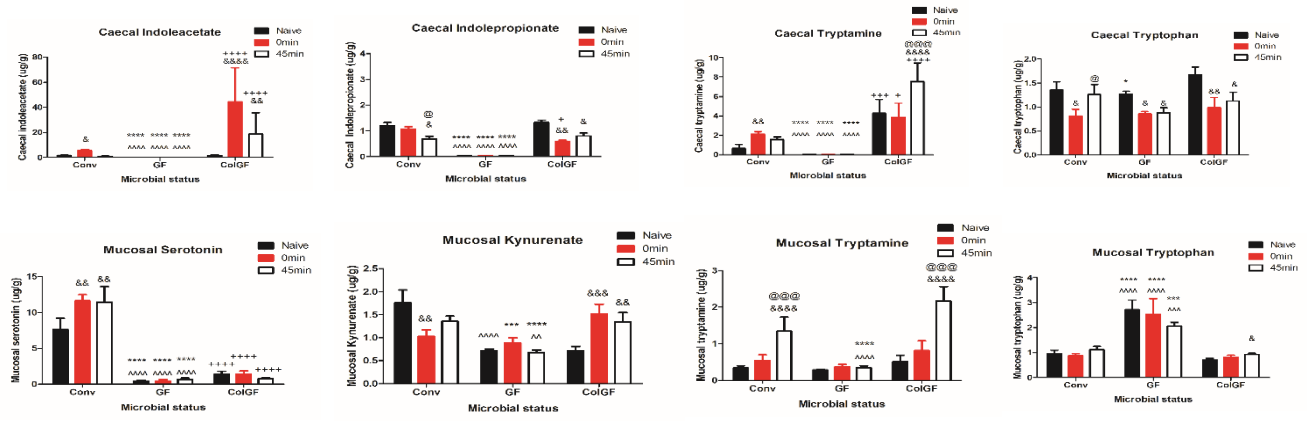

Figure 9: Caecal and mucosal alterations in tryptophan and tryptophan derived metabolites following acute stress exposure. Acute stress caused a significant decrease in caecal tryptophan levels in all groups. There was a stress-induced decrease in colonic kynurenate of conventional mice but this metabolite was increased in response to stress in GF and ex-GF animals. Increases in the microbial metabolites indoleacetate and decreases in indolepropionate were observed post stressor. Mucosal tryptamine levels increased post-stressor only in the presence of a gut microbiota. Symbols indicating significant differences at $p<0.05$, $P<0.01$, $P<0.001$ level: &=difference between timepoint X and respective control; ^ = between GF and Conv; * = between GF and ex-GF; + = between ex-GF and Conv.

Innate Immune System

Acute stress leads to activation of neuroendocrine systems, which in turn orchestrate a large-scale redistribution of innate immune cells. Both these response systems are independently known to be primed by the microbiota, even though much is still unclear about the role of the gastrointestinal microbiota in acute stress-induced immune activation. We investigated whether the microbiota influences acute stress-induced changes in innate immunity using conventionally colonised mice, mice devoid of any microbiota (i.e. germ-free, GF), and colonised GF mice (ex-GF). We also explored the kinetics of stress-induced immune cell mobilisation in the blood, the spleen and mesenteric lymph nodes (MLNs). GF mice had increased baseline levels of adrenaline and noradrenaline, of which adrenaline was normalised in CGF mice. In tandem, GF mice had decreased circulating levels of LY6Chi and LY6Cmid, CCR2+ monocytes, and granulocytes, but not LY6C−, CX3CR1+ monocytes. These deficits were normalised in ex-GF mice. Acute stress decreased blood LY6Chi and LY6Cmid, CCR2+ monocytes while increasing granulocyte levels in all groups 45 min post-stress. However, only GF mice showed stress-induced changes in LY6Chi monocytes and granulocytes 240 min post-stress, indicating impairments in the recovery from acute stress-induced changes in levels of specific innate immune cell types. LY6C−, CX3CR1+ monocytes remained unaffected by stress, indicating that acute stress impacts systemic innate immunity in a cell-type-specific manner. Overall, these data reveal novel cell-type-specific changes in the innate immune system in response to acute stress, which in turn are impacted by the microbiota. The microbiota influences the priming and recovery of the innate

immune system to an acute stressor and may inform future microbiota-targeted therapeutics aimed at modulating stress-induced immune activation in stress-related disorders.

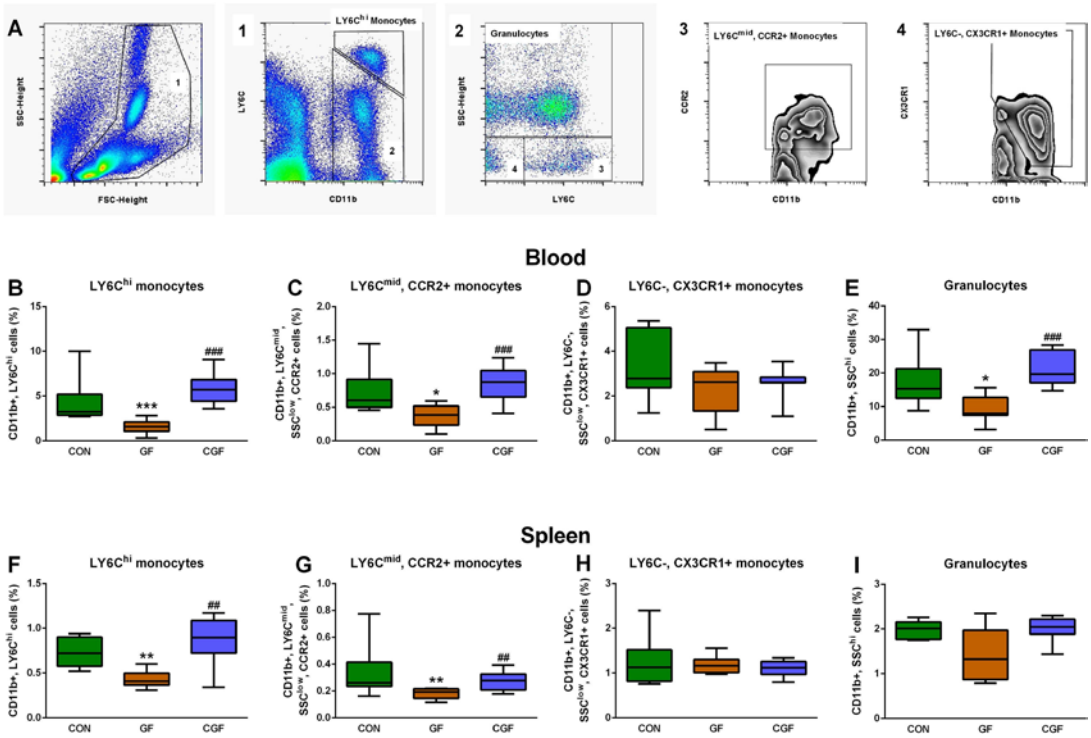

Figure 10: Microbial regulation of innate immune cells Absence of microbiota results in deficits in circulating blood and splenic myeloid cell levels, which are ameliorated by colonisation with microbiota. Gating was performed as following (A). Cells were selected based on FSC-height and SSC-height. 1) LY6Chi monocytes (CD11b+, LY6Chi) were subsequently selected, whereas CD11b+, LY6Clow/- cells were used to select other populations. 2) Granulocytes (CD11b+, SSChi) were then selected. 3) While SSChi, LY6C− cells were used to gate LY6Cmid, CCR2+ monocytes (CD11b+, SSClow, LY6Cmid, CCR2+). 4) SSChi, LY6C− cells were used to gate LY6C−, CX3CR1+ monocytes (CD11b+, SSClow, LY6C−, CX3CR1+). LY6Chi monocyte, LY6Cmid, CCR2+ monocyte, LY6C−, CX3CR1+ monocyte and granulocyte levels were quantified in both blood (B-E) and the spleen (F-I). Data were non-normally distributed and analysed using the Mann-Whitney U test. Significant differences are depicted as: *p < 0.05, **p < 0.01 and ***p < 0.001 compared to CON; #p < 0.05, ##p < 0.01 and ###p < 0.001 compared to GF. Data are presented as boxplots (n = 7–9). Abbreviations: CON = Conventionally raised; GF = Germ-free; CGF = Conventionalized germ-free.

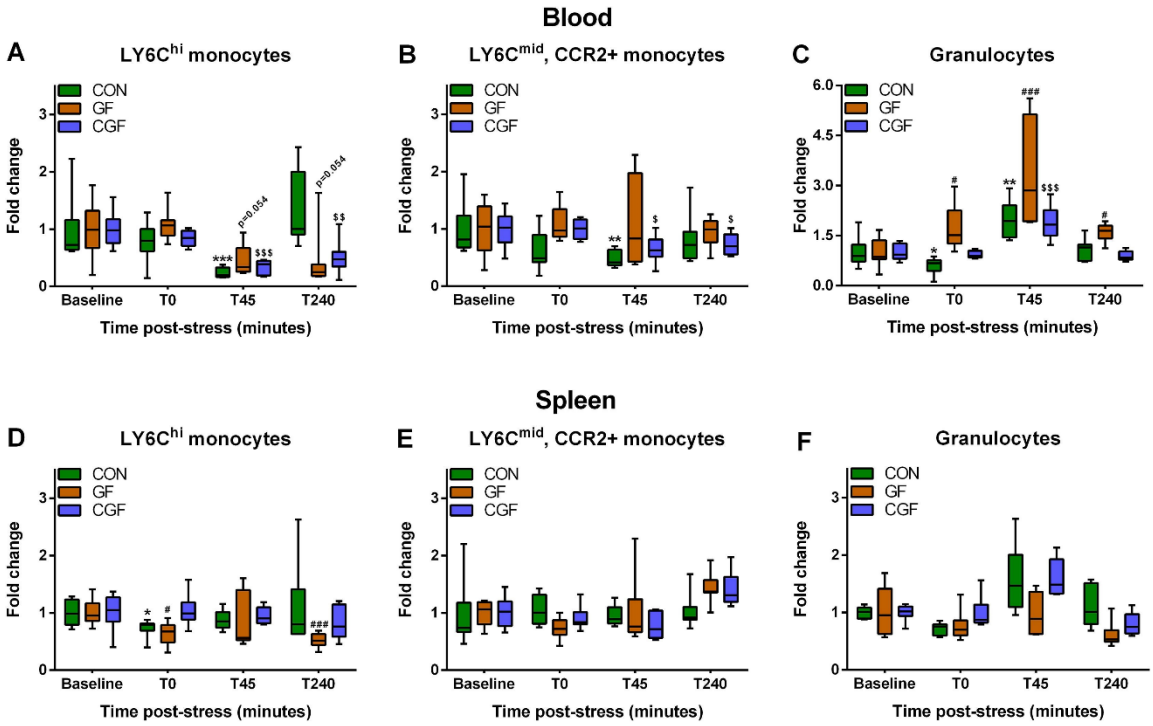

Figure 11: Stress induced alterations in immune cells Stress induced circulating and splenic myeloid cell trafficking, which is affected by microbiota status. LY6Chi monocyte, LY6Cmid, CCR2+ monocyte and granulocte levels were quantified before and after acute stress in the blood (A-C), and spleen (D-F). Data were non-normally distributed and analysed using the Kruskall Wallis test followed by the Mann-Whitney U test. Significant differences are depicted as: *p < 0.05, **p < 0.01 and ***p < 0.001 compared to baseline for CON mice; #p < 0.05 and ###p < 0.001 compared to baseline for GF mice. $p < 0.05, $$p < 0.01 and $$$p < 0.001 compared to baseline for ex-GF mice. Post-stress data from animals were normalized to baseline data (pre-stress). Data are presented as boxplots (n = 7–9) Abbreviations: CON = Conventionally raised; GF = Germ-free; CGF = Conventionalized germ-free.

Intestinal permeability ex-vivo

Stress plays a role in regulating gastrointestinal permeability which may have important implications for health and disease. Out results indicate a region and microbiota-dependent regulation of gastrointestinal permeability following acute stress exposure. In the ileum, this manifests as a rapid and sustained increase in gastrointestinal permeability only in microbiota deficient animals. In contrast, acute stress exposure results in a rapid and sustained reduction in gastrointestinal permeability only in the presence of an intact gut microbiota. Taken together, the data presented here provides novel insights into the role of the gut microbiome in sex-dependent gastrointestinal and central adaptations to acute stress and suggest future analysis including those indicated in table 1.

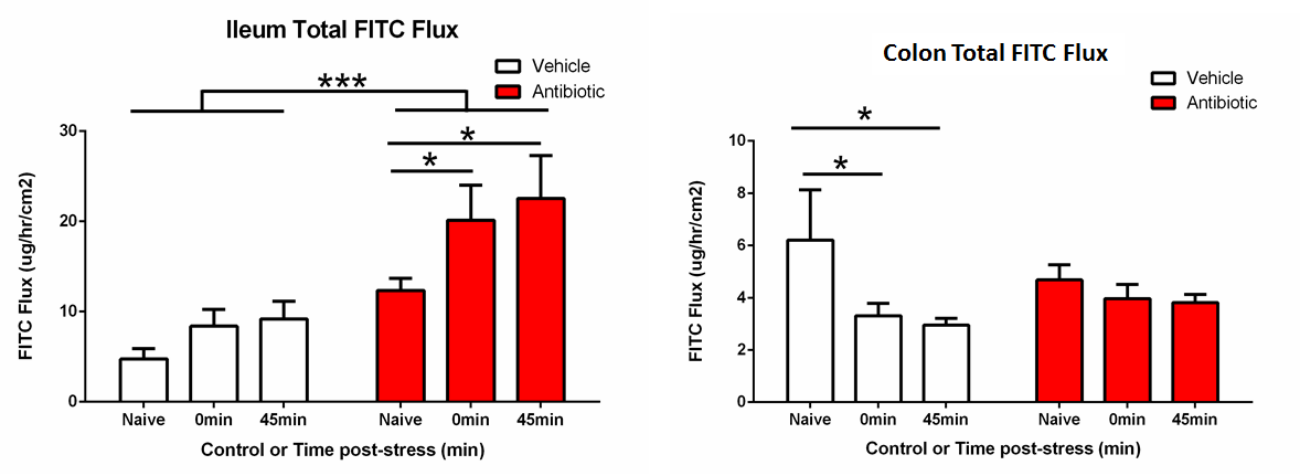

Figure 11: Acute Stress-induced alterations in gastrointestinal permeability Stress induces an increase in ileal permeability only in microbiota-deficient animals. In contrast, there is a post-stressor decrease in colonic permeability only in the presence of the gut microbiota. Symbols indicating significant differences at $p<0.05$, $P<0.01$, $P<0.001$ level.

Table 1. Future analyses of major targets of microbe-host interaction following acute stress

Target	Relevance	Tissue	Reference #
miRNA	Host gut miRNA crosstalk with microbiome after stress	Intestinal tract	15
Aryl hydrocarbon receptor	Xenobiotic receptor which detects indole, a product of bacterial metabolism of L-tryptophan	Intestinal tract/Liver	14

Conclusions

Sex-specific differences between male and female GF and conventional mice in response to acute stress clearly indicate a sex-dependent role for the gut microbiota in modulating the gut and brain serotonergic systems environment following acute stress. On a sex-dependent basis, the colonisation of GF mice with a conventional gut microbiota corrected some differences observed in the gastrointestinal and brain serotonergic systems of GF mice compared to conventional mice. Metabolomic profiling revealed that levels of tryptophan in the cecum decreased after stress in all groups and confirmed that both host and microbial tryptophan metabolic pathways were thus markedly altered by stress. To our knowledge, this is the first time that a direct effect of acute stress on microbial metabolism has been demonstrated, with changes in indoleacetate and indolepropionate, metabolites produced exclusively by gut microbes.

Our data further revealed novel cell-type-specific changes in the innate immune system in response to acute stress, which in turn are impacted by the microbiota. This indicates that the microbiota influences the priming and recovery of the innate immune system to an acute stressor and may inform future microbiota-targeted therapeutics aimed at modulating stress-induced immune activation in stress-related disorders. Microbiota-depleted animals also displayed region-specific alterations in gastrointestinal permeability as a consequence of acute stress exposure.

Cumulatively, our results suggest that acute stress induces rapid changes at the host-microbe interface and along the microbiome-gut-brain axis, and that the microbiota is responsible for directing host and microbial metabolism of tryptophan and other metabolites along specific and physiologically relevant homeostatic pathways. These data meet a major objective of providing novel insights into host-microbe bidirectional interaction following acute stress. The signatures identified to date increase our understanding of the extent and nature of host-microbe dialogue following acute stress. Future work in this area will moves us towards a mechanistic appreciation of how changes in host cognition are mediated by the gut microbiome and whether host-microbe interaction represents a therapeutic target in augmenting cognitive performance. We anticipate the gut neuroendocrine landscape to offer molecular targets which enhance resilience to acute stress.

Table 2. Future directions for investigation into microbe-host interaction following acute stress

1. Implications of acute stress-induced alterations in the microbiome-gut-brain axis for cognition and behaviour

2. How acute stress affect the gut miRNA landscape, and whether these changes are region- and/or sex-dependent.

3. Microbial Regulation of Barrier Function in the Gut and the Brain

4. Examination of microbial metabolism of L-tryptophan, and how these products, such as indole, interact with host xenobiotic receptors to affect host intestinal barrier integrity following an acute stressor.

5. Microbial Metabolites and Neuroinflammation

Table 3. Manuscripts published and under review

Published

1. van de Wouw M, **Lyte JM**, Boehme M, Sichetti M, Moloney G, **Goodson MS**, **Kelley-Loughnane N**, Dinan TG, **Clarke G**, **Cryan JF** The role of the microbiota in acute stress-induced myeloid immune cell trafficking. *Brain Behav Immun*. 2020 Feb;84:209-217. doi: 10.1016/j.bbi.2019.12.003.

2. Gheorghe CE, Martin JA,Villalobos-Manriquez F, Dinan TG, **Cryan JF**, **Clarke G** Focus on the essentials: tryptophan metabolism and the microbiome-gut-brain axis *Curr Opin Pharmacol.* 2019 Oct;48:137-145. doi: 10.1016/j.coph.2019.08.004.

3. Leprun PMB, **Clarke G**. The gut microbiome and pharmacology: a prescription for therapeutic targeting of the gut–brain axis (2019) *Current Opinion in Pharmacology* 49:17-23 *https://doi.org/10.1016/j.coph.2019.04.007*

Under Review

1. Lyte JM, Gheorghe CE, **Goodson MS**, **Kelley-Loughnane N**, Shanahan F, Dinan TG, **Cryan JF**, and **Clarke G**. A Role for The Microbiome in Mediating the Central and Gastrointestinal Serotonergic Responses to Acute Stress Exposure. Submitted to *Neurogastroenterology and Motility*

Table 4. Conference presentations

Oral Presentations

1. The stressed gut: A novel role for short chain fatty acid metabolites in host-microbe dialogue following acute stress exposure (**Martínez-Herrero S**). APC Symposium: Challenges for Microbiome Science. University College Cork, Cork, Ireland. October 2019

2. What Lies Beneath: Microbiome-Gut-Brain Axis Dysregulation in Stress-related Disorders (**Clarke G**) *5th Annual Meeting International Society for Evolution, Public Health and Medicine (ISEMPH) Zurich August 15th 2019*

3. Gut Microbiota and Mental Health- Moving Towards Mechanisms (**Cryan JF**) *FASEB Meeting, The Gastrointestinal Tract XVIII Conference: Integrated Biology of the GI Super-Organ, 2019, Steamboat Springs, Colorado, USA, July 2019*

4. Gut Feelings: The Microbiome as a Key Regulator of Brain and Behaviour Across the Lifespan (**Cryan JF**) *University of Melbourne, Dept. Pharmacology & Therapeutics, July 2019*

5. Microbiomes: A Gut Feeling About Happiness (**Cryan JF**) *World Congress on Positive Psychology, Melbourne, Australia, Plenary July, 2019*

6. Human Physiology- from Gut to Brain (**Cryan JF**) *Topical Meeting: ARO-ONR MURI engagement:Innovations in Academia DoD TriService Microbiome Meeting 03-04th June 2019 in Framingham, MA*

7. Gut Feelings: The Microbiome as a Key Regulator of Brain and Behaviour Across the Lifespan *(***Cryan JF**) *Ohio State University, Columbus, April 2019*

8. What Lies Beneath: Microbiome-Gut-Brain Axis Dysregulation in Psychology (**Clarke G**) *International Convention Psychological Science, Paris, France March 9th 2019*

9. Gut Reactions and the Culture of Stress: Towards Microbial Management of Brain Function and Behaviour (**Clarke G**) *Beneficial Microbes, Amsterdam November 28th 2018*

10. The Gut Microbiome, Brain Function and Behaviour: Focus on Microbial Checkpoints for Host Serotonin and Tryptophan Metabolism (**Clarke G**) *International Society for Serotonin Research (ISSR) 2018 University College Cork, Cork, Ireland July 2018*

11. Crosstalk in the Microbiome-Gut-Brain Axis and Host Behaviour: Focus on Microbial Regulation of Tryptophan Metabolism (**Clarke G**) *Gordon Research Conference, Les Diablerets Conference Center, Switzerland May 30th 2018*

12. Dynamic Gastrointestinal Serotonergic Responses to an Acute Stressor: Role of Host Genetics and the Gut Microbiome (**Clarke G**) *Irish Society of Gastroenterology Naas November 2017*

13. Host-microbe interactions as novel determinants of the stress response (**Lyte JL**) Dayton, Ohio November 2017

Poster presentations

1. **Martínez-Herrero S**, **Lyte JM**, **Goodson MS**, **Kelley-Loughnane N**, Dinan TG, **Cryan JF**, **Clarke G**. Microbial regulation of stress-induced alterations in the gut metabolome *Federation of Neurogastroenterology and Motility (FNM), Adelaide, Australia, March 2020*

2. **Martínez-Herrero S**, **Lyte JM**, **Goodson MS**, **Kelley-Loughnane N**, Dinan TG, **Cryan JF**, **Clarke G**. The gut microbiota shapes acute stress induced alterations in colonic metabolism: Implications for gastrointestinal metabolic homeostasis and microbiota-host signalling New Horizons, University College Cork, December 2019

3. Gheorghe CE, **Lyte JM**, Olavarría-Ramírez L, **Goodson MS**, **Kelley-Loughnane N**, Moloney G, Shanahan F, Dinan TG, **Cryan JF**, **Clarke G**. Microbial Regulation of Tryptophan Metabolism: Focus on Gastrointestinal and Hepatic Gene Expression *New Horizons, University College Cork, December 2019*

4. Martínez-Herrero S, **Lyte JM**, **Goodson MS**, **Kelley-Loughnane N**, Dinan TG, **Cryan JF**, **Clarke G**. The stressed gut: A novel role for short chain fatty acid metabolites in host-microbe dialogue following acute stress exposure. APC Symposium: Challenges for Microbiome Science. University College Cork, Cork, Ireland. October 2019

5. Gheorghe CE, **Lyte JM**, Olavarría-Ramírez L, **Goodson MS**, **Kelley-Loughnane N**, Moloney G, Shanahan F, Dinan TG, **Cryan JF**, **Clarke G**. Microbial Regulation of Tryptophan Metabolism: Focus on Gastrointestinal and Hepatic Gene Expression *Mind, Mood and Microbes Amsterdam January 2019*

6. Olavarría-Ramírez L, O'Donoghue K, Van de Wouw M, Gheorghe C, **Lyte JM**, **Goodson MS**, **Kelley-Loughnane N**, Moloney G, **Cryan JF**, Dinan TG, **Clarke G** The Gut Microbiome and Hepatic Gene Expression: Implications for Tryptophan Metabolism *Federation of Neurogastroenterology and Motility (FNM) 2018 Amsterdam August 2018*

7. Lyte JM, **Goodson MS**, **Kelley-Loughnane N**, Dinan TG, **Cryan JF**, **Clarke G** The microbiota defines the gastrointestinal serotonergic response to acute stress in a sex- and region dependent manner *International Society for Serotonin Research (ISSR) 2018 University College Cork, Cork, Ireland July 2018*

8. Lyte JM, **Goodson MS**, **Kelley-Loughnane N**, Dinan TG, **Cryan JF**, **Clarke G**. Absence of the microbiota alters the gastrointestinal serotonergic response to acute stress in a region-dependent manner *International Human Microbiome Congress. Killarney, Ireland, June 2018.*

9. Lyte JM, **Goodson MS**, **Kelley-Loughnane N**, Dinan TG, **Cryan JF**, and **Clarke G**. Dynamic Gastrointestinal Serotonergic Responses to an Acute Stressor: Role of Host Genetics. *Neurogastro 2017, University College Cork, Cork, Ireland, August 2017.*

10. Lyte JM, **Goodson MS**, **Kelley-Loughnane N**, Dinan TG, **Cryan JF**, and **Clarke G**. Acute stress-induced Gastrointestinal Serotonergic Responses are Region-Dependent and Host Strain Specific. *Society for Neuroscience, Washington DC, USA, November 2017.*

11. Lyte JM, Goodson MS, **Kelley-Loughnane N**, Dinan TG, **Cryan JF**, and **Clarke G**. Dynamic Gastrointestinal Serotonergic Responses to an Acute Stressor: Role of Host Genetics. *New Horizons medical research conference, University College Cork, Cork, Ireland, December 2017.*

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List of Symbols, Abbreviations and Acronyms

5-HT; 5-hydroxytryptamine; 5-HIAA; 5-hydroxyindoleacetic acid; ELISA; enzyme-linked immunosorbent assay GF; germ-free; HPA-axis; hypothalamic-pituitary-adrenal axis; HPLC; high-performance liquid chromatography; TCA: tricarboxylic acid cycle; SCFAs :short chain fatty acids; PCA; Principal component analysis